The Human Growth Hormone Receptor

SECRETION FROM ESCHERICHIA COLI AND DISULFIDE BONDING PATTERN OF THE EXTRACELLULAR BINDING DOMAIN

Germaine Fuh, Michael G. Mulkerrin, Steven Bass, Nancy McFarland, Michael Brochier, James H. Bourell, David R. Light, and James A. Wells

From the Departments of Biomolecular Chemistry, Fermentation Research and Process Development, and Protein Chemistry, Genentech, Inc., South San Francisco, California 94080

A gene fragment encoding the extracellular domain of the human growth hormone (hGH) receptor from liver was cloned into a plasmid under control of the Escherichia coli alkaline phosphatase promoter and the heat-stable enterotoxin (StII) signal peptide sequence. Strains of E. coli expressing properly folded hGH binding protein were identified by blotting colonies with 125I-hGH. The E. coli strain capable of highest expression (KS330) secreted 10 to 20 mg/liter of culture of properly processed and folded hGH receptor fragment into the periplasmic space. The protein was purified to near homogeneity in 70 to 80% yield (in tens of milligram amounts) using ammonium sulfate precipitation, hGH affinity chromatography, and gel filtration. The unglycosylated extracellular domain of the hGH receptor has virtually identical binding properties compared to its natural glycosylated counterpart isolated from human serum, suggesting glycosylation is not important for binding of hGH. The extracellular binding domain codes for 7 cysteines, and we show that six of them form three disulfide bonds. Peptide mapping studies show these disulfides are paired sequentially to produce short loops (9-15 residues long) as follows: Cys<sup>39</sup>-Cys<sup>48</sup>, Cys<sup>63</sup>-Cys<sup>84</sup>, and Cys<sup>108</sup>-Cys<sup>222</sup>. Cys<sup>241</sup> is unpaired, and mutagenic analysis shows that the extreme carboxyl end of the receptor fragment (including Cys<sup>241</sup>) is not essential for folding or binding of the protein to hGH. High level expression of this receptor binding domain and its homologs in E. coli will greatly facilitate their detailed biophysical and structural analysis.

Human growth hormone is a member of a large family of homologous hormones including growth hormones, placental lactogens, and prolactins (for review see Nicoll et al., 1986). These hormones vary widely in their receptor binding properties and biological effects including linear bone growth, lactation, macrophage activation, nitrogen retention, and diabetogenic and insulin-like effects (for reviews see Hughes and Friezon, 1985; Ilesecon et al., 1985). Some tissues even display multiple receptor types; for example, liver contains both prolactin and growth hormone receptors. To understand the molecular basis for these broad pharmacological effects requires purification of the various receptors in amounts suitable for detailed structure-function analysis.

Recently, the cDNA for the GH<sub>1</sub> receptor has been cloned from human, rabbit (Leung et al., 1987), mouse (Smith et al., 1989), and rat (Mathews et al., 1989) livers. In addition, the cDNA for the prolactin receptor has been cloned from rat liver (Boutin et al., 1988), mouse liver (Davis and Linzer, 1989), and rabbit mammary gland (Edery et al., 1989). All of these receptors have one extracellular binding domain (roughly 210-250 residues containing 5 or 7 cysteines for PRL and GH receptors, respectively), one transmembrane domain and a cytoplasmic domain that can vary considerably in length and identity between the PRL and GH receptors.

In addition to the membrane-bound form of the hGH liver receptor, there is also a serum GH binding protein that has been isolated from human (Baumann et al., 1986; Herington et al., 1986), rabbit (Spencer et al., 1988), and mouse (Smith et al., 1989). The liver receptor and serum binding protein from rabbit have the same NH<sub>2</sub>-terminal sequence and the same specificity for natural variants of growth hormone (Spencer et al., 1988). Both proteins are heavily glycosylated (Leung et al., 1987; Spencer et al., 1988) which raises the possibility that glycosylation can affect the affinity for growth hormone.

A truncated form of the GH receptor has been expressed in mammalian cells and found to retain its native binding affinity (Leung et al., 1987). However, the level of expression is low, and the time required for stable high level expression from mammalian cells is very long. Here, we describe high level expression, purification, and characterization of a recombinant hGH binding protein secreted from Escherichia coli. The protein retains the same binding affinity and specificity as the natural hGH serum binding protein, suggesting that glycosylation is irrelevant to binding. Moreover, the high expression levels supplied us with enough protein to map directly the disulfide pattern for the first time in any polypeptide hormone receptor.

The abbreviations used are: GH, growth hormone; hGH, human growth hormone; PRL, prolactin; hPRL, human prolactin; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; FAB ms, fast atom bombardment mass spectrometry; mNBA, m-nitrobenzalcohol; DTT, dithiobis; PAGE, polyacrylamide gel electrophoresis; AP, alkaline phosphatase; StII, E. coli heat-stable enterotoxin; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; C241R designates a mutation in which Cys<sup>241</sup> is converted to Arg; K<sub>d</sub>, dissociation constant; 1/1'NB, 3,5'-dinitrobiose (2-nitrobenzoic acid); bp, base pair(s); ELISA, enzyme-linked immunoabsorbent assay.
Characterization of Recombinant hGH Binding Protein

EXPERIMENTAL PROCEDURES

RESULTS

Binding to hGH and hGH Variants—To compare the specificity of the recombinant hGH binding protein from E. coli with the natural product isolated from human serum, the affinities were determined for wild-type and various hGH mutants (Fig. 4, Table II). Both proteins formed a specific stoichiometric complex with hGH (Fig. 4). The affinities for wild-type and mutants of hGH (Cunningham and Wells, 1989) are nearly identical between the two binding proteins (Table II; right side column). The recombinant hGH binding protein has a marginally higher affinity compared to the natural protein from human serum. This may reflect the greater purity and homogeneity of the recombinant protein. Both proteins had identical specificities as shown by the changes in binding affinities for four alanine mutants of hGH that disrupt binding to the hGH binding protein (Table II; $K_d(wt)/K_d(wt)$). The affinity of hGH for the binding protein that extended to Tyr$^{246}$ ($K_d = 0.36 \pm 0.08 \text{ nM}$) was virtually identical with that terminating after Gln$^{33}$ (0.40 ± 0.03 nM) indicating the last 8 residues (including the 7th cysteine in the molecule) are not essential for binding hGH.

Disulfide Bonding Pattern—The recombinant binding protein (residues 1–238) codes for 6 cysteine residues. DTNB analysis (Ellman, 1959) of the binding protein unfolded in 6 M guanidine HCl or 2% SDS did not detect any free thiols. However, when the protein was reduced in denaturants and the reducing agent was removed by gel filtration, 5.7 ± 0.4 free thiols per binding protein were found by DTNB analysis. The binding protein was subjected to proteolysis in the absence of reductants by digestion with either V8 protease, Asp-N protease (Boehringer Mannheim), or pepsin. Peptides were identified using a strategy outlined by Morris and Pucci (1985) and assigned to the hGH binding protein sequence (Fig. 6). From this we identified the following disulfide linkages: Cys$^5$-Cys$^6$, Cys$^7$-Cys$^{22}$, and Cys$^{33}$-Cys$^{23}$.

DISCUSSION

The unglycosylated hGH binding protein secreted from E. coli retained virtually the same binding affinity and specificity for wild-type and hGH mutants as the glycosylated binding protein isolated from human serum (Baumann et al., 1986). Averaging compensating effects, these data suggest that the recombinant protein is properly folded and that the glycosyl moieties on the serum binding protein are not important binding determinants. This implies that sites of glycosylation are not principally involved in the hormone binding epitope. It is also noteworthy that the clearance rates for complexes digestion. Other control experiments showed the peptides generated by V8 proteolysis were the same with or without treatment with iodoacetate. Cysteine-containing peptides were identified using a strategy outlined by Morris and Pucci (1985) and assigned to the hGH binding protein sequence (Fig. 6). From this we identified the following disulfide linkages: Cys$^5$-Cys$^6$, Cys$^7$-Cys$^{22}$, and Cys$^{33}$-Cys$^{23}$.

<table>
<thead>
<tr>
<th>hGH mutant</th>
<th>$K_d$ (human serum) $/ K_d$ (E. coli)</th>
<th>$K_d$ (mut) $/ K_d$ (wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.55 ± 0.07</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>F16A</td>
<td>21 ± 2</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>R64A</td>
<td>12 ± 1</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>E74A</td>
<td>0.27 ± 0.04</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>F176A</td>
<td>72 ± 7</td>
<td>1.30 ± 0.20</td>
</tr>
</tbody>
</table>

TABLE II

Comparison of dissociation constants ($K_d$) for purified hGH binding proteins isolated from human serum or from E. coli secreting the extracellular portion of the hGH receptor (residues 1–238). Values of $K_d$ and corresponding standard deviations (S.D.) were determined by competitive binding analysis (Fig. 4) with wild-type hGH (wt) and a number of mutants of hGH.

Notes:

* Portions of this paper (including “Experimental Procedures,” Table I, and Figs. 1–3, 5, 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

* hGH variants were produced as described (Cunningham and Wells, 1989). The variants are named according to the single-letter code for the wild-type residue followed by its position and the mutant residue. When the hormone-receptor complex was immunoprecipitated with Mab6 (Cunningham and Wells, 1980), the dissociation in the $K_d$ caused by the hGH mutants was less pronounced than that measured here where Mab263 was used.

* Reduction in binding affinity calculated from the ratio of dissociation constants for the hGH mutant (mut) and wild-type hGH for each hGH binding protein.

* Ratio of dissociation constants for the two hGH binding proteins with a given hGH type.
between the natural binding protein and hGH, or the binding protein expressed from E. coli and hGH are identical from E. coli (Goeddel et al., 1982; Chang et al., 1987) has greatly facilitated their structure-function analysis. The first x-ray structure of a growth hormone (Abdel-Meguid et al., 1987) was determined on porcine GH expressed intracellularly in E. coli. The hGH binding protein secreted from E. coli was used for virtually all of the receptor epitope mapping for mutants of hGH (Cunningham et al., 1989; Cunningham and Wells, 1989). Thus, the high level secretion of properly folded hGH bonding protein and its homologues in E. coli should greatly accelerate analysis of their structure and function relationships.

Acknowledgments—We thank Byron Nevin for amino acid compositional analysis and sequencing, the organic chemistry group for preparation of synthetic oligonucleotides, Ann Rowland and Dr. Way Lee Wong for receptor ELISA data, Brian Cunningham for providing hGH mutants, Michael Waters for providing monoclonal antibodies 5 and 263, Drs. William Wood, Steven Spencer, and David Leung for providing the pCIS2.shGHr plasmid used for construction of phGHr(1-246), and Wayne Anstine, Carol Morta, and Renate Hellmiss for typing and preparation of graphics.

REFERENCES
Eilman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77

The fact that the peptide fragment 239-246 did not copurify with 1-238 suggests that Cys246 is a free thiol. Other evidence for this comes from observations that the 1-246 hGH binding protein will attach to an activated thiol-Sepharose column, whereas the 1-238 binding protein will not and that peptides derived from 1-238 that contain Cys41 are not disulfide-linked to any others. The fact that the binding affinities for hGH of the binding protein extending to either 238 or 246 are identical (Table II) indicates that residues 239 to 246 are not essential for binding hGH. In the construction containing a C241R mutation followed by a stop codon (Table I), the isolated binding protein retained identical affinity for hGH. Thus, Cys41 and the carboxyl-terminal residues are neither crucial for structure nor function in the hGH binding protein.

The hGH binding protein contains a strikingly simple disulfide bonding pattern in which cysteine residues are disulfide-linked. This pattern defines three conserved structural motifs.

Efficient expression of recombinant growth hormones in E. coli.
Characterization of Recombinant hGH Binding Protein


---

Supplementary Materials:

Experimental Procedures


---

To identify E. coli strains that secrete properly folded hGH binding protein, a colony blot assay was developed using [125I]hGH as a probe (Fig. 2).

---

Table 1

| Fltualloc | Membrane DNA | Secretion
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fltualloc (1-246)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino</td>
<td>T14</td>
<td>C13</td>
</tr>
<tr>
<td>Cterminus</td>
<td>C16</td>
<td>C17</td>
</tr>
</tbody>
</table>

---

*Indicates melanotic loss from the wild-type strain.

---

To identify E. coli strains that secrete properly folded hGH binding protein, a colony blot assay was developed using [125I]hGH as a probe (Fig. 2).
Characterization of Recombinant hGH Binding Protein

Fig. 3. Purification of the hGH binding protein containing residues 2-318 from the porcine pancreas of E. coli strain KOS33 harboring the cloned pGHC-1-230 (Fig. 1, Table I). Panel A shows an hGH-affinity chromatogram of the binding protein that was extracted from 6.2 kg of pig pancreas. Protein prior to the wash step was heated to 70 °C for 10 min and then centrifuged at 30000 ×g for 30 min. The supernatant was then sieved through a 0.2-μm filter and pooled. The sample was then diluted with 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to a concentration of 1 mg of protein per ml. The sample was then loaded onto a column of hGH-Sepharose equilibrated with 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), and 20% (v/v) glycerol (pH 8.0). Fractions of 1 ml were collected and assayed for protein content. The hGH-Sepharose was then washed with 2 ml of 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove non-specifically bound material. The column was then eluted with 2 ml of 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), and 20% (v/v) glycerol (pH 8.0) containing 0.5 M NaCl. The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Panel B shows an SDS-PAGE analysis of the purified hGH binding protein. The protein bands were visualized by Coomassie blue staining. The purified hGH binding protein contains the hGH binding activity and is eluted in two major peaks at fractions 20 and 25.

Panel C shows a hGH-affinity chromatogram of the purified hGH binding protein. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 4. hGH-affinity chromatogram of the purified hGH binding protein. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 5. Absorbance spectrum of the purified hGH binding protein. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 6. Sequence of peptides isolated from the soluble binding protein (1-230). The sequence derived from the purified protein (Fig. 3) was confirmed by nucleotide sequencing of the cDNA corresponding to the hGH binding protein. The sequence was consistent with the predicted amino acid sequence of the hGH binding protein containing residues 1-230. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 7. Binding of the purified hGH binding protein to an hGH-Sepharose column. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 8. Comparison of the purified hGH binding protein to the purified hGH binding protein containing residues 1-230. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 9. Comparison of the purified hGH binding protein to the purified hGH binding protein containing residues 1-230. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 10. Comparison of the purified hGH binding protein to the purified hGH binding protein containing residues 1-230. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.

Fig. 11. Comparison of the purified hGH binding protein to the purified hGH binding protein containing residues 1-230. The purified hGH binding protein was applied to an hGH-Sepharose column and eluted with a linear gradient of 0-0.5 M NaCl in 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The fractions containing the binding protein were then pooled and concentrated by ultrafiltration using a 100000 MWCO membrane. The concentrated solution was then dialyzed against 100 mM Tris (pH 8.0) and 20 mM EDTA (pH 8.0) to remove excess NaCl. The purified hGH binding protein was then stored at −80 °C until further use.
The human growth hormone receptor. Secretion from Escherichia coli and disulfide bonding pattern of the extracellular binding domain.
G Fuh, M G Mulkerrin, S Bass, N McFarland, M Brochier, J H Bourell, D R Light and J A Wells


Access the most updated version of this article at http://www.jbc.org/content/265/6/3111

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/6/3111.full.html#ref-list-1