Autologous Biological Response Modification of the Gonadotropin Receptor

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It is generally held with respect to heterotrimeric guanine nucleotide binding protein-coupled receptors that binding of ligand stabilizes a conformation of receptor that activates adenylyl cyclase. It is not formally appreciated if, in the case of G-protein-coupled receptors with large extracellular domains (ECDs), ECDs directly participate in the activation process. The large ECD of the glycoprotein hormone receptors (GPHRs) is 350 amino acids in length, composed of seven leucine-rich repeat domains, and necessary and sufficient for high affinity binding of the glycoprotein hormones. Peptide challenge experiments to identify regions in the follicle-stimulating hormone (FSH) receptor (FSHR) ECD that could bind its cognate ligand identified only a single synthetic peptide corresponding to residues 221–252, which replicated a leucine-rich repeat domain of the FSHR ECD and which had intrinsic activity. This peptide inhibited human FSH binding to the human FSHR (hFSHR) and also inhibited human FSH-induced signal transduction in Y-1 cells expressing recombinant hFSHR. The hFSHR-(221–252) domain was not accessible to anti-peptide antibody probes, suggesting that this domain resides at an interface between the hFSHR ECD and transmembrane domains. CD spectroscopy of the peptide in dodecyl phosphocholine micelles showed an increase in the ordered structure of the peptide. CD and NMR spectroscopies of the peptide in trifluoroethanol confirmed that hFSHR-(221–252) has the propensity to form ordered secondary structure. Importantly and consistent with the foregoing results, dodecyl phosphocholine induced a significant increase in the ordered secondary structure of the purified hFSHR ECD as well. These data provide biophysical evidence of the influence of environment on GPHR ECD subdomain secondary structure and identify a specific activation domain that can autologously modify GPHR activity. 

Follicle-stimulating hormone (FSH), luteinizing hormone

Аbstract

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The abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinizing hormone; ECD, extracellular domain; HPLC, high performance liquid chromatography; RRA, radioreceptor assay; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; hCG, human chorionic gonadotropin; CGR, chorionic gonadotropin receptor; ELISA, enzyme-linked immunosorbent assay; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect correlation spectroscopy; TFE, trifluoroethanol.

hFSH, human follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; LH, luteinizing hormone; LHR, luteinizing hormone receptor; ECD, extracellular domain; HPLC, high performance liquid chromatography; RRA, radioreceptor assay; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; hCG, human chorionic gonadotropin; CGR, chorionic gonadotropin receptor; ELISA, enzyme-linked immunosorbent assay; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect correlation spectroscopy; TFE, trifluoroethanol.
ing domain of the FSHR. This finding demonstrates, in principle, the possibility of identifying autologous biological response modifiers of the G-protein-coupled receptor with knowledge of only the primary structure of the cognate receptor. These data also support the concepts that receptor-active structure is transient, that autologous biological response modifiers can stabilize the inactive state, and that hormone does not bind to the inactive state.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—Peptides from the extracellular domain of the hFSHR or the rat FSHR, where indicated (viz. sequences 9–30, rat; 15–44, rat; 45–72; 72–100; 101–125; 126–150; 150–183; 183–220; 221–252, rat; and 265–296), were selected for the study. Peptides 9–30, 252, and 221–252 derived from FSHR amino and carboxyl termini are encoded primarily within single exons. All other peptides spanned the junctions of adjacent exons. Peptides corresponding to the primary sequence of the FSHR ECD were synthesized by solid-phase Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry using an Applied Biosy-

**Modification of Cysteine**—In some experiments, free thiols in peptides were derivatized to assess the effects on peptide activity. For derivatization, 10 mg of the hFSHR-(221–252) Tyr-Cys peptide was dissolved in 2 ml of buffer (0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, and 0.02% NaN₃). Aliquots (5 µl) of freshly prepared 0.5 M iodoacetic acid in water were added at 5-min intervals. The reaction was monitored by the method of Ellman (17) using 5,5'-dithiobis(2-nitroben-

**Radioimmunoassay (RIA)**—Peptides or anti-peptide antibodies were used, where indicated, to inhibit the binding of hFSH to its receptor in a RRA. Exogenous-resistant Chinese hamster ovary cells (19) stably transfected with the hFSHR (CHO-hFSHR) and obtained from Ares Advanced Technologies (Randolph, MA) were used as a source of hFSHR. CHO-hFSHR cells were washed twice and left for 15 min in EDTA/PBS buffer. Cells were digested, counted, and centrifuged at 3500 rpm for 5 min. The cell pellets were resuspended in RRA buffer (0.05 M Tris and 0.25 M MgCl₂ (pH 7.5)) and stored frozen (10⁵ cells/ml). For competitive displacement, ¹²⁵I-hFSH (150,000 cpn, 25 µCi/µg) in RRA buffer with 0.3% bovine serum albumin was used as the tracer for binding to the hFSHR (2.5 x 10⁵ cells) (20). The RRA was carried out in a reaction volume of 400 µl. Increasing concentrations of hFSH or receptor peptide (100 µl) were preincubated with 125I-hFSH (100 µl) for 1 h at room temperature. CHO-hFSHR cells were then added and incubated overnight at room temperature with shaking. When antibodies to FSHR peptides were used, they were preincubated with 2.5 x 10⁵ CHO-hFSHR cells for 4 h at 4 °C. Radiolabeled hFSH was then added, and the tubes were shaken overnight at room temperature. At all concentrations of peptide or antibody tested, controls were included in which pure hFSH (1 µg) was added to assess nonspecific binding. To separate bound from free hFSH, the assay was terminated by adding 2 ml of ice-cold 0.05 M Tris (pH 7.5) and pelleting cells at 2500 g for 1 h at 4 °C. The supernatant from each tube was aspirated, and the radioactivity in each pellet was determined using a γ-counter (Wallac 1470 Wizard). In the case of the hCG RAA, CHO cells expressing the human LHR obtained from Ares Advanced Technologies were used as the receptor source, and 125I-hCG was used as the tracer (CR127; 10 µCi/µg).

In some experiments, membrane-bound or truncated receptors were solubilized using the detergent Nonidet P-40. Truncated forms of the hFSHR ECD were prepared as previously described (21). A CHO cell line (D7) stably expressing the hFSHR-(1–335) variant was utilized. Cell lysis was performed by freezing the hFSHR-(1–335) (1.5 x 10⁶ cells/100 µl) or hFSHR ECD (clone D7, hFSHR-(1–355) (1 x 10⁶ cells/100 µl) were solubilized using 0.1% Nonidet P-40 (in 0.05 M Tris, 0.025 M MgCl₂, 0.3% bovine serum albumin, and 30% glycerol). Antibodies at different concentrations (100 µl) were incubated with the solubilized receptor preparation (100 µl) at 4 °C for 4 h; then the tracer was added (150,000 cpm/100 µl); and the incubation was continued at 4 °C overnight. The unbound fraction was separated by precipitation of the hFSHR/hFSH complexes using polyethylene glycol. To each tube were added 200 µl of γ-globulin (2% in 0.05 M Tris (pH 7.5)), 500 µl of RRA buffer, and 1 ml of 25% polyethylene glycol in PBS. Tubes were allowed to stand on ice for 15 min and then were spun at 4 °C for 1 h at 2500 g. The supernatant was aspirated, and the radioactivity in the pellets was counted in a γ-counter. The data ob-

**Autologous Biological Response Modification**

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**In Vitro FSH Bioassay**—The effect of hFSHR peptides on signal transduction was evaluated by an in vitro FSH bioassay (19). Y-1 cells stably expressing FSHRs (Ares Advanced Technologies) were cultured in 48-well plates at 3 x 10⁶ cells/50 µlwell in Eagle’s minimum essential medium supplemented with 5% fetal bovine serum and 80 µg/ml bovine insulin. In some experiments, Y-1 cells were re-

**Progesterone secreted in the medium was measured by radioimmu-

**Development of Anti-Receptor Peptide Antibodies**—The hFSHR-(221–

**Characterization of Anti-peptide Antibodies**—Anti-peptide antibody was screened using an ELISA to detect binding to corresponding pep-

**Conclusions**

Characterization of Anti-peptide Antibodies—Anti-peptide antibody was screened using an ELISA to detect binding to corresponding pep-

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the antisera before addition to the microtiter wells.

**Polyacrylamide Gel Electrophoresis and Western Blotting**—To confirm that antibodies to peptide 221–252 bind to the hFSHR, denaturing SDS-polyacrylamide gel electrophoresis in a discontinuous buffer system (46) was carried out. D7 cells (5 x 10⁶) expressing the FSHR ECD were solubilized for 30 min at room temperature in 500 μl of Laemmlí sample buffer (0.25 M Tris (pH 6.8), 4% SDS, 20% glycerol, and 0.24% bromphenol blue) containing 1X protease inhibitor mixture (100X protease inhibitor mixture = 1.6 mg/ml benzamidine HCl, 1 mg/ml phenanthroline, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml peptatin A in 100% ethanol), 5% 2-mercaptoethanol, and 8 μl urea. Electrophoresis chambers were cooled in ice baths. The gels were 7.5% polyacrylamide and made 8% with urea. 18 μl of cell suspension was added per lane. After electrophoretic separation, proteins were transferred to nitrocellulose by electroblotting (47) for 45 min at 250 mA. The nitrocellulose membranes were blocked with Protein Images blocking solution (U. S. Biochemical Corp.) for 2 h at room temperature. Primary antibodies were diluted in Tris-buffered saline containing 1% Tween 20 (TBST), 2% nonfat dry milk, 1% ovalbumin, and plain CHO cell extract (1 x 10⁶ cells/10 ml) and incubated overnight at room temperature. The blots were washed with TBST and incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (BIOSOURCE International, Camarillo, CA) for 2 h at room temperature. The membranes were washed and developed with Western blue-stabilized substrate for alkaline phosphatase (Promega, Madison, WI).

**Flow Cytometry**—To determine whether the hFSHR-(221–252) domain was accessible to antibodies in native receptor present on CHO-hFSHR cells, flow cytometric analysis was carried out. Confluent cells were washed with EDTA/PBS and then incubated with the same buffer for 10–15 min. The cells were dislodged from the culture flasks and centrifuged for 5 min at 3000 rpm. After aspiration of the supernatant, cells were suspended in PBSA (PBS with 0.02% sodium azide). CHO-hFSHR cells (10⁶ cells/100 μl) were added to each tube. Human serum (50 μl) was incubated with the cells on ice for 1 h to block nonspecific sites. Purified rabbit IgG (50 μg/μl) from control serum or antisera against the hFSHR-(221–252) or hFSHR-(265–296) peptide was added, and incubation was continued for 1 h on ice. Cells were washed with 2 ml of PBSA, pelleted, resuspended in fluorescein isothiocyanate-conjugated anti-rabbit IgG (200 μl, 1:40 dilution), and incubated for 1 h on ice. Cells were then pelleted, washed, and resuspended in 2 ml of PBSA. Cell-surface immunofluorescence was measured using a flow cytometer (Becton Dickinson FACScan).

**Circular Dichroism Studies of the hFSHR-(221–254) Peptide**—Circular dichroism analyses were performed to assess the bulk solvent environment of the conformation of the hFSHR-(221–254) peptide. Experiments were carried out on a Jasco J-720 spectropolarimeter at 20 °C using a cell with a path length of 0.05 mm. In one series of experiments, peptides were dissolved in 25 mM phosphate buffer (pH 7.0), and spectra were obtained in a series of solutions containing from 0 to 50% trifluoroethanol, increasing in steps of 5%. In another set of experiments, peptides were dissolved in water; phosphate buffer (pH 7.0) was added to 25 mM, and dodecyl phosphocholine was added to 5.0 or 22.0 mM; the solution was incubated overnight at room temperature; and spectra were obtained at 20 and 37 °C at a peptide concentration of 0.5 mM. All data were analyzed using the computer program SELCON3 (25–28).

**Peptide Synthesis and Characterization**—Peptides were purified by HPLC to homogeneity. The observed molecular mass of each purified peptide as determined by mass spectrometry was, for the most part, as expected. We did note that when a C-terminal cysteine was engineered into the peptide, an additional mass of ~50 Da was always detected. This was not the case, an isotropic mixing time of 120 ms was employed to emphasize correlations between amide and α-protons.

Two-dimensional ¹H NOESY spectra with gradient excitation sculpting for water suppression were recorded (48). In each case, a mixing time of 120 ms was employed for generation of nuclear Overhauser effects. For the two-dimensional spectra, spectral widths were 6000 Hz in both the direct and indirect dimensions. Data sets were 8192 x 512 complex points in the direct and indirect dimensions, respectively. Spectra were processed with exponential/gaussian multiplication in the direct dimension and a quadratic sine-bell in the indirect dimension.

**RESULTS**

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case if the cysteine was at the N terminus. Peptides representing FSHR sequences 45–72 and 150–183 were not soluble in RRA buffer. Sequence listing mis-transcription resulted in synthesis errors in peptides representing FSHR sequences 105–125 and 126–150 of Gln to Gly and of Glu to Val, respectively. The hFSHR sequence of Minegishi et al. (30) was used for peptides representing hFSHR sequences 72–100 and 265–296.

Peptide Activity in RRA and in Vitro Bioassay—Peptides representing FSHR sequences 9–30, 15–44, 101–125, 126–150, 183–220, 221–252, and 265–296 were purified and then screened in the hFSH RRA to determine whether they affected binding of hFSH to the receptor. The remaining peptides were not soluble in RRA buffer and could not be tested. The hFSHR-(221–252) peptide (Fig. 1) was found to inhibit binding of hFSH to CHO-hFSHR cells in a dose-dependent manner (Fig. 2A). This effect was specific to the FSHR, as the peptide did not inhibit hCG binding to the human LHR (Fig. 2B). The hFSHR-(221–252) peptide had a cysteine incorporated at the C terminus for the purpose of conjugation to a carrier protein. To rule out the possibility of involvement of free –SH in the observed inhibition of FSH binding to receptor, this group was modified with iodoacetamide. The alkylated peptide still exhibited binding inhibition (data not shown). There are five lysines in this peptide as compared with two in the corresponding region of the human LHR (Fig. 1). To determine whether these lysines participated in electrostatic interactions that affect peptide activity and specificity, ε-NH₂ groups of lysines were modified with acetic anhydride. Incorporation of acetyl groups was confirmed by mass spectrometry. All five lysine ε-NH₂ groups were modified, and the modification eliminated the activity of the peptide (data not shown). The peptide was also re-synthesized as hFSHR-(221–254) by removing Tyr and Cys at the C terminus (used for iodination and conjugations, respectively) and including two additional residues, Thr and Tyr, as found in the native sequence. This peptide also retained its activity (Fig. 3).

To determine if charge alone could account for the binding inhibition, two subregions of the hFSHR-(221–254) peptide (hFSHR-(221–237) and hFSHR-(238–254)) were synthesized and characterized as described earlier. When tested in the RRA peptide challenge test, peptide 221–237 had no activity, and peptide 238–254 caused high nonspecific binding and had minimal activity at the highest dose tested (Fig. 3). As this daughter peptide was found to be relatively insoluble, higher doses could not be tested. To determine whether peptide 221–254 affected signal transduction, an in vitro bioassay was carried out. It was found that peptide 221–254 inhibited progesterone production induced in Y-1 cells by 1 and 5 ng of highly purified hFSH (Fig. 4).

Ligand Binding Isotherm—To gain a better understanding of the mechanism by which the hFSHR-(221–254) peptide inhibited FSH binding to its receptor, ligand binding isotherms were conducted at three different concentrations of peptide. Inhibition appeared mixed where an apparent decrease in B_max was observed along with an increase in K_d only at higher levels of peptide (Table I). These data do not support a simple competitive mechanism of inhibition where peptide binds to hormone. Nonspecific binding increased with increasing doses of peptide, as is often seen in peptide challenge experiments. The hFSHR-(221–254) peptide inhibits FSH-induced steroidogenesis. Y-1 cells expressing the hFSHR were cultured in 48-well plates at 3 × 10⁴ cells/500 µL well. The cells were stimulated with 1 and 5 ng of hFSH in the presence of different doses of the peptide (pep) or with hFSH at various dose levels in the absence of peptide. Media progesterone was measured using a radioimmunoassay.

Characterization of Antibodies against hFSHR-(221–252)—Following immunization of rabbits with the hFSHR-(221–252) peptide-ovalbumin conjugate, both rabbits (X-180 and X-183) were boosted with hFSH at various dose levels in the absence of peptide. Media progesterone was measured using a radioimmunoassay.

Table I

| Affinity constant (K_a) | Receptor conc Nonspecific binding | Dissociation constant (K_d) |
|-------------------------|----------------------------------|-----------------------------|
| FSH + 0 µg peptide     | 3.7 ± 1.1 × 10^10                | 1.4 ± 0.17 × 10^10         | 1.1 × 10^4 |
| FSH + 160 µg peptide   | 0.7 ± 0.2 × 10^10                | 0.3 ± 0.1 × 10^10          | 3.2 ± 10^10 |
| (400 µM)               |                                  |                             | 15.0 ± 4.0 × 10^11 |
| FSH + 320 µg peptide   | 1.9 ± 0.1 × 10^10                | 0.7 ± 0.02 × 10^10         | 1.9 × 10^11 |
| (200 µM)               |                                  |                             | 5.2 ± 0.3 × 10^11 |
| FSH + 160 µg peptide   | 3.0 ± 0.5 × 10^10                | 1.2 ± 0.1 × 10^10          | 1.3 × 10^11 |
| (100 µM)               |                                  |                             | 3.3 ± 0.6 × 10^11 |
responded with good titers (Fig. 5A). The specificity of the antibodies for binding to the immobilized peptide was shown by competition ELISA, where the free immunizing peptide could inhibit binding of anti-peptide antibodies, whereas an unrelated peptide could not (Fig. 5B). In addition, antisera to this peptide did not bind to other hFSHR peptides coated on an ELISA plate (data not shown). Antibodies were purified using protein A and tested in an hFSH RRA. Membrane-bound FSHR was used to determine whether this region of the FSHR is accessible in situ. Minimal non-dose-related 20–25% inhibition was observed at all concentrations of antiserum X-180 or X-183 tested (Fig. 6A). In contrast, antiserum X-179 showed dose-dependent inhibition, as previously described (24). However, when these hFSHR-containing cells were detergent-solubilized, and the extract was used as a source of receptor, anti-hFSHR-(221–252) antibody (X-180 and X-183) inhibited the binding of hFSH to its receptor in a dose-related manner (Fig. 6B). Antisera could be shown to bind to full-length and truncated receptors by Western blotting (Fig. 7). When truncated hFSHR was extracted from D7 cells expressing the FSHR ECD, antibodies against the hFSHR-(221–252) peptide inhibited binding of FSH to its receptor (data not shown).

To further clarify the epitope recognized by antisera X-180 and X-183, subregions of the hFSHR-(221–254) peptide (hFSHR-(221–237) and hFSHR-(238–254)) were characterized in a competitive ELISA. Antiserum X-180 recognized hFSHR-(238–254) only at very high concentrations of the peptide (Fig. 8A), whereas antiserum X-183 could not recognize peptides 221–237 and 238–254 (Fig. 8B). These results were interpreted to mean that the hFSHR-(221–254) peptide has secondary structure that is essential for the observed inhibitory effect on FSH binding to the FSHR.

Accessibility of peptide 221–252 was further assessed by flow cytometry to determine whether antiserum to hFSHR-(221–252) could bind to the FSHR in situ. Antiserum against hFSHR-(221–252) could not bind the FSHR in situ, further corroborating the inaccessibility of this sequence on the cell surface. However, the positive control (antiserum against hFSHR-(265–296)), as previously described (24), bound with high intensity compared with the nonimmune rabbit serum negative control (data not shown).

Circular Dichroism Analysis of hFSHR-(221–254)—It is clear from the CD studies (Fig. 9) of the peptide in trifluoro-
ethanol (TFE) that the conformational change is very steep. The development of helix-like signature was essentially complete at 25% (when compared with the 50% plot). As little as 10% cosolvent addition resulted in visible changes in the CD spectra. Analysis of the spectra of peptide in 10% TFE using the SELCON program calculated a change from 13.6 to 36.6% α-helix. At 25% TFE, α-helix content was calculated as 58.3%, and no further remarkable change was observed at higher TFE concentrations. At 25% TFE and above, the CD spectra more clearly show the double-negative extrema at ~208 and 222 nm that is the α-helix signature.

The conformation of a peptide portion of a protein in TFE is not a useful predictor of that peptide structure in the protein (32). Moreover, the ECD of the hFSHR is likely to be closely apposed to the plasma membrane. Therefore, additional CD spectra of the active peptide were collected in the presence or absence of 5 and 22 mM dodecyl phosphocholine at 20 and 37 °C. Clearly seen for spectra collected at 20 °C is how addition of 5.0 mM dodecyl phosphocholine induced a marked change in the secondary structure of the active peptide (Fig. 10). The calculated helical content was slightly higher for spectra obtained at 37 °C (26.9%) than for spectra obtained at 20 °C (23.8%). Roughly 50% α-helix was calculated for the peptide at either temperature and at either concentration of detergent. Analysis of the spectra of peptide in 5 mM dodecyl phosphocholine was not greatly different from that in 22 mM dodecyl phosphocholine. The critical micellar concentration of dodecyl phosphocholine, as reported by the manufacturer, is 1.2 mM. Finally, the CD spectra of acetylated, inactive hFSHR-(221–254) in 5 mM dodecyl phosphocholine also contained characteristic signatures of α-helical secondary structure (data not shown).

Although CD spectroscopy is a useful tool to monitor changes in conformation, NMR can be used to verify if the interpretation of secondary structure conformation is reasonable. Indeed, the one-dimensional 1H NMR spectrum of hFSHR-(221–252) in the absence of TFE was characteristic of a peptide with a predominantly random-coil structure (Fig. 11, upper trace). There was little dispersion in the amide proton region of the spectrum (Fig. 11, upper trace); these resonances clustered around the random-coil shift of ~8.3 ppm. Additionally, α-protons were mostly unresolved with a cluster around 4.2 ppm (data not shown). Although a small amount of helix may have been present, it was not ascertainable from this spectrum. In both cases, chemical shift degeneracy in the amide and α-proton regions suggested the peptide to be overwhelmingly unstructured (data not shown). Addition of TFE stimulated a transition from unstructured to some secondary structure in the peptide (Fig. 11, lower trace). CD spectra identified the evolving structural element as a helix. The helix developed fully at ~20–25% TFE and remained unchanged as the level of TFE increased. NMR spectra recorded at 25 and 50% TFE both indicated an essentially identical level of helical content (data not shown). Fig. 11 (lower trace) shows the increase in resonance dispersion in the amide proton region of the peptide upon addition of TFE. This dispersion is indicative of formation of structure. More evidence that the stimulated structure is helical is provided by two-dimensional NMR. To probe more thoroughly for signs of helical content, two-dimensional NOESY and TOCSY spectra were run and analyzed. The extra resolu-
tion afforded by the TOCSY spectra of peptide in TFE shows that there is extra chemical shift dispersion in the \( \alpha \)-proton, again indicating formation of structure (Fig. 12). The \( \alpha \)-protons are all shifted upfield of the water resonance, implying an \( \alpha \)-helix. Corresponding NOESY spectra displayed \( {\delta}_{\text{NN}} \) connectivities between amide protons, again suggestive of a helical structure.

**Circular Dichroism Analysis of the hFSHR ECD**—Recently, we have achieved the purification of the biologically active hFSHR ECD.\(^2\) Since the data to date clearly showed that dodecyl phosphocholine increased the secondary structure of the hFSHR-(221–252) synthetic peptide, we felt it important to determine whether dodecyl phosphocholine exerted similar effects upon the hFSHR ECD protein. Since we succeeded in expressing and purifying the entire ECD of the hFSHR in quantities sufficient for circular dichroism studies, this advance provided an opportunity to examine the influence of the bulk solvent environment of the secondary structure of the purified ECD. These studies demonstrated that the hFSHR ECD undergoes a remarkable change in secondary structure following incubation with dodecyl phosphocholine (Fig. 13 and Table II). This result is reminiscent of the conformational change in the hFSHR-(221–252) synthetic peptide that was observed when the peptide was incubated with dodecyl phosphocholine. The data suggest further that the proximity of the ECD to the plasma membrane may influence the final conformation of the ECD.

**DISCUSSION**

Several homology models of glycoprotein hormone receptors have been published (15, 16, 35). All homology models have been based on the ribonuclease inhibitor three-dimensional structure and a leucine-rich repeat motif. The model of the LHR reported by Jiang et al. (15) is consistent with some of the available mutagenesis data for the LHR/CGR. Charge inversion mutations of LHR Lys\(^{40} \), Lys\(^{104} \), Glu\(^{132} \), and Asp\(^{135} \) carried out by Puett and co-workers (35, 36) led to undetectable binding or activation. An Arg\(^{114} \) mutant had no effect on either hCG binding or signal transduction. Lys\(^{104} \), Glu\(^{132} \), and Asp\(^{135} \) are predicted to be located at or near the predicted \( \beta \)-sheet region; and hence, they are expected to be involved in hormone binding. Lys\(^{40} \) and Arg\(^{114} \) are predicted to be on the outer surface and are not expected to affect hormone receptor binding. Site-directed mutagenesis of Asn residues has shown that the carbohydrate moieties of the glycoprotein hormone receptor are not involved in hormone recognition and high affinity binding (37). The model (15) also predicts that Asn\(^{77} \), Asn\(^{152} \), and Asn\(^{173} \) will be located at the outer surface (helices or loops). Therefore, glycosylation of these residues is not expected to interfere with hormone binding at the \( \beta \)-strand inner face. The region corresponding to the hFSHR-(221–254) peptide was not

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\(^2\) A. Schmidt, R. MacColl, B. Lindau-Shepard, D. R. Buckler, and J. A. Dias, submitted for publication.
included in this model because it did not conform to the authors’ leucine-rich repeat motif. Bhowmick et al. (35) have also reported a model for the hCG receptor. It differs from the model reported by Jiang et al. (15) because it encompasses two leucine-rich repeats encoded by exon 9, including region 220–253 of the human LHR, represented as two \( \beta \)-strands and an \( \alpha \)-helix, and is homologous to hFSHR-(221–252). Analysis of the active peptide in TFE by NMR revealed \( \alpha \)-helical structure that would be expected in a leucine-rich repeat domain.

In this work, the hFSHR-(221–252) peptide appears to be largely disordered in physiologic buffers, but becomes ordered in the presence of dodecyl phosphocholine or TFE. Compared with smaller peptides, the steepness of transition may indicate a higher degree of cooperativity in the transition. It would be expected that a relatively large peptide such as hFSHR-(221–252) would exhibit a sharp transition in the TFE-induced transition if it were to form a single helix along a significant portion of its length.

TFE is not a helix-inducing solvent in the sense that it will induce helix formation independent of the sequence (32). It is rather a helix-enhancing cosolvent that stabilizes helices in regions with some \( \alpha \)-helical propensity (39). This said, it is also clear that solvent can determine the secondary structure of a peptide \textit{in vitro} and can override its propensity for secondary structure due to sequence (32). A conformational change in the presence of TFE does not prove that the peptide is so ordered in the intact protein. For this reason, we collected CD spectra in dodecyl phosphocholine of the hFSHR-(221–252) peptide and found that this detergent, which mimics a membrane environment, caused a conformational change in the peptide. It is tempting to speculate that if the peptide is not ordered in the intact protein, the peptide may undergo a conformational change upon interaction with the receptor or as it nears the plasma membrane. The formation of secondary structural elements by the peptide in the presence of dodecyl phosphocholine suggests that the peptide can become ordered in an appropriate environment. There are examples in the literature of bioactive peptides that adopt a helical conformation upon interaction with a hydrophobic environment (40) or lipid micelles (41) or upon interaction with cognate ligand (42). Micelles and vesicles are thought to induce and stabilize helix formation by binding to residues that fall in a longitudinal, hydrophobic strip (43). A helical wheel projection of the peptide is shown in Fig. 14, illustrating the preponderance of hydrophobic residues and their spatial relationships to other residues in the helix. For clarity, the helical wheel projection is depicted as the first and second halves of the peptide since the presence of a proline would produce a kink in the helix. Therefore, the peptide helical wheel presentation is divided into two wheels at the proline residue. Sequence 221–254 of the hFSHR is very likely in close association with the plasma membrane or is involved in protein-protein contacts that are disrupted upon detergent solubilization. These data suggest that a helical conformation of

![Fig. 12. Two-dimensional proton NMR spectra of the hFSHR-(221–254) peptide made 50% with TFE.](image)

![Fig. 13. CD spectra of the purified hFSHR ECD incubated in the presence or absence of 20 mM dodecyl phosphocholine. The spectra were taken at 6° C using a 0.05-cm path length cell in 0.01 M potassium phosphate buffer after incubation overnight with 20 mM dodecyl phosphocholine (DPC) (—) or buffer (——). The spectra represent the average of two independent experiments. deg, degrees.](image)
hFSHR-(221–254) might be stabilized by association of this part of the extracellular domain with the plasma membrane. The present data also strongly suggest that region 221–252 interfaces with the transmembrane domain in part because it is inaccessible to immunochemical probes without detergent solubilization of membrane-bound receptor, providing an experimental basis for orientation of the hFSHR ECD in the models of the FSHR. Support for these conclusions is derived not only from data collected by immunochemical approaches, but also by combining synthetic peptide studies that identified region 221–254 of the hFSHR as having an effect on signal transduction.

We began these studies by analyzing a series of synthetic peptides of the hFSHR ECD for biological activity because synthetic peptides from the ECDs of the LHR/CGR and thyroid-stimulating hormone receptor were used by others to identify the hormone-binding regions of these receptors. Roche et al. (44) found that three different peptides, 21–38, 102–115, and 253–272, of the LHR/CGR inhibited the binding of labeled hCG to its receptor. Similar studies carried out with the thyroid-stimulating hormone receptor (45) identified four different inhibitory peptides, 16–35, 106–125, 226–245, and 256–275, of which only peptide 226–245 was specific, as other peptides also inhibited binding of hCG to its receptor. An FSHR peptide corresponding to region 9–30 has been shown to affect FSH binding and signal transduction (34). As observed in the LHR/CGR and thyroid-stimulating hormone receptor systems, there could have been other regions in the ECD of the FSHR capable of binding to FSH. Therefore, this study was undertaken to identify the regions of the hFSHR ECD involved in hormone binding. Peptide challenge studies revealed that the hFSHR-(221–254) peptide had remarkable properties of FSH binding inhibition. We selected the hFSHR-(221–254) peptide for further study, as it could provide a model peptide for a better biophysical understanding of the three-dimensional structure of the FSHR ECD. However, in contrast to previous studies, where an implicit assumption was that receptor peptides that block hormone binding do so by binding to hormone, there is no evidence that the activity of peptide 221–252 is due to its binding to hFSH.

A prediction of the models is that charges in the cusp of the receptor ECD may play an important role in electrostatic interactions between hormone and receptor. Four residues in the LHR (Lys158, Lys183, Glu184, and Asp206) and in the hFSHR (Lys161, Lys184, and Asp206) are essential to gonadotropin binding (31). Asp206 is conserved in all glycoprotein hormone receptors. Indeed, modification of ε-NH₂ groups decreased the activity of the peptide, but did not disallow a conformational change in the presence of dodecyl phosphocholine. Concern that basic peptides might have large nonspecific effects in the system used is assuaged by the observation that the inactive daughter peptide we synthesized, which contains four out of the five lysine residues in the parent peptide, has no biological effect. Since the N-terminal fragment 221–237 had no effect on hormone binding and the C-terminal fragment 238–254 caused minimal inhibition at the highest dose tested, correct conformation may be essential for its activity. Since the hFSHR-(221–254) peptide was able to inhibit FSH-induced signal transduction, we reason that this region in the FSHR may be in close contact with one of the extracellular domains making direct contact with the extracellular loops. Since peptide does not appear to bind hormone, but does inhibit binding and signal transduction, the peptide may associate with receptor domains to alter receptor conformation into a nonactive state. This previously unrecognized, autologous acting domain demonstrates, in principle, that discrete proteodomains can have regulating activity.

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| Sample       | α-Helix | β-Sheet | Turns | Unordered |
|--------------|---------|---------|-------|-----------|
| ECD         | 22.7 ± 1.1 | 23.2 ± 1.2 | 22.4 ± 0.9 | 32.7 ± 0.5 |
| ECD + 20 mM DPC | 33.0 ± 3.1 | 15.85 ± 1.45 | 23.6 ± 0.0 | 26.5 ± 0.7 |

* Sum of percentage of regular and distorted structures as calculated by SELCON3.

DPC, dodecyl phosphocholine.
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