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 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: Cys38-Cys48, Cys63-Cys64, and Cys106-Cys222. 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.

*[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; K, dissociation constant; I/1NB, 3,5-dinitrobenzyl(2-nitrobenzoate); bp, base pair(s); ELISA, enzyme-linked immunosorbent assay.]

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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; $K_d(wt)/K_d(mut)$). The affinity of hGH for the binding protein that extended to Tyr246 ($K_d = 0.36 \pm 0.08 \text{ nM}$) was virtually identical with that terminating after Gln238 ($0.40 \pm 0.03 \text{ nM}$) 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 proteinase 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 pre treating 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$^2$–Cys$^3$, Cys$^5$–Cys$^6$, and Cys$^{131}$–Cys$^{132}$.

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 (O) or from E. coli K5330 cultures expressing the plasmid pHGHr(1–238) (●). Bars represent standard deviations from the mean. Inset shows Scatchard plots that were derived from the competitive binding curves (Munson 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 II**

| hGH mutant | Human serum $K_d$/wt | E. coli $K_d$/wt | $K_d$(mut)/$K_d$(wt) | $K_d$(human serum)/$K_d$(E. coli) |
|------------|----------------------|----------------|---------------------|----------------------------------|
| wt         | 0.55 ± 0.07          | 0.40 ± 0.03    | 1.4                 |
| FG5A       | 21 ± 2               | 38 ± 6         | 14 ± 1              | 36 ± 5                           |
| G64A       | 12 ± 1               | 22 ± 4         | 11 ± 1              | 28 ± 0                           |
| E174A      | 0.27 ± 0.04          | 0.40 ± 0.11    | 0.16 ± 0.01         | 0.4 ± 0.1                        |
| F176A      | 71 ± 7               | 130 ± 20       | 48 ± 5              | 120 ± 20                         |

$^a$ hGH mutants 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, 1988), the disruption in the $K_d$ caused by the hGH mutants was less pronounced than that measured here where Mab263 was used.

$^b$ 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.

$^c$ 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 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 Gin236. This suggests the extreme carboxy 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 Cys236 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 Cys236 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, Cys236 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 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 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) 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 are boxed, and residues are numbered according to the hGHr sequence.
Characterization of Recombinant hGH Binding Protein

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Experimental Procedures

Construction and Selection of the hGH Binding Protein in E. coli. (Fig. 1). It was constructed to encode the extracellular domain (residues 1-246) of the hGH receptor. Green and red phytochromes are indicated by thick lines and important residues are indicated by the symbols (Fig. 1). The cDNA for the receptor was inserted into the expression vector pECE in a linearized form. The resulting plasmid was transformed into E. coli. The transformed bacteria were selected for the presence of the plasmid. The recombinant hGH binding protein was purified by affinity chromatography on hGH-Sepharose 4B columns. The purified protein was analyzed by SDS-PAGE and Western blotting.

Table 1

| Mutant | Tertiary DNA structure/function determination |
|--------|-----------------------------------------------|
| 124    | C154E                                      |
| 129    | C154E                                      |

E coli strain: 1603
AP promoter: + + + +

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

![Diagram of hGH binding protein](https://example.com/diagram)

Fig. 2. Heterogeneity of various E. coli strains containing the expression plasmid and a control plasmid pUC18. (A) DNA blot analysis of the expression plasmid (pUC18) and the control plasmid pUC18. The E. coli strains were grown overnight and the supernatant was collected. The DNA was extracted and analyzed by Southern blotting. The results are shown in the figure. (B) SDS-PAGE analysis of the expression plasmid and the control plasmid. The proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. The results are shown in the figure. (C) Western blot analysis of the expression plasmid and the control plasmid. The proteins were separated by SDS-PAGE and visualized by Western blotting. The results are shown in the figure. (D) ELISA analysis of the expression plasmid and the control plasmid. The proteins were separated by SDS-PAGE and visualized by ELISA. The results are shown in the figure.

Supplementary Material

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Materials

All reference entries were taken from New England England (NEB) and Bethesda Research Laboratories (BRL). All chemicals were from Sigma-Aldrich. The following reagents were used: pUC18, a plasmid DNA vector; tetracycline, a genetic marker; KE, a hexokinase; and hGH, a human growth hormone. The following reference entries were taken from BRL: BRL DNA sequence analysis kit, a kit for determining the DNA sequence; pUC18, a plasmid DNA vector; and hGH, a human growth hormone.
Characterization of Recombinant hGH Binding Protein

A. hGH-Affinity Column

B. Characterization of the hGH Affinity Column

C. Elution Profile

Fig. 5. Characterization of the hGH binding protein: A. Equilibrium binding studies with the hGH-affinity column. B. Elution profile of the hGH binding protein. C. Comparison of the elution profile of the hGH binding protein with that of the native hGH.

Analytical Procedures - Protein purity and apparent molecular weight was assessed by SDS-PAGE (Laemmli, 1970). Samples were prepared for SDS-PAGE by acetic acid precipitation and resolubilization in 6% SDS, 3% NaDodSO4, 10 mM Tris (pH 6.8), 5% 2-mercaptoethanol (mercaptoethanol) at 70°C for 10 min. An ELISA was developed (D.W. Wing, unpublished results) for monitoring the activity of the hGH binding protein. Peak fractions containing the hGH binding protein were pooled and lyophilized. The lyophilized samples were resuspended in 6% SDS, 3% NaDodSO4, 10 mM Tris (pH 6.8), and 5% 2-mercaptoethanol (mercaptoethanol) at 70°C for 10 min. The samples were then fractionated by SDS-PAGE (Laemmli, 1970) and the purified hGH binding protein was visualized by silver staining. The apparent molecular weight was determined by comparing the migration of the hGH binding protein with that of known molecular weight standards.

Peptide and Digest Mapping - The binding protein was purified using a Bio-Rad Laboratories Bio-Gel P-250 gel filtration column. The eluate was collected and fractionated into 10 ml fractions. Amino acid sequencing was performed on samples from each fraction. The elution profile of the hGH binding protein was compared to that of the native hGH. The apparent molecular weight of the hGH binding protein was determined by SDS-PAGE (Laemmli, 1970) and the purified hGH binding protein was visualized by silver staining. The apparent molecular weight was determined by comparing the migration of the hGH binding protein with that of known molecular weight standards.

Fig. 5. Characterization of the hGH binding protein: A. Equilibrium binding studies with the hGH-affinity column. B. Elution profile of the hGH binding protein. C. Comparison of the elution profile of the hGH binding protein with that of the native hGH.
The human growth hormone receptor. Secretion from Escherichia coli and disulfide bonding pattern of the extracellular binding domain.

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