Residues in the α Subunit of Human Choriotropin That Are Important for Interaction with the Lutropin Receptor*

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Synthetic peptides were used to probe the structure-function relationships between human choriotropin (hCG) and the lutropin (LH) receptor. Previously, a peptide region of the α subunit of hCG, residues 26–46, had been shown to inhibit binding of $^{125}$I-hCG to the LH receptor in rat ovarian membranes (Charlesworth, M. C., McCormick, D. J., Madden, B., and Ryan, R. J. (1987) J. Biol. Chem. 262, 13409–13416). To determine which residues are important for this inhibitory activity, peptides were truncated from either the amino or carboxyl terminus, or individual residues were substituted with alanine. The amino-terminal boundary was determined to be Gly-30 and the carboxyl-terminal boundary, Lys-44. This core peptide contained all the residues needed for full activity of the parent peptide 26–46. Arg-35 and Phe-33 were particularly important residues; when they were substituted with alanine, the peptide inhibitory potencies were decreased. Ser-43, Arg-42, Cys-32, and Cys-31 were also important but to a lesser degree. These results are consistent with predictions based on chemical and enzymatic modification studies and provide insight into which residues are important for interaction between hCG and the LH receptor.

The family of glycoprotein hormones is the most structurally complex of the peptide hormones. This family includes LH, follicitropin, TSH of pituitary origin, and CG of placental origin. These hormones, through their membrane receptors, activate the adenylylcyclase system (Sullivan and Cooke, 1986) and may be coupled to calcium mobilization via the phosphoinositide breakdown pathway (Lowitt et al., 1982). TSH, follicitropin, LH, and CG are dimers composed of a common α subunit and a unique β subunit associated noncovalently. The intact hormone is required for full biological activity. However, some evidence suggests that the individual subunits can interact with the receptor and exhibit some, albeit low, biological activity. Each subunit is heavily cross-linked with disulfide bonds, and the subunits are glycosylated to varying degrees (for reviews see Pierce and Parsons (1981)).

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The abbreviations used are: LH, lutropin; TSH, thyrotropin; CG, choriotropin; h, human; DMPP, 4-(2',4'-dimethoxyphenyl)Fmoc-aminomethyl)phenoxy; Fmoc-9-fluorenylmethoxycarbonyl; TFS, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

and Ryan et al. (1987, 1989)). Although chemically deglycosylated and enzymatically desialylated hCG have been crystallized (Harris et al., 1989; Lustbader et al., 1989), no high resolution structural data is yet available. Some evidence suggests that the individual amino acids for hormone-receptor interactions, as well as subunit interactions.

Synthetic peptides have been used to study the structure-function relationships between the glycoprotein hormones and their receptors because large amounts of chemically defined and pure material can be generated. Peptides that duplicate defined regions of the α subunit can inhibit binding of $^{125}$I-hCG to the LH receptor in rat ovarian membranes (Charlesworth et al., 1987) and can inhibit binding of $^{125}$I-TSH to FRTL-5 cell membranes (Morris et al., 1988). Peptides representing various regions of the β subunits of hCG (Keutmann et al., 1987, 1989), hTSH (Morris et al., 1990), or human follitropin (Santa Coloma and Reichert, 1990) also inhibit binding of radiiodinated hormone to receptor. Additionally, some α and β peptides exhibit low but significant biological activity (Keutmann et al., 1987; Santa Coloma and Reichert, 1990; Erickson et al., 1990).

Charlesworth et al. (1987) defined a region in the α subunit with high inhibitory activity (IC$_{50}$ value of 10 nM) in a competitive LH/CGR receptor binding assay. This region, α26–46 (Fig. 1), is particularly well conserved across species lines. In several different species, from carp to human, there are 17 of 21 identical residues (Ward and Bousfield, 1990). This region includes Met-29 and Cys-31, which seem to be involved in or near the receptor-binding site, as well as Tyr-37 and Lys-45, which seem to be located in or near the subunit interface region (Pierce and Parsons, 1981). To analyze more precisely which residues are important for receptor interaction, we synthesized a series of peptides truncated from either the amino or carboxyl terminus and a series of peptides in which each residue was individually substituted with alanine. These studies indicate that 1) the core sequence of 30–44 is necessary for full inhibitory activity and 2) Arg-35 and Phe-33 are particularly important for activity, whereas Ser-43, Arg-42, Cys-32, and Cys-31 are necessary but not as crucial for full activity. These results provide insight into which amino acid residues are important for interactions between hCG and the LH receptor and, by analogy, residues important for the interactions between the other glycoprotein hormones and their receptors.

MATERIALS AND METHODS

Peptide Synthesis and Purification—Peptides were synthesized by standard solid phase techniques (Stewart and Young, 1984) either manually or using an automated ABI 431A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). Ten truncation peptides were synthesized on p-methylbenzhydrylamine resin with t-butylox-
peptide that inhibits 50% of the "I-hCG binding to the LH receptor (Heinrikson and Meredith, 1984) and the concentration curves (Fig. 2) were generated, and IC₅₀ values were determined for each concentration of standard hCG or peptide. Competition curves were obtained from Organon Pharmaceuticals and purified as described elsewhere. The potency of peptide 26-46 was underlined.

Arginine was coupled as the 1-hydroxybenzotriazole active ester. The peptides substituted with alanine were synthesized on DMPP resin by a modification of the "tea bag" protocol (Houghten, 1985) using activated pentafluorophenyl esters of Fmoc-L-amino acid derivatives. All peptides synthesized with Fmoc derivatives were cleaved from the resin and depurified with 91% trifluoroacetic acid, containing 2% anisole, 2% ethanedithiol, and 5% H₂O, v/v, for 1.5 h at 25 °C. Peptides removed from p-methylbenzhydrolamine and DMPP resin have carboxyl-terminal amines that are used for internal sequences to avoid the carboxyl-terminal charge. Crude peptides were purified by column chromatography (Sephadex G-25, Pharmacia) in 1 M acetic acid and/or reversed phase high performance liquid chromatography (Vydac C-4, a linear gradient of 5–80% acetonitrile in 0.1% trifluoroacetic acid over 40 min). Amino acid composition was verified by analysis of acid hydrolysates (1 h at 150 °C or 24 h at 100 °C in 6 M HCl, in vacuo) using precolumn derivatization with phenylthiocyanate (Heinrikson and Meredith, 1984) and the Waters PicoTag system (Waters Associates, Millipore Corp., Milford, MA). The composition of each peptide was in good agreement with the expected sequence.

hCG/LH Radioimmunoassay—Competition assays were used to measure the inhibitory activity of each peptide. Briefly, ¹²⁵I-hCG (radioiodinated by the method of Greenwood and Hunter (1963) as modified in Lee and Ryan (1973)) in assay buffer (40 mM Tris, pH 7.3, 0.025% bovine serum albumin, w/v) for 12–16 h at 25 °C in a shaking water bath. Membrane-bound ¹²⁵I-hCG was separated from free ¹²⁵I-hCG by centrifugation. The membrane pellets were washed with assay buffer, then counted in a Beckman Gamma 4000 γ counter. Total assay volume was 500 μl. Nonspecific binding was determined in parallel tubes that were supplemented with 100-fold excess unlabeled hCG. The percentage of specific binding remaining (% Bo) was calculated for each concentration of standard hCG or peptide. Competition curves (Fig. 2) were generated, and IC₅₀ values were determined. The IC₅₀ is that concentration of standard hCG or competitor peptide that inhibits 50% of the ¹²⁵I-hCG binding to the LH receptor in the membrane preparation.

Hormone Preparations and Chemicals—hCG (∼3000 IU/mg) was obtained from Organon Pharmaceuticals and purified as described (Charlesworth et al., 1987). Chemicals used for peptide synthesis and purification were supplied by Aldrich, Applied Biosystems, Inc., and Burdick & Jackson Laboratories Inc. (Muskegon, MI). Amino acids were purchased from Advanced Chemtech (Louisville, KY), Applied Biosystems, Inc., and Bachem (Torrance, CA). Resins were obtained from Applied Biosystems, Inc. and Calbiochem. All other reagents were of the highest reagent grade available.

Circular Dichroic Analysis—CD spectra were recorded using a Jasco (Tokyo, Japan) model J 810 spectropolarimeter in either 5 mM TES, pH 7.0, or 90% trifluoroethanol in the same buffer. Peptide solutions, 150 μg/ml, were scanned from 260 to 175 nm 8 times in a cuvette with a path length of 0.1 cm. Constrained mean percent content of α helix, β sheet, β turn, and unordered structure was calculated by the Jasco analysis program using a set of standard proteins with low α helic content (Compton and Johnsen, 1986; Erickson et al., 1990).

RESULTS

Amino-terminal Truncations—To define the amino-terminal boundary required for inhibitory activity, a nested set of peptides was synthesized. Each peptide was shortened by 1 residue from the amino terminus. In addition, three peptides were synthesized that were extended beyond Leu-26. Table I summarizes the peptides synthesized and tested for this study as well as their IC₅₀ values. Extending the amino terminus to Ile-25, Pro-24, or Ala-23 did not significantly enhance the inhibitory activity of the peptide 26-46 (Table I) (Fig. 3). Furthermore, peptides 27-46, 28-46, 29-46, and 30-46 had activity similar to that of intact 26-46. When Gly-30 was removed, yielding peptide 31-46, the IC₅₀ increased from 10 nM to 40 μM, that is, the potency decreased by 4-fold. When Cys-32 was removed, peptide 32-46 exhibited potency comparable with the control peptide 26-46. Peptide 33-46, which lacked Cys-32, had an IC₅₀ of 78 μM. The potency of 33-46 decreased almost 8-fold, compared with 26-46. The shortest peptides in the series, 34-46 through 41-46, had essentially no activity at the highest concentration tested (extrapolated IC₅₀ values >600 μM, data not shown). These data indicate that the amino-terminal boundary required for inhibitory activity was Gly-30. For further experiments, however, Met-29 was used as the amino-terminal boundary, since it had been previously implicated in hormone-receptor interactions (Cheng, 1976a, 1976b).

Carboxyl-terminal Truncations—A second set of truncated peptides (Table I) (Fig. 4) was synthesized with the carboxyl-terminal boundary defined as Met-29. These peptides were synthesized with the Fmoc-L-amino acid derivatives added as symmetric anhydrides.
peptides 34-46, 35-46, 36-46, 37-46, 38-46, 39-46, and 41-46 were also tested and had essentially no activity at the highest concentration tested, IC_{50} > 600 \mu M. The starred (*) IC_{50} values are significantly different from control values, p < 0.01.

| Peptide     | IC_{50} ± S.E. | Peptide     | IC_{50} ± S.E. |
|-------------|----------------|-------------|----------------|
| 26-46       | 10 ± 3         | M29A        | 13 ± 7         |
| 27-46       | 7 ± 2          | G30A        | 15 ± 6         |
| 24-46       | 6 ± 2          | C31A        | 29 ± 11        |
| 25-46       | 9 ± 1          | C32A        | 32 ± 15        |
| 27-46       | 19 ± 5         | F38A        | 55 ± 9*        |
| 28-46       | 12 ± 5         | S34A        | 15 ± 5         |
| 29-46       | 14 ± 7         | R35A        | 233 ± 62*      |
| 30-46       | 16 ± 2         | Y37A        | 47 ± 4         |
| 31-46       | 40 ± 4*        | P38A        | 43 ± 5         |
| 32-46       | 20 ± 4         | T39A        | 39 ± 11        |
| 33-46       | 78 ± 9*        | P40A        | 47 ± 9         |
| 29-40       | 179 ± 110*     | L41A        | 41 ± 8         |
| 29-41       | 202 ± 92*      | R42A        | 64 ± 13*       |
| 29-42       | 103 ± 18*      | S43A        | 67 ± 20*       |
| 29-43       | 62 ± 15*       | K44A        | 45 ± 3         |
| 29-44       | 32 ± 7         |             |                |
| 29-45       | 24 ± 5         |             |                |

FIG. 3. Amino-terminal truncation peptides. Activity relative to control peptide 26-46 is shown. Peptides were tested in competition assays as described under "Materials and Methods" and in Fig. 2. The starred (*) bars in the graph indicate those values significantly different from the control peptide 26-46, p < 0.01. Peptides 34-46, 35-46, 36-46, 37-46, 38-46, 39-46, 40-46, and 41-46 had less than 5% of control activity. The inset includes the sequences of three selected peptides, which serve as landmarks.

Fig. 4. Carboxyl-terminal truncation peptides. Activity relative to control peptide 29-46 is shown. Peptides were tested in competition assays as described under "Materials and Methods" and in Fig. 2. The starred (*) bars are significantly different from control peptide 29-46, p < 0.01. The inset includes sequences of three selected peptides, which serve as landmarks.

Fig. 5. Alanine-substituted peptides. Activity relative to control peptide 29-46 is shown. Peptides based on the sequence 29-46 were substituted with alanine at residues 29-35. The peptides were then tested in competition assays as described under "Materials and Methods" and in Fig. 2. Significantly different values, compared with the control peptide 29-46, are starred (*), p < 0.01. The inset includes the sequences of three selected peptides which serve as landmarks.

Each residue was substituted individually with alanine. Alanine was chosen because it is an amino acid abundant in proteins and has a fairly small neutral side group that should not disrupt any structure the peptides may adopt. Glycine, on the other hand, with only hydrogen as the side group, allows too much flexibility in the peptide backbone (Schulz and Schirmer, 1979). The first set of peptides was substituted at residues 29-35 in the sequence 29-46. The data in Table I and Fig. 5 show that when Arg-35 was substituted with alanine, the potency of the peptide was decreased to less than 10% of control activity. Substitution of Phe-33 with alanine caused the IC_{50} to increase to 55 \mu M. The potency of the peptide was decreased about 4-fold. Cys-31 and Cys-32 appear to be less important, since the alanine substitutions increased the IC_{50} values somewhat but not significantly. The second set of peptides was substituted at residues 37-44 in the sequence 29-44. The data in Table I indicate that Arg-42 and Ser-43 were important, since substitution with alanine caused the IC_{50} values to increase a small but statistically
Residues Important for hCG-LH Receptor Interactions

Circular dichroic spectra were collected and analyzed as described under “Materials and Methods.” TES represents the data from spectra collected from peptide solutions in 5 mM TES, pH 7.0; TFE represents that data from peptide solutions in 90% trifluoroethanol in TES.

| α Subunit peptides | α Helix | β Sheet | β Turn | Unordered |
|--------------------|---------|---------|--------|-----------|
|                    | TES     | TFE     | TES    | TFE       |
| 29-46              | 1.9     | 9.6     | 45.3   | 33        |
| R35A               | 1.8     | 9.9     | 47.6   | 39.4      |
| F33A               | 3.9     | 11.7    | 45.2   | 25.4      |
| 29-44              | 2.1     | 6.4     | 51.1   | 37.7      |
| R42A               | 2       | 6.5     | 50.9   | 36.6      |
| S45A               | 1.7     | 7.7     | 50.9   | 36.6      |
| P40A               | 1.2     | 13.5    | 48.7   | 32.3      |
| 26-46              | -0.1    | 11.7    | 51.2   | 32        |

α subunit 1.5 6.9 49.2 41.6 22.7 17.6 26.9 34

**Table II**

Analysis of circular dichroic data of selected peptides in aqueous and helicogenic solvents

Cys-31 and Cys-32 are also important but to a lesser degree. Cys-31 has been shown previously to be involved in receptor interactions by chemical modification experiments (Giudice and Pierce, 1979); however, the other 3 residues have not. Arg-42 could be involved in ionic interactions with the receptor. Ser-43 could potentially serve as a hydrogen bond donor with an appropriate residue(s) on the receptor. It is widely accepted that Cys-31 and Cys-32 are disulfide-bonded with Cys-7 and Cys-16, respectively. Since the Cys-7-Cys-31 bond is the first one reduced (Pierce and Parsons, 1981), it must be relatively surface accessible. Cys-31, Cys-32, and Phe-33 are predicted to be in a turn structure, whereas Arg-35 and Ser-34 are predicted to be in the beginning of a β sheet structure (Erickson et al., 1990). These residues and, in fact, most of the amino acids in this region are absolutely conserved among all species analyzed to date (Ward and Bousfield, 1990). Apparently, in order to maintain functional integrity of the glycoprotein α subunits, these residues have been conserved throughout evolution.

Substitution of Pro-38 or Pro-40 with alanine did not affect the IC50 values. This was somewhat unexpected since proline residues can introduce bends in the polypeptide backbone.

Synthetic peptides have proven to be useful probes for the study of structure-function relationships between hCG and the LH receptor. In this study, we have used synthetic peptides to determine the importance of particular amino acid residues for the inhibitory activity of α26-46, a peptide that inhibits binding of [125I]-hCG to rat ovarian membranes (Charnesworth et al., 1987). Our data suggest that the core sequence 30-44 contains all the residues necessary for full inhibitory activity. Met-29, which was used as the amino-terminal boundary in subsequent peptides, is involved in the interaction of hormones with receptors, as indicated by chemical modification experiments (Cheng, 1976a, 1976b). Therefore, our data are consistent with the idea that these residues are in or near the receptor interaction region of the α subunit. The carboxyl-terminal boundary appears to be lys-44, lys-45, and thr-46 can be deleted with little effect on inhibitory activity. Lys-49 in the bovine α subunit can be cross-linked to asp-113 in the β subunit of bovine LH (Weare and Reichert, 1979), so it appears that lys-44 and -45 (equivalent to Lys-48 and -49, respectively, in the bovine α subunit) are in or near the region where the subunit interface and the receptor interaction regions come together. Other data have also suggested that the subunit interface and the receptor interaction regions are, in fact, close together (Ryan et al., 1989). Interestingly, when lys-44 is substituted with alanine, the potency of that peptide is the same as peptide 29-44. So it appears that loss of the residue by truncation is more detrimental than substitution with alanine. This might indicate that Lys-44 is required for a critical peptide length and not necessarily for the positively charged side group. Secondary structure predictions for this region indicate that 9 of 21 residues could be involved in turn structures (Erickson et al., 1990).

Arg-35 and Phe-33 are important. For interaction of α26-46 and hCG with the LH receptor. The positive charge of Arg-35 could be involved in ionic interactions between hCG and the receptor. Phe-33 could be involved in hydrophobic or ring-stacking interactions with hydrophobic or aromatic residues in the receptor. Future experiments using lysine or histidine for Arg-35 and other hydrophobic residues such as tyrosine, tryptophan, or isoleucine for Phe-33 will address these possibilities. Arg-42, ser-43, cys-31, and cys-32 are also important but to a lesser degree. Cys-31 has been shown previously to be involved in receptor interactions by chemical modification experiments (Giudice and Pierce, 1979); however, the other 3 residues have not. Arg-42 could be involved in ionic interactions with the receptor. Ser-43 could potentially serve as a hydrogen bond donor with an appropriate residue(s) on the receptor. It is widely accepted that Cys-31 and Cys-32 are disulfide-bonded with Cys-7 and Cys-16, respectively. Since the Cys-7-Cys-31 bond is the first one reduced (Pierce and Parsons, 1981), it must be relatively surface accessible. Cys-31, Cys-32, and Phe-33 are predicted to be in a turn structure, whereas Arg-35 and Ser-34 are predicted to be in the beginning of a β sheet structure (Erickson et al., 1990). These residues and, in fact, most of the amino acids in this region are absolutely conserved among all species analyzed to date (Ward and Bousfield, 1990). Apparently, in order to maintain functional integrity of the glycoprotein α subunits, these residues have been conserved throughout evolution.

Substitution of Pro-38 or Pro-40 with alanine did not affect the IC50 values. This was somewhat unexpected since proline residues can introduce bends in the polypeptide backbone. Substitution with alanine could relieve this bending. These peptides were also tested for inhibitory activity of TSH binding to porcine thyroid membranes. Peptides active in the ovarian membrane system with hCG were also active in the TSH competition assays. These data are consistent with the fact that the same α subunit interacts with the two different yet related β subunits. One would expect a similar subunit interaction region(s) on the α subunit, and perhaps some similar and some subtly different receptor interaction region(s). In fact, this is what is seen. Leinung et al. demonstrated that Gly-30, Phe-33, Arg-35, Arg-42, and Lys-44 are important for maintaining inhibitory activity of α26-46 in the TSH receptor assay, as was shown in this work for the hCG-LH receptor assay. However, some subtle differences were apparent. Cys-31 and Lys-45 were found to be important in the TSH system but not in the hCG system. In this study, Ser-43 was shown to be somewhat important in the hCG system but not in the TSH system.

The data presented in this report indicate that the core peptide 30-44 has within it two regions of importance. The

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first region seems to lie between residues 30 and 35, the second between residues 42 and 44. Arg-35 and Phe-33 seem to be particularly important in the amino-terminal region, whereas Arg-42 and Ser-43 appear to be the important residues in the carboxyl-terminal region. Previous studies (Charlesworth et al., 1987) showed that two overlapping peptides from the human α subunit, 21-35 and 31-45, had inhibitory activity. However, the potencies were somewhat less than 26-46 (26-46, 10 μM; 21-35, 12 μM; and 31-45, 18 μM). This is explained by these data. Each of the overlapping peptides contains only one of the important regions in the core peptide 30-44. The peptide 26-46 (Charlesworth et al., 1987) and the core peptide 30-44 contain both sites and have higher inhibitory activity than either of the previously mentioned overlapping peptides.

We have defined a core sequence in the α subunit of hCG, 30-44, that seems to be involved in hormone-receptor interactions. In addition, we have provided insight into which residues are particularly important for those interactions. In this work, we have demonstrated the utility of synthetic peptides as probes into structure-function relationships between hCG and LH receptor.

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