Functional Differences of Invariant and Highly Conserved Residues in the Extracellular Domain of the Glycoprotein Hormone Receptors

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Multiple interactions exist between human follicle-stimulating hormone (FSH) and the N-terminal hormone-binding fragment of the human FSH receptor (FSHR) extracellular domain (ECD). Binding of the other human glycoprotein hormones to their cognate human receptors (luteinizing hormone receptor (LHR) and thyroid-stimulating hormone receptor (TSHR)) was expected to be similar. This study focuses on amino acid residues in β-strands 2 (Lys74), 4 (Tyr124, Asn129, and Thr130), and 5 (Asp150 and Asp153) of the FSHR ECD identified in the human FSH:FSHR ECD crystal structure as contact sites with the common glycoprotein hormone α-subunit, and on noncontact residues in β-strands 2 (Ser78) and 8 (Asp224 and Ser226) as controls. These nine residues are either invariant or highly conserved in LHR and TSHR. Mutagenesis and functional characterization of these residues in all three human receptors allowed an assessment of their contribution to binding and receptor activation. Surprisingly, the six reported α-subunit contact residues of the FSHR ECD could be replaced without significant loss of FSH binding, while cAMP signaling potency was diminished significantly with several replacements. Comparative studies of the homologous residues in LHR and TSHR revealed both similarities and differences. The results for FSH/FSHR were analyzed on the basis of the crystal structure of the FSH:FSHR ECD complex, and comparative modeling was used to generate structures for domains, proteins, and complexes for which no structures were available. Although structural information of hormone-receptor interaction allowed the identification of hormone-receptor contact sites, functional analysis of each contact site was necessary to assess its contribution to hormone binding and receptor activation.

The heterodimeric glycoprotein hormones (luteinizing hormone (LH), CG, FSH, and TSH), each consisting of a common α-subunit and a hormone-specific β-subunit, regulate a number of developmental, reproductive, and metabolic processes by activating their cognate GpHRs, LHR, FSHR, and TSHR (1, 2). GpHRs are composed of two approximately equally sized but functionally distinct modules as follows: an N-terminal ECD, responsible for ligand recognition and binding (3–5), linked to a prototypical GPCR domain (6), responsible for signaling. GpHRs belong to the subfamily of LRR-containing GPCRs (7). Within the ECD of a given GpHR, the LRR region that is flanked by N- and C-terminal cysteine-rich domains is responsible for ligand-binding specificity and affinity (8–17). The ECDs of the three GpHRs display a lower sequence identity (~40%), reflecting the observation that hormone specificity is completely encoded within the LRR region, compared with their transmembrane helix portions (~70%).

Structural information related to the transmembrane helix moiety of GpHRs has become available for several GPCRs as follows: rhodopsin in the dark and constitutively active forms (18–21), the β1- and β2-adrenergic receptors (22–25), and the A2a,-adenosine receptor (26). The crystallographic structure of bovine rhodopsin, in particular, has been used for homology modeling of many GPCRs, including the GPCR moiety of GpHRs (27).

Based on models of GpHR ECDs, it was suggested that the LRR region consists of successive β-strands and α-helices that organize themselves into a cusp-shaped structure (8, 28, 29). Isolated ECDs are capable of binding the hormones with high affinity, as demonstrated by the co-purification of FSH with part of the ECD of the FSHR (30). Studies on chimeric glycoproteins and their receptors have proven highly useful in elucidating structure-function relationships of GpHRs, and detailed mutagenesis studies allowed the identification of a relatively small number of positive and negative key hormone-selective amino acid residues in the ECDs of GpHRs (4, 14–17, 31).

The crystallographic structure of an N-terminal hormone-binding fragment of the FSHR ECD complexed with single-chain FSH provided, for the first time, a crystal structure of a glycoprotein hormone bound to its cognate receptor (30). In the complex, electron densities were determined for amino acid residues 18–259 of the ECD, and both subunits of FSH were found to make multiple contacts with several LRRs of the FSHR...
ECD fragment in a clasped hand arrangement. This structure has aided enormously in interpreting mutagenesis data (14–17) and vice versa. Complementing the FSHR ECD structure, the structure of the TSHR ECD complexed with a monoclonal antibody was found to be very similar to that of the FSHR ECD (32).

The goal of this study was to analyze several invariant and highly conserved amino acid residues in the ECDs of human FSHR, LHR, and TSHR (Fig. 1) that have been identified as hormone-receptor contact sites in the FSH-FSHR ECD structure (30). By comparing Ala replacements of these amino acid residues in the three receptors as well as multiple replacements of some, it is possible to obtain a measure of the generality of the FSHR ECD structure too similar or dissimilar manner. Based on their contact with the hormone, the TSHR and LHR at either of the sites chosen.

Our results establish that invariant and highly similar amino acid residues in homologous positions of these three similar human GPCRs have distinct functional roles when comparing FSH/FSHR, CG/LHR, and TSH/TSHR binding and hormone-mediated signaling. Whether these differences reflect distinct binding modalities, microenvironments, and/or localized conformational changes remains to be established.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Cellgro Mediatech, Inc. (Herndon, VA) and Sigma. NCS, antibiotics, Waymouth’s media, and trypsin-EDTA were purchased from Invitrogen, and TransFectin lipid reagent was from Bio-Rad. BSA, isobutylmethylxanthine, a goat anti-rat IgG peroxidase conjugate, and the recombinant human hormones CG and FSH and bovine TSH were purchased from Sigma. Also, human CG, LH, and FSH were obtained from NV Organon (Oss, The Netherlands, courtesy of Dr. W. G. E. J. Schoonen); human TSH was from Sigma and from Dr. A. F. Parlow, National Institutes of Health, Torrance, CA. The high affinity (monoclonal) anti-HA antibody was from Roche Diagnostics.

**Construction of Mutant FSHRs, LHRs, and TSHRs**—The cDNAs encoding human FSHR and LHR were kindly provided by Dr. T. Minegishi (Gunma University School of Medicine, Maebashi, Japan) and Dr. E. Milgrom (INSERM, Paris, France), respectively, and that for human TSHR was obtained from the Missouri S&T cDNA Resource Center. Following subcloning of the cDNAs into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen), PCR-based mutagenesis was used with the FSHR, LHR, and TSHR templates to obtain the desired replacements. An epitope (YPYDVPDYA) from the influenza virus

**Alignment of the three human GpHR ECDs.** The numbering given above is based on the FSHR ECD. The arrows, positioned on the solid line (above the numbering) indicates the part of the FSHR ECD of which no structural information is known yet. The invariant and highly conserved amino acid residues that were studied are shown in boldface, and all cysteine residues are presented in white on a darker background. Residues on a gray background are forming the β-strands facing the hormone in FSHR and are presumed to do the same in LHR and TSHR.
hemagglutinin (HA) was inserted between the C terminus of the signal peptide and the N terminus of the mature receptor, i.e. between Gly<sup>17</sup>–Cys<sup>18</sup> of FSHR, Leu<sup>27</sup>–Arg<sup>28</sup> of LHR, and Gly<sup>20</sup>–Gly<sup>21</sup> of TSHR.

**Cell Culture and Transient Transfection of Cells**—HEK 293 cells (obtained from the American Type Culture Collection, Manassas, VA) and HEK 293T cells (36) were grown at 37 °C in humidified air containing 5% CO<sub>2</sub> in DMEM supplemented with 10% (v/v) newborn calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 125 ng/ml amphotericin B, and 10 mM HEPES, pH 7.4 (HEK 293 cells), or with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B, pH 7.0–7.2 (HEK 293T cells) and transiently transfected as described elsewhere (16, 17, 37, 38). Briefly, the HEK 293 cells were transiently transfected (1 or 5 μg of wild type (WT) cDNA and 5 μg of mutant cDNA) using TransFectin, and the HEK 293T cells were transiently transfected (1 μg of WT and mutant CDAs) with 10 μg of pCRE/β-gal plasmid (39) using the modified bovine serum transfection method (15, 16). To achieve comparable receptor densities between WT and mutant receptors in the HEK 293 cells, each mutant receptor was compared with a WT control in which the B<sub>max</sub> values were within a 2–3-fold range of each other. Hence, in some cases, WT receptor controls, transfected with 1 μg of cDNA, were compared with mutant receptors transfected with 5 μg of cDNA.

**Cell Surface Expression**—Receptor levels at the cell surface were measured by specific hormone binding (described below) and ELISA measurements (15–17). For the latter, the HEK 293T cells were fixed with 10% buffered saline for 30 min and then blocked with 1% dried nonfat milk, 125 mM NaHCO<sub>3</sub> at room temperature for 2 h. A 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma) was used to visualize the peroxidase activity of the WT and mutant receptors. The changes in absorbance values (at 450 nm) of mock transfected cells were subtracted, and mutant receptor expression values were expressed as the percentage of WT receptor expression.

Experiments were repeated a minimum of three times from independent transfections, each performed in duplicate (HEK 293) or triplicate (HEK 293T), and the results are presented as mean ± S.E.

**Hormone Binding**—Binding assays with HEK 293 cells were performed as given elsewhere (37, 38, 40, 41). Briefly, about 16–18 h after transfection, the cells were replated (1 × 10<sup>5</sup> cells per well) into 12-well tissue culture plates, and 24 h later the cells were assayed for 125I-labeled human hormone (CG or FSH) competitive and saturation binding. Competitive binding experiments were performed with a fixed concentration of labeled hormone (50–100 pm) and increasing concentrations of unlabeled hormone, whereas the saturation binding studies were conducted with various concentrations of labeled hormone (50–5000 pm). All binding studies involved incubation of the hormone and cells at 37 °C for 6 h in the presence of binding buffer (278 mM sucrose, 0.1% glucose, 5 mM HEPES, pH 7.4, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, and 0.1% bovine serum albumin (42). Nonspecific binding was determined by addition of a 1000-fold excess of unlabeled hormone. The experiments were repeated 3–5 times, each in duplicate, using independent transfections, and the results are presented as K<sub>d</sub> and B<sub>max</sub> values relative to the WT receptor. As discussed above, transfection conditions were chosen to minimize significant differences between WT and mutant receptors in the HEK 293 cells in which both binding and signaling studies were conducted. Binding studies were not performed for TSH and TSHR because iodination of the human hormone effectively abolishes specific binding to the receptor, and we prefer not to compare data using a nonhomologous system, e.g. bovine TSH.

**Cyclic AMP Assays**—The transfected HEK 293 cells were prepared as described above and incubated with increasing concentrations of the cognate hormone, FSH, CG, and TSH, at 37 °C for 30 min in the presence of 0.8 mM 3-isobutyl-1-methylxanthine (37, 38). The incubation medium was removed, and the cells were lysed in 100% ethanol at −20 °C overnight. The extract was collected, dried under vacuum, and resuspended in the buffer of the 125I-cAMP assay kit. Cyclic AMP concentrations were determined by RIA as recommended by PerkinElmer Life Sciences. All experiments were repeated 3–5 times, each in duplicate, and the results for EC<sub>50</sub> and R<sub>max</sub> are given as means ± S.E., relative to the WT receptor. In separate experiments, signaling was measured with the HEK 293T cells as described elsewhere (15–17). Briefly, 2 days after transfection, the cells were cultured in HEPES-modified DMEM containing 0.1% bovine serum albumin and 0.1 mM 3-isobutyl-1-methylxanthine and then incubated for 6 h with the cognate hormone, including human LH and LHR. The conversion of o-nitrophenyl-β-D-galactopyranoside into o-nitrophenol was measured at 405 nm, reflecting the changes in β-galactosidase activity mediated by the gonadotropins. The β-galactosidase gene used to transfect the HEK 293T cells was under the control of a human vasoactive intestinal peptide promoter containing five cAMP-response elements (39). The collected data were related to 10 μM forskolin-induced changes and were thus expressed in arbitrary units. Experiments were repeated three times, each in triplicate, and the results are given as means ± S.E.

**Data Analysis**—Both binding and cAMP data were analyzed by Prism software (Graph Pad Software, San Diego) using nonlinear regression analysis. Whenever possible, the B<sub>max</sub> and K<sub>d</sub> values, obtained from saturation binding, and the EC<sub>50</sub> and R<sub>max</sub> values, obtained from RIA measurements, were used to determine the coupling efficiency (43),

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Q = \frac{1}{(1/2)(1 + (K_d/EC_{50}))/(R_{max}/B_{max})}
\]

of receptors expressed in HEK 293 cells. Relative values of B<sub>max</sub>, EC<sub>50</sub> and R<sub>max</sub> were also determined independently with the HEK 293T cells. In addition to standard statistical analysis to determine significance (p < 0.05), differences between expression, binding, and signaling of mutant receptors had to exceed that of WT receptor by ≥3- or ≤3-fold. This rather stringent criterion was imposed to correct for inter-
assay variability and to help ensure that stated differences are functionally significant.

**Molecular Modeling**—Models of complexes of human LH, CG, and TSH bound to the LRR domain of their respective human receptors were built using the available structures of FSH-FSHR<sub>B</sub> (Protein Data Bank code 1XWD) (30) and M22-TSHR (Protein Data Bank code 3G04) (32). The high sequence identity between the three GpHRs, the identical α-subunit, and the high sequence identity shared by the three β-subunits of the hormones contributed to this process. The sequences of CG, LHR, TSH, and TSHR were aligned to the stretch of residues present in the crystal structure of the FSH-FSHR ECD complex taking into account sequence identity, the position of disulfide bonds, and structural features. Comparative modeling was done using MODELLE<sub>R</sub> (44), which uses the satisfaction of spatial restraints, along with simultaneous optimization of CHARMM energies, using conjugate gradients and molecular dynamics with simulated annealing (45). A script with increased variable target function method and molecular dynamics optimization was used to generate a model for each complex. The models were evaluated by examination of the MODELLE<sub>R</sub> output log files, their Ramachandran plots, visual inspection, and finally cross-checked with existing mutagenesis data. The programs PISA (46), PROTORP (47), PIC (48), RAMPAGE, LIGPLOT (49), HBPLUS 3.0 (50), and PyMOL (51) were used to identify the residues involved in the interaction between hormone and receptor and to calculate physical and chemical parameters of the protein interaction sites. Models of each glycoprotein hormone-receptor complex were used to generate maps of the electrostatic surface potentials and to study the hormone receptor interface. Visualization and molecular graphics were done using PyMOL and Discovery Studio Visualizer (Accelrys Software, Inc.).

**RESULTS**

**Locations of the FSHR and LHR Mutations**—Fig. 1 shows the aligned amino acid sequences of the three GpHR ECDs and highlights the location of the nine amino acid residues replaced with Ala. Additional replacements with other amino acid residues were also made of the Lys/Arg in β-strand 2 and the Asp/ Glu in β-strands 5 and 8. The structures of β-strands 2, 4, and 5 of FSHR are shown in supplemental Fig. 1, A–C, and the FSH-FSHR contacts (defined as <4 Å) are given schematically in supplemental Fig. 1D. We have determined the details of the FSH-FSHR ECD interactions (supplemental Table 1) based on the coordinates of the FSH-FSHR ECD structure as reported by Fan and Hendrickson (see supplemental material in Ref. 30). The listing in supplemental Table 1 indicates that the six residues bind to the FSH α-subunit via ion-ion, ion-dipole, H-bonding, and hydrophobic interactions. Our assessment of hormone-receptor contacts agree, in general, with those given by Fan and Hendrickson (30). A few differences, however, do exist. Of the nine amino acid residues chosen for Ala replacement, six were reported to be in contact with the FSH α-subunit or β-subunit in Fig. 2 of their paper (30). In our analysis, we find contacts with just the FSH α-subunit, as also concluded in the supplemental material by Fan and Hendrickson (30). Moreover, Ser<sup>78</sup> was reported in Fig. 2 of the Fan and Hendrickson paper (30) to exhibit a fractional solvent accessibility (FSA) of 0.1–0.4, although a direct contact was not indicated in the supplemental material of Ref. 30. Our analysis of the structure indicated no direct contact between the FSH α-subunit and Ser<sup>78</sup> in FSHR, and we have chosen to use this residue as a control in our studies.

**Measured Experimental Parameters (Tables 1–6)**—To fully characterize the mutant receptors and facilitate comparison with the appropriate WT receptor, the following two independent approaches were used to determine cell surface expression (B<sub>max</sub>) of HA-tagged FSHR, LHR, and TSHR: ELISA measurements to determine total receptor numbers (in HEK 293T cells) and saturation binding to determine functional receptor numbers (in HEK 293 cells), i.e. capable of specific binding of cognate hormone. Signaling was also based on two independent approaches as follows: a colorimetric assessment of cAMP-induced β-galactosidase activity (in HEK 293T cells) and RIA of intracellular cAMP (in HEK 293 cells). Both methods permit a quantitative comparison between WT and mutant receptors of the EC<sub>50</sub> (potency) and R<sub>max</sub> (efficacy) values, i.e. the maximal cAMP produced at high ligand concentrations, corrected for the basal concentration. With all receptor mutants investigated, the basal cAMP values were within ~2-fold that of the WT receptor; thus, there is no compelling evidence of constitutive activation in any of the mutants. Each parameter provides information about the mutant receptors, and whenever possible, coupling efficiencies were determined. To facilitate comparisons, the results for K<sub>d</sub>, EC<sub>50</sub>, and Q values are presented as a ratio of the value of the mutant receptor to that of the WT receptor; B<sub>max</sub> and R<sub>max</sub> as per convention, are given as a percentage of that for each WT receptor. Typical results for WT FSHR and LHR (HEK 293 cells transfected with 1 μg of cDNA) are, respectively, as follows for B<sub>max</sub>, K<sub>d</sub>, EC<sub>50</sub>, and Q: 18 ± 4 fmol/well, 2.7 ± 0.5 nm, 0.48 ± 0.07 nm, and 51 ± 13 pmol/well for FSHR (n = 17) and 25 ± 9 fmol/well, 1.2 ± 0.2 nm, 0.49 ± 0.06 nm, and 34 ± 12 pmol/well for LHR (n = 19). For TSHR (HEK 293 cells transfected with 5 μg of cDNA), the EC<sub>50</sub> and R<sub>max</sub> values were 1.5 ± 0.2 and 91 ± 17 pmol/well, respectively (n = 15).

**Functional Characterization of the GpHR Mutants (Tables 1–6 and Figs. 2–6)**—Tables 1–6 summarize the results obtained for the mutants in β-strands 2, 4, 5, and 8 of the three receptors. Figs. 2–5 show signaling dose-response curves for several of the more interesting mutants, and representative binding data for the two aspartic acid residues in β-strand 5 of FSHR and LHR are presented in Fig. 6. The data in Tables 1–5 and Figs. 2–4 are from the studies using HEK 293 cells; the results on expression and signaling with HEK 293T cells, based on different methods of analysis, are given in Table 6 and Fig. 5. Results were also obtained for LH and LHR, but for brevity, these are not presented. They do, however, parallel the results obtained with CG and LHR. Although there are some differences in the expression and signaling results obtained on the two cell types with different methodologies, the agreement between the two methods is good overall.

**Mutants in β-Strand 2** (Tables 1 and 6 and Fig. 6)—We chose Lys<sup>74</sup> in FSHR and the corresponding Lys and Arg in LHR and
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TSHR, respectively, as a contact site to study because it binds to Ser85 and Thr86 of the FSH α-subunit with an FSA of 0.4. The K74A,K74E mutants in FSHR, the K77A,K77E mutants in LHR, and the R80A,R80E mutants in TSHR express well, and all exhibit signaling parameters like those of WT receptor. The $K_d$ values of the FSHR and LHR mutants are also comparable with WT receptors. Thus, even though Lys74 is a contact site for the FSH α-subunit, it can be replaced with Ala or even an oppositely charged residue, Glu, without having an adverse effect on binding affinity. With the exception of the K74E mutant, the Q values are similar to that of WT receptor. Ala replacements of the Ser78 in β-strand 2, a noncontact site in FSHR, also behaved like the WT receptors. In our model of CG-LHR (see below), we find that LHR Lys77 interacts in a very similar way as FSHR Lys74, forming a hydrogen bond with Ser85 and Thr86 in the α-subunit. In our model of TSHR-TSH, we do not observe such an interaction for Arg80, which is not close to any TSH residue within a 4-Å range and has an FSA of 0.67.

Mutants in β-Strand 4 (Tables 2 and 6 and Figs. 2 and 6)—The three following side chains were selected for replacement in β-strand 4: Tyr124, Asn132, and Thr130 in FSHR as well as the corresponding residues in LHR (identical in LHR) and TSHR (Phe, Asn, and Thr, respectively, in TSHR). In the FSH-FSHR ECD structure, Tyr124, Asn132, and Thr130 contact FSH α-subunit residues Tyr38, Leu48,Val49, and Leu46 and have FSAs of 0.1–0.4, 0.1–0.4, and <0.1, respectively. With the exception of the three TSHR mutants, those for FSHR and LHR expressed well. Interestingly, the aromatic side chain could be replaced with a methyl group (on Ala) without any measurable functional change in FSHR and LHR. Likewise, the signaling parameters were similar to those of WT receptor, although the $EC_{50}$ value for TSHR was slightly elevated. Replacement of the invariant Asn in the three receptors with Ala resulted in a reduction in the binding affinities (measured for FSHR and LHR) and a much larger decrease in the signaling potency, particularly for FSHR. At high concentrations of hormone (Table 6), the $R_{max}$ values are like those of the WT receptor; however, the $EC_{50}$ values are greatly increased. Replacement of the invariant Thr with Ala in the three receptors led to a slight increase in the $K_v$ values of FSHR and LHR, but the $EC_{50}$ values of the three receptors were <3-fold higher than that of the WT receptor. The increase in $R_{max}$ of FSHR/T130A may reflect, at least in part, the high level of expression. Despite the low level of expression, TSHR/T130A signals similarly to the WT receptor. With the exception of FSHR/N129A, the coupling efficiencies of the FSHR and LHR mutants are like those of the WT receptors. The conserved Asn in β-strand 4 of FSHR, LHR, and TSHR is important in hormone-mediated signaling, but it seems to have a much larger role in FSHR than in the other two GpHRs. In our models, we also see that the aromatic side chains of Tyr127 (LHR) and Phe130 (TSHR) are involved in hydrophobic interactions with Tyr38 of the α-subunit, as is seen for Tyr124 in FSHR. We also see that the conserved asparagines (Asn129 in FSHR, Asn132 in LHR, and Asn135 in TSHR) are involved in multiple interactions with equivalent residues in the α-subunit. The FSA values for FSHR Asn129, LHR Asn132 and 135, and TSHR Asn135 are 0.13, 0.08, and 0.07, respectively.

Mutants in β-Strand 5 (Tables 3, 4 and 6 and Figs. 3, 4, and 6)—The two acidic residues in β-strand 5 were chosen for replacement in the three receptors. In the FSH-FSHR ECD structure, FSHR/Asp150 and FSHR/Asp153 show FSAs of <0.1 and 0.1–0.4, respectively. In addition to the Ala replacements made in the two acidic residues, we also investigated the effects of replacements with charge retention, e.g. Asp → Glu and Glu → Asp, a polar but nonionizable residue, e.g. Asp → Asn and Glu → Gln, and a positively charged residue, Lys. With the exception of LHR/E154K and TSHR/E157K, which expressed less than 30% that of the WT receptor, the expression levels of the other mutants were reasonably good.

For FSHR, the mutants D150E and D153E have properties comparable with those of WT FSHR. Although the $K_d$ values for D150A,D150K and D153A,D153K are similar to that of WT FSHR, the $EC_{50}$ values are greatly increased, especially for the Lys replacements. The $K_d$ and $EC_{50}$ values of the D150N and
D153N mutants are elevated some 3–6-fold over those of WT FSHR. The two Asp residues in β-strand 5 can thus be interchanged with Glu with no major change in functionality, but replacement with Ala, Asn, or Lys greatly reduces the signaling potency. The coupling efficiency was somewhat elevated for the D150E mutant, reflecting to a large extent the increased expression and $R_{\text{max}}$; the $Q$ values for the D150N, D150A and D153E, D153A mutants are similar to the WT receptor (that for D153N is some 3-fold lower than WT FSHR). The poor signaling of the Lys replacements prohibited an estimate of coupling efficiencies for those two mutants.

For LHR, the mutants E154D and D157E exhibit binding similar to that of WT LHR, but the EC$_{50}$ is increased some 6-fold in the former. The $K_d$ values of the E154A, E154Q and D157A, D157N mutants are increased some 4–13-fold over WT LHR, and the EC$_{50}$ values are increased even more so. Interestingly, the E154K and D157K mutants have $K_d$ values similar to that of WT LHR, although in both cases signaling is nearly abolished. Some of the increase in the EC$_{50}$ values of the E154Q, E154A and D157N, D157A replacements can be attributed to the decreased hormone binding affinity. The coupling efficiencies of the E154D and D157E replacements are similar to WT LHR, but $Q$ values could not be determined for the other replacements at these two positions in β-strand 5 due to the diminished signaling. For TSHR, the EC$_{50}$ values of the E157D, E157Q, E157A and D160A mutants are like that of WT receptor, although those for the D160E, D160N mutants are slightly elevated. Signaling is diminished in both the E157K and D160K mutants.

The two conserved acidic residues in β-strand 5 of the gonadotropin receptors seem to make more of a contribution to binding in LHR than in FSHR. Moreover, these acidic side chains in the three GpHRs appear to be of less importance in hormone-mediated signaling in TSHR than in the other two receptors. In our models (see below), all three complexes show similar interactions between the two acidic residues in the receptor and Lys$^\alpha$ of the α-subunit of the hormones. Interestingly, in our CG-LHR model, an additional hydrogen bond was
between Asp$^{157}$ and Arg$^{95}$ of the β-subunit of CG which, with its large and positively charged side chain of Arg$^{95}$, is prominently present in the interface. In the β-subunits of FSH and TSH, we find the much smaller and polar residues Ser$^{89}$ and Thr$^{90}$, respectively. Ser$^{89}$ of the FSH β-subunit makes contact with FSHR/Lys$^{179}$, a residue of crucial importance for hormone selectivity of the FSH receptor (14, 16, 52). TSH residue Thr$^{90}$ is not involved in a receptor interaction. Arg$^{95}$, Ser$^{89}$, and Thr$^{90}$ have FSA values of 0.4, 0.6, and 0.77, respectively.

**Mutants in β-Strand 8 (Tables 5 and 6)**—The two invariant residues chosen in β-strand 8 for controls are FSHR Asp$^{224}$ and Ser$^{226}$, LHR Asp$^{228}$ and Ser$^{230}$, and TSHR Asp$^{232}$ and Ser$^{234}$. FSHR/S226A and each of the TSHR mutants, D232A, D232K, and S234A, expressed poorly and could not be characterized. The $K_D$ values of FSHR D224A and D224K are comparable with that of WT receptor, but the signaling of D224A is reduced and almost abolished in D224K. The $K_D$ values of LHR D228A and D228K are elevated over WT LHR, and the signaling is greatly reduced in D228K (the increase in $EC_{50}$ of LHR/D228A may result from the increase in $K_D$ values compared with WT LHR). LHR/S230A behaves similarly to WT LHR, except that $R_{max}$ is increased. Although FSHR/Asp$^{224}$ is not a contact site for FSH binding, the side chain evidently contributes to FSH-mediated signaling, as it also does with LHR.

**Molecular Modeling of LHR (Tables 7–9 and Fig. 7)**—To study the possible effects of our mutations on the binding of CG to LHR and TSH to TSHR, and to compare those to the available complex structure of FSH-FSHR, models were generated using comparative modeling. The structures that were used as input are the coordinates of the hormone-binding domain of FSHR in complex with FSH (Protein Data Bank code 1XWD), the structure of the ECD of TSHR from the crystal structure of TSH in complex with a TSHR autoantibody (Protein Data Bank code 3G04), and the structure of human CG (Protein Data Bank code 1HCN). CG-LHR and TSH-TSHR models were built using the program MODELLER version 9.7 (44), checked for a
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TABLE 1
Summary of expression, binding, and signaling results for β-strand 2 mutants in HEK 293 cells

| Mutant | $R_{max}$ | $K_d$ | $EC_{50}$ | $R_{max}$ | $Q$ |
|--------|-----------|--------|-----------|-----------|-----|
|        | % WT      | (mutant/WT) | (mutant/WT) | % WT      |     |
| FSHR   |           |         |           |           |     |
| K74A (n = 5) | 87 ± 33 | 0.8 ± 0.2 | 1.0 ± 0.2 | 84 ± 11 | 0.9 ± 0.2 |
| K74E (n = 2) | 102 ± 24 | 0.8 ± 0.3 | 0.7 ± 0.2 | 210 ± 50 | 2.4 ± 0.1 |
| S78A (n = 5) | 66 ± 26 | 0.9 ± 0.3 | 1.4 ± 0.3 | 89 ± 25 | 0.9 ± 0.1 |
| LHR    |           |         |           |           |     |
| K77A (n = 4) | 143 ± 56 | 1.1 ± 0.2 | 1.0 ± 0.1 | 143 ± 51 | 1.0 ± 0.04 |
| K77E (n = 4) | 159 ± 52 | 1.3 ± 0.5 | 1.7 ± 0.3 | 228 ± 42 | 0.9 ± 0.1 |
| S91A (n = 4) | 217 ± 23 | 1.4 ± 0.3 | 1.3 ± 0.3 | 165 ± 42 | 0.9 ± 0.03 |
| TSHR   |           |         |           |           |     |
| R80A (n = 3) | a       | b       | 0.8 ± 0.1 | 70 ± 18 | a   |
| R80E (n = 3) | a       | b       | 0.9 ± 0.3 | 104 ± 23 | a   |
| S84A (n = 3) | a       |       | 0.5 ± 0.1 | 145 ± 18 | a   |

* Data were not determined by binding (see Table 6).
* Data were not determined.

low overall energy score, the absence of large conformational errors, and the presence of biologically relevant interactions based on the large number of published mutations.

A summary of the interface contacts is presented in Tables 7 and 8; clearly, there are many similarities and a number of potentially important differences in the three complexes. Overall, the three complexes had very low overall Cα root mean square deviation values when superimposed (Table 9), with an average root mean square deviation of 1.1 Å. Fig. 7 shows the crystal structure of the FSHR ECD (Fig. 7A) and that of the structure of bound FSH (Fig. 7D), taken from Fan and Hendrickson (30), both emphasizing the electrostatic surfaces. The hormone and receptor have been separated to enable a better view of the amino acid residues studied herein and the hormone residues that contribute to the hormone-receptor interface. Also shown in Fig. 7 is the model developed in this study of the ECD of the LHR (Fig. 7B) based on the structures of the TSHR ECD (Fig. 7C) (32) and FSHR ECD, together with its modeled hormone CG (Fig. 7E) based on the structure of CG and complexed FSH (30, 55–57). In addition, models for LH (Fig. 7F) and TSH (Fig. 7G) were generated in the same way based on complexed CG and CG structures. The supplementary Table 2 gives the PROTORP outputs for the two forms of the FSH-FSHR ECD complex (30) and the models for CG-LHR ECD and TSH-TSHR ECD complexes. All models were generated as complexes, which might explain why CG has a slightly higher overall root mean square deviation (supplementary Table 3) compared with the structure of free human CG. The root mean square errors of the various structures and models, including those for LH and TSH, are presented in supplementary Table 3, together with the Ramachandran values for each individual molecule (both experimental and modeled structures).

DISCUSSION

Differences in the β-subunits of the glycoprotein hormones (LH, CG, FSH, and TSH) are thought to contain the molecular determinants that are involved in specificity for their respective receptors. However, these hormones share the same α-subunit, suggesting that this subunit mainly contributes to receptor activation (58). The results of this study revealed that some of the identical or highly conserved amino acid residues, present in different β-strands of the LRRs of the GpHRs, do not have the same functions in the three receptors. Because these amino acid residues have been shown to be in contact with the α-subunit of FSH and, by inference, LH, CG, and TSH, these findings suggest that their effects on cell surface expression (presumably reflecting differences in protein folding, post-translational modifications, and/or trafficking), hormone binding affinity, and/or the hormone-mediated cAMP response differ between the GpHRs. The functional differences observed in binding and signaling may result from localized conformational differences in the hormones (see Refs. 55–57 for a comparison of the subtle structural differences in CG and FSH), the receptors, or the complexes, as evidenced by small induced conformational changes when FSH binds to the ECD of FSHR (30).

The amino acid residues in FSHR that were chosen for replacement include ones that bind the FSH α-subunit via hydrogen bonds and salt bridges (e.g. the Nε of Lys74 contacts the Oγ of α-subunit residues Ser85 and Thr86; the Oγ of Asn129 interacts with the N of α-subunit residues Leu48 and Val49; the Oδ2 of Asp150 contacts the Nε of α-subunit residue Lys91; and the Oδ2 of Asp153 interacts with the Nε of α-subunit residue Lys51) and hydrophobic interactions (e.g. Tyr124 with α-subunit residue Tyr88, Thr130 with α-subunit residue Leu48, and Asp153 with α-subunit residue Val49) (see the supplementary Table 1A in Ref. 30 and supplementary Table 1 in this study). The fractional solvent accessibility values are 0.4 (Lys74), 0.1–0.4 (Tyr124 and Asp153), and <0.1 (Asn129, Thr130, and Asp150). The three residues chosen for controls, Ser78 in β-strand 2 and Asp224 and Ser226 in β-strand 8, have no detectable contact with the α-subunit.

Interestingly, replacing FSHR/Lys74 with Ala had no major effect on the functionality of the mutant receptor. Thus, the N-O interactions are not required for WT-like receptor structure and function; indeed, even reversing the charge with K74E has no measurable impact on the parameters measured. This weak interaction is consistent with the FSA of <0.4. Likewise, the hydrophobic interaction of Tyr124 with α-subunit residue Tyr88 can be reduced with no major change in functionality as evidenced by the WT-like properties of the Y124A mutant. In contrast, the side chain interaction of the O on Asn129 is important for WT-like function as indicated by the increase in $K_d$ values associated with the N129A mutant and the very large increase in the $E_{SP}$. This contact site has an FSA <0.1 and clearly contributes significantly to both binding and signaling. The results on the T130A mutant are somewhat enigmatic in that there were differences in the expression and signaling parameters measured on the HEK 293 and 293T cells. With the HEK 293 cells, only a slight increase was noted in the $K_d$ value,
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FIGURE 6. Saturation binding curves of WT and mutant forms of FSHR and LHR with Ala replacements at two positions in β-strand 5. Representative binding curves are shown for the WT receptors and mutant FSHR (D150A and D153A) and LHR (E154A and D157A) forms. All binding studies were done using \( ^{125}\text{I}-\text{CG} \) with HEK 293 cells.

### TABLE 2
Summary of expression, binding, and signaling results for β-strand 4 mutants in HEK 293 cells

| Mutant | \( R_{\text{max}} \) (mutant/WT) | \( K_d \) (mutant/WT) | \( EC_{\text{50}} \) (mutant/WT) | \( R_{\text{max}} \) (mutant/WT) | \( Q \) (mutant/WT) |
|--------|---------------------------------|----------------------|-------------------------------|-----------------------------|-------------------|
| FSHR   |                                 |                      |                               |                             |                   |
| Y124A  | 52 ± 15                         | 0.7 ± 0.2            | 1.1 ± 0.2                     | 72 ± 14                     | 1.1 ± 0.2 |
| N129A  | 45 ± 23                         | 4.8 ± 2.8            | 1.8 ± 0.1                     | 258 ± 47                    | 1.2 ± 0.3 |
| T130A  | 310 ± 52                        | 32.2 ± 0.6           | 1.8 ± 0.1                     | 93 ± 5                      | 1.0 ± 0.7 |
| LHR    |                                 |                      |                               |                             |                   |
| Y127A  | 82 ± 9                          | 1.3 ± 0.2            | 2.1 ± 0.5                     | 83 ± 10                     | 0.9 ± 0.06 |
| N132A  | 112 ± 34                        | 3.4 ± 1.5            | 10.3 ± 1.6                    | 126 ± 34                    | 0.9 ± 0.06 |
| T133A  | 103 ± 36                        | 3.3 ± 1.1            | 2.8 ± 0.6                     | 93 ± 5                      | 1.0 ± 0.7 |
| TSHR   |                                 |                      |                               |                             |                   |
| F130A  | b                               | c                    | 3.3 ± 0.5                     | 71 ± 7                      | c                |
| N135A  | b                               | c                    | 10.5 ± 0.7                    | a                           | c                |
| T136A  | b                               | c                    | 2.2 ± 0.2                     | 75 ± 11                     | c                |

a The \( EC_{\text{50}} \) value was too high to permit accurate measurement of \( R_{\text{max}} \) and \( Q \).
b Data were not determined by binding (see Table 6).

### TABLE 3
Summary of expression, binding, and signaling results for β-strand 5 mutants in HEK 293 cells

| Mutant | \( R_{\text{max}} \) (mutant/WT) | \( K_d \) (mutant/WT) | \( EC_{\text{50}} \) (mutant/WT) | \( R_{\text{max}} \) (mutant/WT) | \( Q \) (mutant/WT) |
|--------|---------------------------------|----------------------|-------------------------------|-----------------------------|-------------------|
| FSHR   |                                 |                      |                               |                             |                   |
| D150E  | 164 ± 28                        | 2.1 ± 0.4            | 0.8 ± 0.2                     | 224 ± 2                     | 3.0 ± 0.6         |
| D150N  | 249 ± 65                        | 2.8 ± 0.6            | 3.0 ± 0.5                     | 231 ± 72                    | 0.8 ± 0.03 |
| D150A  | 60 ± 24                         | 1.2 ± 0.4            | 6.4 ± 1.1                     | 115 ± 9                     | 0.8 ± 0.1         |
| D156K  | 103 ± 2                         | 1.0 ± 0.1            | >200                          | a                           |                   |
| LHR    |                                 |                      |                               |                             |                   |
| E145D  | 36 ± 8                          | 1.9 ± 0.9            | 5.6 ± 0.9                     | 80 ± 13                     | 1.1 ± 0.2         |
| E145Q  | 62 ± 25                         | 12 ± 2              | 27 ± 2                        | a                           | a                |
| E145A  | 33 ± 6                          | 10 ± 4              | 33 ± 3                        | a                           | a                |
| E145K  | 20 ± 6                          | 2.5 ± 0.6           | >100                          | a                           |                   |
| TSHR   |                                 |                      |                               |                             |                   |
| E177D  | b                               | c                    | 0.8 ± 0.1                     | 119 ± 27                    | c                |
| E177Q  | b                               | c                    | 1.4 ± 0.2                     | 84 ± 5                      | c                |
| E177A  | b                               | c                    | 1.0 ± 0.1                     | 77 ± 2                      | c                |
| E177K  | b                               | c                    | 6.6 ± 0.4                     | c                           | a                |

a The \( EC_{\text{50}} \) values were too high to permit accurate measurement of \( R_{\text{max}} \) and \( Q \).
b Data were not determined by binding (see Table 6).

c Data were not determined.
Asn is Ser in FSHR, although in LHR and TSHR it is Cys and the invariant Asn by Thr. The N-terminal side of the invariant TSHR. All three receptors are flanked on the C-terminal side of FSHR (and to a lesser degree in LHR) but greatly reduced the binding affinity in D150N, somewhat in D150A, and significantly in D150K. Binding was not affected in any of the mutants except for a slight reduction in D150N and D153N. In LHR, the signaling potency was reduced considerably with all replacements, although less so with E154D and D157E, and the binding affinity was decreased in E154Q and E154A, and D157N and D157A. Signaling in TSHR was only marginally altered, if at all, except in the Lys replacements of either acidic side chain, which led to decreases in the potency. Overall, signaling potency is reduced by the replacements much more in LHR than in FSHR and TSHR. In the FSH-FSHR ECD complex (30), Asp150 interacts with the α-subunit residues Lys91 and Thr86, and the latter also contacts FSHR Lys74 and Glu99. Asp153, on the other hand, interacts with the α-subunit residues Lys51 and Val139, and one or more of these contacts appear more critical in receptor activation. Our models of the CG-LHR and TSH-TSHR ECD complexes (Fig. 7) predict interactions of Glu154 in LHR and of Glu157 in TSHR with α-subunit residue Lys91 and of Asp157 in the LHR and Asp160 in the TSHR ECD complexes with α-subunit residue Lys51. In the CG-LHR ECD model, we predict that Asp157 interacts with β-subunit residue Arg95, whereas in the TSH-TSHR ECD complex, Asp160 also interacts with its neighboring receptor residue Thr139. The predictions on the TSH-TSHR ECD are, overall, in good agreement with those of Núñez Miguel et al. (58). Thus, disruption of the ionic bridges in the LHR ECD impacts more adversely on signaling than similar changes in the other two GpHRs.

Ala replacements of the invariant serines in the three GpHRs in β-strand 2, a noncontact site in the FSH-FSHR ECD complex, were without significant effects, although others have reported a decrease in binding affinity and signaling potency in LHR/S226A (11). The other noncontact sites in FSHR β-strand 8 that were chosen for controls, FSHR/Asp224 and FSHR/Ser226, gave interesting comparative results. FSHR/D224A and FSHR/D224K, although binding FSH normally, exhibited decreased signaling potency. FSHR/S226A did not express at sufficient levels to permit evaluation. It has been reported that the hinge region of FSHR is important in receptor activation (60, 61). Although structural information is lacking beyond residue Glu259 in FSHR (30), it appears that the last two/three strands are much shorter with fewer hydrogen bonds stabilizing the parallel strands. It is thus possible that changes in this region can more easily cause disorder and perhaps affect the way the LRR domain interacts with the C-terminal cysteine-rich domain. If so, both proper folding (and hence expression) and signaling would be affected. LHR/D228A and LHR/D228K bound hCG with a reduced affinity, and the signaling potency was also decreased, as was observed also with TSHR/D232A and TSHR/D232K. LHR/S230A and TSHR/S234A exhibited properties like those of the WT receptor.

TABLE 4
Summary of expression, binding and signaling results for β-strand 5 mutants in HEK 293 cells

| Mutant | $B_{\text{max}}$ | $K_d$ (mutant/WT) | $EC_{50}$ (mutant/WT) | $R_{\text{max}}$ (mutant/WT) | Q (mutant/WT) |
|--------|------------------|-------------------|----------------------|-----------------------------|---------------|
|        | % WT             | % WT              |                      |                             |               |
| FSHR   |                  |                   |                      |                             |               |
| D153A  | 90 ± 22          | 1.2 ± 0.4         | 1.5 ± 0.4            | 203 ± 1                     | 1.8 ± 0.0     |
| D153N  | 210 ± 51         | 3.2 ± 0.6         | 6.3 ± 1.8            | 130 ± 60                    | 0.3 ± 0.06    |
| D155A  | 49 ± 7           | 1.1 ± 0.2         | 25.0 ± 3.0           | 106 ± 9                     | 0.5 ± 1       |
| D153K  | 47 ± 1           | 0.6 ± 0.1         | >200                 | 0                          |               |
| LHR    |                  |                   |                      |                             |               |
| D157E  | 234 ± 90         | 2.0 ± 0.5         | 2.4 ± 0.3            | 215 ± 76                    | 1.1 ± 0.2     |
| D157N  | 40 ± 8           | 3.9 ± 1.0         | >400                 | a                          | a             |
| D157A  | 95 ± 35          | 13 ± 1            | >95                  | a                          | a             |
| D157K  | 22 ± 8           | 2.4 ± 0.7         | —                    | 0                          |               |
| TSHR   |                  |                   |                      |                             |               |
| D160E  | 3.3 ± 0.5        | 56 ± 15           |                      |                             |               |
| D160N  | 3.3 ± 1.2        | 56 ± 12           |                      |                             |               |
| D160A  | 1.7 ± 0.1        | 68 ± 13           |                      |                             |               |
| D160K  | 6.6 ± 0.4        |                      |                      |                             |               |

| TABLE 5
Summary of expression, binding and signaling results for β-strand 8 mutants in HEK 293 cells

| Mutant | $B_{\text{max}}$ | $K_d$ (mutant/WT) | $EC_{50}$ (mutant/WT) | $R_{\text{max}}$ (mutant/WT) | Q (mutant/WT) |
|--------|------------------|-------------------|----------------------|-----------------------------|---------------|
|        | % WT             | % WT              |                      |                             |               |
| FSHR   |                  |                   |                      |                             |               |
| D224A  | 39 ± 22          | 1.8 ± 0.8         | 3.1 ± 0.4            | 41 ± 30                     | 0.7 ± 0.7     |
| D224K  | 84 ± 8           | 1.3 ± 0.1         | a                    | a                          | a             |
| S226A  | 5 ± 1            | a                  | a                    | a                          | a             |
| LHR    |                  |                   |                      |                             |               |
| D228A  | 37 ± 8           | 4.0 ± 1.5         | 3.9 ± 1.3            | 91 ± 13                     | 2.6 ± 0.3     |
| D228K  | 80 ± 24          | 5.3 ± 1.8         | >20                  | a                          | a             |
| S230A  | 95 ± 19          | 0.7 ± 0.1         | 1.5 ± 0.3            | 244 ± 49                    | 2.1 ± 0.1     |
| TSHR   |                  |                   |                      |                             |               |
| D232A  | 4.0 ± 1.0        | 52 ± 20           |                      |                             |               |
| D232K  | 4 ± 0.5          | a                  | a                    | a                          | a             |
| S234A  | 1.3 ± 0.1        | 90 ± 15           |                      | a                          | a             |

* Data were not determined by binding (see Table 6).
* Data were not determined.
* Data were not determined.
* Data were not determined.
LHR/D232E, but not D232Q, was found to result in decreased binding affinity (9); likewise TSHR/D232A and D232R exhibited $K_d$ increases compared with WT TSHR (34). Thus, the invariant Asp in β-strand 8 appears to be involved in part of the pathway leading to hormone-mediated receptor activation.

The free energy of binding FSH to WT FSHR (and most of the mutants) is estimated to be about $-13 \text{ kcal/mol (}\Delta G = -13 \text{ kcal/mol})$.
Although binding of the FSHR and LHR mutants to their cognate hormones was reduced at most 3–5-fold, much larger decreases were found in the signaling potency and efficacy. Thus, FSHR residues Asn129, Asp150, and Asp153 and LHR residues Asn135, Glu154, and Asp160 appear to be involved, in varying degrees, in receptor activation. In TSHR, Asn135 seems much more important in receptor activation following hormone binding than are Glu154 and Asp160.

In a comprehensive comparative analysis of FSH-FSHR and TSH-TSHR (putative) interactions, Núñez Miguel et al. (58) concluded that of the 43 amino acid residues making contact between the two receptors and the cognate hormones, 22 were equivalent, 13 in FSHR contact FSH, but the corresponding positions in TSHR do not contact TSH, and 8 from TSHR contact TSH, but the corresponding positions in FSHR do not contact FSH. Furthermore, they suggested that just four residues from the two receptors made contact with the respective hormones in a similar fashion as follows: FSHR/Tyr124 and TSHR/Phe130, FSHR/Asn129 and TSHR/Asn135, FSHR/Glu157 and TSHR/Asp150, and FSHR/Asp153 and TSHR/Asp160. Yet our functional studies showed that an Ala substitution of FSHR/Tyr124 had no effect on signaling potency in FSHR but led to a 3-fold reduction in that of TSHR/Phe130. We found that Ala substitution on FSHR/Tyr124 had no effect on the binding affinity, and Sanders et al. (34) reported at most a 3-fold reduction in binding affinity of porcine TSH to TSHR/F130A compared with WT receptor. With FSHR/N129A, we found a much greater reduction in signaling potency than in TSHR/N135A. Ala replacements of Glu157 and Asp160 in TSHR produce at most a 2-fold reduction in signaling potency (Tables 3 and 4) (34), whereas in FSHR the same replacements decrease signaling potency some 6–25-fold. Thus, even though the interactions may be comparable, the functional consequences can vary considerably between receptors. In general, our results on the three GpHRs support the conclusion, based on comparative studies with FSHR and TSHR, that the hormone α-subunit contacts are more involved with signaling than with binding (58).

A careful examination of the FSH-FSHR ECD structure (30) and the models predicted in this study, CG-LHR ECD and TSH-TSHR ECD (Fig. 7), reveals that the number of interface contact residues is similar, averaging 24–28 on the ECDs and 32–36 on the hormones. Furthermore, the interfaces are more polar than hydrophobic in nature. The numbers of polar nonionizable residues, charged residues, and hydrophobic residues on the hormones vary from 11 to 16, 9 to 11, and 9 to 10, respectively, whereas those on the ECDs vary from 9 to 12, 8 to 11, and 4 to 7, respectively. Of interest is a comparison of the surface H-bonds and salt bridges in the three ECDs as follows: FSHR has 7 and 20, respectively (30); LHR has 6 and 16, respectively; and TSHR has 2 and 11, respectively. In addition, in the interface nonionizable polar and charged amino acid residues are more comparable between FSH and FSHR and CG and LHR than between TSH and TSHR. For the latter, our model predicts an excess of nonionizable polar, charged, and hydrophobic residues on the hormones compared with the receptor. This predicted

**TABLE 8** Hydrophobic contacts within 5 Å

Summary of the hydrophobic contacts in the FSH-FSHR ECD (complex 30) and those predicted from the models generated in this study for CG-LHR ECD and TSH-TSHR ECD. The structures of FSH-FSHR ECD (30), TSHR ECD (32), and CG (55, 56) were used for comparative modeling.

| Residues | FSHR | FSH | LHR | CG | TSHR | TSH |
|----------|------|-----|-----|----|------|-----|
| Val54    | LeuB20 |  |     |    |  |    |
| Leu55    | LeuB19 |  |     |    |  |    |
| Tyr124   | TyrB19 |  |     |    |  |    |
| Leu148   | LeuB17 |  |     |    |  |    |
| Ile149   | IleB17 |  |     |    |  |    |
| Trp176   | TrpB16 |  |     |    |  |    |
| Val221   | ValB16 |  |     |    |  |    |
| Leu235   | LeuB15 |  |     |    |  |    |

**TABLE 9** Root mean square error after superposition of Cα atoms (in Å)

FSH1 and FSH2 indicate hormone from complexes 1 and 2, respectively, in Protein Data Bank code 1XWD (30); 1FL71 and 1FL72 indicate the two independent FSH molecules present in Protein Data Bank code 1FL7 (57); Protein Data Bank code 1HCN is the structure of human CG at 2.6 Å resolution (56); LH indicates modeled structure of LH (this study); TSH indicates the modeled structure of TSH (this paper). 1XWD1 and 1XWD2 indicate ECD from complexes 1 and 2, respectively, in Protein Data Bank code 1XWD (30); LHR indicates the modeled structure of the LHR ECD (this study); Protein Data Bank code 3G04 indicates TSHR ECD from 3 GpHRs (32).

| Complexes | FSH1 | FSH2 | 1FL71 | 1FL72 | 1HCN | LH | TSH | CG |
|-----------|------|------|------|------|------|----|-----|----|
| GpHts     | FSH1 | X    | 0.7  | 1.0  | 1.6  | 1.5 | 0.4 | 0.3 | 0.7 |
|           | FSH2 | 0.7  | 1.0  | 1.5  | 1.6  | 0.7 | 0.7 | 0.8 |      |
|           | 1FL71| 1.0  | 1.0  | 1.7  | 1.2  | 1.2 | 1.0 | 1.0 | 0.8 |
|           | 1FL72| 1.6  | 1.5  | 1.2  | X    | 1.6 | 1.7 | 1.6 | 1.3 |
|           | 1HCN | 1.5  | 1.6  | 1.7  | X    | 1.5 | 1.6 | 1.8 |      |
|           | LH   | 0.4  | 0.7  | 1.2  | 1.7  | 1.5 | X   | 0.4 | 0.7 |
|           | TSH  | 0.3  | 0.7  | 1.0  | 1.6  | 1.6 | 0.4 | X   | 0.6 |
|           | CG   | 0.7  | 0.8  | 0.8  | 1.3  | 1.8 | 0.7 | 0.6 | X   |

| ECDs     | 1XWD1 | 1XWD2 | LHR | 3G04 |
|----------|-------|-------|-----|------|
| 1XWD1    | X     | 0.4   | X   | 1.1  |
| 1XWD2    | 0.7   | X     | X   | 1.2  |
| LHR      | 0.4   | 0.5   | X   | 1.1  |
| 3G04     | 1.2   | 1.2   | 1.1 | X    |

\( -RT\ln K_d \), where the association constant \( K_d = 1/K_w \), which arises from many relatively weak interactions spread over a large surface area of 2600 Å² containing an unusually high content of electrostatic charges (30). With LHR, the hormone-receptor affinity is somewhat higher than that of FSH-FSHR leading to a free energy of binding of about \(-13.7 \text{ kcal/mol with hCG.} \) The observation of FSH binding to a negatively charged domain of the ECD validates earlier reports on modeling the hormone binding regions of LHR, which suggested a strong electrostatic component to hormone-receptor binding (⁸, ¹⁴, ²⁸). In studies between proteins interacting with high affinity, such as FSH or hCG binding to the FSHR or LHR ECD, respectively, it is not surprising that disruption of single hydrogen bonds, ion-ion interactions, ion-dipole interactions, or even single hydrophobic interactions by Ala replacement fails to lead to a significant reduction in affinity. (Note that the binding assay used is not perfectly reversible because some hormone-receptor internalization can occur and some of the ¹²⁵I-labeled hormone may be degraded over the time course of the experiment (41).) The \( K_d \) values obtained, however, agree with measurements from numerous other laboratories, and thus confidence can be placed in the validity of the assay, at least when used for comparative purposes, as was done herein.)
**Functional Characterization of the Three GpHR ECDs**

![Structures and models of the three GpHR ECDs and bound cognate hormones.](image)

In summary, these results on mutations of nine selected residues in the C-terminal region, in particular Tyr, Asp, and Lys, as well as certain amino acid residues (Arg, Asp, and Lys) on the β-subunit of CG (66–68), suggest that the modalities of hormone binding and hormone-mediated signaling differ to varying extents in the three homologous receptors, even in identical or highly conserved amino acid residues, a conclusion also reached by others on FSH-FSHR and TSH-TSHR interactions (58). The molecular models we have suggested for the CG-LHR ECD and the TSH-TSHR ECD also exhibit subtle differences with each other and with the FSH-FSHR ECD structure. Finally, it should be emphasized that the C-terminal cysteine-rich region of the ECD has been reported to also function in hormone binding and receptor activation (60, 61, 69–71).

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numbers for LHR and TSHR, coupled with the structural data on FSHR (30), suggest a less constrained structure of TSHR, consistent with its higher basal cAMP compared with FSHR and LHR. The electrostatic surfaces of the hormones and receptors appear to be very important in directing hormone binding and probably specificity. For example, a comparison of some of the hormone-receptor interactions found in the FSH-FSHR ECD complex (30) with our models of the CG-LHR and TSH-TSHR ECD complexes (Fig. 7; see Ref. 58 for TSH-TSHR) reveals a more localized positive surface on CG, arising in part from the localization of α-subunit residues Lys and Lys, and β-subunit residue Arg interacting with a more negative surface on LHR, relative to the FSH-FSHR and TSH-TSHR interactions as can be seen in Fig. 6.

There are examples of earlier work on the hormones that support many of the predicted interactions proposed herein. For example, with human CG binding to and activating LHR, a number of reports have demonstrated the importance of the α-C-terminal region, in particular Tyr, Tyr, and Lys, as well as certain amino acid residues (Arg, Asp, and Lys) on the β-subunit of CG (66–68).

In summary, these results on mutations of nine selected amino acid residues in β-strands 2, 4, 5, and 8 of the three GpHRs show that, of the six reported contact sites of the FSHR with the FSH α-subunit, replacements can be made in FSHR without reducing the binding affinity more than 3–5-fold. On the other hand, signaling is greatly reduced in most mutants at positions Asn, Asp, and Asp. The functional responses of the same replacements in LHR and TSHR as in FSHR were, in many cases, quantitatively and qualitatively different from those of FSHR. In and of themselves, these findings do not necessarily argue against the published crystal structure of the FSH-(truncated) FSHR ECD complex (30), but they do raise interesting questions on the roles of hormone binding and receptor activation. These results strongly suggest that the modalities of hormone binding and hormone-mediated signaling differ to varying extents in the three homologous receptors, even in identical or highly conserved amino acid residues, a conclusion also reached by others on FSH-FSHR and TSH-TSHR interactions (58). The molecular models we have suggested for the CG-LHR ECD and the TSH-TSHR ECD also exhibit subtle differences with each other and with the FSH-FSHR ECD structure. Finally, it should be emphasized that the C-terminal cysteine-rich region of the ECD has been reported to also function in hormone binding and receptor activation (60, 61, 69–71).

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