**Lys** in the Third Extracellular Loop of the Lutropin/Choriogonadotropin Receptor Is Critical for Signaling*

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The lutropin/choriogonadotropin receptor (LH/CG-R) contains a relatively large extracellular domain, in addition to the seven transmembrane helices (TMH), three extracellular loops (ECL), and three intracellular loops typical of G protein-coupled receptors. While high affinity ligand binding has been attributed to the N-terminal extracellular domain, there is evidence that portions of the three ECLs may function in ligand binding and transmembrane signaling. We have investigated the role of several ionizable amino acid residues of rat LH/CG-R in human choriogonadotropin (hCG) binding and hCG-mediated cAMP production. COS-7 cells were transfected with the pSVL expression vector containing cDNAs of either wild-type or mutant rat LH/CG-R. Several point mutants of Lys583, located at the interface of ECL III and TMH VII, bound hCG like wild-type receptor but exhibited greatly diminished ligand-mediated signaling. Neither the point mutant, Lys401→Asp (ECL I), nor the double mutant, Asp997→Lys/Lys583→Asp (ECLs I and III, respectively), showed significant hCG binding to intact cells; in detergent-solubilized cells, only the double mutant bound hCG. The mutants Arg541→Glu (interface of the extracellular domain and TMH I) and Lys408→Glu (ECL II) proved to be similar to wild-type receptor in binding and signaling. Our results establish that Lys583 is important in signaling but not ligand binding. Its location on the opposite side of the membrane from **G** precludes a direct interaction, thus emphasizing the importance of a conformational change in the receptor and suggesting that ligand binding to receptor and ligand-mediated receptor activation are dissociable phenomena.

The LH/CG-R (1) is expressed on several types of gonadal cells (2) and has a crucial role in reproductive processes. Upon binding to their common receptor, LH and CG increase adenylylate cyclase activity. Although cAMP appears to be the principal mediator of the actions of gonadotropins on most gonadal cells, there is evidence to support activation of the phospholipase C pathway as well, which results in the formation of inositol 1,4,5-trisphosphate and increased [Ca**2+**] levels (3–5).

The LH/CG-R, FSH-R, and TSH-R are members of the glycoprotein hormone receptor family, characterized by a relatively large extracellular N-terminal region and a membrane-embedded C-terminal region containing seven TMHs. This C-terminal region is homologous to the small ligand binding members of the G protein-coupled receptor superfamily. However, unlike the small ligand binding receptors, where binding occurs in a clathrin formed by the TMHs, the glycoprotein hormone receptors utilize their extracellular domain as the high affinity binding site for the heterodimeric glycoprotein hormones with molecular masses of 30–37 kDa (6–10). This structural difference classifies these receptors as a distinct subfamily of the G protein-coupled receptor superfamily (11).

The N- and C-terminal domains of the rat LH/CG-R each contain over 300 amino acid residues, the latter distributed over three ECLs, seven TMHs, three intracellular loops, and a cytoplasmic tail (1). If the LH/CG-R spans the membrane similarly to the small ligand binding G protein-coupled receptors, one would expect the seven putative TMHs to form a pocket like that of the bacteriorhodopsin and rhodopsin receptors (12, 13); the six hydrophilic connecting loops are essential in maintaining this conformation. Additionally, although the high affinity binding site of the LH/CG-R is located in the ECD, there is evidence to support the presence of a lower affinity binding site in the C-terminal domain of the receptor (14, 15). Therefore, the ECLs represent potential hormone contact sites.

In an attempt to define the role of the LH/CG-R ECLs in hormone binding and signaling, several Arg and Lys residues were replaced: Arg401 (at the boundary between the ECD and TMH I), Lys401 (ECL I), Lys468 (ECL II) and Lys583 (ECL III). Additionally, a reciprocal mutation of Asp997 and Lys583 was characterized. The relative positions of these amino acid residues are shown in Fig. 1. These particular residues are invariant at the homologous positions in the LH/CG-R and the FSH-R of all known species; in the TSH-R, the positions equivalent to residues 401 and 583 are His and Gly, respectively, and the other residues are invariant. Our results revealed that Lys583 is not involved in hormone binding but is essential for full receptor activation.

**EXPERIMENTAL PROCEDURES**

Materials—**125**I-hCG (100–150 µCi/µg) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA) and DuPont NEN. **125**I-labeled ATP (1000–1500 Ci/mmol) and the **125**I-cAMP radiomunnoassay kit were products of DuPont NEN. hCG was a gift from Dr. Steven Birken (Columbia University, New York, NY). The Transformer™ mutagenesis system was obtained from Clontech (Palo Alto, CA), the Sequenase version 2.0 kit was purchased from United States Biochemical Corp., and the Wizard Miniprep DNA purification system was a product of Promega.
maintained at 37°C in humidified air containing 5% CO₂ in 90% from the American Type Culture Collection (Rockville, MD). The cells, Dr. Nevis Fregien (University of Miami, Miami, FL) and also purchased using the Qiagen plasmid Maxiprep kit.

The single mutations of the full-length LH/CG-R were prepared and characterized in this study are indicated: Arg⁸⁴¹ → Glu (R 341E), Lys⁵⁸³ → Asp (K401D), Lys⁴⁸⁸ → Glu (K488E), Lys⁵⁸³ → Glu (K583E), Lys⁵⁸³ → Gin (K583Q), Lys⁵⁸³ → Arg (K583R), and Lys⁵⁸³ → Pro (K583P) of LH/CG-R. In addition, a reciprocal mutation was investigated: Asp⁸⁴⁰ → Lys/Lys⁵⁸³ → Asp (D397K, K583D) LH/CG-R.

Fig. 1. Schematic representation of the major portion of the C-terminal region of LH/CG-R, including the seven putative transmembrane helices, the three exoplasmic loops, and the three cytoplasmic loops. The single mutations of the full-length LH/CG-R prepared and characterized in this study are indicated: Arg⁸⁴¹ → Glu (R 341E), Lys⁵⁸³ → Asp (K401D), Lys⁴⁸⁸ → Glu (K488E), Lys⁵⁸³ → Glu (K583E), Lys⁵⁸³ → Gin (K583Q), Lys⁵⁸³ → Arg (K583R), and Lys⁵⁸³ → Pro (K583P) of LH/CG-R. In addition, a reciprocal mutation was investigated: Asp⁸⁴⁰ → Lys/Lys⁵⁸³ → Asp (D397K, K583D) LH/CG-R.

Mutant cDNAs of the Rat LH/CG-R—The cDNA for the rat LH/CG-R, inserted into the XbaI-BamHI site of the expression vector pSVL, was the generous gift of Dr. William Moyle (Robert Wood Johnson Medical School, Piscataway, NJ). The 22-base deoxyoligonucleotides coding for the appropriate codon changes were synthesized by Dr. Rudolf Werner (University of Miami, FL) and by the Molecular Genetics Instrumentation Facility at the University of Georgia. In vitro mutagenesis was performed (16) and mutant clones identified by density sequencing (17). Mutant cDNAs were amplified and DNA was obtained from the Qiagen plasmid Maxiprep kit.

The transfected cells were maintained for 16 h after transfection and then replated (5 × 10⁶ cells/dish/swell, six-well tissue culture plates). Some 48–51 h post-transfection, the cells were about 70% confluent. Cells were then washed twice with serum-free Waymouth’s medium containing 1 mg of BSA/ml, and 1 ml of this media was added to each well. Increasing concentrations of unlabeled hCG were then added to each well, followed by addition of 25 pm ¹²⁵I-hCG (approximately 10⁴ cpm). Total and nonspecific binding were determined by addition of ¹²⁵I-hCG in the absence and presence of excess unlabeled hCG (54 nm). The plates were incubated at 25°C for 16–18 h with gentle shaking. The cells were washed twice with cold phosphate-buffered saline, then trypsinized, collected, and counted in a γ counter. All determinations were performed in duplicate. Binding affinities and maximal binding capacities were calculated using the Ligand program (19).

¹²⁵I-hCG Cell-surface Binding to Transfected Cells—The COS-7 cells were maintained for 16 h after transfection and then replated (5 × 10⁶ cells/dish, 10-cm tissue culture dishes). Some 48–51 h post-transfection, the cells were about 70% confluent. Cells were then washed twice with serum-free Waymouth’s medium containing 1 mg of BSA/ml, and 1 ml of this media was added to each well. Increasing concentrations of unlabeled hCG were then added to each well, followed by addition of 25 pm ¹²⁵I-hCG (approximately 10⁴ cpm). Total and nonspecific binding were determined by addition of ¹²⁵I-hCG in the absence and presence of excess unlabeled hCG (54 nm). The plates were incubated at 25°C for 16–18 h with gentle shaking. The cells were washed twice with cold phosphate-buffered saline, then trypsinized, collected, and counted in a γ counter. All determinations were performed in duplicate. Binding affinities and maximal binding capacities were calculated using the Ligand program (19).
ence and absence of excess unlabeled hCG, respectively. Bound radioactivity was separated from unbound by filtration through Whatman GF/B filters that were previously soaked in 0.3% polyethyleneimine in 10 mM Tris-HCl, pH 9.1 (22). The filters were washed five times with 0.1 M NaCl, 10 mM NaN3, 1 mg of BSA/ml in phosphate-buffered saline and counted in a γ counter. All determinations were performed in duplicate.

Indirect cAMP Assay—Some 16–18 h after transfection, the transfected cells were replated (1 × 10^6 cells/well, 12-well tissue culture plates). At 48–51 h post-transfection, the cells were washed twice with DMEM containing 1 mg of BSA/ml and incubated in 0.5 ml of this medium with 0.8 mM isobutylmethylxanthine for 15 min at 37°C. Increasing concentrations of hCG were then added and the incubation was continued for 30 min at 37°C. The cells were washed twice with fresh medium without isobutylmethylxanthine and then lysed in the buffer of the 125I-cAMP assay kit, and cAMP concentrations were determined by radioimmunoassay. All measurements were performed in duplicate; means and standard errors were calculated using the Prism program.

Partial Purification of the LH/CG-R—Transfected cells were maintained for 16–20 h after transfection and then replated (2–2.5 × 10^6 cells/10-cm tissue culture dishes). Some 48–51 h post-transfection, detergent-soluble extracts were prepared as described above. The procedures for cell lysis and partial purification of wild-type and mutant LH/CG-Rs were based on reports from other laboratories (21, 23, 24). Following cell lysis with 1% Nonidet P-40 and centrifugation as described above, the supernatant was collected and this buffer, the LH/CG-R was eluted with one column volume of 3 M NaCl, 10 mM NaN3, 1 mg of BSA/ml in phosphate-buffered saline. The buffers of the 125I-cAMP assay kit, and cAMP concentrations were determined by radioimmunoassay. All measurements were performed in duplicate; means and standard errors were calculated using the Prism program.

SDS-PAGE and Western Blots—Equal amounts of purified cell lysate in sample buffer with no reducing agent were applied to a 7% SDS-polyacrylamide gel without loading. After the electrophoresis, the proteins were electrophoretically transferred to polyvinylidene difluoride membranes, which were then washed twice in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and blocked for 2 h at room temperature in blocking solution (10% glycerol, 5% nonfat dry milk, 0.2% Tween 20 in phosphate-buffered saline). The filters were incubated overnight at room temperature with the same blocking solution containing 3 μg/ml rabbit anti-LH/CG-R IgGs, obtained by protein A-Sepharose purification of anti-LH/CG-R antiserum (23). The membranes were washed five times for 5 min each with blocking solution and then incubated for 1 h at room temperature with a 15,000 dilution of a horseradish peroxidase-labeled donkey anti-rabbit IgG whole antibody. Following washing, the membranes were washed twice with TBS, twice with 1% Nonidet P-40 in TBS, then once each with TBS, 1% Nonidet P-40 in TBS, and TBS alone. The membranes were exposed to an Amersham Enhanced Chemiluminescence developer solution for 1 min, wrapped in Saran Wrap, and exposed to Kodak XAR-5 film for 1 min. The films were densitometrically scanned (PDI System, Huntington, NY).

RESULTS

The mean Kd from multiple independent transfections (n = 9) for hCG binding to wild-type LH/CG-R on transfected intact COS-7 cells was 0.14 nM, with a range of 0.07–0.25 nM (Table I), there being no difference between transfections with DEAE-dextran and Lipofectamine. On the other hand, receptor numbers/cell (uncorrected for transfection efficiencies) were greater with Lipofectamine transfection (mean of 2 × 10^6) than with DEAE-dextran transfection (mean of 0.5 × 10^6). The difference in receptor number/cell, however, had no significant impact on the maximal cAMP production elicited by hCG at 100 ng/ml in COS-7 cells transfected with the cDNA to wild-type LH/CG-R; the mean value was 6.7 pmol cAMP/10^5 cells and that of control cells in the presence of 100 ng/ml hCG was 0.40(4) pmol cAMP/10^5 cells. The Lys583→Glu mutation resulted in a LH/CG-R that specifically bound 125I-hCG, and this binding was inhibited by unlabeled hCG in a concentration-dependent manner (Fig. 2A). Although the estimated number of receptors varied in the transfected cells, e.g. 2 × 10^5 for wild-type and 1 × 10^5 for mutant LH/CG-R (uncorrected for transfection efficiency), the mutant LH/CG-R yielded a binding affinity equivalent to that of wild-type LH/CG-R (Table I).

The Lys583→Glu replacement resulted in markedly decreased production of cAMP in response to added hCG, e.g. 15% that of wild-type LH/CG-R at 100 ng/ml hCG (Fig. 3A, Table I). Since this result could be attributed to the decreased number of cell surface receptors, this mutant LH/CG-R was also transfected using Lipofectamine to obtain higher efficiency and increased receptor expression. The mutant receptor bound hCG with an affinity comparable to wild-type LH/CG-R (Table I). There was an increase in cell surface expression, e.g. 2 × 10^4 receptors/cell (uncorrected for transfection efficiency), for both wild-type and mutant LH/CG-R, but the mutant receptor again stimulated cAMP production only 15% that of wild-type LH/CG-R at 100 ng/ml hCG (Table I).
To investigate the side-chain specificity of Lys583, replacements were also made with Arg, Gin, and Pro. In each case the mutations yielded LH/CG-Rs that bound 125I-hCG with affinities comparable to that of wild-type LH/CG-R (Fig. 2B, Table I), but these mutant receptors also resulted in cAMP accumulation, 30% that of wild-type LH/CG-R upon addition of hCG to transfected cells (Fig. 3B, Table I).

Competitive binding assays were unable to detect significant cell surface binding of 125I-hCG to cells transfected with cDNAs to the Lys401 → Asp single mutation and the Asp397 → Lys/Lys583 → Asp reciprocal mutation (Table II). 125I-hCG binding to detergent-soluble extracts of transfected cells was found for the reciprocal mutant but not the point mutant (Table II). However, Western blot analysis indicated that the (Lys401 → Asp) LH/CG-R was expressed (Fig. 4). Wild-type and mutant LH/CG-Rs were about equally distributed among three bands of apparent molecular mass 101, 93, and 82 kDa; the total protein in the mutant LH/CG-R was much less than that of wild-type LH/CG-R.
wild-type LH/CG-R.

Arg<sup>341</sup> and Lys<sup>583</sup> were each replaced with Glu; both mutations yielded LH/CG-Rs that bound 125<sup>i</sup>-hCG with affinities comparable to that of wild-type LH/CG-R (Fig. 2, C and D, Table I). These mutant LH/CG-Rs were capable of stimulating cAMP production in a dose-dependent manner, quite similar to wild-type LH/CG-R (Fig. 3, C and D, Table I).

**DISCUSSION**

Our results on the substitution of Lys<sup>583</sup> of the rat LH/CG-R with 4 amino acid residues, positively charged Arg, negatively charged Glu, polar but nonionizable Gln, and nonpolar Pro (an imino acid), show that the mutant LH/CG-Rs bind hCG as well as wild-type receptor, but coupling to adenylate cyclase is greatly diminished. These findings are particularly intriguing since Lys<sup>583</sup> is located extracellularly, at the boundary of ECL III and TMH VII, and is unable to interact directly with the ECD. Thus, one can conclude that ligand-mediated transmembrane signal transduction is involved in receptor binding, but that this change accompanies, or perhaps even triggers, a change in the conformation of the unoccupied receptor (32). We found that the mutant TSH-R bound TSH with high affinity, but the sensitivity of TSH-stimulated cAMP production and the maximal level of TSH-mediated cAMP production were significantly diminished in the mutant TSH-R (32). Coupled with our studies on Lys<sup>583</sup> in ECL III of the LH/CG-R, these results indicate an important role of ECL III in glycoprotein hormone signal transduction and a possible region of specificity delineating gonadotropin receptors from the TSH-R.

Two other positively charged residues of LH/CG-R, which are invariant in the glycoprotein hormone receptors, Arg<sup>341</sup> located...
ed in the ECD at the interface with TMH I) and Lys\(^{401}\) (ECL I) were each replaced with Glu with no observable effect on hCG binding or receptor activation. Lys\(^{401}\) (ECL I) was replaced with Asp, and the mutant receptor failed to exhibit significant hCG binding to intact or detergent-solubilized cells, suggesting that this amino acid residue may be involved in hormone binding. However, binding may occur but is difficult to detect if there is a low level of mutant receptor expression, if the receptor is rapidly degraded or if the K_d is significantly increased. Lys is present at this position in LH/CG-R and FSH-R, while in TSH-R it is occupied by His; depending upon the pH of the His, the potential exists for retention of a positive charge at this location in ECL I of all glycoprotein hormone receptors.

Our results on Western analysis of wild-type LH/CG-R and (Lys\(^{401}\) → Asp) LH/CG-R revealed the presence of three bands of apparent molecular mass 101, 93, and 82 kDa. The original Western blot experiments on expressed LH/CG-R were performed using human 293 cells stably transfected with the wild-type LH/CG-R cDNA (24). A major 85-kDa form, considered the mature receptor, and a minor 68-kDa form, which appeared to be the incompletely glycosylated form, were found. The apparent 93-kDa band observed in transiently transfected COS-7 cells corresponds to the M_1 of purified rat ovarian LH/CG-R (2), although wide variations have been reported in the M_1 of LH/CG-R from various sources. In another study from our laboratory using a slightly different set of protein standards, we found major and minor bands of apparent molecular mass 93 kDa (80%) and 78 kDa for expressed wild-type LH/CG-R (25). The greater amount of protein loaded in that study could easily prevent resolution of the bands of 93 and 101 kDa reported herein. Interestingly, the Lys\(^{401}\) → Asp LH/CG-R mutant also gave the same apparent M_1 forms as wild-type LH/CG-R, but at much lower levels.

In summary, these results enable us to conclude that Lys\(^{583}\) of the rat LH/CG-R is critical in receptor activation following hormone binding. Since Lys\(^{583}\) (ECL III) is located on the opposite side of the membrane from G proteins and does not appear to be involved in hormone binding, these findings offer considerable weight to the concept that receptor binding and activation are dissociable phenomena. Of interest are other observations we recently made that certain amino acid residues in TMH VII are also critical for signal transduction (33). Thus, this region of the receptor appears to be important in transmembrane signaling subsequent to hormone binding.

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