

Insulin-like growth factor 1 receptor binding peptides

Field of the Invention

The present invention relates to insulin-like growth factor 1 receptor binding peptides, polynucleotides encoding them, and methods of making and using the foregoing.

Background of the Invention

Recent advances in biomedical research and high-throughput drug screening have generated numerous potential therapeutics for the treatment of CNS related illnesses. However, many therapeutics fail *in vivo* testing because of inadequate transport through the blood-brain barrier (BBB).

Therapeutics can cross the BBB using several pathways, including saturable transporter systems, adsorptive transcytosis wherein the therapeutic to be transported is internalized by a cell in the BBB and routed to the abluminal surface for deposition into the brain intracellular fluid compartment, transmembrane diffusion wherein the therapeutic dissolves into the lipid bilayer which forms the membranes of the cells comprising the BBB, and extracellular pathways wherein the therapeutic exploits the residual leakiness of the BBB.

Several approaches to modify therapeutic agents to alter their BBB permeability have been attempted, including conjugation with proteins that naturally cross the BBB, for example insulin, insulin-growth factor 1 and 2 (IGF-1, IGF-2), leptin and transferrin (US Pat. Appl. No. US2007/0081992), linking the polypeptides to cationized antibodies binding to certain cellular receptors, such as insulin receptor (US Pat. No. 7,388,079) or transferrin receptor (US Pat. No. 6,329,508; Zhang and Pardridge, Brain Res. 889:49-56, 2001); coupling the therapeutics with synthetic polymers such as poly(butyl cyanoacrylate) or polyacrylamide covered with Polysorbate 80 (US Pat. Appl. No.

2002/0009491, US Pat. Appl. No. 2002/0013266; US Pat. Appl. No. 2006/0051317), and using liposomes or immunoliposomes.

The current approaches to improve the transport of therapeutic agents across the BBB include ineffectiveness due to competition with the endogenous ligand, lack of transport of the therapeutic to brain parenchyma, and degradation of the therapeutics due to lysosomal targeting.

Thus, there is a need to develop methods to transport therapeutic agents through the BBB.

#### **Brief Description of the Drawings**

Fig. 1. shows binding of select phage lysates to IGF1R and IR.

Fig. 2. shows binding of peptide-AP fusions to IGF1R.

#### **Summary of the Invention**

One aspect of the invention is an isolated polypeptide comprising a polypeptide having the sequence shown in SEQ ID NOs: 1-13.

Another aspect of the invention is an isolated polynucleotide comprising a polynucleotide encoding the polypeptide comprising the amino acid sequence shown in SEQ ID NOs: 1-13.

Another aspect of the invention is an isolated polynucleotide comprising a polynucleotide having the sequence shown in SEQ ID NOs: 14-26, or a complementary sequence thereof.

Another aspect of the invention is an isolated vector comprising a polynucleotide having the sequence shown in SEQ ID NOs: 14-26.

Another aspect of the invention is an isolated host cell comprising the vector of the invention.

Another aspect of the invention is an isolated fusion protein comprising a polypeptide having the sequence shown in SEQ ID NOs: 1-13 fused to a second polypeptide.

Another aspect of the invention is a method of expressing a polypeptide comprising the steps of:

- a. providing the host cell of the invention; and
- b. culturing the host cell under conditions sufficient for the expression of the polypeptide having the sequence shown in SEQ ID NOs: 1-13.

Another aspect of the invention is a method for delivery of a therapeutic agent across endothelial cells, comprising

- a. conjugating the therapeutic agent to a polypeptide comprising the polypeptide having the sequence shown in SEQ ID NOs: 1, 2, 4, 8, or 12 to form a conjugate;
- b. contacting the conjugate with the endothelial cells; and
- c. measuring the amount of the conjugate delivered across the endothelial cells.

#### **Detailed Description of the Invention**

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

As used herein and in the claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a polypeptide" is a reference to one or more polypeptides and includes equivalents thereof known to those skilled in the art.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which an invention belongs. Although any compositions and methods similar or equivalent to those described herein can be used in the practice or testing of the invention, exemplary compositions and methods are described herein.

The term "polypeptide" means a molecule that comprises at least two amino acid residues linked by a peptide bond to

form a polypeptide. Small polypeptides of less than 50 amino acids may be referred to as "peptides". Polypeptides may also be referred as "proteins."

The term "polynucleotide" means a molecule comprising a chain of nucleotides covalently linked by a sugar-phosphate backbone or other equivalent covalent chemistry. Double and single-stranded DNAs and RNAs are typical examples of polynucleotides.

The term "complementary sequence" means a second isolated polynucleotide sequence that is antiparallel to a first isolated polynucleotide sequence and that comprises nucleotides complementary to the nucleotides in the first polynucleotide sequence. Typically, such "complementary sequences" are capable of forming a double-stranded polynucleotide molecule such as double-stranded DNA or double-stranded RNA when combined under appropriate conditions with the first isolated polynucleotide sequence.

The term "vector" means a polynucleotide capable of being duplicated within a biological system or that can be moved between such systems. Vector polynucleotides typically contain elements, such as origins of replication, polyadenylation signal or selection markers, that function to facilitate the duplication or maintenance of these polynucleotides in a biological system. Examples of such biological systems may include a cell, virus, animal, plant, and reconstituted biological systems utilizing biological components capable of duplicating a vector. The polynucleotides comprising a vector may be DNA or RNA molecules or hybrids of these.

The term "expression vector" means a vector that can be utilized in a biological system or a reconstituted biological system to direct the translation of a polypeptide encoded by a polynucleotide sequence present in the expression vector.

The term "blood-brain barrier" or "BBB" as used herein refers to the barrier between the peripheral circulation and

the brain and spinal cord which is formed by tight junctions within the brain capillary endothelial plasma membranes, creates an extremely tight barrier that restricts the transport of molecules into the brain, even molecules as small as urea having a molecular weight of 60 Da. The blood-brain barrier within the brain, the blood-spinal cord barrier within the spinal cord, and the blood-retinal barrier within the retina, are contiguous capillary barriers within the central nervous system (CNS), and are collectively referred to as the blood-brain barrier.

The term "antibody" refers to a molecule specifically binding to an antigen, and includes dimeric, trimeric and multimeric antibodies, and chimeric, humanized and fully human antibodies. Also, an antibody may be a whole antibody or a functional fragment of an antibody molecule, such as a fragment retaining at least its antigen binding function, and include Fab, F(ab'), F(ab')<sub>2</sub>, scFv, dsFv, and diabodies. For example, antibody fragments may be obtained using proteolytic enzymes (e.g., a whole antibody is digested with papain to produce Fab fragments, and pepsin treatment results in the production of F(ab')<sub>2</sub> fragments). Techniques for the preparation and use of the various antibodies are well known in the art (Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY 1987-2001; Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Edition, Cold Spring Harbor, NY, 1989; Harlow and Lane, Antibodies, a Laboratory Manual, Cold Spring Harbor, NY, 1989; Colligan, et al., ed., Current Protocols in Immunology, John Wiley & Sons, Inc., NY 1994-2001; Colligan et al., Current Protocols in Protein Science, John Wiley & Sons, NY, NY, 1997-2001; Kohler et al., Nature 256:495-497, 1975; US 4,816,567, Queen et al., Proc. Natl. Acad. Sci. 86:10029-10033, 1989). For example, fully human monoclonal antibodies lacking any non-human sequences can be prepared from human immunoglobulin transgenic mice or from phage display

libraries (Lonberg et al., Nature 368:856-859, 1994; Fishwild et al., Nature Biotech. 14:845-851, 1996; Mendez et al., Nature Genetics 15:146-156, 1997; Knappik et al., J. Mol. Biol. 296:57-86, 2000; Krebs et al., J. Immunol. Meth. 265:67-84, 2001).

An antibody molecule or preparation "specifically binds" a given antigen when it binds this antigen with higher affinity and in a specific, as opposed to non-specific fashion, relative to a second non-identical antigen. Stated differently, the "specific binding" of an antibody molecule or preparation can be used to distinguish between two different polypeptides.

The term "insulin-like growth factor receptor 1" or "IGF1R" as used herein refers to a human IGF1R (GenBank Acc. No. NP\_000866) having an amino acid sequence shown in SEQ ID NO: 27. The IGF1R pro-polypeptide is cleaved into an alpha and a beta chain to form a mature protein. The alpha chain has amino acid residues 31-740 of SEQ ID NO: 27, and the beta chain has amino acid residues 741-1367 of SEQ ID NO: 27. "Soluble IGF1R" or "sIGF1R" as used herein refers to the extracellular domain of the IGF1R (amino acids 31 - 932 of SEQ ID NO: 27). The soluble IGF1R may be an extracellular domain of an uncleaved pro-polypeptide, or an extracellular domain of a mature IGF1R (amino acid residues 31-740 forming the alpha chain, and amino acid residues 741-932 forming the extracellular portion of the beta chain).

The term "conjugate" as used herein refers to a chimeric molecule comprising a peptide of the invention having an amino acid sequence shown in SEQ ID NOs: 1-13 and a therapeutic agent. The term "conjugated" or "conjugating" means that the therapeutic agent(s) and the peptides of the invention are physically linked by, for example, by covalent chemical bonds, physical forces such as van der Waals or hydrophobic interactions, encapsulation, embedding, or combinations thereof. The therapeutic agent(s) and the

peptides of the invention can be linked by chemical bonds through alcohol, acid, carbonyl, thiol or amine groups using well known chemical synthesis methods (see e.g. US Pat. Appl. No. US2010/0028370). The therapeutic agent can be connected to the peptides of the invention by a linker. Exemplary linkers are glycine-rich linkers such as Gly<sub>3</sub>SerGly<sub>3</sub>Ser (SEQ ID NO: 28) or Gly<sub>4</sub>SerGly<sub>4</sub>SerGly<sub>4</sub>Ser (SEQ ID NO: 29). When the therapeutic agent and the peptides of the invention are conjugated via a covalent bond or a peptide and the therapeutic agent is a polypeptide, then the entire conjugate is a "fusion protein". Thus, the term "fusion protein" refers to a polypeptide made up of two (or more) heterologous polypeptides which are not normally fused together in a single amino acid sequence. Fusion proteins may generally be prepared using either recombinant nucleic acid methods, i.e., as a result of transcription and translation of a recombinant gene fusion product, which fusion comprises a segment encoding a polypeptide of the invention and a segment encoding a heterologous polypeptide.

The term "therapeutic agent" as used herein refers to a molecule administered to induce a desired therapeutic effect in a subject. A subject is a human or non-human animal, including a mammal or a primate. Exemplary therapeutic agents are proteins, antibodies, peptides, small molecules, or polynucleotides. Therapeutic agents may also be toxins or radioisotopes, where the therapeutic effect intended is, for example, the killing of a cancer cell.

The present invention provides isolated polypeptides that bind IGF1R, polynucleotides encoding the polypeptides, vectors comprising the polynucleotides, isolated host cells, polypeptides obtainable from expression of the polynucleotides, methods for expressing the polypeptides of the invention, and methods of using the polynucleotides and polypeptides of the invention. The polypeptides of the invention bind to the IGF1R, and are transcytosed across

endothelial cells. Since IFG1R is expressed on the endothelial cells within the blood-brain barrier (BBB), the polypeptides of the invention can provide means for delivering therapeutic agents across the BBB.

One aspect of the invention is an isolated polypeptide comprising a polypeptide having the sequence shown in SEQ ID NOs: 1-13.

The polypeptides of the invention may be produced by chemical synthesis, such as solid phase peptide synthesis, on an automated peptide synthesizer. Alternatively, the polypeptides of the invention can be obtained from polynucleotides encoding these polypeptides by the use of cell-free expression systems such as reticulocyte lysate based expression systems, wheat germ extract based expression systems, and *Escherichia coli* extract based expression systems. The polypeptides of the invention can also be obtained by expression and isolation from cells harboring a nucleic acid sequence of the invention by techniques well known in the art, such as recombinant expression of easily isolated affinity labeled polypeptides. Those skilled in the art will recognize other techniques for obtaining the polypeptides of the invention.

Another aspect of the invention is an isolated fusion protein comprising a polypeptide having the sequence shown in SEQ ID NOs: 1-13 fused to a second polypeptide. Such second polypeptide may be a leader or a secretory signal sequences. Such second polypeptide may be a therapeutic agent fused to the peptides of the invention. The therapeutic agent and the peptide of the invention can be fused to one another in a variety of ways. The C-terminus or the N-terminus of the peptide of the invention may be directly linked to the N-terminus or the C-terminus, respectively of the therapeutic agent via an amide bond or a peptide linker. Therapeutic agents may be linked to the peptide of the invention using chemical crosslinking well known in the art.

Another aspect of the invention is an isolated polynucleotide comprising a polynucleotide encoding the polypeptides of the invention.

The polynucleotides of the invention may be produced by chemical synthesis such as solid phase polynucleotide synthesis on an automated polynucleotide synthesizer. Alternatively, the polynucleotides of the invention may be produced by other techniques such as PCR based duplication, vector based duplication, or restriction enzyme based DNA manipulation techniques. Techniques for producing or obtaining polynucleotides of a given known sequence are well known in the art.

The polynucleotides of the invention may also comprise at least one non-coding sequence, such as transcribed but not translated sequences, termination signals, ribosome binding sites, mRNA stabilizing sequences, introns and polyadenylation signals. The polynucleotide sequences may also comprise additional sequences encoding additional amino acids. These additional polynucleotide sequences may, for example, encode a marker or tag sequence such as a hexahistidine peptide (Gentz et al., Proc. Natl. Acad. Sci. (USA) 86:821-284, 1989) or the HA peptide tag (Wilson et al., Cell 37:767-778, 1984) which facilitate the purification of fused polypeptides. Exemplary polynucleotides are polynucleotides having a sequence shown in SEQ ID NOs: 14-26.

Another embodiment of the invention is a vector comprising an isolated polynucleotide having a sequence shown in SEQ ID NOs: 14-26. The vectors of the invention are useful for maintaining polynucleotides, duplicating polynucleotides, or driving expression of a polypeptide encoded by a vector of the invention in biological systems, including reconstituted biological systems. Vectors may be chromosomal-, episomal- and virus-derived such as vectors derived from bacterial plasmids, bacteriophages, transposons, yeast episomes, insertion elements, yeast chromosomal

elements, baculoviruses, papova viruses such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses, picornaviruses and retroviruses and vectors derived from combinations thereof, such as cosmids and phagemids.

The vectors of the invention can be formulated in microparticles, with adjuvants, lipid, buffer or other excipients as appropriate for a particular application.

In one embodiment of the invention the vector is an expression vector. Expression vectors typically comprise nucleic acid sequence elements that can control, regulate, cause or permit expression of a polypeptide encoded by such a vector. Such elements may comprise transcriptional enhancer binding sites, RNA polymerase initiation sites, ribosome binding sites, and other sites that facilitate the expression of encoded polypeptides in a given expression system. Such expression systems may be cell-based, or cell-free systems well known in the art. Nucleic acid sequence elements and parent vector sequences suitable for use in the expression of encoded polypeptides are also well known. An exemplary plasmid-derived expression vector useful for expression of the polypeptides of the invention comprises an *E. coli* origin of replication, a chloramphenicol acetyltransferase (CAT) gene, a bacteriophage T7 promoter, a *pelB* signal sequence, and a T7 terminator sequence.

Another embodiment of the invention is an isolated host cell comprising a vector of the invention. Representative host cell examples include Archaea cells; bacterial cells such as *Streptococci*, *Staphylococci*, *Enterococci*, *E. coli*, *Streptomyces*, cyanobacteria, *B. subtilis* and *S. aureus*; fungal cells such as *Kluveromyces*, *Saccharomyces*, Basidiomycete, *Candida albicans* or *Aspergillus*; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293, CV-1, Bowes melanoma and myeloma; and plant cells, such as gymnosperm or

angiosperm cells. The host cells in the methods of the invention may be provided as individual cells, or populations of cells. Populations of cells may comprise an isolated or cultured population of cells or cells present in a matrix such as a tissue.

Introduction of a polynucleotide, such as a vector, into a host cell can be effected by methods well known to those skilled in the art (Davis et al., Basic Methods in Molecular Biology, 2<sup>nd</sup> ed., Appleton & Lange, Norwalk, CT, 1994; Sambrook et al., Molecular Cloning: A Laboratory Manual, 3<sup>rd</sup> ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001). These methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection.

It is possible to modify the structure of the polypeptides or fragments of the invention for such purposes as enhancing substrate specificity, stability, solubility, and the like. For example, a modified polypeptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition. It is contemplated that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*i.e.*, conservative mutations) will, in some instances but not all, not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine); (3) nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); and (4) uncharged

polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. Alternatively, the amino acid repertoire can be grouped as (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine histidine), (3) aliphatic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic (phenylalanine, tyrosine, tryptophan); (5) amide (asparagine, glutamine); and (6) sulfur-containing (cysteine and methionine) (Stryer (ed.), Biochemistry, 2nd ed, WH Freeman and Co., 1981). Whether a change in the amino acid sequence of a polypeptide or fragment thereof results in a functional homolog can be readily determined by assessing the ability of the modified polypeptide or fragment to produce a response in a fashion similar to the unmodified polypeptide or fragment using the assays described herein. Peptides, polypeptides or proteins in which more than one replacement has taken place can readily be tested in the same manner.

The polypeptides of the invention can also be formulated in a pharmaceutically acceptable carrier or diluent. A variety of aqueous carriers may be employed, e.g., 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well-known sterilization techniques (e.g., filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents. The concentration of the polypeptides of the invention in such pharmaceutical formulation can vary widely, i.e., 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 and other factors, according to

the particular mode of administration selected. The appropriate therapeutically effective dose can be determined readily by those of skill in the art. A determined dose may, if necessary, be repeated at appropriate time intervals selected as appropriate by a physician or other person skilled in the relevant art (e.g. nurse, veterinarian, or veterinary technician) during the treatment period.

The polypeptides 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 protein preparations. Lyophilization and reconstitution techniques are well known in the art.

Another embodiment of the invention is a method for expressing a polypeptide comprising the steps of providing a host cell of the invention; culturing the host cell under conditions sufficient for the expression of at least one polypeptide of the invention.

Host cells can be cultured under any conditions suitable for maintaining or propagating a given type of host cell and sufficient for expressing a polypeptide. Culture conditions, media, and related methods sufficient for the expression of polypeptides are well known in the art. For example, many mammalian cell types can be aerobically cultured at 37°C using appropriately buffered DMEM media while bacterial, yeast and other cell types may be cultured at 37°C under appropriate atmospheric conditions in LB media.

In the methods of the invention the expression of a polypeptide can be confirmed using a variety of well known methods. For example, expression of a polypeptide can be confirmed using detection reagents, such as antibodies using for example FACS or immunofluorescent techniques, or using SDS-PAGE or HPLC.

Another aspect of the invention is a method for delivery of a therapeutic agent across endothelial cells, comprising:

- a. conjugating the therapeutic agent to a polypeptide comprising the polypeptide having the sequence shown in SEQ ID NOs: 1, 2, 4, 8, or 12 to form a conjugate;
- b. contacting the conjugate with the endothelial cells; and
- c. measuring the amount of the conjugate delivered across the endothelial cells.

The polypeptides of the invention facilitate delivery of a therapeutic agent across endothelial cells through the binding of the polypeptides of the invention to an IGF1R. The peptides are selected so that the conjugated therapeutic agent does not interfere with binding of the polypeptides of the invention to IGF1R. The present invention describes conjugation of a protein having a molecular weight of approximately 100 kD (921 amino acids) to the polypeptides of the invention without loss of transcytosis activity. Other polypeptides of comparable size are also likely to be successfully conjugated to the polypeptides of the invention and delivered across endothelial cell to the brain.

Delivery of the conjugate across the endothelial cells can be measured using well known *in vitro* or *in vivo* methods. Exemplary *in vitro* measurements can be done using polarized monolayers of endothelial cells and measuring the transcytosis of the conjugate using for example antibodies to the conjugate. *In vivo* measurements can be done in a subject using for example radioactive conjugates and measuring their distribution in brain after administration. Good correlation has been observed between the *in vitro* and *in vivo* method. For example, Perrier et al., demonstrated good correlation ( $R=0.94$ ) between the permeability coefficients of a series of compounds and their corresponding *in vivo* rodent blood-brain transfer coefficients using co-cultures of astrocytes and rat brain endothelial cells (Perrier et al., Brain Res. 1150:1-13, 2007).

The present invention will now be described with reference to the following specific, non-limiting examples.

### Example 1

#### Identification of IGF1R binding peptides

##### Phage panning

The pIX phage libraries displaying random peptides were generated according to methods described in US Pat. Appl. No. US2010/0021477, and used as a source of human IGF1R binding peptides. This library was panned in solution against a biotinylated form of purified soluble IGF1R (sIGF1R) having a carboxy-terminal hexahistidine tag (R&D Systems, Minneapolis, MN) for three rounds. Biotinylation of sIGF1R was done using the EZ-Link No-Weigh Sulfo-NHS-LC-Biotin Microtubes (Pierce, Rockford, IL). Because of the large size of sIGF1R (~330 kDa), Tetralink Avidin beads were used for round 1 selection due to their ~ 10 fold greater binding capacity than Dynal magnetic beads.

Total of 384 individual phage lysates from the panning were tested for binding specificity towards sIGF1R in a solid phase phage ELISA. Briefly, 100 µl/well of 5 µg/ml sIGF1R (R&D Systems, Minneapolis, MN) was bound to Black Maxisorp Plates (Nunc, Rochester, NY), 25 µl of phage lysate added, and the reaction was detected using anti-M13-HRP antibody (EMD Biosciences, Gibbstown, NJ) and POD substrate (Roche, Indianapolis, IN), and the signal detected using a TEKAN plate reader. A non-related protein demonstrated not to cross the BBB was used as a negative control. Positive lysates were defined as clones with a sIGF1R specific signal that was three fold above the background of the negative control. A total of 13 clones with unique peptide sequences were obtained with confirmed binding to the sIGF1R (Table 1). From these 13 clones, 3 (clones 5, 13 and 16) cross-reacted with the insulin receptor.

The peptides from clones 5-14, 16 and 17 were cloned in-frame as peptide-Alkaline phosphatase-His6 (peptide-AP) fusion proteins into a modified pET20b+ vector having a Chloramphenicol acetyltransferase (CAT) gene cloned within. The resulting peptide-AP fusions were expressed in bacteria and purified using Ni-NTA (EMD Biosciences, Gibbstown, NJ) according to manufacturer's instructions. The amino acid sequence of the alkaline phosphatase used is shown in SEQ ID NO: 30.

Table 1a. Polypeptide sequences of identified IGF1R binding peptides

| Peptide Sequence         | Clone number: | Peptide SEQ ID NO: |
|--------------------------|---------------|--------------------|
| TGCDFPCLCRGCHP           | 5             | 1                  |
| AECEWPWLTLELCQS          | 6             | 2                  |
| PFCYSGGPLPYPCTY          | 7             | 3                  |
| PVCPSFCYDQYVCPT          | 8             | 4                  |
| FTCAVYSLSELDCRD          | 9             | 5                  |
| LSCYDPTLRTLYCHV          | 10            | 6                  |
| HTCFYPTLMPPELCFD         | 11            | 7                  |
| SNCPLDMRLTELCVM          | 12            | 8                  |
| WHCTPLTQIADPGSIHILECTV   | 13            | 9                  |
| VECDTPSITFSPGLEALFWNTCSP | 14            | 10                 |
| MTCAWHTLHTDPGLTPQLTLPCY  | 15            | 11                 |
| AGCPSPMPVDPGFYSAMQLCRE   | 16            | 12                 |
| DDIDEFLHQLHNLVNNVH       | 17            | 13                 |

The binding of purified peptide-AP fusion proteins to sIGF1R was assessed using ELISA against immobilized sIGF1R. Briefly, bacteria transformed with each peptide-AP fusion expression vector were grown overnight and the following day

the cultures were clarified by centrifugation at 4,500 rpm, 4°C. The supernatants were collected and 75 µl of each supernatant was assayed for binding to 2 µg/ml sIGF1R immobilized on ELISA plates. The bound peptides were detected using the Attophos Substrate (Roche, Indianapolis, IN) following the manufacturer's recommendation and read using a Molecular Devices M5 plate reader. Fusions with peptide clones 7 and 10 did not express well. All other peptide-AP fusions showed binding activity to sIGF1R (Figure 2).

**Table 1b.** Polynucleotide sequences of the IGF1R binding peptides

| Clone number: | SEQ ID NO: | Sequence                                                                     |
|---------------|------------|------------------------------------------------------------------------------|
| 5             | 14         | ACGGGTTGTGATTTCCGGAGTTGTGTCGTGGTTGTCATCCG                                    |
| 6             | 15         | GCTGAGTGTGAGTGGCCGTGGCTTACGCTGGAGCTTTGTCAGTCT                                |
| 7             | 16         | CCTTTTGTATTCTGGTGGGCCGCTGCCGTATCCTTGACGTAT                                   |
| 8             | 17         | CCTGTGTGCCGTCGTTTTGTATGATCAGTATGTGTGTCCGACT                                  |
| 9             | 18         | TTACGTGTGCTGTTTATTCGTTGTCTGAGCTGGATTGTAGGGAT                                 |
| 10            | 19         | TTGAGTTGTTATGATCCGACGCTGCGTACGTTGTATTGTCATGTT                                |
| 11            | 20         | CATACGTGTTTTATCCTACGCTGATGCCTCCTGAGCTGTGTTTCG<br>AT                          |
| 12            | 21         | AGTAATTGCCTCCGTTGGATATGCGGCTGACTGAGCTTTGTGTTAT<br>G                          |
| 13            | 22         | TGGCATTGTACTCCGCTGACGCAGATTGCTGATCCAGGCCAGAGT<br>ATTATTCATATTTGGAGTGTACTGTT  |
| 14            | 23         | GTTGAGTGTGATACGCCGTCTATTACTTTTCGCCAGGCCTGGAGG<br>CGCTGTTTGGAAACGTGTTCTCCT    |
| 15            | 24         | ATGACGTGTGCGTGGCATACTTTCATACGGATCCAGGCCTTACT<br>CCGCAGCTGAGGCTGCCTTGATTTAT   |
| 16            | 25         | GCGGGGTGTCCGAGTCCGATGCCGCCGGTGGATCCAGGCTTTTAT<br>AGTGCTATTGTGCAGCTGTGTAGGGAG |
| 17            | 26         | GATGACATAGACGAATTTCTTCATCAACTCCACAACCTAGTAAACA<br>ATGTTTAC                   |

## Example 2

### Characterization of the IGF1R binding peptides

The identified IGF1R binding peptides were cloned in-frame to the C-terminus of the protein G IgG domain (PG) in a modified pET17b vector (EMD Chemicals, Gibbstown, NJ) having a ligation independent cloning site (LIC) to generate PG-peptide fusions. The IgG binding domain of protein G is stable and thus enabled easy purification of the fusion protein from bacterial lysates. The amino acid sequence of the protein G IgG domain used is shown in SEQ ID NO: 31. The PG-peptide fusions were expressed in bacteria upon 1 mM IPTG induction and purified using IgG Sepharose beads (GE Healthcare Life Sciences, Piscataway, NJ) from the bacterial lysates cleared by centrifugation at 16,000g, 4°C for 20 min.

Relative binding affinities of the PG-peptide fusions for sIGF1R were measured using ELISA. The K<sub>d</sub>s were determined using GraphPad Prism 4 software with a one site binding equation. 100 µl of 2 µg/ml sIGF1R (R&D Systems, Minneapolis, MN) in Dulbecco's Phosphate Buffer Saline (DPBS (-/-)) was coated onto a 96 well Black Maxisorp plates (Nunc, Rochester, NY) overnight at 4°C. The plates were washed with TBST and the wells blocked with 200 µl/well Starting Block T20 (TBS) (Pierce, Rockford, IL) for 1 hr, room temperature with 300 rpm shaking. 75 µl/well of purified, serially diluted PG-peptide fusions, starting concentration 40 µM, was added into the wells and incubated for 1 h, room temperature with 300 rpm mixing; the volume was brought up to 100 µl with Starting Block T20. The plates were washed 3X with TBST and then probed with 100 µl/well Peroxidase labeled-Rabbit antibody (1:5000) (Rockland, Gilbertsville, PA), washed, and the signal was detected using 100 µl/well POD substrate (Roche, Indianapolis, IN). Chemiluminescence was detected using a Molecular Devices M5 plate reader.

For competition assays, insulin-like growth factor 1 (IGF-1) (R&D Systems, Minneapolis, MN) was added to the Starting Block T20 used to bring the volume up to 100  $\mu$ l at a concentration of 400 nM final.

Characterization of the PG-peptide fusions are summarized in Table 2. The majority of the peptides bound with relative affinities in the low  $\mu$ M range (0.75-8  $\mu$ M). The fusion protein with peptide clone 17 was unique in its binding profile in that the binding curve was bellshaped. This is similar to insulin binding to the insulin receptor and IGF-1 to the IGF1R, the ligands inducing conformational shifts in the receptors upon binding and having negative cooperativity profiles at higher concentrations of the ligands. The results of the competition studies with IGF-1 are shown in Table 2.

Table 2.

| Clone number: | Peptide SEQ ID NO: | EC <sub>50</sub> ( $\mu$ M) | Competed by IGF-1 | Transcytosis |
|---------------|--------------------|-----------------------------|-------------------|--------------|
| 5             | 1                  | 0.75                        | -                 | yes          |
| 6             | 2                  | 1                           | -                 | yes          |
| 7             | 3                  | 1.8                         | -                 | not done     |
| 8             | 4                  | 1.6                         | -                 | yes          |
| 9             | 5                  | 3.5                         | -                 | no           |
| 10            | 6                  | 4.3                         | -                 | not done     |
| 11            | 7                  | 1.2                         | yes               | no           |
| 12            | 8                  | 1.8                         | -                 | yes          |
| 13            | 9                  | >50                         | yes               | no           |
| 14            | 10                 | 0.85                        | yes               | no           |
| 15            | 11                 | 2.6                         | -                 | not done     |
| 16            | 12                 | 8.2                         | yes               | yes          |
| 17            | 13                 | not done                    | yes               | no           |

Example 3.In vitro BBB model

Select sIGF1R binding peptides were further characterized in the *in vitro* blood-brain barrier model, rat brain microvascular endothelial cell model.

Rat Brain capillary endothelial cells were prepared as described (Perriere et al., J. Neurochem. 93:279-289, 2005). Briefly, brains from 6-8 week old male Sprague Dawley rats were rolled on 3MM chromatography paper to remove the meninges, cut sagittally, and the white matter dissected leaving the cortices, which were then minced thoroughly. The minced cortices were transferred to a 50 ml polypropylene conical tube with 20 ml DMEM supplemented with 39 units/ml DNase I (Worthington, Lakewood, NJ) and 0.7 mg/ml Collagenase type 2 (Worthington, Lakewood, NJ) at final concentration and incubated at 37°C with gentle mixing for 1.25 hrs. After a brief centrifugation the resultant pellet was resuspended in 20 ml 20% BSA (Sigma, St. Louis, MO) in DMEM, centrifuged and the microvessel enriched pellet was isolated and digested a second time with 20 ml DMEM supplemented with 39 units/ml DNase I and 1 mg/ml Collagenase/Dispase (Roche, Indianapolis, IN) at 37°C for 1 hr. The digestion was briefly centrifuged and the resulting cell pellet was resuspended in 2 ml DMEM and subsequently layered onto the top of a 33% continuous Percoll gradient, centrifuged, and the enriched microvessel fraction was removed and briefly centrifuged a second time. The cell pellet was resuspended in 10 ml complete rat brain microvascular endothelial cell growth media (DMEM, 20% Plasma Derived Serum (PDS), 100 µg/ml Heparin, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin) and plated onto a 10 cm tissue culture dish for 4 hrs at 37 °C, 5% CO<sub>2</sub>. After 4 hrs cells that remained unattached were pipetted off and counted in a hemacytometer using Trypan Blue. The cells were plated into the top

chamber of the Transwells (0.4  $\mu\text{m}$  pore size, diameter 1.12 cm, Corning, Acton, MA) treated with 400  $\mu\text{g/ml}$  collagen type IV (Sigma, St. Louis, MO) and 100  $\mu\text{g/ml}$  fibronectin (Sigma, St. Louis, MO) at a density of  $6 \times 10^5$  cells/ml, 500  $\mu\text{l}$  per well. 1ml of growth media was placed into the bottom chamber. Both chambers were supplemented with 4  $\mu\text{g/ml}$  puromycin (Clontech, Mountain View, CA) and the plates were incubated at 37 °C, 5%  $\text{CO}_2$  overnight. The next day the media was changed with fresh complete culture media with 4 $\mu\text{g/ml}$  puromycin and the cells were placed back into the incubator overnight. The next day the media was changed with complete culture media and again two days after. Cultures were monitored by eye till they reached 100% confluence, ~6-7 days after seeding. The developed *in vitro* BBB model had high transendothelial electrical resistance ( $>100 \text{ ohms-cm}^2$ ), measured using Millicell-ERS (Millipore, Billerica, MA) and very low Na-Fluorescein permeability (about  $1-5 \times 10^{-6} \text{ cm/s}$ ).

25  $\mu\text{g}$  of purified peptide-AP fusions were added to the top chamber of the *in vitro* BBB model and transcytosed peptide-AP fusions were detected at 15 and 30 minute timepoints in the bottom chamber using ELISA. Briefly, 75  $\mu\text{l}$  of each sample was transferred to plates coated with 5  $\mu\text{g/ml}$  mouse monoclonal anti-bacterial AP antibody (Sigma, St. Louis, MO). The plate was incubated for 1 hr, washed, the signal was developed with Attophos Substrate (Roche, Indianapolis, IN) according to manufacturer's recommendation, and read using a Molecular Devices M5 plate reader with 440 nm excitation and 550 nm emission. The results are shown in Table 2.

The present invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

**We claim:**

1. An isolated polypeptide comprising a polypeptide having the sequence shown in SEQ ID NOs: 1-13.
2. An isolated polynucleotide comprising a polynucleotide encoding the polypeptide comprising the amino acid sequence shown in SEQ ID NOs: 1-13.
3. An isolated polynucleotide comprising a polynucleotide having the sequence shown in SEQ ID NOs: 14-26, or a complementary sequence thereof.
4. An isolated vector comprising a polynucleotide having the sequence shown in SEQ ID NOs: 14-26.
5. The vector of claim 4, wherein the vector is an expression vector.
6. An isolated host cell comprising the vector of claim 4.
7. An isolated fusion protein comprising a polypeptide having the sequence shown in SEQ ID NOs: 1-13 fused to a second polypeptide.
8. The fusion protein of claim 7, wherein the second polypeptide encodes an immunoglobulin or fragment thereof.
9. A method of expressing a polypeptide comprising the steps of :
  - a. providing the host cell of claim 6; and
  - b. culturing the host cell under conditions sufficient for the expression of the polypeptide having the sequence shown in SEQ ID NOs: 1-13.
10. A method of for delivery of a therapeutic agent across endothelial cells, comprising:
  - a. conjugating the therapeutic agent to a polypeptide comprising the polypeptide having the sequence shown in SEQ ID NOs: 1, 2, 4, 8, or 12 to form a conjugate;
  - b. contacting the conjugate with the endothelial cells; and
  - c. measuring the amount of the conjugate delivered across the endothelial cells.

11. The method of claim 10, wherein the endothelial cells form a blood-brain barrier.