Identification of Two Amino Acid Residues on the Extracellular Domain of the Lutropin/Choriogonadotropin Receptor Important in Signaling*

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The lutropin/choriogonadotropin receptor (LH/CG-R) is a G protein-coupled receptor with a relatively large extracellular domain. The cDNAs of LH/CG-R wild type and 15 point and double mutants, which encoded residues of opposite charge to that of wild type, were transiently transfected into COS-7 cells. Human choriogonadotropin (hCG) binding was determined, as was hCG-mediated cAMP production. Most of the replacements resulted in no substantive effect on the binding affinity of hCG to LH/CG-R or on hCG-stimulated cAMP production, although the mutants expressed at a lower level than LH/CG-R wild type. The most interesting observation was noted with two point mutants of LH/CG-R, Glu332→Lys and Asp333→Lys, which bound hCG but failed to give increased cAMP production. Several of the mutant forms of LH/CG-R that expressed at low levels were further analyzed by soluble binding assays and Western blots. There was no evidence of any significant degree of intracellular trapping of hCG-binding mutant receptors. The expected major (93 kDa) forms were found for LH/CG-R wild type and several of the mutants. The Lys325→Asp and Asp333→Lys mutants exhibited primarily the lower M, form, indicating that receptor processing was impaired or that the mutant higher M form was more rapidly degraded than LH/CG-R wild type. These results demonstrate that Glu332 and Asp333, which are located near the first transmembrane helix, are important in receptor activation, while other conserved ionizable residues of LH/CG-R appear important in cell surface expression or stability but not in binding or signaling.

Considerable data have been obtained to delineate specific amino acid residues and regions on the hCG α and β subunits that participate in receptor binding and signal transduction. The recent availability of a crystal structure of partially de-glycosylated hCG (Lapthorn et al., 1994; Wu et al., 1994) adds a new dimension to the analysis of glycoprotein hormone structure-function relationships. In contrast, relatively little is known about the conformation of the receptor and specific amino acid residues that may be involved in hormone binding and signaling, although mutagenesis has been used to map certain of these regions of LH/CG-R (Xie et al., 1990; Ji and Ji, 1991, 1993; Segaloff and Ascoli, 1993; Ji et al., 1993; Quintana et al., 1993; Wang et al., 1993a, 1993b). A perusal of the available amino acid sequences of LH/CG-R from several species shows the presence of a number of conserved ionizable amino acid residues, many of which are also conserved in the follicle-stimulating hormone receptor and the thyrotropin receptor. Such conserved residues may be important in signaling or as hormone contact sites, e.g. to oppositely charged conserved side chains on LH, CG, follitropin, and thyrotropin.

The present study addresses 11 of the ionizable groups on the extracellular portion of rat LH/CG-R: Arg21 and Arg71 (encoded by exon 1), Lys55 (encoded by exon 2), Glu68 and Glu68 (encoded by exon 3), Arg144 and Lys121 (encoded by exon 5), Glu221 and Lys235 (encoded by exon 9), and Glu322 and Asp333 (encoded by exon 11). Site-directed mutagenesis was used to prepare single and double mutants where each ionizable side chain was replaced by one of opposite charge. The data suggest that two point mutants of LH/CG-R, Lys322 and Lys333, bind hCG with nearly the same affinity as LH/CG-R wild type but exhibit diminished signaling.

MATERIALS AND METHODS

Supplies—The Transformer™ site-directed mutagenesis kit was obtained from Clontech Laboratories, Inc, the Sequanase version 2.0 kit was a product of U. S. Biochemical Corp., the plasmid kit was from Qiagen Inc. (Chatsworth, CA), and the Magic™ Minipreps DNA Purification System was from Promega. Bovine serum albumin, isobutylmethylxanthine, DEAE-dextran, chloroquine, dextran sulfate, dimethyl sulfoxide, and Nonidet P-40 were purchased from Sigma. hCG (CR-121) was provided by National Institutes of Health and by Dr. Steven Birken (Columbia University, NY); [125I]hCG (100–150 μCi/μg) was from ICN (Horsham, PA). The cAMP radioimmunoassay kit was from DuPont NEN. Dulbecco’s modified Eagle’s medium and fetal bovine serum were obtained from Life Technologies, Inc.; NuSerum was from Collaborative Biomedical Products (Bedford, MA).

Mutagenesis of the LH/CG-R cDNA—The cDNA for the rat LH/CG receptor was kindly provided by Dr. William Moyle (Piscataway, NJ) in the expression vector pSVL. The desired 21-base deoxynucleotides were synthesized by Dr. Rudolf Werner (University of Miami, FL) on an Applied Biosystems model 380B DNA synthesizer and by the Molecular Genetics Core Facility of the University of Georgia. In vitro mutagenesis was performed using the Transformer™ site-directed mutagenesis kit as described (Deng and Nickoloff, 1992), and mutated plasmid clones were identified by double-stranded sequencing.
Expression of LH/CG-R—COS-7 Cells (African green monkey kidney cells), obtained from ATCC CRL, were grown at 37 °C in humidified air containing 5% CO₂ in the following medium: Dulbecco’s modified Eagle’s medium (90%), fetal bovine serum (10%), and 100 units/ml each of penicillin and streptomycin. Cells were transiently transfected at 60–80% confluency by the DEAE-dextran method. Transfection efficiencies were determined by cotransfection of pSV-LH/CG-R wild type and mutant cDNAs. 48 h after transfection, the cells were stained for β-galactosidase. This was accomplished by first washing the cells with PBS five times, then fixing them in 0.05% (v/v) glutaraldehyde solution in PBS for 10–15 min. After three additional washes with PBS, the cells were incubated in a solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl β-d-galactosidase 1 mg/ml MgCl₂, 20 mM potassium ferricyanide/ ferrocyanide in PBS for at least 1 h at 37 °C. The cells transfected with the pSV-β-galactosidase control plasmid were readily visualized under the microscope and counted. From the total number of cells in the field, transfection efficiencies were obtained.

[125I]hCG Binding to COS-7 Cells—At 48 h post-transfection, the cells were assayed for [125I]hCG binding, along with untransfected control cells. Cells (1 × 10⁶ cells/well) were washed twice with serum-free Waymouth’s medium containing 1 mg of bovine serum albumin/ml, then 1 ml of this medium was added to each well. For competitive binding assays, [125I]hCG (10⁷ cpm) and different concentrations of unlabeled hCG were added to each well, while for direct binding assays, cells were incubated with increasing concentrations of [125I]hCG. Specific binding was determined by inclusion of a 1000-fold excess of unlabeled hormone. The cells were incubated overnight with gentle shaking at room temperature. On the following day, cells were washed twice with PBS, then trypsinized, collected, and counted in a γ counter. Specific binding of [125I]hCG in the absence of unlabeled hormone gave 4,000–5,000 cpm, and nonspecific binding was about 5%. The results were analyzed by In-Plot (Graphpad Software, San Diego, CA), by Ligand Program (Munson and Rodbard, 1980), and by graphical analysis, the agreement between the various methods being good. Unless indicated otherwise, the data from a typical experiment are shown as mean ± S.E.

[125I]hCG Binding to Detergent-solubilized COS-7 Cell Extracts—This procedure is based on that described by others (Xie et al., 1990; Thomas and Segaloff, 1994). Control cells and transfected cells (48 h) were placed on ice and washed twice with cold 0.15M NaCl, 20 mM Hepes, pH 7.4. They were then scraped into a small volume of the same buffer containing 5% CO₂ in the following medium: Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin/ml, and 1 ml of this medium was added to each well. For competitive binding assays, [125I]hCG (10⁷ cpm) and different concentrations of unlabeled hCG were added to each well, while for direct binding assays, cells were incubated with increasing concentrations of [125I]hCG. Specific binding was determined by inclusion of a 1000-fold excess of unlabeled hormone. The cells were incubated overnight with gentle shaking at room temperature. On the following day, cells were washed twice with PBS, then trypsinized, collected, and counted in a γ counter. Specific binding of [125I]hCG in the absence of unlabeled hormone gave 4,000–5,000 cpm, and nonspecific binding was about 5%. The results were analyzed by In-Plot (Graphpad Software, San Diego, CA), by Ligand Program (Munson and Rodbard, 1980), and by graphical analysis, the agreement between the various methods being good. Unless indicated otherwise, the data from a typical experiment are shown as mean ± S.E.

RESULTS

The DNA sequences of the rat LH/CG-R wild type and mutants were determined for the mutated and adjacent regions in all clones by dideoxynucleotide sequencing. Following this confirmation of the desired wild type and mutant clones, transfection efficiencies were monitored by transient cotransfection of pSV-LH/CG-R wild type and mutants into COS-7 cells with pSV-L-β-galactosidase. The cotransfected cells were stained in situ, and transfection efficiencies of 5–10% were estimated.

[125I]hCG binding to transfected cells was determined via competition of [125I]hCG with increasing concentrations of unlabeled hCG. The results from multiple studies using competition and saturation binding with LH/CG-R wild type yielded an apparent Kᵦ of 0.17 ± 0.02 nM (n = 11) and an average of about 1.0 ± 10⁴ receptors/cell, uncorrected for transfection efficiencies (Table I). We also found that untransfected COS-7 cells, as expected (Loosfelt et al., 1989), do not contain LH/CG-R (data not shown).

Figs. 1–3 show the results of competition and saturation binding of the LH/CG-R point mutants, and the Kᵦ values and receptor numbers are given in Table I. Generally, the Kᵦ values are within severalfold of that obtained for wild type LH/CG-R, indicating that no major alteration in hormone affinity accompanied the mutations. Several of the receptor point mutants, however, had Kᵦ values slightly lower than that of wild type, e.g. Lys⁻⁵⁵ → Arg, Lys⁻⁵⁵ → Asp, and Lys⁻¹²¹ → Asp. In contrast, the number of receptors/cell is, with the exception of the Arg⁻¹¹¹ replacements and the Lys⁻⁵⁵ → Arg replacement, considerably less than that obtained for the wild type receptor. Most of the other LH/CG-R mutants yield on the order of 1.0 × 10⁴ receptors/cell, although receptors with the Lys⁻¹²¹→ Asp and Asp⁻²³¹→ Lys replacements contained < 1.0 × 10³ receptors/cell, again uncorrected for transfection efficiencies.

Two double mutants of LH/CG-R, E65K,E68K and
Parameters of hCG binding and stimulation of cAMP production in COS-7 cells expressing LH/CG-R wild type and mutants

Cells were transiently transfected with pSVL containing the cDNA to rat LH/CG-R wild type or mutant. After 48 h, competitive binding studies with hCG and [125I]hCG were performed to determine IC50 values, Kd values, and average receptor numbers/cell; direct binding studies were also conducted with [125I]hCG in several cases to determine Kd values and average receptor numbers/cell. The production of cAMP in response to hCG was determined in transfected cells, and the ED50 is given. The results are presented as mean ± S.E. when n ≥ 2; for n = 1, the results are given as mean ± range, i.e. upper and lower values.

### Table I

| LH/CG-R        | Kd (nM) | cAMP ED50 (nM) | Receptors/cell* |
|----------------|---------|----------------|-----------------|
| Wild type      | 0.17 ± 0.02 (11) | 0.05 ± 0.02 (10) | 100 ± 14 |
| Arg114→Asp    | 0.12 ± 0.04 (5)  | 0.08 ± 0.03 (3)  | 62 ± 4  |
| Arg111→Lys    | 0.15 ± 0.06 (2)  | 0.01 ± 0.00 (2)  | 115 ± 22 |
| Arg11→Asp     | 0.10 ± 0.02 (2)  | 0.05 ± 0.03 (2)  | 96 ± 14 |
| Lys55→Arg     | 0.05 ± 0.01 (2)  | 0.02 ± 0.00 (2)  | 76 ± 18 |
| Lys55→Asp     | 0.04 ± 0 (3)     | 0.05 ± 0.02 (2)  | 17 ± 4  |
| Glu68→Lys     | 0.09 ± 0.07 (2)  | 0.02 ± 0.01 (2)  | 42 ± 12 |
| Glu68→Lys     | 0.07 ± 0.05 (3)  | 0.02 ± 0.01 (2)  | 32 ± 15 |
| E65K,E68K     | 0.05 ± 0.02 (3)  | 0.07 ± 0.02 (3)  | 6 ± 1   |
| Arg114→Glu    | 0.08 ± 0.04 (2)  | 0.02 ± 0.01 (2)  | 33 ± 8  |
| Lys221→Asp    | 0.05 ± 0.03 (6)  | 0.09 ± 0.03 (3)  | 7 ± 2   |
| Glu221→Lys    | 0.19 ± 0.14 (4)  | 0.03 ± 0.01 (3)  | 45 ± 6  |
| Lys235→Asp    | 0.17 ± 0.04 (4)  | 0.10 ± 0.01 (2)  | 17 ± 5  |
| Glu332→Lys    | 0.16 ± 0.04 (3)  | >2.7 (3)*        | 20 ± 7  |
| Asp333→Lys    | 0.24 ± 0.06 (2)  | >2.7 (2)*        | 9 ± 4   |
| E332K,D333K   | >2.7 (1)*        | >2.7 (1)*        | 0*     |

*The average value for wild type receptors (9.8 ± 1.3 × 10^6 receptors/cell) was normalized to 100%, and the numbers for mutant receptors are presented as a percentage of that for wild type. The numbers are not corrected for transfection efficiencies (5–10%) and thus reflect approximate relative values.

*Represents the highest concentration of hCG used.

*No detectable receptors under the conditions used.

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**Fig. 1.** hCG binding to COS-7 cells transfected with pSVL vectors containing the wild type and mutant LH/CG-R cDNAs. Competition binding of [125I]hCG and hCG to seven point mutant forms of LH/CG-R with replacements of Arg21, Arg31, Lys55, Glu65, and Glu68 is shown. In each case wild type (Wt) receptor was included for comparison.

**Fig. 2.** hCG binding to COS-7 cells transfected with pSVL vectors containing the wild type and mutant LH/CG-R cDNAs. Competition binding of [125I]hCG and hCG to six point mutant forms of LH/CG-R with replacements of Arg114, Lys221, Lys235, Lys332, Glu332, and Asp333 is shown. Wild type (Wt) receptor was also included for comparison.

E332K,D333K, were also characterized by direct and competitive binding (Fig. 4). The Kd of the former double mutant was somewhat lower than that of LH/CG-R wild type, and the average number of receptors/cell was 625 ± 106 (n = 3) (Fig. 4 and Table I). No specific binding was detected with LH/CG-R (Lys332, Lys333) under the conditions used.

Soluble binding assays were performed on several of the point LH/CG-R mutants that exhibited a relatively low number of surface receptors (Table II). Compared to wild type LH/CG-R, the Lys121 → Asp and Lys235 → Asp mutants yielded only very low levels of specific binding. In contrast, the Glu332 → Lys and the Asp333 → Lys mutants gave specific binding intermediate to that of control and wild type LH/CG-R.

To assess the relative amounts of mutant receptors in cells with a low number of surface receptors, e.g. <25% LH/CG-R wild type, Western blots were obtained. As reported by others (Segaloff and Ascoli, 1993), purified rat LH/CG-R gives an apparent molecular mass of about 93 kDa. Transiently transfected COS-7 cells express a major band of about 93 kDa (Fig. 5), which is presumed to correspond to the 85-kDa form obtained by Hipkin et al. (1992) using stably transfected human
embryonic kidney 293 cells. In addition, a minor band of apparent molecular mass of 78 kDa is also detected from LH/CG-R wild type, probably corresponding to the 68-kDa band reported by Hipkin et al. (1992), as are apparent higher and lower Mₘ lower and upper Mₘ bands, which may be nonspecifically detected. Interestingly, the LH/CG-R point mutants, Lys¹²¹ → Asp and Glu³³² → Lys, as well as the double mutant, E₆₅₅K-E₆₈₆₈K, yielded both the 93- and 78-kDa forms in about equal amounts, while the point mutants, Lys¹²¹ → Asp, Lys³³⁵ → Asp, and Asp³³³ → Lys, exhibit predominationally lower Mₘ form (Fig. 5 and Table II). Equal amounts of cell lysate protein were added from cells transfected with wild type and mutant LH/CG-R cDNAs to facilitate comparison of the relative total amount of immunoreactive receptor in each case. This amount of protein, which was necessary to visualize the relatively low levels of mutant receptors, resulted in overloading the gel with wild type LH/CG-R and obscuring the resolution of the major and minor bands. These were resolved, however, by densitometric scanning of the blots and by adding reduced amounts of cell lysate protein (data not shown). Comparable studies on cell lysates that were not purified by lectin chromatography gave similar results (data not shown), indicating that no other non-glycosylated forms of LH/CG-R are present.

The functionality of each of the mutant receptors was evaluated by cAMP production in response to added hCG. Figs. 6 and 7 show typical results for those receptor point mutants that exhibited ED₅₀ values and maximal cAMP levels similar to LH/CG-R wild type. Interestingly, the LH/CG-R(Aasp¹²¹) mutant, which gave a low level of expression, exhibited an ED₅₀ and maximal cAMP response like that of wild type receptor. In contrast, the LH/CG-R point mutants Lys³³² and Lys³³³ were non-responsive to added hCG (Fig. 8). Likewise, the double mutant, LH/CG-R(Lys³³², Lys³³³), for which no receptors could be detected, exhibited no hCG-responsive cAMP production. The ED₅₀ values for cAMP production by hCG in the transiently transfected COS-7 cells are given in Table I.

**DISCUSSION**

The results presented herein enable us to conclude that replacement of either Glu³³² or Asp³³³ with Lys in LH/CG-R yields mutant receptors that bind hCG as well as LH/CG-R wild type but exhibit diminished signaling. Thus, this region of the extracellular domain of the receptor, which is <10 amino acid residues from the first transmembrane helix, appears critical in gonadotropin-mediated receptor activation. While receptor numbers are reduced with these particular mutants relative to LH/CG-R wild type, other single and double receptor mutants with comparable receptor densities, e.g. Asp²⁵⁵, Asp¹²¹, Lys⁵⁵⁵, and Lys⁶⁸₈ gave cAMP responses like those of LH/CG-R wild type. Thus, the reduced hormone-mediated signaling noted with Lys³³² and Lys³³³ is believed to be an intrinsic property of the mutant receptors and not a result of diminished receptor number.

The two Mₘ forms of LH/CG-R expressed in COS-7 cells, i.e. 93 and 78 kDa as obtained herein, were first reported by Ascoli and colleagues (Hipkin et al., 1992) who expressed the receptor in stably transfected human embryonic kidney 293 cells, al-
though the molecular mass values they determined were somewhat different than the values we obtained, e.g. 85 and 68 kDa, respectively. They reported data showing that the lower $M_r$ form was a precursor to the higher $M_r$ species; in addition, they suggested that the lower $M_r$ form bound hormone with reduced affinity relative to the major 85-kDa form. Since a variety of $M_r$ values has been reported for LH/CG-R purified from various tissues/species and expressed in cell lines (cf. Segaloff and Ascoli, 1993), the glycosylation patterns may differ depending upon the source of the receptor. In any case, we find that the lower $M_r$ form of rat LH/CG-R expressed in COS-7 cells is predominant for most of the mutants that lead to low receptor densities. Since there is no evidence of significant intracellular accumulation of these mutant forms, this observation suggests that the mature mutant receptors have a short half-life relative to LH/CG-R wild type, that processing proceeds less efficiently, or that the mRNA is degraded more rapidly or translated less efficiently.

Single replacements of LH/CG-R at several conserved sites with oppositely charged amino acid residues have no significant effect on the apparent $K_d$ relative to LH/CG-R wild type. That these particular replacements in LH/CG-R result in little or no change in the apparent $K_d$ of hCG binding does not necessarily mean that they are not contact sites. For example, Clackson and Wells (1995) replaced each of the 33 side chains on the extracellular domain of human growth hormone recep-

Fig. 6. Effects of hCG on cAMP production in COS-7 cells transfected with pSVL vectors containing mutant and seven wild type LH/CG-R cDNAs. Results are given for replacements of Arg$^{21}$, Arg$^{31}$, Lys$^{55}$, Arg$^{114}$, and Lys$^{221}$ of LH/CG-R. Data of wild type (WT) LH/CG-R and untransfected (control) cells are shown for comparison.

Fig. 7. Effects of hCG on cAMP production in COS-7 cells transfected with pSVL vectors containing five point mutants and one double mutant of LH/CG-R. Data are presented for lysine replacements of Glu$^{65}$, Glu$^{68}$ (single mutants of each and a double mutant), and Glu$^{221}$ and for aspartic acid replacement of Lys$^{235}$. Results are also shown for wild type (WT) LH/CG-R and untransfected (control) cells. The cAMP response to LH/CG-R(Asp$^{235}$) was lower than that of wild type receptor in this experiment, but this finding was not reproducible.
Mutagenesis of LH/CG-R

Fig. 8. Lack of an effect of hCG on cAMP production in COS-7 cells transfected with pSVL vectors containing mutant LH/CG-R cDNAs. Results are shown for two point mutants and one double mutant with lysine replacements of Glu332 and Asp333. The data are given as combined means ± S.E. from several independent experiments. The cAMP values at each concentration of hCG are slightly higher than control values (not significant), i.e. in untransfected cells, and significantly lower than values obtained with wild type LH/CG-R (cf. Figs. 6 and 7 for typical responses).

tor, known to be involved in single ligand binding (de Vos et al., 1992), and found that fewer than half of the mutant receptors exhibited any significant loss in binding affinity. The greatest contributions to hormone-receptor binding were attributed to six hydrophobic amino acid residues on the receptor and, to a lesser extent, to five ionizable side chains. These particular 11 contact sites form a contiguous region in which the hydrophobic groups are surrounded by the charged groups. Thus, about two-thirds of the receptor amino acid residues that are rendered solvent-inaccessible upon hormone binding contribute little to binding affinity. Whether a similar relationship will emerge for LH/CG-R remains to be determined.

With the exception of the replacements on Glu332 and Asp333 with Lys, the charge reversals we made on LH/CG-R did not alter receptor functionality as judged by cAMP production in response to added hCG. There was, however, a dramatic effect of most of these mutations on receptor density. A reduction in receptor number could result from inefficient translation of the mRNA, instability of the mutant receptor, and/or if inefficient assembly into the plasma membrane, as has been noted for several point and deletion mutants of LH/CG-R (Segaloff and Ascoli, 1993).

It is interesting and somewhat surprising that cAMP responsiveness appears independent of receptor density over a fairly wide concentration range. Excluding replacements at positions 332 and 333, LH/CG-R wild type and the various mutants are present at average receptor numbers/cell ranging over 2 orders of magnitude; yet, the maximal amount of cAMP produced at high concentrations of hCG is about the same. These results suggest that the amount of Gs proteins or adenylate cyclase is limiting with regard to hCG-LH/CG-R-mediated cAMP production.

In summary, Glu332 and Asp333, which are located <10 amino acid residues from the beginning of the first transmembrane helix and are invariant in the known LH/CG, follitropin, and thyrotropin receptors, appear to participate in transmembrane signaling. Thus, these particular amino acid residues may have fundamental roles in signaling for all of the glycoprotein hormone receptors.

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