

Modified Polypeptide

5 The invention relates to chimeric polypeptides wherein said polypeptides comprise a modified binding domain of growth hormone linked to a receptor binding domain of growth hormone receptor; and tandems/oligomers of said modified growth hormone binding domains.

10 GH is a member of a large family of hormones involved in the regulation of mammalian growth and development. Human GH is a 22kDa polypeptide which is involved in a number of biological processes. For example, cell growth, lactation, the activation of macrophages and the regulation of energy metabolism. GH interacts sequentially with two membrane bound GHR's via two separate sites on GH referred as site 1 and site 2. Site 1 is a high affinity binding site and site 2 a low affinity site.
15 A single GH molecule binds 1 GHR via site 1. A second GHR is then recruited via site 2 to form a GHR:GH:GHR complex. The complex is then internalised and activates a signal transduction cascade leading to changes in gene expression.

20 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 (b) being closest to the cell surface and the N-terminal SD-100 domain (a) 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.

25 Modified GH's are disclosed in US 5, 849, 535 which is incorporated by reference. The modification to GH is at both site 1 and site 2 binding sites. The modifications to site 1 produce a GH molecule which has a higher affinity for GHR compared to wild-type GH. These modified GH molecules act agonists. There is also disclosure of site 2 modifications which result in the creation of GH antagonists. Further
30 examples of modifications to GH which alter the binding affinity of GH for site 1 are disclosed in US 5,854,026; US 6,004,931; US6,022,711; US6,057,292; and

US6136563 each of which are incorporated by reference. A summary of the modifications made to site 1 is provided in Table 1. Modifications to site 2 are also disclosed, in particular amino acid residue G120 which when modified to either arginine, lysine, tryptophan, tyrosine, phenylalanine, or glutamic acid creates a GH molecule with antagonistic properties.

In addition, the modified GH is coated in polyethylene glycol (PEG) by a process known as "pegylation" this has several beneficial effects. Firstly, the PEG coat increases the effective molecular weight of GH from 22kD to approximately 40kD. The effect this has is to decrease glomerular filtration of GH thereby increasing the half-life of GH *in vivo* which reduces the dose administered to produce the desired effect. In addition pegylation is thought to reduce both the immunogenicity and toxicity of proteins which are treated in this way, see Abuchowski *et al* J Biol Chem., 252, 3578-3581, (1977).

However, a consequence of pegylation is to reduce the affinity of the modified GH molecule for GHR. This means that an increased dose is required to counter the reduced affinity. This is undesirable since it counteracts the advantageous effect of pegylation with respect to increasing the half life of modified GH. It would be desirable to provide a modified GH molecule which does not require pegylation but has an increased half-life and also has the added benefits of reduced immunogenicity and lacks toxicity.

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

- i) at least one modified binding domain of growth hormone wherein said modification is the addition, deletion or substitution of at least one amino acid residue; and
- ii) a growth hormone binding domain of a growth hormone receptor.

In a preferred embodiment of the invention said polypeptide is modified in the site 1 binding domain of growth hormone.

In a further preferred embodiment of the invention said polypeptide is modified in the
5 site 2 binding domain of growth hormone.

In a yet further preferred embodiment of the invention said polypeptide is modified at both site 1 and site 2 of growth hormone.

10 As previously described, site 1 mutations are known in the art which increase the affinity of growth hormone for its binding domain on growth hormone receptor. Such modified growth hormone acts as an agonist. If a site 1 modification is combined with a site 2 modification wherein the latter modification results in an inactive or partially active site 2 binding site then such a molecule is an antagonist.
15 A modification just to site 2 which exploits a wild-type site 1 binding site also creates an antagonist.

In a further preferred embodiment of the invention there is provided a polypeptide comprising a site 1 binding domain which has been modified by amino acid
20 substitution wherein said modification is selected from the group consisting of: histidine 18 with alanine or aspartic acid; and/or histidine 21 with asparagine; and/or glutamine 22 with alanine; and/or phenylalanine 25 with alanine; and/or aspartic acid 26 with alanine; and/or glutamine 29 with alanine; and/or glutamic acid 167 with alanine; and/or aspartic acid 171 with serine; and/or lysine 172 with serine or alanine;
25 and/or isoleucine 179 with tyrosine, of the sequence represented in Figure 13.

Preferably said modification to increase the affinity of site 1 for its binding domain in GHR consists of the amino acid substitutions: histidine 18 aspartic acid; histidine 21 asparagine; arginine 167 asparagine; aspartic acid 171 arginine; glutamic acid 174
30 serine; and isoleucine 179 threonine; as represented by the GH amino acid sequence in Figure 13.

In a further preferred embodiment of the invention said modification to increase the affinity of site 1 for its binding domain in GHR consists of the amino acid substitutions: histidine 18 alanine; glutamine 22 alanine; phenylalanine 25 alanine; 5 aspartic acid 26 alanine; glutamine 29 alanine; glutamic acid 65 alanine; lysine 168 alanine; and glutamic acid 174 alanine; as represented by the GH amino acid sequence in Figure 13.

10 In a further preferred embodiment of the invention said site 2 modification is to amino acid residue 120 of the sequence presented in Figure 13. Preferably said site 2 modification is combined with site 1 modifications as herein disclosed. Alternatively, GH is modified only at amino acid residue glycine 120.

15 In a preferred embodiment of the invention said site 2 modification is a substitution of glycine for an amino acid selected from the group consisting of: arginine; alanine; lysine; tryptophan; tyrosine; phenylalanine; and glutamic acid. Preferably said substitution is glycine 120 for arginine or lysine or alanine.

20 In a further preferred embodiment of the invention the growth hormone binding domain of GHR is the extracellular domain of GHR. More preferably the binding domain is the C-terminal SD-100 domain of GH.

Alternatively said binding domain is the full length GHR.

25 In a preferred embodiment of the invention said chimeric polypeptide is a fusion protein wherein the modified GH is an inframe translational fusion with GHR, or part thereof. Preferably, said fusion polypeptide comprises modified GH and the C-terminal SD-100 domain of GHR.

In an alternative further preferred embodiment of the invention, the modified binding domain of GH is linked by a linker to the GH binding domain of GHR. The linker may be flexible.

- 5 The linker could be at any residue within the extracellular domain of the receptor which would allow the modified GH to flexibly bind with the free receptor at the cell surface. Preferably the linkage is made between a residue close to the C-terminus of the modified GH molecule and a residue close to the N-terminus of GHR. More preferably the linkage is made between a residue close to the C-terminus of modified
- 10 GH molecule and a residue close to the N-terminal of the N-terminal of the C-terminal SD-100. More preferably the linkage is made at any of residues 126-128 of the N-terminus of the C-terminal SD-100 of the GHR. In one embodiment of the invention, the linkage is made at residue 127 of the N-terminus of the C-terminal SD-100. Preferably the linker is a peptide.

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The crystal structure of the GHR:GH:GHR complex reveals that the distance between the C-terminus of GH (residue 191) and N-terminus of the C-terminal SD-100 (residue 126-128) is 10A. This provides invaluable information with respect to linker design.

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Preferably the linker is a polypeptide which comprises 5 to 30 amino acid residues. More preferably the linker comprises 10 to 20 amino acid residues. More preferably still the linker comprises at least one copy of the peptide:

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Gly Gly Gly Gly Ser (hereinafter referred to as "Gly4Ser").

- In one embodiment of the invention the linker is 10 amino acids in length and comprises two copies of the Gly4Ser linker. In an alternative embodiment of the invention, the linker is 15 amino acids in length and comprises three copies of the
- 30 Gly4Ser linker. In yet an alternative embodiment, the linker is 20 amino acids in length and comprises four copies of the Gly4Ser linker.

In a preferred embodiment of the invention said polypeptide is derived from human GH and human GHR.

5 According to a further aspect of the invention there is provided a nucleic acid molecule which encodes a polypeptide according to the invention selected from the group consisting of:

- 10 i) a nucleic acid molecule as represented by the nucleic acid sequence in Figure 13; and
- ii) a nucleic acid molecule which hybridises to the nucleic acid sequence in (i).

Nucleic acid molecules which encode a modified growth hormone according to the invention can typically be synthesized by molecular techniques known in the art and
15 include recombinant methods as well as the synthesis of nucleic acid molecules using oligonucleotide synthesizers.

In a preferred embodiment of the invention said nucleic acid molecule hybridises under stringent hybridisation.

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The term "stringent hybridisation conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be 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
25 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. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH₂PO₄(pH7), 0.5% SDS,
30 2mM EDTA). SSC is 0.15M sodium chloride/0.015M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After

hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 - 0.5 X SSC/0.1 x SDS at temperatures up to 68°C.

- 5 According to a further aspect of the invention there is provided a vector comprising the nucleic acid molecule according to the invention.

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

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Typically said adaptation includes, by example and not by way of limitation, 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.

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- 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 is 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 factor 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 and/or environmental effectors.
- 20
- 25

- Promoter elements also include so called TATA box and RNA polymerase initiation selection (RIS) sequences which function to select a site of transcription initiation.
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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
5 replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors which are maintained autonomously are referred to as episomal vectors. Episomal vectors are desirable since these molecules can incorporate large DNA fragments (30-50kb DNA). Episomal vectors of this type are described in WO98/07876 which is incorporated by reference.

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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
15 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
20 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).

25 According to a further aspect of the invention there is provided the use of the polypeptide according to the invention as a pharmaceutical. In a preferred embodiment of the invention said polypeptide is for use in the manufacture of a medicament for the treatment of a condition selected from the group consisting of: gigantism, acromegaly; cancer (e.g. Wilm's tumour, osteogenic sarcoma, breast,
30 colon, prostate, thyroid); diabetic retinopathy; diabetic nephropathy and other complications of diabetes and GH excess.

The polypeptides and compositions of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, 5 intracavity, intraocular, subcutaneous, or transdermal. 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.

When administered, the pharmaceutical preparations of the invention are applied in 10 pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents.

15 The compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" 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 20 inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. 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; 25 chlorobutanol; parabens and thimerosal.

According to a yet further aspect of the invention there is provided a cell transformed or transfected with the nucleic acid or vector according to the invention.

30 In a preferred embodiment of the invention said cell is a eukaryotic cell. Preferably said cell is selected from the group consisting of: a slime mould (e.g. *Dictyostelium*

spp) a yeast cell (e.g. *Saccharomyces cerevisiae*; *Pichia spp*); a mammalian cell (e.g. Chinese Hamster Ovary); a plant cell; an insect cell (e.g. *Spodoptera spp*).

In an alternative preferred embodiment said cell is a prokaryotic cell, preferably
5 *Escherchia coli* or *Bacillus spp*.

According to a further aspect of the invention there is provided a method to manufacture the polypeptide according to the invention comprising:

- 10 i) providing a cell according to the invention;
- ii) incubating said cell under conditions conducive to the production of the polypeptide according to the invention; and optionally
- iii) isolating the polypeptide from the cell or the cell culture medium.

15 In a preferred method of the invention said polypeptide is provided with a secretion signal to facilitate the purification of the polypeptide from said cell. More preferably still said polypeptide is provided with an affinity tag to facilitate the purification of the polypeptide from said cell or the cell culture medium.

20 According to a yet further aspect of the invention there is provided a method of treatment of a mammal, preferably a human, comprising administering to said mammal the polypeptide according to the invention.

According to a further aspect of the invention there is provided a chimeric
25 polypeptide comprising more than two modified growth hormone binding domains wherein said modification is the addition, deletion or substitution of at least one amino acid residue.

In a preferred embodiment of the invention there is provided a chimeric polypeptide
30 comprising a plurality of modified growth hormone binding domains.

In a further preferred embodiment of the invention there is provided a chimeric polypeptide comprising at least two modified site 2 growth hormone binding domains.

- 5 In a further preferred embodiment of the invention there is provided a chimeric polypeptide comprising 3, 4, 5, 6, 7, 8, 9, 10 modified site 2 growth hormone binding domains.

- 10 In a yet further preferred embodiment of the invention said chimeric polypeptide comprises more than two modified growth hormone binding domains linked together by a linker molecule. Preferably said linker molecule is as hereinbefore disclosed.

- 15 According to a yet further aspect of the invention said chimeric polypeptide comprising more than two modified growth hormone binding domains further comprises at least one growth hormone binding domain of a growth hormone receptor.

- 20 Preferably said chimeric polypeptide consists of two modified growth hormone binding domains and one growth hormone binding domain of a growth hormone receptor.

Preferably said chimeric polypeptide consists of at least two modified site 2 growth hormone binding domains.

- 25 Aspects and embodiments which relate to a chimeric polypeptide comprising growth a hormone binding domain linked to a receptor binding domain are applicable to chimeric polypeptides comprising more than or a plurality of growth hormone binding domains. For example, vectors comprising nucleic acids encoding said chimeric polypeptides, pharmaceutical compositions comprising said polypeptides,
30 cell-lines expressing said chimeric polypeptides, methods to manufacture said

polypeptides and methods of treatment utilising said polypeptides are all within the scope of the invention with respect to this species of chimeric polypeptide.

An embodiment of the invention will now be described by example only and with
5 reference to the following table and figures:

Table 1 represents a summary of the amino acid substitutions to site 1 and site 2 of human GH;

10 Figure 1 Plasmid map of pHEAT.GH.G120R, which was generated by ligating the GH.G120R gene, synthesised by PCR, between the *Bam*HI and *Not*I restriction sites. The selective marker on the plasmid is Amp^R;

Figure 2 Plasmid map of pTrcHis-TOPO. 1A7, which was generated by ligating the
15 GH.G120R gene between the *Bam*HI and *Not*I sites in pTrcHis 1A1. The linker is (G₄S)₄, and the selective marker on the plasmid is Amp^R;

Figure 3 Plasmid map of pTrcHis-TOPO. 1B2, which was generated by ligating the
20 GH.G120R gene between the *Bam*HI and *Not*I sites in pTrcHis 1B1. The linker is (G₄S)₄, and the selective marker on the plasmid is Amp^R;

Figure 4 Plasmid map of pTrcHis-TOPO. 1C3, which was generated by ligating the
GH.G120R gene between the *Eco*RI and *Hin*DIII sites in pTrcHis 1A7. The linker is (G₄S)₄, and the selective marker on the plasmid is Amp^R;

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Figure 5. Sequence of the GH.G120R gene, showing the start codon, 6xHis tag, relevant restriction sites, stop codons and the G120R mutation (CGC). The actual GH.G120R component is shown in CAPITALS, and the sequenced regions are shown in **bold**

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Figure 6. Sequence of the 1A7 gene, showing the start codon, 6xHis tag, relevant restriction sites, stop codons and the G120R mutation (CGC). The actual GH.G120R-(G₄S)₄-GHR(b) component is shown in CAPITALS, and the sequenced regions are shown in **bold**;

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Figure 7. Sequence of the 1B2 gene, showing the start codon, 6xHis tag, relevant restriction sites, stop codons and the G120R mutation (CGC). The actual GH.G120R-(G₄S)₄-GHR(flec) component is shown in CAPITALS, and the sequenced regions are shown in **bold**;

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Figure 8. Sequence of the 1C3 gene, showing the start codon, 6xHis tag, relevant restriction sites, stop codons and the G120R mutation (CGC). The actual GH.G120R-(G₄S)₄-GH.G120R component is shown in CAPITALS, and the sequenced regions are shown in **bold**;

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Figure 9. Western blots using anti-human GH as the primary antibody of 15% SDS PAGE gels for the expression studies of GH.G120R, 1A7, 1B2 and 1C3. Expression was from the pTrcHis vector in *E. coli* XL1 Blue or *E. coli* SURE cells, these samples were taken 4 hours after induction with 1mM (final concentration) IPTG. The blots show that GH.G120R and 1C3 produce single bands, while the samples of 1A7 and 1B2 contain cleavage products;

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Figure 10. Coomassie stained [C] 15% SDS PAGE gels of purified GH.G120R, 1A7 and 1C3. Western blots [W] of these samples using anti-human GH as the primary antibody are also shown. The coomassie stained gels show that the purified protein samples are >95% pure, however the western blots show that only GH.G120R and 1C3 produce single bands, while the sample of 1A7 contain cleavage products;

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Figure 11 Graphs showing the results of the GH bioassay from GH.G120R, 1A7 and 1C3. Each graph shows a standard curve, the assay with the construct alone at

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different concentrations and the assay with the construct at different concentrations with 25ng/ml hGH. These show that none of the proteins have inherent agonistic activity, but all have antagonistic activity with the GH.G120R being the most active, and 1A7 the least;

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Figure 12 is the amino acid sequence of unmodified GH;

Figure 13 is the nucleic acid sequence of unmodified GH.

10 **Materials and Methods**

The methods to generate modified GH at site 1 and/or site 2 are disclosed in US 5, 849, 535; US 5,854,026; US 6,004,931; US6,022,711; US6,057,292; and US6136563 each of which is incorporated by reference.

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DNA constructs

GENERATION OF SITE 2 MUTATED GH ANTAGONIST (GHa)

20 The cDNA for human GH (Fig 1) has been PCR amplified from human pituitary tissue and cloned into the vector pTrcHis-Topo (pTrcHis-TOPO-GHstop). The GHR extracellular domain was amplified from human liver cDNA using PCR.

Growth Hormone Antagonist (G120R) Constructs

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G120R Mutation of Growth Hormone

The growth hormone (GH) gene was mutated using the phagemid ssDNA mutation method. The GH gene was first sub-cloned from pTrcHisGH into pT7T318 between
30 *Bam*HI and *Hind*III sites, to produce pT7T318-GH. This plasmid was then transformed into *E. coli* CJ236 and single stranded ssDNA produced.

The ssDNA pT7T318-GH was then mutated by changing the codon for Gly120 from GGC to CGC, the primer GH.(G120R)For was used (Table 1).

5 The dsDNA pT7T318-GH.G120R produced after the mutation process was then used to sub-clone the GH.G120R into a pHEAT vector, this gave pHEAT.GH.G120R (Fig.1).

Generation of GH.G120R Constructs

10 χ 1A7 [GH.G120R-(G₄S)₄-GHR(b)] = GHa linked to b domain of GHR.

The GH.G120R gene was excised from pHEAT.GH.G120R (Fig. 1) using the restriction sites *Bam*HI and *Not*I. The gene was then ligated in place of the GH gene in pTrcHis χ 1A1 [GH-(G₄S)₄-GHR(b)] (Fig. 2). The resulting plasmid was transformed into *Escherichia coli* XL1 Blue and plated on LB (0.3% glucose, 15 50 μ g/ml ampicillin, 12.5 μ g/ml tetracycline) agar plates.

χ 1B2 [GH.G120R-(G₄S)₄-GHRflec] = GHa linked to full length extracellular domain of the GHR.

The strategy used to generate the χ 1A7 gene was repeated, however the 20 recipient vector was pTrcHis χ 1B1 [GH-(G₄S)₄-GHRflec] (Fig. 3). The resulting plasmid was transformed into *E. coli* XL1 Blue and plated on LB (0.3% glucose, 50 μ g/ml ampicillin, 12.5 μ g/ml tetracycline) agar plates.

χ 1C3 [GH.G120R-(G₄S)₄-GH.G120R] = GHa tandem.

25 A PCR reaction was performed on pTrcHisGH using the primers DiGHEcoF1 and DiGHHinR1 (Table 1). The PCR product was then digested with *Eco*RI and *Hind*III, this was then ligated in place of the GHR(b) domain in pTrcHis χ 1A1 [GH-(G₄S)₄-GHR(b)] (Fig. 4). The resulting plasmid was transformed into the recombinant deficient *E. coli* SURE and plated on LB (0.3% glucose, 50 μ g/ml 30 ampicillin, 12.5 μ g/ml tetracycline, 50 μ g/ml kanamycin) agar plates.

Sequencing Results

Plasmids containing the construct genes were sequenced. The sequences of the genes and the regions sequenced for GH.G120R, χ 1A7, χ 1B2 and χ 1C3 are shown in Figs. 5-8, respectively.

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Expression Studies

Single colonies were used to inoculate 3ml LB (0.3% glucose, 50 μ g/ml ampicillin, 12.5 μ g/ml tetracycline) broth for *E. coli* XL1 Blue cells and LB (0.3% glucose, 10 50 μ g/ml ampicillin, 12.5 μ g/ml tetracycline, 50 μ g/ml kanamycin) broth for *E. coli* SURE cells. These were grown, shaking, overnight at 37°C.

4mls of 4YT media, containing the appropriate antibiotics, were then inoculated with 200 μ l of the overnight LB culture. These were grown for 3 hours, 1ml samples were 15 then taken (T_0 samples).

The 4YT cultures were then induced with IPTG to a final concentration of 1mM and then further incubated for another 4 hours (T_4 samples).

20 The T_0 and T_4 samples were processed immediately after they had been taken. They were first centrifuged to pellet the cells, the supernatant was then discarded and the pellet processed for running on a SDS PAGE gel. Protein was visualised on these PAGE gels by either Coomassie staining or by western blot using an anti-GH primary antibody to probe for the construct.

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In all cases the Coomassie stained PAGE gels do not show over-expression of the construct. However, the constructs are observed on the western blots (Fig. 9). These show that in all cases protein of the correct size is expressed.

30 **Purification**

In general protein was purified from 4 x 250ml cultures grown in 4YT, containing the appropriate antibiotics, and induced for 4-5 hours with IPTG to a final

concentration of 1mM. The cells were harvested by centrifugation and lysed by treatment with lysozyme and sodium deoxycholate followed by sonication.

The lysed cells were centrifuged to remove cell debris and the supernatant initially
5 purified using Invitrogen ProBond Resin (Ni-column). Protein was eluted using 5ml 0.5M imidazole.

The protein sample was further purified by diluting the eluant from the Ni-column 10 times in a suitable buffer and then passing it through a MonoQ ion-exchange column.

10 Protein was eluted using a salt gradient of 0-1M NaCl over 20ml at a rate of 0.5ml/min; 0.5ml fractions were collected. The fractions were then analysed for the presence of the construct, and the fractions containing the construct pooled.

The purified protein was analysed by SDS PAGE (Coomassie staining and western
15 blot) (Fig. 10) and assayed to measure its concentration. The protein was then submitted for the bioassay.

In the cases of χ 1A7 and χ 1B2, which showed cleaved products by western blot, the constructs were submitted to the Rapid Translation System (RTS) for *in vitro*
20 transcription. Previous, studies on χ 1A1 and χ 1B1 have shown that cleavage was greatly reduced using the RTS system in conjunction with protease inhibitors and chaperones for expression.

Bioassay

25 The purified constructs were submitted to the Asterion standard GH bioassay. Prepared 293 Hi, which stably express growth hormone receptor, were stimulated with the construct using a range of doses. A second duplicate plate was also prepared, but 25ng/ml GH was added 30min. after adding the construct to observe the
30 antagonistic capability of the construct.

All the GH.G120R constructs had antagonistic activities (Fig. 11).

Screening of Antagonist Activity

An established bioassay is used to screen for antagonist activity (9). A permanent cell
 5 line expressing the full length GHR is transiently transfected with a luciferase
 reporter that binds activated Stat5 (9). 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 (9).

10 Testing metabolic clearance rate in vivo

Sprague-Dawley rats are anaesthetised and cannulae implanted in femoral and jugular
 veins. Two days later GH chimera or tandem is administered by intravenous or
 subcutaneous injection. Blood samples are collected via the femoral cannula and
 15 chimera and tandem or oligomer protein levels measured by radio-immunoassay.
 Pharmacokinetic parameters are estimated using available computer programs fitting
 hormone concentration against time.

Table 1 represents a summary of amino acid substitutions made to site 1 of GH.
 20 Modifications to site 2 include the substitution of G120 for any of arginine; alanine;
 lysine; tryptophan; tyrosine; phenylalanine; or glutamic acid.

H18	H2 1	Q22	F25	D26	Q29	E65	R167	K168	D171	K172	E174	I179
D	N						N	A	S	R	S	T
A		A	A	A	A	A		A			A	
A		A	A	A	A	A					A	
D		A	A	A	A	A		A			S	
A		A	A	A	A	A		A			S	
D		A	A	A	A	A		A			A	
A		A	A	A	A	A		A			A	
D	N						N	A	S	R	S	T
A		A	A	A	A	A		A			A	

CLAIMS

1. A chimeric polypeptide comprising:
 - 5 i) at least one modified binding domain of growth hormone wherein said modification is the addition, deletion or substitution of at least one amino acid residue; and
 - ii) a ligand binding domain of growth hormone receptor.
- 10 2. A polypeptide according to Claim 1 wherein said binding domain is modified in site 1 of growth hormone.
3. A polypeptide according to Claim 1 or wherein said binding domain is modified in site 2 of growth hormone.
- 15 4. A polypeptide according to Claim 1 wherein said modification is to both site 1 and site 2 of growth hormone.
5. A polypeptide according to Claim 1 or 2 or 4 wherein said modification is
20 selected from the group consisting of: histidine 18 with alanine or aspartic acid; and/or histidine 21 with asparagine; and/or glutamine 22 with alanine; and/or phenylalanine 25 with alanine; and/or aspartic acid 26 with alanine; and/or glutamine 29 with alanine; and/or glutamic acid 167 with alanine; and/or aspartic acid 171 with serine; and/or lysine 172 with serine or alanine; and/or isoleucine 179
25 with tyrosine as represented in Figure 13.
- 6 A polypeptide according to Claim 5 wherein said modification consists of the amino acid substitutions: histidine 18 aspartic acid; histidine 21 asparagine; arginine 167 asparagine; aspartic acid 171 arginine; glutamic acid 174 serine; and isoleucine
30 179 threonine.

7. A polypeptide according to Claim 5 wherein said modification consists of the amino acid substitutions: histidine 18 alanine; glutamine 22 alanine; phenylalanine 25 alanine; aspartic acid 26 alanine; glutamine 29 alanine; glutamic acid 65 alanine; lysine 168 alanine; and glutamic acid 174 alanine.
- 5 8. A polypeptide according to Claim 3 wherein said site 2 modification is to amino acid residue glycine 120 of the sequence represented in Figure 12.
9. A polypeptide according to Claim 8 wherein said site 2 modification is a
10 substitution of glycine for an amino acid selected from the group consisting of: arginine; alanine; lysine; tryptophan; tyrosine; phenylalanine; and glutamic acid.
10. A polypeptide according to Claim 9 wherein said site 2 substitution is glycine 120 for arginine or lysine or alanine.
- 15 11. A polypeptide according to any of Claims 1-10 wherein the growth hormone binding domain of GHR is the extracellular domain of GHR.
12. A polypeptide according to Claim 11 wherein the extracellular domain of
20 GHR is the C-terminal SD-100 domain of GHR.
13. A polypeptide according to any of Claims 1-12 wherein said polypeptide is a fusion protein.
- 25 14. A polypeptide according to Claim 13 wherein said fusion polypeptide comprises modified GH according to any of Claims 1-10 and the C-terminal SD-100 domain of GHR.
15. A polypeptide according to any of Claims 1-14 wherein, the modified binding
30 domain of GH is linked by a linker to the GH binding domain of GHR.

16. A polypeptide according to Claim 15 wherein the linker is a polypeptide which comprises 5 to 30 amino acid residues.
17. A polypeptide according to Claim 16 wherein the linker comprises 10 to 20 amino acid residues.
18. A polypeptide according to Claim 16 or 17 wherein the linker comprises at least one copy of the peptide Gly Gly Gly Gly Ser.
19. A nucleic acid molecule which encodes a polypeptide according to any of Claims 1-18 selected from the group comprising:
- i) a nucleic acid molecule as represented by the nucleic acid sequence in Figure 13; and
 - iii) a nucleic acid molecule which hybridises to the nucleic acid sequence in (i).
20. A nucleic acid molecule according to Claim 19 which hybridises under stringent hybridisation conditions to the nucleic acid sequence in Figure 13.
21. A nucleic acid molecule according to Claim 19 or 20 consisting of the DNA in Figure 13.
22. A vector comprising the nucleic acid molecule according to any of Claims 19-21.
23. A vector according to Claim 22 wherein said vector is an expression vector adapted for recombinant expression.
24. Use of the polypeptide according to any of Claims 1-18 as a pharmaceutical.
25. Use of the polypeptide according to any of Claims 1-18 for the manufacture

of a medicament for the treatment of a condition selected from the group consisting of: gigantism; acromegaly; cancer (e.g. Wilm's tumour, osteogenic sarcoma, breast, colon, prostate, thyroid); diabetic retinopathy; diabetic nephropathy, diabetic complications and any disease of GH excess.

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26. Use of the polypeptide according to Claim 25 for the treatment of acromegaly.

27. A pharmaceutical composition comprising a polypeptide according to any of
10 Claims 1-18.

28. A cell transformed or transfected with the nucleic acid or vector according to any of Claims 19-23.

15 29. A cell according to Claim 28 wherein said cell is a eukaryotic cell selected from the group consisting of: a slime mould (e.g. *Dictyostelium spp*) a yeast cell (e.g. *Saccharomyces cerevisiae*; *Pichia spp*); a mammalian cell (e.g. Chinese Hamster Ovary); a plant cell; an insect cell (e.g. *Spodoptera spp*).

20 30. A cell according to Claim 28 wherein said cell is a prokaryotic cell.

31. A method to manufacture a polypeptide according to any of Claims 1-18 comprising:

- i) providing a cell according to any of Claims 28-30;
- 25 ii) incubating said cell under conditions conducive to the production of said polypeptide; and optionally
- iii) isolating the polypeptide from the cell or the cell culture medium.

32. A method according to Claim 31 wherein said polypeptide is provided with a
30 secretion signal to facilitate the purification of the polypeptide from said cell.

33. A method according to Claim 31 or 32 wherein said polypeptide is provided with an affinity tag to facilitate the purification of the polypeptide from said cell or the cell culture medium.

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