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 (10–15 residues long) as follows: Cys20-Cys48, Cys63-Cys84, and Cys108-Cys212. Cys241 is unpaired, and mutagenic analysis shows that the extreme carboxyl end of the receptor fragment (including Cys241) 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, macophage activation, nitrogen retention, and diabetogenic and insulin-like effects (for reviews see Hughes and Friecon, 1985; Isaksson 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 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 NH2-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, fast atom bombardment mass spectrometry; mNBA, m-nitrobenzylalcohol; 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 Cys241 is converted to Arg; Av, dissociation constant; 1/2TNB, 3,5-ditnitol-s2-nitrobenzoic acid; bp, base pair(s); ELISA, enzyme-linked immuno sorbent 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; $K_d$(mut)/$K_d$(wt)). The affinity of hGH for the binding protein that extended to Tyr246 ($K_d = 0.36 \pm 0.08$ nM) was virtually identical with that terminating after Gln33 (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$^{31}$-Cys$^{8}$, Cys$^{8}$-Cys$^{44}$, and Cys$^{31}$-Cys$^{77}$.

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

![Fig. 4. Competitive binding curves of $^{125}$I-hGH (Spencer et al., 1988) and unlabeled hGH to the hGH binding protein isolated from either human serum (○) or from *E. coli* KS330 cultures expressing the plasmid phGHRr(1–238) (●). Bars represent standard deviations from the mean. Inset shows Scatchard plots that were derived from the competitive binding curves (Monson and Rodbard, 1980). The concentrations of the binding protein from human serum and *E. coli* were 0.1 and 0.08 nM, respectively.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>hGH mutant</th>
<th>$K_d$ (mut)/$K_d$(wt)</th>
<th>$K_d$(mut)/$K_d$(wt)</th>
<th>$K_d$(mut)/$K_d$(E.coli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.55 ± 0.07</td>
<td>0.40 ± 0.03</td>
<td>1.4</td>
</tr>
<tr>
<td>F58A</td>
<td>21 ± 2</td>
<td>38 ± 6</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>R64A</td>
<td>12 ± 1</td>
<td>22 ± 4</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>E174A</td>
<td>0.27 ± 0.04</td>
<td>0.49 ± 0.11</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>P176A</td>
<td>130 ± 20</td>
<td>48 ± 5</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

* 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 disruption 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.

Downloaded from http://www.jbc.org/
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 Glu246. 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 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 1-246 that contain Cys246 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, Cys246 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 cysteines nearest 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 73%, 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 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.sghGH plasmid used for construction of phGH(1-246), and Wayne Anstine, Carol Morta, and Renate Hellmis for typing and preparation of graphics.

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Characterization of Recombinant hGH Binding Protein


Supplementary Material:


Experimental Procedures

Fig. 1. Pulsed-field diagram of pSCHR (2-1246) used to direct omission in E. coli of the entire extra-cytoplasmic domain (residues 2-768) of the EGF receptor. Genes and replication origins are indicated by thick boxes and important restriction sites used in the construction of the plasmid. The 3' termini were derived from the E. coli plasmid pBR322 (see text). The 5' termini were derived from the plasmid pHC101 [see ref. 3]. These 3' and 5' termini were ligated together to create the final vector, pSCHR (2-1246). The E. coli strain used was HB101, which is proficient in the E. coli fusion protein. The plasmid was transformed into HB101 transformed by electroporation. The transformants were selected on agar plates containing ampicillin. The E. coli strain used was HB101, which is proficient in the E. coli fusion protein. The plasmid was transformed into HB101 transformed by electroporation. The transformants were selected on agar plates containing ampicillin. The E. coli strain used was HB101, which is proficient in the E. coli fusion protein. The plasmid was transformed into HB101 transformed by electroporation. The transformants were selected on agar plates containing ampicillin.

Fig. 2. Pulsed-field diagram of various E. coli strains containing expression plasmids pSCHR (2-1246) on a control plasmid pJCM8 (Lee, A. E., 1987, which contains the HGH binding gene under a control of a baculovirus promoter which is not introduced in E. coli. E. coli strains were constructed by transforming the E. coli strain HB101 with pSCHR (2-1246) as described in the legend.

Fig. 3. Diagram of the expression plasmid pSCHR (2-1246). The plasmid was constructed as follows. The EcoRI site at position 768 of the hGH receptor was purified from the periplasmic space of E. coli. The presence of the EcoRI site in the expression vector in pSCHR (2-1246) was confirmed by Southern blot analysis. The EcoRI site was then used to clone the hGH receptor into pUC19. The resulting plasmid, pSCHR (2-1246), was transformed into E. coli HB101. The transformants were selected on agar plates containing ampicillin. The E. coli strain used was HB101, which is proficient in the E. coli fusion protein. The plasmid was transformed into HB101 transformed by electroporation. The transformants were selected on agar plates containing ampicillin.

Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Target Site</th>
<th>Expected Restriction Patterns</th>
<th>Observed Restriction Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSCHR(2-1246)</td>
<td>BglII</td>
<td>EcoRI, HindIII, PstI, BamHI</td>
<td>EcoRI, HindIII, PstI, BamHI</td>
</tr>
<tr>
<td>pSCHR(2-1246)</td>
<td>BamHI</td>
<td>EcoRI, HindIII, PstI, BglII</td>
<td>EcoRI, HindIII, PstI, BglII</td>
</tr>
</tbody>
</table>

* Indicates EcoRI sites from the wild-type template
Characterization of Recombinant hGH Binding Protein

Fig. 3. Purification of the hGH binding protein containing residues 2-218 from the periplasmic space of E. coli strain K220 harboring the pTHC6-trialg gene construct (Fig. 1, Table 1). Panel A shows an SDS-PAGE chromogram of the binding protein that was extracted from 6.2 kg of culture. Protein pellets were treated with 40 mM Tris (pH 8.0) and 0.1% SDS and subjected to size exclusion chromatography on a Sephacryl S-200 column. The protein was eluted with a linear gradient of 0.1 M NaCl in 10 mM Tris (pH 8.0). Panel B shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel C shows a FACScan analysis of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel D shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue.

Analytical Protocols - Purified and apparent molecular weight was measured by SDS-PAGE (Laemmli, 1970). Samples were prepared for SDS-PAGE by protein precipitation and solubilization in 1% SDS, 50 mM Tris (pH 8.0), 5% 2-mercaptoethanol (sulfur reduction), and 40°C-80°C. Panel A shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel B shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel C shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel D shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue.

Fig. 4. Sequence of peptides isolated from the soluble binding protein (1-218). The sequences identified indicate that the protein from Fig. 3.d was found in the fraction from the periplasmic space of E. coli strain K220 harboring the pTHC6-trialg gene construct (Fig. 1, Table 1). The protein was isolated by size exclusion chromatography on a Sephacryl S-200 column. The protein was eluted with a linear gradient of 0.1 M NaCl in 10 mM Tris (pH 8.0) and 0.1% SDS and subjected to size exclusion chromatography on a Sephacryl S-200 column. The protein was eluted with a linear gradient of 0.1 M NaCl in 10 mM Tris (pH 8.0). Panel A shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel B shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel C shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue. Panel D shows an SDS-PAGE separation of the purified protein from the hGH affinity column. The elution profile was monitored by staining with Coomassie blue.

Fig. 5. Disulfide mapping of the soluble human growth hormone receptor. Peptides generated by proteolytic digestion were applied directly to an HPLC column (Vydac C18, 4.6 × 250 mm, 5-μm particle size, 10-μl loop) and eluted with a gradient of 0-100% acetonitrile in 0.1% trifluoroacetic acid. Panel A shows the HPLC profile of peptides generated by digester of the binding protein (13 to 218) with Lysine-arginine or cysteine-lysyl dipeptide groups. Panel B is the endoglycosidase HaseA-A-Glycogenase I. Panel C is the HPLC trace of the peptide digest of fraction 27 from Panel B. Panel D shows the elution profile of the HPLC profile, the fractions in which the peptides containing disulfide bridges were collected.

Panels A and B show the HPLC profile of the soluble human growth hormone receptor. Peptides generated by proteolytic digestion were applied directly to an HPLC column (Vydac C18, 4.6 × 250 mm, 5-μm particle size, 10-μl loop) and eluted with a gradient of 0-100% acetonitrile in 0.1% trifluoroacetic acid. Panel A shows the HPLC profile of peptides generated by digester of the binding protein (13 to 218) with Lysine-arginine or cysteine-lysyl dipeptide groups. Panel B is the endoglycosidase HaseA-A-Glycogenase I. Panel C is the HPLC trace of the peptide digest of fraction 27 from Panel B. Panel D shows the elution profile of the HPLC profile, the fractions in which the peptides containing disulfide bridges were collected.
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


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