The Human Growth Hormone Receptor

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

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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 uncleaved 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 (10-15 residues long) as follows: Cys36-Cys46, Cys83-Cys94, and Cys106-Cys122. Cys124 is unpaired, and mutagenic analysis shows that the extreme carboxyl end of the receptor fragment (including Cys124) is not essential for folding or binding of the protein to hGH. High level expression of the receptor binding domain and its homologs in E. coli will greatly facilitate their detailed biophysical and structural analysis.

The abbreviations used are: GH, growth hormone; hGH, human growth hormone; PRL, prolactin; hPRL, human prolactin; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; FAB, fast atom bombardment mass spectrometry; mNBA, m-nitrobenzyloalcohol; DTT, dithiothreitol; 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 Cys124 is converted to Arg; Kd, dissociation constant; 1/1TNB, 5,5'-dithiobis(2-nitrobenzoic acid); bp, base pair(s); ELISA, enzyme-linked immuno-sorbent assay.

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**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; $K_d$(mut)/$K_d$(wt)). The affinity of hGH for the binding protein that extended to Tyr246 ($K_d$ = 0.36 ± 0.08 nM) was virtually identical with that terminating after Gln238 (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 isolated by reverse-phase HPLC (Fig. 5, A–C) and analyzed by mass spectrometry. Peptides covering nearly the entire sequence (1–238) were isolated including those containing the amino and carboxyl termini. Precautions were taken to prevent thiol-catalyzed disulfide shuffling by pretreating the protein exhaustively with iodoacetate prior to proteolytic 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$^{52}$-Cys$^{15}$, Cys$^{87}$-Cys$^{15}$, and Cys$^{196}$-Cys$^{212}$.

**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). Barring 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

\[
\frac{K_d \text{(mut)}}{K_d \text{(wt)}} = \frac{1.7 \text{ (human serum)}}{1.5 \text{ (E. coli)}}
\]

*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).

<table>
<thead>
<tr>
<th>hGH mutant</th>
<th>$K_d$ (mut)/$K_d$ (wt)</th>
<th>$K_d$ (mut)/$K_d$ (wt)</th>
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<tbody>
<tr>
<td>wt</td>
<td>0.55 ± 0.07</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>F176A</td>
<td>0.12 ± 0.2</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>E174A</td>
<td>0.27 ± 0.04</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>F176A</td>
<td>0.71 ± 0.12</td>
<td>0.49 ± 0.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>hGH mutant</th>
<th>$K_d$ (human serum)/$K_d$ (E. coli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F176A</td>
<td>1.20 ± 0.20</td>
</tr>
<tr>
<td>E174A</td>
<td>1.00 ± 0.10</td>
</tr>
</tbody>
</table>

*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.*
between the natural binding protein and hGH, or the binding protein expressed from E. coli and hGH are identical in rats (Moore et al., 1989) suggesting that glycosylation does not affect clearance rate in vivo.

Although it is possible to isolate the hGH binding protein extending from residues 1-246, much of it was cleaved after Glu238. This suggests the extreme carboxyl terminus from 238 to 246 is surface-accessible because susceptibility to proteolysis is strongly correlated to surface accessibility and segmental mobility (Fontana et al., 1986). It has been proposed (Leung et al., 1987; Spencer et al., 1988) that the hGH binding protein in serum derives from proteolysis of the membrane-bound form of the receptor near the transmembrane anchor.

The fact that the peptide fragment 239-246 did not copurify with 1-238 suggests that Cys239 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 1-246 that contain Cys241 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, Cys241 and the carboxyl-terminal residues are neither crucial for structure nor function of the hGH binding protein.

The hGH binding protein contains a strikingly simple disulfide bonding pattern in which cysteine residues in primary structure are disulfide-linked. This pattern defines three short disulfide loops (Fig. 7) that represent some of the most conserved residues among the GH and PRL receptor family. For example, the rabbit GH and hGH receptors are 68% identical from residues 1-238 (excluding insertions and deletions; Leung et al., 1987); over a similar stretch, the rabbit GH and rabbit PRL receptors are 32% identical (Boutin et al., 1988). By comparison, sequence identity between the rabbit GH and hGH receptors within the three disulfide loop regions are 91%, 92%, and 80%, respectively; identities between the rabbit GH and rabbit PRL receptors in these regions are 75%, 67%, and 13%, respectively (Fig. 7). Thus, there is remarkable sequence conservation within the first two disulfide loops. The third disulfide loop is considerably more variable and is even completely absent in the hPRL receptor. We infer from this analysis that all these receptors have identical disulfide bonding patterns in which cysteine pairs are sequentially linked. Moreover, the hydrophilic character and strong sequence conservation of the first two disulfide loops suggest that they are solvent-accessible and highly conserved structural motifs.

Efficient expression of recombinant growth hormones in 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 binding protein and its homologs in E. coli should greatly accelerate analysis of their structure and function relationships.

Acknowledgments—We thank Byron Nevins 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 phGH(1-246), and Wayne Anstine, Carol Morita, and Renee Hell- miss for typing and preparation of graphics.

REFERENCES

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

Fig. 7. Sequence comparisons of the extracellular disulfide loops between the human growth hormone receptor (hGHr) (Leung et al., 1987), rabbit GH receptor (rabGHr) (Leung et al., 1987), mouse GH receptor (mGHr) (Smith et al., 1989), rat growth hormone receptor (ratGHr) (Mathews et al., 1989), and rat prolactin receptor (ratPRLr) (Boutin et al., 1988). Sequences that exactly match the hGHr are boxed, and residues are numbered according to the hGHr sequence.
Characterization of Recombinant hGH Binding Protein


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Supplementary Material:


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Experimental Procedures

Materials: All restriction enzymes were from New England Biolabs (NEB) or Bethesda Research Laboratories (BRL). DNA polymerase large fragment (Klenow) and polymerase chain reaction were from BRL. 32P-γ-ATP was from New England Nuclear (Park Ridge, NJ). All other reagents were obtained from commercial sources. Restriction enzymes were used as recommended by the suppliers unless otherwise indicated. Affinity purified hGH and recombinant rhodamine antibodies, Dr. Michael Waters provided MAb 263 for binding studies and IGF-I for immunizations. Brian Cunningham provided hGH mutants. Dr. W. O. Strauch provided E.coli strain K1290.

Fig. 1. Diagram of the DNA encoding the cDNA for the entire extracellular domain residues 1-246 of the hGH receptor. Genes and restriction sites are indicated by thick lines and important restriction sites are indicated in the construction are indicated. The 32P by Shoufler fragment from PGL-2 was cloned into the Sall site of pBR322 using T4 DNA ligase (Maniatis et al., 1982) containing the signal sequence, residues 55-72 of the hGH receptor and 9 base sequence was cloned into a similar cut fragment (Klenow) and polymerase chain reaction (Cohen et al., 1982). Site directed mutagenesis was performed (Cohen et al., 1982) to generate a single site in the hGH receptor sequence using the oligonucleotides 5'-AGCTGCGCCAT GACCTGCAC-3' and 5'-CGCAGTGCGCCAGGACTGAC-3' at the 3' end of the cloning site to produce the underlined Nsi I site. Bacteriotherapy (Maniatis et al., 1982) confirmed this site and all subsequent mutations. The 5' and 3' adjacent fragment containing residues 3-186 plus 3-186 of the signal sequence was subcloned into the SalI-SmaI site of pBR322 using T4 DNA ligase (Maniatis et al., 1982) to give pGEM-hGH (1-246). The full transcription of the hGH binding protein gene under control of the E. coli Ap promoter and initiation of the coding sequence for the signal sequence (Chang et al., 1978) as well as being dispensable for proper cleavage by signal peptide polypeptide in E. coli, see Table 2.

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Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Termination</th>
<th>DNA sequence/Recombinant Construct</th>
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<tbody>
<tr>
<td>pGEM-hGH</td>
<td>TAC</td>
<td>5'-AGCTGGCGCCATGACCTGCAC-3'</td>
</tr>
<tr>
<td>pGEM-hGH</td>
<td>TAC</td>
<td>5'-CGCAGTGCGCCAGGACTGAC-3'</td>
</tr>
<tr>
<td>pGEM-hGH</td>
<td>TAC</td>
<td>5'-AGCTGGCGCCATGACCTGCAC-3'</td>
</tr>
<tr>
<td>pGEM-hGH</td>
<td>TAC</td>
<td>5'-CGCAGTGCGCCAGGACTGAC-3'</td>
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<tr>
<td>pGEM-hGH</td>
<td>TAC</td>
<td>5'-AGCTGGCGCCATGACCTGCAC-3'</td>
</tr>
<tr>
<td>pGEM-hGH</td>
<td>TAC</td>
<td>5'-CGCAGTGCGCCAGGACTGAC-3'</td>
</tr>
</tbody>
</table>

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To identify E.coli strains that successfully encoded hGH binding protein, a colony blot assay was developed using [32P]GTP in a probe (Fig. 2).

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Fig. 2. Purification of various E.coli strains containing the expression plasmid pGEM-hGH (1-246) a control plasmid pGEM-128 (Lewg et al., 1987) that contains no hGH binding protein gene under control of an SV40 promoter which is not expressed in E.coli. Protein and peptide mapping is done in SDS-10% polyacrylamide gels and visualized by autoradiography. Spc. [32P]GTP was used as a probe for the hGH binding protein. The hGH binding protein was significantly higher in the strain pGEM-128 (K1290) than in the strain pGEM-hGH (1-246).
Characterization of Recombinant hGH Binding Protein

Fig. 3. Purification of the hGH binding protein containing residues 2-218 from the pepsin-active space of E. coli strains KS100 harboring the plasmid pGHC-218 (Fig. A). Panel A shows an hGH affinity chromatogram of the binding protein that was extracted from 6.2 kg of cell free. Protein peaks were determined to mean temperature and assayed in five fractions of bovine testicular T3-001 (100 Ml of T3-001) at pH 7.5 dissolved in a cocktail of protein binding inhibitors (1 mm phenylmethyl sulfone fluoride (Sigma), 2 mm EDTA, 10 mm NaF, 0.1 mm N-ethylmaleimide, and 0.1 mm mercaptoethanol). The mixture was gently stirred for 1.5 h at 4°C and the supernatant clarified by centrifugation (10700 x g for 30 min). Solid Ni-NTA-gel, was added to molar volume (100 ml) and the mixture stirred at 4°C for 30 min. Protein precipitates (including the binding protein) were removed by centrifugation (12000 x g for 20 min). The protein pellet was re-dissolved in one tenth volume of buffer T (100 Ml of buffer T) and the supernatant was clarified by centrifugation (12000 x g for 10 min). The supernatant was combined and dialyzed against buffer T to a final volume of 300 ml. The dialyzed fraction was then purified by affinity chromatography using an hGH affinity column (1.5 x 10 cm) containing 50 ml affinity gel from which the column was washed with buffer T (100 Ml of buffer T) and the binding protein was eluted with buffer T containing 500 mm NaCl (27°C). Protein concentrations were determined by absorbance at 280 nm, and purity was determined by SDS-PAGE analysis and Coomassie blue staining. The peaks were pooled and concentrated by ultrafiltration at 4°C. The purified binding protein was stored at -80°C until used for further characterization.

Fig. 5. Disulfide mapping of the soluble human growth hormone receptor. Peptides generated by protease digestion were applied directly an HPLC column (Vydac C18, 50 x 4.6 mm). Purity was reduced by reduced, etc.

Fig. 6. Sequence of peptides isolated from the soluble binding protein (2-218). The sequences indicated the peptides from the cell free fraction of the culture (X, N, P) or the supernatant (Y, M, D) of the culture (X, N, P) or the supernatant (Y, M, D) of the culture. The peptides were sequenced by HPLC (Fig. 3), cleaved manually, iodoacetylated and subjected to mass spectrometry analysis (peptide mapping).

Fig. 7. Analysis of the hGH binding protein by gel filtration. Panel A shows the elution pattern of the hGH binding protein from a Sephadex G-100 column. Panel B shows the elution pattern of the hGH binding protein from a Sepharose 4B column. Panel C shows the elution pattern of the hGH binding protein from a Superose 6 column. Panel D shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel E shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel F shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel G shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel H shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel I shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel J shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel K shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel L shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel M shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel N shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel O shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel P shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel Q shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel R shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel S shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel T shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel U shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel V shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel W shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel X shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel Y shows the elution pattern of the hGH binding protein from a Superose 12 column. Panel Z shows the elution pattern of the hGH binding protein from a Superose 12 column.
The human growth hormone receptor. Secretion from Escherichia coli and disulfide bonding pattern of the extracellular binding domain.
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