# Identification of Amino Acid Residues in Transmembrane Helices VI and VII of the Lutropin/Choriogonadotropin Receptor Involved in Signaling†

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ABSTRACT: The lutropin/choriogonadotropin receptor (LH/CG-R) is a member of the G protein-coupled receptor superfamily containing a relatively large extracellular domain responsible for high affinity ligand binding. There is a paucity of information on the mechanism of ligand-mediated transmembrane signaling via the seven transmembrane helices (TMH) of these receptors. In the present study we have used site-directed mutagenesis to replace each of nine conserved amino acid residues in the TMHs of rat LH/CG-R. COS-7 cells were transiently transfected with the eukaryotic expression vector pSVL-LH/CG-R, wild-type and mutants, followed by measurements of human CG binding and human CG-mediated cAMP production. Three point mutants of LH/CG-R were prepared that diminished signaling but not binding: Pro-562-Leu (TMH VI), Pro-591-Leu (TMH VII), and Tyr-601-Ala (TMH VII). Two point mutants of LH/CG-R, Pro-479-Leu (TMH IV) and Pro-598-Leu (TMH VII), resulted in impaired localization, and four receptor mutants, Thr-424-Ala (TMH III), Ser-562-Ala (TMH VI), Met-560-Leu (TMH VI), and Tyr-590-Ala (TMH VII), were similar to wild-type LH/CG-R. In summary, these findings indicate a critical role of Tyr-601 in transmembrane signaling of LH/CG-R since an Ala replacement results in almost total abolition of cAMP production in response to human CG; prolines 562 and 591 also appear to be important for full signaling.

The gonadotropin receptor LH/CG-R[1] (McFarland et al., 1989; Loosfelt et al., 1989), essential for gametogenesis and steroidogenesis, is a member of the glycoprotein hormone receptor family, which also includes FSH-R and TSH-R (Segaloff & Ascoli, 1993). The glycoprotein hormone receptors belong to the G protein-coupled receptor superfamily (Probst et al., 1992) and are characterized by a relatively large N-terminal domain followed by a C-terminal transmembrane-embedded domain that is comprised of three extracellular loops, seven transmembrane segments believed to span the lipid bilayer as α-helices, three intracellular loops, and a cytoplasmic tail. This C-terminal domain is presumably responsible for effecting intracellular signaling when the appropriate ligand binds to its respective cell-surface receptor.

Whereas most small ligand binding G protein-coupled receptors, e.g., rhodopsin and $\beta$-adrenergic receptor, utilize the TMHs as a binding pocket to transmit extracellular stimuli to the cytoplasm, the glycoprotein hormone receptors utilize their hydrophilic N-terminal domain as a high affinity binding site (Xie et al., 1990; Tsai-Morris et al., 1990; Braun et al., 1991; Ji & Ji, 1991; Moyle et al., 1991) for the relatively large gonadotropins, e.g., 30−40 kDa. Binding of LH and CG to their common receptor may modulate the TMHs of the LH/CG-R resulting in subsequent receptor activation and signal transduction via increases in the activity of adenylyl cyclase (Segaloff & Ascoli, 1993) or phospholipase C (Davis et al., 1987; Gudermann et al., 1992a,b). Studies on glycophorin A and bacteriorhodopsin have shown that sequence-specific helical interactions can provide much of the energy necessary for interhelical packing (Lemmon et al., 1992; Kahn & Engelman, 1992). Additionally, work on the $\beta$-adrenergic receptor (Suryanarayana et al., 1992), the gonadotropin-releasing hormone receptor (Zhou et al., 1994) and the muscarinic receptor (Liu et al., 1995) have established the importance of helix−helix interactions in ligand binding and subsequent signaling events.

If the LH/CG-R inserts into the plasma membrane similar to the small ligand binding G-protein coupled receptors, the seven membrane-spanning segments may in fact adopt a conformation like that observed for bacteriorhodopsin or rhodopsin (Henderson et al., 1990; Baldwin, 1993). Therefore, this putative helical pocket could provide the necessary conformations for cell-surface expression, hormone binding and/or signal transduction.

In an attempt to define the role of the TMHs of the LH/CG-R in these processes, Thr-424 (TMH III), Ser-564 (TMH VI), Tyr-590 (TMH VII), and Tyr-601 (TMH VII) were each replaced with Ala. Pro-479 (TMH IV), Met-560 (TMH VI), Pro-562 (TMH VI), Pro-591 (TMH VII), and Pro-598 (TMH VII) were individually substituted by Leu. The relative positions of these amino acid residues are shown in Figure 1. These studies indicated that Pro-562, Pro-591, and Tyr-

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[1] Abbreviations: BSA, bovine serum albumin; DMEM, Dubecco's modified essential medium; EDTA, ethylenediaminetetracetic acid; FBS, fetal bovine serum; FSH-R, follitropin receptor; hCG, human choriogonadotropin; IBMX, isobutylmethylxanthine; LH, lutropin; LH/CG-R, lutropin/choriogonadotropin receptor; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMH, transmembrane helix; TSH-R; thyrotropin receptor; X-gal, 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside.

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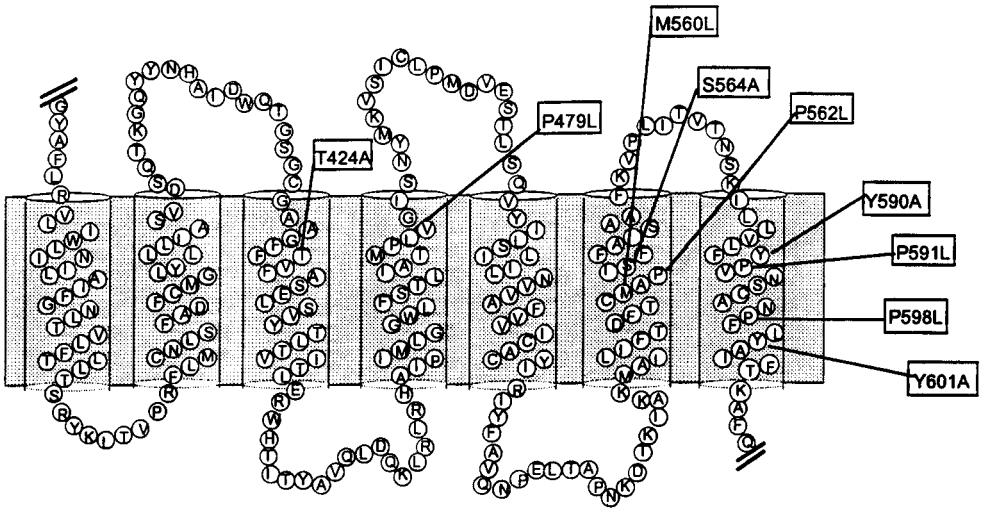


FIGURE 1: Schematic diagram of a portion of the rat LH/CG-R showing the amino acid sequence of the seven TMHs, the three extracellular loops, and the three intracellular loops. The amino acid residues replaced in the current study are indicated.

601 are not required for hormone binding; they are, however, necessary for proper signaling via $G_s$ and adenylyl cyclase.

## MATERIALS AND METHODS

*Materials.* [α-$^{35}$S]dATP (1000−1500 Ci/mmol) and the [$^{125}$I]cAMP RIA kit were purchased from DuPont NEN (Boston, MA). [$^{125}$I]hCG (100−150 $\mu$Ci/$\mu$g) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA) and from DuPont NEN (Boston, MA). Highly purified hCG was kindly provided by Dr. Steven Birken (Columbia University, New York, NY) and the NIDDK, NIH (Bethesda, MD). The Transformer mutagenesis system was a product of Clontech (Palo Alto, CA); the SEQUENASE Version 2.0 kit was obtained from USB (Cleveland, OH); and the Wizard Minipreps DNA purification system was purchased from Promega (Madison, WI). The plasmid maxiprep DNA purification system was obtained from Qiagen, Inc. (Chatsworth, CA). Waymouth's media, Lipofectamine, trypsin-EDTA, FBS, gentamicin, and penicillin-streptomycin were purchased from Gibco-BRL (Gaithersburg, MD). PBS and DMEM were products of Fisher (Norcross, GA). DEAE-dextran, IBMX, chloroquine, BSA, triacetylchitotriose, wide range protein molecular weight markers, and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO). The BCA protein assay system was from Pierce Chemical Co. (Rockford, IL), and the Enhanced Chemiluminescence Western blotting analysis system was a product of Amersham Life Sciences (Arlington heights, IL). Centricon-30 columns were obtained from Amicon (Beverly, MA), and agarose-bound wheat germ agglutinin was purchased from Vector Laboratories (Burlingame, CA). Tissue culture plates, flasks, and tubes were products of Costar (Cambridge, MA), Falcon (Franklin Lakes, NJ), and Corning (Corning, NY). Immobilon P transfer membranes were obtained from Millipore (Bedford, MA), and glass fiber filters were from Whatman (Maidstone, U.K.). Most other reagents were purchased from Gibco-BRL (Gaithersburg, MD) and Sigma Chemical Co. (St. Louis, MO).

*Preparation and Expression of Wild-Type and Mutant cDNAs of the Rat LH/CG-R.* The cDNA for the rat LH/CG-R, inserted into the *Xba*1-*Bam*H1 site of the expression vector pSVL, was a gift from Dr. William Moyle (Robert Wood Johnson Medical School, Piscataway, NJ). The 21-base deoxyoligonucleotides encoding the appropriate codon changes were synthesized by Dr. Rudolf Werner (University of Miami, Miami, FL) and by the Molecular Genetics Instrumentation Facility at the University of Georgia. *In vitro* site-directed mutagenesis was performed as recommended by Clontech (Deng & Nikoloff, 1992), and mutant clones were identified by sequencing (Sanger et al., 1977). Mutant cDNAs were then amplified, and the Qiagen plasmid maxi kit was used to obtain purified DNA. COS-7 cells (African green monkey SV40 transformed kidney cells) (Gluzman, 1981) were the generous gift of Dr. Nevis Fregien (University of Miami, Miami, FL) and were also obtained from the American Type Culture Collection (Rockville, MD). These cells were kept at 37 °C in humidified air containing 5% $CO_2$ in 90% DMEM, 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin.

The COS-7 cells were transiently transfected with the eukaryotic expression vector pSVL-LH/CG-R, wild-type and mutants, using DEAE-dextran (Selden, 1992) or Lipofectamine as recommended by Gibco-BRL. Briefly, for transfection with DEAE-dextran, 0.4 mL of a solution containing 10 mg/mL of DEAE-dextran and 2.5 mM chloroquine was added to 9.6 mL of 90% DMEM/10% NuSerum transfection medium. Then, 20 $\mu$g of pSVL-LH/CG-R cDNA and 20 $\mu$g of pSV-$\beta$-gal (to monitor transfection efficiency) were added, and washed cells (ca. $4 \times 10^6$) were incubated with the mixture for 3.5−4 h at 37 °C. The DNA transfection medium was aspirated, and the cells were shocked in 10% DMSO for 2 min at room temperature. This was removed, the cells were washed, and then COS-7 growth medium was added. For Lipofectamine transfection, 45 $\mu$L of Lipofectamine were combined with 0.4 mL of Opti-mem media, and this was mixed with 0.4 mL of media containing 10 $\mu$g of the pSVL-LH/CG-R constructs and 10 $\mu$g of the pSV-$\beta$-gal plasmid. This solution was then added to 7 mL of serum-free DMEM, and the mixture was placed on washed cells (ca. $2-3 \times 10^6$) and incubated for 5 h at 37 °C in humidified air containing 5% $CO_2$. After removal of the transfection medium, COS-7 growth medium was added. Following DEAE-dextran and Lipofectamine transfections, the cells were incubated overnight at 37 °C in humidified air containing 5% $CO_2$.

Transfection efficiencies were estimated to be about 10 and 40% for DEAE-dextran and Lipofectamine, respectively, as judged by X-gal staining. For this, the cells were extensively washed with PBS, and then a solution of 0.05% glutaraldehyde in PBS was added for 15 min at room temperature. After removal of glutaraldehyde, the X-gal staining solution [40 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 40 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, and 1 mg/mL of X-gal in PBS] was added to the cells followed by incubation for at least 2 h at 37 °C before counting.

*Hormone Binding to Transfected Cells.* For binding to intact cells, the COS-7 cells were maintained for 16 h posttransfection and then replated ($5 \times 10^5$ cells per well) into six-well tissue culture plates. Some 48−51 h after transfection, the cells were approximately 70% confluent. At this time, the cells were washed twice with serum-free Waymouth's medium containing 1 mg BSA/mL, and 1 mL of this media was added to each well. Increasing concentrations of unlabeled hCG were added to each well followed by the addition of 25 pM [$^{125}$I]hCG (approximately $10^5$ cpm). Nonspecific and total binding were determined by addition of [$^{125}$I]hCG in the presence and absence of 2 $\mu$g of unlabeled hCG, respectively. The plates were then incubated at 25 °C overnight with gentle shaking. After 16−18 h, the medium was aspirated, and the cells were washed twice with cold PBS, trypsinized, and collected. Then, each well was washed with an equal volume of cold PBS, which was pooled with the trypsinized cells and counted in a gamma counter (LKB/Wallac Instruments, Rockville, MD). All determinations were performed in duplicate, and two independent transfections were analyzed. Binding affinities and maximal binding capacities were calculated using the Ligand program (Munson & Rodbard, 1980).

The procedure for hormone binding to detergent-soluble extracts of cells was taken, with only minor modifications, from that developed by Segaloff and co-workers (Xie et al., 1990; Wang et al., 1993; Thomas & Segaloff, 1994) and described elsewhere (Fernandez & Puett, 1996). Transfected cells were maintained for 16 h posttransfection and then replated ($2-2.5 \times 10^6$ cells per dish) into 10 cm tissue culture dishes. Some 48 h after transfection, the transfected cells were placed on ice for 15 min and then washed twice with 5 mL of ice-cold Hepes-buffered saline (20 mM Hepes, pH 7.4, 0.1 mM NaCl). Next, the cells were scraped into 2 mL of cold Hepes-buffered saline containing protease inhibitors (1 mM PMSF, 2 mM EDTA, and 5 mM NEM) and pelleted by centrifugation at 2000*g* for 20 min at 4 °C. This pellet was then resuspended in 0.25 mL of 1% Nonidet P-40/20% glycerol in Hepes-buffered saline containing protease inhibitors and maintained on ice for 15 min. This mixture was centrifuged at 16000*g* for 15 min at 4 °C. The supernatant was diluted with 2.25 mL of 20% glycerol in Hepes-buffered saline; 0.5 mL of the diluted extract was incubated with 50 pM [$^{125}$I]hCG ($10^5$ cpm) for 16−18 h at 4 °C. Total and nonspecific binding were determined in the presence and absence of 2 $\mu$g of unlabeled hCG, respectively. The binding extracts were added to Whatman GF/B filters, which had been soaked overnight in 0.3% polyethylenimine in 10 mM Tris-HCl, pH 9.1 (Roche et al., 1985), and then washed five times with 0.1 M NaCl, 10 mM $NaN_3$, and 1 mg of BSA/mL in PBS and counted in a gamma counter. Duplicate determinations were obtained for each measurement, and the results of two independent transfections are reported.

*cAMP Assay.* About 16−18 h posttransfection, the transfected cells were replated ($1 \times 10^5$ cells per well) into 12-well tissue culture plates. Approximately 48−51 h after transfection, the cells were washed twice with DMEM containing 1 mg of BSA/mL and incubated in 0.5 mL of this medium containing IBMX (0.8 mM) for 15 min at 37 °C. Then, increasing concentrations of hCG were added, and the incubation was continued for 30 min at 37 °C. The incubation medium was removed, the cells washed twice with fresh medium without IBMX and then lysed in 100% ethanol at −20 °C overnight. The extract was collected, dried under a gentle stream of nitrogen gas, and resuspended in the sodium acetate buffer of the [$^{125}$I]cAMP assay kit. cAMP concentrations were determined by RIA as recommended by DuPont NEN. Duplicate determinations were performed for each measurement; means and SEMs were calculated using the Prism program. Results are given for at least two and sometimes three independent transfections. The maximal cAMP produced in response to a saturating concentration of hCG varied between 18−32 pmol/$10^5$ cells.

*Partial Purification of the LH/CG-R and Western Analysis.* The procedures for partial purification of the LH/CG-R (wild-type and mutants) were adapted from that described by Ascoli, Segaloff, and co-workers (Rosemblit et al., 1988; Hipkin et al., 1992; Thomas & Segaloff, 1994). Following lysis of transiently transfected cells as described above, the supernatant from the 16000*g* centrifugation step was diluted 2-fold with 10% glycerol in Hepes-buffered saline and then loaded onto an agarose-bound wheat germ agglutinin column equilibrated with the same buffer. The column was rotated at 4 °C overnight and then washed with 40 volumes of buffer. The LH/CG-R fraction was eluted with one volume of 3 mM triacetylchitotriose (Hipkin et al., 1992) containing protease inhibitors (1 mM PMSF, 2 mM EDTA, and 5 mM NEM) and concentrated to about 0.1 mL in a Centricon-30 spin column; the BCA assay was used to determine protein concentration.

Equal amounts of purified cell lysates in sample buffer, but with no reducing agent, were applied to a 7% SDS−polyacrylamide gel without boiling. At the end of the electrophoresis, the gel was equilibrated in transfer buffer, and the proteins were electrophoretically transferred via the Semi-Dry Transfer Cell (Bio-Rad) to polyvinylidine difluoride membranes (Immobilon-P). The membranes were washed twice in Tris-buffered saline (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% instant nonfat dry milk, 0.2% Tween-20 in PBS). Next, the membranes were incubated overnight at room temperature with the same blocking solution containing 3 $\mu$g/mL of rabbit anti-LH/CG-R IgGs. The anti-LH/CG-R antiserum, generously provided by Dr. Deborah Segaloff (Rosemblit et al., 1988), was purified using protein A-Sepharose to obtain the IgG fraction. The membranes were then washed five times for 5 min each with blocking solution and incubated for 1 h at room temperature with a 1:5000 dilution of a horseradish peroxidase-labeled donkey anti-rabbit IgG whole antibody in blocking solution. The membranes were washed twice with fresh Tris-buffered saline, then twice with 1% Nonidet P-40 in Tris-buffered saline, once with Tris-buffered saline, once with Nonidet P-40 in the same buffer, and finally with

Table 1: Lack of Correlation between Receptor Density and Maximal cAMP Production in COS-7 Cells Transfected with Wild-Type LH/CG-R cDNA

| receptors/cell ($\times 10^{-3}$)[a] | $cAMP_{max}$ (pmol/$10^5$ cells)[b] |
|---|---|
| $22.7 \pm 7.3$[c,d] | $14.5 \pm 3.8$ |
| $19.7 \pm 2.8$[c,e] | $18.7 \pm 2.5$ |
| $19.6 \pm 3.7$[c,d] | $16.8 \pm 1.7$ |
| $19.0 \pm 2.2$[c,e] | $15.3 \pm 2.8$ |
| $15.0 \pm 3.4$[c,d] | $17.0 \pm 2.4$ |
| $2.9 \pm 1.1$[d,f] | $25.0 \pm 3.7$ |
| $1.8 \pm 0.4$[e,d] | $24.9 \pm 3.6$ |
| $1.6 \pm 0.6$[d,f] | $18.3 \pm 8.8$ |
| $1.0 \pm 0.2$[d,f] | $15.8 \pm 7.7$ |

[a] Not corrected for transfection efficiencies. Different transfection conditions and slightly varying amounts of cDNA were used to obtain these results. [b] The amount of cAMP produced by 100 ng/mL of hCG after correction for basal production. [c] Based on transfection with Lipofectamine. [d] This work (cf. Table 2) and unpublished data. [e] From Fernandez and Puett (1996). [f] Based on transfection with DEAE-Dextran.

buffer alone. Next, the membranes were exposed to an Amersham Enhanced Chemiluminescence developer solution (1 min), wrapped in Saranwrap, and exposed to Kodak XAR-5 film (1 min). The PDI system (Huntington, NY) was used to densitometrically scan the films.

## RESULTS

All pSVL-LH/CG-R constructs were confirmed by sequencing prior to transient transfection in COS-7 cells. The mean $K_d$ for binding hCG to wild-type LH/CG-R from six independent transfections was 0.15 nM; however, $K_d$'s were compared between the mutant LH/CG-Rs and wild-type LH/CG-R within each experiment. The mean receptor density obtained from two independent DEAE-dextran transfections using the pSVL-LH/CG-R wild-type construct was $1.8 \times 10^3$ receptors/cell (uncorrected for transfection efficiency); approximately a 10-fold greater number of receptors/cell was obtained with Lipofectamine, $1.7 \times 10^4$ (mean of four independent transfections, also uncorrected for transfection efficiencies). As documented in Table 1, the cAMP response to hCG is independent of receptor number/cell over at least a 20-fold range, confirming our earlier conclusion (Huang & Puett, 1995).

Individual replacements of Pro-562 (TMH VI), Pro-591 (TMH VII), and Tyr-601 (TMH VII), invariant among the glycoprotein hormone receptors, with Leu, Leu, and Ala, respectively, resulted in LH/CG-R mutants that bound hCG with $K_d$'s similar to that of wild-type LH/CG-R (Figure 2A,B and Table 2). In contrast, hCG-mediated signaling, determined via intracellular cAMP concentrations, was greatly diminished with the two Pro→Leu mutants (Figure 3A and Table 2). For example, the maximal cAMP produced at high ligand concentrations, corrected for basal production, was only about 50−55% and 20−25% that of wild-type LH/CG-R with (Pro-562-Leu) and (Pro-591-Leu) LH/CG-R, respectively. (Tyr-601-Ala) LH/CG-R, on the other hand, was minimally responsive to bound hCG in terms of cAMP production (Figure 3B and Table 2). Even at an hCG concentration of 100 ng/mL, where receptor occupancy should be near 100%, the intracellular concentration of cAMP is only marginally above that of control cells. Although the receptor density of the (Pro-591-Leu) and (Tyr-601-Ala) LH/CG-R mutants are less than the corresponding values for the control wild-type LH/CG-R in each experiment, e.g., ca. 40% and 7%, respectively, this reduction in receptor number has no significant effect on maximal cAMP production since the numbers are well within the range we have found to yield maximal cAMP production (cf. Table 1).

Prolines 479 (TMH IV) and 598 (TMH VII), also invariant among the glycoprotein hormone receptors, were each replaced with Leu, but no significant binding of 50 pM [$^{125}$I]-hCG to intact cells was detected with either mutant, e.g., the specific binding of cells transfected with each mutant cDNA was <0.05% that of wild-type LH/CG-R binding (Table 3). Binding assays were then performed on detergent-solubilized extracts of the transfected cells, and the (Pro-598-Leu) LH/CG-R specifically bound [$^{125}$I]hCG, although the amount of bound hormone was <50% that of wild-type LH/CG-R. In contrast, only minimal binding of [$^{125}$I]hCG was detected with solubilized extracts from cells transfected with the (Pro-479-Leu) LH/CG-R cDNA.

In view of the negligible binding of [$^{125}$I]hCG to cells transfected with the cDNA to (Pro-479-Leu) LH/CG-R, Western blots were obtained for the mutant and wild-type receptors (Figure 4). Since equivalent amounts of transfected cell protein were analyzed, there is somewhat less (Pro-479-Leu) LH/CG-R than wild-type LH/CG-R; the major difference, however, is in the distribution of the various molecular mass components. Although not obvious from the blot with the amount of protein loaded, densitometric scanning of the wild-type receptor indicated the presence of three bands of apparent molecular masses of 101 and 93 kDa, which are not well resolved and appear as one major band, and 82 kDa; these three bands exhibit approximately the same intensity. The (Pro-479-Leu) LH/CG-R mutant, on the other hand, exhibits primarily the lower molecular mass band, although the two higher molecular mass bands are present. The work of Hipkin et al. (1992) demonstrated that the lower molecular mass component is a precursor to the higher molecular mass band(s), which presumably correspond to the mature form(s) of the receptor. From this information, we can conclude from our results that the (Pro-479-Leu) LH/CG-R mutant is expressed, although the relative amount of mature receptor is considerably less than that of wild-type receptor.

Other amino acid residues in the LH/CG-R TMHs, which are also invariant among the glycoprotein hormone receptors, were individually replaced with Ala, Thr-424 (TMH III), Ser-564 (TMH VI), and Tyr-590 (TMH VII); Met-560 (TMH VI) was replaced with Leu. hCG binding affinities to the four mutant LH/CG-Rs were comparable to wild-type LH/CG-R (Figure 2C,D and Table 2), as was cAMP production in response to hCG (Figure 3C,D and Table 2). Receptor densities were comparable between wild-type LH/CG-R and the mutant receptors with the exception of (Thr-424-Ala) LH/CG-R for which the number of receptors/cell was only about 23% that of wild-type LH/CG-R. This lower number, however, had no functional consequence.

## DISCUSSION

This work has identified three amino acid residues, Pro-562 (TMH VI), Pro-591 (TMH VII), and Tyr-601 (TMH VII), that appear important in ligand-mediated transmembrane signaling but not ligand binding. These results offer fairly compelling evidence that receptor binding and activation can be dissociated. A similar conclusion was reached based on mutations of Lys-583, located at the interface

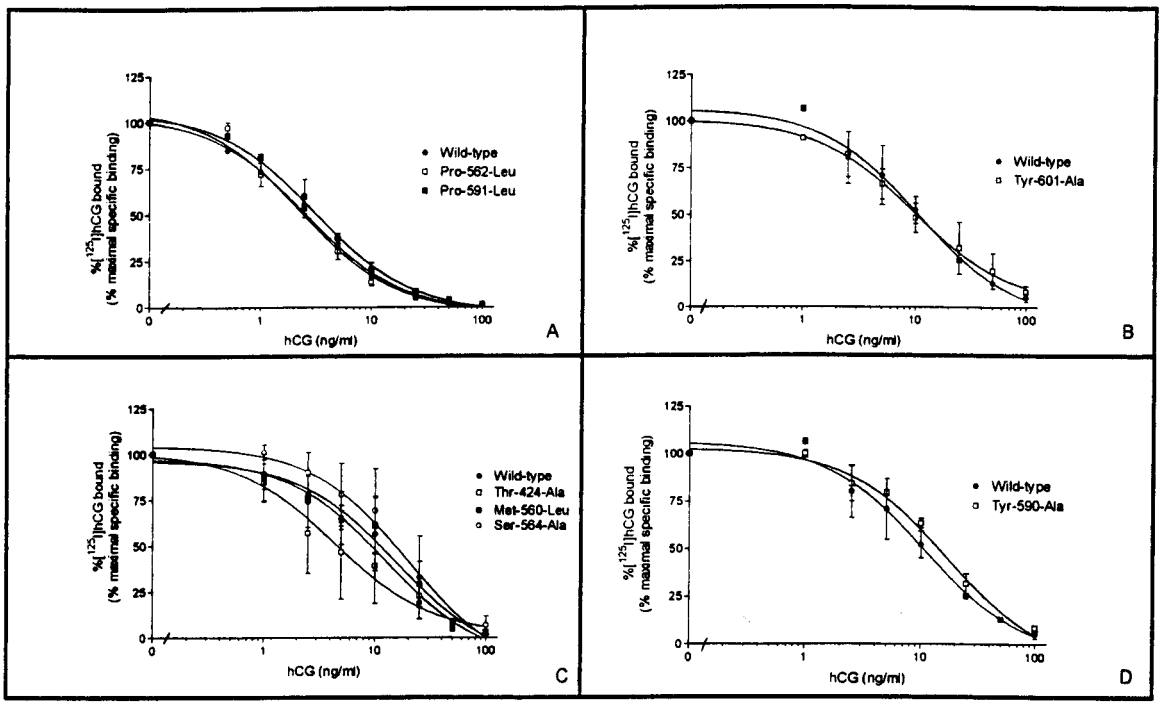


FIGURE 2: Competitive specific binding of [$^{125}$I]hCG (25 pM) and hCG (0.5−100 ng/mL in A; 1−100 ng/mL in B−D) to COS-7 cells transfected with wild-type and mutant pSVL-LH/CG-Rs as indicated. The specific binding of [$^{125}$I]hCG in the absence of unlabeled hCG was normalized to 100%, and the binding at various concentrations of hCG is given as mean ± SEM relative to that value; results are averaged from two independent transfections and binding experiments. There is no significant difference between the $K_d$'s describing the hCG-LH/CG-R interaction of wild-type (0.15 nM) and mutant receptors. For the results in panel A, DEAE-dextran was used for the transfections, and the number of receptors/cell (uncorrected for transfection efficiency) was $(1.8 \pm 0.4) \times 10^3$ for wild-type LH/CG-R; the (Pro-562-Leu) and (Pro-591-Leu) LH/CG-Rs were, respectively, 65 ± 24% and 39 ± 7% of that for wild-type receptor. For the data in panels B−D, Lipofectamine was used for the transfections, and the number of receptors/cell (uncorrected for transfection efficiency) was $1.5-2 \times 10^4$. There was no significant difference in the number of receptors/cell for the (Met-560-Leu) and (Ser-564-Ala) LH/CG-Rs and that of wild-type LH/CG-R; the (Thr-424-Ala), (Tyr-590-Ala) and (Tyr-601-Ala) LH/CG-Rs were present at 23 ± 2%, 69 ± 7%, and 7 ± 1% that of wild-type LH/CG-R, respectively.

Table 2: Binding and cAMP Parameters in Intact COS-7 Cells Transfected with Wild-Type and Mutant LH/CG-R cDNAs[a]

| LH/CG-R type | $K_d$ (nM) | receptors/cell ($\times 10^{-3}$) | $EC_{50}$[b] (nM) | $cAMP_{max}$[c] (%) |
|---|---|---|---|---|
| wild-type[d] | 0.13 ± 0.08 | 15.0 ± 3.4 | 0.09 ± 0.04 | 100 |
| Thr-424-Ala[d] | 0.12 ± 0.10 | 3.5 ± 0.3 | 0.14 ± 0.06 | 99 |
| Met-560-Leu[d] | 0.16 ± 0.12 | 24.8 ± 12.5 | 0.13 ± 0.04 | 105 |
| Ser-564-Ala[d] | 0.23 ± 0.17 | 24.1 ± 9.5 | 0.11 ± 0.05 | 106 |
| wild-type[e] | 0.03 ± 0.01 | 1.8 ± 0.4 | 0.23 ± 0.04 | 100 |
| Pro-562-Leu[e] | 0.02 ± 0.01 | 1.2 ± 0.4 | 1.71 ± 0.11 | 52 |
| Pro-591-Leu[e] | 0.05 ± 0.02 | 0.7 ± 0.1 | 0.33 ± 0.37 | 23 |
| wild-type[d] | 0.28 ± 0.16 | 19.6 ± 3.7 | 0.07 ± 0.04 | 100 |
| Tyr-590-Ala[d] | 0.27 ± 0.09 | 13.4 ± 1.3 | 0.23 ± 0.05 | 90 |
| Tyr-601-Ala[d] | 0.32 ± 0.18 | 1.3 ± 0.1 | >3 | 8 |

[a] Cells were transfected with either Lipofectamine or DEAE-dextran, followed by competitive binding studies with 25 pM [$^{125}$I]hCG and hCG to determine $K_d$ and the average number of receptors/cells (not corrected for transfection efficiencies) and measurements of cAMP production in response to hCG. [b] $EC_{50}$ is defined as the effective concentration of hCG required to achieve 50% of the maximal cAMP production, above basal, in response to added hCG. [c] $cAMP_{max}$ is the maximum amount of cAMP produced in response to added hCG (the highest concentration used was 100 ng/mL); basal values have been subtracted. The $cAMP_{max}$ for each experiment with wild-type LH/CG-R was normalized to 100%, and the results for the mutant receptors are given relative to that value. [d] Transfection via Lipofectamine. [e] Transfection via DEAE-dextran.

between extracellular loop III and TMH VII (Fernandez & Puett, 1996), and of Glu$^{332}$ and Asp$^{333}$, located in the extracellular domain just prior to TMH I (Huang & Puett, 1995).

Prolines at positions 562 and 591 are invariant in the glycoprotein hormone receptors, and Pro-562 is invariant in all members of the G protein-coupled receptor superfamily (Probst et al., 1992). It seems quite possible that equivalent Pro→Leu mutations in the other G protein-coupled receptors would diminish ligand-mediated receptor activation. Pro→Leu mutations have been associated with several G protein-coupled receptor-linked disorders. For example, Stein et al. (1993) showed that the naturally occurring Pro-556-Leu mutation in TMH IV of the mouse TSH-R, corresponding to Pro-479 in the rat LH/CG-R, resulted in hypothyroidism in *hyt/hyt* mice. Moreover, cells transfected with a cDNA containing the (Pro-556-Leu) TSH-R exhibited no cell surface hormone binding (Stein et al., 1993), although the mutant receptor is expressed on the plasma membrane (Gu et al., 1995). Another well documented case is that of autosomal dominant retinitis pigmentosa, attributable to a Pro-171-Leu mutation in the rhodopsin gene (Sung et al., 1993); this proline in TMH IV corresponds to Pro-479 in LH/CG-R. Since the (Pro-171-Leu) rhodopsin mutant accumulates in the endoplasmic reticulum, the replacement presumably interferes with proper folding or localization.

Tyr-601 is also invariant in the G protein-coupled receptor superfamily (Probst et al., 1992). Site-directed mutagenesis of Tyr-678 of TSH-R, corresponding to Tyr-601 of LH/CG-R, yielded a mutant receptor that bound TSH like wild-type receptor, but TSH-mediated cAMP production was virtually eliminated (Shi et al., 1994), as we found with the (Tyr-601-Ala) LH/CG-R mutant. These results, coupled with the

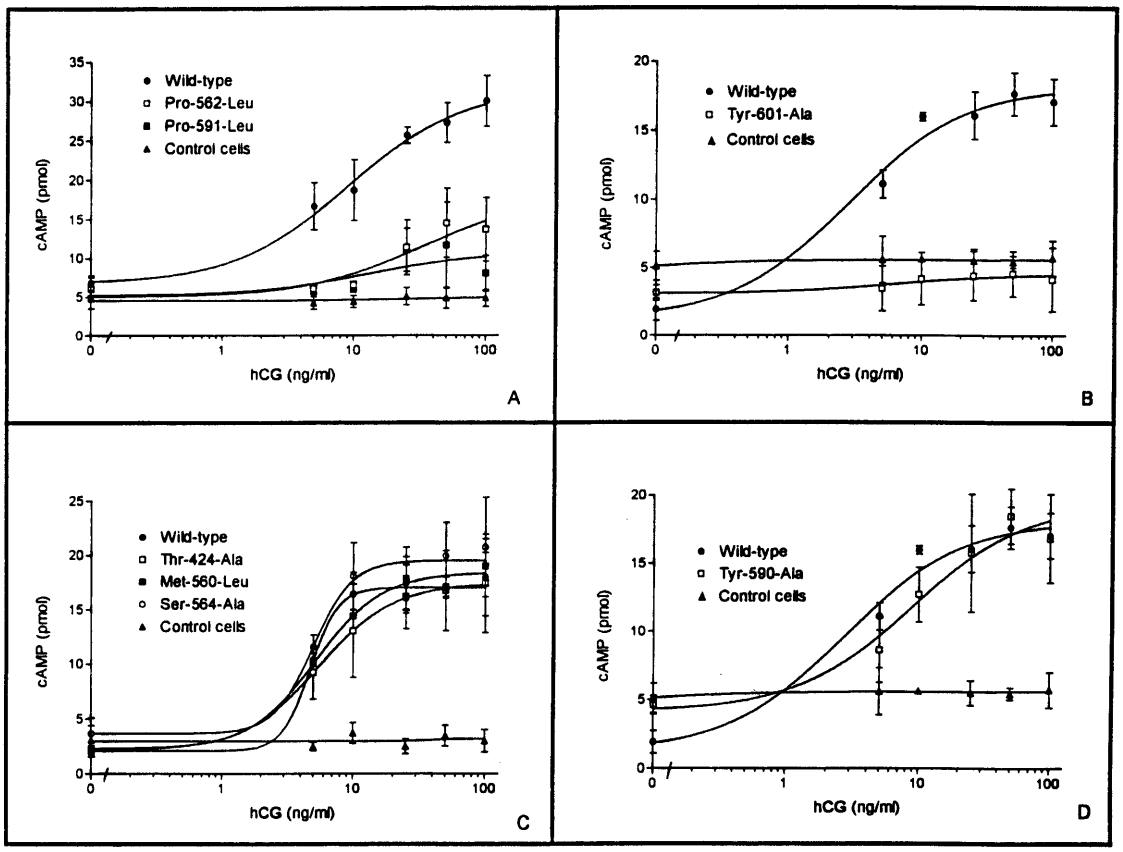


FIGURE 3: cAMP production in COS-7 cells transfected with wild-type and mutant pSVL-LH/CG-Rs at different concentrations of hCG (5−100 ng/mL). The results are given as mean ± SEM and represent the results from three (panels A and B) and two (panels C and D) independent transfections. Similar to the data presented in Figure 2, results are from transfections with DEAE-dextran (panel A) and Lipofectamine (panels B−D). The maximal amount of cAMP produced in cells transfected with the wild-type LH/CG-R cDNA varied from 18−32 pmol/$10^5$ cells under the conditions used, and basal and control levels were between 1 and 6 pmol/$10^5$ cells; these values were independent of the transfection method. Only the (Pro-562-Leu), (Pro-591-Leu), and (Tyr-601-Ala) LH/CG-Rs yielded maximal cAMP levels, i.e., at 100 ng/mL of hCG, significantly less than control. For those receptor mutants exhibiting hCG-mediated maximal cAMP levels like that of wild-type LH/CG-R, the average effective hCG concentrations for a 50% increase in cAMP over basal were similar.

Table 3: [$^{125}$I]hCG Binding to (Pro-479-Leu) LH/CG-R and (Pro-598-Leu) LH/CG-R in Intact and Lysed COS-7 Cells[a]

| LH/CG-R type | intact cells | lysed cells |
|---|---|---|
| wild-type | 100 ± 7% | 100 ± 24% |
| Pro-479-Leu | 0.04 ± 0.04% | 4 ± 1% |
| Pro-598-Leu | 0.03 ± 0.02% | 43 ± 6% |

[a] Cells were transfected with Lipofectamine, and specific binding was determined using 50 pM [$^{125}$I]hCG with intact and solubilized cells. For purposes of comparison, the specific binding to wild-type LH/CG-R was normalized to 100%, and the specific binding of each of the two mutants is given relative to that value. The total binding of [$^{125}$I]hCG to wild-type LH/CG-R varied from about 4000 to 9000 cpm, and nonspecific binding was generally <5% of the total binding to intact cells and about 20% in the soluble binding assay. The results are given as mean ± SEM from two transfections.

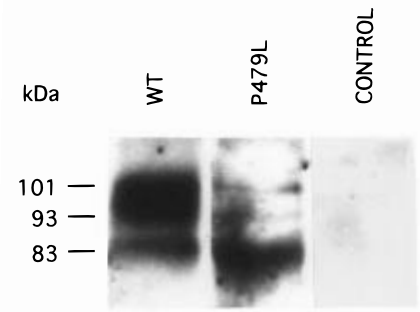


FIGURE 4: Western blots of COS-7 cells transfected with pSVL-LH/CG-R wild-type and the (Pro-479-Leu) mutant (P479L) (lanes 1 and 2, respectively). Lane 3 shows a blot obtained using control cells. The wild-type and mutant LH/CG-R apparent molecular masses of 101, 93, and 83 kDa were determined with a set of prestained standards [rabbit muscle myosin (205 kDa), *E. coli* $\beta$-galactosidase (116 kDa), BSA (66 kDa), chicken egg ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), bovine milk α-lactalbumin (14.2 kDa), and bovine milk aprotinin (6.5 kDa)].

stringent conservation of Tyr at this position in TMH VII of all G protein-coupled receptors, suggest a critical and fundamental role of this aromatic residue in transmembrane signaling.

In view of the intriguing results noted with single replacements of amino acid residues in TMH VII of LH/CG-R, a helical wheel projection was made (Figure 5), with the realization of course that the actual structure will exhibit kinks at positions 591 and 598 due to the prolines. This projection suggests that the two point mutants of LH/CG-R which resulted in diminished signal transduction, (Pro-591-Leu) and (Tyr-601-Ala), may map on the same face of the helix as does (Pro-598-Leu) LH/CG-R, which fails to localize properly. In addition, a naturally occurring mutation of Ser-616 of the human LH/CG-R, corresponding to Ser-594 of

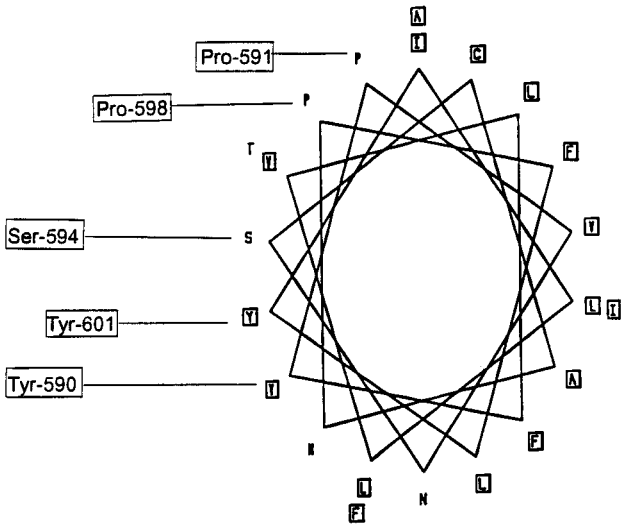


FIGURE 5: Helical wheel projection of TMH VII of rat LH/CG-R. Ile-584 and Ala-602 are shown at the top, and the amino acid residues replaced in this study are indicated: Tyr-590, Pro-591, Pro-598, and Tyr-601. Ser-594 is also highlighted since Laue et al. (1995) reported a naturally occurring mutation at this equivalent position in human LH/CG-R (residue 616 in the human LH/CG-R sequence) in a patient presenting with hypogonadism. The (Pro-591-Leu) and (Tyr-601-Ala) LH/CG-Rs bind hCG like wild-type LH/CG-R but signaling is diminished; the (Pro-598-Leu) LH/CG-R is not properly expressed at the cell surface; and the (Tyr-590-Ala) LH/CG-R is indistinguishable from wild-type LH/CG-R in the assays used. The actual structure of TMH VII would be kinked from the presence of the two prolines at positions 591 and 598.

the rat LH/CG-R, was found which resulted in hypogonadism (Laue et al., 1995). This serine is invariant in the glycoprotein hormone receptors and maps within the arc of the helix between Pro-591 and Tyr-601. Also, a Glu replacement of Lys-583, which occupies the interface between the third extracellular loop and TMH VII, had no effect on ligand binding, but signaling was greatly diminished (Fernandez & Puett, 1996). Aside from the kinks introduced by prolines 591 and 598, Lys-583 would map 12 Å above Tyr-601 if it began TMH VII. Thus, our results and those of Laue et al. (1995) on Ser-616 of human LH/CG-R strongly implicate a critical role for several amino acid residues in TMH VII in transmembrane signaling. If one projects these amino acid residues to the corresponding ones in rhodopsin and uses the Baldwin (1993) model, they would be oriented toward the internal cleft formed by the TMHs. Additional experimentation is required to identify other critical amino acid residues in TMH VII. It is also not known whether the effect of the replacements is attributable to an alteration of helix−helix contacts or of the intracellular loops, which could reduce signaling.

Studies on bacteriorhodopsin have shown that TMH associations can be driven by sequence-specific helix−helix interactions (Kahn & Engelman, 1992). Such interactions may include electrostatic interactions within the membrane, packing effects, and constraints imposed by interactions outside the plasma membrane (Treutlein et al., 1992). Also, work on the dimerization of the TMH of glycophorin A indicates that very subtle changes in the side-chain structure at certain positions, which occur at approximately every 3.9 residues along the helix, disrupt interhelical interactions (Lemmon et al., 1992).

Since helix−helix contact surfaces can contain both polar and nonpolar amino acid residues, we targeted several polar but nonionizable residues for mutation in LH/CG-R: Thr-424-Ala (TMH III), Met-560-Leu (TMH VI), and Ser-564-Ala (TMH VI). These particular amino acid residues were selected since they are invariant in the glycoprotein hormone receptors; also, modeling studies based on the structure of bacteriorhodopsin have indicated that these three residues could be appropriately situated in the internal cleft formed by the seven TMHs (Hoflack et al., 1993), and thus at least Thr and Ser may form electrostatic interactions. However, neither of the point mutants at these positions exhibited any significant differences relative to wild-type LH/CG-R in ligand binding or transmembrane signaling. Likewise, an Ala replacement of Tyr-590 (TMH VII) of LH/CG-R, which is invariant in the glycoprotein hormone receptors, yielded a mutant that exhibited ligand binding and cAMP responsiveness comparable to that of wild-type LH/CG-R.

Replacement of Pro-479 (TMH IV) and of Pro-598 (TMH VII) with Leu in LH/CG-R led to mutant forms of the receptor that exhibited no significant cell surface binding of hCG, although binding was found with (Pro-598-Leu) LH/CG-R in detergent-solubilized transfected cells. (Under the conditions used in the soluble assays, we cannot distinguish between a higher $K_d$ or a lower number of receptors for the mutants relative to wild-type LH/CG-R.) Since (Pro-479-Leu) LH/CG-R bound hCG very poorly, Western analysis was performed to ensure expression of the mutant receptor. On the basis of the assignments of Hipkin et al. (1992), we found that, relative to wild-type receptor, the mutant receptor exhibited a greater amount of putative receptor precursor than mature form. Since the wild-type receptor precursor binds hCG with a lower affinity than the mature form (Hipkin et al., 1992), the Western blot of the (Pro-479-Leu) LH/CG-R can account, at least qualitatively, for the diminished binding: there is somewhat less total receptor relative to wild-type, and the proportion of precursor-to-mature forms is much greater in the mutant. These results indicate that the Pro-479-Leu replacement in TMH IV of LH/CG-R interferes with proper receptor folding, localization, or posttranslational processing; it may also lead to decreased translation of the receptor mRNA or to increased degradation of the mature form of the receptor. Since the (Pro-598-Leu) LH/CG-R specifically binds roughly half of the [$^{125}$I]hCG compared to wild-type LH/CG-R in solubilized cells but not intact cells, we can suggest that the major effect of the replacement is on receptor localization and perhaps processing.

## CONCLUSIONS

We have identified two prolines and one tyrosine in rat LH/CG-R that appear critical for proper ligand-mediated signaling, Pro-562 (TMH VI), Pro-591 (TMH VII), and Tyr-601 (TMH VII), and two prolines that seem important in chain folding, stability, proper localization, or processing, Pro-479 (TMH IV) and Pro-598 (TMH VII). We would predict that naturally occurring mutations of these amino acid residues in the corresponding positions of human LH/CG-R would result in hypogonadism.

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