NOVEL MODULATORS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application claims the priority benefit of US Provisional Patent Application No. 61/246,067, filed September 25, 2009, US Provisional Patent Application No. 61/306,321, filed February 19, 2010, and US Provisional Patent Application No. 61/358,749, filed June 25, 2010, each of which is incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present disclosure relates to novel modulators and/or agonists of the insulin-insulin receptor signaling complex and to methods of screening for such modulators and/or agonists. Such modulators and/or agonists may, for example, be used to treat a mammalian subject suffering from Type 2 diabetes, obesity, hyperglycemia, hyperinsulinemia, insulin overdose, chronic kidney disease, Type 1 diabetes, insulin resistance and disease states and conditions characterized by insulin resistance or to prevent occurrence of the same in an at risk subject.

BACKGROUND OF THE INVENTION

[0003] The present disclosure relates to novel modulators and/or agonists of the insulin-insulin receptor signaling complex, to methods of screening for such modulators and/or agonists and to the use of such modulators and/or agonists in the treatment or prevention of disease states and conditions characterized by abnormal production and/or utilization of insulin.

[0004] The peptide hormone insulin is a major regulator of glucose homeostasis and cell growth. The first step in insulin action is the binding of the hormone to the insulin receptor (INSR), an integral membrane glycoprotein, also designated as CD220 or HHF5. The INSR belongs to the tyrosine kinase growth factor receptor superfamily and is composed of two extracellular α subunits that bind insulin, and two transmembrane β subunits with intrinsic tyrosine kinase activity. The amino acid sequence of the INSR is described in US 4,761,371 and as NCBI Reference Sequence NP_000199.2. The INSR is expressed in two isoforms, INSR-A and INSR-B. The three-dimensional structure of the intact homodimeric ectodomain fragment of human INSR has been elucidated using X-ray crystallography (WO07/147213). INSR isoforms also form heterodimers, INSR-A/INSR-B, and hybrid INSR/IGF-1R

receptors, whose role in physiology and disease is not yet fully understood (Belfiore et al, Endocrine Rev., 30(6):586-623, 2009).

[0005] When insulin binds to the INSR, the receptor is activated by tyrosine autophosphorylation and the INSR tyrosine kinase phosphorylates various effector molecules, including the insulin receptor substrate-1 (IRS-1), leading to hormone action (Ullrich et al, Nature 313: 756-761, 1985; Goldfine et al, Endocrine Reviews 8: 235-255, 1987; White and Kahn, Journal Biol. Chem. 269: 1-4, 1994). IRS-1 binding and phosphorylation eventually leads to an increase in the high affinity glucose transporter (Glut4) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissue, and therefore to an increase in the uptake of glucose from blood into these tissues. Glut4 is transported from cellular vesicles to the cell surface, where it then can mediate the transport of glucose into the cell. A decrease in INSR signaling, leads to a reduction in the uptake of glucose by cells, hyperglycemia (an increase in circulating glucose), and all the sequelae which result.

[0006] Reduction in glucose uptake can result in insulin resistance, which describes a condition in which physiological amounts of insulin are inadequate to produce a normal insulin response from cells or tissues. Severe insulin resistance is associated with diabetes, while less severe insulin resistance is also associated with a number of disease states and conditions present in approximately 30-40% of non-diabetic individuals (reviewed in Woods et al, End, Metab & Immune Disorders –Drug Targets 9: 187-198, 2009).

[0007] Current treatments for diabetes and insulin resistance are directed toward improving insulin secretion, reducing glucose production, and enhancing insulin action.

[0008] Currently, there are various pharmacological approaches for the treatment of Type 2 diabetes (Scheen et al, Diabetes Care, 22(9):1568-1577, 1999; Zangeneh et al, Mayo Clin. Proc. 78: 471-479, 2003; Mohler et al, Med Res Rev 29(1): 125-195, 2009). They act via different modes of action: 1) sulfonylureas (*e.g.*, glimepiride, glisentide, sulfonylurea, AY31637) essentially stimulate insulin secretion; 2) biguanides (*e.g.*, metformin) act by promoting glucose utilization, reducing hepatic glucose production and diminishing intestinal glucose output; 3) alpha-glucosidase inhibitors (*e.g.*, acarbose, miglitol) slow down carbohydrate digestion and consequently absorption from the gut and reduce postprandial hyperglycemia; 4) thiazol-idinediones (*e.g.*, troglitazone, pioglitazone, rosiglitazone, glipizide, balaglitazone, rivoglitazone, netoglitazone, troglitazone, englitazone, AD 5075, T

174, YM 268, R 102380, NC 2100, NIP 223, NIP 221, MK 0767, ciglitazone, adaglitazone, CLX 0921, darglitazone, CP 92768, BM 152054) enhance insulin action, thus promoting glucose utilization in peripheral tissues; 5) glucagon-like-peptides and agonists (e.g. exendin) or stabilizers thereof (e.g. DPP4 inhibitors, such as sitagliptin) potentiate glucose-stimulated insulin secretion; and 6) insulin or analogues thereof (e.g. LANTUS®) stimulate tissue glucose utilization and inhibits hepatic glucose output. The above mentioned pharmacological approaches may be utilized individually or in combination therapy. However, each approach has its limitations and adverse effects. Over time, a large percentage of Type 2 diabetic subjects lose their response to these agents. 63% of Type 2 diabetes patients fail to reach global HbA_{1c} levels of <7% as advised by the American Diabetes Association, and are thus at high risk of developing complications. Moreover, almost invariably patients progress through the stages of declining pancreatic function. Insulin treatment is typically instituted after diet, exercise, and oral medications have failed to adequately control blood glucose. The drawbacks of insulin treatment are the need for drug injection, the potential for hypoglycemia, and weight gain. Consequently there is still an urgent need for novel anti-diabetic agents.

[0009] Antibodies binding to human INSR have been reported in Soos et al, Biochem. J. 235: 199-208, 1986; Taylor et al, Biochem. J. 242: 123-129, 1987; Prigent et al, J. Biol. Chem. 265(17):9970-9977, 1990; Brindle et al, Biochem. J. 268: 615-620, 1990; Steele-Perkins and Roth, J. Biol. Chem. 265(16): 9458-9463, 1990; McKern et al, Nature 443(14): 218-221; Boado et al, Biotech and BioEng. 96(2): 381-391; WO04/050016; Roth et al, Proc. Natl. Acad. Sci. USA 79: 7312-7316, 1982; Morgan et al, Proc. Natl. Acad. Sci. USA 83: 328-332, 1986; Lebrun et al, J. Bl. Chem. 268(15): 11272-11277, 1993; Forsayeth et al, Proc. Natl. Acad. Sci. USA 84: 3448-3451, 1987; Forsayeth et al, J. Biol. Chem. 262(9): 4134-4140, Goodman et al, J. Receptor Res. 14(6-8), 381-398, 1994; Ganderton et al, Biochem J. 288: 195-205, 1992; Spasov et al, Bull. of Exp. Biol. and Med. 144(1): 46-48, 2007; EP 2 036 574 A1.

SUMMARY OF THE INVENTION

[0010] The present disclosure is directed to polypeptide binding agents, e.g., antibodies or fragments thereof, that modulate and/or agonize the insulin-INSR signaling complex by

binding to extracellular regions of the INSR uncomplexed to inslin, to the INSR complexed with insulin, or to both. INSR is a membrane-bound cell surface receptor.

[0011] In one aspect, the invention provides an antibody that binds to insulin receptor and/or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant K_D of 10^{-5}M or less that is capable of strengthening binding affinity or binding rate parameter between insulin and insulin receptor (INSR) by about 5-fold to 200-fold. In one embodiment, the antibody is capable of strengthening the binding affinity or binding rate parameter between insulin and insulin receptor by about 1.5-fold to about 100-fold, or about 2-fold to 25-fold. It is further contemplated that the modulation is about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g., at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, or up to 30-fold, or up to 20-fold, or up to 10-fold. In a further embodiment, the antibody strengthens binding affinity or binding rate parameter by 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 fold or more, or any range between any of these values. In some embodiments, the binding affinity is any one of K_A, K_D, the ratio of on rate divided by off rate, or the ratio of off rate divided by on rate. In specific exemplary embodiments, the antibody increases K_A by the desired fold, or decreases K_D by the desired fold, or increases the ratio of on rate to off rate by the desired fold, or decreases the ratio of off rate to on rate by the desired fold. In some embodiments, the binding rate parameter is the on rate or off rate. In specific exemplary embodiments, the antibody increases the on rate or decreases the off rate. Alternatively, in some embodiments where the binding affinity does not change detectably or significantly, increasing the on rate and increasing the off rate may shift the signaling pathway away from mitogenic signaling towards metabolic signaling (glucose uptake).

[0012] In one embodiment, an antibody that strengthens the binding affinity between insulin and INSR is a positive modulator.

[0013] In another aspect, the antibody is an agonist.

[0014] In a related aspect, an antibody that activates the INSR without dependence on insulin is an allosteric agonist. In certain embodiments, the invention provides an allosteric agonist antibody that binds to insulin receptor with an affinity of at least 10^{-5},10^{-6}, 10^{-7}, 10^{-8},

10^{-9} M and (a) exhibits maximal agonist activity that is 20%-80% that of insulin's maximal agonist activity when measured in a pAKT assay, (b) when present does not alter the EC50 of insulin for INSR by more than 2-fold, and (c) when present does not alter the K_D of insulin for INSR by more than 2-fold .

[0015] In a related embodiment, the allosteric agonist exhibits a maximal agonist response that is 80% or less of the maximal agonist response of insulin, for example 15%-80%, 20-60%, 20%-40% or 15%-30%. In certain embodiments, the antibodies constitutively activate INSR with a maximal agonist response that is at least about 15%, 20%, 25%, 30%, 35%, 40%; and up to 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% of the maximal agonist response of insulin. It is understood that any combination of any of these range endpoints is contemplated without having to recite each possible combination.

[0016] In another aspect, the invention provides an antibody that binds to insulin receptor and/or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant K_D of 10^{-5}M or less that is capable of weakening the binding affinity or binding rate parameter between insulin and insulin receptor by about 1.5-fold to 100-fold. In one embodiment, the antibody is capable of weakening the binding affinity or binding rate parameter between insulin and insulin receptor by about 2-fold to 25-fold, or 1.5-fold to 25 fold, or 2-fold to 50-fold. It is further contemplated that the modulation is about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, or up to 30-fold, or up to 20-fold, or up to 10-fold. In a further embodiment, the antibody weakens binding affinity or binding rate parameter by 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 fold or more, or any range between any of these values. In some embodiment, the binding affinity is any one of K_A, K_D, the ratio of on rate divided by off rate, or the ratio of off rate divided by on rate. In specific exemplary embodiments, the antibody decreases K_A by the desired fold, or increases K_D by the desired fold, or decreases the ratio of on rate to off rate by the desired fold, or increases the ratio of off rate to on rate by the desired fold. In some embodiments, the binding rate parameter is the on rate or off rate. In specific exemplary embodiments, the antibody decreases the on rate or increases the off rate.

[0017] In one embodiment, an antibody that weakens the binding affinity between insulin and INSR is a negative modulator. In some specific embodiments, an antibody that weakens the binding affinity between insulin and INSR is an antagonist.

[0018] In still another embodiment, an antibody that strengthens or weakens binding affinity or binding rate parameter between insulin and insulin receptor comprises at least one heavy chain CDR (HCDR1, HCDR2 and HCDR3) set out in SEQ ID NOS: 151-303. In a related embodiment, the antibody comprises a mature heavy chain variable region of SEQ ID NO: 151-303. It is contemplated that any of the above antibodies further comprises a suitable human or human consensus or human-derived constant region, e.g. IgG1, IgG2, IgG3, or IgG4 or a hybrid thereof.

[0019] In a further embodiment, the antibody that strengthens or weakens binding affinity or binding rate parameter between insulin and insulin receptor comprises at least one light chain CDR (LCDR1, LCDR2, or LCDR3) set out in SEQ ID NOS: 1-150. In still another embodiment, the antibody comprises a mature light chain variable region of SEQ ID NO: 1-150. It is contemplated that any of the above antibodies further comprises a human kappa or lambda light chain constant region.

[0020] In one embodiment, the antibody binds insulin receptor. In a related embodiment, the antibody binds the α subunit of INSR. In a further embodiment, the antibody binds the β subunit of INSR. In yet another embodiment, the antibody binds the α and β subunit of the receptor. In a related embodiment, the antibody binds an insulin/ insulin receptor complex. In another embodiment, the antibody that binds the insulin/INSR complex does not detectably bind insulin receptor alone, e.g., in the absence of insulin, or insulin alone.

[0021] In another aspect, the invention provides an antibody that specifically binds insulin receptor and/or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant K_D of 10^{-5}M or less, comprising at least one heavy chain CDR (HCDR1, HCDR2 and HCDR3) of SEQ ID NOS: 151-303. In a related embodiment, the antibody comprises a heavy chain variable region of SEQ ID NO: 151-303. It is contemplated that any of the above antibodies further comprises a suitable human or human consensus or human-derived constant region, e.g. IgG1, IgG2, IgG3, or IgG4 or a hybrid thereof.

[0022] In a further embodiment, the antibody that specifically binds insulin receptor and/or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant

K_D of 10^{-5}M or less, comprises at least one light chain CDR (LCDR1, LCDR2, or LCDR3) set out in SEQ ID NOS: 1-150. In still another embodiment, the antibody comprises a light chain variable region of SEQ ID NO: 1-150. It is contemplated that any of the above antibodies further comprises a human kappa or lambda light chain constant region.

[0023] It is further contemplated that any of the antibodies described above comprises one, two, three, four, five or six CDRs. In one embodiment, the antibody comprises one, two or three heavy chain CDRs set out in SEQ ID NO: 151-303. In another embodiment, the antibody comprises one, two or three light chain CDRs set out in SEQ ID NO: 1-150.

[0024] In one embodiment, the antibody binds insulin receptor. In a related embodiment, the antibody binds an insulin/INSR complex. In another embodiment, the antibody that binds the insulin/INSR complex does not detectably bind insulin receptor alone or insulin alone.

[0025] It is further contemplated that an antibody that strengthens binding affinity or binding rate parameter of insulin and insulin receptor activates insulin receptor by at least 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay. In a related embodiment, the INSR is activated by at least 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, or 80% of the maximal signal of insulin. In another embodiment, the antibody that strengthens binding affinity or binding rate parameter of insulin/INSR activates less than 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay. In a related embodiment, the INSR is activated by less than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% of the maximal signal of insulin. In some embodiments, the INSR is not detectably activated by the antibody.

[0026] It is further contemplated that the antibody reduces fasting blood glucose levels, in a subject having elevated blood glucose, hyperglycemia or disorder associated with insulin resistance, toward the normal range of glucose levels. In one embodiment, the positive modulating antibody or agonist reduces fasting blood glucose levels in the subject by approximately 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more.

[0027] In one embodiment, antibody refers to an antibody or fragment thereof, or a polypeptide comprising an antigen binding domain of an antibody. Exemplary antibodies or antibody fragments include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, multispecific antibodies, Fab, Fab', F(ab')2, Fv, domain antibody (dAb), complementarity determining region (CDR) fragments, CDR-grafted antibodies, single-chain antibodies (scFv), single chain antibody fragments,

chimeric antibodies, diabodies, triabodies, tetrabodies, minibody, linear antibody; chelating recombinant antibody, a tribody or bibody, an intrabody, a nanobody, a small modular immunopharmaceutical (SMIP), a antigen-binding-domain immunoglobulin fusion protein, a camelized antibody, a VHH containing antibody, or a variant or a derivative thereof, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide, such as one, two, three, four, five or six CDR sequences. In one embodiment, the antibody is a monoclonal antibody. In a related embodiment, the antibody is a human antibody.

[0028] In some specific embodiments, the invention excludes rodent antibodies, i.e. antibodies produced by a hybridoma of rodent (e.g. murine, rat) cells. Such antibodies, whether produced by the hybridoma or recombinantly, would have rodent framework amino acid sequence and be immunogenic if administered to humans. In some specific embodiments, the invention excludes the rodent antibodies disclosed in any one of the following references, hereby incorporated by reference in their entirety: Soos et al, Biochem. J. 235: 199-208, 1986; Taylor et al, Biochem. J. 242: 123-129, 1987; Prigent et al, J. Biol. Chem. 265(17):9970-9977, 1990; Brindle et al, Biochem. J. 268: 615-620, 1990; Steele-Perkins and Roth, J. Biol. Chem. 265(16): 9458-9463, 1990; McKern et al, Nature 443(14): 218-221; Boado et al, Biotech and BioEng. 96(2): 381-391; WO04/050016; Roth et al, Proc. Natl. Acad. Sci. USA 79: 7312-7316, 1982; Morgan et al, Proc. Natl. Acad. Sci. USA 83: 328-332, 1986; Lebrun et al, J. Bl. Chem. 268(15): 11272-11277, 1993; Forsayeth et al, Proc. Natl. Acad. Sci. USA 84: 3448-3451, 1987; Forsayeth et al, J. Biol. Chem. 262(9): 4134-4140, Goodman et al, J. Receptor Res. 14(6-8), 381-398, 1994; Ganderton et al, Biochem J. 288: 195-205, 1992; Spasov et al, Bull. of Exp. Biol. and Med. 144(1): 46-48, 2007; EP 2 036 574 A1. However, the invention may include humanized versions of such rodent antibodies, treatment methods using such humanized antibodies, and sterile pharmaceutical compositions comprising such humanized antibodies. In some specific embodiments, the invention excludes the humanized antibody 83-14 reported in Boado et al, Biotech and BioEng. 96(2): 381-391 or WO04/050016.

[0029] In exemplary embodiments, the invention contemplates:

[0030] a monoclonal antibody that retains any one, two, three, four, five, or six of HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, or LCDR3 of any one of SEQ ID NOs: 151-303 and SEQ ID NOs: 1-150, respectively, optionally including one or two mutations in such CDR(s),

e.g., a conservative or non-conservative substitution, and optionally paired as set forth in Table 3;

[0031] a monoclonal antibody that retains all of HCDR1, HCDR2, HCDR3, or the heavy chain variable region of any one of SEQ ID NOs: 151-303, optionally including one or two mutations in any of such CDR(s), optionally further comprising any suitable heavy chain constant region, e.g., IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, or IgE or hybrid thereof;

[0032] a monoclonal antibody that retains all of LCDR1, LCDR2, LCDR3, or the light chain variable region of any one SEQ ID NOs: 1-150, optionally including one or two mutations in any of such CDR(s), optionally further comprising to any suitable light chain constant region, e.g. a kappa or lambda light chain constant region;

[0033] a purified preparation of a monoclonal antibody, comprising the light chain variable region and heavy chain variable regions as set forth in SEQ ID NOs: 1-303 and paired as set forth in Table 3;

[0034] a monoclonal antibody that binds to the same linear or three-dimensional epitope of INSR as an antibody comprising a variable region set out in SEQ ID NO: 1-303 a, e.g., as determined through X-ray crystallography or other biophysical or biochemical techniques such as deuterium exchange mass spectrometry, alanine scanning and peptide fragment ELISA;

[0035] a monoclonal antibody that competes with an antibody comprising a variable region set out in SEQ ID NO: 1-303, optionally paired as in Table 3, for binding to human INSR by more than about 75%, more than about 80%, or more than about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% or 95%.

[0036] In some embodiments, the antibody comprises all three light chain CDRs, all three heavy chain CDRs, or all six CDRs of an antibody comprising a variable region set out in SEQ ID NO: 1-303. In some exemplary embodiments, two light chain CDRs from an antibody may be combined with a third light chain CDR from a different antibody. Alternatively, a LCDR1 from one antibody can be combined with a LCDR2 from a different antibody and a LCDR3 from yet another antibody, particularly where the CDRs are highly homologous. Similarly, two heavy chain CDRs from an antibody may be combined with a third heavy chain CDR from a different antibody; or a HCDR1 from one antibody can be combined with a HCDR2 from a different antibody and a HCDR3 from yet another antibody, particularly where the CDRs are highly homologous.

[0037] Consensus CDRs may also be used. Any one of the consensus CDRs derived herein may be combined with two other CDRs from the same chain (e.g., heavy or light) of any of the antibodies described herein, e.g. to form a suitable heavy or light chain variable region.

[0038] In another aspect, the invention provides variants or derivatives of the antibodies described herein. For example, in one embodiment the antibody is labeled with a detectable moiety as described herein. In a further embodiment, the antibody is conjugated to a hydrophobic moiety described herein.

[0039] Variants of the antibodies include antibodies having a mutation or alteration in an amino acid sequence provided herein, including an amino acid insertion, deletion or substitution, e.g., a conservative or non-conservative substitution.

[0040] In some embodiments, an antibody is provided that comprises a polypeptide having an amino acid sequence at least about 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96% , 97%, 98%, 99% or more identical to the heavy chain variable region set out in SEQ ID NO: 151-303 and/or an amino acid sequence an amino acid sequence at least about 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96% , 97%, 98%, 99% or more identical to the light chain variable region set out in SEQ ID NO: 1-150, the antibody further comprising at least one, two, three, four, five or all of CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 or CDRL3. In some embodiments, the amino acid sequence with percentage identity to the light chain variable region may comprise one, two or three of the light chain CDRs. In other embodiments, the amino acid sequence with percentage identity to the heavy chain variable region may comprise one, two, or three of the heavy chain CDRs.

[0041] It is contemplated that the antibodies of the invention may have one, or two or more amino acid substitutions in the CDR regions of the antibody, e.g. conservative substitutions.

[0042] In a related embodiment, the residues of the framework are altered. The heavy chain framework regions which can be altered lie within regions designated H-FR1, H-FR2, H-FR3 and H-FR4, which surround the heavy chain CDR residues, and the residues of the light chain framework regions which can be altered lie within the regions designated L-FR1, L-FR2, L-FR3 and L-FR4, which surround the light chain CDR residues. An amino acid within the framework region may be replaced, for example, with any suitable amino acid identified in a human framework or human consensus framework.

[0043] It is further contemplated that the invention provides a purified polypeptide comprising any one of the amino acid sequences of SEQ ID NO: 1-150 fused to any one of the amino acid sequences of SEQ ID NO: 151-303, optionally paired as the heavy/light chain variable regions set forth in Table 3, or fragments thereof that include at least a portion of SEQ ID NO: 1-150 and SEQ ID NO: 151-303, optionally paired as set forth in Table 3, wherein the polypeptide binds insulin receptor, insulin or the insulin/insulin receptor complex.

[0044] It is contemplated that antibodies of the invention, including polypeptides comprising all or a portion of an antigen binding fragment in any one of SEQ ID NOs: 1-303, retain binding affinity, e.g. as measured by K_D, to insulin receptor, insulin or a complex of insulin/INSR of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M or less (wherein a lower value indicates a higher binding affinity), optionally as measured by surface plasmon resonance.

[0045] In some of the preceding embodiments, the invention contemplates an antibody that binds to insulin receptor and/or a complex comprising insulin and insulin receptor, with an equilibrium dissociation constant K_D of 10^{-5}M or less, that is capable of strengthening the binding affinity between insulin and insulin receptor by about 5-fold to 500-fold. In one embodiment, the antibody is characterized by the following equilibrium dissociation constant K_D binding properties: (i) said antibody binds with an equilibrium dissociation constant K_D of about 10^{-5}M, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10} , 10^{-11} M or less, to a complex comprising insulin (C1) and insulin receptor (C2); and (ii) any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is at least about 5-fold lower than any of K_{AC2} or K_{AC1}. In a related embodiment, any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is about 5-fold to 200-fold lower than any of K_{AC2} or K_{AC1}.

[0046] In some embodiments, the antibody binds an insulin/ insulin receptor complex. In further embodiments, the antibody binds insulin receptor alone, in an uncomplexed form. In a related embodiment, the antibody does not detectably bind insulin receptor alone, e.g., in the absence of insulin. In certain embodiments, the antibody is capable of strengthening the binding affinity between insulin and insulin receptor by at least about 5-fold, optionally to about 200-fold, optionally to about 100-fold. It is further provided that in some embodiments, the binding affinity is any one of K_A, K_D, the ratio of on rate to off rate, or the ratio of off rate to on rate.

[0047] In some embodiments, for any of the antibodies described herein, the difference in binding affinity or binding rate parameter ranges from about 1.5-fold to about 1000-fold, or

about 1.5-fold to about 500-fold, about1.5-fold to about 100-fold, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, about 5-fold to about 500-fold, or about 5-fold to about 200-fold, e.g. at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 500-fold, or up to 200-fold, or up to 150-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, up to 30-fold, up to 20-fold, or up to 10-fold, or up to 5-fold or up to 3-fold.

[0048] In some embodiments, the invention provides an agonist antibody that binds to insulin receptor with an affinity, e.g. K_D, of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10} , 10^{-11} M or less, optionally that exhibits maximal agonist activity that is 20%-100% that of insulin's maximal agonist activity when measured in pAKT assay. In a related aspect, the invention contemplates an allosteric agonist antibody that binds to insulin receptor with an affinity, e.g., K_D of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10} , 10^{-11} M or less and (a) exhibits maximal agonist activity that is 20%-80% that of insulin's maximal agonist activity when measured in pAKT assay, (b) when present does not alter the EC50 of insulin for INSR by more than 2-fold or 3-fold, and (c) when present does not alter the K_D of insulin for INSR by more than 2-fold or 3-fold. It is further provided that in some embodiments, the binding affinity is any one of K_A, K_D, the ratio of on rate to off rate, or the ratio of off rate to on rate.

[0049] It is contemplated, in certain embodiments, that any of the above antibodies may also exhibit weak agonist activity, e.g., activates insulin receptor by at least 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay. In a further embodiment, the antibody activates insulin receptor by less than 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay.

[0050] In some embodiments, the antibody comprises a heavy chain variable region selected from the group consisting of the mature heavy chain variable region sequences set forth in SEQ ID NOs:, 281, 278, 277, 209, 275, 223, 284, 276, and 236 and a light chain variable region selected from the group consisting of the mature light chain variable sequences set forth in SEQ ID NOs: 141, 138, 137, 35, 135, 57, 144, 136, and 98, optionally paired as set forth in Table 3.

[0051] In another embodiment, the antibody comprises (a) the heavy chain variable region of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010,

Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 291, 196, 239, 267 and 271 and the light chain variable region of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 76, 80, 101, 128, and 132, optionally paired as set forth in Table 3 and preferably the mature portions thereof, or (b) one, two or three heavy chain CDRs of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 291, 196, 239, 267 and 271 and/or one, two or three light chain CDRs of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 76, 80, 101, 128, and 132, optionally including one or two mutations in any one, two or three of such heavy or light chain CDRs, e.g., a conservative or non-conservative substitution, optinoally paired as set forth in Table 3; or (c) all six CDRs of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or antibodies having the variable regions set out in SEQ ID NO: 76, 80, 101, 128, 132, 291, 196, 239, 267, and 271, optionally paired as set forth in Table 3.

[0052] In other embodiments, the invention provides an antibody that competes with any of the antibodies described herein, e.g. by at least 70%, 75%, or 80%. In certain embodiments, the antibody exhibits greater than or equal to 70% competition, e.g. at least 75% or at least 80% competition, with any one, two, three or all antibodies selected from the group consisting of Ab079, Ab076, Ab083, Ab080, Ab062, Ab020, Ab019, Ab088, and Ab089, and optionally exhibits greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab086, Ab064, Ab001, and Ab018. Optionally, the antibody does not compete with one or more of Ab062 and Ab086, and optionally may bind both human and murine insulin receptor or complex.

[0053] In a related embodiment, the antibody exhibits greater than or equal to 70% competition, e.g., at least 75% or at least 80% competition, with any one, two, three or all antibodies selected from the group consisting of Ab040, Ab062, Ab030, Ab001, and Ab018, and optionally exhibits greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab037, Ab078, Ab083, Ab080, and

Ab085. In a related embodiment, the antibody does not compete with any one, two, three or more of antibodies selected from the group consisting of Ab053, Ab064, 83-7, Ab019, Ab088, and Ab089. Optionally, the antibody binds both human and murine insulin receptor or complex. In another embodiment, the antibody exhibits greater than or equal to 70% competition, e.g. at least 75% or at least 80% competition, with any one, two, three or all antibodies selected from the group consisting of Ab064, Ab062, Ab085, and Ab078. Optionally, the antibody exhibits no competition with any one, two, three or more of antibodies selected from the group consisting of Ab077, Ab001, Ab018, Ab030, Ab037, Ab079, Ab076, Ab083, Ab019, Ab088, Ab089, and Ab040. Optionally, the antibody binds both human and murine insulin receptor or complex.

[0054] In a further aspect, the invention provides an antibody that binds to insulin receptor and/or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant K_D of 10^{-5}M or less that is capable of weakening the binding affinity between insulin and insulin receptor by at least about 3-fold, optionally up to 1000-fold. In certain emeobidments, the antibody weakens the affinity between said insulin and insulin receptor by about 3-fold to 500-fold. In some embodiments, the binding affinity is any one of K_A, K_D, the ratio of on rate to off rate, or the ratio of off rate to on rate.

[0055] In some embodiments, for any of the antibodies described herein, the difference in binding affinity or binding rate parameter ranges from about 1.5-fold to about 1000-fold, or about 1.5-fold to about 500-fold, about1.5-fold to about 100-fold, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, about 5-fold to about 500-fold, or about 5-fold to about 200-fold, e.g. at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 500-fold, or up to 200-fold, or up to 150-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, up to 30-fold, up to 20-fold, or up to 10-fold, or up to 5-fold or up to 3-fold.

[0056] In a related embodiment, the antibody increases the EC50 of insulin signaling activity by about 2-fold to 1000-fold, optionally in a pAKT assay. In certain embodiments, the antibody increases the EC50 by at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000-fold.

[0057] In certain embodiments, the antibody comprises a heavy chain variable region selected from the group consisting of the mature heavy chain variable region sequences set forth in SEQ ID NOs: 241, 279, 258, 155, and 228 and a light chain variable region selected from the group consisting of the mature light chain variable region sequences set forth in SEQ ID NOs: 103, 139, 119, 8, and 89, optinoally paired as set forth in Table 3.

[0058] In a further embodiment, the antibody comprises (a) the heavy chain variable region of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081 and the light chain variable region of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081, preferably the mature portions thereof, or (b) one, two or three heavy chain CDRs of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081 and/or one, two or three light chain CDRs of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081, optionally including one or two mutations in any one, two or three of such heavy or light chain CDRs, e.g., a conservative or non-conservative substitution; or (c) all six CDRs of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081.

[0059] In a further embodiment, the invention provides an antibody that competes with the above antibodies, wherein the antibody exhibits greater than or equal to 70% competition, e.g., at least 75% or at least 80% competition, with any one, two, three or all antibodies selected from the group consisting of Ab079, Ab076, Ab083, Ab080, Ab062, and Ab020, Ab019, Ab088, Ab089. Optionally, the antibody does not exhibit competition with any one, two, three or more of the antibodies selected from the group consisting of Ab062, Ab086, Ab001, Ab018, Ab030, Ab037, Ab064; and optionally, the antibody is human reactive only, and does not bind murine insulin receptor or complex.

[0060] In one embodiment, the invention provides an antibody that is an agonist, wherein the antibody comprises a heavy chain variable region selected from the group consisting of the mature heavy chain variable region sequences set forth in SEQ ID NOs: 195, 220, 303, 197, 208, 243, 245 and 251 and a light chain variable region selected from the group consisting of the mature light chain variable region sequences set forth in SEQ ID NOs: 77, 50, 90, 84, 34, 104, 106 and 112, optionally paired as set forth in Table 3.

[0061] In a related embodiment, the antibody comprises (a) the heavy chain variable region of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 set out in SEQ ID NOs: 252, 253, 263, 265 and 269 and the light chain variable region of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 7, 113, 114, 124, 126 and 130, optionally paired as set forth in Table 3 and preferably the mature portions thereof, or (b) one, two or three heavy chain CDRs of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 252, 253, 263, 265 and 269 and/or one, two or three light chain CDRs of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 7, 113, 114, 124, 126 and 130, optionally including one or two mutations in any one, two or three of such heavy or light chain CDRs, e.g., a conservative or non-conservative substitution, optionally paired as set forth in Table 3; (c) all six CDRs of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 7, 113, 114, 124, 126, 130, 252, 253, 263, 265 and 269, optionally paired as set forth in Table 3.

[0062] In a related embodiment, the invention provides an antibody that competes with the above antibodies for binding to target, wherein the antibody exhibits greater than or equal to 70% competition, e.g., at least 75% or at least 80% competition, with any one, two, three or all antibodies selected from the group consisting of Ab030, Ab037, Ab053, Ab001, Ab018, Ab064, Ab040 and optionally exhibits greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab085 and Ab086. Optionally, the antibody exhibits no competition with any one, two, three or more of the antibodies selected from the group consisting of Ab079, Ab076 and Ab088; and optionally binds to both human and murine insulin receptor or complex.

[0063] In a another aspect, the invention provides polynucleotides encoding antibodies and polypeptides of the invention, vectors comprising such polynucleotides, host cells comprising such polynucleotides or vectors, and methods of producing antibodies and polypeptides of the invention comprising growing such host cells in culture medium under suitable conditions and optionally isolating the encoded antibody or polypeptide from the host cells or culture medium, optionally followed by further purification of the antibody or polypeptide, e.g., as described herein.

[0064] Antibodies having the properties described herein may be isolated using a screening method to determine binding to the INSR and modulation of the insulin/INSR complex.

[0065] In one embodiment, the invention provides a positive modulating antibody that strengthens the binding of a first component (C1) to a second component (C2) of a signaling complex, said antibody characterized by the following equilibrium dissociation constant K_D binding properties: (i) said antibody binds with an equilibrium dissociation constant K_D of about 10^{-5}M or less, e.g., 10^{-6}M or less, or 10^{-7}M or less, or 10^{-8}M or less, to any one of C1, C2, or a complex comprising C1 and C2 (C1C2); and (ii) any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is at least about 50% (1.5-fold) lower than any of K_{AC2} or K_{AC1}. In some embodiments any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is about 1.5-fold to about 100-fold lower than any of K_{AC2} or K_{AC1}; or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to about 100-fold, or up to about 90-fold, or up to about 80-fold, or up to about 70-fold, or up to about 60-fold, or up to about 50-fold, or up to about 40-fold, or up to about 30-fold, or up to about 20-fold, or up to about 10-fold lower. In some embodiments, any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is at least about 1.5-fold

lower than both of K_{AC2} or K_{AC1}; or 1.5-fold to about 100-fold lower, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to about 100-fold, or up to about 90-fold, or up to about 80-fold, or up to about 70-fold, or up to about 60-fold, or up to about 50-fold, or up to about 40-fold, or up to about 30-fold, or up to about 20-fold, or up to about 10-fold lower.

[0066] In some embodiments, the invention provides a negative modulating antibody that weakens the binding of a first component (C1) to a second component (C2) of a signaling complex, said antibody characterized by the following equilibrium dissociation constant K_D binding properties: (i) said antibody binds with an equilibrium dissociation constant K_D of about 10^{-5}M or less, e.g., 10^{-6}M or less, or 10^{-7}M or less, or 10^{-8}M or less, to any one of C1, C2, or a complex comprising C1 and C2 (C1C2), and (ii) any of K_{AC2} or K_{AC1} is at least about 50% (1.5-fold) lower than any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$. In some embodiments, any of K_{AC2} or K_{AC1} is at least about 1.5-fold to 100-fold lower than any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$; or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to about 100-fold, or up to about 90-fold, or up to about 80-fold, or up to about 70-fold, or up to about 60-fold, or up to about 50-fold, or up to about 40-fold, or up to about 30-fold, or up to about 20-fold, or up to about 10-fold lower. In some embodiments, any of K_{AC2} or K_{AC1} is at least about 1.5-fold lower than all of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$; or 1.5-fold to about 100-fold lower, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to about 100-fold, or up to about 90-fold, or up to about 80-fold, or up to about 70-fold, or up to about 60-fold, or up to about 50-fold, or up to about 40-fold, or up to about 30-fold lower, or up to about 20-fold, or up to about 10-fold.

[0067] In specific embodiments, C1 and C2 are selected from the group consisting of insulin and insulin receptor.

[0068] In another aspect, the invention contemplates a method of preparing a sterile pharmaceutical composition, comprising adding a sterile pharmaceutically acceptable diluent

to an antibody of the invention. Optionally small amounts of a preservative such as a bactericidal or bacteriostatic agent are also included in the composition.

[0069] Also contemplated is a sterile composition comprising an antibody of the invention and a sterile pharmaceutically acceptable diluent.

[0070] The invention further contemplates that the antibodies of the invention modulate binding between the INSR and insulin or insulin analogs or insulin mimetics. The antibodies of the invention preferably also exhibit desirable biological properties, including but not limited to enhancing glucose uptake in vitro or in vivo in animal models, and preferably the glucose uptake induced by exogenous insulin. In some embodiments, the antibodies are capable of increasing the rate or total amount of glucose uptake, or both.

[0071] In a further aspect, the invention contemplates a method of treating a disorder associated with insulin resistance, comprising administering to a subject in need thereof a positive modulating antibody or agonist antibody of the invention in an amount effective to treat insulin resistance. In a related embodiment, the treatment enhances glucose uptake. In a further embodiment, the enhanced glucose uptake is selected from the group consisting of an increase in the rate of glucose uptake, an increase in the total amount of glucose uptake, or both. It is further contemplated that the treatment reduces fasting blood glucose levels, in a subject having elevated levels of blood glucose, hyperglycemia or a disorder associated with insulin resistance, back toward the normal range of fasting blood glucose levels. In a related embodiment, the fasting blood glucose is reduced by approximately 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more compared to an untreated subject.

[0072] In related aspects, the treatment reduces elevated HbA1c levels, which are a marker of elevated glucose levels over the preceding several-month period, and are indicative of diabetes. In further embodiments, treatment improves impaired glucose tolerance. In one embodiment glucose tolerance is measured by glucose tolerance test (GTT).

[0073] In other embodiments, the treatment slows, reduces, or normalizes weight gain of a subject. In one embodiment, the treatment reduces or slows weight gain by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% compared to an untreated subject. In some embodiments, the treatment slows, reduces, or normalizes weight loss of a subject. In one embodiment, the treatment reduces or slows weight loss by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% compared to an untreated subject.

[0074] In a related aspect, it is contemplated that the antibodies or polypeptides described herein promote or induce weight loss in a subject. In one embodiment, the invention provides a method to promote or induce weight loss by administration of a modulating antibody, fragment thereof or polypeptide as described herein. In one embodiment, the modulating antibody is a positive modulator or partial agonist. In a related embodiment, the modulating antibody is a negative modulator.

[0075] It certain embodiments, the treatment further results in improvement of one, two, three or more symptoms of diabetes or insulin resistance selected from the group consisting of dyslipidemia, elevated plasma triglycerides, elevated HOMA-IR, elevated plasma unesterified cholesterol, plasma total cholesterol elevated plasma insulin (indicative of insulin resistance), low non-HDL/HDL cholesterol ratio (or low total cholesterol/HDL cholesterol ratio), and elevated plasma leptin levels (indicative of leptin resistance).

[0076] It is further provided that the effects of treatment are also measured using in vitro and in vivo analysis using factors as described in the Detailed Description.

[0077] In one embodiment, the disorder associated with insulin resistance is selected from the group consisting of hyperglycemia, pre-diabetes, metabolic syndrome (also referred to as insulin resistance syndrome), Type 2 diabetes mellitus, polycystic ovary disease (PCOS), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), steatosis, obesity, dyslipidemia, Rabson-Mendenhall syndrome, Donohue syndrome or Leprechaunism.

[0078] In another aspect, the invention provides a method of treating a condition or disorder associated with hyperinsulinemia, abnormal production and/or sensitivity to insulin that manifests as excess insulin signaling, comprising administering to a subject in need thereof a negative modulating antibody or an antagonist antibody of the invention in an amount effective to treat insulin overproduction and/or sensitivity. In one embodiment, the disorder associated with insulin sensitivity is selected from the group consisting of cancer, Kaposi's sarcoma, insulinoma, diabetic renal disease, hypoglycemia, nesidioblastosis (KATP-Hl Diffuse Disease, KATP-Hl Focal Disease, or "PHHI"), GDH-Hl (Hyperinsulinism/Hyperammonaemia Syndrome (HI/HA), leucine-sensitive hypoglycemia, or diazoxide-sensitive hypoglycemia), islet cell dysregulation syndrome, idiopathic hypoglycemia of infancy, Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), and Congenital Hyperinsulinism, insulin overdose, hypoglycemia due to renal failure (acute or chronic), and chronic kidney disease, e.g., type III, IV or V.

[0079] Use of any of the foregoing antibodies or polypeptides of the invention that modulate the insulin-INSR signaling interaction in preparation of a medicament for treatment of any of the disorders described herein is also contemplated. Syringes, e.g., single use or pre-filled syringes, sterile sealed containers, e.g. vials, bottle, vessel, and/or kits or packages comprising any of the foregoing antibodies or polypeptides, optionally with suitable instructions for use, are also contemplated.

[0080] Any of the foregoing antibodies or polypeptides of the invention may be concurrently administered with any anti-diabetic agents known in the art or described herein, as adjunct therapy. Compositions comprising any of the foregoing antibodies or polypeptides of the invention together with other anti-diabetic agents are also contemplated.

[0081] A number of anti-diabetic agents are known in the art, including but not limited to: 1) sulfonylureas (*e.g.*, glimepiride, glisentide, sulfonylurea, AY31637); 2) biguanides (*e.g.*, metformin); 3) alpha-glucosidase inhibitors (*e.g.*, acarbose, miglitol); 4) thiazol-idinediones (*e.g.*, troglitazone, pioglitazone, rosiglitazone, glipizide, balaglitazone, rivoglitazone, netoglitazone, troglitazone, englitazone, AD 5075, T 174, YM 268, R 102380, NC 2100, NIP 223, NIP 221, MK 0767, ciglitazone, adaglitazone, CLX 0921, darglitazone, CP 92768, BM 152054); 5) glucagon-like-peptides (GLP) and GLP analogs or agonists of GLP-1 receptor (e.g. exendin) or stabilizers thereof (e.g. DPP4 inhibitors, such as sitagliptin); and 6) insulin or analogues or mimetics thereof (e.g. LANTUS®).

[0082] In a related aspect, the invention provides a method of diagnosing insulin resistance or insulin sensitivity using antibodies as described herein. In one embodiment, the method comprises measuring levels of insulin or insulin receptor in a sample from a subject using an insulin receptor antibody described herein, wherein an increased level of insulin or free insulin receptor, or a decreased level of membrane-bound insulin receptor indicates the subject has or is at risk for diabetes or insulin resistance, and optionally administering a diabetes therapeutic to said subject who has or is at risk of diabetes or insulin resistance. In another embodiment, the method comprises measuring levels of insulin receptor in a sample from a subject using an insulin receptor antibody described herein, wherein an increased level of free insulin receptor or a decreased level of membrane-bound insulin receptor indicates the subject has or is at risk for cancer, and optionally administering a cancer therapeutic to said subject.

[0083] In another aspect, the invention provides methods for identifying antibodies that modulate the binding of insulin to the insulin receptor as described herein.

[0084] It is understood that each feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the invention and, as such, is meant to be combinable with any other feature or embodiment, or combination, described herein. For example, where features are described with language such as "one embodiment", "some embodiments", "further embodiment", "specific exemplary embodiments", and/or "another embodiment", each of these types of embodiments is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination. Such features or combinations of features apply to any of the aspects of the invention. Similarly, where a method describes identifying polypeptide binding agents, such as antibodies, characterized by certain features, polypeptide binding agents characterized by those features are also contemplated by the invention. Where examples of values falling within ranges are disclosed, any of these examples are contemplated as possible endpoints of a range, any and all numeric values between such endpoints are contemplated, and any and all combinations of upper and lower endpoints are envisioned.

BRIEF DESCRIPTION OF THE FIGURES

[0085] Figure 1 depicts representative results from an INSR receptor occupancy screen showing test antibodies binding to IM-9 cells expressing the INSR in the presence and absence of insulin.

[0086] Figure 2 shows representative results from a biotinylated ligand screen showing the effects of test antibodies on insulin binding to insulin receptor.

[0087] Figure 3 shows results from an assay measuring the ability of test antibodies to modulate insulin dependent pIRS-1 phosphrylation.

[0088] Figure 4 depicts results from a pIRS-1 activity assay showing binding of representative antibodies to INSR from different functional classes. A) Positive Modulators; B) Positive modulators with significant agonism; C) Non-modulators; D) Agonist Antibodies; E) Negative modulators.

[0089] Figure 5 is a table showing insulin EC50 values for representative antibodies from the pIRS-1 assay ranked according to EC50 ratio +Ab/-Ab.

[0090] Figure 6 shows results of a pAKT assay for representative antibodies: A) Positive modulator with very low agonism; B) Positive modulator with agonism; C) Agonist antibodies; D) 83-7; E) Insulin and background response in the absence of antibody.

[0091] Figure 7 is a table showing agonism and mouse cross reactivity properties of representative test antibodies. (nd = not determined).

[0092] Figure 8 shows pAKT assay results showing changes in sensitivity (EC50, fold-change in EC50) and cooperativity (Hillslope) of the insulin dose response effected by a positive modulator INSR antibody at four different concentrations. Figure 8A shows the results graphically while Figure 8B shows the results in tabular form.

[0093] Figure 9 illustrates the enhancement of insulin dependent glucose uptake by a positive modulator antibody. ^{3}H-2-deoxyglucose uptake in 3T3-L1 cells was induced by 0.8 nM insulin in the presence of 10 ug/ml test antibody Ab001 or anti-KLH isotype control.

[0094] Figure 10 shows blood glucose levels in 20 week old DIO mice fed a high fat diet and treated with partial agonist anti-INSR antibodies: A. Line graph of glucose levels. B. Bar chart of glucose levels showing statistically significant reduction in blood glucose after injection of partial agonist anti-INSR antibody.

[0095] Figure 11 illustrates that administration of a partial agonist anti-INSR antibody improves glycemic control in DIO mice: A. Glucose tolerance test timecourse; B. Fasting blood glucose levels; C. Glucose tolerance test; area under curve (AUC).

[0096] Figure 12 shows that a positive modulator anti-INSR antibody improves insulin sensitivity in DIO mice: A. Insulin tolerance test timecourse; B. Fasting blood glucose levels; C. Insulin tolerance test; area under curve (AUC).

[0097] Figure 13 shows that a positive modulator anti-INSR antibody improves glycemic control in DIO mice: A. Glucose tolerance test timecourse; B. Fasting blood glucose levels; C. Glucose tolerance test; area under curve (AUC).

[0098] Figure 14 demonstrates that positive modulator and partial agonist anti-INSR antibodies improve triglyceride and cholesterol levels in DIO Mice. Plasma triglyceride and cholesterol levels were measured in 30-week old DIO mice injected intraperitoneally (IP)

with Ab001, Ab037 or isotype control (10 mg/kg; * $p < 0.05$ relative to isotype control/HFD): A. Plasma triglyceride levels; B. Plasma cholesterol levels.

[0099] Figure 15 illustrates improvement of glycemic control in DIO mice after administration of positive modulator and partial agonist anti-INSR antibodies. Glycemic control measurements were observed in DIO mice injected intraperitoneally (IP) with Ab001, Ab037, Ab083, Ab085 or isotype control (10 mg/kg; * $p < 0.05$ relative to isotype control/HFD): A. Glucose tolerance test timecourse; B. Glucose tolerance test; area under curve (AUC); C. Fasting blood glucose levels.

[00100] Figure 16 shows positive modulator and partial agonist anti-INSR antibodies improve glycemic parameters, insulin resistance and and/or dyslipidemia in 18-week old DIO mice treated with mAb for 4 weeks. Analysis of plasma from DIO mice injected intraperitoneally (IP) with Ab001, Ab037, Ab083, Ab085 or isotype control for 4 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control/HFD) was performed. A. Plasma glucose levels B. Plasma insulin levels; C. HOMA-IR; D. Plasma triglyceride levels; E. Plasma unesterified cholesterol levels; F. Plasma total cholesterol levels; G. Plasma non-HDL cholesterol levels; H. Plasma non-HDL/HDL cholesterol ratio.

[00101] Figure 17 demonstrates that positive modulator and partial agonist anti-INSR antibodies reduce weight gain in DIO mice as assessed by body weight measurements in 18-week old DIO mice injected intraperitoneally (IP) with Ab001, Ab037, Ab083, Ab085 or isotype control for 3 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control/HFD): A. Percent change in body weight relative to pre-dose weight; B. Percent change in body weight relative to pre-dose weight: Area under the curve.

[00102] Figure 18 depicts positive modulator and partial agonist anti-INSR antibody-induced normalization of weight gain in db/db mice analyzed by body weight measurements in 5 week old db/db mice injected intraperitoneally (IP) with Ab001 (1 mg/kg or 10 mg/kg), Ab037 (10 mg/kg) or isotype control (1 mg/kg or 10 mg/kg) for 14 weeks (* $p < 0.05$ relative to isotype control): A. Percent change in body weight relative to pre-dose weight until day 35 of study; B. Percent change in body weight relative to weight at day 35 of study; C. Percent change in body weight relative to pre-dose weight until day 35 of study: Area under the curve; D. Percent change in body weight relative to weight at day 35 of study: Area under the curve.

[00103] Figure 19 shows that positive modulator antibody reduces fasting blood glucose and HbA1c in db/db mice as assessed using glycemic control measurements in 5 week old db/db mice injected intraperitoneally (IP) with Ab001 (1 mg/kg or 10 mg/kg), Ab037 (10 mg/kg) or isotype control (1 mg/kg or 10 mg/kg) for 14 weeks (* $p < 0.05$ relative to isotype control at same dose): A. Fasting blood glucose levels; B. % HbA1c levels.

[00104] Figure 20 illustrates that administration of positive modulator antibody improves dyslipidemia in db/db mice. Analysis of plasma from 5-week old db/db mice injected intraperitoneally (IP) with Ab001 (1 mg/kg or 10 mg/kg), Ab037 (10 mg/kg) or isotype control (1 mg/kg or 10 mg/kg) for 14 weeks (* $p < 0.05$ relative to isotype control at same dose) was carried out. A. Plasma insulin levels; B. Plasma triglyceride levels; C. Plasma unesterified cholesterol levels; D. Plasma total cholesterol levels; E. Plasma non-HDL cholesterol levels; F. Plasma non-HDL/HDL cholesterol ratio.

[00105] Figure 21 shows that administration of positive modulator antibody reduces fasting blood glucose in db/db Mice. Weekly fasting blood glucose assessment of 5-week old db/db mice injected intraperitoneally (IP) with Ab001 (1 mg/kg or 10 mg/kg), Ab037 (10 mg/kg) or isotype control (1 mg/kg or 10 mg/kg) for 14 weeks (* $p < 0.05$ for Ab085 relative to isotype control at same dose).

[00106] Figure 22 illustrates that positive modulator antibodies improve insulin resistance in db/db mice as assessed by analysis of plasma from 5-week old db/db mice injected intraperitoneally (IP) with Ab001, Ab037, Ab083, Ab085 or isotype control for 4 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control at same dose) and shows Homeostatic model assessment for Insulin Resistance (HOMA-IR) after 4 weeks of dosing.

[00107] Figure 23 illustrates that positive modulator and partial agonist anti-INSR antibodies improve glycemic control in MLDS/HFD mice. Glycemic control measurements were taken from 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab037 or isotype control (10 mg/kg; * $p < 0.05$ relative to isotype control): A. Glucose tolerance test timecourse; B. Glucose tolerance test; area under curve (AUC); C. Fasting blood glucose levels.

[00108] Figure 24 shows administration of a partial agonist antibody reduces fed blood glucose and HbA1c in MLDS/HFD mice. Glycemic control measurements were taken in 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab037 or isotype

control for 6 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control): A. Fed blood glucose levels; B. % HbA1c levels.

[00109] Figure 25 shows positive modulator and/or partial agonist anti-INSR antibodies partially correct insulin, leptin and non-HDL/HDL cholesterol levels in MLDS/HFD mice. Plasma cholesterol, insulin and leptin levels were measured in 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab037 or isotype control for 6 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control): A. Plasma non-HDL/HDL cholesterol ratio; B. Plasma insulin levels; C. Plasma leptin levels.

[00110] Figure 26 illustrates that positive modulator and partial agonist anti-INSR antibodies do not affect body weight in MLDS/HFD mice. Body weight measurements were taken in 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab037 or isotype control for 6 weeks (10 mg/kg;) and results expressed as percent change in body weight relative to pre-dose weight.

[00111] Figure 27 illustrates that positive modulator and partial agonist anti-INSR antibodies improve glycemic control in MLDS/HFD mice. A glucose tolerance test (GTT) was carried out in 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab083, Ab085, Ab037 or isotype control for 3 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control). A. Glucose tolerance test timecourse B. Glucose tolerance test; area under curve (AUC).

[00112] Figure 28 shows that positive modulator and partial agonist anti-INSR antibodies improve glycemic control in MLDS/HFD mice as determined by a weekly fasting blood glucose assessment of 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab083, Ab085, Ab037 or isotype control for 6 weeks (10 mg/kg; * $p < 0.05$ for Ab083 and Ab037 relative to isotype control).

[00113] Figure 29 illustrates that positive modulator and partial agonist anti-INSR antibodies improve dyslipidemia in MLDS/HFD mice. Analysis of plasma from of 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab083, Ab085, Ab037 or isotype control for 6 weeks (10 mg/kg; * $p < 0.05$ relative to isotype control) was carried out. A. Plasma triglyceride levels; B. Plasma free fatty acid levels; C. Plasma unesterified cholesterol levels; D. Plasma total cholesterol levels; E. Plasma non-HDL cholesterol levels; F. Plasma non-HDL/HDL cholesterol ratio.

[00114] Figure 30 shows that positive modulator and partial agonist anti-INSR antibodies improve glycemic control (HbA1c) in MLDS/HFD mice as determined by a blood HbA1c evaluation in 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab083, Ab085, Ab037 or isotype control for 6 weeks (10 mg/kg; * $p < 0.05$ for Ab083 and Ab037 relative to isotype control).

[00115] Figure 31 shows that positive modulator and partial agonist anti-INSR antibodies generally do not affect body weight in MLDS/HFD mice as determined using body weight measurements in 10-week old MLDS/HFD mice injected intraperitoneally (IP) with Ab001, Ab083, Ab085, Ab037 or isotype control for 6 weeks (10 mg/kg).

[00116] Figure 32 shows that positive modulator and partial agonist anti-INSR antibodies augment insulin signaling in vivo. Ten week-old C56BL/6 male mice were injected with Ab083, Ab085, Ab037 or isotype control (10 mg/kg) for 24 hours, and effects on liver (A) and muscle (B) INSR tyrosine phosphorylation were determined by ELISA after an insulin bolus.

[00117] Figure 33 is a table showing the binding characteristics of INSR-specific antibodies reformatted with an IgG2 constant region.

[00118] Figure 34 illustrates the dose response from a partial allosteric agonist anti-INSR antibody in comparison to the dose response to the endogenous ligand (A) or activation by ligand in the presence or absence of the allosteric agonist antibody (B).

[00119] Figure 35 shows the dose response from a positive modulator antibody in comparison to the dose response to the endogenous ligand (A) or the dose response of an endogenous ligand in the presence and absence of a positive modulator antibody (B).

[00120] Figure 36 illustrates the activation parameters for a set of partial allosteric agonists alone relative to the endogenous ligand insulin. Data obtained from measurements of percent Akt phosphorylation at Ser473.

[00121] Figure 37 illustrates the activation properties of insulin in the presence of 10 ug/ml partial allosteric agonist antibodies relative to the maximal response to the endogenous ligand in the presence of a negative control antibody. Data obtained from measurements of percent Akt phosphorylation at Ser473.

[00122] Figures 38-40 depict pAkt activation by antibodies in the absence of insulin or in the presence of a sub-maximal concentration of insulin for parental CHO-K1 cells, CHO-K1

cells expressing human insulin receptor and CHO-K1 cells expressing mouse insulin receptor. Figures 38A-C show the effects of sensitizer Abs (Ab077, Ab078, Ab085) with little or no agonism of pAkt activity (<10% pAkt activation in the absence of insulin with 50 ug/ml antibody). Figures 39A-C show the effects of sensitizer Abs (Ab001, Ab079, Ab083) with weak to moderate agonism of pAkt activity (10-20% pAkt activation in the absence of insulin with 50 ug/ml antibody). Figure 40 illustrates the effects of a sensitizer Ab (Ab080) with moderate agonism of pAkt activity (>20% pAkt activation in the absence of insulin with 50 ug/ml antibody).

[00123] Figure 41 shows insulin dependent pAkt activation in CHO cells expressing the human (A and C) or mouse INSR (B) in the presence of fixed concentrations of sensitizing anti-INSR antibodies.

[00124] Figure 42 demonstrates pAkt activation in CHO cells expressing the human (A) or mouse INSR (B) by partial allosteric agonist antibodies in the absence of insulin compared to insulin-alone.

[00125] Figure 43 depicts the results of insulin dependent pAkt activation in CHO cells expressing the human INSR in the presence of fixed concentrations of partial allosteric agonist antibodies.

[00126] Figure 44 shows pAKT assay results for antibody 83-7 and Ab001 on CHOK1 cells expressing: (A) human INSR, or; (B) mouse INSR.

[00127] Figure 45 shows free insulin percentage plotted against estimated insulin receptor concentration. The insulin level was fixed at 50pM and the antibody concentration was 10ug/mL (67nM) for all clones except Ab078 which was tested at 25ug/mL (167nM). Curves shown are the non-linear regression Prism fit used to calculate EC50.

[00128] Figure 46 shows free insulin percentage plotted against estimated insulin receptor concentration. The insulin level was fixed at 50pM and the antibody concentration was 10ug/mL (67nM) for all clones. Curves shown are the non-linear regression Prism fit used to calculate EC50.

[00129] Figure 47 shows that TNFα-induces desensitization of insulin-mediated fatty acid uptake in 3T3-L1 adipocytes in the presence of anti-INSR antibody Ab085.

[00130] Figures 48 and Figure 49 illustrate the effects of purified positive modulator anti-INSR antibodies Ab001, Ab037, Ab077, Ab079, AB080, Ab083 on human INSR (Figure 48) and mouse INSR (Figure 49).as measured in the pAKT assay.

[00131] Figures 50 and 51 demonstrates the relative % pAKT of purified agonist antibodies Ab037, Ab030, Ab053 and Ab062 on human INSR (Figure 50) and mouse INSR (Figure 51).

[00132] Figure 52 demonstrates that the purified anti-INSR antibodies Ab030, Ab037, Ab053, Ab001, Ab079, AB080 and Ab083 are capable of inducing AKT phosphorylation (relative % pAKT) after activation of monkey INSR.

[00133] Figure 53 shows the relative % pAKT of negative modulator antibodies Ab061, Ab070 and Ab081 measured in CHOK1 cells expressing human INSR.

[00134] Figure 54 is a table showing cross-reactivity of insulin receptor antibodies, and illustrates that certain antibodies that bind to the human insulin receptor also bind to the rabbit and the cynomolgous insulin receptor and that this binding was modulated by the presence of insulin.

DETAILED DESCRIPTION

[00135] The invention provides antibodies specific for the insulin receptor (INSR) or the insulin receptor-insulin complex and uses thereof in the treatment of disorders related to aberrant glucose levels, e.g. hyperglycemia or hypoglycemia, aberrant insulin levels or aberrant insulin sensitivity, e.g. disorders of insulin resistance or disorders of insulin sensitivity. These antibodies can induce either a positive or negative effect on the cellular response in the INSR by altering the kinetic rate constants for assembly and dissociation of INSR-INS signaling complex components or by other mechanisms including altering the structural state of the signaling complex, e.g., by binding to a transition state and accelerating the activation of signaling.

[00136] Modulation of a signaling complex can result in an increase or decrease in sensitivity to signal input and concomitant increases or decreases in signal transduction. Administration of modulator antibodies increases or decreases the sensitivity of the cellular pathway and/or absolute levels of the cellular response. The modulators of the invention,

depending on their properties, can function as a modulator, potentiator, regulator, effector or sensitizer.

[00137] Many antibody drugs act to block signaling pathways by binding to either a cell-surface receptor or its cognate ligand and eliminating the ability of the ligand to bind to and activate the receptor. Such blocking drugs mediate their effect stoichiometrically by preventing the formation of receptor-ligand complex.

[00138] Successful treatment of some diseases may require attenuation rather than complete inhibition of signaling pathways to restore a normal physiological state with acceptable side-effect profiles. The antibodies provided by the invention are expected to provide such advantages.

[00139] Other therapeutic drugs affect cellular signaling pathways by binding to a cell-surface receptor and altering the activity of the receptor. Such direct agonist drugs may mediate their effects by mimicking the natural activity of the ligand and thus have inherent activity, i.e., they do not require the presence of ligand to mediate their effects. Further therapeutic drugs affect cellular signaling pathways by binding to a ligand. Such indirect agonist drugs may mediate their effects by altering ligand stability or valency.

[00140] Biological processes are generally regulated in a continuous rather than binary manner, and thus in many cases modulation of pathway activity may be a more appropriate therapeutic strategy than complete pathway blockade or stimulation. Performing functional, cell-based screens for modulation of pathway activity, rather than for complete pathway blockade or stimulation, is laborious and may not readily be performed in a high throughput manner, since such screens generally require a known concentration of test compound and may be sensitive to any impurities in the test compound preparation. In particular, the ability to perform high throughput functional, cell-based screens for modulation of pathway activity is restricted for cell-impermeable molecules which are unable to enter the intracellular environment, and especially for recombinant biological molecules which may have different expression levels, degrees of purity and stability in the production system used. In addition, some binding interactions may have no signaling output to measure in a functional screen (e.g. in the case of decoy receptors, decoy substrates, or inactive forms of a target) making it difficult to identify agents that perturb these interactions.

[00141] The present invention overcomes these disadvantages and provides a means for identifying positive and negative modulators of the INSR activity and desired potency of

drug in a high throughput manner. The present invention also provides positive and negative modulators of the INSR activity with a desired range of modulation of activity, and provides data showing that these modulators exhibit the desired biological effect of altering glucose uptake.

Definitions

[00142] The term "compound" refers to any chemical compound, organic or inorganic, endogenous or exogenous, including, without limitation, polypeptides, proteins, peptides, small molecules, nucleic acids (e.g. DNA and RNA), carbohydrates, lipids, fatty acids, steroids, purines, pyrimidines, peptidomimetics, polyketides and derivatives, structural analogs or combinations thereof. "Endogenous" means naturally occurring in a mammal, while "exogenous" means not naturally occurring in the mammal, e.g. an administered foreign compound.

[00143] The term "polypeptide binding agent" refers to a polypeptide that is capable of specifically binding an antigen, e.g. a target or its signaling partner, or that is capable of binding an antigen with a measurable binding affinity. Examples of polypeptide binding agents include antibodies, peptibodies, polypeptides and peptides, optionally conjugated to other peptide moieties or non-peptidic moieties. Antigens to which a polypeptide binding agent may bind include any proteinaceous or non-proteinaceous molecule that is capable of eliciting an antibody response, or that is capable of binding to a polypeptide binding agent with detectable binding affinity greater than non-specific binding. The antigen to which a modulating polypeptide binding agent binds may include a target, a signaling partner of a target, and/or a complex comprising the target and its signaling partner.

[00144] The term "antibody" is used in the broadest sense and includes fully assembled antibodies, tetrameric antibodies, monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), antibody fragments that can bind an antigen (e.g., Fab', F'(ab)2, Fv, single chain antibodies, diabodies), and recombinant peptides comprising the forgoing as long as they exhibit the desired biological activity. An "immunoglobulin" or "tetrameric antibody" is a tetrameric glycoprotein that consists of two heavy chains and two light chains, each comprising a variable region and a constant region. Antigen-binding portions may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. Antibody fragments or antigen-binding portions include, inter alia, Fab, Fab', F(ab')2, Fv, domain antibody (dAb), complementarity determining region (CDR) fragments, CDR-grafted antibodies, single-chain antibodies (scFv), single chain

antibody fragments, chimeric antibodies, diabodies, triabodies, tetrabodies, minibody, linear antibody; chelating recombinant antibody, a tribody or bibody, an intrabody, a nanobody, a small modular immunopharmaceutical (SMIP), a antigen-binding-domain immunoglobulin fusion protein, a camelized antibody, a VHH containing antibody, or a variant or a derivative thereof, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide, such as one, two, three, four, five or six CDR sequences, as long as the antibody retains the desired biological activity.

[00145] "Monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts.

[00146] "Antibody variant" as used herein refers to an antibody polypeptide sequence that contains at least one amino acid substitution, deletion, or insertion in the variable region of the natural antibody variable region domains. Variants may be substantially homologous or substantially identical to the unmodified antibody.

[00147] A "chimeric antibody," as used herein, refers to an antibody containing sequence derived from two different antibodies (see, e.g., U.S. Patent No. 4,816,567) which typically originate from different species. Most typically, chimeric antibodies comprise human and rodent antibody fragments, generally human constant and mouse variable regions.

[00148] A "neutralizing antibody" is an antibody molecule which is able to eliminate or significantly reduce a biological function of an antigen to which it binds. Accordingly, a "neutralizing" antibody is capable of eliminating or significantly reducing a biological function, such as enzyme activity, ligand binding, or intracellular signaling.

[00149] An "isolated" antibody is one that has been identified and separated and recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver

stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

[00150] "Heavy chain variable region" as used herein refers to the region of the antibody molecule comprising at least one complementarity determining region (CDR) of said antibody heavy chain variable domain. The heavy chain variable region may contain one, two, or three CDR of said antibody heavy chain.

[00151] "Light chain variable region" as used herein refers to the region of an antibody molecule, comprising at least one complementarity determining region (CDR) of said antibody light chain variable domain. The light chain variable region may contain one, two, or three CDR of said antibody light chain, which may be either a kappa or lambda light chain depending on the antibody.

[00152] As used herein, an antibody that "specifically binds" is "antigen specific", is "specific for" antigen target or is "immunoreactive" with an antigen refers to an antibody or polypeptide binding agent of the invention that binds an antigen with greater affinity than other antigens of similar sequence. In one aspect, the polypeptide binding agents of the invention, or fragments, variants, or derivatives thereof, will bind with a greater affinity to human antigen as compared to its binding affinity to similar antigens of other, i.e., non-human, species, but polypeptide binding agents that recognize and bind orthologs of the target are within the scope of the invention.

[00153] For example, a polypeptide binding agent that is an antibody or fragment thereof "specific for" its cognate antigen indicates that the variable regions of the antibodies recognize and bind the desired antigen with a detectable preference (e.g., where the desired antigen is a polypeptide, the variable regions of the antibodies are able to distinguish the antigen polypeptide from other known polypeptides of the same family, by virtue of measurable differences in binding affinity, despite the possible existence of localized sequence identity, homology, or similarity between family members). It will be understood that specific antibodies may also interact with other proteins (for example, S. aureus protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and in particular, in the constant region of the molecule. Screening assays to determine binding specificity of a polypeptide binding agent, e.g. antibody, for use in the methods of the invention are well known and routinely practiced in

the art. For a comprehensive discussion of such assays, see Harlow et al. (Eds), Antibodies A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor, NY (1988), Chapter 6. Antibodies for use in the invention can be produced using any method known in the art.

[00154] The term "epitope" refers to that portion of any molecule capable of being recognized by and bound by a selective binding agent at one or more of the antigen binding regions. Epitopes usually consist of chemically active surface groupings of molecules, such as, amino acids or carbohydrate side chains, and have specific three-dimensional structural characteristics as well as specific charge characteristics. Epitopes as used herein may be contiguous or non-contiguous.

[00155] The term "derivative" when used in connection with polypeptide binding agents and polypeptides of the invention refers to polypeptides chemically modified by such techniques as ubiquitination, conjugation to therapeutic or diagnostic agents, labeling (e.g., with radionuclides or various enzymes), covalent polymer attachment such as pegylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins. Derivatives retain the binding properties of underivatized molecules of the invention.

[00156] "Detectable moiety" or a "label" refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include 32P, 35S, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin-streptavadin, dioxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to another labeled nucleic acid molecule. The detectable moiety often generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantitate the amount of bound detectable moiety in a sample.

[00157] "Peptides" or "oligopeptides" are short amino acid sequences, typically between 3 and 100 amino acid residues in length and encompass naturally occurring amino acid residues and non-naturally occurring analogs of residues which may be used singly or in combination with naturally occurring amino acid residues in order to give the peptide a particular conformational specificity or a particular biological activity, such as resistance to proteolysis. Peptides include repeats of peptide sequences and may include 2, 3, 4, 5, 6, 7, 8, 9, 10 or more copies of an amino acid sequence arranged head-to-tail or head-to-head. Peptides may

be conjugated to non-peptidic moieties, e.g. [expand]. Peptides include dimers, trimers or higher order multimers, e.g. formed through conjugation to other polymeric or non-polymeric moieties, such as PEG.

[00158] "Polypeptides" are longer amino acid sequences, typically 100 or more amino acid residues in length, and encompass naturally occurring amino acid residues and non-naturally occurring analogs of residues which may be used singly or in combination with naturally occurring amino acid residues in order to give the polypeptide a particular conformational specificity or a particular biological activity, such as resistance to proteolysis.

[00159] As used herein, a "peptibody" is a fusion polypeptide comprising one or more peptides fused to all or a portion of an immunoglobulin (Ig) constant region. See, e.g., U.S. Pat. No. 6,660,843. The peptide may be any naturally occurring or recombinantly prepared or chemically synthesized peptide that binds to the antigen. The peptide may be repeated and may include 2, 3, 4, 5, 6, 7, 8, 9, 10 or more copies of an amino acid sequence arranged head-to-tail or head-to-head. The portion of the Ig constant region may include at least one constant region domain (e.g., CH1, CH2, CH3, and/or CH4), multiple domains (e.g., CH2 with CH3), multiple copies of domains (e.g., CH2-CH2), any fragment of a constant domain that retains the desired activity, e.g. the salvage receptor epitope responsible for the prolonged half-life of immunoglobulins in circulation, or any combinations thereof.

[00160] A "small" molecule or "small" organic molecule is defined herein as a non-polymeric organic chemical compound having a molecular weight of about 1000 Daltons or less.

[00161] As used herein, a "signaling complex" is an assembly of proteins and/or endogenous or exogenous compounds that mediate the transduction of a cellular signal. Examples of a signaling complex include, but are not limited to, a ligand bound to a membrane bound receptor, an enzyme bound to a substrate or any cellular molecules that associate to propagate biochemical reactions that are involved in a signal cascade. Signaling complexes can also include coreceptors, cofactors, scaffold proteins, allosteric modulators and numerous other types of proteins and molecules that are involved in cellular signal transduction. Signaling complexes can be formed transiently or can be long lived. The molecular constituents or components of a signaling complex can vary over time and can be dependent on activation state of each component and the cellular environment. Signaling complexes can undergo chemical modification and regulation that can induce a spectrum of

effects on the complex including subtle changes in transduction activity, complete inactivation and constitutive activation or both positive and negative modulation.

[00162] The term "therapeutically effective amount" is used herein to indicate the amount of target-specific composition of the invention that is effective to ameliorate or lessen symptoms or signs of disease associated with abnormal (e.g. abnormally high or abnormally low) signaling of the signaling complex.

[00163] As used herein "binding" is the physical association between two or more distinct molecular entities that results from a specific network of non-covalent interactions consisting of one or more of the weak forces including hydrogen bonds, Van der Waals, ion-dipole and hydrophobic interactions and the strong force ionic bonds. The level or degree of binding may be measured in terms of affinity. Affinity is a measure of the strength of the binding interaction between two or more distinct molecular entities that can be defined by equilibrium binding constants or kinetic binding rate parameters. Examples of suitable constants or parameters and their measurement units are well known in the art and include but are not limited to equilibrium association constant (K_A), e.g. about 10^5M^{-1} or higher, about 10^6M^{-1} or higher, about 10^7M^{-1} or higher, about 10^8M^{-1} or higher, about 10^9M^{-1} or higher, about $10^{10}M^{-1}$ or higher, about $10^{11}M^{-1}$ or higher or about $10^{12}M^{-1}$ or higher; equilibrium dissociation constant (K_D), e.g., about $10^{-5}M$ or less, or about $10^{-6}M$ or less, or about $10^{-7}M$ or less, or about $10^{-8}M$ or less, or about $10^{-9}M$ or less, or about $10^{-10}M$ or less, or about $10^{-11}M$ or less, or about $10^{-12}M$ or less; on-rate (e.g., sec^{-1}, mol^{-1}) and off-rate (e.g., sec-^{1})). In the case of K_A, higher values mean "stronger" or "strengthened" binding affinity while in the case of K_D, lower values mean "stronger" or "strengthened" binding affinity. As used herein, a "strengthened" binding rate parameter means increased residency time, stronger association or weaker dissociation. As used herein, a "weakened" binding rate parameter means decreased residency time, weaker association or stronger dissociation. In the case of on-rate, higher values mean faster or more frequent association and thus generally result in strengthened binding affinity. In the case of off-rate, lower values generally mean slower dissociation and thus generally result in stronger binding affinity. However, it is the ratio of the on-rate and off-rate that indicates binding affinity, as explained in further detail later.

[00164] Affinity between two compounds, e.g., between an antibody and an antigen, or between first and second components of a signaling complex, may be measured directly or indirectly. Indirect measurement of affinity may be performed using surrogate properties that are indicative of, and/or proportional to, affinity. Such surrogate properties include: the

quantity or level of binding of a first component to a second component of a signaling complex, or a biophysical characteristic of the first component or the second component that is predictive of or correlated to the apparent binding affinity of the first component for the second component. Specific examples include measuring the quantity or level of binding of first component to a second component at a subsaturating concentration of either the first or the second component. Other biophysical characteristics that can be measured include, but are not limited to, the net molecular charge, rotational activity, diffusion rate, melting temperature, electrostatic steering, or conformation of one or both of the first and second components. Yet other biophysical characteristics that can be measured include determining stability of a binding interaction to the impact of varying temperature, pH, or ionic strength.

[00165] Measured affinity is dependent on the exact conditions used to make the measurement including, among many other factors, concentration of binding components, assay setup, valence of binding components, buffer composition, pH, ionic strength and temperature as well as additional components added to the binding reaction such as allosteric modulators and regulators. Quantitative and qualitative methods may be used to measure both the absolute and relative strength of binding interactions.

[00166] Apparent affinity is a measure of the strength of the binding interaction between two or more distinct molecular entities under conditions where the affinity is altered by conditions or components in the binding reaction such as allosteric modulators, inhibitors, binding component valence etc.

[00167] As used herein a "subsaturating concentration" is a concentration of one or more components in a binding reaction that is significantly below the binding affinity K_D and/or a concentration of one component in a binding reaction that is less than is required to occupy all of the binding sites of the other component(s). Under subsaturating conditions a significant percentage of one of the binding components in the binding reaction has available binding sites.

[00168] As used herein a "biophysical assay" is any method that measures, in an absolute or relative fashion, the binding, association, dissociation, binding affinity, binding level, or binding rate parameters between at least two compounds. Biophysical assays are generally performed in vitro and may be conducted with purified binding components, unpurified components, cell associated components as well as a combination of purified and cell associated components.

[00169] An "agonist" is a term used to describe a type of ligand or drug that binds and activates signaling of a receptor. The ability to alter the activity of a receptor, also known as the agonist's efficacy, is a property that distinguishes it from antagonists, a type of receptor ligand which also binds a receptor but which does not activate signaling of the receptor. The efficacy of an agonist may be positive, causing an increase in the receptor's activity, or negative causing a decrease in the receptor's activity. Full agonists bind and activate a receptor, displaying full efficacy at that receptor. Partial agonists also bind and activate a given receptor, but have only partial efficacy at the receptor relative to a full agonist. An inverse agonist is an agent which binds to the same receptor binding-site as an agonist for that receptor and reverses constitutive activity of receptors. Inverse agonists exert the opposite pharmacological effect of a receptor agonist. A co-agonist works with other co-agonists to produce the desired effect together.

[00170] Competitive, or orthosteric, agonists reversibly bind to receptors at the same binding site (active site) as the ligand, thereby competing with ligand for the same binding site on the receptor.

[00171] In a different aspect, antibodies disclosed herein act as allosteric agonists. They bind to a portion of INSR that is distinct from the active insulin-binding site, and do not appreciably change the binding affinity of insulin and INSR by more than 2-fold. They also do not appreciably affect the EC50 of insulin activation of INSR, e.g. alter EC50 by less than 2-fold. Such antibodies constitutively activate INSR with a maximal agonist response that is 80% or less of the maximal agonist response of insulin, for example 15%-80%, 20-60%, 20%-40% or 15%-30%. In certain embodiments, the antibodies constitutively activate INSR with a maximal agonist response that is at least about 15%, 20%, 25%, 30%, 35%, 40%; and up to 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% of the maximal agonist response of insulin. It is understood that any combination of any of these range endpoints is contemplated without having to recite each possible combination. In some embodiments, maximal agonist response is measured by Akt assay. In further embodiments, the invention provides an allosteric agonist antibody that binds to insulin receptor with an affinity of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} , 10^{-10} , 10^{-11} M or less M. Without being bound by a theory of the invention, the weak agonist activity of allosteric agonists serves to mimic the effect of natural basal insulin secretion levels, while permitting exogenously administered insulin to have its normal glucose-lowering effect. In certain embodiments, an allosteric agonist is a partial allosteric agonist. An antagonist blocks a receptor from activation by agonists. A selective

agonist is selective for one certain type of receptor. It can additionally be of any of the aforementioned types.

[00172] The potency of an agonist is usually defined by the inverse of its EC50 value. This can be calculated for a given agonist by determining the concentration of agonist needed to elicit half of the maximum biological response of the agonist. The lower the EC50, the greater the potency of the agonist.

[00173] A receptor "antagonist" is a type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses. Antagonists may have affinity but no efficacy for their cognate receptors, and binding will disrupt the interaction and inhibit the function of an agonist or inverse agonist at receptors. Antagonists mediate their effects by binding to the active site or to allosteric sites on receptors, or they may interact at unique binding sites not normally involved in the biological regulation of the receptor's activity. Antagonist activity may be reversible or irreversible depending on the longevity of the antagonist–receptor complex, which, in turn, depends on the nature of antagonist receptor binding. The majority of antagonists achieve their potency by competing with endogenous ligands or substrates at structurally-defined binding sites on receptors.

[00174] Antagonists display no efficacy to activate the receptors they bind. Once bound, however, antagonists may inhibit the function of agonists, inverse agonists and partial agonists. In functional antagonist assays, a dose-response curve measures the effect of the ability of a range of concentrations of antagonists to reverse the activity of an agonist. The potency of an antagonist is usually defined by its IC50 value. This can be calculated for a given antagonist by determining the concentration of antagonist needed to elicit half inhibition of the maximum biological response of an agonist. The lower the IC50, the greater the potency of the antagonist.

[00175] Competitive, or orthosteric, antagonists reversibly bind to receptors at the same binding site (active site) as the ligand or agonist, but without activating the receptor, thereby competing with agonist for the same binding site on the receptor. Non-competitive, or allosteric, antagonists bind to a separate binding site from the agonist, exerting their action to that receptor via that separate binding site. Thus, they do not compete with agonists for binding. Uncompetitive antagonists differ from non-competitive antagonists in that they

require receptor activation by an agonist before they can bind to a separate allosteric binding site.

[00176] "Insulin resistance" describes a condition in which physiological amounts of insulin are inadequate to produce a normal insulin response from cells or tissues.

[00177] "Insulin sensitizer" is a compound or drug that increases cell- or tissue- sensitivity to insulin resulting in greater levels of glucose uptake for a given subsaturating concentration of insulin.

Advantages

[00178] The present invention relates to the discovery that it is possible to develop therapeutic agents that modulate the insulin-INSR signaling complex by binding to extracellular regions of the INSR. Novel selection and screening methods are employed to identify, for example, insulin-sensitizers that target extracellular regions of the INSR and potentiate insulin action. In particular, some of the antibodies identified herein are non-agonistic antibodies which bind to extracellular regions of the INSR and positively or negatively modulate the insulin-INSR signaling complex.

[00179] The present invention encompasses insulin-INSR signaling complex modulators that offer unique advantages over existing therapies. They act at the level of the INSR, which should allow induction of the entire range of actions of insulin while minimizing unwanted side effects. Avoiding INSR agonism should reduce the risk of functional hypoglycemia. Additionally, more precise control of glucose levels might be achieved. Thus, when blood glucose levels increase, leading to elevation of insulin levels, such a modulator would have a greater effect. Targeting the extracellular region of the INSR allows for the use of biological molecules as insulin-INSR signaling complex modulators that modulate the effect of endogenous or exogenous insulin, insulin analogs or insulin mimetics; these may have advantages such as improved half-life, reduced dosage or frequency of dosage, reduced toxicity and greater ease of manufacture. The present invention encompasses insulin-INSR signaling complex modulators that are expected to reduce peripheral insulin resistance and improve glycemic control. The sensitizing effect of the modulators should allow for improved levels of glucose uptake by peripheral tissues in patients whose insulin levels are not high enough to stimulate adequate glucose uptake in the absence of exogenous insulin therapy. Thus, administration of the antibodies of the invention may be used in the early stages of insulin resistance in place of other drugs, or as adjunct therapy to other anti-diabetic

agents. When administered as an adjunct therapy to other anti-diabetic agents, the antibodies of the invention may reduce the total daily amount of anti-diabetic agent required to maintain blood glucose levels closer to normal range, or may reduce the frequency of dosing of the anti-diabetic agent, or may achieve improved glycemic control with the same dose and/or frequency, e.g. with fewer episodes of hyperglycemia or a reduced level of maximal hyperglycemia (reduction in the highest aberrant glucose level observed).

The Insulin Receptor (INSR)

[00180] The INSR is a tyrosine kinase receptor found in organisms as primitive as cnidarians and insects. In higher organisms it is essential for glucose homeostasis. Mouse knockout studies have also shown the INSR to be important in adipogenesis, neovascularization, the regulation of hepatic glucose synthesis and glucose-induced pancreatic insulin secretion (Kitamura et al, Ann. Rev. Physiol., 65: 313-332 2003). INSR signaling is also important in the brain, where it is involved in the regulation of food intake, peripheral fat deposition and the reproductive endocrine axis as well as in learning and memory (Wada et al, J. Pharmacol. Sci. 99: 128-143, 2005). Dysfunctional INSR signaling has been implicated in diseases including type I and type II diabetes, dementia and cancer.

[00181] The domains of the closely related insulin-like growth factor receptor (IGFR-1) exhibit high (47-67%) amino acid identity with the INSR. While similar in structure, IGF-IR and INSR serve different physiological functions. IGF- IR is expressed in almost all normal adult tissue except for liver, which is itself the major site of IGF-I production. INSR is primarily involved in metabolic functions whereas IGF-IR mediates growth and differentiation (Adams et al, Cell. Mol. Life Sci. 57: 1050-1093, 2000).

[00182] INSR exists as two splice variant isoforms, INSR-A and INSR-B, which respectively lack or contain the 12 amino acids coded by exon 11. The longer variant, INSR-B, is the isoform responsible for signaling metabolic responses. In contrast, INSR-A signals predominantly mitogenic responses, is the preferentially expressed isoform in several cancers (Denley et al., Horm. Metab. Res. 35: 778-785, 2003) and is capable of binding insulin-like growth factor 2 (IGF-II) with high affinity (Denley et al, Mol. Endocrinol. 18: 2502-2512, 2004).

[00183] The mature human INSR is a homodimer comprising two α subunits and two β subunits (chains). The α and β chains are encoded by a single gene and arise from the post-translational cleavage of a 1370 amino acid precursor at a furin cleavage site located at

residues 720-723. The α-chain and 194 residues of the β-chain comprise the extracellular protion of the INSR. There is a single transmembrane sequence and a 403 residue cytoplasmic domain containing a tyrosine kinase. The N-terminal half of each ectodomain monomer consists of two homologous leucine-rich repeat domains (L1 and L2) of approximately 150 amino acids, separated by a cysteine-rich region (CR), also approximately 150 amino acids in size. The C-terminal half of each ectodomain monomer (approximately 460 residues) consists of three fibronectin type III domains (FnIII-1, FnIII-2 and FnIII-3). The FnIII-2 domain contains an insert domain (ID) of approximately 120 residues, within which lies the furin cleavage site that generates the α and β chains of the mature receptor . Intracellularly, each monomer contains a tyrosine kinase catalytic domain flanked by two regulatory regions (the juxtmembrane region and the C-tail) that contain the phosphotyrosine binding sites for signaling molecules (Ward et al, Acta Physiol. 192: 3-9, 2008).

[00184] Current models for insulin binding proposes that, in the basal state, the INSR homodimer contains two identical pairs of binding sites (referred to as Site 1 and Site 2) on each monomer (De Meyts, Bioessays, 26: 1351-1362, 2004). Binding of insulin to a low affinity site (Site 1) on one α-subunit is followed by a second binding event between the bound insulin and a different region of the second INSR α-subunit (Site 2). This ligand-mediated bridging between the two α subunits generates the high affinity state that results in signal transduction. In contrast, soluble INSR ectodomain, which is not tethered at its C-terminus, cannot generate the high affinity receptor-ligand complex. It can bind two molecules of insulin simultaneously at its two Site 1's, but only with low affinity (Adams et al, Cell. Mol. Life Sci. 57: 1050-1093, 2000). Site 1 is thought to be comprised of elements from the central β-sheet of the L1 domain and the last 16 residues of the α-chain (referred to as the CT peptide). Site 2 most likely includes the loops at the junction of FnIII-1 and FnIII-2. Insulin binding is thought to involve structural changes in both insulin and its receptor (Ward and Lawrence, BioEssays 31: 422-434, 2009).

[00185] Once an insulin molecule has docked onto the receptor and effected its action, it may be released back into the extracellular environment or it may be degraded by the cell. Degradation normally involves endocytosis of the insulin-INSR complex followed by the action of insulin degrading enzyme. Most insulin molecules are degraded by liver cells. It has been estimated that a typical insulin molecule is finally degraded about 71 minutes after its initial release into circulation (Duckworth et al, Endocr. Rev. 19(5): 608–24, 1998).

Insulin signaling

[00186] Insulin induces a signaling network of molecules, carrying the information from the INSR to the effector proteins involved in metabolism and growth. Insulin binding to INSR induces a conformational change that promotes activation of an intrinsic tyrosine kinase activity, leading to autophosphorylation of the INSR β-subunit. Insulin receptor substrate (IRS) proteins are recruited to the plasma membrane through an interaction with the phosphorylated INSR, and these also become phosphorylated on tyrosine residiues, promoting recruitment of additional signaling proteins to the complex resulting in signaling through two major pathways (1) the PI3 kinase/PDK1/PKB pathway which primarily regulates metabolism, with some influence on growth ad (2) the Ras/ERK mitogenic pathway which primarily regulates cell growth.

[00187] Certain marketed insulin analogues have been reported to display IGF-1-like mitogenic and anti-apoptotic activities in cultured cancer cells, raising questions over their long-term safety in humans (Weinstein et al, Diabetes Metab Res Rev 25: 41-49, 2009). Therefore, it would be desirable to obtain an INSR agonist that did not alter the balance in metabolic vs. mitogenic INSR signaling, or promoted metabolic signaling preferentially over mitogenic INSR signaling.

Methods of identifying antibodies that are modulators

[00188] The invention provides methods of identifying a candidate polypeptide binding agent, e.g., an antibody, that modulates binding between first and second components of a signaling complex, e.g., a receptor such as the insulin receptor and its ligand insulin.

[00189] Without being bound by a theory of the invention, the present disclosure provides that kinetic perturbation of an interaction between two components (first component, C1 and second component, C2) of a signaling complex with a modulator (M) can be described mathematically as:

$$K'_{C1C2} = K_{C1C2} \quad \frac{(1 + M/K_{MC1})\,(1 + M/K_{MC2})}{(1 + M/K_{[C1C2]M})}$$

where the change in binding equilibrium constant between the components (K'_{C1C2}) is a function of equilibrium constant between the components (K_{C1C2}), modulator concentration (M), modulator affinity for the complex ($K_{[C1C2]M}$) and modulator affinity for either the first component (K_{MC1}) or the second component (K_{MC2}).

[00190] In cases where the signaling complex is a receptor-ligand complex, and the modulator is an antibody, the kinetic perturbation of the receptor-ligand interaction with an antibody can be described mathematically as:

$$K'_{RL} = K_{RL}\frac{\left(1+\frac{A}{K_{AR}}\right)\left(1+\frac{A}{K_{AL}}\right)}{\left(1+\frac{A}{K_{RLA}}\right)}$$

where the change in receptor-ligand binding equilibrium constant (K'_{RL}) is a function of receptor-ligand equilibrium constant (K_{RL}), antibody concentration (A), antibody affinity for the complex ($K_{[RL]A}$) and antibody affinity for either the receptor (K_{AR}) or ligand (K_{AL}).

[00191] A modulator binds the target, or its signaling partner, or a complex of the target and signaling partner, in such a manner that the binding affinity or binding rate parameter of the target for its signaling partner is weakened or strengthened. For example, where the target is either a receptor or ligand, the binding affinity or binding rate parameter of the ligand for its receptor is weakened or strengthened in the presence of the modulator. A modulator with complete blocking activity represents a boundary condition in this analysis, since when $K_{[C1C2]M}$ is sufficiently high, K'_{C1C2} approaches infinity. One implication of this model is that the degree of signaling modulation is independent of modulator concentration when the concentration of modulator ([M]) is sufficiently above the equilibrium dissociation constant (K_D) for the modulator/antigen interaction to be saturating for binding ligand. Hence, modulation of the interaction is related to the ratio of affinities for the complex versus the components where [M] > K_D for the modulator and its antigen.

[00192] The present disclosure provides that the biophysical properties of a modulator's interactions with a target and/or its signaling partner can be used to predict the functional effect of the modulator on the target signaling pathway. Modulators which alter the signaling pathway can therefore be identified based on their relative affinity for target (and/or its signaling partner) in complexed versus uncomplexed form. The invention contemplates that kinetic perturbation of an interaction between two components (first component, C1 and second component, C2) of a signaling complex with a modulator (M) can be predicted in the following manner:

$K_{[C1C2]M}$ or $K_{[MC2]C1}$ or $K_{[MC1]C2} < K_{MC2}$ or K_{MC1} leads to positive modulation

$K_{[C1C2]M}$ or $K_{[MC2]C1}$ or $K_{[MC1]C2} = K_{MC2}$ or K_{MC1} leads to no modulation

$K_{[C1C2]M}$ or $K_{[MC2]C1}$ or $K_{[MC1]C2} > K_{MC2}$ or K_{MC1} leads to negative modulation

[00193] In cases where the signaling complex is a receptor (R)-ligand(L) complex, and the kinetic modulator is an antibody (A), the kinetic perturbation can be predicted in the following manner:

$K_{[RL]A}$ or $K_{[AL]R}$ or $K_{[AR]L} < K_{AL}$ or K_{AR} leads to positive kinetic modulation

$K_{[RL]A}$ or $K_{[AL]R}$ or $K_{[AR]L} = K_{AL}$ or K_{AR} leads to no kinetic modulation

$K_{[RL]A}$ or $K_{[AL]R}$ or $K_{[AR]L} > K_{AL}$ or K_{AR} leads to negative kinetic modulation

[00194] In some embodiments, a modulator, such as an antibody (A) can be identified by its ability to alter a binding interaction, such as a receptor(R)-ligand(L) interaction at any given sub-saturating concentration of the first or second component (e.g. ligand (L) concentration). A modulator antibody or polypeptide could effectively shift the affinity and the corresponding dose response of the receptor ligand interaction from the 500pM interaction to either the 10pM (positive modulator) or 10nM (negative modulator) as depicted. In some embodiments the modulator will produce a higher level of R-L binding at a given ligand concentration, shifting the assay curve to the left (positive modulation). In other embodiments the modulator will produce a lower level of R-L binding at a given ligand concentration, shifting the assay curve to the right (negative modulation). In some embodiments the shift is uniform. In other embodiments the shift is non-uniform, reflecting the involvement of other factors e.g. accessory proteins in the complex, receptor internalization, etc.

[00195] The binding properties of the interaction(s) between the modulator and the target, its signaling partner and/or a complex comprising the target and its signaling partner, are generally predictive of the functional effect of the kinetic modulator on the target signaling pathway. Depending on the target being studied, certain other factors may need to be considered. These include: (1) the concentration of the kinetic modulator, the concentration of the target, and/or the concentration of its signaling partner (e.g., the prediction is optimized if the modulator concentration ([M]) is significantly greater than the K_D of the binding between modulator and its antigen), (2) the structural form of the modulator used e.g. monovalent vs. divalent or bivalent, (3) inter/intra target crosslinking, which may restrict the conformation of target and/or cause target activation, (4) the modulator's ability to alter assembly or docking, or to alter additional components of the signaling complex by steric or allosteric mechanisms, (5) signaling pathway specific properties such as alterations in the

signal pathway due to disease that introduce a "bottleneck," (6) negative/positive feedback regulation of the signaling pathway, (7) alteration of clearance/internalization rates of the components of the signaling complex, (8) alterations in the target that uncouple or differentially alter ligand binding and activation e.g. a modulator enhances ligand binding but traps its receptor in a desensitized state, or a modulator attenuates ligand binding but induces a conformational change in its receptor that is activating.

[00196] In some aspects the invention provides methods for measuring the differential binding of a first component of a signaling complex for a second component of the signaling complex in the presence or absence of a test polypeptide agent. In these aspects, differential binding is preferably observed when there are sub-saturating concentrations of the first or second component. In some preferred embodiments the concentration of the first or second component may be reduced to provide sub-saturating conditions.

[00197] In some aspects the invention provides methods for measuring the differential binding of a test polypeptide binding agent, e.g. antibody, to target and/or its signaling partner, in complexed and uncomplexed form. In these aspects, differential binding is preferably observed when there are sub-saturating concentrations of test polypeptide binding agent. In some preferred embodiments the concentration of test polypeptide binding agent may be reduced to provide sub-saturating conditions.

[00198] In some embodiments, testing in the absence of a test polypeptide agent is performed using a control compound which is preferably a compound belonging to a similar structural class as the test polypeptide agent, but which binds to a different antigen that has no effect on the signaling complex being tested. For example, a control for a test antibody may be an isotype-matched antibody binding to an unrelated antigen, e.g. keyhole limpet hemocyanin (KLH).

[00199] For positive modulators, at a given sub-saturating concentration of C1, higher C1 affinity will be reflected in a higher signal for C1 binding to C2 in the presence of the positive modulator. Preferential binding of the modulator will be reflected in a higher signal for the complex comprising C1 and C2, compared to the signal for either C1 alone or C2 alone. In some aspects, there may be binding of the modulator to the complex of C1 and C2, but no measurable binding to either C1 alone or C2 alone.

[00200] For negative modulators, at a given sub-saturating concentration of C1, lower C1 affinity will be reflected in a lower signal for C1 binding to C2 in the presence of the

modulator. Preferential binding of the modulator will be reflected in a higher signal for binding of the modulator to C1 alone, or to C2 alone, compared to the signal for binding of the modulator to the complex of C1 and C2.

[00201] The invention provides methods of identifying a candidate polypeptide binding agent, e.g., an antibody, that modulates binding between first and second components of a signaling complex. In some embodiments, the first and second components are polypeptides. In exemplary specific embodiments, the first and second components are endogenous.

[00202] In one aspect, the methods of identifying a candidate modulating antibody include (a) measuring a binding affinity or binding rate parameter of said first component for said second component, in the presence of a test polypeptide binding agent, e.g. antibody, (b) measuring a binding affinity or binding rate parameter of said first component for said second component in the absence of said test polypeptide binding agent; and (c) identifying said test polypeptide binding agent as a candidate modulating drug when said test polypeptide binding agent exhibits at least a 1.5-fold difference in the binding affinity or binding rate parameter measured in steps (a) and (b). In some embodiments, the difference in binding affinity or binding rate parameter ranges from about 1.5-fold (i.e., 50%) to about 1000-fold, or about 1.5-fold to about 100-fold, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold.

[00203] In some embodiments, the test polypeptide binding agent is identified as a candidate positive modulator if the test polypeptide agent strengthens the binding affinity or binding rate parameter between said first component and said second component. In other embodiments, the test polypeptide agent is identified as a candidate negative modulator if the test polypeptide agent weakens the binding affinity or binding rate parameter between said first component and said second component.

[00204] Whether a change (increase or decrease) in a particular binding affinity value or binding rate parameter value represents "strengthened" (or stronger) or "weakened" (or weaker) binding affinity or binding rate parameter depends on the value of the parameter and its units, and is well known in the art. For example, in the case of the parameter K_A, higher values mean "strengthened" binding affinity, such that a K_A of about 10^6M^{-1} is stronger than a K_A of about 10^5M^{-1}. As another example, in the case of the parameter K_D, lower values mean "strengthened" binding affinity, such that a K_D of about $10^{-6}M$ is stronger than a K_D of about $10^{-5}M$. Conversely, in the case of K_A, lower values mean "weakened" binding affinity,

such that a K_A of about $10^5 M^{-1}$ is a weakened binding affinity compared to a K_A of about $10^6 M^{-1}$. As another example, in the case of K_D, higher values mean "weakened" binding affinity, such that a K_D of about 10^{-5}M is weakened binding affinity compared to a K_D of about 10^{-6}M.

[00205] As used herein, a "strengthened" binding rate parameter means increased residency time, stronger association or weaker dissociation. As used herein, a "weakened" binding rate parameter means decreased residency time, weaker association or stronger dissociation.

[00206] Binding affinity can also be determined through the ratio of the on-rate and off-rate binding rate parameters. Generally, in the case of on-rate, higher values mean faster or stronger association or increased residence time, and typically result in stronger binding affinity. Conversely, lower values for on-rate mean slower or weaker association or decreased residence time, and typically result in weaker binding affinity. Generally, in the case of off-rate, higher values mean faster dissociation or decreased residence time, and typically result in weaker binding affinity. Conversely, lower values for off-rate mean slower dissociation or increased residence time, and typically result in stronger binding affinity. This is because the ratio of off-rate to on-rate, or on-rate to off-rate, indicates binding affinity as displayed in the equations below.

$$\text{Affinity}\begin{cases} K_D = \dfrac{[A][L]}{[AL]} = \dfrac{\text{off}-\text{rate}}{\text{on}-\text{rate}} \\ K_A = \dfrac{[AL]}{[A][L]} = \dfrac{\text{off}-\text{rate}}{\text{on}-\text{rate}} \end{cases}$$

where

$$A + L \underset{K_{off}}{\overset{K_{on}}{\rightleftharpoons}} AL$$

[00207] Even when binding affinity is not detectably or significantly altered, however, the change in residence time, i.e. an increased residence time via increased on-rate or decreased off-rate, or a decreased residence time via a decreased on-rate or increased off-rate, may still result in differential activation of signaling pathways. For example, in some instances where a receptor may activate two different pathways, the pathways differ in the degree of receptor activation required for a full effect. One signaling pathway can be fully activated at low levels of receptor activation or residence time, while full activation of the second pathway requires higher levels of receptor activation or residence time.

[00208] The predicted correlation of binding characteristics to functional effect is depicted in the table below.

Target Binding Characteristics			**KD ratios**	**Functional effect (pAKT assay shift)**
R	L	R-L		
-	-	+	$K_{[RL]A} < K_R, K_L$	Positive modulation
-	+	+	$K_{[AL]R} < K_L$	Positive modulation
+	-	+	$K_{[AR]L} < K_R$	Positive modulation
-	+	+	$K_{[AL]R} > K_L$	Negative modulation
+	-	+	$K_{[AR]L} > K_R$	Negative modulation

[00209] Illustrative examples of data showing that the functional effects of anti-INSR antibodies correlate with their binding characteristics are shown in the following table.

Ab	**Target Binding Characteristics**			**KD ratios**	**Functional effect (pAKT assay, fold-decrease in insulin EC_{50} relative to isotype control Ab)** [#]
	R	**L**	**R-L**		
Predicted	-	-	+	$K_{[RL]A} < K_R, K_L$	Positive modulation
Ab078	Out of Range*		3.4e-10		3.3
Ab085	No Binding		2e-10		8.9
Predicted	+	-	+	$K_{[AR]L} < K_R$	Positive modulation
Ab001	1.2e-8		1.16e-10	103.4	9.7
Ab079	9.6e-9		4.96e-10	19.4	6.7
Ab080	1.2e-8		6.8e-10	17.6	8.4
Ab083	7.6e-9		3.76e-10	20.2	8.5
Predicted	+	-	+	$K_{[AR]L} = K_R$	Non-Modulators
Ab037	1.08e-10		8e-11	1.4	No change
Ab053	1.48e-10		9.6e-11	1.5	No change
Ab062	1.24e-10		1.08e-10	1.1	No change

[00210] Thus, the binding properties of the interaction(s) between the modulator and the target, its signaling partner and/or a complex comprising the target and its signaling partner, are generally predictive of the functional effect of the modulator polypeptide on the target signaling pathway.

[00211] In another aspect, the methods of identifying a candidate modulating antibody include (a) (i) measuring a binding affinity or binding rate parameter of a test polypeptide binding agent, e.g. antibody, for said first component in the presence of said second component, or (ii) measuring a binding affinity or binding rate parameter of a test polypeptide binding agent for said second component in the presence of said first component; and (b) (i) measuring a binding affinity or binding rate parameter of said test polypeptide binding agent for said first component in the absence of said second component, or (ii) measuring a binding affinity or binding rate parameter of said test polypeptide binding agent for said second component in the absence of said first component; and (c) identifying said test polypeptide binding agent as a candidate kinetic modulating drug when said test polypeptide binding agent exhibits at least a 1.5-fold (i.e., 50%) difference in the binding affinity or binding rate parameters measured in steps (a) and (b).

[00212] In some embodiments, the test polypeptide binding agent is identified as a candidate positive modulator if the binding affinity or binding rate parameter measured in step (a) is at least 1.5-fold (i.e., 50%) stronger than the binding affinity or binding rate parameter measured in step (b). In specific embodiments, the binding affinity or binding rate parameter measured in step (a) compared to that measured in step (b) is about 1.5-fold (i.e., 50%) to about 1000-fold stronger for step (a) vs. step (b), or about 1.5-fold to about 100-fold, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g., at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 500-fold, or up to 200-fold, or up to 150-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, up to 30-fold, up to 20-fold, or up to 10-fold.

[00213] In other embodiments, the test polypeptide binding agent is identified as a candidate negative modulator if the binding affinity or binding rate parameter measured in step (b) is at least 1.5-fold (i.e., 50%) stronger than the binding affinity or binding rate parameter measured in step (a). In specific embodiments, the binding affinity or binding rate parameter measured in step (b) compared to that measured in step (a) is about 1.5-fold (i.e., 50%) to about 1000-fold stronger for step (b) vs. step (a), or about 1.5-fold to about 100-fold, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold,

18-fold, 19-fold or 20-fold, or up to 500-fold, or up to 200-fold, or up to 150-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, up to 30-fold, up to 20-fold, or up to 10-fold.

[00214] In some embodiments, the binding affinity or binding rate parameter of the test polypeptide binding agent for the first component alone is measured. In some embodiments, the binding affinity or binding rate parameter of the test polypeptide binding agent for the second component alone is measured.

[00215] In some embodiments, the test polypeptide binding agent is identified as a candidate positive modulator if one or more binding affinity or binding rate parameters selected from the group consisting of (A) the binding affinity or binding rate parameter of the test polypeptide binding agent for a complex comprising the first and second components, optionally $K_{[C1C2]M}$, (B) the binding affinity or binding rate parameter of the first component for a complex comprising the polypeptide binding agent and the second component, optionally $K_{[MC2]C1}$, or (C) the binding affinity or binding rate parameter of the second component for a complex comprising the polypeptide binding agent and the first component, optionally $K_{[MC1]C2}$, is at least about 1.5-fold stronger than one or more binding affinity or binding rate parameter selected from the group consisting of (1) the binding affinity or binding rate parameter of the test polypeptide binding agent for the second component alone, optionally K_{MC2} or (2) the binding affinity or binding rate parameter of the test polypeptide binding agent for the first component alone, optionally K_{MC1}. In some embodiments, the specific binding affinity or binding rate parameter of any one or more of (A), (B) or (C) is about 1.5-fold (i.e., 50%) to about 1000-fold stronger than the binding affinity or binding rate parameter of any one or more of (1) or (2); or alternatively, about 1.5-fold to about 100-fold stronger, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g. at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 500-fold, or up to 200-fold, or up to 150-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, up to 30-fold, up to 20-fold, or up to 10-fold. For example, in some embodiments, the binding affinity or binding rate parameter of any one or more of (A), (B) or (C) is stronger than the binding affinity or binding rate parameter of both (1) and (2). In some embodiments, the binding affinity or binding rate parameter of (1) is stronger than the binding affinity or binding rate parameter of (2). In other embodiments, the binding

affinity or binding rate parameter of (2) is stronger than the binding affinity or binding rate parameter of (1). In some embodiments, two or more binding affinity or binding rate parameters are measured and compared, e.g. off-rate and on-rate, or K_A and K_D, or any combination thereof.

[00216] In specific embodiments, wherein the binding affinity measured is the equilibrium dissociation constant K_D, any of $K_{[C1C2]M}$, $K_{[MC2]C1}$, or $K_{[MC1]C2}$ is lower, e.g., about 1.5-fold to 1000-fold lower, than any of K_{MC2} or K_{MC1}. Similarly, wherein the binding affinity measured is the off-rate, any of the off-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2 are lower, e.g. about 1.5-fold to 1000-fold lower, than any of the off-rates between (1) M and C2 or (2) M and C1. In one exemplary embodiment, $K_{[C1C2]M}$ is about 1.5-fold to 1000-fold lower than K_{MC2}. In another exemplary embodiment, $K_{[MC2]C1}$ is about 1.5-fold to 1000-fold lower than K_{MC2}. In another exemplary embodiment, $K_{[MC1]C2}$ is about 1.5-fold to 1000-fold lower than K_{MC2}. In another exemplary embodiment, $K_{[C1C2]M}$ is about 1.5-fold to 1000-fold lower than K_{MC1}. In another exemplary embodiment, $K_{[MC2]C1}$ is about 1.5-fold to 1000-fold lower than K_{MC1}. In yet another exemplary embodiment, $K_{[MC1]C2}$ is about 1.5-fold to 1000-fold lower than K_{MC1}. Similar examples can be envisioned for each of the off-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2, compared to each of the off-rates between (1) M and C2 or (2) M and C1.

[00217] Conversely, where the binding affinity measured is the equilibrium association constant K_A, any of $K_{[C1C2]M}$, $K_{[MC2]C1}$, or $K_{[MC1]C2}$ is higher, e.g., about 1.5-fold to 1000-fold higher, than any of K_{MC2} or K_{MC1}. Similarly, wherein the binding affinity measured is the on-rate, any of the on-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2 are higher, e.g. about 1.5-fold to 1000-fold higher, than any of the on-rates between (1) M and C2 or (2) M and C1. In one exemplary embodiment, $K_{[C1C2]M}$ is about 1.5-fold to 1000-fold higher than K_{MC2}. In another exemplary embodiment, $K_{[MC2]C1}$ is about 1.5-fold to 1000-fold higher than K_{MC2}. In another exemplary embodiment, $K_{[MC1]C2}$ is about 1.5-fold to 1000-fold higher than K_{MC2}. In another exemplary embodiment, $K_{[C1C2]M}$ is about 1.5-fold to 1000-fold higher than K_{MC1}. In another exemplary embodiment, $K_{[MC2]C1}$ is about 1.5-fold to 1000-fold higher than K_{MC1}. In yet another exemplary embodiment, $K_{[MC1]C2}$ is about 1.5-fold to 1000-fold higher than K_{MC1}. Similar examples can be envisioned for each of the on-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2, compared to each of the on-rates between (1) M and C2 or (2) M and C1.

[00218] In some embodiments, the test polypeptide binding agent is identified as a candidate negative modulator if one or more binding affinity or binding rate parameters selected from the group consisting of (1) the binding affinity or binding rate parameter of the test polypeptide binding agent for the second component alone, optionally K_{MC2}, or (2) the binding affinity or binding rate parameter of the test polypeptide binding agent for the first component alone, optionally K_{MC1}, is at least about 1.5-fold stronger than one or more binding affinity or binding rate parameter selected from the group consisting of (A) the binding affinity or binding rate parameter of the test polypeptide binding agent for a complex comprising the first and second components, optionally $K_{[C1C2]M}$, (B) the binding affinity or binding rate parameter of the first component for a complex comprising the polypeptide binding agent and the second component, optionally $K_{[MC2]C1}$, or (C) the binding affinity or binding rate parameter of the second component for a complex comprising the polypeptide binding agent and the first component, optionally $K_{[MC1]C2}$. In some embodiments, the specific binding affinity or binding rate parameter of any one or more of (1) or (2) is about 1.5-fold (i.e., 50%) to about 1000-fold stronger than the binding affinity or binding rate parameter of any one or more of (A), (B) or (C); or alternatively, about 1.5-fold to about 100-fold stronger, or about 2-fold to 25-fold, or about 2-fold to about 50-fold, or about 1.5-fold to about 25-fold, or about 1.5-fold to about 50-fold, e.g., at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold or 20-fold, or up to 500-fold, or up to 200-fold, or up to 150-fold, or up to 100-fold, or up to 90-fold, or up to 80-fold, or up to 70-fold, or up to 60-fold, or up to 50-fold, or up to 40-fold, up to 30-fold, up to 20-fold, or up to 10-fold. In some embodiments, the binding affinity or binding rate parameter of any of (1) or (2) is stronger than the binding affinity or binding rate parameter of all of (A), (B) and (C). In some embodiments, the binding affinity or binding rate parameter of (1) is stronger than the binding affinity or binding rate parameter of (2). In other embodiments, the binding affinity or binding rate parameter of (2) is stronger than the binding affinity or binding rate parameter of (1). In some embodiments, two or more binding affinity or binding rate parameters are measured and compared, e.g. off-rate and on-rate, or K_A and K_D, or any combination thereof.

[00219] In specific embodiments, where the binding affinity measured is the equilibrium dissociation constant K_D, any of K_{MC2} or K_{MC1} is lower, e.g., about 1.5-fold to 1000-fold lower, than any of $K_{[C1C2]M}$, $K_{[MC2]C1}$, or $K_{[MC1]C2}$. Similarly, wherein the binding affinity measured is the off-rate, any of the off-rates between (1) M and C2 or (2) M and C1 are

lower, e.g. about 1.5-fold to 1000-fold lower, than any of the off-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2. In one exemplary embodiment K_{MC2} is about 1.5-fold to 1000-fold lower than $K_{[C1C2]M}$. In another exemplary embodiment, K_{MC2} is about 1.5-fold to 1000-fold lower than $K_{[MC2]C1}$. In another exemplary embodiment, K_{MC2} is about 1.5-fold to 1000-fold lower than $K_{[MC1]C2}$. In another exemplary embodiment, K_{MC1} is about 1.5-fold to 1000-fold lower than $K_{[C1C2]M}$. In another exemplary embodiment, K_{MC1} is about 1.5-fold to 1000-fold lower than $K_{[MC2]C1}$. In yet another exemplary embodiment, K_{MC1} is about 1.5-fold to 1000-fold lower than $K_{[MC1]C2}$. Similar examples can be envisioned for each of the off-rates between (1) M and C2 or (2) M and C1, compared to each of the off-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2.

[00220] Conversely, wherein the binding affinity is the equilibrium association constant K_A, any of K_{MC2} or K_{MC1} is higher, e.g., about 1.5-fold to 1000-fold higher, than any of $K_{[C1C2]M}$, $K_{[MC2]C1}$, or $K_{[MC1]C2}$. Similarly, wherein the binding affinity measured is the on-rate, any of the on-rates between (1) M and C2 or (2) M and C1 are higher, e.g. about 1.5-fold to 1000-fold higher, than any of the on-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2. In one exemplary embodiment K_{MC2} is about 1.5-fold to 1000-fold higher than $K_{[C1C2]M}$. In another exemplary embodiment, K_{MC2} is about 1.5-fold to 1000-fold higher than $K_{[MC2]C1}$. In another exemplary embodiment, K_{MC2} is about 1.5-fold to 1000-fold higher than $K_{[MC1]C2}$. In another exemplary embodiment, K_{MC1} is about 1.5-fold to 1000-fold higher than $K_{[C1C2]M}$. In another exemplary embodiment, K_{MC1} is about 1.5-fold to 1000-fold higher than $K_{[MC2]C1}$. In yet another exemplary embodiment, K_{MC1} is about 1.5-fold to 1000-fold higher than $K_{[MC1]C2}$. Similar examples can be envisioned for each of the on-rates between (1) M and C2 or (2) M and C1, compared to each of the on-rates between (A) [C1C2] and M, or (B) [MC2] and C1, or (C) [MC1] and C2.

[00221] In certain embodiments, the modulator is an antibody and C1 and C2 are selected from the group consisting of insulin and insulin receptor.

[00222] In any of these embodiments, the test polypeptide binding agent and second component can be contacted with multiple different concentrations of said first component. In any of these embodiments, the test polypeptide binding agent and first component can be contacted with multiple different concentrations of said second component. In any of these embodiments, multiple different concentrations of the test polypeptide binding agent can be contacted with said first component and said second component.

[00223] When the effect of test polypeptide binding agent on the binding interaction between the first component and second component is determined, in some specific embodiments, when the antigen for the test polypeptide binding agent is the first component, e.g., ligand, the test polypeptide binding agent is at a saturating concentration compared to the concentration of the first component. Alternatively, when the antigen for the test polypeptide binding agent is the second component, e.g., receptor, the test polypeptide binding agent is at a saturating concentration compared to the concentration of the second component. In some embodiments, the concentration of the test polypeptide binding agent is greater than or equal to the K_D of the test polypeptide binding agent for a complex comprising the first component and the second component. In further embodiments, the concentration of the second component is less than the K_D of the test polypeptide binding agent for the first component, e.g., ligand. In yet further embodiments, the concentration of the first component, e.g., ligand, is at a subsaturating concentration for the binding of first component to second component, e.g., receptor. In some embodiments, the concentration of the first component, e.g., ligand is within the range of about EC_{20} to EC_{80} for the interaction of the first component with the second component. In some embodiments, one or more concentrations of the test polypeptide binding agent is contacted with multiple different concentrations of the first component, e.g., ligand, in the presence of one or more concentrations of the second component, e.g., receptor. In some embodiments, one or more concentrations of the test polypeptide binding agent is contacted with multiple different concentrations of the second component, e.g., receptor, in the presence of one or more concentrations of the first component, e.g., ligand.

[00224] When differential binding of test polypeptide binding agent to complexed vs. uncomplexed target and/or signaling partner is determined in order to identify a positive modulator, in some embodiments, the test polypeptide binding agent is at a saturating concentration for a complex comprising the first component and the second component. In some embodiments, the concentration of test polypeptide binding agent is greater than or equal to the K_D of the test polypeptide binding agent for a complex comprising the first component, e.g., ligand, and the second component, e.g., receptor. In further embodiments, the concentration of the second component, e.g., receptor is greater than the K_D of the second component, e.g., receptor, for the first component, e.g., ligand. In further embodiments, the concentration of the first component, e.g., ligand, is a saturating concentration for the second component, e.g., receptor. In yet further embodiments, the test polypeptide binding agent is

at a subsaturating concentration for a complex comprising the first component and the second component. In some embodiments, the concentration of the polypeptide binding agent is within the range of about EC_{20} to EC_{80} for the interaction of the first component with the second component. In some embodiments, the concentration of the second component, e.g., receptor, is greater than the K_D of the second component, e.g., receptor, for the first component, e.g., ligand. In some embodiments, the concentration of the first component, e.g., ligand, is a saturating concentration for the second component, e.g., receptor.

[00225] When differential binding of test polypeptide binding agent to complexed vs. uncomplexed target and/or signaling partner is determined in order to identify a negative modulator, in some embodiments, when the antigen to which the test polypeptide binding agent binds is the first component, e.g., ligand, the test polypeptide binding agent is at a subsaturating concentration for the first component. When the antigen to which the test polypeptide binding agent binds is the second component, e.g., receptor, the test polypeptide binding agent is at a subsaturating concentration for the second component. In further embodiments, the concentration of the polypeptide binding agent is within the range of about EC_{20} to EC_{80} for the interaction of the first component with the second component. In further embodiments, the concentration of the second component, e.g., receptor, is greater than the K_D of the second component, e.g., receptor, for the first component, e.g., ligand. In further embodiments, the concentration of the first component, e.g., ligand, is a saturating concentration for the second component, e.g., receptor.

[00226] In some embodiments, the methods further involve assaying a plurality of test polypeptide binding agents, e.g. antibodies, for binding affinity to any one of (a) the first component, (b) the second component, or (c) a complex comprising the first component and second component. In some specific embodiments, the polypeptide binding agents have a binding affinity characterized, e.g., by an equilibrium dissociation constant K_D of about 10^{-5}M or less, or about 10^{-6}M or less, or about 10^{-7}M or less, or about 10^{-8}M or less, where a lower K_D means stronger binding affinity. In some embodiments, the plurality of test polypeptide binding agents screened are variants of a parent polypeptide binding agent made by introducing one or more different mutations into a parent polypeptide binding agent.

[00227] In further embodiments, the polypeptide binding agents may be screened for selectivity of effect for the first or second component, compared to a different binding partner such as a decoy receptor, clearance receptor, or alternate signal pathway component. Such methods may involve identifying a polypeptide binding agent that does not significantly

change the binding affinity or binding rate parameter of the first or second component for a different binding partner, such binding partner being neither the first nor second component. In some embodiments, the presence of the polypeptide binding agent changes the binding affinity or binding rate parameter of the first or second component for a different binding partner no more than 5-fold, or no more than 10-fold, or no more than 20-fold, or no more than 30-fold, or no more than 40-fold, or no more than 50-fold.

[00228] Any of the preceding methods may further include measuring the level of signaling mediated by the signaling complex in the presence and absence of the test polypeptide binding agent, and determining whether the test polypeptide binding agent is additionally an agonist, partial agonist, antagonist or partial antagonist. Antagonism or agonism can be measured in any in vitro or in vivo assay known in the art, including but not limited to signaling in a phosphorylation assay, ion flux assay, molecular transport assay, or gene expression assay.

[00229] In some embodiments, the test polypeptide binding agent shifts (positively or negatively) the dose-response curve of the interaction of the first component, e.g. ligand, with the second component, e.g. receptor. The shift may manifest as an increased or decreased EC_{50} by at least about 1.5-fold, e.g. about 1.5-fold to about 100-fold. In some embodiments, the test polypeptide binding agent does not significantly change the maximal agonist response of the signal produced by interaction of the first and second components of the signaling complex. In other embodiments, the test polypeptide binding agent itself acts as an antagonist (e.g., reduces the maximal agonist response of the signaling produced by said signaling complex) or agonist (e.g. increases the maximal agonist response of the signaling produced by said signaling complex).

[00230] Where the test polypeptide binding agent acts as an antagonist or partial antagonist, the maximal agonist response may be decreased, e.g., by about 1.5-fold to about 100-fold, or about 2-fold to about 25-fold, or about 1.5-fold to about 50-fold; or, decreased by about 10%, 25%, 50% (1.5-fold), 75%, 2-fold, 3-fold, or 4-, 5-, 6-, 7-, 8-, 9- or 10-fold. Alternatively, where the test polypeptide binding agent acts as an agonist or partial agonist, the maximal agonist response may be increased, e.g. by at least about 10%, 25%, 50% (1.5-fold), 75%, 2-fold, 3-fold, or 4-, 5-, 6-, 7-, 8-, 9- or 10-fold. Moreover, when the test polypeptide binding agent acts as an antagonist or partial antagonist, the IC50 may be $1x10^{-5}$ or less. The test polypeptide binding agent may exhibit further desirable characteristics, e.g. the test polypeptide binding agent does not significantly decrease clearance of said first

component, or said second component, or said signaling complex comprising said first and second components.

[00231] Methods of identifying modulating agents, e.g., kinetic modulating agents, are described further in co-pending, co-owned U.S. Patent Application No. 61/246,079, filed September 25, 2009, U.S. Patent Application No. 61/306,324, filed February 19, 2010, and International Patent Application No. _____, filed September 24, 2010 (Docket No. 27129/41726).

[00232] The test polypeptide binding agent may exhibit further desirable characteristics, e.g. the test polypeptide binding agent does not significantly decrease clearance of said first component, or said second component, or said signaling complex comprising said first and second components.

[00233] In a related aspect, the invention provides methods of identifying modulators of the insulin/insulin receptor signaling complex and an antibody or other modulator identified by any of the methods described above or anywhere in the present application.

Types and Sources of Antibodies

[00234] The present invention encompasses target specific antibodies that bind to insulin, insulin receptor or the insulin/insulin receptor complex. In exemplary embodiments, a target specific antibody of the invention can comprise a human kappa (κ) or a human lambda (λ) light chain or an amino acid sequence derived therefrom, or a human heavy chain or a sequence derived therefrom, or both heavy and light chains together in a single chain, dimeric, tetrameric or other form. In some embodiments, a heavy chain and a light chain of a target specific immunoglobulin are different amino acid molecules. In other embodiments, the same amino acid molecule contains a heavy chain variable region and a light chain variable region of a target specific antibody.

[00235] The term "antibody" is used in the broadest sense and includes fully assembled antibodies, tetrameric antibodies, monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), human and humanized antibodies, antibody fragments that can bind an antigen (e.g., Fab', F'(ab)2, Fv, single chain antibodies, diabodies), and recombinant peptides comprising the forgoing as long as they exhibit the desired biological activity. An "immunoglobulin" or "tetrameric antibody" is a tetrameric glycoprotein that consists of two heavy chains and two light chains, each comprising a variable region and a constant region. Antigen-binding portions may be produced by recombinant DNA techniques

or by enzymatic or chemical cleavage of intact antibodies. Antibody fragments or antigen-binding portions include, inter alia, Fab, Fab', F(ab')2, Fv, domain antibody (dAb), complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single chain antibody fragments, chimeric antibodies, diabodies, triabodies, tetrabodies, minibody, linear antibody; chelating recombinant antibody, a tribody or bibody, an intrabody, a nanobody, a small modular immunopharmaceutical (SMIP), a antigen-binding-domain immunoglobulin fusion protein, a camelized antibody, a VHH containing antibody, or a variant or a derivative thereof, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide, as long as the antibody retains the desired biological activity.

[00236] In a naturally-occurring immunoglobulin, each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. Human light chains are classified as kappa (κ) and lambda (λ) light chains. Heavy chains are classified as mu (μ), delta (Δ), gamma (γ), alpha (α), and epsilon (ε), and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, Fundamental Immunology, Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)) (incorporated by reference in its entirety for all purposes). The variable regions of each light/heavy chain pair form the antibody binding site such that an intact immunoglobulin has two binding sites.

[00237] Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Chothia et al., *J. Mol. Biol.* 196:901-917, 1987).

[00238] Immunoglobulin variable domains exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions or CDRs. From N-

terminus to C-terminus, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk, (*J. Mol. Biol.* 196:901-917, 1987); Chothia et al., (*Nature* 342:878-883, 1989).

[00239] The hypervariable region of an antibody refers to the CDR amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a CDR [i.e., residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain as described by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)] and/or those residues from a hypervariable loop (*i.e.*, residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain as described by [Chothia et al., *J. Mol.Biol. 196*: 901-917 (1987)]. However, one of skill in the art understands that the actual location of the CDR residues may vary from the projected residues described above when the sequence of the particular antibody is identified.

[00240] Framework or FR residues are those variable domain residues other than the hypervariable region residues.

[00241] Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes, IgA, IgD, IgE, IgG and IgM, which may be further divided into subclasses or isotypes, e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Different isotypes have different effector functions; for example, IgG1 and IgG3 isotypes have ADCC activity. An antibody of the invention, if it comprises a constant domain, may be of any of these subclasses or isotypes.

[00242] In exemplary embodiments, an antibody of the invention can comprise a human kappa (κ) or a human lambda (λ) light chain or an amino acid sequence derived therefrom, or a human heavy chain or a sequence derived therefrom, or both heavy and light chains together in a single chain, dimeric, tetrameric or other form.

[00243] Monoclonal antibody refers to an antibody obtained from a population of substantially homogeneous antibodies. Monoclonal antibodies are generally highly specific,

and may be directed against a single antigenic site, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes). In addition to their specificity, monoclonal antibodies are advantageous in that they are synthesized by the homogeneous culture, uncontaminated by other immunoglobulins with different specificities and characteristics.

[00244] Monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., (*Nature*, 256:495-7, 1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in, for example, Clackson et al., (*Nature* 352:624-628, 1991) and Marks et al., (*J. Mol. Biol.* 222:581-597, 1991).

[00245] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization (Harlow & Lane; Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1988).

Recombinant production of antibodies

[00246] The present invention also encompasses nucleic acid molecules encoding antibodies of the invention. In some embodiments, different nucleic acid molecules encode a heavy chain variable region and a light chain variable region of an antigen-specific antibody. In other embodiments, the same nucleic acid molecule encodes a heavy chain and a light chain variable regions of an antigen-specific antibody.

[00247] DNA encoding a monoclonal antibody of the invention may be isolated and sequenced from a hybridoma cell secreting the antibody using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). Sequence determination will generally require isolation of at least a portion of the gene or cDNA of interest. Usually this requires cloning the DNA or, preferably, mRNA (i.e., cDNA) encoding the monoclonal antibodies. Cloning is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, which is incorporated herein by reference). For example, a cDNA library may be constructed by reverse transcription of polyA+ mRNA, preferably membrane-associated mRNA, and the library

screened using probes specific for human immunoglobulin polypeptide gene sequences. Nucleotide probe reactions and other nucleotide hybridization reactions are carried out at conditions enabling the identification of polynucleotides which hybridize to each other under specified conditions. The hybridization conditions can be calculated as described in Sambrook, et al., (Eds.), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51

[00248] In a preferred embodiment, the polymerase chain reaction (PCR) is used to amplify cDNAs (or portions of full-length cDNAs) encoding an immunoglobulin gene segment of interest (e.g., a light chain variable segment). The amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used is not critical, so long as it is possible to determine the sequence of some portion of the immunoglobulin polypeptide of interest. As used herein, an "isolated" nucleic acid molecule or "isolated" nucleic acid sequence is a nucleic acid molecule that is either (1) identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid or (2) cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the sequence of the nucleic acid of interest can be determined, is considered isolated. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[00249] One source for RNA used for cloning and sequencing is a hybridoma produced by obtaining a B cell from the transgenic mouse and fusing the B cell to an immortal cell. Alternatively, RNA can be isolated from B cells (or whole spleen) of the immunized animal. When sources other than hybridomas are used, it may be desirable to screen for sequences encoding immunoglobulins or immunoglobulin polypeptides with specific binding characteristics. One method for such screening is the use of phage display technology. Phage display is described further herein and is also well-known in the art. See e.g., Dower et al., WO 91/17271, McCafferty et al., WO 92/01047, and Caton and Koprowski, (*Proc. Natl. Acad. Sci. USA*, 87:6450-54 (1990)), each of which is incorporated herein by reference. In one embodiment, cDNA from an immunized transgenic mouse (e.g., total spleen cDNA) is

isolated, the polymerase chain reaction is used to amplify a cDNA sequences that encode a portion of an immunoglobulin polypeptide, e.g., CDR regions, and the amplified sequences are inserted into a phage vector. cDNAs encoding peptides of interest, e.g., variable region peptides with desired binding characteristics, are identified by standard phage display techniques such as panning.

[00250] The sequence of the amplified or cloned nucleic acid is then determined. Typically the sequence encoding an entire variable region of the immunoglobulin polypeptide is determined, however, it will sometimes by adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Typically the portion sequenced will be at least 30 bases in length, more often based coding for at least about one-third or at least about one-half of the length of the variable region will be sequenced.

[00251] Sequencing can be carried out on clones isolated from a cDNA library, or, when PCR is used, after subcloning the amplified sequence or by direct PCR sequencing of the amplified segment. Sequencing is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoma immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

[00252] Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, human embryonic kidney 293 cells (e.g., 293E cells), Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies is well known in the art.

[00253] Expression control sequences refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an

operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

[00254] In an alternative embodiment, the amino acid sequence of an immunoglobulin of interest may be determined by direct protein sequencing. Suitable encoding nucleotide sequences can be designed according to a universal codon table.

[00255] Amino acid sequence variants of the desired antibody may be prepared by introducing appropriate nucleotide changes into the encoding DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibodies. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

[00256] Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

[00257] The invention also provides isolated nucleic acid encoding antibodies of the invention, optionally operably linked to control sequences recognized by a host cell, vectors and host cells comprising the nucleic acids, and recombinant techniques for the production of the antibodies, which may comprise culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture or culture medium. Various systems and methods for antibody production are reviewed by Birch & Racher (Adv. Drug Deliv. Rev. 671-685 (2006)).

[00258] For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or

more of the following: a signal sequence, an origin of replication, one or more selective marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[00259] Suitable host cells for cloning or expressing the DNA in the vectors herein are prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41 P disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

[00260] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g., K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastors* (EP 183,070); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g., Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

[00261] Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

[00262] Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, tobacco, lemna, and other plant cells can also be utilized as hosts.

[00263] Examples of useful mammalian host cell lines are Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, (Graham et al., *J. Gen Virol. 36*: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, (*Biol. Reprod.* 23: 243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y Acad. Sci.* 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[00264] Host cells are transformed or transfected with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In addition, novel vectors and transfected cell lines with multiple copies of transcription units separated by a selective marker are particularly useful and preferred for the expression of antibodies that bind the desired antigen.

[00265] Host cells containing desired antibody nucleic acid sequences may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., (*Meth. Enz.* 58: 44, 1979), Barnes et al., *Anal. Biochem.* 102: 255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO90103430; WO 87/00195; or U.S. Patent Re. No. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the

micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[00266] When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium, including from microbial cultures. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Better et al. (*Science* 240:1041-43, 1988; ICSU Short Reports 10:105 (1990); and *Proc. Natl. Acad. Sci. USA* 90:457-461 (1993) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. [*See* also, (Carter et al., *Bio/Technology* 10:163-167 (1992)].

[00267] The antibody composition prepared from microbial or mammalian cells can be purified using, for example, hydroxylapatite chromatography cation or avian exchange chromatography, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth. 62*: 1-13, 1983). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H 3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Antibodies of the Invention

[00268] The present invention encompasses target specific antibodies that bind insulin, insulin receptor and/or the insulin/insulin receptor complex, and preferably alter (e.g. increase or decrease) signaling of the insulin receptor and/or its effect on glucose levels and glucose uptake. In exemplary embodiments, a target specific antibody of the invention can comprise a human kappa (κ) or a human lambda (λ) light chain or an amino acid sequence derived therefrom, or a human heavy chain or a sequence derived therefrom, or both heavy and light chains together in a single chain, dimeric, tetrameric or other form. In some embodiments, a heavy chain and a light chain of a target specific immunoglobulin are different amino acid molecules. In other embodiments, the same amino acid molecule contains a heavy chain variable region and a light chain variable region of a target specific antibody.

[00269] In some embodiments, the amino acid sequence of the anti-target antibody comprises one or more CDRs of the amino acid sequence of the mature (i.e., missing signal sequence) light chain variable region (V_L) of antibodies in SEQ ID NO: 1-150 or variants thereof. In some embodiments, the V_L comprises the amino acid sequence from the beginning of the CDR1 to the end of the CDR3 of the light chain of any one of the foregoing antibodies.

[00270] In one embodiment, the target specific antibody comprises a light chain CDR1, CDR2 or CDR3 (LCDR1, LCDR2, LCDR3), each of which are independently selected from the CDR1, CDR2 and CDR3 regions of an antibody having a light chain variable region comprising the amino acid sequence of the V_L region set out in SEQ ID NOs: 1- 150. In one aspect the light chain CDR1 is within residues 24-36, CDR2 is within residues 50-56 and CDR3 is within residues 89-101, according to Chothia numbering. A polypeptide of the target specific antibody may comprise the CDR1, CDR2 and CDR3 regions of an antibody having the amino acid sequence of the V_L region selected from the group consisting of SEQ ID NOs: 1- 150.

[00271] In some embodiments, the target specific antibody comprises one or more CDRs of the amino acid sequence of the mature (i.e., missing signal sequence) heavy chain variable region (V_H) of antibodies set out in SEQ ID NOs: 151-303 or variants thereof. In some embodiments, the V_H comprises the amino acid sequence from the beginning of the CDR1 to the end of the CDR3 of any one of the heavy chain of the foregoing antibodies.

[00272] In one embodiment, the target specific antibody comprises a heavy chain CDR1, CDR2 or CDR3 (HCDR1, HCDR2, HCDR3), each of which are independently selected from

the CDR1, CDR2 and CDR3 regions of an antibody having a heavy chain variable region comprising the amino acid sequence of the V_H region set out in SEQ ID NOs: 151-303. It is further contemplated that a target specific antibody comprises a heavy chain CDR1, CDR2 or CDR3, each of which are independently selected from the CDR1, CDR2 and CDR3 regions of an antibody having a heavy chain variable region having the amino acid sequence of the V_H region set out in SEQ ID NOs: 151-303. In one aspect the heavy chain CDRs are located according to Chothia numbering: CDR1 is within residues 26-35, CDR2 is within residues 50-58 and CDR3 is within residues 95-111 or 97-118. A polypeptide of the target specific antibody may comprise the CDR1, CDR2 and CDR3 regions of an antibody having the amino acid sequence of the V_H region selected from the group consisting of SEQ ID NOs: 151-303.

[00273] CDRs in Tables 1 and 2 and SEQ ID NO: 1-303 were determined according to the IMGT system, LeFranc et al IMGT, the INTERNATIONAL IMMUNOGENETICS INFORMATION SYSTEM®, Nucl. Ac. Res. 33 D593-597 (2005).

[00274] In another embodiment, the antibody comprises a mature light chain variable region as disclosed above and a mature heavy chain variable region as disclosed above, paired as set forth in Table 3. In another embodiment, the invention contemplates a purified preparation of a monoclonal antibody, comprising the light chain variable region and heavy chain variable regions of any of antibodies as set forth in SEQ ID NOs: 1-303 and paired as set forth in Table 3.

[00275] In exemplary embodiments, the invention contemplates:

[00276] a monoclonal antibody that retains any one, two, three, four, five, or six of HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, or LCDR3 of any one of SEQ ID NOs: 151-303 and SEQ ID NOs: 1-150, respectively, optionally including one or two mutations in any of such CDR(s), e.g., a conservative or non-conservative substitution, and optionally paired as set forth in Table 3;

[00277] a monoclonal antibody that retains all of HCDR1, HCDR2, HCDR3, or the heavy chain variable region of any one of SEQ ID NOs: 151-303, optionally including one or two mutations in any of such CDR(s), optionally further comprising any suitable heavy chain constant region, e.g., IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, or IgE, a human sequence thereof, or a hybrid thereof or a human consensus thereof;

[00278] a monoclonal antibody that retains all of LCDR1, LCDR2, LCDR3, or the light chain variable region of any one SEQ ID NOs: 1-150, optionally including one or two mutations in any of such CDR(s), optionally further comprising to any suitable light chain constant region, e.g. a kappa or lambda light chain constant region, a human sequence thereof, or a hybrid thereof or a human consensus thereof;

[00279] a monoclonal antibody that binds to the same linear or three-dimensional epitope of INSR as an antibody comprising variable regions set out in SEQ ID NO: 1-303, e.g., as determined through X-ray crystallography or other biophysical or biochemical techniques such as deuterium exchange mass spectrometry, alanine scanning and peptide fragment ELISA;

[00280] a monoclonal antibody that competes with an antibody comprising variable regions set out in SEQ ID NO: 1-303 for binding to human INSR by more than about 75%, more than about 80%, or more than about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% or 95%.

[00281] In some embodiments, the antibody comprises all three light chain CDRs, all three heavy chain CDRs, or all six CDRs of the light and heavy chain, paired as set forth in Table 3. In some exemplary embodiments, two light chain CDRs from an antibody may be combined with a third light chain CDR from a different antibody. Alternatively, a LCDR1 from one antibody can be combined with a LCDR2 from a different antibody and a LCDR3 from yet another antibody, particularly where the CDRs are highly homologous. Similarly, two heavy chain CDRs from an antibody may be combined with a third heavy chain CDR from a different antibody; or a HCDR1 from one antibody can be combined with a HCDR2 from a different antibody and a HCDR3 from yet another antibody, particularly where the CDRs are highly homologous.

[00282] Consensus CDRs may also be used. Any one of the consensus CDRs derived herein may be combined with two other CDRs from the same chain (e.g. heavy or light) of any of antibodies, e.g. to form a suitable heavy or light chain variable region.

[00283] In some embodiments, an antibody is provided that comprises a polypeptide having an amino acid sequence at least about 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96% , 97%, 98%, 99% or more identical to the heavy chain variable region set out in SEQ ID NO: 148-284 and/or an amino acid sequence an amino acid sequence at least about 65%, 70%, 75%, 80%, 81%,

82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96% , 97%, 98%, 99% or more identical to the light chain variable region set out in SEQ ID NO: 1-150, the antibody further comprising at least one, two, three, four, five or all of CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 or CDRL3. In some embodiments, the amino acid sequence with percentage identity to the light chain variable region may comprise one, two or three of the light chain CDRs. In other embodiments, the amino acid sequence with percentage identity to the heavy chain variable region may comprise one, two, or three of the heavy chain CDRs.

[00284] It is contemplated that the antibodies of the invention may have one, or two or more amino acid substitutions in the CDR regions of the antibody, e.g., non-conservative or conservative substitutions.

[00285] In a related embodiment, the residues of the framework are altered. The heavy chain framework regions which can be altered lie within regions designated H-FR1, H-FR2, H-FR3 and H-FR4, which surround the heavy chain CDR residues, and the residues of the light chain framework regions which can be altered lie within the regions designated L-FR1, L-FR2, L-FR3 and L-FR4, which surround the light chain CDR residues. An amino acid within the framework region may be replaced, for example, with any suitable amino acid identified in a human framework or human consensus framework.

[00286] It is further contemplated that the invention provides a purified polypeptide comprising any one of the amino acid sequences of SEQ ID NO: 1-150 fused to any one of the amino acid sequences of SEQ ID NO: 151-303, optionally paired as the heavy/light chain variable regions set forth in Table 3, or fragments thereof that include at least a portion of SEQ ID NO: 1-150 and SEQ ID NO: 151-303, optionally paired as set forth in Table 3, wherein the polypeptide binds insulin receptor, insulin or the insulin/insulin receptor complex.

[00287] In another aspect, the invention provides a purified polypeptide comprising at least one CDR of a light chain variable region described herein, wherein the light chain variable region comprises an amino acid sequence at least 90% identical to the LCDR sequences set out in SEQ ID NO: 1-150. In one embodiment, the polypeptide may be 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of the LCDRs set out in SEQ ID NO: 1-150. In a further aspect, the invention provides a purified polypeptide comprising at least one CDR of a heavy chain variable region described herein, wherein the heavy chain variable region comprises an amino acid sequence at least 90% identical to the HCDR sequences set out in

SEQ ID NO: 151-303. In one embodiment, the polypeptide may be 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of the HCDRs set out in SEQ ID NO: 151-303.

[00288] It is further contemplated that the CDR of the antibody heavy and light chains comprise variant amino acid sequences which may improve antibody binding affinity and are derived through, for example, affinity maturation. In one aspect it is contemplated that an antibody of the invention comprises a heavy chain HCDR2 sequence having about 35% identity to a HCDR2 of a parent antibody sequence set out in SEQ ID NOs: 151-303. In a related aspect it is contemplated that an antibody of the invention comprises a heavy chain HCDR3 sequence having about 50% identity to a HCDR3 of a parent antibody sequence set out in SEQ ID NOs: 151-303.

[00289] In one embodiment the invention provides antigen-binding compounds, including functional fragments, having a variable region amino acid sequence set forth in any one of SEQ ID NOs: 1-150 and 151-303. In a related embodiment, an aforementioned antigen binding compound is selected from the group consisting of a fully assembled tetrameric antibody, a polyclonal antibody, a monoclonal antibody including a HUMAN ENGINEERED™ antibody; a humanized antibody; a human antibody; a chimeric antibody; a multispecific antibody, an antibody fragment, Fab, $F(ab')_2$; Fv; scFv or single-chain antibody fragment; a diabody; triabody, tetrabody, minibody, linear antibody; chelating recombinant antibody, a tribody or bibody, an intrabody, a nanobody, a small modular immunopharmaceutical (SMIP), a binding-domain immunoglobulin fusion protein, a camelized antibody, a V_{HH} containing antibody, or a variant or derivative of any one of these antibodies, that comprise one or more CDR sequences of the invention and exhibit the desired biological activity. The antigen binding compounds of the invention preferably retain binding affinity of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, $10^{-9,}$ 10^{-10} , 10^{-11} M or less as measured by surface plasmon resonance.

[00290] In one aspect, the antibodies of the invention comprise a heavy chain variable region or light chain variable region as set out in amino acid sequences SEQ ID NO: 151-303 and SEQ ID NO: 1-150, respectively, as paired in Table 3. It is further contemplated that the antibodies may comprise all or part of the antibodies set out in the above amino acid sequences. In one embodiment, the antibodies comprise at least one of CDR1, CDR2, or CDR3 of the heavy chain of SEQ ID NOs: 151-303, or at least one of CDR1, CDR2 or CDR3 of the light chain of SEQ ID NOs: 1-150, as paired in Table 3.

[00291] In one embodiment, the heavy chain comprises an amino acid sequence identified as a heavy chain CDR3 sequence. Such a "heavy chain CDR3 sequence" (HCDR3) includes an amino acid sequence identified as a heavy chain CDR3 sequence set out in Table 2 and SEQ ID NOs: 151-303. Alternatively, the HCDR3 sequence comprises an amino acid sequence that contains one or more amino acid changes compared to any HCDR3 amino acid sequence identified in Table 2, i.e., a substitution, insertion or deletion. Preferable substitutions include a substitution to an amino acid at the corresponding position within another HCDR3 of Table 2. Alternatively, the HCDR3 sequence may comprise a consensus amino acid sequence of the HCDR3 described herein.

[00292] Alternatively, the heavy chain comprising a HCDR3 sequence of the invention described above may further comprise a "heavy chain CDR2 sequence" (HCDR2) of the invention, which includes any of the amino acid sequences identified as an HCDR2 in SEQ ID NO: 151-303 and Table 2, amino acid sequences that contain one or more amino acid changes compared to any HCDR2 identified in SEQ ID NO: 151-303 and Table 2, preferably a substitution to an amino acid at the corresponding position within another HCDR2 of Table 2, or a consensus sequence of the HCDR2 described herein.

[00293] The heavy chain comprising a heavy chain CDR3 sequence of the invention described above may also comprise both (a) a heavy chain CDR1 sequence of the invention described above and (b) a heavy chain CDR2 sequence of the invention described above.

[00294] One aspect of the invention provides an antibody that binds target antigen comprising a heavy chain that comprises any one, two, and/or three of the heavy chain CDR sequences of the invention described below.

[00295] Any of the heavy chain CDR sequences described above may also include amino acids added to either end of the CDRs. Preparation of variants and derivatives of antibodies and antigen-binding compounds of the invention, including affinity maturation or preparation of variants or derivatives containing amino acid analogs, is described in further detail herein. Exemplary variants include those containing a conservative or non-conservative substitution of a corresponding amino acid within the amino acid sequence, or a replacement of an amino acid with a corresponding amino acid of a human antibody sequence.

[00296] Antibodies comprising any one of the heavy chains described above may further comprise a light chain, preferably a light chain that binds to target antigen, and most

preferably a light chain comprising light chain CDR sequences of the invention described below.

[00297] Another aspect of the invention provides an antibody that binds target antigen comprising a light chain that comprises any one, two, and/or three of the light chain CDR sequences of the invention described below.

[00298] Preferably the light chain comprises an amino acid sequence identified as a light chain CDR3 sequence. Such a "light chain CDR3 sequence" (LCDR3) includes an amino acid sequence identified as a light chain CDR3 sequence in Table 1 and within SEQ ID NOs: 1-150. Alternatively, the light chain CDR3 sequence comprises an amino acid sequence that contains one or more amino acid changes compared to any light chain CDR3 amino acid sequence identified in Table 1, i.e. a substitution, insertion or deletion. Preferable substitutions include a substitution to an amino acid at the corresponding position within another light chain CDR3 of Table 1. Alternatively, the light chain CDR3 sequence may comprise a consensus amino acid sequence of light chain CDR3 shown in Table 1.

[00299] The light chain comprising a light chain CDR3 sequence of the invention described above may further comprise a "light chain CDR1 sequence" of the invention, which includes any of the amino acid sequences identified as a light chain CDR1 in SEQ ID NO: 1-150 or Table 1, amino acid sequences that contain one or more amino acid changes compared to any light chain CDR1 identified in SEQ ID NO: 1-150 or Table 1, preferably a substitution to an amino acid at the corresponding position within another light chain CDR1 of Table 1, or a consensus sequence of light chain CDR1 described herein.

[00300] Alternatively, the light chain comprising a light chain CDR3 sequence of the invention described above may further comprise a "light chain CDR2 sequence" of the invention, which includes any of the amino acid sequences identified as a light chain CDR2 in SEQ ID NO: 1-150 or Table 1, amino acid sequences that contain one or more amino acid changes compared to any light chain CDR2 identified in Table 1, preferably a substitution to an amino acid at the corresponding position within another light chain CDR2 of SEQ ID NO: 1-150 or Table 1, or a consensus sequence of light chain CDR2 shown in Table 1.

[00301] In a related aspect, the invention contemplates a purified polypeptide comprising at least one HCDR of SEQ ID NO: 151-303 or LCDR of SEQ ID NO: 1-150, wherein the framework regions of the heavy chain variable region and the framework regions of the light chain variable region comprise framework regions from a human antibody. In another

embodiment, the framework regions of the heavy chain variable region and the framework regions of the light chain variable region are chemically altered by amino acid substitution to be more homologous to a human antibody sequence. For example, within each heavy chain framework region (H-FR1-4) it is contemplated that at least one, at least two, at least three, at least four, at least five, or at least six native framework region residues of the murine heavy chain variable region have been altered by amino acid substitution, and wherein within each light chain framework region (L-FR1-4), at least one, at least two, at least three, at least four, at least five or at least six native framework residues of the murine light chain variable region have been altered by amino acid substitution.

[00302] The light chain comprising a light chain CDR3 sequence of the invention described above may also comprise both (a) a light chain CDR1 sequence of the invention described above and (b) a light chain CDR2 sequence of the invention described above.

[00303] Antibodies comprising any one of the light chain variable regions described above may further comprise a heavy chain variable region, optionally paired as described in Table 3, preferably a heavy chain variable region that binds to target antigen, and most preferably a heavy chain variable region comprising heavy chain CDR sequences of the invention described above.

[00304] In one aspect, the antibody binds to insulin receptor or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant K_D of 10^{-5}M or less that is capable of strengthening the binding affinity between insulin and insulin receptor by about 5-fold to 200-fold. In one embodiment, the antibody is a positive modulator antibody, e.g., that strengthens the binding affinity between insulin and insulin receptor. In some embodiments, the positive modulator antibody includes, but is not limited to Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or any polypeptide comprising one or more of the CDRs corresponding to any one of the above antibodies as set out in Tables 1 and 2, or in an antibody variable region set out in SEQ ID NOs: 76, 80, 101, 128, 132 and SEQ ID NOs: 291, 196, 239, 267, 271.

[00305] In further embodiments, the positive modulator antibody binds to insulin receptor, the insulin/insulin receptor complex, or binds both insulin receptor and the insulin/insulin receptor complex. In a related embodiment, the positive modulator antibody that binds to insulin receptor or insulin/insulin receptor complex, or both, includes, but is not limited to

Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083 or any polypeptide comprising one or more of the CDRs corresponding to any one of the above antibodies as set out in Tables 1 and 2, or in an antibody variable region set out in SEQ ID NOs: 76, 80, 101 and SEQ ID NOs: 291, 196 and 239.

[00306] In a further embodiment, the positive modulator antibody binds to the insulin/insulin receptor complex but not detectably to uncomplexed insulin receptor. In a related embodiment, the positive modulator antibody that binds to the insulin/insulin receptor complex includes, but is not limited to, Ab059, Ab078, Ab085 or any polypeptide comprising one or more of the CDRs corresponding to any one of the above antibodies as set out in Tables 1 and 2, or in an antibody variable region set out in SEQ ID NOs: 128, 132 and SEQ ID NOs: 267 and 271.

[00307] In a related aspect, the antibody is an agonist antibody. In one embodiment, the antibody is an agonist antibody that binds to insulin receptor with an affinity of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10} , 10^{-11} M or less, optionally that exhibits maximal agonist activity that is 20%-100% that of insulin's maximal agonist activity when measured in pAKT assay. In a further embodiment, the antibody is an allosteric agonist antibody that binds to insulin receptor with an affinity of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10} , 10^{-11} M or less and (a) exhibits maximal agonist activity that is 20%-80% that of insulin's maximal agonist activity when measured in pAKT assay, (b) when present does not alter the EC50 of insulin for INSR by more than 2-fold, and (c) when present does not alter the K_D of insulin for INSR by more than 2-fold.

[00308] In certain embodiments, the agonist antibody includes, but is not limited to, Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or any polypeptide comprising one or more of the CDR corresponding to any one of the above antibodies as set out in Tables 1 and 2, or in an antibody variable region set out in SEQ ID NOs: 7, 113, 114, 124, 126, 130 and SEQ ID NOs: 164, 252, 253, 263, 265 and 269.

[00309] In a further aspect, the antibody binds to insulin receptor or a complex comprising insulin and insulin receptor with an equilibrium dissociation constant K_D of 10^{-5}M or less that

is capable of weakening the binding affinity between insulin and insulin receptor by at least about 3-fold, optionally up to 1000-fold. In one embodiment, the antibody is a negative modulator antibody that weakens the binding affinity between insulin and the insulin receptor. In a related embodiment, the negative modulator antibody includes, but is not limited to the following antibodies: Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074 and Ab081.

[00310] In a further aspect, the antibody is an antibody that competes with any of the antibodies described herein for binding to the insulin receptor or insulin/insulin receptor complex. In certain embodiments, the antibody exhibits partial competition. In a related embodiment, partial competition is competition of about 30% to 70%, about 30% to 80%, or about 30%, 35%, 40%,45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80%. In some embodiments, the antibody exhibits complete competition. In one embodiment, complete competition is competition of greater than 70%, 75%, 80%, 85%, 90%, 95% or 100%. Exemplary assays for measuring antibody competition include, but are not limited to, receptor loading assays and epitope binning assays as described herein and in the art.

[00311] In one embodiment, the antibody exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab079, Ab076, Ab083, Ab080, Ab062, Ab020, Ab019, Ab088, and Ab089, and optionally, exhibit greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab086, Ab064, Ab001, and Ab018. In a further embodiment, the antibody optionally does not compete with one or more ofAb062 and Ab086. In certain embodiments, the antibody binds to both human and murine insulin receptor or complex.

[00312] In a further embodiment, the antibody that competes with an antibody described herein exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab040, Ab062, Ab030, Ab001, and Ab018, and optionally exhibit greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of AB037, Ab078, AB083, AB080, and AB085. In a related embodiment, the antibody does not compete with Ab053, Ab064, 83-7, Ab019, Ab088, and Ab089. Optionally, the antibody binds to human and murine insulin receptor or complex.

[00313] In a further embodiment, the antibody that competes with an antibody described herein exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab030, Ab037, Ab053, Ab001, Ab018, Ab064, Ab040, and optionally exhibit greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab085 and Ab086. Optionally, the antibody exhibits no competition with Ab079, Ab076 and Ab088, and optionally binds to both human and murine insulin receptor or complex.

[00314] In a further embodiment, the antibody that competes with an antibody described herein that exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab064, Ab062, Ab085, and Ab078, and optionally exhibits no competition with Ab077, Ab001, Ab018, Ab030, Ab037, Ab079, Ab076, Ab083, Ab019, Ab088, Ab089, and Ab040. Optionally, the antibody binds both human and murine insulin receptor or complex.

[00315] In a further embodiment, the antibody that competes with an antibody described herein exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab079, Ab076, Ab083, Ab080, Ab062, Ab020, Ab019, Ab088, Ab089. Optionally, the antibody does not exhibit competition with Ab062, Ab086, Ab001, Ab018, Ab030, Ab037, Ab064; and optionally, the antibody is human reactive only, and does not bind murine insulin receptor or complex.

[00316] In a further embodiment, the antibody shows greater than or equal to 30% competition with any antibody. Optionally, the antibody shows greater than or equal to 30% competition with Ab061, and optionally has less than 30% competition with Ab019 and Ab074, optionally shows no competition with Ab088. Optionally, the antibody binds with both human and murine receptor or complex.

[00317] In yet another embodiment, the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 281, 278, 277, 209, 275, 223, 284, 276, and 236 and a light chain variable region selected from the group consisting of SEQ ID NOs: 141, 138, 137, 35, 135, 57, 144, 136, and 98.

[00318] In yet another embodiment, the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 195, 220, 303, 197, 208, 243, 245 and 251 and a light chain variable region selected from the group consisting of SEQ ID NOs: 77, 50, 90, 84, 34, 104, 106 and 112.

[00319] In yet another embodiment, the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 241, 279, 258, 155, and 228 and a light chain variable region selected from the group consisting of SEQ ID NOs: 103, 139, 119, 8, and 89.

Antibody Nucleic Acids of the Invention

[00320] The present invention also encompasses nucleic acid molecules encoding target specific antibodies as described above. In some embodiments, different nucleic acid molecules encode a heavy chain variable region and a light chain variable region of a target specific antibody. In other embodiments, the same nucleic acid molecule encodes a heavy chain and a light chain variable regions of a target specific antibody. In one embodiment, the nucleic acid encodes a target specific antibody of the invention.

[00321] In one aspect, a nucleic acid molecule of the invention comprises a nucleotide sequence that encodes the V_L amino acid sequence set out in any one of SEQ ID NOs: 1-150 or a portion thereof. In a related aspect, the V_L amino acid sequence is a consensus sequence. In some embodiments, the nucleic acid encodes the amino acid sequence of the light chain CDRs of said antibody. In some embodiments, said portion is a contiguous portion comprising CDR1-CDR3. In one embodiment, said portion comprises at least one, two or three of a light chain CDR1, CDR2, or CDR3 region.

[00322] In some embodiments, the nucleic acid molecule encodes a V_L amino acid sequence that is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96 97, 98 or 99% identical to a V_L amino acid sequence set out in SEQ ID NOs: 1-150. Nucleic acid molecules of the invention include nucleic acids that hybridize under highly stringent conditions.

[00323] It is further contemplated that a nucleic acid molecule of the invention comprises a nucleotide sequence that encodes the V_H amino acid sequence of any one of SEQ ID NO: 151-303, or a portion thereof. In a related aspect, the V_H amino acid sequence is a consensus sequence. In some embodiments, the nucleic acid encodes the amino acid sequence of the heavy chain CDRs of said antibody. In some embodiments, said portion is a contiguous portion comprising heavy chain CDR1-CDR3. In one embodiment, said portion comprises at least one, two or three of a heavy chain CDR1, CDR2, or CDR3 region.

[00324] In some embodiments, the nucleic acid molecule encodes a V_H amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical

to a V_H amino acid sequence set out in SEQ ID NOs: 151-303. Nucleic acid molecules of the invention include nucleic acids that hybridize under highly stringent conditions.

[00325] It is further contemplated that the nucleic acids of the invention encode a full-length light chain or heavy chain of an antibody comprising a heavy chain or light chain variable region set out in SEQ ID NOs:1-303 and optionally paired as described in Table 3, wherein a full-length light chain or full-length heavy chain comprises a light chain constant region or a heavy chain constant region, respectively.

[00326] The invention further contemplates nucleic acids encoding antibody variants and polypeptides comprising antigen binding regions of the invention as described above.

[00327] Methods of preparing and isolating polynucleotide encoding antibodies of the invention are well-known to those of skill in the art. A polynucleotide according to the invention can be joined to any of a variety of other nucleotide sequences by well-established recombinant DNA techniques (see Sambrook et al., (2d Ed.; 1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Useful nucleotide sequences for joining to polypeptides include an assortment of vectors, *e.g.*, plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art. Accordingly, the invention also provides a vector including a polynucleotide of the invention and a host cell containing the polynucleotide. In general, the vector contains an origin of replication functional in at least one organism, convenient restriction endonuclease sites, and a selectable marker for the host cell. Vectors according to the invention include expression vectors, replication vectors, probe generation vectors, sequencing vectors, and retroviral vectors. A host cell according to the invention can be a prokaryotic or eukaryotic cell and can be a unicellular organism or part of a multicellular organism. Large numbers of suitable vectors and promoters are known to those of skill in the art and are commercially available for generating the recombinant constructs of the present invention.

[00328] A variety of expression vector/host systems may be utilized to contain and express the coding sequence. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, phagemid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (*e.g.*, baculovirus); plant cell systems transfected with virus expression vectors (*e.g.*, Cauliflower Mosaic Virus, CaMV; Tobacco Mosaic Virus, TMV) or transformed with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmid); or even animal

cell systems. Mammalian cells that are useful in recombinant protein productions include, but are not limited to, VERO cells, HeLa cells, Chinese hamster ovary (CHO) cells, COS cells (such as COS-7), WI38, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562 and HEK 293 cells.

[00329] Polynucleotide variants and antibody fragments may be readily generated by a worker of skill to encode biologically active fragments, variants, or mutants of the naturally occurring antibody molecule that possess the same or similar biological activity to the naturally occurring antibody. This may be done by PCR techniques, cutting and digestion of DNA encoding the antibody heavy and light chain regions, and the like. For example, point mutagenesis, using PCR and other techniques well-known in the art, may be employed to identify with particularity which amino acid residues are important in particular activities associated with antibody activity. Thus, one of skill in the art will be able to generate single base changes in the DNA strand to result in an altered codon and a missense mutation.

Antibody Fragments

[00330] Antibody fragments comprise a portion of an intact full length antibody, preferably an antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules (e.g., scFv); multispecific antibody fragments such as bispecfic, trispecific, etc. antibodies (e.g., diabodies, triabodies, tetrabodies); minibody; chelating recombinant antibody; tribodies or bibodies; intrabodies; nanobodies; small modular immunopharmaceuticals (SMIP), binding-domain immunoglobulin fusion proteins; camelized antibodies; V_{HH} containing antibodies; and other polypeptides formed from antibody fragments. See for example Holliger & Hudson (Nat. Biotech. 23(9) 1126-36 (2005))

[00331] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, monovalent fragments consisting of the V_L, V_H, C_L and C_H domains each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region, that has two "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the Fv to form the desired structure for antigen binding, resulting in a single-chain

antibody (scFv), in which a V_L and V_H region are paired to form a monovalent molecule via a synthetic linker that enables them to be made as a single protein chain (Bird et al., *Science* 242:423-426, 1988, and Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988). For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 1 13, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). An Fd fragment consists of the V_H and C_H1 domains.

[00332] Additional antibody fragments include a domain antibody (dAb) fragment (Ward et al., *Nature* 341:544-546, 1989) which consists of a V_H domain. Diabodies are bivalent antibodies in which V_H and V_L domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., EP 404,097; WO 93/11161; Holliger et al., *Proc. Natl. Acad. Sci. USA* 90:6444-6448, 1993, and Poljak et al., *Structure* 2:1121-1123, 1994). Diabodies can be bispecific or monospecific.

[00333] Functional heavy-chain antibodies devoid of light chains are naturally occurring in nurse sharks (Greenberg et al., *Nature* 374:168-73, 1995), wobbegong sharks (Nuttall et al., *Mol Immunol.* 38:313-26, 2001) and *Camelidae* (Hamers-Casterman et al., *Nature* 363: 446-8, 1993; Nguyen et al., *J. Mol. Biol.* 275: 413, 1998), such as camels, dromedaries, alpacas and llamas. The antigen-binding site is reduced to a single domain, the VHH domain, in these animals. These antibodies form antigen-binding regions using only heavy chain variable region, i.e., these functional antibodies are homodimers of heavy chains only having the structure H_2L_2 (referred to as "heavy-chain antibodies" or "HCAbs"). Camelid V_{HH} reportedly recombines with IgG2 and IgG3 constant regions that contain hinge, CH2, and CH3 domains and lack a CH1 domain (Hamers-Casterman et al., *supra*). For example, llama IgG1 is a conventional (H_2L_2) antibody isotype in which V_H recombines with a constant region that contains hinge, CH1, CH2 and CH3 domains, whereas the llama IgG2 and IgG3 are heavy chain-only isotypes that lack CH1 domains and that contain no light chains. Camelid V_{HH} domains have been found to bind to antigen with high affinity (Desmyter et al., *J. Biol. Chem.* 276:26285-90, 2001) and possess high stability in solution (Ewert et al., *Biochemistry* 41:3628-36, 2002). Classical V_H-only fragments are difficult to produce in soluble form, but improvements in solubility and specific binding can be obtained when framework residues are altered to be more VH_H-like. (See, e.g., Reichman, et al., J Immunol Methods 1999, 231:25-38.) Methods for generating antibodies having camelid heavy chains

are described in, for example, in U.S. Patent Publication Nos. 20050136049 and 20050037421.

[00334] The variable domain of an antibody heavy-chain is has a molecular mass of 15 kDa, and is referred to as a nanobody (Cortez-Retamozo et al., *Cancer Research* 64:2853-57, 2004). A nanobody library may be generated from an immunized dromedary as described in Conrath et al., (*Antimicrob Agents Chemother* 45: 2807-12, 2001) or using recombinant methods as described in Revets et al, Expert Opin. Biol. Ther. 5(1): 111-24 (2005).

[00335] Production of bispecific Fab-scFv ("bibody") and trispecific Fab-(scFv)(2) ("tribody") are described in Schoonjans et al. (*J Immunol.* 165:7050-57, 2000) and Willems et al. (*J Chromatogr B Analyt Technol Biomed Life Sci.* 786:161-76, 2003). For bibodies or tribodies, a scFv molecule is fused to one or both of the VL-CL (L) and VH-CH_1 (Fd) chains, e.g., to produce a tribody two scFvs are fused to C-term of Fab while in a bibody one scFv is fused to C-term of Fab.

[00336] A "minibody" consisting of scFv fused to CH3 via a peptide linker (hingeless) or via an IgG hinge has been described in Olafsen, et al., Protein Eng Des Sel. 2004 Apr;17(4):315-23.

[00337] Intrabodies are single chain antibodies which demonstrate intracellular expression and can manipulate intracellular protein function (Biocca, et al., *EMBO J.* 9:101–108, 1990; Colby et al., *Proc Natl Acad Sci U S A.* 101:17616-21, 2004). Intrabodies, which comprise cell signal sequences which retain the antibody construct in intracellular regions, may be produced as described in Mhashilkar et al (*EMBO J* 14:1542-51, 1995) and Wheeler et al. (*FASEB J.* 17:1733-5. 2003). Transbodies are cell-permeable antibodies in which a protein transduction domain (PTD) is fused with single chain variable fragment (scFv) antibodies Heng et al., (*Med Hypotheses.* 64:1105-8, 2005).

[00338] Further contemplated are antibodies that are SMIPs or binding domain immunoglobulin fusion proteins specific for an antigen. These constructs are single-chain polypeptides comprising antigen binding domains fused to immunoglobulin domains necessary to carry out antibody effector functions. See e.g., WO03/041600, U.S. Patent publication 20030133939 and US Patent Publication 20030118592.

[00339] One or more CDRs may be incorporated into a molecule either covalently or noncovalently to make it an immunoadhesin. An immunoadhesin may incorporate the CDR(s) as part of a larger polypeptide chain, may covalently link the CDR(s) to another

polypeptide chain, or may incorporate the CDR(s) noncovalently. The CDRs permit the immunoadhesin to specifically bind to a particular antigen of interest.

[00340] In yet another embodiment, the antibody or antigen-binding compound comprises a constant region and one or more heavy and light chain variable framework regions of a human antibody sequence. In a related embodiment, the antibody comprises a modified or unmodified constant region of a human IgG1, IgG2, IgG3 or IgG4.

[00341] Alternatively, antibody fragments may be fused to a protein scaffold. Libraries of protein scaffolds include, but are not limited to, Adnectins, Affibodies, Anticalins, DARPins, engineered Kunitz-type inhibitors, tetranectins, A-domain proteins, lipocalins, repeat proteins such as ankyrin repeat proteins, immunity proteins, α2p8 peptide, insect defensin A, PDZ domains, charybdotoxins, PHD fingers, TEM-1 β-lactamase, fibronectin type III domains, CTLA-4, T-cell resptors, knottins, neocarzinostatin, carbohydrate binding module 4-2, green fluorescent protein, thioredoxin (Gebauer & Skerra, Curr. Opin. Chem. Biol. 13:245-55 (2009); Gill & Damle, Curr. Opin. Biotech 17: 653-58 (2006); Hosse et al, Protein Sci. 15:14-27 (2006); Skerra, Curr. Opin. Biotech 18: 295-3-4 (2007)).

[00342] Thus, a variety of compositions comprising one, two, and/or three CDRs of a heavy chain variable region or a light chain variable region of an antibody may be generated by techniques known in the art.

Multispecific Antibodies

[00343] In some embodiments, it may be desirable to generate multispecific (e.g. bispecific) antibodies of the invention having binding specificities for at least two different epitopes of the same or different molecules. Exemplary bispecific antibodies may bind to two different epitopes of the antigen. Alternatively, an antigen-specific antibody arm may be combined with an arm which binds to a cell surface molecule, such as a T-cell receptor molecule (e.g., CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the desired antigen. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express or take up the desired antigen. These antibodies possess an antigen-binding arm and an arm which binds the cytotoxic agent (e.g., saporin, anti-interferon-60, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g., F(ab')2 bispecific antibodies).

[00344] According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published Sep. 6, 1996.

[00345] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[00346] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., (*Science* 229:81-83, 1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from E. coli can be chemically coupled in vitro to form bispecific antibodies. (Shalaby et al., *J. Exp. Med.* 175:217-225 (1992))

[00347] Shalaby et al., J. Exp. Med. 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E.coli* and subjected to directed chemical coupling in vitro to form the bispecfic antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the

HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor antigens.

[00348] Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. (Kostelny et al., *J. Immunol.* 148:1547-1553, 1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Holliger et al. (*Proc. Natl. Acad. Sci. USA* 90:6444-48, 1993) has provided an alternative mechanism for making bispecific antibody fragments.

[00349] The fragments comprise a heavy chain variable region (V_H) connected to a light-chain variable region (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (scFv) dimers has also been reported. *See* Gruber et al., *J. Immunol.* 152: 5368 (1994).

[00350] Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata et al. Protein Eng. 8:1057-62 (1995). Linear antibodies comprise a pair of tandem Fd segments (V_H -C_H1-V_H -C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[00351] In a further embodiment, the bispecific antibody may be a chelating recombinant antibody (CRAb). A chelating recombinant antibody recognizes adjacent and non-overlapping epitopes of the antigen, and is flexible enough to bind to both epitopes simultaneously (Neri et al., *J Mol Biol.* 246:367-73, 1995).

[00352] Antibodies with more than two valencies are also contemplated. For example, trispecific antibodies can be prepared. (Tutt et al., *J. Immunol.* 147:60, 1991).

Chimeric and humanized antibodies

[00353] Because chimeric or humanized antibodies are less immunogenic in humans than the parental mouse monoclonal antibodies, they can be used for the treatment of humans with far less risk of anaphylaxis.

[00354] Chimeric monoclonal antibodies, in which the variable Ig domains of a mouse monoclonal antibody are fused to human constant Ig domains, can be generated using standard procedures known in the art (See Morrison et al., *Proc. Natl. Acad. Sci. USA* 81, 6841-6855 (1984); and, Boulianne et al, *Nature* 312, 643-646, (1984)). Although some chimeric monoclonal antibodies have proved less immunogenic in humans, the mouse variable Ig domains can still lead to a significant human anti-mouse response.

[00355] Humanized antibodies may be achieved by a variety of methods including, for example: (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as humanizing through "CDR grafting") (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering"), or, alternatively, (3) substituting human amino acids at positions determined to be unlikely to adversely effect either antigen binding or protein folding, but likely to reduce immunogenicity in a human environment (a process referred to in the art as HUMAN ENGINEERING$^{TM)}$. In the present invention, humanized antibodies will include both "humanized", "veneered" and "HUMAN ENGINEERED^{TM}" antibodies. These methods are disclosed in, e.g., Jones et al., *Nature* 321:522 525 (1986); Morrison et al., *Proc. Natl. Acad. Sci., U.S.A.*, 81:6851-6855 (1984); Morrison and Oi, *Adv. Immunol.*, 44:65-92 (1988); Verhoeyer et al., *Science* 239:1534-1536 (1988); Padlan, *Molec. Immun.* 28:489-498 (1991); Padlan, *Molec. Immunol.* 31:169-217 (1994); Kettleborough et al., *Protein Eng.* 4:773-783 (1991); Studnicka et al. U.S. Patent No. 5,766,886; Studnicka et al., (*Protein Eng* 7: 805-814, 1994) each of which is incorporated herein by reference.

Human antibodies from transgenic animals

[00356] Human antibodies to antigen can also be produced using transgenic animals that have no endogenous immunoglobulin production and are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and light chain loci. WO 91/00906 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin encoding loci are substituted or inactivated. WO 96/30498 and US Patent No. 6,091,001 disclose the use of the Cre/Lox system to modify the immunoglobulin locus in a mammal, such as to replace all or a portion of the constant or

variable region to form a modified antibody molecule. WO 94/02602 discloses non-human mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Patent No. 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy chains, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions. See also, U.S. Patent Nos. 6,114,598 6,657,103 and 6,833,268.

[00357] Using a transgenic animal described above, an immune response can be produced to a selected antigen, and antibody producing cells can be removed from the animal and used to produce hybridomas that secrete human monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735. This publication discloses monoclonal antibodies against a variety of antigens including IL-6, IL-8, TNFa, human CD4, L selectin, gp39, and tetanus toxin. The monoclonal antibodies can be tested for the ability to inhibit or neutralize the biological activity or physiological effect of the corresponding protein. WO 96/33735 discloses that monoclonal antibodies against IL-8, derived from immune cells of transgenic mice immunized with IL-8, blocked IL-8 induced functions of neutrophils. Human monoclonal antibodies with specificity for the antigen used to immunize transgenic animals are also disclosed in WO 96/34096 and U.S. patent application no. 20030194404; and U.S. patent application no. 20030031667.

[00358] Additional transgenic animals useful to make monoclonal antibodies include the Medarex HuMAb-MOUSE®, described in U.S. Pat. No. 5,770,429 and Fishwild, et al. (*Nat. Biotechnol.* 14:845-851 (1996)), which contains gene sequences from unrearranged human antibody genes that code for the heavy and light chains of human antibodies. Immunization of a HuMAb-MOUSE® enables the production of fully human monoclonal antibodies to the antigen.

[00359] Also, Ishida et al. (*Cloning Stem Cells.* 4:91-102 (2002)) describes the TransChromo Mouse (TCMOUSE™) which comprises megabase-sized segments of human DNA and which incorporates the entire human immunoglobulin (hIg) loci. The TCMOUSE™ has a fully diverse repertoire of hIgs, including all the subclasses of IgGs (IgG1-G4). Immunization of the TCMOUSE™ with various human antigens produces antibody responses comprising human antibodies.

[00360] See also Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immunol.*, 7:33 (1993); and U.S. Pat. No. 5,591,669, U.S. Patent No. 5,589,369, U.S. Patent No. 5,545,807; and U.S. Patent Publication No. 20020199213. U.S. Patent Publication No. 20030092125 describes methods for biasing the immune response of an animal to the desired epitope. Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

Human antibodies from display technology

[00361] The development of technologies for making repertoires of recombinant human antibody genes, and the display of the encoded antibody fragments on the surface of filamentous bacteriophage, has provided a means for making human antibodies directly. The antibodies produced by phage technology are produced as antigen binding fragments-usually Fv or Fab fragments-in bacteria and thus lack effector functions. Effector functions can be introduced by one of two strategies: The fragments can be engineered either into complete antibodies for expression in mammalian cells, or into bispecific antibody fragments with a second binding site capable of triggering an effector function.

[00362] The invention contemplates a method for producing antigen-specific antibody or antigen-binding portion thereof comprising the steps of synthesizing a library of human antibodies on phage, screening the library with antigen or a portion thereof, isolating phage that bind antigen, and obtaining the antibody from the phage. By way of example, one method for preparing the library of antibodies for use in phage display techniques comprises the steps of immunizing a non-human animal comprising human immunoglobulin loci with antigen or an antigenic portion thereof to create an immune response, extracting antibody producing cells from the immunized animal; isolating RNA from the extracted cells, reverse transcribing the RNA to produce cDNA, amplifying the cDNA using a primer, and inserting the cDNA into a phage display vector such that antibodies are expressed on the phage. Recombinant antigen-specific antibodies of the invention may be obtained in this way. In another example, antibody producing cells can be extracted from non-immunized animals, RNA isolated from the extracted cells and reverse transcribed to produce cDNA, which is amplified using a primer, and inserted into a phage display vector such that antibodies are expressed on the phage. Phage-display processes mimic immune selection through the display of antibody repertoires on the surface of filamentous bacteriophage, and subsequent selection of phage by their binding to an antigen of choice. One such technique is described

in WO 99/10494, which describes the isolation of high affinity and functional agonistic antibodies for MPL and msk receptors using such an approach. Antibodies of the invention can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human V_L and V_H cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. See e.g., U.S. Patent No. 5,969,108. There are commercially available kits for generating phage display libraries (e.g., the Pharmacia Recombinant Phage Antibody System, catalog no. 27-9400-01; and the Stratagene SurfZAP.TM. phage display kit, catalog no. 240612). There are also other methods and reagents that can be used in generating and screening antibody display libraries (see, e.g., Ladner et al. U.S. Pat. No. 5,223,409; Kang et al. PCT Publication No. WO 92/18619; Dower et al. PCT Publication No. WO 91/17271; Winter et al. PCT Publication No. WO 92/20791; Markland et al. PCT Publication No. WO 92/15679; Breitling et al. PCT Publication No. WO 93/01288; McCafferty et al. PCT Publication No. WO 92/01047; Garrard et al. PCT Publication No. WO 92/09690; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) Science 246:1275-1281; McCafferty et al., *Nature* (1990) 348:552-554; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J. Mol. Biol.* 226:889-896; Clackson et al. (1991) Nature 352:624-628; Gram et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) Nuc Acid Res 19:4133-4137; and Barbas et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982.

[00363] In one embodiment, to isolate human antibodies specific for an antigen, with the desired binding characteristics, a human V_H and V_L library are screened to select for antibody fragments having the desired specificity. The antibody libraries used in this method are preferably scFv libraries prepared and screened as described herein and in the art (McCafferty et al., PCT Publication No. WO 92/01047, McCafferty et al., (*Nature* 348:552-554 (1990)); and Griffiths et al., (*EMBO J* 12:725-734 (1993)). The scFv antibody libraries preferably are screened using the antigen.

[00364] Alternatively, the Fd fragment (V_H-C_H1) and light chain (V_L-C_L) of antibodies are separately cloned by PCR and recombined randomly in combinatorial phage display libraries, which can then be selected for binding to a particular antigen. The Fab fragments are expressed on the phage surface, i.e., physically linked to the genes that encode them. Thus, selection of Fab by antigen binding co-selects for the Fab encoding sequences, which can be

amplified subsequently. Through several rounds of antigen binding and re-amplification, a procedure termed panning, Fab specific for the antigen are enriched and finally isolated.

[00365] In 1994, an approach for the humanization of antibodies, called "guided selection", was described. Guided selection utilizes the power of the phage display technique for the humanization of mouse monoclonal antibody (See Jespers, L. S., et al., *Bio/Technology* 12, 899-903 (1994)). For this, the Fd fragment of the mouse monoclonal antibody can be displayed in combination with a human light chain library, and the resulting hybrid Fab library may then be selected with antigen. The mouse Fd fragment thereby provides a template to guide the selection. Subsequently, the selected human light chains are combined with a human Fd fragment library. Selection of the resulting library yields entirely human Fab.

[00366] A variety of procedures have been described for deriving human antibodies from phage-display libraries (See, for example, Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol*, 222:581-597 (1991); U.S. Pat. Nos. 5,565,332 and 5,573,905; Clackson, T., and Wells, J. A., *TIBTECH* 12, 173-184 (1994)). In particular, in vitro selection and evolution of antibodies derived from phage display libraries has become a powerful tool (See Burton, D. R., and Barbas III, C. F., *Adv. Immunol.* 57, 191-280 (1994); Winter, G., et al., *Annu. Rev. Immunol.* 12, 433-455 (1994); U.S. patent publication no. 20020004215 and WO 92/01047; U.S. patent publication no. 20030190317; and U.S. Patent Nos. 6,054,287 and 5,877,293.

[00367] Watkins, "Screening of Phage-Expressed Antibody Libraries by Capture Lift," Methods in Molecular Biology, Antibody Phage Display: Methods and Protocols 178:187-193 (2002), and U.S. patent publication no. 20030044772, published March 6, 2003, describe methods for screening phage-expressed antibody libraries or other binding molecules by capture lift, a method involving immobilization of the candidate binding molecules on a solid support.

[00368] Fv fragments are displayed on the surface of phage, by the association of one chain expressed as a phage protein fusion (e.g., with M13 gene III) with the complementary chain expressed as a soluble fragment. It is contemplated that the phage may be a filamentous phage such as one of the class I phages: fd, M13, f1, If1, lke, ZJ/Z, Ff and one of the class II phages Xf, Pf1 and Pf3. The phage may be M13, or fd or a derivative thereof.

[00369] Once initial human V_L and V_H segments are selected, "mix and match" experiments, in which different pairs of the initially selected V_L and V_H segments are screened for antigen binding, may be performed to select preferred V_L/V_H pair combinations. Additionally, to further improve the quality of the antibody, the V_L and V_H segments of the preferred V_L/V_H pair(s) can be randomly mutated, preferably within the any of the CDR1, CDR2 or CDR3 region of V_H and/or V_L, in a process analogous to the in vivo somatic mutation process responsible for affinity maturation of antibodies during a natural immune response. This in vitro affinity maturation can be accomplished by amplifying V_L and V_H regions using PCR primers complimentary to the V_H CDR1, CDR2, and CDR3, or V_L CDR1, CDR2, and CDR3, respectively, which primers have been "spiked" with a random mixture of the four nucleotide bases at certain positions such that the resultant PCR products encode V_L and V_H segments into which random mutations have been introduced into the V_H and/or V_L CDR3 regions. These randomly mutated V_L and V_H segments can be rescreened for binding to antigen.

[00370] Following screening and isolation of an antigen-specific antibody from a recombinant immunoglobulin display library, nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques. If desired, the nucleic acid can be further manipulated to create other antibody forms of the invention, as described below. To express a recombinant human antibody isolated by screening of a combinatorial library, the DNA encoding the antibody is cloned into a recombinant expression vector and introduced into a mammalian host cell, as described herein.

[00371] It is contemplated that the phage display method may be carried out in a mutator strain of bacteria or host cell. A mutator strain is a host cell which has a genetic defect which causes DNA replicated within it to be mutated with respect to its parent DNA. Example mutator strains are NR9046mutD5 and NR9046 mut T1.

[00372] It is also contemplated that the phage display method may be carried out using a helper phage. This is a phage which is used to infect cells containing a defective phage genome and which functions to complement the defect. The defective phage genome can be a phagemid or a phage with some function encoding gene sequences removed. Examples of helper phages are M13K07, M13K07 gene III no. 3, hyperphage; and phage displaying or encoding a binding molecule fused to a capsid protein.

[00373] Antibodies may also be generated via phage display screening methods using the hierarchical dual combinatorial approach as disclosed in WO 92/01047 in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain specific binding member is selected in accordance with phage display techniques such as those described therein. This technique is also disclosed in Marks *et al*, (*Bio/Technology*, 10:779-783 (1992)).

[00374] Methods for display of polypeptides on the surface of viruses, yeast, microbial and mammalian cells have also been used to identify antigen specific antibodies. See, for example, U.S. Patent Nos. 5,348,867; 5,723,287; 6,699,658; Wittrup, Curr Op. Biotech. 12:395-99 (2001); Lee et al, Trends in Biotech. 21(1) 45-52 (2003); Surgeeva et al, Adv. Drug Deliv. Rev. 58: 1622-54 (2006). Antibody libraries may be attached to yeast proteins, such as agglutinin, effectively mimicking the cell surface display of antibodies by B cells in the immune system.

[00375] In addition to phage display methods, antibodies may be isolated using in vitro display methods including ribosome display and mRNA display (Amstutz et al, Curr. Op. Biotech. 12: 400-05 (2001)). Selection of polypeptide using ribosome display is described in Hanes et al., (*Proc. Natl Acad Sci USA*, 94:4937-4942 (1997)) and U.S. Pat. Nos. 5,643,768 and 5,658,754 issued to Kawasaki. Ribosome display is also useful for rapid large scale mutational analysis of antibodies. The selective mutagenesis approach also provides a method of producing antibodies with improved activities that can be selected using ribosomal display techniques.

Altered glycosylation

[00376] Antibody variants can also be produced that have a modified glycosylation pattern relative to the parent antibody, for example, deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

[00377] Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. The presence of either of these tripeptide

sequences in a polypeptide creates a potential glycosylation site. Thus, N-linked glycosylation sites may be added to an antibody by altering the amino acid sequence such that it contains one or more of these tripeptide sequences. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. O-linked glycosylation sites may be added to an antibody by inserting or substituting one or more serine or threonine residues to the sequence of the original antibody.

[00378] Fc glycans influence the binding of IgG to Fc receptors and C1q, and are therefore important for IgG effector functions. Antibody variants with modified Fc glycans and altered effector function may be produced. For example, antibodies with modified terminal sugars such as sialic acids, core fucose, bisecting N-acetylglucosamine, and mannose residues may have altered binding to the FcγRIIIa receptor and altered ADCC activity. In a further example, antibodies with modified terminal galactose residues may have altered binding to C1q and altered CDC activity (Raju, Curr. Opin. Immunol. 20: 471-78 (2008).

[00379] Also contemplated are antibody molecules with absent or reduced fucosylation that exhibit improved ADCC activity. A variety of ways are known in the art to accomplish this. For example, ADCC effector activity is mediated by binding of the antibody molecule to the FcγRIII receptor, which has been shown to be dependent on the carbohydrate structure of the N-linked glycosylation at the Asn-297 of the CH2 domain. Non-fucosylated antibodies bind this receptor with increased affinity and trigger FcγRIII-mediated effector functions more efficiently than native, fucosylated antibodies. For example, recombinant production of non-fucosylated antibody in CHO cells in which the alpha-1,6-fucosyl transferase enzyme has been knocked out results in antibody with 100-fold increased ADCC activity (Yamane-Ohnuki et al., *Biotechnol Bioeng.* 87:614-22 (2004)). Similar effects can be accomplished through decreasing the activity of this or other enzymes in the fucosylation pathway, e.g., through siRNA or antisense RNA treatment, engineering cell lines to knockout the enzyme(s), or culturing with selective glycosylation inhibitors (Rothman et al., *Mol Immunol.* 26:1113-23 (1989)). Some host cell strains, e.g. Lec13 or rat hybridoma YB2/0 cell line naturally produce antibodies with lower fucosylation levels. (Shields et al., *J Biol Chem.* 277:26733-40 (2002); Shinkawa et al., *J Biol Chem.* 278:3466-73 (2003)). An increase in the level of bisected carbohydrate, e.g. through recombinantly producing antibody in cells that overexpress GnTIII enzyme, has also been determined to increase ADCC activity (Umana et

al., *Nat Biotechnol.* 17:176-80 (1999)). It has been predicted that the absence of only one of the two fucose residues may be sufficient to increase ADCC activity (Ferrara et al., *Biotechnol Bioeng.* 93:851-61 (2006)).

Variants with altered effector function

[00380] Other modifications of the antibody are contemplated. In one aspect, it may be desirable to modify the antibody of the invention with respect to effector function, for example, to enhance the effectiveness of the antibody in treating cancer (Natsume et al, Drug Design Dev't & Ther. 3: 7-16 (2009). Exemplary effector functions include Clq binding; CDC; Fc receptor binding; ADCC; phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. One method for modifying effector function teaches that cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). *See* Caron et al., (*J. Exp Med.* 176: 1191-1195 (1992)) and Shopes, B. (*J. Immunol.* 148: 2918-2922 (1992)). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., (*Cancer Research* 53: 2560-2565 (1993)). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. *See* Stevenson et al., (*Anti-Cancer Drug Design 3*: 219-230 (1989)). In addition, it has been shown that sequences within the CDR can cause an antibody to bind to MHC Class II and trigger an unwanted helper T-cell response. A conservative substitution can allow the antibody to retain binding activity yet lose its ability to trigger an unwanted T-cell response. Also see Steplewski et al., (*Proc Natl Acad Sci U S A.* 85:4852-56 (1998)), which described chimeric antibodies wherein a murine variable region was joined with human gamma 1, gamma 2, gamma 3, and gamma 4 constant regions.

[00381] In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half-life, for example, adding molecules such as PEG or other water soluble polymers, including polysaccharide polymers, to antibody fragments to increase the half-life. This may also be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g., by mutation of the appropriate region in the antibody fragment or by

incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis) (see, e.g., WO96/32478).

[00382] The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of an Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or VH region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment. See also International applications WO 97/34631 and WO 96/32478 which describe Fc variants and their interaction with the salvage receptor.

[00383] Thus, antibodies of the invention may comprise a human Fc portion, a human consensus Fc portion, or a variant thereof that retains the ability to interact with the Fc salvage receptor, including variants in which cysteines involved in disulfide bonding are modified or removed, and/or in which the a met is added at the N-terminus and/or one or more of the N-terminal 20 amino acids are removed, and/or regions that interact with complement, such as the C1q binding site, are removed, and/or the ADCC site is removed [see, e.g., Sarmay et al., *Molec. Immunol.* 29:633-9 (1992)].

[00384] Previous studies mapped the binding site on human and murine IgG for FcR primarily to the lower hinge region composed of IgG residues 233-239. Other studies proposed additional broad segments, e.g. Gly316-Lys338 for human Fc receptor I, Lys274-Arg301 and Tyr407-Arg416 for human Fc receptor III, or found a few specific residues outside the lower hinge, e.g., Asn297 and Glu318 for murine IgG2b interacting with murine Fc receptor II. The report of the 3.2-Å crystal structure of the human IgG1 Fc fragment with human Fc receptor IIIA delineated IgG1 residues Leu234-Ser239, Asp265-Glu269, Asn297-Thr299, and Ala327-Ile332 as involved in binding to Fc receptor IIIA. It has been suggested based on crystal structure that in addition to the lower hinge (Leu234-Gly237), residues in IgG CH2 domain loops FG (residues 326-330) and BC (residues 265-271) might play a role in binding to Fc receptor IIA. See Shields et al., (*J. Biol. Chem.*, 276:6591-604 (2001)), incorporated by reference herein in its entirety. Mutation of residues within Fc receptor binding sites can result in altered effector function, such as altered ADCC or CDC activity, or altered half-life. As described above, potential mutations include insertion, deletion or substitution of one or more residues, including substitution with alanine, a conservative

substitution, a non-conservative substitution, or replacement with a corresponding amino acid residue at the same position from a different IgG subclass (e.g. replacing an IgG1 residue with a corresponding IgG2 residue at that position).

[00385] Shields et al. reported that IgG1 residues involved in binding to all human Fc receptors are located in the CH2 domain proximal to the hinge and fall into two categories as follows: 1) positions that may interact directly with all FcR include Leu234-Pro238, Ala327, and Pro329 (and possibly Asp265); 2) positions that influence carbohydrate nature or position include Asp265 and Asn297. The additional IgG1 residues that affected binding to Fc receptor II are as follows: (largest effect) Arg255, Thr256, Glu258, Ser267, Asp270, Glu272, Asp280, Arg292, Ser298, and (less effect) His268, Asn276, His285, Asn286, Lys290, Gln295, Arg301, Thr307, Leu309, Asn315, Lys322, Lys326, Pro331, Ser337, Ala339, Ala378, and Lys414. A327Q, A327S, P329A, D265A and D270A reduced binding. In addition to the residues identified above for all FcR, additional IgG1 residues that reduced binding to Fc receptor IIIA by 40% or more are as follows: Ser239, Ser267 (Gly only), His268, Glu293, Gln295, Tyr296, Arg301, Val303, Lys338, and Asp376. Variants that improved binding to FcRIIIA include T256A, K290A, S298A, E333A, K334A, and A339T. Lys414 showed a 40% reduction in binding for FcRIIA and FcRIIB, Arg416 a 30% reduction for FcRIIA and FcRIIIA, Gln419 a 30% reduction to FcRIIA and a 40% reduction to FcRIIB, and Lys360 a 23% improvement to FcRIIIA. See also Presta et al., (*Biochem. Soc. Trans.* 30:487–490, 2001), incorporated herein by reference in its entirety, which described several positions in the Fc region of IgG1 were found which improved binding only to specific Fc gamma receptors (R) or simultaneously improved binding to one type of Fc gamma R and reduced binding to another type. Selected IgG1 variants with improved binding to Fc gamma RIIIa were then tested in an in vitro antibody-dependent cellular cytotoxicity (ADCC) assay and showed an enhancement in ADCC when either peripheral blood mononuclear cells or natural killer cells were used.

[00386] For example, U.S. Patent No. 6,194,551, incorporated herein by reference in its entirety, describes variants with altered effector function containing mutations in the human IgG Fc region, at amino acid position 329, 331 or 322 (using Kabat numbering), some of which display reduced C1q binding or CDC activity. As another example, U.S. Patent No. 6,737,056, incorporated herein by reference in its entirety, describes variants with altered effector or Fc-gamma-receptor binding containing mutations in the human IgG Fc region, at amino acid position 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270,

272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439 (using Kabat numbering), some of which display receptor binding profiles associated with reduced ADCC or CDC activity. Of these, a mutation at amino acid position 238, 265, 269, 270, 327 or 329 are stated to reduce binding to FcRI, a mutation at amino acid position 238, 265, 269, 270, 292, 294, 295, 298, 303, 324, 327, 329, 333, 335, 338, 373, 376, 414, 416, 419, 435, 438 or 439 are stated to reduce binding to FcRII, and a mutation at amino acid position 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 293, 294, 295, 296, 301, 303, 322, 327, 329, 338, 340, 373, 376, 382, 388, 389, 416, 434, 435 or 437 is stated to reduce binding to FcRIII.

[00387] U.S. Patent No. 5,624,821, incorporated by reference herein in its entirety, reports that Clq binding activity of an murine antibody can be altered by mutating amino acid residue 318, 320 or 322 of the heavy chain and that replacing residue 297 (Asn) results in removal of lytic activity.

[00388] U.S. Patent Publication No. 20040132101, incorporated by reference herein in its entirety, describes variants with mutations at amino acid positions 240, 244, 245, 247, 262, 263, 266, 299, 313, 325, 328, or 332 (using Kabat numbering) or positions 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 267, 269, 296, 297, 298, 299, 313, 325, 327, 328, 329, 330, or 332 (using Kabat numbering), of which mutations at positions 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 267, 269, 296, 297, 298, 299, 313, 325, 327, 328, 329, 330, or 332 may reduce ADCC activity or reduce binding to an Fc gamma receptor.

[00389] Chappel et al. (*Proc Natl Acad Sci U S A.* 88:9036-40 (1991)), incorporated herein by reference in its entirety, report that cytophilic activity of IgG1 is an intrinsic property of its heavy chain CH2 domain. Single point mutations at any of amino acid residues 234-237 of IgG1 significantly lowered or abolished its activity. Substitution of all of IgG1 residues 234-237 (LLGG) into IgG2 and IgG4 were required to restore full binding activity. An IgG2 antibody containing the entire ELLGGP sequence (residues 233-238) was observed to be more active than wild-type IgG1.

[00390] Isaacs et al. (*J Immunol.* 161:3862-9 (1998)), incorporated herein by reference in its entirety, report that mutations within a motif critical for Fc gammaR binding (glutamate

233 to proline, leucine/phenylalanine 234 to valine, and leucine 235 to alanine) completely prevented depletion of target cells. The mutation glutamate 318 to alanine eliminated effector function of mouse IgG2b and also reduced the potency of human IgG4.

[00391] Armour et al. (*Mol Immunol.* 40:585-93 (2003)), incorporated by reference herein in its entirety, identified IgG1 variants which react with the activating receptor, FcgammaRIIa, at least 10-fold less efficiently than wildtype IgG1 but whose binding to the inhibitory receptor, FcgammaRIIb, is only four-fold reduced. Mutations were made in the region of amino acids 233-236 and/or at amino acid positions 327, 330 and 331. See also WO 99/58572, incorporated by reference herein in its entirety.

[00392] Xu et al. (*J Biol Chem.* 269:3469-74 (1994)), incorporated by reference herein in its entirety, report that mutating IgG1 Pro331 to Ser markedly decreased C1q binding and virtually eliminated lytic activity. In contrast, the substitution of Pro for Ser331 in IgG4 bestowed partial lytic activity (40%) to the IgG4 Pro331 variant.

[00393] Schuurman et al. (*Mol Immunol.* 38:1-8 (2001)), incorporated by reference herein in its entirety, report that mutating one of the hinge cysteines involved in the inter-heavy chain bond formation, Cys226, to serine resulted in a more stable inter-heavy chain linkage. Mutating the IgG4 hinge sequence Cys-Pro-Ser-Cys to the IgG1 hinge sequence Cys-Pro-Pro-Cys also markedly stabilizes the covalent interaction between the heavy chains.

[00394] Angal et al. (*Mol Immunol.* 30:105-8 (1993)), incorporated by reference herein in its entirety, report that mutating the serine at amino acid position 241 in IgG4 to proline (found at that position in IgG1 and IgG2) led to the production of a homogeneous antibody, as well as extending serum half-life and improving tissue distribution compared to the original chimeric IgG4.

Covalent modifications

[00395] Covalent modifications of the polypeptide binding agents of the invention, e.g., antibodies, are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the polypeptide binding agent, if applicable. Other types of covalent modifications of the polypeptide binding agent are introduced into the molecule by reacting targeted amino acid residues of the polypeptide binding agent with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

[00396] Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, .alpha.-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[00397] Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

[00398] Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing .alpha.-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

[00399] Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

[00400] The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

[00401] Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-

dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[00402] Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

[00403] Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[00404] Another type of covalent modification involves chemically or enzymatically coupling glycosides to the polypeptide binding agent. These procedures are advantageous in that they do not require production of the polypeptide binding agent in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO87/05330 and in Aplin and Wriston, (*CRC Crit. Rev. Biochem.*, pp. 259-306 (1981)).

[00405] Removal of any carbohydrate moieties present on the polypeptide binding agent may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide binding agent to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide binding agent intact. Chemical deglycosylation is described by Hakimuddin, et al., (*Arch. Biochem. Biophys.* 259: 52 (1987)) and by Edge et al., (*Anal. Biochem.* 118: 131 (1981)). Enzymatic cleavage of carbohydrate moieties on polypeptide binding agents can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., (*Meth. Enzymol.* 138: 350 (1987)).

[00406] Another type of covalent modification of the polypeptide binding agent comprises linking the polypeptide binding agent to one of a variety of hydrophobic moieties or

nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyethylated polyols, polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol, polyoxyalkylenes, or polysaccharide polymers such as dextran. Such methods are known in the art, see, e.g. U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192, 4,179,337, 4,766,106, 4,179,337, 4,495,285, 4,609,546 or EP 315 456.

Derivatives

[00407] Derivative refers to polypeptide binding agents, including antibodies, chemically modified by such techniques as ubiquitination, labeling (*e.g.*, with radionuclides or various enzymes), covalent polymer attachment such as pegylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of amino acids such as ornithine. Derivatives of the polypeptide binding agents of the invention, such as an antibody, are also useful as therapeutic agents and may be produced by the method of the invention

[00408] The conjugated moiety can be incorporated in or attached to a polypeptide binding agent either covalently, or through ionic, van der Waals or hydrogen bonds, *e.g.*, incorporation of radioactive nucleotides, or biotinylated nucleotides that are recognized by streptavadin.

[00409] Polyethylene glycol (PEG) may be attached to the polypeptide binding agents to provide a longer half-life *in vivo*. The PEG group may be of any convenient molecular weight and may be linear or branched. The average molecular weight of the PEG will preferably range from about 2 kiloDalton ("kD") to about 100 kDa, more preferably from about 5 kDa to about 50 kDa, most preferably from about 5 kDa to about 10 kDa. The PEG groups will generally be attached to the polypeptide binding agents of the invention via acylation or reductive alkylation through a natural or engineered reactive group on the PEG moiety (e.g., an aldehyde, amino, thiol, or ester group) to a reactive group on the polypeptide binding agent (e.g., an aldehyde, amino, or ester group). Addition of PEG moieties to polypeptide binding agents can be carried out using techniques well-known in the art. See, e.g., International Publication No. WO 96/11953 and U.S. Patent No. 4,179,337.

[00410] Ligation of the polypeptide binding agent with PEG usually takes place in aqueous phase and can be easily monitored by reverse phase analytical HPLC. The PEGylated substances are purified by preparative HPLC and characterized by analytical HPLC, amino acid analysis and laser desorption mass spectrometry.

Antibody Conjugates

[00411] A polypeptide binding agent may be administered in its "naked" or unconjugated form, or may be conjugated directly to other therapeutic or diagnostic agents, or may be conjugated indirectly to carrier polymers comprising such other therapeutic or diagnostic agents. In some embodiments the polypeptide binding agent is conjugated to a cytotoxic agent such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate). Suitable chemotherapeutic agents include: daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., (1986) supra). Suitable toxins include: bacterial toxins such as diphtheria toxin; plant toxins such as ricin; small molecule toxins such as geldanamycin (Mandler et al J. Natl. Cancer Inst. 92(19):1573-81 (2000); Mandler et al., Bioorg. Med. Chem. Letters 10:1025-1028 (2000); Mandler et al., Bioconjugate Chem. 13.786-91 (2002)), maytansinoids (EP 1391213; Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-23 (1996)), auristatins (Doronina et al., Nat. Biotech. 21: 778-84 (2003) and calicheamicin (Lode et al., Cancer Res. 58:2928 (1998); Hinman et al., Cancer Res. 53:3336-3342 (1993)).

[00412] Polypeptide binding agents can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.) fluorescent or luminescent or bioluminescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, and the like. Procedures for accomplishing such labeling are well known in the art; for example, see (Sternberger, L.A. et al., J. Histochem. Cytochem. 18:315 (1970); Bayer, E.A. et al., Meth. Enzym. 62:308 (1979); Engval, E. et al., Immunol. 109:129 (1972); Goding, J.W. J. Immunol. Meth. 13:215 (1976)).

[00413] Conjugation of polypeptide binding agent moieties is described in U.S. Patent No. 6,306,393. General techniques are also described in Shih et al., Int. J. Cancer 41:832-839 (1988); Shih et al., Int. J. Cancer 46:1101-1106 (1990); and Shih et al., U.S. Pat. No. 5,057,313. This general method involves reacting a polypeptide binding agent component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function and that is loaded with a plurality of drug, toxin, chelator, boron addends, or other therapeutic agent. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

[00414] The carrier polymer may be, for example, an aminodextran or polypeptide of at least 50 amino acid residues. Various techniques for conjugating a drug or other agent to the carrier polymer are known in the art. A polypeptide carrier can be used instead of aminodextran, but the polypeptide carrier should have at least 50 amino acid residues in the chain, preferably 100-5000 amino acid residues. At least some of the amino acids should be lysine residues or glutamate or aspartate residues. The pendant amines of lysine residues and pendant carboxylates of glutamine and aspartate are convenient for attaching a drug, toxin, immunomodulator, chelator, boron addend or other therapeutic agent. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, e.g., serines, to confer desirable solubility properties on the resultant loaded carrier and conjugate.

[00415] Alternatively, conjugated polypeptide binding agents can be prepared by directly conjugating a polypeptide binding agent component with a therapeutic agent. The general procedure is analogous to the indirect method of conjugation except that a therapeutic agent is directly attached to an oxidized polypeptide binding agent component. For example, a carbohydrate moiety of a polypeptide binding agent can be attached to polyethyleneglycol to extend half-life.

[00416] Alternatively, a therapeutic agent can be attached at the hinge region of a reduced antibody component via disulfide bond formation, or using a heterobifunctional cross-linker, such as N-succinyl 3-(2-pyridyldithio)proprionate (SPDP). Yu et al., Int. J. Cancer56:244 (1994). General techniques for such conjugation are well-known in the art. See, for example, Wong, Chemistry Of Protein Conjugation and Cross-Linking (CRC Press 1991); Upeslacis et al., "Modification of Antibodies by Chemical Methods," in Monoclonal Antibodies: Principles and Applications, Birch et al. (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in Monoclonal Antibodies: Production, Engineering and Clinical Application, Ritter et al. (eds.), pages 60-84 (Cambridge University Press 1995). A variety of bifunctional protein coupling agents are known in the art, such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as

tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene).

Antibody Fusion Proteins

[00417] Methods of making antibody fusion proteins are well known in the art. See, *e.g.*, U.S. Patent No. 6,306,393. Antibody fusion proteins comprising an interleukin-2 moiety are described by Boleti et al., Ann. Oncol. 6:945 (1995), Nicolet et al., Cancer Gene Ther. 2:161 (1995), Becker et al., Proc. Nat'l Acad. Sci. USA 93:7826 (1996), Hank et al., Clin. Cancer Res. 2:1951 (1996), and Hu et al., Cancer Res. 56:4998 (1996). In addition, Yang et al., (*Hum. Antibodies Hybridomas* 6:129 (1995)), describe a fusion protein that includes an $F(ab')_2$ fragment and a tumor necrosis factor alpha moiety. Further examples of antibody fusion proteins are described by Pastan et al, Nat. Reviews Cancer 6: 559-65 (2006).

[00418] Methods of making antibody-toxin fusion proteins in which a recombinant molecule comprises one or more antibody components and a toxin or chemotherapeutic agent also are known to those of skill in the art. For example, antibody-*Pseudomonas* exotoxin A fusion proteins have been described by Chaudhary et al., Nature 339:394 (1989), Brinkmann et al., Proc. Nat'l Acad. Sci. USA 88:8616 (1991), Batra et al., Proc. Nat'l Acad. Sci. USA 89:5867 (1992), Friedman et al., J. Immunol. 150:3054 (1993), Wels et al., Int. J. Can. 60:137 (1995), Fominaya et al., J. Biol. Chem. 271:10560 (1996), Kuan et al., Biochemistry 35:2872 (1996), and Schmidt et al., Int. J. Can. 65:538 (1996). Antibody-toxin fusion proteins containing a diphtheria toxin moiety have been described by Kreitman et al., Leukemia 7:553 (1993), Nicholls et al., J. Biol. Chem. 268:5302 (1993), Thompson et al., J. Biol. Chem. 270:28037 (1995), and Vallera et al., Blood 88:2342 (1996). Deonarain et al., Tumor Targeting 1:177 (1995), have described an antibody-toxin fusion protein having an RNase moiety, while Linardou et al., Cell Biophys. 24-25:243 (1994), produced an antibody-toxin fusion protein comprising a DNase I component. Gelonin was used as the toxin moiety in the antibody-toxin fusion protein of Wang et al., Abstracts of the 209th ACS National Meeting, Anaheim, Calif., Apr. 2-6, 1995, Part 1, BIOT005. As a further example, Dohlsten et al., Proc. Nat'l Acad. Sci. USA 91:8945 (1994), reported an antibody-toxin fusion protein comprising *Staphylococcal* enterotoxin-A.

[00419] Illustrative of toxins which are suitably employed in the preparation of such fusion proteins are ricin, abrin, ribonuclease, DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, Pseudomonas exotoxin, and Pseudomonas endotoxin. See, for example, Pastan et al., Cell 47:641 (1986), and Goldenberg, CA--A

Cancer Journal for Clinicians 44:43 (1994). Other suitable toxins are known to those of skill in the art.

[00420] Antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.,* a peptidyl chemotherapeutic agent, *See* WO81/01145) to an active anti-cancer drug. *See,* for example, WO88/07378 and U.S. Patent No. 4,975,278.

[00421] The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

[00422] Enzymes that are useful in the this invention include, but are not limited to: alkaline phosphatase; arylsulfatase; cytosine deaminase, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L); D-alanylcarboxypeptidases,; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase; β-lactamase; and penicillin amidases, such as penicillin V amidase or penicillin G amidase. Alternatively, antibodies with enzymatic activity, also known in the art as abzymes, can be used to convert the prodrugs of the invention into free active drugs (*See*, *e.g.*, Massey, *Nature* 328: 457-458 (1987). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

[00423] The enzymes above can be covalently bound to the antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (*See, e.g.,* Neuberger et al., *Nature* 312: 604-608 (1984))

Preparing amino acid sequence variants

[00424] It is contemplated that modified polypeptide compositions comprising one, two, three, four, five, and/or six CDRs of an antibody or polypeptide binding agent are generated, wherein a CDR or non-CDR region is altered to provide increased specificity or affinity to the antigen, or to provide increased modulation of binding affinity between the target and its signaling partner. For example, sites within antibody CDRs are typically modified in series, e.g., by substituting first with conservative choices (e.g., hydrophobic amino acid substituted for a non-identical hydrophobic amino acid) and then with more dissimilar choices (e.g.,

hydrophobic amino acid substituted for a charged amino acid), and then deletions or insertions may be made at the targeted site. It is contemplated that conservative substitutions within the CDR allow the variable region to retain biological activity. For example, using the conserved framework sequences surrounding the CDRs, PCR primers complementary to these consensus sequences are generated to amplify the antigen-specific CDR sequence located between the primer regions. Techniques for cloning and expressing nucleotide and polypeptide sequences are well-established in the art [see e.g. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, New York (1989)]. The amplified CDR sequences are ligated into an appropriate plasmid. The plasmid comprising one, two, three, four, five and/or six cloned CDRs optionally contains additional polypeptide encoding regions linked to the CDR.

[00425] Polypeptide binding agents comprising the modified CDRs are screened for binding affinity for the original antigen. Additionally, the antibody or polypeptide is further tested for its ability to neutralize the activity of its antigen. For example, antibodies of the invention may be analyzed as set out in the Examples to determine their ability to interfere with the biological activity of the target.

[00426] Modifications may be made by conservative or non-conservative amino acid substitutions described in greater detail below. "Insertions" or "deletions" are preferably in the range of about 1 to 20 amino acids, more preferably 1 to 10 amino acids. The variation may be introduced by systematically making substitutions of amino acids in an antibody polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity. Nucleic acid alterations can be made at sites that differ in the nucleic acids from different species (variable positions) or in highly conserved regions (constant regions). Methods for altering antibody sequences and expressing antibody polypeptide compositions useful in the invention are described in greater detail below.

[00427] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intra-sequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody (including antibody fragment) fused to an epitope tag or a salvage receptor epitope. Other insertional variants of the antibody molecule include the fusion to a polypeptide which increases the serum half-life of the antibody, e.g. at the N-terminus or C-terminus.

[00428] The term "epitope tagged" refers to the antibody fused to an epitope tag. The epitope tag polypeptide has enough residues to provide an epitope against which an antibody there against can be made, yet is short enough such that it does not interfere with activity of the antibody. The epitope tag preferably is sufficiently unique so that the antibody there against does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu hemagglutinin (HA) tag polypeptide and its antibody 12CA5 (Field et al., *Mol. Cell. Biol. 8*: 2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., *Mol. Cell. Biol.* 5:3610-16 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., *Protein Engineering* 3:547-53 (1990)). Other exemplary tags are a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAG® tag (Eastman Kodak, Rochester, NY), well known and routinely used in the art, are embraced by the invention.

[00429] As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

[00430] Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule removed and a different residue inserted in its place. Substitutional mutagenesis within any of the hypervariable or CDR regions or framework regions is contemplated. Conservative substitutions involve replacing an amino acid with another member of its class. Non-conservative substitutions involve replacing a member of one of these classes with a member of another class.

[00431] Conservative amino acid substitutions are made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine (Ala, A), leucine (Leu, L), isoleucine (Ile, I), valine (Val, V), proline (Pro, P), phenylalanine (Phe, F), tryptophan (Trp, W), and methionine (Met, M); polar neutral amino acids include glycine (Gly, G), serine (Ser, S), threonine (Thr, T), cysteine (Cys, C), tyrosine (Tyr, Y), asparagine (Asn, N), and glutamine (Gln, Q); positively charged (basic) amino acids include arginine (Arg, R), lysine (Lys, K), and histidine (His, H); and negatively charged (acidic) amino acids include aspartic acid (Asp, D) and glutamic acid (Glu, E).

[00432] Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

Affinity Maturation

[00433] Affinity maturation generally involves preparing and screening antibody variants that have substitutions within the CDRs of a parent antibody and selecting variants that have improved biological properties such as stronger binding affinity relative to the parent antibody. A convenient way for generating such substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity). See e.g., WO 92/01047, WO 93/112366, WO 95/15388 and WO 93/19172.

[00434] Current antibody affinity maturation methods belong to two mutagenesis categories: stochastic and nonstochastic. Error prone PCR, mutator bacterial strains (Low et al., *J. Mol. Biol.* 260, 359-68 (1996)), and saturation mutagenesis (Nishimiya et al.,. *J. Biol. Chem.* 275:12813-20 (2000); Chowdhury, P. S. *Methods Mol. Biol.* 178, 269-85 (2002)) are typical examples of stochastic mutagenesis methods (Rajpal et al., *Proc Natl Acad Sci U S A.* 102:8466-71 (2005)). Nonstochastic techniques often use alanine-scanning or site-directed mutagenesis to generate limited collections of specific variants. Some methods are described in further detail below.

[00435] *Affinity maturation via panning methods*—Affinity maturation of recombinant antibodies is commonly performed through several rounds of panning of candidate antibodies in the presence of decreasing amounts of antigen. Decreasing the amount of antigen per round selects the antibodies with the highest affinity to the antigen thereby yielding antibodies of high affinity from a large pool of starting material. Affinity maturation via panning is well known in the art and is described, for example, in Huls et al. (*Cancer Immunol Immunother.* 50:163-71 (2001)). Methods of affinity maturation using phage display technologies are described elsewhere herein and known in the art (see e.g., Daugherty et al., *Proc Natl Acad Sci U S A.* 97:2029-34 (2000)).

[00436] *Look-through mutagenesis*—Look-through mutagenesis (LTM) (Rajpal et al., *Proc Natl Acad Sci U S A.* 102:8466-71 (2005)) provides a method for rapidly mapping the antibody-binding site. For LTM, nine amino acids, representative of the major side-chain chemistries provided by the 20 natural amino acids, are selected to dissect the functional side-chain contributions to binding at every position in all six CDRs of an antibody. LTM generates a positional series of single mutations within a CDR where each "wild type" residue is systematically substituted by one of nine selected amino acids. Mutated CDRs are combined to generate combinatorial single-chain variable fragment (scFv) libraries of increasing complexity and size without becoming prohibitive to the quantitative display of all variants. After positive selection, clones with stronger binding affinity are sequenced, and beneficial mutations are mapped.

[00437] *Error-prone PCR*—Error-prone PCR involves the randomization of nucleic acids between different selection rounds. The randomization occurs at a low rate by the intrinsic error rate of the polymerase used but can be enhanced by error-prone PCR (Zaccolo et al.,. *J. Mol. Biol.* 285:775-783 (1999)) using a polymerase having a high intrinsic error rate during transcription (Hawkins et al., *J Mol Biol.* 226:889-96 (1992)). After the mutation cycles, clones with stronger binding affinity for the antigen are selected using routine methods in the art.

[00438] *DNA Shuffling*—Nucleic acid shuffling is a method for *in vitro* or *in vivo* homologous recombination of pools of shorter or smaller polynucleotides to produce variant polynucleotides. DNA shuffling has been described in US Patent No. 6,605,449, US Patent 6,489,145, WO 02/092780 and Stemmer, *Proc. Natl. Acad. Sci. USA*, 91:10747-51 (1994). Generally, DNA shuffling is comprised of 3 steps: fragmentation of the genes to be shuffled with DNase I, random hybridization of fragments and reassembly or filling in of the fragmented gene by PCR in the presence of DNA polymerase (sexual PCR), and amplification of reassembled product by conventional PCR.

[00439] DNA shuffling differs from error-prone PCR in that it is an inverse chain reaction. In error-prone PCR, the number of polymerase start sites and the number of molecules grows exponentially. In contrast, in nucleic acid reassembly or shuffling of random polynucleotides the number of start sites and the number (but not size) of the random polynucleotides decreases over time.

[00440] In the case of an antibody, DNA shuffling allows the free combinatorial association of all of the CDR1s with all of the CDR2s with all of the CDR3s, for example. It is contemplated that multiple families of sequences can be shuffled in the same reaction. Further, shuffling generally conserves the relative order, such that, for example, CDR1 will not be found in the position of CDR2. Rare shufflants will contain a large number of the best (e.g. highest affinity) CDRs and these rare shufflants may be selected based on their superior affinity.

[00441] The template polynucleotide which may be used in DNA shuffling may be DNA or RNA. It may be of various lengths depending on the size of the gene or shorter or smaller polynucleotide to be recombined or reassembled. Preferably, the template polynucleotide is from 50 bp to 50 kb. The template polynucleotide often should be double-stranded.

[00442] It is contemplated that single-stranded or double-stranded nucleic acid polynucleotides having regions of identity to the template polynucleotide and regions of heterology to the template polynucleotide may be added to the template polynucleotide, during the initial step of gene selection. It is also contemplated that two different but related polynucleotide templates can be mixed during the initial step.

[00443] *Alanine scanning* - Alanine scanning mutagenesis can be performed to identify hypervariable region residues that contribute significantly to antigen binding. Cunningham and Wells, (*Science* 244:1081-1085 (1989)). A residue or group of targeted residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution.

[00444] *Computer-aided design* - Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen, or to use computer software to model such contact points. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Formulation of Pharmaceutical Compositions

[00445] To administer polypeptide binding agents of the invention to human or test mammals, it is preferable to formulate the polypeptide binding agent in a sterile composition comprising one or more sterile pharmaceutically acceptable carriers. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce allergic, or other adverse reactions when administered using routes well-known in the art, as described below. "Pharmaceutically acceptable carriers" include any and all clinically useful solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like.

[00446] The polypeptide binding agent is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intravenous, intraarterial, intraperitoneal, intramuscular, intradermal or subcutaneous administration. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Other administration methods are contemplated, including topical, particularly transdermal, transmucosal, rectal, oral or local administration e.g. through a catheter placed close to the desired site.

[00447] Pharmaceutical compositions of the present invention containing a polypeptide binding agent of the invention as an active ingredient may contain sterile pharmaceutically acceptable carriers or additives depending on the route of administration. Examples of such carriers or additives include water, a pharmaceutical acceptable organic solvent, collagen, polyvinyl alcohol, polyvinylpyrrolidone, a carboxyvinyl polymer, carboxymethylcellulose sodium, polyacrylic sodium, sodium alginate, water-soluble dextran, carboxymethyl starch sodium, pectin, methyl cellulose, ethyl cellulose, xanthan gum, gum Arabic, casein, gelatin, agar, diglycerin, glycerin, propylene glycol, polyethylene glycol, Vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, a pharmaceutically acceptable surfactant and the like. Additives used are chosen from, but not limited to, the above or combinations thereof, as appropriate, depending on the dosage form of the present invention. For solutions or emulsions, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles can include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replenishers. A

variety of aqueous carriers are suitable, e.g., sterile phosphate buffered saline solutions, bacteriostatic water, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include other proteins for enhanced stability, such as albumin, lipoprotein, globulin, etc., subjected to mild chemical modifications or the like.

[00448] Therapeutic formulations of the polypeptide binding agent are prepared for storage by mixing the polypeptide binding agent having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[00449] The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

[00450] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

[00451] Aqueous suspensions may contain the active compound in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyl-eneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or n-propyl, p-hydroxybenzoate.

[00452] The antibodies of the invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins. Any suitable lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss and that use levels may have to be adjusted to compensate.

[00453] Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active compound in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above.

[00454] The concentration of polypeptide binding agent in these formulations can vary widely, for example from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Thus, a typical pharmaceutical composition for parenteral injection could be made up to contain 1 ml sterile buffered water, and 50 mg of polypeptide binding agent. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of polypeptide binding agent. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more

detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa. (1980). An effective dosage of polypeptide binding agent is within the range of 0.01 mg to 1000 mg per kg of body weight per administration.

[00455] The pharmaceutical compositions may be in the form of a sterile injectable aqueous, oleaginous suspension, dispersions or sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. The suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butane diol. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, vegetable oils, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[00456] In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[00457] Compositions useful for administration may be formulated with uptake or absorption enhancers to increase their efficacy. Such enhancers include for example, salicylate, glycocholate/linoleate, glycholate, aprotinin, bacitracin, SDS, caprate and the like. See, *e.g.*, Fix (*J. Pharm. Sci.*, 85:1282-1285 (1996)) and Oliyai and Stella (*Ann. Rev. Pharmacol. Toxicol.*, 32:521-544 (1993)).

Biophysical Assays

[00458] Complex biological events can be studied via molecular biophysical approaches which consider them as systems of interacting units which can be understood in terms of statistical mechanics, thermodynamics and chemical kinetics

[00459] In certain embodiments, the assays of the present invention may employ a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a measurable signal, such as a radioactive, chromogenic, luminescence, or fluorescent signal, which can be used to quantitate the amount of bound detectable moiety or label in a sample. Detectable labels known in the art include radioisotopes, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, electrochemiluminescent labels (such as Ruthenium (Ru)-based catalyst in conjunction with substrates, etc.), luminescent or bioluminescent labels (e.g., Europium, Vanadium), fluorescent or chemiluminescent compounds, such as fluorescein isothiocyanate, rhodamine, or luciferin, enzymes (e.g., enzyme, such as alkaline phosphatase, ß-galactosidase, or horseradish peroxidase), colorimetric labels such as colloidal gold, colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.), paramagnetic atoms or magnetic agents, electron-dense reagents, a nano- or micro-bead containing a fluorescent dye, nanocrystals, a quantum dot, a quantum bead, a nanotag, dendrimers with a fluorescent label, a micro-transponder, an electron donor molecule or molecular structure, or a light reflecting particle. the microparticles may be nanocrystals or quantum dots. Nanocrystals are substances that absorb photons of light, then re-emit photons at a different wavelength (fluorophores). In addition, additional florescent labels, or secondary antibodies may be conjugated to the nanocrystals. Nanocrystals are commercially available from sources such as Invitrogen and Evident Technologies (Troy, N.Y.). Other labels include E)-5-[2-(methoxycarbonyl)ethenyl]cytidine, which is a nonfluorescent molecule that when subjected to ultraviolet (UV) irradiation yields a product, 3-.beta.-D-ribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine, which displays a strong fluorescence signal. Bar code labels are described in U.S. Patent Publication No. US 20070037195.

[00460] A variety of assay methods known in the art may be employed in the present invention, such as competitive binding assays, direct and indirect sandwich assays, immunoprecipitation assays, fluorescent resonance energy transfer (FRET), electroimmunoassays surface plasmon resonance (SPR), and nanoparticle-derived techniques

[00461] Competitive binding assays rely on the ability of a labeled standard (e.g., an antigen or a fragment thereof to which a polypeptide binding agent binds) to compete with antigen in the test sample for binding to the polypeptide binding agent. The amount of antigen in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies typically are insolubilized before or after the competition, so that the bound antigen may conveniently be separated from the unbound antigen. In alternative embodiments, competitive binding assays measure the ability of a labeled polypeptide binding agent to compete with unlabeled polypeptide binding agent for binding to antigen or a fragment thereof.

[00462] Sandwich assays typically involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected and/or quantitated. In a sandwich assay, the analyte in the test sample is typically bound by a first polypeptide binding agent which is immobilized on a solid phase, and thereafter a second polypeptide binding agent binds to the analyte, thus forming an insoluble three-part complex. See, e.g., U.S. Patent No. 4,376,110. The second polypeptide binding agent may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assays). For example, one type of sandwich assay is an enzyme-linked immunosorbent assay (ELISA), in which case the detectable moiety is an enzyme. See, for example, chapter 18, Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, New York, NY (1995).

[00463] Yet another example of an assay method involves fluorescent resonance energy transfer (FRET) emissions. For example, one compound is labeled with a FRET donor molecule and its binding partner is labeled with a FRET acceptor molecule, or vice versa. When binding occurs between the binding partners, the FRET donor and FRET acceptor molecules are brought into proximity and emit fluorescence at a certain wavelength. A narrow band pass filter can be used to block all wavelengths except that of the label. FRET molecule pairs are commercially available in the art (e.g., from Invitrogen), and may be used according to the manufacturer's protocol. FRET emissions are detected using optical imaging techniques, such as a CCD camera.

[00464] Yet another example of an assay method is bioluminescence resonance energy transfer (BRET), for example using biosensors as described in WO/06/086883.

[00465] Another type of assay involves labeling with an electron donor. One molecule is labeled with an electron donor and the interacting molecule is bound to an electrical contact, or vice versa. When binding occurs between the binding partners, the label donates electrons to the electrical contact. See, for example, Ghindilis, Biochem Soc Trans. 28:84-9, (2000) and Dai et al., Cancer Detect Prev. 29:233-40 (2005), which describe methods for electro immunoassays. The electron contact would then be read by an A to D (analog to digital) converter and quantified. The higher the electron count the more interactions took place.

[00466] One embodiment of a label capable of single molecule detection is the use of plasmon-resonant particles (PRPs) as optical reporters, as described in Schultz et al., Proc. Natl. Acad. Sci. USA 97:996-1001 (2000), incorporated herein by reference. PRPs are metallic nanoparticles, e.g. 40-100 nm in diameter, which scatter light because of a collective resonance of the conduction electrons in the metal (i.e., the surface plasmon resonance). The magnitude, peak wavelength, and spectral bandwidth of the plasmon resonance associated with a nanoparticle are dependent on the particle's size, shape, and material composition, as well as the local environment. By influencing these parameters during preparation, PRPs can be formed that have scattering peak anywhere in the visible range of the spectrum. For spherical PRPs, both the peak scattering wavelength and scattering efficiency increase with larger radius, providing a means for producing differently colored labels. Populations of silver spheres, for example, can be reproducibly prepared for which the peak scattering wavelength is within a few nanometers of the targeted wavelength, by adjusting the final radius of the spheres during preparation. Because PRPs are bright, yet nanosized, they are used as indicators for single-molecule detection; that is, the presence of a bound PRP in a field of view can indicate a single binding event. An example of a surface plasmon resonance detector system is the BIAcore assay system. See, e.g., Malmquist, J Molec Recognition, 7:1-7 (1994).

[00467] Molecular interactions may also be detected using nanoparticle-derived techniques. See, for example, Ao et al., Anal Chem. 78:1104-6 (2006), which describes gold nanoparticle quenching, Tang et al., Biosens Bioelectron. 2005 Nov 30, which describes SiO(2)/Au nanoparticle surfaces in antibody detection, and Lieu et al., J Immunol Methods. 307:34-40 (2005), which describes silicon dioxide nanoparticles containing dibromofluorescein for use in solid substrate-room temperature phosphorescence immunoassay (SS-RTP-IA).

[00468] A KinExA assay is also useful to measure the affinity of a modulating antibody for its antigen. An exemplary KinExA assay is described in Example 20. For example, a KinExA assay measures very low levels of ligand in cell culture media. This assay allows the binding of ligand to cells expressing the cognate receptor to be measured by determining the level of ligand depletion from the cell culture media. As the ligand becomes bound to the cells, the concentration of ligand in the cell culture media drops. By using a titration of cells expressing the receptor and measuring the percent free ligand, the affinity of the ligand-receptor interaction is estimated using KinExA software (Sāpidyne, Boise ID). This assay is used to measure the degree of modulation of ligand binding activity shown by various anti-receptor antibodies.

[00469] Any of the preceding measurements of binding affinity or binding rate parameters may be carried out in assays where one or more of the first component, second component and polypeptide binding agent are in solution, or in assays where one or more of the first component, second component and polypeptide binding agent are linked to a solid phase (covalently or noncovalently), or in assays where one or more of the first component, second component and polypeptide binding agent are expressed on a cell surface. The first and/or second components may each themselves be complexes of multiple compounds.

Administration and Dosing

[00470] In one aspect, methods of the invention include a step of administering a pharmaceutical composition.

[00471] Methods of the invention are performed using any medically-accepted means for introducing a therapeutic directly or indirectly into a mammalian subject, including but not limited to injections, oral ingestion, intranasal, topical, transdermal, parenteral, inhalation spray, vaginal, or rectal administration. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, and intracisternal injections, as well as catheter or infusion techniques. Administration by, intradermal, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary injection and or surgical implantation at a particular site is contemplated as well. Suitable delivery devices may include those developed for the delivery of insulin (see e.g. Owens et al Diabetic Med. 20(11):886-898, 2003).

[00472] In one embodiment, administration is performed at the site of a cancer or affected tissue needing treatment by direct injection into the site or via a sustained delivery or sustained release mechanism, which can deliver the formulation internally. For example,

biodegradable microspheres or capsules or other biodegradable polymer configurations capable of sustained delivery of a composition (*e.g.*, a soluble polypeptide, antibody, or small molecule) can be included in the formulations of the invention implanted at the site.

[00473] Therapeutic compositions may also be delivered to the patient at multiple sites. The multiple administrations may be rendered simultaneously or may be administered over a period of time. In certain cases it is beneficial to provide a continuous flow of the therapeutic composition. Additional therapy may be administered on a period basis, for example, hourly, daily, weekly, every 2 weeks, every 3 weeks, or monthly.

[00474] Also contemplated in the present invention is the administration of multiple agents, such as an antibody composition in conjunction with a second agent as described herein.

[00475] The amounts of antibody composition in a given dosage will vary according to the size of the individual to whom the therapy is being administered as well as the characteristics of the disorder being treated. In exemplary treatments, it may be necessary to administer about 1 mg/day, 5 mg/day, 10 mg/day, 20 mg/day, 50 mg/day, 75 mg/day, 100 mg/day, 150 mg/day, 200 mg/day, 250 mg/day, 500 mg/day or 1000 mg/day. These concentrations may be administered as a single dosage form or as multiple doses. Standard dose-response studies, first in animal models and then in clinical testing, reveal optimal dosages for particular disease states and patient populations.

Combination Therapy

[00476] It one embodiment, an antibody of the invention is administered with a second agent useful to treat a disease or disorder as described herein. It is contemplated that two or more antibodies to different epitopes of the target antigen may be mixed such that the combination of antibodies together to provide still improved efficacy against a condition or disorder to be treated associated with the target polypeptide. Compositions comprising one or more antibody of the invention may be administered to persons or mammals suffering from, or predisposed to suffer from, a condition or disorder to be treated associated with the target polypeptide.

[00477] Concurrent administration of two therapeutic agents does not require that the agents be administered at the same time or by the same route, as long as there is an overlap in the time period during which the agents are exerting their therapeutic effect. Simultaneous or sequential administration is contemplated, as is administration on different days or weeks.

[00478] A second agent may be other therapeutic agents, such as anti-diabetic agents, cytokines, growth factors, other anti-inflammatory agents, anti-coagulant agents, agents that will lower or reduce blood pressure, agents that will reduce cholesterol, triglycerides, LDL, VLDL, or lipoprotein(a) or increase HDL, agents that will increase or decrease levels of cholesterol-regulating proteins, anti-neoplastic drugs or molecules. For patients with a hyperproliferative disorder, such as cancer or a tumor, combination with second therapeutic modalities such as radiotherapy, chemotherapy, photodynamic therapy, or surgery is also contemplated.

[00479] Exemplary anti-diabetic agents include, but are not limited to, 1) sulfonylureas (*e.g.*, glimepiride, glisentide, sulfonylurea, AY31637); 2) biguanides (*e.g.*, metformin); 3) alpha-glucosidase inhibitors (*e.g.*, acarbose, miglitol); 4) thiazol-idinediones (*e.g.*, troglitazone, pioglitazone, rosiglitazone, glipizide, balaglitazone, rivoglitazone, netoglitazone, troglitazone, englitazone, AD 5075, T 174, YM 268, R 102380, NC 2100, NIP 223, NIP 221, MK 0767, ciglitazone, adaglitazone, CLX 0921, darglitazone, CP 92768, BM 152054); 5) glucagon-like-peptides (GLP) and GLP analogs or agonists of GLP-1 receptor (e.g. exendin) or stabilizers thereof (e.g. DPP4 inhibitors, such as sitagliptin); and 6) insulin or analogues or mimetics thereof (e.g. LANTUS®).

[00480] It is contemplated the antibody of the invention and the second agent may be given simultaneously, in the same formulation. It is further contemplated that the agents are administered in a separate formulation and administered concurrently, with concurrently referring to agents given within 30 minutes of each other.

[00481] In another aspect, the second agent is administered prior to administration of the antibody composition. Prior administration refers to administration of the second agent within the range of one week prior to treatment with the antibody, up to 30 minutes before administration of the antibody. It is further contemplated that the second agent is administered subsequent to administration of the antibody composition. Subsequent administration is meant to describe administration from 30 minutes after antibody treatment up to one week after antibody administration.

[00482] It is further contemplated that other adjunct therapies may be administered, where appropriate. For example, the patient may also be administered a diabetic diet or food plan, surgical therapy, or radiation therapy where appropriate.

[00483] It will also be apparent that dosing may be modified if traditional therapeutics are administered in combination with therapeutics of the invention.

Methods of Use

Therapeutic indications for INSR agonists/positive modulators

[00484] In another embodiment, the invention provides a method for inhibiting target activity by administering a target-specific antibody to a patient in need thereof. Any of the types of antibodies described herein may be used therapeutically. In exemplary embodiments, the target specific antibody is a human, chimeric or humanized antibody. In another exemplary embodiment, the target is human and the patient is a human patient. Alternatively, the patient may be a mammal that expresses a target protein that the target specific antibody cross-reacts with. The antibody may be administered to a non-human mammal expressing a target protein with which the antibody cross-reacts (i.e. a primate) for veterinary purposes or as an animal model of human disease. Such animal models may be useful for evaluating the therapeutic efficacy of target specific antibodies of the invention.

[00485] Insulin resistance describes a condition in which physiological amounts of insulin are inadequate to produce a normal insulin response from cells or tissues. Insulin resistance is associated with a number of disease states and conditions and is present in approximately 30-40% of non-diabetic individuals. These disease states and conditions include, but are not limited to, pre-diabetes, metabolic syndrome (also referred to as insulin resistance syndrome), Type 2 diabetes mellitus, polycystic ovary disease (PCOS) and non-alcoholic fatty liver disease (NAFLD) (reviewed in Woods et al, End, Metab & Immune Disorders –Drug Targets 9: 187-198, 2009).

[00486] Pre-diabetes is a state of abnormal glucose tolerance characterized by either impaired glucose tolerance (IGT) or impaired fasting glucose (IFG). Patients with pre-diabetes are insulin resistant and are at high risk for future progression to overt Type 2 diabetes. Metabolic syndrome is an associated cluster of traits that include, but is not limited to, hyperinsulinemia, abnormal glucose tolerance, obesity, redistribution of fat to the abdominal or upper body compartment, hypertension, dysfibrinolysis, and a dyslipidemia characterized by high triglycerides, low HDL-cholesterol, and small dense LDL particles. Insulin resistance has been linked to each of the traits, suggesting metabolic syndrome and insulin resistance are intimately related to one another. The diagnosis of metabolic syndrome is a powerful risk factor for future development of Type 2 diabetes, as well as accelerated atherosclerosis resulting in heart attacks, strokes, and peripheral vascular disease.

[00487] Diabetes mellitus is a metabolic disorder in humans with a prevalence of approximately one percent in the general population (Foster, D. W., Harrison's Principles of Internal Medicine, Chap. 114, pp. 661-678, 10th Ed., McGraw-Hill, New York). The disease manifests itself as a series of hormone-induced metabolic abnormalities that eventually lead to serious, long-term and debilitating complications involving several organ systems including the eyes, kidneys, nerves, and blood vessels. Pathologically, the disease is characterized by lesions of the basement membranes, demonstrable under electron microscopy. Diabetes mellitus can be divided into two clinical syndromes, Type 1 and Type 2 diabetes mellitus.

[00488] Type 1, or insulin-dependent diabetes mellitus (IDDM), also referred to as the juvenile onset form, is a chronic autoimmune disease characterized by the extensive loss of beta cells in the pancreatic Islets of Langerhans, which produce insulin. As these cells are progressively destroyed, the amount of secreted insulin decreases, eventually leading to hyperglycemia (abnormally high level of glucose in the blood) when the amount of secreted insulin drops below the normally required blood glucose levels. Although the exact trigger for this immune response is not known, patients with IDDM have high levels of antibodies against proteins expressed in pancreatic beta cells. However, not all patients with high levels of these antibodies develop IDDM. Type 1 diabetics characteristically show very low or immeasurable plasma insulin with elevated glucagon. Regardless of what the exact etiology is, most Type 1 patients have circulating antibodies directed against their own pancreatic cells including antibodies to insulin, to Islet of Langerhans cell cytoplasm and to the enzyme glutamic acid decarboxylase. An immune response specifically directed against beta cells (insulin producing cells) leads to Type 1 diabetes. The current treatment for Type 1 diabetic patients is the injection of insulin, and may also include modifications to the diet in order to minimize hyperglycemia resulting from the lack of natural insulin, which in turn, is the result of damaged beta cells. Diet is also modified with regard to insulin administration to counter the hypoglycemic effects of the hormone.

[00489] Type 2 diabetes (also referred to as non-insulin dependent diabetes mellitus (NIDDM), maturity onset form, adult onset form) develops when muscle, fat and liver cells fail to respond normally to insulin. This failure to respond (called insulin resistance) may be due to reduced numbers of insulin receptors on these cells, or a dysfunction of signaling pathways within the cells, or both. The beta cells initially compensate for this insulin resistance by increasing insulin output. Over time, these cells become unable to produce

enough insulin to maintain normal glucose levels, indicating progression to Type 2 diabetes. Type 2 diabetes is brought on by a combination of genetic and acquired risk factors, including a high-fat diet, lack of exercise, and aging. Greater than 90% of the diabetic population suffers from Type 2 diabetes and the incidence continues to rise, becoming a leading cause of mortality, morbidity and healthcare expenditure throughout the world (Amos et al., Diabetic Med. 14:S1-85, 1997).

[00490] Type 2 diabetes is a complex disease characterized by defects in glucose and lipid metabolism. Typically there are perturbations in many metabolic parameters including increases in fasting plasma glucose levels, free fatty acid levels and triglyceride levels, as well as a decrease in the ratio of HDL/LDL. As discussed above, one of the principal underlying causes of diabetes is thought to be an increase in insulin resistance in peripheral tissues, principally muscle and fat. The causes of Type 2 diabetes are not well understood. It is thought that both resistance of target tissues to the action of insulin and decreased insulin secretion ("β-cell failure") occur. Major insulin-responsive tissues for glucose homeostasis are liver, in which insulin stimulates glycogen synthesis and inhibits gluconeogenesis; muscle, in which insulin stimulates glucose uptake and glycogen stimulates glucose uptake and inhibits lipolysis. Thus, as a consequence of the diabetic condition, there are elevated levels of glucose in the blood, which can lead to glucose-mediated cellular toxicity and subsequent morbidity (nephropathy, neuropathy, retinopathy, etc.). Insulin resistance is strongly correlated with the development of Type 2 diabetes.

[00491] Currently, there are various pharmacological approaches for the treatment of Type 2 diabetes (Scheen et al, Diabetes Care, 22(9):1568-1577, 1999; Zangeneh et al, Mayo Clin. Proc. 78: 471-479, 2003; Mohler et al, Med Res Rev 29(1): 125-195, 2009). They act via different modes of action: 1) sulfonylureas (*e.g.*, glimepiride, glisentide, sulfonylurea, AY31637) essentially stimulate insulin secretion; 2) biguanides (*e.g.*, metformin) act by promoting glucose utilization, reducing hepatic glucose production and diminishing intestinal glucose output; 3) alpha-glucosidase inhibitors (*e.g.*, acarbose, miglitol) slow down carbohydrate digestion and consequently absorption from the gut and reduce postprandial hyperglycemia; 4) thiazol-idinediones (*e.g.*, troglitazone, pioglitazone, rosiglitazone, glipizide, balaglitazone, rivoglitazone, netoglitazone, troglitazone, englitazone, AD 5075, T 174, YM 268, R 102380, NC 2100, NIP 223, NIP 221, MK 0767, ciglitazone, adaglitazone, CLX 0921, darglitazone, CP 92768, BM 152054) enhance insulin action, thus promoting glucose utilization in peripheral tissues; 5) glucagon-like-peptides and agonists (e.g. exendin)

or stabilizers thereof (e.g. DPP4 inhibitors, such as sitagliptin) potentiate glucose-stimulated insulin secretion; and 6) insulin or analogues thereof (e.g. LANTUS®) stimulate tissue glucose utilization and inhibits hepatic glucose output. The above mentioned pharmacological approaches may be utilized individually or in combination therapy. However, each approach has its limitations and adverse effects. Over time, a large percentage of Type 2 diabetic subjects lose their response to these agents. 63% of Type 2 diabetes patients fail to reach global HbA_{1c} levels of <7% as advised by the American Diabetes Association, and are thus at high risk of developing complications. Moreover, almost invariably patients progress through the stages of declining pancreatic function. Insulin treatment is typically instituted after diet, exercise, and oral medications have failed to adequately control blood glucose. The drawbacks of insulin treatment are the need for drug injection, the potential for hypoglycemia, and weight gain. Consequently there is still an urgent need for novel anti-diabetic agents.

[00492] Schaffer et al. used phage display to identify a series of peptides binding to two discrete hotspots on the INSR, which showed agonistic or antagonistic activity when covalently linked to form homodimers or heterodimers (Schäffer et al, Proc. Natl. Acad. Sci. USA, 100(8):4435-4439, 2003).

[00493] A further pharmacological approach for the treatment of Type 2 diabetes is the use of non-peptide small molecules that can activate the INSR, or potentiate INSR activation by insulin (Moller, Nature 414: 821-827). Such molecules have proved elusive to identify, but two groups have reported examples. L783281 (DMAQ-B1, L7) and its derivative, compound 2, are insulin mimetics identified from a screen for small molecules that activate the INSR tyrosine kinase (Zhang et al, Science 284: 974-977, 1999; Qureshi et al, J. Biol. Chem. 275(47): 36590-36595, 2000). TLK16998 and TLK19780 are insulin sensitizers identified by their ability to increase autophosphorylation of isolated, naturally expressed human INSR (Manchem et al, Diabetes 50: 824-830, 2001; Pender et al, J. Biol. Chem. 277(46): 43565-43571, 2002). Both L783281 and TLK16998 potentiate insulin action in insulin-resistant cells by acting on the intracellular portion of the INSR β-subunit, enhancing β-subunit autophosphorylation and subsequent downstream signaling (Li et al, Diabetes 50: 2323-2328, 2001). Compound 2 and TLK16998 have been shown to reduce blood glucose levels in mouse models of diabetes when given continuously at high doses (Strowski et al, Endocrinology 145(11):5259-5268, 2004; Manchem et al, Diabetes 50: 824-830, 2001). However, none of these compounds appears to have entered clinical testing. Agents that

target the INSR tyrosine kinase domain are expected to have side effects due to non-specific activation homologous tyrosine kinase domains in other molecules. The intracellular portion of the INSR β-subunit is not a suitable target for larger molecules, such as antibodies, which are unable to diffuse into the cell.

[00494] Polyclonal autoantibodies from the sera patients with insulin-resistant diabetes have been identified and used as probes to study insulin action. These autoantibodies inhibited insulin binding to INSR and bivalent (but not monovalent) forms produced insulin-like biological effects when exposed to tissues in vitro (Kahn et al, Proc. Natl. Acad. Sci. USA 75(9): 4209-4213, 1978; Heffetz and Zick, J. Biol. Chem. 261(2): 889-894, 1986).

[00495] Jacobs and Cuatrecasas described two rabbit polyclonal antibodies and reported that these antibodies, as well as a number of polyclonal antibodies produced by other investigators, were able to mediate various insulin-like effects (Jacobs and Cuatrecasas, CIBA Found. Symp. 90: 82-90, 1982).

[00496] Kull *et al* described three mouse monoclonal antibodies, αIR-1, αIR-2 and αIR-3 and a polyclonal, A410, and their use to investigate the immunochemical cross-reactivity of, and identify the subunits of, the insulin and somatomedin-C (IGF-1) receptors (Kull et al, J. Biol. Chem. 258(10): 6561-6566, 1983). Herrera *et al* also made antibodies (rabbit polyclonal anti-INSR peptide antibodies P4 and P5) to study the relationship between the human INSR and IGF-1 receptors (Herrera et al, J. Biol. Chem. 261(6): 2489-2491, 1986).

[00497] A positive modulator antibody that increases the on-rate or decreases the off-rate of insulin (insulin analog or INSR agonist) for the INSR could result in an increased residency time of receptor bound insulin (insulin analog or INSR agonist), a change in the rate of INSR internalization and/or a change in the degree of phosphorylation of signaling proteins activated or deactivated by the INSR. These changes could significantly alter both the metabolic and mitogenic activity of insulin (insulin analog or INSR agonist) and the level and frequency of dosing of exogenous insulin (insulin analog or INSR agonist).

[00498] A negative modulating antibody that increases the on-rate or decreases the off-rate of insulin (insulin analog or INSR agonist) for the receptor could result in an decreased residency time of receptor bound insulin (insulin analog or INSR agonist), a change in the rate of INSR internalization and/or a change in the degree of phosphorylation of signaling proteins activated or deactivated by the INSR. These changes could significantly alter both

the metabolic and mitogenic activity of insulin (insulin analog or INSR agonist) and the level and frequency of dosing of exogenous insulin (insulin analog or INSR agonist).

[00499] It is contemplated that diabetic patients receiving a positive modulating antibody of the invention would have improvement in blood glucose levels, glucose tolerance test, and other measures of insulin sensitivity compared to patients not receiving treatment. For example, administration of a positive modulator antibody of the invention is expected to reduce elevated blood glucose levels toward normal glucose levels, which are between approximately 70 mg/dL to 125 mg/dL for fasting blood glucose levels according to the American Diabetes Association. In one embodiment, administration of an antibody of the invention reduces blood glucose levels by approximately 15%, 20%, 25%, 30%, 35%, 40% or greater compared to a patient not receiving antibody treatment.

[00500] According to the criteria of the World Health Organization and the American Diabetes Association, normal glucose tolerance is defined as glucose levels of below 140 mg per dL measured two hours after ingesting 75 g of oral glucose. Impaired glucose tolerance is defined as two-hour glucose levels of 140 to 199 mg per dL (7.8 to 11.0 mmol) after ingesting 75-g oral glucose. A patient is said to have impaired glucose tolerance when the glucose level is elevated (compared to a normal healthy patient) after 2 hours, but less elevated than would qualify for a diagnosis type 2 diabetes mellitus. A patient with impaired glucose tolerance may still have a fasting glucose that is either normal or only mildly elevated. In one embodiment, administration of an antibody of the invention reduces two-hour glucose levels (after the 75-g oral glucose dose) by approximately 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or greater compared to a patient not receiving antibody treatment.

[00501] The ADA also recommends a hemoglobin A1c target level of less than 7% in adults. For children, the ADA recommends higher target levels of A1c. In children younger than 6 years old, the recommended level is from 7.5% to 8.5%. In children 6 to 12 years old, the recommended level is less than 8%. the recommended level for teens 13 to 19 years old, is less than 7.5%. A1c is a measure of how well blood sugar levels have remained within a target range over the previous 2 to 3 months. (American Diabetes Association, Diabetes Care, 28(1): 186–212, 2005.) It is contemplated that administration of an antibody of the invention to treat diabetes reduces A1c levels towards that observed in a non-diabetic individual. In one embodiment, administration of an antibody of the invention reduces A1c levels in a patient by an absolute HbA1c percentage measurement of at least 0.5%, 0.7%, 1.0% or 1.5%.

[00502] Beta cells in the pancreatic islets of Langerhans make and release insulin, a hormone that controls the level of glucose in the blood. There is a baseline level of insulin maintained by the pancreas, but it can respond quickly to spikes in blood glucose by releasing stored insulin while simultaneously producing more. The response time is fairly rapid. In Type 1 diabetes, progressive and extensive loss of beta cells results in decreased levels of secreted insulin, eventually leading to hyperglycemia (abnormally high level of glucose in the blood). In Type 2 diabetes, beta cells initially compensate for insulin resistance in a subject by increasing insulin output, but, over time, the cells become unable to produce enough insulin to maintain normal glucose levels. It is thought that both resistance of target tissues to the action of insulin and decreased insulin secretion, in part due to beta cell failure, occur. Administration of antibodies or polypeptides described herein which improve glucose uptake and other diabetic symptoms are also useful to improve beta cell function in a subject in need thereof. Such improvement includes, but is not limited to, preservation of beta cell viability or reduction of beta cell turnover, increased beta cell proliferation, or enhanced insulin secretion. Additional methods for and results of improvement of beta cell function are disclosed in co-owned international application no. WO 2010/028273.

[00503] In certain embodiments, treatment with a positive modulating antibody or partial agonist antibody results in an improvement of one, two, three or more symptoms of diabetes or insulin resistance selected from the group consisting of elevated plasma triglycerides, elevated plasma unesterified cholesterol, elevated plasma total cholesterol elevated plasma insulin (indicative of insulin resistance), elevated HOMA-IR, high non-HDL/HDL cholesterol ratio (or high total cholesterol/HDL cholesterol ratio), improved beta cell function, and elevated plasma leptin levels (indicative of leptin resistance). Where elevated levels are indicative of diabetes, insulin resistance or increased risk for cardiovascular complications, an "improvement" manifests as a reduced level, and vice versa. "Improvement" as used herein refers to a normalization of a level toward the level seen in healthy subjects.

[00504] Although the "normal" levels determined upon testing vary on a laboratory-by-laboratory basis, and each laboratory has its own normal range, in general, normal triglyceride levels are less than 150 mg/dl in diabetes (borderline high 150 - 199 mg/dL); normal cholesterol levels are less than 200 mg/dL, a normal or target non-HDL/HDL cholesterol ratio is approximately <3.25 (based on <130 mg/dL non-HDL target and >41 ng/dL target HDL), a normal or target range for fasting insulin is approximately 5-20

microU/m, and a normal or target range for leptin (usually also associated with body mass index (BMI) or hyperinsulinemia) is between 3-25 ng/ml, e.g., 3 ng/mL appears to be required for normal metabolic function and 20-25 ng/mL appears to be associated with disease. In some embodiments, treatment normalizes any one or more of the above symptoms by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more.

[00505] Polycystic ovary syndrome (PCOS) is the most common gynecological endocrine disorder and is present in approximately 5-10% of women of childbearing age. Clinical presentations include menstrual disorders, obesity, infertility and hirsutism. Insulin resistance in PCOS results from a post-insulin binding defect in signaling. INSR and insulin receptor substrate (IRS)-1 serine hyperphosphorylation by an unidentified kinase(s) contributes to this defect. Mitogenic signaling was observed to be enhanced in skeletal muscle from women with PCOS (Corbould et al, Diabetes 55: 751-59, 2006). Agonists and/or positive modulators of insulin binding to INSR may therefore be useful for treating and/or reducing the likelihood of the onset of disorders and symptoms related to PCOS. Agonists and/or positive modulators of insulin binding to INSR that do not increase the ratio of mitogenic to metabolic signaling may be particularly useful for treating PCOS.

[00506] Non-alcoholic steatohepatitis (NASH) is part of a spectrum of pathology (known as NAFLD) ranging from simple steatosis (fatty infiltration) to NASH, through to cirrhosis and hepatocellular carcinoma (Farrell and Larter, Hepatol. 43, S99-112, 2006). Insulin resistance is associated with fat accumulation in the liver and this organ is now recognized as a major target of injury in patients with insulin resistance. It is estimated that about 20% of all adults have NAFLD, and 2-3% of adults have NASH. Up to one third of patients with NASH will develop cirrhosis over longer follow up. Liver disease is a significant complication of Type 2 diabetes.

[00507] Individuals with obesity and dyslipidemia exhibit poorer insulin sensitivity than that found in the average population. Obesity is a chronic disease that is highly prevalent and is associated not only with a social stigma, but also with decreased life span and numerous medical problems including adverse psychological development, dermatological disorders such as infections, varicose veins, exercise intolerance, diabetes mellitus, insulin resistance, hypertension, hypercholesterolemia, and coronary heart disease (Rissanen et al., British Medical Journal, 301: 835-837, 1990). Obesity is highly correlated with insulin resistance and diabetes in experimental animals and humans. Indeed, obesity and insulin resistance, the latter of which is generally accompanied by hyperinsulinemia or hyperglycemia, or both, are

hallmarks of Type 2 diabetes. In addition, Type 2 diabetes is associated with a two- to four-fold risk of coronary artery disease. Despite decades of research on these serious health problems, the etiology of obesity and insulin resistance is unknown. It is disclosed herein that positive modulator antibodies and partial agonist antibodies can reduce or slow the weight gain, i.e., normalize weight gain, observed in diabetic animals. It is contemplated that the antibodies have the same effect on weight gain in obese patients. It has also been demonstrated that administration of positive modulator antibodies can slow or reduce weight loss, i.e., normalize weight loss, in diabetic animals whose beta cell population is depleted, which often results in significant weight loss and wasting.

[00508] In some embodiments it is contemplated that administration of positive modulator antibodies or partial agonist antibodies described herein can reduce or slow weight gain in a subject by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% compared to an untreated subject.

[00509] In an alternate embodiment, it is contemplated that administration of positive modulator antibodies or partial agonist antibodies described herein can reduce or slow weight loss in an individual, such as a diabetic patient or an individual having at least partial beta cell depletion, by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% compared to an untreated subject.

[00510] In some embodiments, it is contemplated that administration of positive modular antibodies or partial agonist antibodies described herein can promote or induce weight loss relative to untreated subjects, e.g. by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% compared to an untreated subject.

[00511] Protease inhibitors used for the treatment of HIV patients are associated with development of a group of metabolic disorders, including insulin resistance (Graham, JAIDS 25: S4-S11, 2000). HIV protease inhibitor- induced insulin resistance may lead to hyperglycemia that can progress to diabetes and ultimately life threatening ketoacidosis. (Carr et al, Lancet 351:1881-1883, 1998). For some patients, these metabolic side-effects greatly limit the use of these life sustaining drugs. Murata et al (J. Biol. Chem. 275(27): 20251-54, 2000) reported that at least three of the commercialized HIV protease inhibitor drugs also inhibit the glucose transporter from localizing to the cell membrane of in 3T3 Ll adipocytes, with the subsequent inhibition of glucose uptake by these cells. This inhibition of cellular glucose transport into cells by these HIV protease inhibitors is consistent with the

elevation of glucose and lipids observed in the clinic for some patients being treated with these protease inhibitor drugs. Thus agonists and/or positive modulators of insulin binding to INSR may be useful for treating the metabolic side-effects of HIV protease inhibitors.

[00512] Insulin resistance is also one of the pathological features in patients with hepatitis C virus (HCV) infection and plays a crucial role in the development of various complications and events associated with HCV infection (Kawaguchi and Sata, World J. Gastroenterol. 16: 1943-52, 2010). Thus agonists and/or positive modulators of insulin binding to INSR may be useful for treating complications and events associated with HCV infection.

[00513] INSR signaling may also play a role in other diseases. For example, it has been speculated that INSR/IGF-1R signaling may play a role in amyloid-beta metabolism (Freude et al, Curr. Alzheimer Res. 6(3): 213-23, 2009). Activation of IR has been postulated to be an essential element of photoreceptor neuroprotection (Rajala et al, J. Biol. Chem. 283(28):19781-92, 2008). Insulin signaling has also been suggested to promote bone formation (Rosen and Motyl, Cell 142: 198-200). Treatment with insulin sensitizers has been reported to improve pulmonary function in patients with both chronic obstructive pulmonary disease and diabetes mellitus (Kim et al, Int. J. Tuberc. Lung Dis. 14(3): 362-67, 2010).

[00514] A few patients with homozygous mutations in the INSR gene have been described, which causes Donohue syndrome or Leprechaunism. This autosomal recessive disorder results in a totally non-functional insulin receptor. These patients have low set, often protuberant, ears, flared nostrils, thickened lips, and severe growth retardation. In most cases, the outlook for these patients is extremely poor with death occurring within the first year of life. Other mutations of the INSR gene cause the less severe Rabson-Mendenhall syndrome, in which patients have characteristically abnormal teeth, hypertrophic gingiva (gums) and enlargement of the pineal gland. Both diseases present with fluctuations of the glucose level: after a meal the glucose is initially very high, and then falls rapidly to abnormally low levels (Longo et al, Hum. Mol. Genet. 11(12): 1465–75, 2002).

Therapeutic indications for INSR antagonists/negative modulators

[00515] The INSR has also been implicated in cancer. Several epidemiological studies have shown that insulin resistance states, characterized by hyperinsulinemia, are associated with an increased risk for a number of malignancies, including carcinomas of the breast, prostate, colon and kidney. INSR, particularly the INSR-A form, is overexpressed in several human malignancies. INSR forms hybrid receptors with IGF-IR, which is also commonly

overexpressed in cancer. Hybrid receptors containing INSR-A hemidimers have broad binding specificity as they bind IGF-I and also IGF-II and insulin. By binding to hybrid receptors, insulin may stimulate specific IGF-IR signaling pathways. Antagonists and/or negative modulators of insulin binding to INSR and/or to hybrid INSR/IGF-1R receptors may therefore be useful as novel anti-cancer therapies (Belfiore Current Pharm. Design 13 (7): 671-686, 2007). INSR has been reported to be essential for virus-induced tumorigenesis of Kaposi's sarcoma (Rose et al, Onogene 26: 1995-2005, 2007).

[00516] Hyperinsulinemia is a condition defined by abnormally high levels of insulin in the blood. Causes of hyperinsulinemia include insulinoma and insulin resistance, which may be caused by congenital hyperinsulinemia or other conditions, such as a lack of activity, obesity, polycystic ovary syndrome or insulin overdose. An insulinoma is a tumor of the pancreas that produces excessive amounts of insulin. High insulin levels cause hypoglycemia, or low blood glucose (sugar). Hyperinsulinemia is the most common cause of neonatal hypoglycemia following the first few hours of life. Treatment of such a condition may often be necessary to prevent onset of seizures and neurologic sequelae.

[00517] Insulin overdose may be caused, for example by: administration of too much insulin; by administration of the right amount of insulin but the wrong type, such as of short acting insulin instead of long-acting insulin; by administration of insulin followed by a failure to eat; or by intentional insulin over-administration.

[00518] In general, hypoglycemia may be mild and lead to symptoms such as anxiety and hunger, but patients are also at risk for severe hypoglycemia, which can cause seizures, coma, and even death. Typical symptoms associated with hypoglycemia that patients complain about include tiredness, weakness, tremulous and hunger. Many patients have to eat frequently to prevent symptoms from the low blood sugar. Some patients may develop psychiatric symptoms because of the low blood sugar.

[00519] Currently, patients with insulinomas or other severe forms of hyperinsulinemia are treated by surgery such as partial pancreatectomy or by administration of drugs such as diazoxide or somatostatin which in some cases reduces insulin production. In some cases glucose must be infused continuously. Although peptide INSR antagonists have been described (Schaffer et al, BBRC 376: 380-383, 2008), there is no existing treatment which reduces the effects of circulating insulin. Antagonists and/or negative modulators of insulin binding to INSR may be useful for stabilizing patients with insulinomas before surgery or as

part of the therapeutic armamentarium. Antagonists and/or negative modulators are also useful to treat Kaposi's sarcoma.

[00520] Additionally, a significant number of patients (25,000-100,000) in the US who undergo dialysis present with hypoglycemia due to renal failure (chronic kidney disease, chronic renal disease, chronic kidney failure, chronic renal failure, established chronic kidney disease) and may benefit from treatment with an antagonist or negative modulator of INSR described herein.

[00521] Antagonists and/or negative modulators of insulin binding to INSR may be useful for treating and/or reducing the likelihood of the onset of disorders and symptoms related to hyperinsulinemia in a subject, such as reducing anxiety, abnormal hunger, abnormal fatigue, overeating, psychiatric symptoms associated with low blood sugar, and/or hypoglycemia (including hypoglycemia-related seizure, coma, and death). Antagonists and/or negative modulators of insulin binding to INSR may therefore be used to treat various types of persistent hyperinsulinemia conditions, such as nesidioblastosis (KATP-Hl Diffuse Disease, KATP-Hl Focal Disease, or "PHHI"), GDH-Hl (Hyperinsulinism/Hyperammonaemia Syndrome (HI/HA), leucine-sensitive hypoglycemia, or diazoxide-sensitive hypoglycemia), islet cell dysregulation syndrome, idiopathic hypoglycemia of infancy, Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), Congenital Hyperinsulinism, insulinoma, insulin overdose, hypoglycemia due to renal failure (acute or chronic), and chronic kidney disease, e.g., type III, IV or V.

Diagnostic indications for INSR agonists/positive modulators

[00522] Antibodies specific for insulin receptor have been used as tools to diagnose diabetes. US Patent 7,732,154 describes polyclonal antibodies to insulin receptor subunit A (IR-A) as a diagnostic for diabetes, and reports that elevated levels of free IR-A were detected in sera of diabetes and cancer patients. The INSR antibodies disclosed herein are useful to measure insulin receptor, e.g. soluble insulin receptor-A, or insulin levels in a sample from a patient to determine if the levels of INSR or insulin are indicative of diabetes or insulin resistance in the patient. A subject with altered levels of insulin or insulin receptor compared to normal acceptable levels of these factors in an otherwise healthy individual may have or be at risk of diabetes or insulin resistance. The INSR antibodies disclosed herein are also useful to measure insulin receptor, e.g. soluble insulin receptor A, or insulin levels in a sample from a patient to determine if the levels of INSR or insulin are indicative of cancer in the patient. A subject with altered levels of insulin or insulin receptor compared to normal

acceptable levels of these factors in an otherwise healthy individual may have or be at risk of cancer.

[00523] In one embodiment, the invention provides a method of diagnosing insulin resistance or insulin sensitivity using any of the antibodies as described herein. In one embodiment, the method comprises measuring levels of insulin or insulin receptor, e.g. soluble insulin-receptor–A, in a sample from a subject using an antibody described herein, wherein an altered level of insulin or insulin receptor indicates the subject has or is at risk for diabetes, insulin resistance, insulin sensitivity or cancer, and optionally, administering a therapeutic to said subject who has or is at risk of diabetes, insulin resistance, insulin sensitivity or cancer. In certain embodiments, the sample is a biological sample. In some embodiments, the biological sample is selected from the group consisting of blood, serum, plasma, urine, papillary secretions, cerebrospinal fluid and tumor biopsy. Methods of measuring insulin receptor in a sample include, but are not limited to, immunoassays, competitive inhibition assays, immunoprecipitation assays, and other assays as described herein.

Assays Useful to Measure the Effects of Modulator Administration

[00524] Effects of administration of positive or negative modulator antibodies to subjects are measured in vivo and in vitro. In one embodiment, it is contemplated that antibodies that positively modulate insulin/insulin receptor activity decrease in vivo levels of HbA1c, cholesterol, LDL, triglycerides, or non-esterified fatty acids, and HDL in a subject. These factors are measured using techniques common to those of skill in the art.

[00525] Subjects receiving a positive modulator antibody also may show reduced weight or reduced weight gain, a decreased frequency and/or number of hypoglycemic or hyperglycemic events, and improved: HDL/LDL ratio, insulin secretion, glycemic control (as measured by glucose tolerance test GTT)), insulin sensitivity as measured by insulin tolerance test (ITT)), beta-cell function (as measured by, e.g., cell mass, insulin secretion, C-peptide levels), beta-cell dormancy, dyslipidemia.

[00526] Improved insulin resistance is measured by normalized gene expression of any of the following in liver, adipose tissue and/or muscle: Pck1 (PEPCK), G6pc (G6Pase), Srebf1 (SREBP-1), Gck (GK) , Ppargc1a (PGC-1), Abca1 (ABC-1), Acaca (acetyl-CoA carboxylase), IL1b (IL-1beta) , IL6 (IL-6), Tnf (TNF-alpha), Ccl2 (MCP-1), Slc2a4

(GLUT4), Il-1rn (IL-1ra), CD68, SAA1, SAA2, FAS (fatty acid synthase), Emr1 (F4/80), Irs1, Irs2. The above are measured by well-known techniques in the art.

[00527] In vitro assays are also useful to measure the effects of administration of a modulator of insulin/insulin receptor activity. Positive modulator antibodies are expected to result in increased translocation of GLUT4 to the cell surface. Methods for measuring the translocation of GLUT4 from an intracellular location to the plasma membrane are provided for example in US 6,632,924, US 2007/0141635, US 2003/0104490 and Liu et al, Biochem. J. 418(2), 413-20 (2009). Effects of positive modulators may also be assessed by analyzing enhanced glucose uptake by liver, adipose and/or muscle cells, enhanced depletion of glucose from liver, adipose and/or muscle cell culture medium, and measuring the ratio of metabolic to mitogenic INSR signaling increased or unchanged, pAKT activation, and pIRS-1 activation. The relative Hill slope of insulin-INSR interaction is also measurable. Some dose response curves, however, are steeper or shallower than the standard curve. The steepness is quantified by the Hill slope, also called a slope factor. A dose response curve with a standard slope has a Hill slope of 1.0. A steeper curve has a higher slope factor and a shallower curve has a lower slope factor. Exemplary assays to analyze these factors are described in the Examples.

Use of INSR antibodies as drug delivery agents

[00528] An antibody to INSR, 83-14, has been humanized for the purpose of creating a "molecular Trojan horse" to deliver protein and non-viral gene therapies across the blood-brain-barrier. 83-14 binding drives rapid internalization of the INSR. Hence, further antibodies with this property, or improved properties, may be useful for drug delivery to the brain and central nervous system (Boado et al, Biotech and BioEng. 96(2): 381-391; WO04/050016).

Kits

[00529] As an additional aspect, the invention includes kits which comprise one or more compounds or compositions packaged in a manner which facilitates their use to practice methods of the invention. In one embodiment, such a kit includes a compound or composition described herein (e.g., a composition comprising a insulin receptor or insulin/insulin receptor complex-specific antibody alone or in combination with a second agent), packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. Preferably, the compound or composition is packaged in a unit dosage

form. The kit may further include a device suitable for administering the composition according to a specific route of administration or for practicing a screening assay. Preferably, the kit contains a label that describes use of the antibody composition.

[00530] Additional aspects and details of the invention will be apparent from the following examples, which are intended to be illustrative rather than limiting.

EXAMPLES

EXAMPLE 1
Isolation of anti-INSR antibodies from antibody phage display libraries

(1)Phage Panning and Rescue

A. Naïve antibody phage display libraries

[00531] Human insulin receptor (hINSR) (R&D Systems, MN) was biotinylated with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) using the manufacturer's protocol and 16-fold molar excess of biotin reagent. The biotinylation of hINSR was confirmed by surface plasmon resonance (SPR).

[00532] For the first round of phage panning, $1.6x10^{11}$ cfu of phage particles from an scFv phage display library (BioInvent, Lund, Sweden) were blocked for 1h at room temperature (RT) in 1ml of 5% milk/PBS (Teknova, Hollister, CA) with gentle rotation. Blocked phage were twice deselected for 30 minutes against streptavidin-coated magnetic Dynabeads® M-280 (Invitrogen Dynal AS, Oslo, Norway). To form the biotin-hINSR-hINS complex, 100 pmoles of biotinylated hINSR was preincubated with excess (2,100 pmoles) human insulin (hINS) (Sigma, MO) dissolved in 5% milk/PBS, for 1h at RT with gentle rotation. For the second round of panning, 50 pmoles of biotin-hINSR was used with 1050 pmoles hINS. For the final round of panning, 25 pmoles of biotin-hINSR was incubated with 525 pmoles hINS.

[00533] The biotin-hINSR/hINS solution was incubated with blocked streptavidin-coated magnetic Dynabeads® M-280 (Invitrogen Dynal AS, Oslo, Norway) for 30 minutes with gentle rotation in order to immobilize the biotin-hINSR-hINS complex. The deselected phage were incubated with the biotin-hINSR-hINS streptavidin beads for 2h at RT. In order to saturate the hINSR with hINS, additional hINS (2,100 pmoles) was added to the solution. The beads were washed. For the first round of panning, beads were quickly washed (i.e. beads were pulled out of solution using a magnet and resuspended in 1 ml wash buffer) three times with PBS-0.1% TWEEN, followed by three times with PBS. For the second round of

panning, beads were quickly washed five times with PBS-0.1% TWEEN followed by a one 5 minute wash (in 1 ml wash buffer at room temperature with gentle rotation) with PBS-0.1% TWEEN and then five times with PBS followed by one 5 minute wash with PBS. For the third round of panning, beads were quickly washed four times with PBS-0.1% TWEEN, followed by two washes for five minutes with PBS-0.1% TWEEN and then four quick washes with PBS, followed by two 5 minute washes with PBS.

[00534] The hINSR-hINS- bound phage were eluted with 100 mM triethylamine (TEA) (30 min incubation at RT) which was then neutralized with 1M Tris-HCl (pH 7.4). The eluted phage were used to infect TG1 bacterial cells (Stratagene, CA) when they reached an OD_{600} of ~0.5. Following infection for 30 min at 37°C without shaking, and for 30 min at 37°C with shaking at 90 rpm, cells were pelleted and resuspended in 2YT media supplemented with 100 ug/ml ampicillin and 2% glucose. The resuspended cells were plated on 2YT agar plates with 100 ug/ml carbenicillin and 2% glucose and incubated overnight at 30°C.

[00535] Phage was then rescued with helper phage VCSM13 (New England Biolabs, MA) at a multiplicity of infection (MOI) ~ 10. Following helper phage infection at an OD_{600} of 0.6 at 37°C for 30 min without rotation and 30 min incubation at 37°C at 150 rpm, cell pellets were resuspended in 2YT media supplemented with 100 ug/ml ampicillin and 50 ug/ml kanamycin and allowed to grow overnight at 30°C. Phage in the supernatant were recovered after rigorous centrifugation and used for the next round of panning. In order to monitor the enrichment resulting from the phage selections, the amount of input and output phage was titered for the three rounds of panning

Gene III excision and generation of bacterial periplasmic extracts

[00536] Before screening the phage panning output scFv clones for binding to the hINSR-hINS complex, the gene III gene was first excised from the phagemid vectors to enable production of secreted scFv. In order to do this, a plasmid midi prep (Qiagen, Valencia, CA) of the third panning round output pool of clones was digested with the restriction enzyme *Eag*I (New England Biolabs, MA). The digestion product without the gene III was then allowed to self-ligate with T4 DNA ligase (New England Biolabs, MA) and used to transform chemically-competent TOP10 E. *coli* cells (Invitrogen, Carlsbad, CA). Individual transformed colonies in 96-well plates were then used to generate bacterial periplasmic extracts according to standard methods, with a 1:3 volume ratio of ice-cold PPB solution (Teknova, Hollister, CA) and double distilled water (ddH2O) and two protease inhibitor

cocktail tablets (Roche, IN). The lysate supernatants were assayed by ELISA, as described below.

B. Immunized antibody phage display libraries

[00537] An Omniclonal™ phage display library was generated from mice hyperimmunized with hINSR-hINS complex according to the methods described in US 6,057,098. The immunization material consisted of approximately equal molar amounts of recombinant human insulin (cat # I9278, Sigma-Aldrich, Inc. St. Louis, MO) and recombinant human INSR (28-956) (cat # 1544-IR/CF, R&D Systems, MN). The protein concentration of the complex was around 0.24 mg/ml. Single colonies, obtained from the Omniclonal™ library according to the protocol in U.S. Patent No. 6,057,098, were screened for binding activity in an ELISA assay as described below.

(2)ELISA screening of antibody clones on hINSR/hINS complex

[00538] ELISA Maxisorp® plates (Thermo Fisher Scientific, Rochester, NY) were coated overnight at 4°C with 3 ug/ml hINSR in PBS. Plates were then blocked for 1h at RT with 400 ul/well 5% milk/PBS. To generate wells containing the hINSR-hINS complex, 50 ul/well of hINS (2.1 uM) was allowed to bind to the hINSR for 30 min at RT. Bacterial periplasmic extracts were also blocked with 5% milk/PBS for 1h and then added to the coated ELISA plate (50 ul/well) and allowed to bind to either hINSR or hINSR-hINS complex on the ELISA plate for 2h at RT. The murine 83-7 anti-hINSR mAb was used as a positive ELISA screening control (Soos et al, Biochem. J. 235: 199-208, 1986). Bound scFv fragments were detected with murine anti-c-myc mAb (Roche, IN) for 1h at RT followed by goat anti-mouse HRP-conjugated antisera (Thermo Scientific, Rockford, IL). Three washes with PBS-0.1% TWEEN-20 (Teknova, Hollister, CA) were performed following every stage of the ELISA screens. The positive control 83-7 mAb was detected by goat-anti-mouse HRP (Thermo Scientific, Rockford, IL) following incubation for 1h at RT. Color was developed at 450nm absorbance with 50 ul/well soluble 3.3', 5.5'-tetramethylbenzidine (TMB) substrate (EMD chemicals, Calbiochem, NJ) and stopped with 1M H_2SO_4 (50 ul/well).

Results

[00539] ELISA screening of the bacterial periplasmic extracts identified multiple hINSR or hINSR-hINS complex binders that originated from the phage panning selection. Fifty-eight percent (868 out of 1,488) of the clones selected from the naïve library were able to bind the hINSR or hINSR-hINS complex. Forty-three percent (200 out of 465) of the clones selected from the immunized library were able to bind the hINSR or hINSR-hINS complex.

Periplasmic extracts from the selected clones were also assayed by FACS (see Example 2). Selected clones were reformatted as IgG1 or IgG2 antibodies. The variable heavy (VH) and light (VL) chains of the selected scFv fragments were PCR-amplified, cloned into plasmid vectors containing antibody constant genes, and transfected into 293E EBNA human cells using standard methods.

EXAMPLE 2
Receptor occupancy screen to determine antibody binding to INSR in the presence or absence of human insulin

[00540] This example describes the use of flow cytometric (FACS) based assays to measure differential antibody binding to cells in the presence or absence of human insulin (hINS). Anti-insulin receptor (INSR) antibodies from phage display libraries were screened in the assays to identify modulators of INS-INSR binding.

[00541] IM-9 cells were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640+10% FBS. Prior to use in assays cells were washed in serum-free RPMI 1640, counted and the concentration adjusted to $2x10^6$cells/ml in RPMI 1640+0.5% BSA (Sigma-Aldrich). The cells were cultured overnight in this media and as such were designated as "serum-starved." These cells were washed once and resuspended at $2x10^6$cells/ml in PBS containing 0.5% BSA and 0.01% sodium azide (FACS buffer).

[00542] Cells exposed to insulin were resuspended in FACS buffer supplemented with 70nM human insulin (Sigma-Aldrich, St. Louis, MO). Both cell populations (+hINS) or (-hINS) were incubated at 4°C for 30 minutes, washed once with FACS buffer and resuspended at $2x10^6$cells/ml in FACS buffer. Twenty five microliter aliquots of cells were plated into 96 well plates, mixed with 25ul of antibody or PPE and incubated on ice for 1h .

[00543] The cells were then washed once with FACS buffer and the binding of the antibody was detected by the addition of 25ul of an appropriate fluorochrome-conjugated secondary antibody. If the initial incubation had been with PPE containing a myc-tagged antibody, 25ul of a 1/1000 dilution of an anti-c-myc antibody (Roche) was added to the wells and the cells incubated on ice for 30 mins. The cells were then washed once with FACS buffer and the binding of the anti-c-myc revealed by the addition of a phycoerythrin-conjugated anti-mouse IgG. After a final 15 min incubation on ice the cells were washed and the pellets resuspended in FACS buffer. The cells were analyzed on a FACSCAN™

(Becton-Dickinson, Milipitas, CA) and the data analyzed in both FLOWJO™ (Treestar, Ashland, OR) and Microsoft Excel™.

[00544] This assay allowed the detection of four types of antibody, examples of which are shown in figure 1:

1. Antibodies that only bind to IM-9 cells if they have been exposed to human insulin (bind exclusively to INS/INSR complex)
2. Antibodies that bind better to IM-9 cells if they have been exposed to human insulin (bind preferentially to INS/INSR complex)
3. Antibodies that bind less well to IM-9 cells if they have been exposed to human insulin (bind preferentially to uncomplexed INSR).

[00545] Antibodies were scored as predicted positive modulators if the ratio of antibody binding to INS/INSR complex: antibody binding to uncomplexed INSR was greater than 1.3. Antibodies were scored as predicted negative modulators if the ratio of antibody binding to INS/INSR complex: antibody binding to uncomplexed INSR was less than 0.6. Antibodies were scored as predicted non-modulators if the ratio of antibody binding to INS/INSR complex: antibody binding to uncomplexed INSR was greater than 0.9 but less than 1.1.

EXAMPLE 3
Biotinylated ligand screen to determine the effects of anti-INSR antibodies on insulin binding to INSR

[00546] This example describes the use of FACS based assays to measure differential ligand (human insulin) binding to cells in the presence or absence of anti-INSR antibodies. Anti-INSR antibodies from phage display libraries were screened in the assays to identify modulators of the INS-INSR complex.

[00547] IM 9 cells were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640+10% FBS. Prior to use in assays cells were washed in serum-free RPMI 1640, counted and the concentration adjusted to $2x10^6$cells/ml in RPMI 1640+0.5% BSA (Sigma-Aldrich). The cells were cultured overnight in this media and as such were designated as "serum-starved." These cells were washed once and resuspended at $2x10^6$cells/ml in PBS containing 0.5% BSA (binding buffer).

[00548] Serum-starved cells were pre-exposed to INSR antibodies at room temperature for 15 minutes and then incubated with various concentrations of biotinylated human insulin

purchased from R&D Systems for a further 30 minutes at room temperature. The binding of the biotinylated insulin was revealed by the addition of a 1/100 dilution of streptavidin-phycoerythrin to this mixture for a further 15 minutes at room temperature. The cells were then washed once with binding buffer and resuspended in equal volumes of PBS containing 0.5% BSA, 0.1% sodium azide and 2% paraformaldehyde. The cells were analyzed on a FACSCAN™ (Becton-Dickinson, Milipitas, CA) and the data analyzed in both FLOWJO™ (Treestar, Ashland, OR) and Microsoft Excel™.

[00549] Figure 2 shows the binding of biotinylated insulin to IM9 cells in the presence or absence of anti-INSR antibodies at different insulin concentrations. Antibody 83-7 enhanced binding of biotinylated insulin; antibody MA-20 diminished binding of biotinylated insulin; control mouse IgG had no effect on binding of biotinylated insulin.

EXAMPLE 4
Assay to determine the ability of anti-INSR antibodies to stimulate pIRS-1 phosphorylation

[00550] The substrate proteins which are phosphorylated by the INSR include a protein called insulin receptor substrate 1 (IRS-1). IRS-1 phosphorylation to form pIRS-1 eventually leads to an increase in the high affinity glucose transporter (Glut4) molecules on the outer membrane of insulin-responsive tissues, and therefore to an increase in the uptake of glucose from blood into these tissues. A pIRS-1 assay was developed using the Luminex® technology platform (Luminex Corp., Austin, TX). Two modes of assay were developed: (a) titration of test antibody at a fixed concentration of insulin, and (b) titration of insulin at a fixed concentration of antibody. Anti-INSR antibodies selected on the basis of their differential binding to complexed and uncomplexed INSR were tested in the assays to identify modulators of the INS-INSR complex signaling.

Cell treatment and lysis

[00551] IM-9 cells were serum starved for 16-20 hours by counting, centrifuging, washing once with PBS and re-suspending at about $2x10^6$cells/ml in RPMI + 0.5% Sigma Cohn V BSA (10% stock in RPMI, filter sterilized, stored 4°C).

[00552] 2X concentrated solutions of insulin (Sigma I-9278 (10mg/ml) 1.77mM liquid stock stored at 4'C) dilutions were prepared in RPMI +0.5% BSA. A standard insulin titration may include 4-fold serial dilutions of for example: 6.25nM, 1.56nM, 0.39nM, 0.097nM, 0.024nM, 0.006nM, 0.0015nM, 0nM.

[00553] Milliplex MAP Cell Signaling Buffer and Detection Kit (Millipore catalog # 48-602) and Phospho-IRS-1 MAP Mates (Millipore catalog # 46-627) were employed for the detection of pIRS-1 levels, according to the manufacturer's instructions. Briefly, V-bottomed plates containing 50ul/well of 2X treatment media (RPMI containing 0.5% BSA +/- test antibody) were prepared and 1 x 10^6 cells serum-starved IM-9 cells resuspended in 50ul RPMI+0.5%BSA were added per well. Antibody pretreatment was performed for 15 minutes prior to insulin treatment, either (a) as a bulk antibody/cell mixture at a single antibody concentration that was then applied to wells containing serial dilutions of insulin, or (b) by adding cells directly to wells containing serial dilutions of antibody and spiking in insulin at 0.1nM. Plates were placed in a 37°C incubator and centrifuged at 1500rpm at RT for the last 3 minutes of treatment time (total of 15 minutes). Supernatant was removed by inversion and gentle blotting and treated cell pellets were lysed by triturating 3 times using a multi channel pipette with 100ul Lysis Buffer prepared according to Table 4 below (labile components, i.e. protease inhibitors and benzonase, were added just prior to use). Plates were placed on a shaker at RT for 30 minutes and centrifuged at 3000 rpm for 10 minutes to clarify the lysate and remove any air bubbles that may have occurred during trituration. 50ul of cleared lysate was removed and diluted 1:1 in 50uL Assay Buffer-1 (AB-1) from the Detection Kit, triturated 2-3 times to mix and 50ul was loaded onto a filter plate membrane on top of the 25ul/well of diluted beads (see below).

[00554] *Table 4: Lysis buffer components*

Lysis Buffer	10 wells 1 ml	20 wells 2 mls	25 wells 2.5 mls	30 wells 3 mls	40 wells 4 mls	50 wells 5 mls	60 wells 6 mls	100 wells 10 mls
Lysis Buffer (Millipore cat. # 43-040)	1	2	2.5	3	4	5	6	10
SDS 20% stock	0.045	0.09	0.1125	0.135	0.18	0.225	0.27	0.45
MgCl 50mM (Invitrogen cat. # Y02016)	0.02	0.04	0.05	0.06	0.08	0.1	0.12	0.2
Protease inhibitors (50X) (Millipore cat. # 20-201)	0.02	0.04	0.05	0.06	0.08	0.1	0.12	0.2
Benzonase EMD 1.01697.0002 @ 250ug/ml	0.004	0.008	0.01	0.012	0.016	0.02	0.024	0.04

[00555] Filter plate membranes (Millipore Catalog# MABVN1250) were pre-wet with 25ul AB-1/well. Pre-wetting buffer was aspirated from the filter plate using a Millipore

vacuum manifold, being careful not to dry the membranes, and any remaining liquid was blotted from the bottom of the filter plate. 25ul of 1X bead suspension was added per well (pIRS-1 beads (Millipore catalog # 46-627) were pre-prepared by diluting from 20X concentrate into AB-1 buffer and alternately vortexing and sonicating for 5 seconds 3 times each).

[00556] Filter plate wells were covered with a plate sealer, covered in aluminum foil to prevent light exposure, and incubated on a plate shaker (setting 7-8 on a Labline, Bellco plate shaker or similar model) at either RT for 2 hours or alternatively at 4°C overnight.

Luminex Detection

[00557] The filter plates were aspirated and their bottoms blotted. The beads remained in the well and were washed with 100ul of AB-1 and placed on shaker for 1-2 minutes. Plates were aspirated, and the wash step was repeated.

[00558] 25ul per well 1X biotinylated detection antibody, diluted from a 20X stock into AB-1 buffer, was added and plates were incubated on a shaker at RT for 1 hour. Plates were aspirated and their bottoms blotted. 25ul per well 1X streptavidin phycoerythrin diluted from a 25X stock into AB-1 buffer, was added and plates were incubated on a shaker at RT for 15 minutes. 25ul of Amplification Buffer (Millipore catalog # 48-602) was added to each well, and plates were incubated on a shaker at RT for further 15 minutes. The plates were aspirated and the beads were resuspended in 150uL AB-1 and read on the Luminex® instrument.

Results

[00559] Figure 3 shows pIRS-1 assay results from titrations of insulin in the presence of fixed concentrations of representative test antibodies. MFIs were normalized such that the curve fit maximum was adjusted to 100%. Some antibodies (positive modulators) shifted the insulin titration curve to the left. Other antibodies (negative modulators) shifted the insulin titration curve to the right. Varying magnitudes of modulation were observed. The data in Figure 3 shows antibodies producing up to a 9-fold increase, or up to a 24-fold decrease, in insulin sensitivity.

[00560] Figure 4 shows representative examples of the various functional classes of antibody based on pIRS-1 assay data. In each case results from the two modes of assay are shown: (i) titration of insulin at a fixed concentration of antibody, and (ii) titration of test antibody at a fixed concentration of insulin.

[00561] Figure 5 is a table showing insulin EC50 values from the pIRS-1 assay in the presence or absence of fixed concentrations of various test antibodies. The results are ranked according to EC50 ratio +Ab/-Ab.

EXAMPLE 5
Measurement of effects of anti-INSR antibodies on INSR-induced phosphorylation of AKT and MAPK

[00562] The INSR is a tyrosine kinase that undergoes autophosphorylation after insulin binding and subsequently catalyzes the phosphorylation of intracellular proteins such as insulin receptor substrate (IRS) family members, Shc, and Gab1. Each of these proteins serves as a docking site for the recruitment of downstream signaling molecules resulting in the activation of various signaling pathways including the PI(3)K/AKT and MAP kinase (MAPK) pathways. These pathways ultimately coordinate to regulate cell growth and differentiation, gene expression, glycogen, protein and lipid synthesis, and glucose metabolism.

[00563] The effects of a test antibody on signaling via the INS/INSR complex can be measured by assessing the ability of the antibody to augment insulin-induced serine or tyrosine phosphorylation of specific intracellular proteins, such as AKT and MAPK (ERK1/2), which are specific to the INSR signaling pathway. The phosphorylation of these proteins can be measured and quantified by electrochemiluminescence, Western blotting, ELISA, and other techniques known in the art.

[00564] In this example assay, CHOK1 cells, engineered to express either the human or mouse INSR, were used. These cells were maintained in Growth Medium containing EX-CELL 302 Serum-Free Medium for CHO Cells (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, and 0.4 mg/mL GENETICIN® (Invitrogen, Carlsbad, CA). The parental CHOK1 cells were used as a control and were maintained in Growth Medium without GENETICIN®.

[00565] On the day before the assay, the cells were washed with PBS, resuspended at 1 x 10^6 cells/mL in Starvation Medium containing RPMI 1640 (Invitrogen), 2 mM L-Glutamine, 0.4 mg/mL GENETICIN®, and 0.5% BSA, and incubated for 16-20 hours in a 37 °C, 5% CO_2 incubator. The parental CHOK1 cells were incubated in Starvation Medium without GENETICIN®. The next day, cells were resuspended in PBS with 0.5% BSA and 1 x 10^5 cells were added to wells of a 96-well plate. The test antibody was added at 0, 1, or 10 ug/ml, approximately 10 minutes prior to the addition of insulin. After incubation for 5-60 minutes

in a 37 °C, 5% CO_2 incubator, the treated cells were centrifuged and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich), and Complete Mini Protease Inhibitor (Roche Diagnostics Corporation, Indianapolis, IN) for 1 hour with shaking at 4 °C. The lysates were clarified by centrifugation at 485 x g for 3 minutes. Electrochemiluminescence using the MesoScale Discovery Multi-spot Assay System (Meso Scale Discovery, Gaithersburg, MD) was used to quantify the amount of phosphorylated AKT or MAPK present within the lysates. Data were analyzed using GraphPad Prism® (GraphPad Software Inc., La Jolla, CA) software to calculate EC50 values from a 4-parameter logistic equation.

[00566] For analysis of agonist activity, the assay was performed as follows. On the day before the assay, cells are washed with PBS, resuspended at 1 x 10^6 cells/mL in Starvation Medium containing RPMI 1640 (Invitrogen), 2 mM L-Glutamine, 0.4 mg/mL GENETICIN®, and 0.5% BSA, and incubated for 16-20 hours in a 37 °C, 5% CO_2 incubator. The parental CHOK1 cells were incubated in Starvation Medium without GENETICIN®. The next day, cells were resuspended in PBS with 0.5% BSA and 1 x 10^5 cells are added to wells of a 96-well plate. After incubation with test antibody for 5-60 minutes in a 37 °C, 5% CO_2 incubator, the treated cells were centrifuged and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich), and Complete Mini Protease Inhibitor (Roche Diagnostics Corporation, Indianapolis, IN) for 1 hour with shaking at 4 °C. The lysates were clarified by centrifugation at 485 x g for 3 minutes. Electrochemiluminescence using the MesoScale Discovery Multi-spot Assay System (Meso Scale Discovery, Gaithersburg, Maryland) was used to quantify the amount of phosphorylated AKT or MAPK is present within the lysates. Data were analyzed using GraphPad Prism® (GraphPad Software Inc., La Jolla, CA) software to calculate EC50 values from a 4-parameter logistic equation.

[00567] Figure 6 shows pAKT assay results for antibodies representative of: (A) positive modulators (increase insulin-induced signal transduction); (B) positive modulators with agonism (increase insulin-induced signal transduction and increase insulin-independent signal transduction) (C) non-modulators (no significant effect on insulin-induced signal transduction); (D) agonists (increase signal transduction independently of insulin; may or may not have modulatory activity) (E) negative modulators (decrease insulin-induced signal

transduction). The assay results also indicate whether the antibodies show functional cross-reactivity i.e. have effects on both human and mouse INSR-mediated signaling.

EXAMPLE 6
Anti-INSR antibodies exhibit a spectrum of agonism

[00568] The pIRS-1 assay of Example 4 and the pAKT assay of Example 5 were used to measure the degree of agonism of the selected anti-INSR antibodies. Rather than using titrations of antibodies or insulin, 5 ug/ml anti-INSR antibody was added to the assay in the absence of insulin. The assay measured the level of antibody-induced activation of signaling through INSR in the absence of insulin (agonism).

[00569] Figure 7 shows tabulated results to illustrate that the selected antibodies exhibit a spectrum of agonism.

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EXAMPLE 7
Change in cooperativity of insulin binding to INSR effected by a positive modulator INSR antibody

[00570] The pAKT assay of Example 5 was performed on one of the positive modulator antibodies, using various antibody concentrations and adding a serial dilution of insulin. The results are shown in Figure 8. Figure 8A shows there is a dose response of INSR binding in the presence of differing concentrations of antibody and insulin. Figure 8B shows the relative Hill slope of insulin-INSR interaction in the presence of varying concentrations of antibody.

EXAMPLE 8
Enhancement of glucose uptake by a positive modulator INSR antibody

[00571] The effects of a positive modulator INSR antibody on glucose uptake in 3T3-L1 adipocytes were measured. Upon insulin treatment, INSR is phosphorylated, activating a signal transduction pathway which leads to increased glucose uptake by glucose transporter 4 (GLUT4) in adipocytes (fat) or myocytes (muscle). Measuring glucose uptake provides a relevant end point assay for insulin sensitivity.

[00572] An assay using ^{3}H-2-deoxyglucose as a substrate for GLUT4 was employed (Zen-Bio, Inc., Research Triangle Park, NC). Briefly, 3T3-L1 preadipocytes were differentiated in

96-well isoplates. After maturation, the cells were washed 2 times with assay buffer and the cells were allowed to rest in Assay buffer for 4 hours. The cells were treated with anti-INSR antibody, or control antibody (10 ug/ml) and serial concentrations of insulin, or insulin at 0.8 nM for 15 minutes. After 15 minutes glucose uptake was initiated by adding ^{3}H-2-deoxyglucose cocktail and the cells were incubated at 37°C, 5% CO_2 for 10 minutes. After 10 minutes, the cells were washed with PBS, lysed, and mixed with scintillation fluid. The CPM of each well measured. Cytochalasin B (10μM) was used as a negative control.

[00573] Results are shown in Figure 9. Figure 9 shows the enhancement of insulin dependent glucose uptake by a positive modulator antibody. The positive modulator antibody results in approximately a 2-fold increase in ^{3}H-2-deoxyglucose uptake by 3T3-L1 cells in the presence of 10 ug/ml test antibody Ab001 compared to insulin alone.

[00574] These results suggest that positive modulator antibodes are useful to induce glucose uptake in vivo and treat patients exhibiting insulin resistance.

EXAMPLE 9
Measurement of the effects of anti-INSR antibodies on depletion of glucose in cell culture media

[00575] Depletion of glucose from cell culture media can be used a surrogate measurement of glucose uptake. The effects of anti-INSR antibodies on media depletion of glucose is measured as follows.

[00576] To measure glucose depletion, a Wako autokit glucose (Cat# 439-90901, Autokit C) is used according to the manufactures directions. Briefly, CHOK1 cell lines adapted to adherent with DMEM +10% FBS in 24 or 96 well plates are plated at an appropriate concentration. The cells are glucose and serum starved overnight in 0.5% BSA DMEM (no glucose) before use. The starvation media is aspirated and media consisting of the following is added in the presence and absence of test antibody or isotype control antibody: group 1, 4 parts DMEM no glucose:1 part DMEM high glucose (0.9 mg/mL); group 2, 4 parts DMEM no glucose:1 part DMEM high glucose (0.9 mg/mL) + insulin. At each desired time point, 2uL samples of media from each well are removed and added to 118 uL of Wako working solution . In some embodiments, sampleas are taken at 0, 1, 2-5, 5, 10, and 24 hours. Glucose uptake is evaluated with the FLEXSTATION at absorbance 505nm and 600nM. The amount of glucose is determined as follows: [Average reading for similar samples]/[average

reading for standards]. Cell count is obtained before and at the end of the experiment to normalize for cell growth.

EXAMPLE 10
Measurement of the effects of anti-INSR antibodies on the balance between mitogenic and metabolic INSR signaling

[00577] INSR signals through two major pathways (1) the PI3 kinase/PDK1/PKB pathway which primarily regulates metabolism, with some influence on growth and (2) the Ras/ERK mitogenic pathway which primarily regulates cell growth. The effects of anti-INSR antibodies on the balance between mitogenic and metabolic INSR signaling is measured as described in the art. See, e.g., Jensen et al. (Vitam Horm. 80:51-75, 2009), De Meyts and Shymko, (Novartis Found. Symp. 227:46–57, 2000); and Rakatzi et al. (Diabetes 52:2227–2238, 2003).

EXAMPLE 11
Measurement of the in vivo effects of anti-INSR antibodies

[00578] Anti-INSR antibodies found to be cross-reactive with mouse INSR are measured in a number of in vivo models. In the DIO model, C57BL/6J (B6) male mice (The Jackson Laboratory, Maine) are fed a high fat diet (HFD) for twelve weeks, becoming obese, mildly to moderately hyperglycemic and impaired for glucose tolerance. This model is used to evaluate the ability of INSR antibodies to affect insulin sensitivity in a tightly controlled setting. This system also allows a direct comparison of INSR action and modulation under normal versus diseased conditions. In this experiment, DIO or age-matched B6 mice are dosed with INSR antibody 24 hours prior to administration of a pre-defined sub-maximal dose of insulin in an insulin tolerance test (ITT). Control IgG or maximal insulin serve as negative and positive controls, respectively. Responsiveness to insulin is assessed by measuring plasma glucose; a greater decrease in glucose over 60 minutes is suggestive of an increased INSR response. In a separate study, DIO or B6 mice are dosed with antibody 24 hours prior to a glucose tolerance test (GTT). By this measure, lowered fasting glucose and area under the curve (AUC) indicates improved insulin sensitivity.

[00579] Two murine models are used to assess the impact of INSR antibodies on type-2 diabetes progression. ob/ob mice (The Jackson Laboratory, ME) are leptin deficient, becoming obese and only mildly hyperglycemic due to compensatory hyperinsulemia. In this

model, animals receive INSR antibodies beginning at 6 weeks of age or rosiglitazone (PPAR-gamma agonist), an agent previously shown to improve glycemic control in these animals. As in the DIO study, glycemic control is assessed by ITT and GTT, every 2 weeks for 6 weeks. In addition, hemoglobin A1c (HbA1c), a key indicator of prolonged elevated plasma glucose, and a lipid panel, is evaluated at the end of the study. In the second model, the streptozotocin (STZ)/HFD model, pancreatic beta cells in Swiss Albino mice (The Jackson Laboratory, Maine) are ablated via multiple low-dose of streptozotocin, while insulin resistance is induced through HFD feeding. In this model, animals are severely hyperglycemic due to impairment of pancreatic insulin output, a situation analogous to late stage T2D (Dakshinamoorty et al, J. Pharm. and Pharmacology 60: 1167-73 (2008)). STZ/HFD animals are treated and evaluated in a manner similar to the ob/ob model to measure the effect of INSR antibodies on disease progression.

EXAMPLE 12
Effects of partial agonist anti-INSR antibodies on glycemic control in DIO mice

[00580] In the diet-induced obesity (DIO) model, C57BL/6 mice can become insulin resistant after approximately 12-14 weeks on a high-fat diet (HFD). Anti-INSR antibodies demonstrated to behave as partial agonists or positive modulators *in vitro* were evaluated in this model to determine if these antibodies improved insulin sensitivity and/or glycemic control *in vivo*.

[00581] To determine whether partial agonist anti-INSR antibodies reduce fasted blood glucose, 20 week-old DIO mice (14 weeks on HFD; n =8/group) were fasted for 5 hours and challenged intravenously with partial agonist antibodies Ab030 and Ab037, or an isotype control (5 mg/kg). In additional control studies, DIO mice were treated with insulin (0.5 U/kg), or age-matched mice fed a normal diet (ND) were dosed with isotype control (5 mg/kg). Blood glucose was sampled prior to injection (time = 0) and 1, 2 and 4 hours post-administration. Compared to age-matched controls, increased blood glucose was observed in DIO mice (HFD-fed/isotype control) at the 1-hour time point, consistent with insulin resistance in animals fed HFD (Figure 10A). Administration of insulin or either of the partial agonist antibodies resulted in a statistically significant reduction ($p < 0.05$; one-tailed t-test) in blood glucose (Figure 10B). Neither antibody induced hypoglycemia at any time point (defined as blood glucose < 36 mg/dL). These results suggest that anti-INSR partial agonist antibodies safely and effectively reduce fasting blood glucose.

[00582] To further evaluate the effect of a partial agonist anti-INSR antibody on glycemic control, 18-week old DIO mice (12 weeks on HFD; n =8/group) were injected intraperitoneally (IP) with Ab037 (0.1, 1.0 or 9 mg/kg) or isotype control (1.0 mg/kg). As additional controls, age-matched control mice were dosed with isotype control (1.0 mg/kg) or DIO animals were given insulin (0.75 U/kg; IP). A glucose tolerance test (GTT) was performed 24 hours after antibody administration (30 min after insulin) by fasting the animals for 16 hours (beginning approximately 8 hours after antibody administration), injecting glucose (1.0 U/kg) and following blood glucose over 2 hours. In this experiment, HFD did not have a significant impact on fasting glucose (Figure 11B) or post-bolus peak glucose (Figure 11A). Nevertheless, in DIO mice, partial agonist antibody significantly reduced fasting blood glucose relative to isotype control when dosed at or above 1.0 mg/kg (Figure 11B) and reduced GTT area under the curve (AUC) at 9.0 mg/kg (Figure 11C).

[00583] This outcome demonstrates that an anti-INSR partial agonist antibody can reduce fasting glucose and improve glycemic control *in vivo*.

EXAMPLE 13
Effects of positive modulator anti-INSR antibodies on glycemic control in DIO mice

[00584] To determine if a positive modulator anti-INSR antibody improves insulin sensitivity *in vivo*, 18-week old DIO mice (*n* =8/group) were given IP injections of Ab001 (positive modulator)(0.1, 1.0 or 10 mg/kg), partial agonist antibody (Ab037) (10 mg/kg) or isotype control (1.0 mg/kg). Age-matched mice fed ND dosed with isotype control (1.0 mg/kg) served as an additional control (Figure 12A). Twenty-four hours later, an insulin tolerance test (ITT) was carried out by administering insulin (0.5 U/kg) after a 5 hour fast and monitoring blood glucose levels over 2 hours. A HFD did not have a significant impact on fasting glucose (Figure 12B) or ITT AUC (Figure 12C) relative to regular diet, and neither partial agonist antibody (Ab037) nor positive modulator antibody (Ab001) administration resulted in a statistically significant lower AUC ITT, relative to isotype control treated DIO animals (Figure 12C). Partial agonist antibody Ab037 significantly reduced fasting glucose, while positive modulator antibody Ab001 induced a non-statistically significant, dose-dependent trend towards reduced fasting glucose.

[00585] The following week, a GTT was carried out on the same animals after an additional dose of antibody (Figure 13A). In this study, HFD resulted in a non-statistical

increase in fasting glucose (Figure 13B) and GTT AUC (Figure 13C) compared to control fed animals. Compared to isotype control-treated DIO mice, partial agonist antibody and positive modulator antibody significantly reduced fasting glucose at all doses tested. In addition, both partial agonist antibody and positive modulator antibody significantly reduced GTT AUC at 10 mg/kg relative to isotype control.

[00586] The effect of Ab001 and Ab037 on lipid parameters was investigated by treating 18-week old DIO mice IP twice weekly (BIW) with antibody (10 mg/kg; n =5/group) for twelve weeks. In this experiment, similar efficacy to the two-week study (as described above) was observed with respect to fasting glucose, GTT and ITT. At the end of the study, plasma was collected to measure lipids using standard ELISA-based techniques. Relative to isotype control, both Ab001 and Ab037 reduced fasting triglyceride and total cholesterol levels in DIO mice ($p < 0.05$; Figure 14A and 14B), suggesting that these antibodies are able to improve lipid dysregulation associated with insulin resistance.

[00587] Two additional positive modulator anti-INSR antibodies were evaluated for improvement of glycemic parameters in vivo using 18-week old DIO mice (n =10/group). In this study, positive modulator antibodies Ab083 and Ab085 were compared against Ab001 and Ab037 and an isotype control antibody. A ND fed, age-matched group treated with isotype control antibody served as an additional control. All antibodies were dosed IP at 10 mg/kg BIW. A day after the third dose of antibody, fasting blood glucose was measured and a GTT was performed. Glycemic control was significantly impaired in isotype control-treated DIO mice relative to similarly treated age-matched ND fed animals, as reflected by a GTT time course assessment and the corresponding AUC determination (Figure 15A and 15B). In this experiment, Ab037 and Ab083 improved AUC to levels indistinguishable from normal ($p < 0.05$ relative to HFD/isotype control), whereas Ab001 did not produce significant improvements. Similarly, with respect to fasting glucose, a significant difference was observed between isotype control treated DIO and age-matched ND-fed mice and both Ab037 and Ab001 exerted statistically significant normalizing effects ($p < 0.05$; Figure 15C). Ab083 yielded a small, non-statistically significant improvement in fasting blood glucose, whereas Ab085 did not elicit any change in this parameter.

[00588] Another measure of the effects of anti-INSR antibodies on in vivo function is by Homeostasis model assessment–insulin resistance (HOMA-IR). HOMA-IR is an empirical mathematical formula based on fasting plasma glucose and fasting plasma insulin levels that was developed as a surrogate measurement of in vivo insulin sensitivity: HOMA-IR =

fasting plasma insulin (μIU/mL) x fasting plasma glucose (mmol/L)/22.5, or alternatively using the formula: Insulin (ng/mL) x Glucose (mM), which incorporates the 22.5 conversion factor. Examples of HOMA-IR are described in Owyang et al., Endocrinology 151:2515-27, 2010 and Matthews et al., Diabetologia. 28:412-9, 1985.

[00589] After 4 weeks of dosing, plasma glucose, insulin and lipids were evaluated. Ab083 and Ab037 reduced plasma glucose at this time point, whereas Ab083 and Ab085 reduced insulin ($p < 0.05$; Figures 16A and 16B). These effects translated into improved insulin sensitivity in this model of insulin resistance for Ab083 and Ab085, as determined by HOMA-IR ($p < 0.05$; Figure 16C). With respect to lipids, Ab085 significantly improved only triglycerides ($p < 0.05$; Figure 16D), while Ab083 and Ab037 significantly reduced unesterified, total and non-HDL cholesterol ($p < 0.05$; Figure 16E-G). The latter two antibodies also improved the non-HDL/HDL cholesterol ratio (Figure 16H). Ab001 significantly reduced both total and non-HDL cholesterol.

[00590] Surprisingly, all four antibodies reduced weight gain in DIO mice relative to isotype control over 3 weeks of treatment, without reducing body weight to below baseline (Figure 17A and 17B). These results demonstrate that the positive modulator antibody Ab083 and agonist antibody Ab037 correct impaired glucose tolerance in DIO mice, that modulator antibodies Ab083 and Ab085 improve insulin sensitivity and suggest that all four antibodies have the capacity to decrease weight gain resulting from HFD.

[00591] These results suggest that partial agonist and positive modulator antibodies specific for the INSR improve glycemic control in diabetic subjects.

EXAMPLE 14
Effects of partial agonist and positive modulator anti-INSR antibodies on glycemic control and disease in db/db mice

[00592] Mice homozygous for the spontaneous $Lepr^{db}$ allele lack leptin receptor function and become progressively insulin resistant and obese beginning at three to four weeks of age. In these mice, insulin levels rise, until about 8-10 weeks of age, at which time the animals are severely insulin resistant and hyperinsulinemic. This genetic background nevertheless results in uncontrolled hyperglycemia, leading to pancreatic beta cell dysfunction after approximately 10 weeks of age and ultimately to beta cell failure. Anti-INSR antibodies demonstrated to behave as partial agonists or positive modulators *in vitro* were evaluated in

this model to determine if these antibodies improved insulin sensitivity, glycemic control and/or disease progression *in vivo*.

[00593] The db/db mouse was utilized to assess the activity of Ab001 and Ab037 in a setting of progressive insulin resistance and beta cell dysfunction, combined with severe obesity. In this experiment, Ab001 (1 mg/kg or 10 mg/kg), Ab037 (10 mg/kg) or isotype control antibody (1 mg/kg or 10 mg/kg) were dosed IP, BIW to 5 week-old db/db mice (n =10/group). As an additional control, a group of age-matched heterozygous littermates, which are generally phenotypically normal, were dosed similarly with 10 mg/kg isotype control antibody. As in the DIO model, weight gain was significantly reduced in animals treated with 10 mg/kg of either Ab001 or Ab037 relative to isotype control-treated mice over the first five weeks of treatment ($p < 0.05$; Figure 18A and 18C). Importantly, after 5 weeks of treatment, which corresponds to 10 weeks of age, when db/db mice generally begin losing weight as a result of pancreatic beta-cell depletion, both antibodies reduce weight loss ($p < 0.05$; Figure 18B and 18D). After 10 weeks of treatment, treatment with either Ab001 or Ab037 at 10 mg/kg significant improvements were observed in fasting blood glucose relative to corresponding isotype control-treated groups ($p < 0.05$; Figure 19A). In addition, at this time point, HbA1c was significantly reduced in the 1 mg/kg Ab001 group, and also reduced to a lesser degree in the 10 mg/kg Ab001 group ($p < 0.05$; Figure 19B).

[00594] Plasma insulin and lipids were evaluated after 14 weeks of dosing. At this time, both Ab001 (10 mg/kg) and Ab037 increased circulating insulin at an age (approximately 20 weeks old) at which these animals would be expected to have pancreatic beta-cell failure ($p < 0.05$; Figure 20A), suggesting that both mAbs are capable of restoring insulin output in insulinopenic animals. In addition, Ab001 (10 mg/kg) significantly reduced plasma triglycerides, total cholesterol, non-HDL cholesterol, unesterified cholesterol and the non-HDL/HDL cholesterol ratio ($p < 0.05$; Figure 20B-F). A significant reduction in unesterified cholesterol and a trend towards lowered triglycerides was observed in plasma from Ab037-treated animals ($p < 0.05$ and $p = 0.08$, respectively; Figure 20B and 20C).

[00595] Interestingly, the reduction in weight gain occurred early, while the animals were insulin resistant, but not expected to have severe beta cell depletion, as is the case in the DIO model. However, in this experiment, Ab001-induced changes in glycemic control and glycated hemoglobin occurred only during the late phase, when the animals would be expected to have beta cell dysfunction. Moreover, during this time period, both antibodies reduced pathological weight loss. Not to be bound by theory, this outcome suggests that the

anti-INSR antibody effects on weight and glycemic control can occur in tandem, but are separable. These data suggest that Ab001 is capable of normalizing weight, improving glycemic control and partially correcting dyslipidemia under conditions of combined insulin resistance and beta cell depletion.

[00596] To evaluate the activity of antibodies under severely insulin resistant and insulinopenic conditions, 10-week old db/db mice, which would be expected to manifest with progressive pancreatic beta cell dysfunction, were treated with Ab001, Ab037, Ab083, Ab085 or isotype control antibody, at 10 mg/kg IP, BIW for eight weeks. Fasting blood glucose was measured weekly for the duration of the study. In this study, Ab085 significantly reduced fasting blood glucose relative to isotype control ($p < 0.05$; Figure 21). This demonstrates that Ab085 improves disease under insulin resistant, hypoinsulinemic conditions.

[00597] Two additional positive modulator anti-INSR antibodies were evaluated for improvement of insulin resistance in 5-week old db/db mice, which would be expected to manifest with severe insulin resistance. Mice were treated with Ab001, Ab037, Ab083, Ab085 or isotype control antibody, at 10 mg/kg IP, BIW for four weeks to evaluate the effect of antibodies on insulin resistance before the onset of beta cell dysfunction. Fasting plasma glucose and fasting plasma insulin were measured at the end of the study and HOMA-IR was calculated. In this study, Ab083 and Ab085 significantly improved insulin resistance compared to isotype control ($p < 0.05$; Figure 22), demonstrating that these antibodies improves insulin sensitivity in this model of diabetes.

EXAMPLE 15
Effects of partial agonist and positive modulator anti-INSR antibodies on glycemic control and disease in MLDS/HFD mice

[00598] In the multi-low dose streptozotocin (MLDS)/HFD model, insulin resistance is achieved by feeding 6-week old ICR mice a HFD (40 kcal% fat) for four weeks, during which time 5 daily doses of streptozotocin (40 mg/kg, during the third week) are administered IP to partially ablate beta cell function. Anti-INSR antibodies demonstrated to behave as partial agonists or positive modulators *in vitro* were evaluated in this model to determine if these antibodies improved insulin sensitivity, glycemic control and/or disease progression *in vivo*.

[00599] To evaluate the effect of Ab001 and Ab037 on disease in a model of combined insulin resistance and beta cell dysfunction, MLDS/HFD mice (n =10/group) were dosed with Ab001, Ab037 or isotype control antibody, at 10 mg/kg IP, BIW for six weeks. One week after the first dose, a three-fold increase in fasting blood glucose was observed in isotype control treated diseased mice, relative to age-matched normal animals, confirming that a diabetic phenotype was achieved. At this time, a GTT was carried out, revealing significant improvements in glycemic control for both Ab001 and Ab037 ($p < 0.05$; Figure 23A and 23B). Fasting blood glucose was also significantly reduced in the group of mice treated with Ab037 ($p < 0.05$), whereas no significant change was elicited by Ab001 (Figure 23C). One week later, fed glucose was evaluated. Similar to fasting glucose, disease in MLDS/HFD mice was manifested by significantly elevated fed glucose levels, which was ameliorated by Ab037 ($p < 0.05$; Figure 24A). Consistent with these improvements in GTT and fed/fasting glucose, Ab037 reduced HbA1c by approximately 1.5% after six weeks of dosing ($p < 0.05$; Figure 24B). End of study plasma analysis revealed that Ab037 treatment led to a statistically significant normalization in plasma insulin and a smaller reduction in non-HDL/HDL cholesterol ratio, whereas Ab001 significantly improved plasma leptin levels, with a similar, but smaller corrective impact on plasma insulin ($p < 0.05$; Figure 25A-C). This model does not present with consistent, disease-related weight change as observed in the db/db model, and neither Ab001 nor Ab037 impacted body weight in this model (Figure 26), suggesting that the reduced weight gain observed with these antibodies in the other in vivo models was not a non-specific effect. This data demonstrates Ab037 improves multiple manifestations of disease in MLDS/HFD mice, while Ab001 also corrects some parameters of impaired glycemic control in this model.

[00600] Two additional positive modulator anti-INSR antibodies were evaluated for improvement of glycemic parameters *in vivo*. MLDS/HFD mice (n =10/group) were dosed with Ab001, Ab037, Ab083, Ab085 or isotype control antibody, at 10 mg/kg IP, BIW for six weeks. After 3 weeks of treatment, a GTT was performed, revealing that Ab037 and Ab083 a completely normalize glycemic control, relative to isotype control ($p < 0.05$; Figure 27A and 27B). Fasting blood glucose was also significantly reduced in mice treated with Ab037 or Ab083 over the duration of the 6-week study ($p < 0.05$), whereas no significant change was elicited by Ab001 or Ab085 (Figure 28). At the end of the study, plasma lipids were evaluated. Ab083 significantly improved plasma triglycerides, unesterified cholesterol, total cholesterol, non-HDL cholesterol, non-HDL/HDL cholesterol ratio and free fatty acids ($p <$

0.05; Figure 29A-F). In addition, Ab001 significantly reduced total, non-HDL and unesterfied cholesterol, as well as non-HDL/HDL cholesterol ratio. Ab037 improved non-HDL cholesterol, unesterified cholesterol, non-HDL/HDL cholesterol ratio and free fatty acids. In this experiment, Ab085 significantly reduced only free fatty acids. Consistent with the observed improvements in GTT and fasting glucose, Ab037 and Ab083 significantly reduced HbA1c after six weeks of dosing ($p < 0.05$; Figure 30). In addition, Ab001 and Ab085, which exerted less of an effect on fasting glucose and glucose tolerance, but did improve certain lipid parameters, also reduced HbA1c. As in the previous experiment, none of the mAbs meaningfully impacted body weight in this model, except Ab085, with reduced weight gain over the first 3 weeks of dosing (Figure 31). This data demonstrates that all four antibodies tested improve multiple manifestations of disease in MLDS/HFD mice, without impacting body mass in this weight neutral model.

EXAMPLE 16
Effects of 24 hour administration of partial agonist and positive modulator anti-INSR antibodies on INSR phosphorylation *in vivo*

[00601] The increase in INSR tyrosine phosphorylation in insulin-sensitive tissues such as liver and muscle by short-term administration of anti-INSR antibodies confirms that the antibodies are bioavailable and capable of acting similarly on INSR in vivo as observed in vitro. In this experiment, anti-INSR antibodies identified as partial agonists or positive modulators in vitro were dosed for 24 hours in C56BL/6 male mice and evaluated for their effects on basal and insulin-induced liver and muscle INSR phosphorylation.

[00602] To determine if INSR partial agonist and positive modulators increase INSR phosphorylation in liver and muscle, 10 week-old C56BL/6 male mice (n=3) were given anti-INSR or isotype control antibodies (10 mg/kg) for 24 hours, and effects on liver and muscle INSR tyrosine phosphorylation were determined by ELISA in mice given an insulin bolus (1 U/kg) or PBS for 10 minutes. Phosphorylated INSR concentrations were normalized to total insulin receptor concentrations and expressed as a percentage.

[00603] Exogenous insulin (1 U/kg) did not significantly increase INSR phosphorylation in control animals (although there was a positive trend) in either liver or muscle (Figures 32A, B). However, in liver, significant increases in insulin-stimulated INSR phosphorylation were observed in Ab083- and Ab037-treated mice ($p < 0.05$) as well as a nearly significant increase in Ab085-treated mice ($p = 0.07$; Figure 32A). This outcome suggests that partial

agonist and positive modulator antibodies are capable of increasing responsiveness to insulin in vivo. Interestingly, in liver, Ab083 significantly increased INSR phosphorylation even in the basal state (no exogenous insulin), suggesting that Ab083 is able to sensitize the response to insulin even in presence of low, fasting levels of endogenous insulin.

[00604] The most pronounced effects from anti-INSR partial agonist and positive modulator antibodies were seen in the muscle. While all three anti-INSR antibodies positively-modulated insulin signaling in mice receiving an insulin bolus, Ab083, and to a greater extent, Ab085, also sensitized muscle INSR signaling to endogenous, fasting levels of insulin when compared to control animals (Figure 32B).

[00605] These results suggest that both partial agonist and positive modulator anti-INSR antibodies improve responsiveness to insulin-mediated signaling in liver and muscle in vivo. Relative to the effects of Ab037, antibodies Ab083 and Ab085 sensitize INSR at relatively low insulin concentrations.

EXAMPLE 17
Isolation of anti-INSR antibodies from additional antibody phage display libraries

[00606] Additional naïve antibody libraries were screened for antibodies specific for INSR.

(1) Phage Panning and Rescue

[00607] Human insulin receptor (hINSR) (R&D Systems, Minneapolis, MN) was biotinylated as described in Example 1 and used for panning of additional naïve antibody phage display libraries.

A. scFv library

[00608] scFv Naïve Library: For the first round of phage panning, $4.5x10^{12}$ cfu of phage particles from an scFv lambda phage display library or $4.12x10^{12}$cfu of phage particles from an scFv kappa phage display library (XOMA LLC, Berkeley, CA) were blocked for 1h at room temperature (RT) in 1ml of 5% milk/PBS (Teknova, Hollister, CA) with gentle rotation. This represents two separate pannings, scFv-kappa and scFv-lambda. Blocked phage were deselected twice for 30 minutes against streptavidin-coated magnetic Dynabeads® M-280 (Invitrogen Dynal AS, Oslo, Norway). To form the biotin-hINSR-hINS complex 103 pmoles of biotinylated hINSR was preincubated with excess (2,100 pmoles) human insulin (hINS) (Sigma, St Louis, MO) dissolved in 5% milk/PBS, for 1h at RT with gentle rotation. For the

second round of panning, 50 pmoles of biotin-hINSR was used with 1050 pmoles hINS. For the final round of panning, 25 pmoles of biotin-hINSR was incubated with 525 pmoles hINS.

B. Fab library

[00609] Fab Naïve Library: For the first round of phage panning, $1.2x10^{13}$cfu of phage particles or $1.8x10^{13}$cfu of phage particles from two different rescues of an Fab lambda library (XOMA LLC, Berkeley, CA), or $7.2x10^{12}$cfu of phage particles or $1.8x10^{13}$cfu of phage particles from two different rescues of an Fab kappa library (XOMA LLC, Berkeley, CA) were blocked for 1h at room temperature (RT) in 1ml of 5% milk/PBS (Teknova, Hollister, CA) with gentle rotation. This represents four separate pannings. Blocked phage were twice deselected for 30 minutes against streptavidin-coated magnetic Dynabeads® M-280 (Invitrogen Dynal AS, Oslo, Norway). To form the biotin-hINSR-hINS complex, 103 pmoles of biotinylated hINSR was preincubated with excess (2,100 pmoles) human insulin (hINS) (Sigma, St. Louis, MO) dissolved in 5% milk/PBS, for 1h at RT with gentle rotation. For the second round of panning, 50 pmoles of biotin-hINSR was used with 1050 pmoles hINS. For the final round of panning, 25 pmoles of biotin-hINSR was incubated with 525 pmoles hINS.

[00610] The biotin-hINSR/hINS solution was incubated with blocked streptavidin-coated magnetic Dynabeads® M-280 (Invitrogen Dynal AS, Oslo, Norway) for 30 minutes with gentle rotation in order to immobilize the biotin-hINSR-hINS complex. The deselected phage were incubated with the biotin-hINSR-hINS streptavidin beads for 2h at RT. In order to saturate the hINSR with hINS, additional hINS (2,100 pmoles) was added to the solution. The beads were washed. For the first round of panning, beads were quickly washed (i.e. beads were pulled out of solution using a magnet and resuspended in 1 ml wash buffer) three times with 0.5% milk-PBS-0.1% TWEEN, followed by three washes with 0.5% milk-PBS followed by one quick wash with PBS. For the second round of panning, beads were quickly washed five times with 0.5% milk-PBS-0.1% TWEEN followed by one 5 minute wash (in 1 ml wash buffer at room temperature with gentle rotation) with 0.5% milk-PBS-0.1% TWEEN and then five washes with 0.5% milk-PBS followed by one 5 minute wash with 0.5% milk-PBS and then one quick wash with PBS. For the third round of panning, beads were quickly washed four times with 0.5% milk-PBS-0.1% TWEEN, followed by two washes for five minutes with 0.5% milk-PBS-0.1% TWEEN and then four quick washes with 0.5% milk-PBS, followed by two 5 minute washes with 0.5% milk-PBS and then one quick wash with PBS.

C. Elution and rescue

[00611] The hINSR-hINS-Streptavidin bead-bound phage were eluted with 0.5ml 100 mM triethylamine (TEA) for 30 minutes at RT with gentle rotation. The beads were separated from the eluate. The eluate was removed and neutralized with 0.5ml 1M Tris-HCl (pH 7.4). The beads were neutralized with 1ml 1M Tris-HCl (pH 7.4). The eluted phage from beads or eluate, were used separately to infect TG1 bacterial cells (Stratagene, La Jolla, CA) when they reached an OD_{600} of ~0.5. Following infection for 30 min at 37°C without shaking, and for 30 min at 37°C with shaking at 90 rpm, cells were pelleted and resuspended in 2YT media supplemented with 100 ug/ml carbenicillin and 2% glucose. The resuspended cells were plated on 2YT agar plates with 100 ug/ml carbenicillin and 2% glucose and incubated overnight at 30°C.

[00612] Phage was then rescued with helper phage M13KO7 (New England Biolabs, MA) at a multiplicity of infection (MOI) ~ 20. Following helper phage infection of TG1 cells at an OD_{600} of 0.5 at 37°C for 30 min without shaking and 30 min incubation at 37°C at 100 rpm, cell pellets were resuspended in 2YT media supplemented with 100 ug/ml carbenicillin and 50 ug/ml kanamycin and allowed to grow overnight at 25°C and 250rpm. Phage in the supernatant were recovered after rigorous centrifugation and used for the next round of panning. In order to monitor the enrichment resulting from the phage selections, the amount of input and output phage was titered for the three rounds of panning.

(2) FACS screening of antibody clones on human INSR/hINS or murine INSR/hINS complex

[00613] Individual colonies were picked and grown in 96-well plates and were then used to generate bacterial periplasmic extracts according to standard methods, with a 1:3 volume ratio of ice-cold PPB solution (Teknova, Hollister, CA) and ddH2O and protease inhibitor (Roche, Indianapolis, IN). The lysate supernatants were assayed by FACS on hINSR/hINS or murine INSR/hINS complex, using the protocol described in Example 2, except that suspension adapted CHO-K1 transfected with either hINSR or muINSR were used instead of IM-9 cells, and cells exposed to insulin were resuspended in FACS buffer supplemented with 150nM rather than 70nM human insulin. This assay allowed the detection of at least 6 types of antibody:

1. Antibodies that only bind to hINSR-CHO cells if they have been exposed to human insulin (bind exclusively to INS/INSR complex in a species specific manner)

2. Antibodies that only bind to muINSR-CHO cells if they have been exposed to human insulin (bind exclusively to INS/INSR complex in a species specific manner)

3. Antibodies that bind to both hINSR-CHO and muINSR-CHO cells if they have been exposed to human insulin (bind exclusively to INS/INSR complex in a species cross-reactive manner)

4. Antibodies that only bind to hINSR-CHO cells (bind exclusively to INSR in a species specific manner)

5. Antibodies that only bind to muINSR-CHO cells (bind exclusively to INSR in a species specific manner)

6. Antibodies that bind to both hINSR-CHO and muINSR-CHO cells (bind exclusively to INSR in a species cross-reactive manner)

[00614] Antibodies were scored as described in Example 2. Light chain and heavy chain sequences of the isolated antibodies were sequenced and are set out in SEQ ID NOs: 87-147 (light chain) and SEQ ID NOs: 223-284 (heavy chain).

Results

[00615] FACS screening of the bacterial periplasmic extracts identified multiple antibodies that bound human receptor or receptor/ligand complex, hINSR or hINSR-hINS, or murine receptor or receptor/ligand complex, muINSR or muINSR/hINS. Thirty-three percent (484 out of 1,488) of the clones selected from these naïve libraries were able to bind the hINSR or hINSR-hINS complex. Twenty-five percent (370 out of 1,488) of the clones selected from these naïve libraries were able to bind the muINSR or muINSR-hINS complex. Sixteen percent (234 out of 1,488) of the clones bound to both hINSR or hINSR-hINS and muINSR or muINSR/hINS complexes by FACS.

[00616] Selected clones were reformatted as IgG2 antibodies. The variable heavy (VH) and light (VL) chains of the selected scFv fragments were PCR-amplified, cloned into plasmid vectors containing antibody constant genes, and transfected into 293E EBNA human cells using standard methods. Binding of the reformatted antibodies to hINSR or hINSR-hINS or muINSR or muINSR/hINS were assessed by FACS as described above. Results are set out in Figure 33.

[00617] Results show that certain reformatted antibodies bind to both mouse and human INSR. Figure 33 also shows that certain reformatted antibodies bind differentially to INSR

in the presence and absence of insulin and are therefore predicted to modulate insulin binding to INSR.

EXAMPLE 18
Panning for Allosteric Agonist Antibodies Against INSR

[00618] Selection of agonist antibodies that exhibit greater binding to the complex of receptor/ligand than to the free receptor enhances the probability of identifying antibodies that are noncompetitive with the ligand and do not block or diminish binding of the ligand to the orthosteric site of the receptor. An antibody of this type, that binds to a site on the target receptor distinct from the endogenous binding site, is known as an allosteric agonist (Kenakin et al., Journal of Receptors and Signal Transduction, 27:247-259, 2007; Jahns et al., J Am Coll Cardiol. 36:1280-87, 2000; May et al., Ann Rev Toxicol. 47: 1-51, 2007).

[00619] Methods described above to screen for agonist antibodies are also useful to screen for allosteric agonists. Preferential binding of the test antibody to the receptor ligand complex is consistent with allosteric activity whereas preferential binding of the test antibody to the free receptor is consistent with an antibody that competes with insulin for the orthosteric site. The screen is useful to enrich the pool of candidate clones for allosteric agonists by eliminating the some if not all competitive agonists.

[00620] Allosteric antibodies are less likely to interfere with the binding affinity and efficacy of the ligand and therefore, are less likely to interfere with the maximum ligand signaling or maximum sensitivity to ligand. Allosteric antibodies can exhibit a range of agonism from weak partial agonists to agonism levels similar to the endogenous ligand. A partial allosteric agonist will elicit a maximum signaling response that is of significantly lower in magnitude than the maximum response of the endogenous ligand. In some applications, where sustained sub maximal signal activation is preferred over maximum signal activation, a partial agonist antibody is preferable to a full agonist antibody. The distinguishing characteristics between a partial allosteric agonist and a positive modulator (sensitizer) are evident from a comparison of the dose response curves shown in Figures 34 and 17, which show the different binding curves for a partial allosteric agonist (Figure 34) and a positive modulator (sensitizer) antibody (Figure 35).

[00621] Figure 34A illustrates an example of the dose response from a partial allosteric agonist in comparison to the dose response to the endogenous ligand and Figure 34B

demonstrates activation by ligand in the presence or absence of the allosteric agonist. Figure 35A shows the dose response from a positive allosteric modulator antibody in comparison to the dose response to the endogenous ligand while Figure 35B shows a dose response curve of an endogenous ligand in the presence and absence of a positive modulator antibody. Figure 36 provides the activation parameters for a set of partial allosteric agonists relative to the endogenous ligand. The nature of signal activation by the partial allosteric agonists is distinct from that of a positive modulator obtained from the same primary screening approach.

[00622] A non-competitive partial allosteric agonist antibody may offer a therapeutic advantage over a competitive agonist where it is beneficial to have independent signal activation by both the partial agonist and an endogenous ligand simultaneously. For example, and not to be bound by theory, a partial allosteric agonist can be used to elevate the basal activation of a signaling pathway while still allowing response from transient fluctuations in endogenous ligand levels. In certain instances, under conditions where a partial allosteric agonist of this sort is present, the endogenous ligand dose response will exhibit an increase in the baseline (constitutive or basal) signaling level and will achieve the same or greater maximal response to the endogenous ligand with little or no significant change in the ligand EC50. For example, Figure 34B shows the dose response of an endogenous ligand in the presence and absence of a partial allosteric agonist and Figure 37 shows the maximal activation of insulin in the presence partial allosteric agonist antibodies relative to the maximal response to the endogenous ligand in the presence of a negative control antibody. Figure 37 demonstrates that the partial allosteric agonist antibodies Ab037 and Ab040 have little or no significant impact on the EC50 of the dose response and maximum phosphorylation of Akt at Ser473 by insulin when compared to a negative control antibody within the same assay.

EXAMPLE 19
Examples of functional classes of anti-INSR antibodies: differential effects on insulin-induced phosphorylation of Akt

[00623] The effects of test antibodies on signaling via the insulin/insulin receptor complex were measured by assessing the ability of the antibodies to sensitize and agonize insulin-induced phosphorylation of Akt. Assays were performed using the method described in Example 5. In all data shown, the percent pAtk pSer473 values are relative and do not necessarily represent absolute cellular pAkt pSer473 levels.

[00624] Figures 38-40 show pAkt antibody dose response curves in the absence of insulin or in the presence of a sub-maximal concentration of insulin for parental CHO-K1 cells, CHO-K1 cells expressing human insulin receptor and CHO-K1 cells expressing mouse insulin receptor. The titrations of antibodies in the absence of insulin (open symbols) provide an indication of antibody agonism activity. Parallel titration of antibodies in the presence of a sub-maximal level of insulin (closed symbols) provides an indication of sensitizing activity relative to the agonism actity. The sensitizing activity can be seen as an increase in the pAkt levels above that caused by an EC30 concentration of insulin (dashed line) which is greater in magnitude than the agonism activity at the same antibody concentration. Antibodies Ab077, Ab078 and Ab 085 (Figure 38A-C) do not exhibit significant agonism in the absence of insulin. Antibodies Ab001, Ab079 and Ab083 are weak agonists (Figure 39A-C) and antibody Ab080 shows a moderate level of agonism (Figure 40). The assay results also indicate whether the antibodies show functional cross-reactivity, i.e., have effects on both human and mouse INSR-mediated signaling. Note that antibodies Ab078 and Ab085 only bind the insulin receptor in the presence of insulin, i.e., they do not detectably bind unoccupied insulin receptor as assessed by binding to insulin receptor expressed in CHO-K1 cells in a FACS based assay.

[00625] Figure 41A-C shows insulin induced pAkt activation in the presence of fixed concentrations of sensitizing antibodies in comparison to insulin in the presence of IgG2 isotype control antibody anti-KLH (solid lines). pAkt activation levels for antibodies in the absence of insulin at the concentrations used in the insulin dose response titrations are shown as dashed lines. EC50 values for insulin induced pAkt activation in the presence of the sensitizing antibodies and fold change in EC50 values relative to isotype control are listed in Table 5.

Table 5. EC50 values for insulin induced activation of pAkt in the presence of sensitizer antibodies.

Experiment	*Antibody*	*Antibody Concentration (ug/ml)*	*EC50 (pM)*	*Fold-change in EC50 relative to isotype control*
Human INSR CHO-K1 cells	**Ab001**	**2**	**59**	**12**
	Ab077	**10**	**81**	**9**
	Ab078	**20**	**221**	**3**

	Ab079	10	100	7
	Ab083	2	68	11
	Ab085	1	207	3
	Ab085	10	81	9
	anti-KLH.G2	10	724	
Mouse INSR CHO-K1 cells	Ab001	2	354	7
	Ab077	20	301	8
	Ab078	20	990	2
	Ab079	20	300	8
	Ab083	10	276	9
	Ab085	20	312	8
	anti-KLH.G2	10	2414	

[00626] Figures 42A-B show pAkt activation activity of partial allosteric agonist antibodies in the absence of insulin in comparison to insulin alone. Antibodies Ab037, Ab053 and Ab062 all act as agonists of pAkt activity having maximal activation plateaus that are significantly less than insulin in CHO-K1 cells expressing either human or mouse insulin receptor. The assay results also indicate whether the antibodies show functional cross-reactivity i.e. have effects on both human and mouse insulin receptor-mediated signaling. Antibody EC50 values and maximum activation levels are given in Table 6.

Table 6. Maximum activation levels and EC50 values for partial allosteric agonists

		Hu Insulin	Ab037	Ab053	Ab062
Human INSR CHO-K1	*Relative maximum activation*	100%	79%	64%	52%
	EC_{50} (nM)	0.15	0.65	0.42	2.43
Mouse INSR CHO-K1	*Relative maximum activation*	100%	42%	48%	34%
	EC_{50} (nM)	1.70	1.42	0.69	1.10

[00627] Figure 43 shows insulin dependent pAkt activation in the presence of fixed concentrations of partial allosteric agonist antibodies in comparison to insulin alone. Agonist activity of antibodies is seen as an increase in the baseline of the insulin dose response. The

agonist antibodies Ab037 and Ab053 have little effect on insulin sensitivity which is reflected in the lack of significant change in the insulin EC50 and Hill coefficient in the presence of these antibodies (see Table 7). Antibody Ab062 appears to reduce insulin sensitivity as the EC50 for insulin in the presence of Ab062 is 6.6-fold higher (see Table 7).

Table 7. Insulin activation parameters in the presence and absence of agonist antibodies

Engineered insulin receptor cell line used in the assay	***Assay parameter determined from sigmoidal dose-response curve fit***	***Hu Insulin with 2 ug/ml control antibody (95% confidence interval)***	***Hu Insulin with 2 ug/ml Ab037 (95% confidence interval)***	***Hu Insulin with 2 ug/ml Ab053 (95% confidence interval)***	***Hu Insulin with 2 ug/ml Ab062 (95% confidence interval)***
Human INSR CHO-K1	Relative maximum activation of pAkt in the presence of 2 ug/ml antibody	100% (93% to 108%)	109% (106% to 112%)	99% (97% to 102%)	106% (100% to 112%)
	EC_{50} of insulin in the presence of 2 ug/ml antibody (nM)	0.58 (0.35 to 0.96)	1.11 (0.84 to 1.5)	0.92 (0.68 to 1.3)	3.91 (2.7 to 5.7)
	Hill coeff. of insulin in the presence of 2 ug/ml antibody	0.74 (0.48 to 1.0)	0.79 (0.63 to 0.95)	0.93 (0.69 to 1.2)	0.71 (0.54 to 0.89)

EXAMPLE 20
Anti-INSR antibody 83-7 is not a positive modulator of insulin binding to hINSR

[00628] Anti-INSR antibody 83-7 has been identified previously as specific for human insulin receptor, however, the 83-7 antibody has not been demonstrated to have any modulating abilities on insulin-insulin receptor binding. In order to assess the ability of the 83-7 antibody to kinetically modulate insulin-insulin receptor interactions, insulin-induced serine phosphorylation of AKT was measured in the presence of 83-7.

[00629] The VH and VL sequences encoding antibody 83-7 (McKern et al., Nature 443: 218-221, 2006) were synthesized and the antibody (IgG1, lambda light chain) was transiently expressed in HEK293 EBNA cells. The antibody was purified using protein A capture and size exclusion chromatography. The ability of 83-7 to augment insulin-induced serine phosphorylation of AKT was measured using the method described in Example 5. Figure 44 shows pAKT assay results for 83-7 and Ab001 on CHOK1 cells expressing: (A) human INSR, or; (B) mouse INSR. Antibody 83-7 did not positively modulate insulin-dependent INSR signaling, showing only agonist activity on human INSR and did not exhibit agonism on mouse INSR. In contrast, Ab001 positively modulated insulin-dependent INSR signaling by about 10-fold on both human INSR and mouse INSR.

EXAMPLE 21
Assay to measure modulation of insulin binding affinity for INSR by anti-INSR antibodies

[00630] To determine the ability of the modulating antibodes to affect the binding of insulin to the insulin receptor, the affinity of unmodified insulin binding to human INSR expressed on the surface of serum starved CHOK1 cells (hINSR8-CHOK1) was measured in the presence and absence of monoclonal antibodies to INSR. A KinExA assay was developed to measure very low levels of insulin in cell culture media. This assay allowed the binding of insulin to cells expressing INSR to be measured by determining the level of insulin depletion from the cell culture media. As insulin became bound to the cells, the concentration of insulin in the cell culture media dropped. By using a titration of cells expressing INSR and measuring the percent free insulin, the affinity of the INS-INSR interaction could be estimated using KinExA software. This assay was used to measure the degree of modulation of insulin binding activity shown by various anti-INSR antibodies.

[00631] hINSR8-CHOK1 cells were serum starved overnight and then prepared for assay by pelleting cells and resuspending at a concentration of 2X the final assay concentration for the highest dilutions (between $3.5x10^7$ and $2.0x10^7$cells/mL in assay dilution buffer of PBS (Teknova, Hollister CA) with 500µg/mL BSA and 0.1% sodium azide (Sigma Aldrich, St. Louis, MO)). A two-fold serial dilution of cells was prepared creating a ten-point dilutions series and a no-cells control was also used. Cell suspensions were aliquoted into polypropylene assay tubes in 2mL volume each. To these cell suspensions 1mL of 40ug/mL test antibody (or 100ug/mL for Ab078) was added to each tube, gently mixed and incubated for 30-45 minutes on ice. The antibodies used were tested in comparison to the negative control human IgG2 anti-KLH antibody. 1mL of 200pM insulin was added to each tube to establish a final insulin concentration of 50pM (300pg/mL) (Sigma-Aldrich, St. Louis, MO). Samples were incubated overnight at 4°C for 18 hours then centrifuged to pellet cells and supernatants were removed for testing.

[00632] KinExA 3000 analysis was performed using beads coated with an anti-insulin monoclonal antibody. 2 grams of poly(methyl methacrylate) (PMMA) beads (Sāpidyne, Boise, ID) was suspended in 9mL of assay buffer PBS containing 65 ug/mL of clone D6C4 mouse anti-insulin monoclonal antibody (Fitzgerald Industries, Acton MA). Beads were rotated at room temperature for 6 hours then allowed to settle. Supernatant was replaced with

PBS with 50mg/mL BSA Fraction V (Sigma-Aldrich, St. Louis, MO) and rotated overnight at 4°C. Detection solution used was biotinylated mouse anti-insulin clone D3E7 (Fitzgerald Industries, Acton MA) at 0.15µg/mL in assay dilution buffer with Streptavidin-PE at 1 ug/mL (Invitrogen, Carlsbad, CA). On the KinExA 3000 the sample was injected at 0.25mL/minute for 240 seconds, then rinsed for 60 seconds in running buffer (PBS with 0.05% sodium azide), then 240 seconds of the detection solution was injected, followed by a final 90 second wash at 1 mL/minute. The difference in voltage from an early initial time-point and a time point near the end of the run was measured and used to calculate affinities. The INSR concentration on the cells was estimated at $2.5x10^5$ receptors/cell. Affinity was determined using the KinExA software (Sāpidyne, Boise ID) and EC50's were calculated by non-linear fit in Prism (GraphPad Software, La Jolla CA.).

[00633] A number of anti-INSR antibodies enhanced the affinity of insulin for the cells. Other antibodies had no effect on insulin affinity for the cells (Table 8). One of the tested antibodies decreased the affinity of insulin for the cells by approximately three-fold. Figure 45 shows free insulin percentage plotted against estimated insulin receptor concentration. The insulin level was fixed at 50pM and the antibody concentration was 10ug/mL (67nM) for all clones except Ab078 which was tested at 25ug/mL (167nM). Curves shown are the non-linear regression Prism fit used to calculate EC50.

[00634] Figure 46 shows free insulin percentage plotted against estimated insulin receptor concentration. The insulin level was fixed at 50pM and the antibody concentration was 10ug/mL (67nM) for all clones. Curves shown are the non-linear regression Prism fit used to calculate EC50.

Table 8

Insulin Affinity and IC50 Table			
Antibody	K_D (pM)	EC50 (pM)	Fold Shift in Affinity
IgG2-KLH	**272**	**365**	**1.0**
Ab037	**271**	**471**	**1.0**
Ab001	**49**	**104**	**+5.6**
Ab053	**228**	**33**	**+1.**
Ab062	**762**	**760**	**22.8**
Ab078	**41**	**80**	**+ 6.6**
Ab079	**12.1**	**40**	**+22.5**
Ab080	**11.2**	**34**	**+24.3**
Ab083	**13.7**	**39**	**+19.9**
Ab085	**34**	**70**	**+ 8.0**

EXAMPLE 22

Assay to measure insulin, IGF-1, and IGF-2 mediated proliferation of MCF-7 cells in the presence or absence of anti-INSR antibodies

[00635] Insulin, IGF-1, and IGF-2 promote mitogenesis in MCF-7 human mammary adenocarcinoma cells. Previous studies have shown that insulin analogs promote mitogenic signaling in addition to metabolic signaling following binding to INSR. The positive modulator and agonist anti-INSR antibodies described herein were expected to promote INSR-mediated mitogenic signaling in parallel to their activation of INSR-mediated metabolic signaling. The effects of the modulating antibodies on insulin-mediated mitogenic stimuli were measured using MCF-7 cells expressing the receptors.

[00636] MCF-7 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing glucose at 4.5 g/L supplemented with 10% FBS and 2 mM glutamine (Invitrogen) for normal maintenance. For the proliferation assay, cells were seeded in 96 well white opaque microtiter plates at a density of 1 x 10^4 cells/well (Costar 3917) and allowed to re-attach for 24 hrs. After 24 hrs, the cells were washed 2x with pre-warmed PBS and incubated in DMEM containing glucose at 1 g/L and no phenol red supplemented with 0.1 % FBS and 2 mM glutamine (Invitrogen), which will be referred to as "starvation media," for another 24 hrs. Insulin (Sigma), IGF-1 (R&D Systems), and IGF-2 (R&D Systems) were prepared as 10x stocks in starvation media and serially diluted 5-fold starting from 1 uM down to 64 nM (6 dilutions), and added to the cells after the 24 hr starvation period. For the co-incubation experiments that include the anti-INSR antibodies along with the growth factor, a 50 ug/ml stock of each antibody was prepared in starvation media and added to the cells prior to addition of growth factor to a final concentration of 5 ug/ml. The cells were incubated at

37°C for 48 hrs and cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The results are shown in Table 9.

Table 9: MCF-7 proliferation results

	EC_{50} and 95% confidence interval values					
	Insulin (nM)		IGF-1 (nM)		IGF-2 (nM)	
	EC_{50}	95% CI	EC_{50}	95% CI	EC_{50}	95% CI
No antibody	0.80	0.48 - 1.33	1.73	1.07 - 2.80	1.30	0.80 - 2.11
KLH*	1.54	1.03 - 2.29	2.10	1.33 - 3.30	2.66	1.75 - 4.06
Ab001*	3.46	2.22 - 5.40	4.71	3.28 - 6.75	2.54	1.87 - 3.43
Ab037*	1.54	1.00 - 2.37	2.33	1.51 - 3.58	2.09	1.44 - 3.05
Ab083*	1.08	0.49 - 2.38	0.91	0.58 - 1.43	1.47	0.77 - 2.81
Ab085*	0.48	0.28 - 0.81	2.11	1.38 - 3.21	1.84	1.30 - 2.60
	* antibody concentration @ 5 ug/ml					

[00637] These results show that, surprisingly, in the presence anti-INSR antibodies, no significant changes in the mitogenic responses to any of the aforementioned growth factors were observed within a 95% confidence interval. It is possible that these antibodies may cross-react and weakly bind IGF-1 and IGF-2, but the above assay demonstrates that any possible crossreactive binding does not elicit a functional effect, i.e., does not promote signaling through the receptor. This suggests the antibodies are able to increase the ratio of metabolic to mitogenic INSR-mediated signaling.

EXAMPLE 23
The effects of anti-INSR antibodies to reverse insulin resistant fatty acid uptake in differentiated 3T3-L1 adipocytes

[00638] TNFα can inhibit insulin dependent fatty acid uptake. Since TNFα is known to cause insulin resistance by deactivation of insulin signaling pathway intermediates such as IRS-1 (Nguyen et al, J. Biol. Chem. 280(42): 35361-71, 2005; Luca and Olefsky, FEBS Let. 582: 97-105, 2008) that are also part of the insulin dependent glucose uptake pathway, reversal of TNFα inhibition of insulin dependent fatty acid uptake by anti-INSR antibodies is indicative of the ability of these antibodies to reverse TNFα mediated inhibition of insulin dependent glucose uptake.

[00639] 3T3-L1 mouse embryonic fibroblasts can be induced to differentiate into adipocytes, after which they become highly responsive to insulin-mediated fatty acid uptake. High fat feeding has been established as a cause of adipose tissue insulin resistance. To examine this condition in vitro, 3T3-L1 adipocytes have been treated with free fatty acids (FFA) which result in impaired insulin receptor-mediated signal transduction and ultimately decreased insulin-stimulated glucose uptake. One of the downstream effector molecules induced by FFA treatment that contributes to insulin resistance is TNFα. TNFα has also been shown to inhibit insulin-mediated fatty acid uptake and provides a well defined in vitro system to assess whether anti-INSR antibodies can reverse insulin-resistant fatty acid transport.

[00640] 3T3-L1 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing glucose at 4.5 g/L supplemented with 10% newborn calf serum (NCS; Invitrogen) and 2 mM glutamine (Invitrogen) for normal maintenance. To differentiate cells into adipocytes in 96-well microtiter plates, the following protocol was used: (1) at day -5, 2 x 10^3 cells per well were seeded in a black/clear bottom 96-well plate (BD Falcon 353948), (2) at day -2, cells reach confluency and are left for 2 additional days, (3) at day 0 differentiation mediais added, (4) at day 3, media is changed to normal growth media containing 0.425 uM insulin, (5) at day 7 media is changed to normal growth media. To induce insulin resistance, 10x stocks of TNFα (R&D Systems) were prepared in normal growth media and added to cells on day 9 of the differentiation process. Working concentrations of TNFα used were between 1-10 ng/ml. Fatty acid uptake was run on cells at day 10. The fatty acid uptake protocol used was as follows. Cells were washed in 2x in Hank's Balanced Salt Solution (HBSS; Invitrogen) containing 0.2% fatty acid-free BSA (FAF-BSA; Sigma) and 20 mM HEPES (Invitrogen), and then serum starved in HBSS for 1-2 hrs at 37°C. Anti-INSR antibodies or other relevant controls were added from a 10x stock or HBSS alone and incubate at 37°C for 30 minutes, and insulin added at dilutions from a 10x stock and incubated at 37°C for 30 minutes. An equal volume of reconstituted QBT Fatty Acid Uptake Assay (Molecular Devices) loading buffer was then added and incubated at 37°C for up to 3 hours, and the plates read on a fluorescent plate reader to measure internalized fluorescent fatty acid analogs.

[00641] Figure 47 shows that TNFα-induces desensitization of insulin-mediated fatty acid uptake in 3T3-L1 adipocytes in the presence of anti-INSR antibody Ab085. Table 10 shows relative EC50 for the antibodies for fatty acid uptake, demonstrating that Ab085 decreases the

EC50 for fatty acid uptake. In the presence of anti-INSR antibody Ab085, the TNFα-induced desensitization of insulin-mediated fatty acid uptake was completely reversed back to the untreated control values. Similar results were observed for Ab083.

Table 10

EC_{50} and 95% confidence interval values

	Insulin (nM)	
	EC_{50}	95% CI
Insulin only	0.77	0.37 - 1.60
+ TNFα	2.89	1.37 - 6.08
+ TNFα, + anti-KLH	3.39	1.42 - 8.11
+ TNFα, + Ab085	0.32	0.14 - 0.75

TNFα concentration @ 1.25 ng/ml
Ab085 concentration @ 50 ug/ml

[00642] These results demonstrate that the positive- modulator antibody can increase fatty acid uptake in adipocytes, which suggests the antibody is useful to treat a disorder or condition that would benefit from increasing fatty acid uptake.

EXAMPLE 24
Characterization of highly purified anti-INSR antibodies by insulin dependent pAkt Activation

[00643] A certain amount of assay-to-assay variation was noted in the functional pIRS-1 and pAKT assays. It was determined that this variation could be reduced when the test antibodies were purified using a further step in addition to protein-A purification, e.g., size-exclusion chromatography, resulting in antibodies that were approximately >95% pure. This purification step reduced or eliminated aggregates and contaminating growth factors thought to interfere with the functional assay.

[00644] A number of highly purified anti-INSR antibodies were tested in the pAKT assay described in Example 5, using CHOK1 cells expressing either the human INSR or mouse INSR. In addition, certain anti-INSR antibodies were tested for activity on a CHOK1 cell line transfected with cynomolgus monkey INSR (CHOK1-cynoINSR-4).

[00645] The effects of positive modulator anti-INSR antibodies Ab001, Ab037, Ab077, Ab079, AB080, Ab083 were measured in the pAKT assay and results are shown in Figure 48 (human INSR) and Figure 49 (mouse INSR).

[00646] The relative % pAKT of agonist antibodies Ab037, Ab030, Ab053 and Ab062 on human INSR and mouse INSR are shown in Figures 50 and 51, respectively.

[00647] The relative % pAKT of positive modulator antibodies and agonist antibodies were also measured in CHOK1 cells expressing cynomolgus monkey INSR4. Figure 52 demonstrates that the anti-INSR antibodies Ab030, Ab037, Ab053, Ab001, Ab079, AB080 and Ab083 are capable of inducing AKT phosphorylation after activation of monkey INSR.

[00648] Additionally, the relative % pAKT of negative modulator antibodies Ab061, Ab070 and Ab081 were also measured in CHOK1 cells expressing human INSR. The results are shown in Table 11 and Figure 53.

Table 11

	10ug/mL Ab061	20ug/mL Ab070	20ug/mL Ab081	20ug/mL Isotype control mAb
Insulin EC50	32.43	2.09	4.53	0.12
Fold change in EC50 relative to Isotype control mAb	269	17	38	
EC50 95% Confidence Intervals	14.36 to 73.25	1.686 to 2.598	3.503 to 5.843	0.09691 to 0.1496

[00649] These results demonstrate that negative modulator antibodies increase the EC50 of insulin, in some cases by several hundred-fold.

EXAMPLE 25
Assessment of Species Cross Reactivity of Anti-INSR Antibodies

[00650] This example describes the use of a FACS based assay to assess the binding of insulin receptor antibodies to cells of species such as rabbit and cynomolgus monkey that are often used in toxicological studies. Anti-INSR antibodies from phage display libraries were screened for both binding to peripheral blood monocytes of human, rabbit and cynomolgus

monkeys and for differential binding in the presence or absence of the ligand (insulin) to the monocytes of the above named species.

[00651] Cynomolgus monkey whole blood was obtained from California National Primate Research Center (Davis, CA) and rabbit whole blood was obtained from LifeSource Biomedical, LLC (Moffett Field, CA). Human PBMC were purified using Ficoll Hypaque from buffy coats obtained from the American Red Cross. Cynomolgus and Rabbit PBMC were than purified using Ficoll Hypaque gradients (Pharmacia). Purified PBMC were frozen and stored in liquid nitrogen prior to use in the assay. Human, cynomolgus and rabbit PBMC were thawed and washed with FACS Buffer (0.5% BSA and 0.1%NaN3 in PBS). Once the cells were prepared, they were used in the FACS staining assay at a final concentration of $2x10^6$ cells/ml.

[00652] To look at differential binding, cells were incubated in the presence or absence of the insulin with decreasing concentrations of anti-INSR antibody at 4°C for 1 hour and washed once with FACS Buffer. The binding of anti-INSR antibody was revealed by the addition of goat anti-human IgG Alexa647 (Jackson ImmunoResearch) for 30 minutes at 4°C. After washing twice with FACS buffer, cells were stained with various markers to capture monocytes population. Human and cynomolgus cells were stained with CD45 and CD14. Rabbit cells were stained with CD11b and CD14. Antibodies were than incubated for 20 minutes and washed twice with FACS Buffer. Cells were than fixed with 2% paraformaldehyde and equal volume of FACS Buffer was added prior to cell analysis. The cells were analyzed on a FACScan™ (Becton-Dickinson, Franklin Lakes, NJ) and the data was analyzed using both FloJo™ (Tristar, Paso Robles, CA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

[00653] The binding seen on human, rabbit or cynomolgus monkey PBMC was confirmed by generating CHO cell-lines that expressed the appropriate species insulin receptor and repeating the binding assay described above. Data shown in Figure 54 shows that many of the antibodies that bound to the human insulin receptor also bound to the rabbit and the cynomolgous insulin receptor and that this binding was modulated by the presence of insulin.

EXAMPLE 26
Measurement of the Affinity of Anti-INSR Antibodies in the Presence and Absence of Human Insulin

[00654] The affinity of various anti-INSR antibodies for recombinant human INSR expressed on the surface of serum starved CHOK1 cells (hINSR8-CHOK1) was measured in the presence and absence of insulin. A KinExA assay was developed to measure very low levels of antibody in an incubation buffer. This assay allowed the binding of antibodies to cells expressing INSR to be measured by determining the level of antibody depletion from the incubation buffer. As antibody became bound to the cells, the concentration of antibody in the buffer solution dropped. By using a titration of cells expressing INSR and measuring the percent free antibody, the affinity of the antibody to INSR interaction could be estimated using KinExA software. This assay was used to determine the relative affinities of the tested antibody clones in the presence or absence of insulin and demonstrated insulin-dependent modulation of antibody binding to the cells.

[00655] hINSR8-CHOK1 cells were serum starved overnight and then prepared for assay as described in Example 20. One mL of 4 ug/mL insulin or buffer was added to each tube of cells to establish a final insulin concentration of 0 or 175nM (1 ug/mL). Then 1 mL of 40 ng/mL antibody was added to each tube to yield a final antibody concentration of 10 ng/mL or 66.6 pM. Samples were incubated overnight at 4°C for 18 hours then centrifuged to pellet cells and supernatants were removed for testing on the KinExA. The KinExA 3000 analysis was performed as described in Example 20 using beads coated with an (Fab')2 fragment goat anti-human IgG (H+L) (Jackson Immuno Research, West Grove PA). Detection solution used was R-PE-(Fab')2 fragment goat anti-Human IgG(H+L) (Jackson Immuno Research, West Grove PA). For the 83-7 murine antibody the beads were conjugated as above with a rabbit anti-mouse F(ab')2 antibody (Jackson Immuno Research, West Grove PA) and the detection solution used was an R-PE-(Fab')2 fragment Goat anti-Mouse IgG(H+L) (Jackson Immuno Research). The INSR concentration on the cells was estimated at 2.5×10^5 receptors/cell and bivalent antibody binding to INSR was assumed.

[00656] The affinities of a number of anti-INSR antibodies in the presence and absence of insulin are shown in Table 12. The agonist antibodies Ab037, Ab053, and Ab062 have binding that is independent of insulin and showed less than a two-fold affinity shift in the presence or absence of insulin. The 83-7 mouse antibody had a modest three-fold affinity shift in the presence of insulin, where as the positive modulator antibodies Ab001, Ab079, Ab080, and Ab083 all showed positive binding modulation in the presence of insulin ranging from seventeen-fold for Ab080 to over 100-fold for Ab001. The positive modulators Ab077 and Ab078 have a weaker affinity in the absence of insulin than the other clones and, as a

result, their "without insulin" affinity was beyond the range of the assay, which is limited in maximum receptor concentration given the use of the cells as a receptor source. Although binding can be seen with these clones in the absence of insulin, it is substantially weaker than in the presence of insulin and modulated to a much greater extent than 83-7, but the degree of modulation cannot be accurately estimated with these assay conditions. Ab085 showed little to no evidence of binding in the absence of insulin and its binding is considered insulin dependent.

Table 12

Affinity of Antibodies to hINSR8-CHOK1 Cells			
mAb	**With Insulin**	**Without Insulin**	**Fold Improvement with Insulin**
Ab001	1.16E-10	1.20E-08	103
Ab037	8.00E-11	1.08E-10	1.4
Ab053	9.60E-11	1.48E-10	1.5
Ab062	1.08E-10	1.24E-10	1.1
Ab077	6.40E-09	Out of Range*	
Ab078	3.40E-10	Out of Range*	
Ab079	4.96E-10	9.60E-09	19.4
Ab080	6.80E-10	1.20E-08	17.6
Ab083	3.76E-10	7.60E-09	20.2
Ab085	2.00E-10	No Binding	
83-7	1.60E-10	4.80E-10	3.0

EXAMPLE 27
Epitope Binning of Anti-INSR Antibodies

[00657] A multifactorial approach was taken to epitope binning to determine if various anti-INSR antibodies bind to potentially similar epitopes or if they have demonstrated differential binding properties and different epitope recognition. Competitive binding or "binning" experiments were performed as well as analysis of the antibodies' ability to bind to different human and murine species of the insulin receptor and their ability to bind in the presence and absence of insulin. All of these are factors in determining the potential similarity or difference of antibody binding epitopes. Flow cytometry assays were performed by analyzing the binding of biotinylated IgG's to serum starved hINSR8-CHOK1 cells and mINSR-CHOK1 cells in the presence and absence of insulin

[00658] For the competitive binding assay, hINSR8-CHOK1 cells and mINSR-CHOK1 cells were serum starved overnight and then prepared for assay as described in Example 20. In some embodiments, it is useful to calculate the number of receptors on the cell surface to carry out the competition binding assays. For example, hINSR8-CHOK1 receptor expression levels were determined initially by standard cell staining and flow cytometry techniques. Briefly this was carried out by staining the cells with a saturating concentration of MA-20 monoclonal Ab (ThermoFisher Scientific, Waltham MA) and detecting with R-Phycoerythrin conjugated goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove PA) and then comparing relative fluorescence with BD Quantibrite™ PE Beads (BD Biosciences, Franklin Lakes NJ) to provide an estimation of number of Phycoerythrin molecules bound and extrapolate the number of insulin receptors based on the number of phycoerythrin molecules bound. This number was then further tested and refined using KinExA as described in Rathanaswami et al, (Analytical Biochemistry 373:52-60, 2008). Briefly, KinExA experiments were performed looking at both antibody and insulin binding where the ligand concentration used was much higher than that described herein for the determination of affinities which creates a more stoichiometrically limited dose response. This was then analyzed in the KinExA software (Sapidyne, Boise ID) using an unknown ligand model and determination of a ligand multiplier parameter that was used to confirm binding receptor concentration. In the present assay, for example, it is estimated that the hINSR8-CHOK1 cells when serum starved express roughly 250,000 tetrameric INSR receptors per cell. For the antibody affinity, this means the stoichiometry of 2 antibodies per receptor tetramer and for the high affinity insulin binding site, a 1:1 tetramer to insulin ratio.

[00659] The antibodies to be tested were biotinylated using standard amine chemistry and the activated PEG4-biotin (Thermo-Fisher, Waltham MA). Mouse antibodies 83-7 and 83-14 were also tested. These antibodies have been reported to bind to amino acids 233-281 of the CR domain and to the FnIII-I domain of INSR, respectively (McKern et al, 2006; Nature 443: 218-21). After serum starving the transfected cells overnight, the cells were stained with a titration of the biotinylated antibodies in the presence of 1 ug/mL insulin. Antibodies were incubated on cells at 4°C for approximately 30 minutes. Samples were then washed 2x in FACS buffer and Streptavidin-phycoerythrin (Jackson ImmunoResearch Labs, West Grove, PA, USA) was used to detect biotinylated antibody. The concentrations of biotinylated antibodies used in the binning experiment were selected based on them having a subsaturating, but still strong, signal to the human cell line in the presence of insulin. Once the concentrations of the biotinylated antibodies to be used were experimentally determined, the competition assay was performed as below.

[00660] The cells were serum starved overnight and then resuspended in cold FACS buffer with or without 1 ug/mL human insulin. The cells were then mixed 1:1 with 60 ug/mL cold or unlabelled competitor antibody and incubated at 4°C for approximately 30 minutes establishing a cold Ab concentration of 30 ug/mL. The biotinylated antibodies were then added in a 1:2 dilution as a 3x concentration and incubated at 4°C for approximately 30 minutes. The cells were then washed 2x in FACS buffer and detected with Streptavidin-phycoerythrin and assayed on a FACS analyzer (Becton Dickinson, San Jose, CA).

[00661] MFI was compared between the biotinylated antibodies when mixed with a non-binding control antibody or with a competitor antibody. The extent of binding was measured on the human and the murine cell lines and in the presence or absence of insulin. A matrix approach was used where each biotinylated antibody was tested against each cold competitor. Antibodies with the same competition profiles are considered to be in the same bin. Exemplary bin groupings as presented in Table 13 are derived from the hINSR8-CHOK1 cells in the presence of insulin as virtually all clones had the strongest binding under those conditions. Clones shown in Table 13 are labeled to reflect other binding properties such as insulin dependence and murine reactivity.

[00662] Results of the experiment resulted in approximately seven different competition bins among the anti-INSR antibodies. An antibodiy with no competition is defined as one exhibiting less than 30% competition, partial competition is competition greater than 30% and less than 80%, and complete competition is greater than 80% competition using the method described above with hINSR8-CHOK1.

[00663] The antibodies that map to Bin 1, which are human and murine reactive, exhibited no competition with AB079, AB076, AB083, partial to complete competition with AB085 and AB086 and complete competition with AB030, AB037, AB053, AB001, AB018, and AB064, AB040.

[00664] The antibodies of Bin 2, which are human and murine reactive, exhibited the same profile as those antibodies in Bin 1, but demonstrated no competition with AB086 and partial competition with AB078.

[00665] The antibodies in Bin 3, which bind to both human and murine INSR, showed no competition with Ab062 and Ab086, partial Competition with Ab086, Ab064, Ab001, Ab018 and complete competition with Ab079, Ab076, Ab083, Ab080, Ab062, and Ab020, Ab019, Ab088, Ab089.

[00666] Bin 4 antibodies, which bind to hman receptor only, exhibited no competition with Ab062, Ab086, Ab001, Ab018, Ab030, Ab037, Ab064 and complete competition with Ab079, Ab076, Ab083, Ab080, Ab062, and Ab020, Ab019, Ab088, Ab089

[00667] Bin 5 antibodies exhibit no competition with AB077, AB001, AB018, AB030, AB037, AB079, AB076, AB083, AB019, AB088, AB089, and AB040 and show complete competition with AB064, AB062, AB085, and AB078. These antibodies react with both human and murine receptor.

[00668] Bin 6 antibodies showed complete to partial competition with almost all clones tested. Clone Ab061 had less than 30% competition with Ab019 and clone Ab074 showed no competition with Ab088. These antibodies react with both human and murine receptor.

[00669] The antibodies grouped in Bin 7 showed no competition with Ab053, Ab064, 83-7, Ab019, Ab088, and Ab089, showed partial competition with Ab037, Ab078, Ab083, Ab080, and Ab085, and showed complete competition with Ab040, Ab062, Ab030, Ab001, and Ab018. These antibodies react with both human and murine receptor.

[00670] Competition Bin 4 which contains the murine 83-7 clone contained all of the clones that lacked murine reactivity. The antibody groupings correlated with their functional properties. All of the human agonist antibodies grouped into Bin 1. Positive modulator antibodies grouped into Bins 3 and 5 with the exception of Ab004. The Bin 3 antibodies bind both INSR-insulin complex and INSR alone, whereas the Bin 5 antibodies bind INSR-insulin complex but do not bind INSR alone.

Table 13

Epitope Bins						
1	2	3	4	5	6	7
Ab030	83-14	Ab079	Ab020	Ab078	Ab061	Ab004
Ab037		Ab080	Ab019	Ab085	Ab074	
Ab053		Ab083	Ab088		Ab077	
Ab001			Ab089			
Ab018			Ab087			
Ab086			83-7			
Ab062						
Ab064			Human Specific			
Ab040						
			Agonist			
			Positive modulator (complex-specific binding)			
			Positive modulator (binds complexed and free INSR)			
			Negative Modulator			

[00671] Numerous modifications and variations in the invention as set forth in the above illustrative examples are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.

CLAIMS

We claim:

1. An antibody that binds to (i) insulin receptor or (ii) a complex comprising insulin and insulin receptor or both (i) and (ii), with an equilibrium dissociation constant K_D of 10^{-5}M or less that is capable of strengthening the binding affinity between insulin and insulin receptor by about 5-fold to 200-fold.

2. An allosteric agonist antibody that binds to insulin receptor with an affinity of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} M or less and (a) exhibits maximal agonist activity that is 20%-80% that of insulin's maximal agonist activity when measured in pAKT assay, (b) when present does not alter the EC50 of insulin for INSR by more than 2-fold, and (c) when present does not alter the K_D of insulin for INSR by more than 2-fold.

3. An agonist antibody that binds to insulin receptor with an affinity of 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10}, 10^{-11} M or less, optionally that exhibits maximal agonist activity that is 20%-100% that of insulin's maximal agonist activity when measured in pAKT assay.

4. An antibody that binds to (i) insulin receptor or (ii) a complex comprising insulin and insulin receptor, or both (i) and (ii), with an equilibrium dissociation constant K_D of 10^{-5}M or less that is capable of weakening the binding affinity between insulin and insulin receptor by at least about 3-fold, optionally up to 1000-fold.

5. The antibody of claim 1 characterized by the following equilibrium dissociation constant K_D binding properties:

(i) said antibody binds with an equilibrium dissociation constant K_D of about 10^{-5}M, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 10^{-10} , 10^{-11} M or less, to a complex comprising insulin (C1) and insulin receptor (C2); and

(ii) any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is at least about 5-fold lower than any of K_{AC2} or K_{AC1}.

6. The antibody of claim 5 in which any of $K_{[C1C2]A}$, $K_{[AC2]C1}$, or $K_{[AC1]C2}$ is about 5-fold to 200-fold lower than any of K_{AC2} or K_{AC1}.

7. The antibody of any one of claims 1 and 5 to 6 wherein the antibody binds insulin receptor.

8. The antibody of any one of claims 1 and 5 to 6 wherein the antibody binds an insulin/ insulin receptor complex.

9. The antibody of any one of claim 1, claims 5 to 6 and claim 8 wherein the antibody does not detectably bind insulin receptor alone.

10. The antibody of any of claims 1 and 5 to 9, wherein the antibody is capable of strengthening the binding affinity between insulin and insulin receptor by about 5-fold to 25-fold.

11. The antibody of claim 10, wherein the binding affinity is any one of K_A, K_D, the ratio of on rate to off rate, or the ratio of off rate to on rate.

12. The antibody of any of claims 1 to 8 and 10 to 11 that activates insulin receptor by at least 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay.

13. The antibody of any of claims 1 to 8 and 10 to 11 that activates insulin receptor by less than 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay.

14. The antibody of any of claims 1, 3 or 5 to 13, wherein the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs:, 281, 278, 277, 209, 275, 223, 284, 276, and 236 and a light chain variable region selected from the group consisting of SEQ ID NOs: 141, 138, 137, 35, 135, 57, 144, 136, and 98.

15. The antibody of any of claims 1, 3 or 5 to 14, wherein the antibody comprises

(a) the heavy chain variable region of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 291, 196, 239, 267 and 271 and the light chain variable region of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 76, 80, 101, 128, and 132, or

(b) one, two or three heavy chain CDRs of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 291, 196, 239, 267 and 271 and/or one, two or three light chain CDRs of any of Ab006, Ab030, Ab004,

Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or set out in SEQ ID NO: 76, 80, 101, 128, and 132, optionally including one or two mutations in any one, two or three of such heavy or light chain CDRs, e.g., a conservative or non-conservative substitution; or

(c) all six CDRs of any of Ab006, Ab030, Ab004, Ab013, Ab009, Ab007, Ab011, Ab001, Ab012, Ab010, Ab003, Ab008, Ab002, Ab005, Ab076, Ab077, Ab079, Ab080, Ab083, Ab059, Ab078, Ab085 or SEQ ID NO: 76, 80, 101, 128, 132, 291, 196, 239, 267, and 271.

16. The antibody of any of claims 1, 2, 3 and 5 to 15 that exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab079, Ab076, Ab083, Ab080, Ab062, and Ab020, Ab019, Ab088, and Ab089, and optionally

exhibits greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab086, Ab064, Ab001, and Ab018,

17. The antibody of any of claims 1,2, 3 and 5 to 15 that exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab040, Ab062, Ab030, Ab001, and Ab018, and optionally

exhibits greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab037, Ab078, Ab083, Ab080, and Ab085

18. The antibody of any of claims 1, 3, 5 to 6 and 8 to 9 that exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab064, Ab062, Ab085, and Ab078.

19. The antibody of claim 4 wherein the antibody is capable of weakening the binding affinity between said insulin and insulin receptor by about 3-fold to 500-fold.

20. The antibody of claim 4, wherein the binding affinity is any one of K_A, K_D, the ratio of on rate to off rate, or the ratio of off rate to on rate.

21. The antibody of claim 4, wherein the antibody increases the EC50 of insulin signaling activity by about 2-fold to 1000-fold, optionally in a pAKT assay.

22. The antibody of any of claims 4 or 19 to 21, wherein the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 241, 279,

258, 155, and 228 and a light chain variable region selected from the group consisting of SEQ ID NOs: 103, 139, 119, 8, and 89.

23. The antibody of any of claims 4 or 19 to 22, wherein the antibody comprises

(a) the heavy chain variable region of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081 and the light chain variable region of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081, or

(b) one, two or three heavy chain CDRs of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081and/or one, two or three light chain CDRs of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081, optionally including one or two mutations in any one, two or three of such heavy or light chain CDRs, e.g., a conservative or non-conservative substitution; or

(c) all six CDRs of any of Ab087, Ab019, Ab088, Ab089, Ab020, Ab050, Ab052, Ab055, Ab057, Ab061, Ab063, Ab065, Ab070, Ab072, Ab074, Ab081.

24. The antibody of any of claims 4 and 19 to 23 that exhibits greater than or equal to 70% competition with any one, two, three or all antibodies selected from the group consisting of Ab079, Ab076, Ab083, Ab080, Ab062, and Ab020, Ab019, Ab088, Ab089, optionally wherein the antibody binds human receptor or complex and not murine receptor or complex.

25. The antibody of claim 2 or 3, wherein the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 195, 220, 303, 197, 208, 243, 245 and 251 and a light chain variable region selected from the group consisting of SEQ ID NOs: 77, 50, 90, 84, 34, 104, 106 and 112.

26. The antibody of any of claims 2 or 3, wherein the antibody comprises

(a) the heavy chain variable region of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 set out in SEQ ID NOs: 252, 253, 263, 265 and 269and the light chain variable region

of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 7, 113, 114, 124, 126 and 130, or

(b) one, two or three heavy chain CDRs of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 252, 253, 263, 265 and 269 and/or one, two or three light chain CDRs of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 7, 113, 114, 124, 126 and 130, optionally including one or two mutations in any one, two or three of such heavy or light chain CDRs, e.g., a conservative or non-conservative substitution; or

(c) all six CDRs of any of Ab021, Ab029, Ab022, Ab017, Ab023, Ab024, Ab025, Ab026, Ab031, Ab035, Ab027, Ab036, Ab037, Ab028, Ab038, Ab039, Ab040, Ab041, Ab042, Ab032, Ab043, Ab044, Ab045, Ab046, Ab047, Ab018, Ab033, Ab048, Ab014, Ab015, Ab049, Ab034, Ab051, Ab053, Ab054, Ab056, Ab058, Ab062, Ab064, Ab066, Ab067, Ab068, Ab086, Ab069, Ab071, Ab073, Ab075, Ab082, Ab084 or set out in SEQ ID NOs: 7, 113, 114, 124, 126, 130, 252, 253, 263, 265 and 269.

27. The antibody of any of claims 2, 3, 25 and 26 that exhibits greater than or equal to 70%, 75% or 80% competition with any one, two, three or all antibodies selected from the group consisting of Ab030, Ab037, Ab053, Ab001, Ab018, Ab064, Ab040, and optionally

exhibits greater than or equal to 30% competition with any one, two, three or all antibodies selected from the group consisting of Ab085 and Ab086.

28. The antibody of any of claims 1 to 27 that comprises at least one heavy chain CDR HCDR1, HCDR2 and HCDR3 set out in SEQ ID NOS: 151-303.

29. The antibody of any of claims 1 to 28 that comprises a heavy chain variable region of SEQ ID NOS: 151-303.

30. The antibody of any of claims 1 to 29 that comprises at least one light chain CDR LCDR1, LCDR2, or LCDR3 set out in SEQ ID NOS: 1-150.

31. The antibody of any of claims 1 to 30 that comprises a light chain variable region of SEQ ID NOS: 1-150.

32. The antibody of any of claims 1 to 31, further comprising a human IgG1, IgG2, IgG3, or IgG4 heavy chain constant region.

33. The antibody of claim 32, further comprising a human light chain constant region.

34. An antibody that specifically binds (i) insulin receptor or (ii) a complex comprising insulin and insulin receptor or both (i) and (ii), comprising at least one of HCDR1, HCDR2 and HCDR3 of SEQ ID NOS: 151-303 and having an equilibrium dissociation constant K_D of 10^{-5}M or less.

35. The antibody of claim 34 that comprises a heavy chain variable region of SEQ ID NOS: 151-303.

36. The antibody of claim 34, further comprising at least one of LCDR1, LCDR2, or LCDR3 of SEQ ID NOS: 1-150.

37. The antibody of any one of claims 34 to 36, wherein the antibody comprises one, two, three, four, five or six CDRs.

38. The antibody of any one of claims 34 to 37, wherein the antibody comprises one, two or three heavy chain CDRs set out in SEQ ID NOS: 151-303.

39. The antibody of any one of claims 37 to 38, wherein the antibody comprises one, two or three light chain CDRs set out in SEQ ID NOS: 1-150.

40. The antibody of claim 34 that comprises a light chain variable region of SEQ ID NOS: 1-150.

41. The antibody of any one of claims 34 to 40, further comprising a human IgG1, IgG2, IgG3 or IgG4 constant region.

42. The antibody of any one of claims 34 to 41, wherein the antibody binds insulin receptor.

43. The antibody of any one of claims 34 to 41, wherein the antibody binds an insulin/ insulin receptor complex.

44. The antibody of claim 43, wherein the antibody does not detectably bind insulin receptor alone.

45. The antibody of any one of claims 1 to 3, 5 to 18, and 25 to 44, wherein the antibody reduces fasting blood glucose levels in a subject having elevated blood glucose toward normal glucose levels.

46. The antibody of any of claims 34 to 44 that activates insulin receptor by at least 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay.

47. The antibody of any of claims 34 to 44 that activates insulin receptor by less than 10% of the maximal signal of insulin, optionally in a phosphorylated AKT assay.

48. The antibody of any of the preceding claims, wherein said antibody is a monoclonal antibody.

49. The antibody of any of the preceding claims, wherein said antibody is a human antibody.

50. The antibody of any one of claims 1 to 49, wherein the antibody is conjugated to a hydrophobic moiety.

51. A method of preparing a sterile pharmaceutical composition, comprising adding a sterile pharmaceutically acceptable diluent to an antibody of any of claims 1 to 50.

52. A sterile composition comprising the antibody of any of claims 1 to 50 and a sterile pharmaceutically acceptable diluent.

53. A method of treating a disorder associated with insulin resistance, comprising administering an antibody of any of claims 1, 2, 3, 5 to 18 and 25 to 50 in an amount effective to treat insulin resistance.

54. The method of claim 53, wherein the disorder is selected from the group consisting of hyperglycemia, pre-diabetes, metabolic syndrome (also referred to as insulin

resistance syndrome), Type 2 diabetes mellitus, polycystic ovary disease (PCOS), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), steatosis, obesity, dyslipidemia, Rabson-Mendenhall syndrome, Donohue syndrome or Leprechaunism.

55. The method of claim 54, wherein the treatment enhances glucose uptake.

56. The method of claim 55, wherein the enhanced glucose uptake is selected from the group consisting of an increase in the rate of glucose uptake, an increase in the total amount of glucose uptake, or both.

57. The method of claim 56, wherein the treatment slows or reduces weight gain in the subject compared to an untreated subject.

58. The method of claim 53, wherein the treatment results in a decrease in HbA1c levels compared to an untreated subject.

59. The method of claim 53, wherein the treatment results in an improved glucose tolerance test, or reduced 2-hour glucose level during a glucose tolerance test.

60. The method of claim 53, wherein the treatment results in an improvement in a symptom associated with insulin resistance selected from the group consisting of dyslipidemia, plasma triglycerides, HOMA-IR, plasma unesterified cholesterol, plasma total cholesterol, plasma insulin, non-HDL/HDL cholesterol ratio, total/HDL cholesterol ratio, beta cell function and plasma leptin levels.

61. A method of treating a condition associated with hyperinsulinemia or excess insulin signaling, comprising administering an antibody of claims 4 and 19 to 24 in an amount effective to treat hypoglycemia or insulin sensitivity.

62. The method of claim 61, wherein the condition is selected from the group consisting of cancer, insulinoma, Kaposi's sarcoma, insulin overdose, hypoglycemia, nesidioblastosis (KATP-Hl Diffuse Disease, KATP-Hl Focal Disease, or "PHHI"), GDH-Hl (Hyperinsulinism/Hyperammonaemia Syndrome (HI/HA), leucine-sensitive hypoglycemia, or diazoxide-sensitive hypoglycemia), islet cell dysregulation syndrome, idiopathic hypoglycemia of infancy, Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), Congenital Hyperinsulinism, hypoglycemia due to renal failure (acute or chronic), and hypoglycemia due to chronic kidney disease.