# Glycosylphosphatidylinositol Containing Polypeptides

This invention relates to polypeptides which comprise a receptor binding domain of a cytokine and a domain which includes a signal sequence for the attachment of glycosylphosphatidylinositol (GPI) anchors; methods to manufacture said polypeptides; nucleic acids molecules encoding said polypeptides; and therapeutic compositions comprising said polypeptides.

GPI-anchors are post-translational modifications to proteins which add glycosyl – phosphatidylinositol which enable these proteins to anchor to extracellular side of cell membranes. Typically proteins which have a GPI anchor do not have transmembrane or a cytoplasmic domain. GPI anchor proteins form a diverse family of proteins that includes by example and not by way of limitation, membrane associated enzymes, adhesion molecules and proteins which coat the outer surface of protozoan parasites such as *Trypanosoma brucei* spp. The kidney includes a number of examples of GPI-anchored proteins ie uromodulin, carbonic anhydrase type IV, alkaline phosphatase, Thy-1, BP-3, amino peptidase P, and dipeptidylpeptidase.

All GPI-anchor proteins are initially synthesized with a transmembrane anchor which, after translocation across the endoplasmic reticulum, is cleaved and covalently linked to a preformed GPI- anchor by a specific transamidase enzyme. The modification of proteins by the addition of a GPI-anchor confers important properties on the protein since the addition of the lipid moiety allows the protein to be inserted into cell membranes thereby anchoring the protein.

There are some general requirements for creating a synthetic GPI anchor sequence. These are a hydrophobic region at the C-terminus of the molecule (10-20 amino acids) not followed by a cluster of basic residues, a"spacer domain" of 7-10 residues preceding the hydrophobic region and small amino acids after the spacer region, where cleavage of the precursor and attachment of the anchor occurs. The GPI anchor is preassembled and added to nascent protein in the endoplasmic reticulum. Concomitant with this step, the initial C-terminal peptide is removed so that the GPI anchor is covalently attached to a new C-terminal amino acid on the protein.

Ligands which interact with receptors to bring about a suitable biochemical response are known as agonists and those that prevent, or hinder, a biochemical response are known as antagonists. For example, and not by way of limitation, cell specific growth factors are ligands that act as agonists and bind receptors located in cell membranes.

A large group of growth factors, referred to as cytokines, are involved in a number of diverse cellular functions. These include, by example and not by way of limitation, modulation of the immune system, regulation of energy metabolism and control of growth and development. Cytokines mediate their effects via receptors expressed at the cell surface on target cells.

Receptors of the cytokine receptor family possess a single transmembrane domain and lack intrinsic enzyme activity (Kishimoto *et al.*, 1994). When a cytokine binds to its cognate receptor the complex is internalised and signaling occurs through the activation of signaling cascades that include the Jak/Stat and Mapk pathways. Internalisation is followed by a recycling step whereby the receptor molecule is regenerated for further use within the cell. An example of the above is described with respect to growth hormone (GH) and its binding to the growth hormone receptor (GHR).

It is known that a single molecule of growth hormone (GH) associates with two receptor molecules (Cunningham *et al.*, 1991; de Vos *et al.*, 1992; Sundstrom *et al.*, 1996; Clackson *et al.*, 1998). This occurs through two unique receptor-binding sites on GH and a common binding pocket on the extracellular domain of two receptors. Site 1 on the GH molecule has a higher affinity than site 2, and receptor dimerization is thought to occur sequentially with one receptor binding to site 1 on GH followed by recruitment of a second receptor to site 2.

The extracellular domain of the GHR exists as two linked domains each of approximately 100 amino acids (SD-100), the C-terminal SD-100 domain being closest to the cell surface and the N-terminal SD-100 domain being furthest away. It is a conformational change in these two domains that occurs on hormone binding with the formation of the trimeric complex GHR-GH-GHR.

Moreover, truncated GH receptors, which lack the cytoplasmic domain of the receptor, act as dominant negative inhibitors of GH signalling (Ross *et al.*, 1997). The truncated receptor is expressed at a high level on the cell surface because it lacks the cytoplasmic domain essential

for internalisation (Maamra *et al.*, 1999) . In the presence of GH, the truncated receptor heterodimerises with the full length receptor and blocks signalling because it lacks the cytoplasmic domain. As the truncated receptor fails to internalise it acts as a dominant negative inhibitor preventing internalisation of the GH receptor complex.

We have synthesised cytokine receptor variants which comprise a domain of a GPI-anchor protein. The variants lack a cytoplasmic domain and therefore do not have the capability to signal. The provision of a GPI-anchor domain means the variant inserts into membranes and acts as an effective inhibitor of GH signalling by competing for circulating GH and binding GH at the cell surface in a heterodimeric complex that consists of the chimeric truncated GPI anchored receptor, GH, and the native receptor. In addition truncated GPI anchored GH receptor generates a large amount of soluble GH receptor which will bind GH.

Many cytokines activate their cognate receptors via dimerisation or oligomerisation and the invention relates to the provision of cytokine receptors which have been modified by the provision of a polypeptide domain which comprises a sequence which is modified by the addition of glycosylphosphatidylinositol.

According to a first aspect of the invention there is provided a chimeric polypeptide comprising:

i) a ligand binding domain of a cytokine receptor; and

ii) a domain which includes a signal sequence for the attachment of glycosylphosphatidylinositol.

Preferably said chimeric polypeptide consists of the extracellular domain of a cytokine receptor and a domain which includes a signal sequence for the attachment of glycosylphosphatidylinositol.

Preferably the polypeptide is modified by the addition of glycosylphosphatidylinositol.

Preferably the modified polypeptide is a modulator of cytokine mediated cell signalling.

Preferably the signal sequence for the attachment of glycosylphosphatidylinositol is selected from the group consisting of:

DKLVKCGGIS LLVQNTSWML LLLLSLSLLQ ALDFISL;

PSPTPTETAT PSPTPKPTST PEETEAPSSA TTLISPLSLI VIFISFVLLI;

LVPRGSIEGR GTSITAYNSE GESAEFFFLL ILLLLLVLV; and

TSITAYKSE GESAEFFFLL ILLLLLVLV.

In a preferred embodiment of the invention said polypeptide is an antagonist.

The invention exploits the high affinity of a cytokine for its receptor and the ability of the lipophilic GPI tail to reinsert into a plasma membrane. The polypeptide of the invention is a "chimera" comprising a cytokine receptor ligand binding domain and a domain of a protein which includes a site for the addition of a GPI anchor. The chimeric molecule will consist of the extracellular cytokine hormone binding domain with a C-terminal GPI anchor.

It is expected that in the circulation the chimeric protein will form a micelle of a large number of chimeric proteins with the GPI anchors in the centre. On contact with the cell membrane the GPI will reinsert into the cell membrane. The invention has the important advantage that the binding of the cytokine to the chimera results in a receptor:hormone:chimera complex. In the example of GH this will be GHR:GH:chimera. This complex will not signal as the chimera is a truncated receptor and will therefore block both receptor signaling and internalisation.

In an alternative preferred embodiment of the invention said polypeptide chimera acts as a circulating antagonist. It is expected that the micellar molecule in the circulation will have the GPI anchors at its centre and the binding domain of the receptor pointing outwards and therefore able to bind and antagonise the action of the hormone.

In an alternative preferred embodiment of the invention said polypeptide chimera acts as an antagonist following local or transgenic expression through gene therapy. It would be expected that transfection or gene therapy could be used either at the cellular level or whole body. Thus, the local expression in cells could antagonise the action of cytokines or the

injection of DNA into a body compartment such as an inflamed knee would block the actions of inflammatory cytokines such as TNF.

In a preferred embodiment of the invention the ligand binding domain of the cytokine receptor is derived from the receptors selected from the group consisting of: growth hormone (GH); leptin; erythropoietin; prolactin; TNF, interleukins (IL), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, the p35 subunit of IL-12, IL-13, IL-15; granulocyte colony stimulating factor (G-CSF); granulocyte macrophage colony stimulating factor (GM-CSF); ciliary neurotrophic factor (CNTF); cardiotrophin-1 (CT-1); leukemia inhibitory factor (LIF); oncostatin M (OSM); interferon, IFNα and IFNγ. .

Preferably the ligand binding domain of the cytokine receptor is derived from the growth hormone receptor.

In one embodiment of the invention, the polypeptide is a fusion protein.

In a further preferred embodiment of the invention said polypeptide comprises the amino acid sequence shown in Figures 3 or Figure 9 or Figure 14 or Figure 15.

In a further preferred embodiment of the invention there is provided a polypeptide according to the invention which has been modified by addition, deletion or substitution of at least one amino acid residue to provide a sequence variant of the polypeptide according to the invention.

Typically, variants include chimeras which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of the chimera by eliminating proteolysis by proteases in an expression system.

Variant chimeras are expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with the desired properties. Further mutations can be made to variants which are silent as to the amino acid sequence of the polypeptide, but which

provide preferred codons for translation in a particular host, for example in yeast and slime molds such as *Dictyostelium spp.*

The skilled person will also realize that conservative amino acid substitutions may be made in the chimeric polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e, the variants retain the functional capabilities of the chimeras. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made.

Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of chimeric polypeptides to produce functionally equivalent variants of these polypeptides typically are made by alteration of a nucleic acid encoding the chimera. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985.

Alternatively, or preferably, said modification includes the use of modified amino acids in the production of recombinant or synthetic forms of chimeric polypeptides according to the invention.

It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, $N^6$-acetyllysine, $N^6$-methyllysine, $N^6$,$N^6$-dimethyllysine, $N^6$,$N^6$,$N^6$-trimethyllysine, cyclohexyalanine, D-amino acids, ornithine.

The incorporation of modified amino acids may confer advantageous properties on polypeptides according to the invention. For example, the incorporation of modified amino acids may confer increased *in vivo* stability on the chimeric polypeptide thus allowing a decrease in the effective amount of polypeptide administered to a patient.

According to a further aspect of the invention there is provided a nucleic acid molecule comprising a nucleic acid sequence which encodes a polypeptide according to the invention.

In a preferred embodiment of the invention said nucleic acid molecule comprises a nucleic acid sequence as represented in Fig 3 or Fig 9.

In a preferred embodiment of the invention there is provided a nucleic acid sequence which hybridises under stringent hybridisation conditions to the sequence presented in Fig 3 or Fig 9.

According to a further aspect of the invention there is provided a polypeptide encoded by a nucleic acid molecule as represented in Fig 3 or Fig 9.

According to a yet further aspect of the invention there is provided a vector including a DNA molecule encoding a polypeptide according to any preceding aspect or embodiment of the invention.

In a preferred embodiment of the invention said vector is an expression vector adapted for eukaryotic gene expression.

Typically said adaptation includes, the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive.

Promoter is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only, and not by way of limitation. Enhancer elements are *cis* acting nucleic acid sequences often found 5' to the transcription initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences and are therefore position independent). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to *trans* acting transcription factors (polypeptides) which have been shown to bind specifically to enhancer

elements. The binding/activity of transcription factors (please see Eukaryotic Transcription Factors, by David S Latchman, Academic Press Ltd, San Diego) is responsive to a number of environmental cues which include, by example and not by way of limitation, intermediary metabolites (eg glucose, lipids), environmental effectors (eg light, heat).

Promoter elements also include so called TATA box and RNA polymerase initiation selection (RIS) sequences which function to select a site of transcription initiation. These sequences also bind polypeptides which function, *inter alia*, to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers and autonomous replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell. Vectors which are maintained autonomously are referred to as episomal vectors.

Adaptations which facilitate the expression of vector encoded genes include the provision of transcription termination/polyadenylation sequences. This also includes the provision of internal ribosome entry sites (IRES) which function to maximise expression of vector encoded genes arranged in bicistronic or multi-cistronic expression cassettes.

These adaptations are well known in the art. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.(1994).

It will be apparent to one skilled in the art that the vectors according to the invention could be gene therapy vectors. Gene therapy vectors are typically viral based. A number of viruses are commonly used as vectors for the delivery of exogenous genes. Commonly employed vectors include recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridiae, parvoviridiae, picornoviridiae, herpesveridiae, poxviridae, adenoviridiae, or picornaviridiae. Chimeric vectors may also be employed

which exploit advantageous elements of each of the parent vector properties (See e.g., Feng, et al.(1997) Nature Biotechnology 15:866-870). Such viral vectors may be wild-type or may be modified by recombinant DNA techniques to be replication deficient, conditionally replicating or replication competent.

Preferred vectors are derived from the adenoviral, adeno-associated viral and retroviral genomes. In the most preferred practice of the invention, the vectors are derived from the human adenovirus genome. Particularly preferred vectors are derived from the human adenovirus serotypes 2 or 5. The replicative capacity of such vectors may be attenuated (to the point of being considered "replication deficient") by modifications or deletions in the E1a and/or E1b coding regions. Other modifications to the viral genome to achieve particular expression characteristics or permit repeat administration or lower immune response are preferred.

Alternatively, the viral vectors may be conditionally replicating or replication competent. Conditionally replicating viral vectors are used to achieve selective expression in particular cell types while avoiding untoward broad spectrum infection. Examples of conditionally replicating vectors are described in Pennisi, E. (1996) Science 274:342-343; Russell, and S.J. (1994) Eur. J. of Cancer 30A(8):1165-1171. Additional examples of selectively replicating vectors include those vectors wherein an gene essential for replication of the virus is under control of a promoter which is active only in a particular cell type or cell state such that in the absence of expression of such gene, the virus will not replicate. Examples of such vectors are described in Henderson, et al., United States Patent No. 5,698,443 issued December 16, 1997 and Henderson, et al., United States Patent No. 5,871,726 issued February 16, 1999 the entire teachings of which are herein incorporated by reference.

Additionally, the viral genome may be modified to include inducible promoters which achieve replication or expression only under certain conditions. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, et al. (1996) J. Virol. 70(9):6054-6059; Hwang, et al.(1997) J. Virol 71(9):7128-7131; Lee, et al. (1997) Mol. Cell. Biol. 17(9):5097-5105; and Dreher, et al.(1997) J. Biol. Chem 272(46); 29364-29371.

Vectors may also be non-viral and are available from a number of commercial sources readily available to the man-skilled in the art. For example the vectors may be plasmids which can be episomal or integrating.

In a further aspect of the invention there is provided a method to prepare a polypeptide according to the invention comprising:

(i) growing a cell transfected with a vector or nucleic acid of the present invention in conditions conducive to the manufacture of said polypeptide; and
(ii) purifying said polypeptide from said cell, or its growth environment.

In a preferred method of the invention said vector encodes, and thus said recombinant polypeptide is provided with, a secretion signal to facilitate purification of said polypeptide.

In yet a further embodiment of the invention there is provided a cell transfected with the vector or nucleic acid according to the invention.

Preferably said eukaryotic cell is selected from the group consisting of: a fungal cell eg *Saccharomyces cerevisiae, Pichia spp*; slime mold (eg *Dictyostelium spp*); insect (eg *Spodoptera frugiperda*); a plant cell; or a mammalian cell.

In a further preferred embodiment of the invention said eukaryotic cell is *Dictyostelium spp.*

According to a further aspect of the invention there is provided the use of the polypeptide according to the invention as a pharmaceutical. Preferably said polypeptide is used in a pharmaceutical composition.

When administered the polypeptide of the present invention is administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents.

The polypeptide of the invention can be administered by any conventional route, including injection. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal.

Pharmaceutical compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a composition that alone, or together with further doses, produces the desired response. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods or can be monitored according to diagnostic methods.

The doses of the polypeptide administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject (ie age, sex). When administered, the pharmaceutical compositions of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

The pharmaceutical compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such

that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may 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. Among the acceptable solvents that may be employed are water, 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 di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

According to a further aspect of the invention there is provided the use of the polypeptide according to the invention for the manufacture of a medicament for use in the treatment of a disease selected from the group consisting of: acromegaly; gigantism; diabetes mellitus, cancer; anorexia ; autoimmune and infectious disease; inflammatory disorders including rheumatoid arthritis.

In a preferred embodiment of the invention said disease is acromegaly.

In a further preferred embodiment of the invention said disease is gigantism.

In a yet further preferred embodiment of the invention said disease is cancer.

The invention also provides for a method of treating a human or animal subject comprising administering an effective amount of the polypeptide pharmaceutical composition or medicament to said subject.

The invention also provides a method of reduced renal clearance of a molecule comprising forming an agent according to any embodiment of the invention. The size of the micelles reduce renal clearance.

An embodiment of the invention will now be described by example only and with reference to the following figures wherein;

Figure 1 shows the nucleotide sequence of vector pAc6-LP-MCS-GPI used to clone the extracellular domain of the GHR for expression in Dictyostelium;

Figure 2 shows part of the multiple cloning site of pAc6-LP-MCS-GPI with the extracellular domain of the GHR cloned in and the new vector is called pAc6GHRGPI. The extracellular domain of the GHR is in capitals;

Figure 3 represents the GHRGPI fusion nucleic acid and amino acid sequence from vector pAc6GHRGPI. The nucleotide for the extracellular domain of the GHR is in capitals and the amino acid sequence is in bold;

Figure 4 illustrates screening for GHRGPI in cell lysates from Dictyostelium clones transfected with pAc6GHRGPI. Screening was performed by western blotting using a monoclonal antibody to the GHR, Mab5. Lane AX2 is a negative control of untransfected cells and lanes 1-7 different screened clones. Lanes 1, 2, 3, & 5 show a positive band at the correct size and lane 3 was selected for purification of GHRGPI;

Figure 5 illustrates immunostaining for GHRGPI and demonstrates that the protein is expressed at the Dictyostelium cell surface. The upper panels show surface staining with a negative control on the left of untransfected cells, on the right the cells transfected with pAc6GHRGPI show positive immunostaining on the cell surface. In the lower panels the cells have been permeabilised to study intracellular staining. Again the negative control is on the left and the positive control on the right shows intracellular staining;

Figure 6 Elution profile (Sephadex G100) of iodinated GH alone, and GHRGPI (purified from stable Dictyostelium clones expressing the GHRGPI identified in figure 5) with iodinated GH plus or minus cold GH. GHR-GPI forms single complex of around 60-65 Kda with 125 I-GH (blue line). The yellow line is iodinated GH alone and the pink line GHR-GPI in the presence of excess cold GH. The result shows functional binding of GH by purified GHRGPI;

Figure 7 shows a bioassay for growth hormone in the presence or absence of purified GHRGPI. Cells expressing the growth hormone receptor are transfected with a luciferase reporter activated by growth hormone signalling. The cells were then stimulated with increasing doses of growth hormone in the presence or absence of GHRGPI (approximately 10 nM). In the presence of GHRGPI the growth hormone signal was abolished at low doses of growth hormone or reduced a high doses;

Fig 8 is pCR-3GPI_Thy-1 a mammalian expression vector with mammalian GPI signal;

Figure 9 is the nucleotide and amino acid sequence of a mammalian GHRGPI anchor polypeptide in pCR-3GPI_Thy-1 and called pCR3GHRGPI. The extracellular domain of the GHR is ligated into the BamHI and EcorI site. For the amino acid sequence bold is extracellular domain GHR, bold italics underlined is GHR signal and underlined is start of the GPI signal which is cleaved;

Figure 10 FACS analysis of CHO cells transfected with vector only (thin line) and with pCR3GHRGPI thick line immunostained with GHR specific monoclonal antibody and demonstrating that almost all CHO cells are expressing a high level of GHRGPI;

Figure 11 GH activation of Stat5 (fold induction of luciferase) in HEK293 cells expressing the GH receptor (GH only) and cells transfected with GHRGPI plasmid. Demonstrates that transgenic expression of GHRGPI (extracellular domain GHR linked to GPI) completely inhibits GH signalling;

Figure 12 Measurement of soluble GH receptor (GHBP) in media from mammalian cells undoing going transfection with various vectors. The results were obtained by the GHBP-LIFA (ligand immunofunctional assay) and "<70" means below the lowest standard of the assay. The results demonstrate that expression of GHRGPI in cells results in very high levels of soluble receptor in the medium;

Figure 13 represents the amino acid and nucleotide sequence of TNF receptor type 1. Protein sequence P19438, signal in green and transmembrane helix in red. Nucleic acid sequence X55313 H.sapiens TNF-R m...[gi:37223]. 256-318 is signal and 319-888 extracellular domains;

Figure 14 represents the amino acid sequence of a TNF receptor type 1-GPI fusion polypeptide from vector pCR3TNFGPI after cloning of extracellular domain TNF receptor into the vector pCR-3GPI_Thy-1 (Figure 8). Bold is extracellular domain TNF receptor, bold italics underlined is TNF receptor signal and underlined is start of the GPI signal which is cleaved;

Figure 15 represents the amino acid sequence of the leptin receptor-GPI fusion from pCR3ObRGPI after cloning of extracellular domain leptin receptor (ObR) into the vector pCR-3GPI_Thy-1 (Figure 8). Italics ObR signal, bold ObR extracellular domain, then the link to GPI then underlined the cleavage site for GPI

Figure 16 is a table of cytokine accession numbers.

## MATERIALS AND METHODS

### Purification of extracellular domain cytokine receptor fusion with GPI from Dictyostelium and demonstration of antagonist activity.

**Transfection and Maintenance of Dictylostelium Strains**

Dictylostelium were transfected by the calcium phosphate method or electroporation and maintained in culture medium with G418 to select stable clones.

**Cloning and Expression of Chimeric Polypeptides**

The cDNA extracellular domain of the human GHR (bases 98-834, accession number X06562) was ligated into a vector (pAc6-LP-MCS-GPI) containing the *Dictyostelium* actin 6 gene promoter, a *Dictyostelium* signal peptide coding region, multiple cloning site and the signal (PSPTPTETAT PSPTPKPTST PEETEAPSSA TTLISPLSLI VIFISFVLLI )for a GPI anchor. The resultant vector (pAc6-GHR-GPI) was transfected into *Dictyostelium* cells.

*Dictyostelium* adds N and O-glycosylations. Clones expressing GHR-GPI were then selected by immunohistochemistry and western blotting using an anti-GHR antibody Mab5. GHR-GPI was purified from cell lysates using a GH affinity column. Purified GHR-GPI was demonstrated to bind GH and in a bioassay for GH signalling GHR-GPI acted as an antagonist inhibiting signalling.

The vector pAc6-LP-MCS-GPI (Fig 1) was digested with BamHI and EcoRI. Extracellular domain GHR from base 98-834 (accession X06562) was amplified by PCR with BamHI and EcoRI restriction sites 5' and 3' respectively and then ligated into the MCS of pAc6-LP-MCS-GPI to give the following MCS: cag gat cca ttt...GHR 98-834..... tac cga att cca (Fig 2). The encoded GHR protein is from the first residue after the mammalian signal peptide to the last residue before the transmembrane domain. The resultant vector was called pAc6-GHR-GPI.

**Purification of GHR-GPI**

The cDNA for pAc6-GHR-GPI was transfected into *Dictyostelium* cells and clones were then selected and screened by western blotting using Mab5, a monoclonal antibody to the extracellular domain of the human GHR. Five clones gave a clear signal at 40kDa, the

expected size of glycosylated GHR-GPI (Fig 4). Immunostaining of one clone clearly identified GHR-GPI on the cell surface (Fig 5). Cell lysate in 0.1% Triton X100 were then purified on a GH affinity column in PBS/0.1% Triton X100 (Na-phosphate 0.01 M, NaCl 0.15M, 0.02% NaNO3, 0.1% Triton X-100 Ph 7.4). The approximate concentration of GHR-GPI was 120 ug/ml or 3μM. Functional activity of the purified GHRGPI was confirmed by binding of iodinated GH (Fig 6).

***GH bioassay***

An established bioassay was used to screen for antagonist activity (Ross *et al.*, 1997). A permanent cell line expressing the full length GHR was transiently transfected with a luciferase reporter that binds activated Stat5. Twenty-four hours later the cells are stimulated with GH for 6 hours with or without antagonist. The cells are then lysed and luciferase activity measured. The bioassay was performed in the presence or absence of GHR-GPI at a concentration of approximately 10nM (Fig 7). The GHR-GPI completely blocked signalling at low concentration of GH and caused an approximate 50% reduction in signalling at higher concentrations of GH confirming the antagonistic action of GHR-GPI on GH signalling.

**Cloning of extracellular domain cytokine receptor GPI fusion into mammalian expression vector and demonstration of antagonist activity when expressed in cytokine responsive cell line.**

**GHR-GPI can act as a transgenic therapy.**

To demonstrate that GHR-GPI can act as a transgenic therapy we have cloned the extracellular domain of the GHR upstream of a human GPI signal sequence in a mammalian expression vector (Figs 8-9). This construct was then transfected into CHO cells and expression demonstrated by FACS analysis using a monoclonal antibody specific for the human GHR (Fig 10). The level of binding on CHO cells was extremely high. To test whether the GHR-GPI expression on the cell surface was acting as antagonist the GHR-GPI was transfected into the Hek293 cells expressing the wild-type GHR and stimulated with GH. This co-expression of GHR-GPI completely blocked GH signalling (Fig 11). In addition the media from cells transfected with GHR-GPI was measured for soluble receptor (GHBP) and high levels were found (Fig 12) providing another mechanism by which the transgenic expression

of cytokine GPI fusions could act as antagonists. Other examples of cytokine receptors that could be expressed as GPI fusions are the TNF and leptin receptors (Figs 13-16).

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## CLAIMS

1. A chimeric polypeptide comprising:

i) an extracellular cytokine binding domain of a cytokine receptor; and

ii) a domain which includes a signal sequence for the attachment of glycosylphosphatidylinositol.

2. A chimeric polypeptide comprising:

(i) an extracellular cytokine binding domain of a cytokine receptor; and

(ii) a domain which includes a signal sequence for the attachment of glycosylphosphatidylinositol, for use as a pharmaceutical.

3. A polypeptide according to claim 1 or 2 wherein the polypeptide is modified by the addition of glycosylphosphatidylinositol.

4. A polypeptide according to any of claims 1-3 wherein the signal sequence for the attachment of glycosylphosphatidylinositol is selected from the group consisting of:

PSPTPTETAT PSPTPKPTST PEETEAPSSA TTLISPLSLI VIFISFVLLI;
DKLVKCGGIS LLVQNTSWML LLLLSLSLLQ ALDFISL;
LVPRGSIEGR GTSITAYNSE GESAEFFFLL ILLLLLVLV;
TSITAYKSE GESAEFFFLL ILLLLLVLV.

5. A polypeptide according to any of claims 1-4 wherein the binding domain of the cytokine receptor is derived from the receptors selected from the group consisting of: growth hormone (GH); leptin; erythropoietin; prolactin; TNF, interleukins (IL), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, the p35 subunit of IL-12, IL-13, IL-15; granulocyte colony stimulating factor (G-CSF); granulocyte macrophage colony stimulaing factor (GM-CSF); ciliary neurotrophic factor (CNTF); cardiotrophin-1 (CT-1); leukemia inhibitory factor (LIF); oncostatin M (OSM); interferon, IFN$\alpha$ and IFN$\gamma$.

6. A polypeptide according to claim 5 wherein the ligand binding domain of the cytokine receptor is derived from the growth hormone receptor.

7. A polypeptide according to any of claims 1-6 wherein the polypeptide is a fusion protein.

8. A polypeptide according to any of claims 1-7 wherein the polypeptide has been modified by addition, deletion or substitution of at least one amino acid residue or by the use of modified amino acids to provide a sequence variant.

9. An isolated nucleic acid molecule wherein said molecule encodes a polypeptide according to any of claims 1-8.

10. A nucleic acid molecule according to claim 9 comprising a nucleic acid sequence as represented in Fig 3 or Fig 9.

11. A nucleic acid sequence according to claim 10 which hybridises under stringent hybridisation conditions to the sequence presented in Fig 3 or Fig 9.

12. A polypeptide encoded by a nucleic acid molecule of claim 10 or 11.

13. A vector comprising a nucleic acid molecule according to any of claims 9-11.

14. A vector according to claim 13 wherein said vector is an expression vector adapted for eukaryotic gene expression.

15. A vector according to claim 13 or 14 wherein said vector encodes, and thus said recombinant polypeptide is provided with, a secretion signal to facilitate purification of said polypeptide.

16. A method to prepare a polypeptide according to claim 1 or 12 comprising:

(i) growing a cell transfected with a nucleic acid of any of claims 9-11, or a vector of any of claims 13-15 in conditions conducive to the manufacture of said polypeptide; and

(ii) purifying said polypeptide from said cell, or its growth environment.

17. A method to prepare and purify a polypeptide according to claim 1 or 12 wherein said purified polypeptide does not include a GPI anchor, comprising the steps of:

(i) growing a cell transfected with a nucleic acid of any of claims 9-11, or a vector of any of claims 13-15 in conditions conducive to the manufacture of said polypeptide; and

(ii) purifying said polypeptide from its growth environment.

18. A eukaryotic cell transfected with a nucleic acid according to any of claims 9-11, or a vector according to any Claims 13-15.

19. A eukaryotic cell according to claim 18 selected from the group consisting of: fungal, slime mold, insect, plant or mammalian cell.

20. A cell according to claim 19 wherein said cell is a slime mold cell and is *Dictyostelium spp.*

21. A pharmaceutical composition comprising a polypeptide according to any of claims 1-8 or 12 and optionally, a pharmaceutically-acceptable carrier.

22. The use of a polypeptide according to any of claims 1-8 or 12 for the manufacture of a medicament for the treatment of a disease or condition selected from the group consisting of: acromegaly; gigantism; diabetes mellitus, cancer; anorexia; autoimmune and infectious disease; inflammatory disorders including rheumatoid arthritis.

23. The use of a polypeptide according to any of claims 1-8 or 12 for the manufacture of a medicament for the treatment of a disease or condition in a human or animal subject

24. The use of an effective amount of nucleic acid according to any of claims 9-11 and/or a vector according to any of claims 13-15 for the manufacture of a medicament for treating a disease or condition in a human or animal subject.

25. Use according to claim 24 wherein the disease is selected from the group consisting of: acromegaly; gigantism; diabetes mellitus, cancer; anorexia; autoimmune and infectious disease; inflammatory disorders including rheumatoid arthritis.

26. The use of an agent comprising a polypeptide according to any of claims 1-8 or 12 for the manufacture of a medicament for reducing renal clearance of a molecule.

27. A micelle comprising a plurality of polypeptides according to any of claims 1-8 or 12.