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 columns 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: Cys3'-Cys4', Cys3'-Cys9', and Cys10'-Cys122. Cys41 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, macrophage activation, nitrogen retention, and diabetogenic and insulin-like effects (for reviews see Hughes and Friesen, 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.

1 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-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 CYSTIC is converted to Arg; Kd, dissociation constant; 1/ITNB, 3.5·dinitrobenzoic acid; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay.

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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; \( K_d^{\text{mut}}/K_d^{\text{wt}} \)). 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\(^{238} \) (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\(^6\)-Cys\(^8\), Cys\(^9\)-Cys\(^12\), and Cys\(^33\)-Cys\(^37\).

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
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 Cys240 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 Cys240 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, Cys240 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 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 PRL 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.

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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 sequence are boxed, and residues are numbered according to the hGHr sequence.
Characterization of Recombinant hGH Binding Protein


Supplementary Material

Fig. 1. Diagram of pG4Hr (1-246) used to direct expression of hGH in E. coli. The entire extracellular domain (residues 1-246) of the hGH receptor was inserted between the cloning sites of the AP promoter and the pBR322 AP selectable marker. The AP promoter contains 157 bp of the AP coding sequence and 224 bp downstream. The hGH gene was inserted into the pG4Hr vector as a 2.9 kb HindIII-EcoRI fragment, resulting in the plasmid pHGr (1-246).

Table 1

<table>
<thead>
<tr>
<th>Sequence of amino- and carboxyl-terminal amino acids of hGHbinding protein construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
</tr>
<tr>
<td>Yeast</td>
</tr>
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To identify E. coli strains containing the expression plasmid, pG4Hr (1-246) was used as a probe (Fig. 2).
Characterization of Recombinant hGH Binding Protein

Fig. 3. Purification of the hGH binding protein containing residues 3-218 from the periplasmic space of E. coli strain K12(harborizing the plasmid pBEH1-218) (Fig. 1). Panel A shows an SDS-PAGE chromogram of the binding protein that was extracted from 6.2 x 10^7 of cell mass. Protein pellets were stained in stain solution and subjected to electrophoresis under reducing conditions on 10% SDS-PAGE gels contailing a cocktail of protein standards (G protein, 66 kDa; EEF, 21 kDa; actin, 42 kDa). The purity of the protein band was estimated from the gel scan. Panel B shows the absorbance of the fractions collected over the column. Panel C shows the absorbance of the fractions collected over the column. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer.

A. hGH-Affinity Column
B. G-100 Column
C. Fractions

Fig. 4. Absorbance at 280 nm of the fractions collected over the column. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer.

Fig. 5. A. Absorbance at 280 nm of the fractions collected over the column. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer.

Fig. 6. A. Absorbance at 280 nm of the fractions collected over the column. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer. The absorbance was determined by measuring the absorbance at 280 nm in a Beckman DU-2 spectrophotometer.
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