Hormone-induced Conformational Change of the Purified Soluble Hormone Binding Domain of Follitropin Receptor Complexed with Single Chain Follitropin*

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Human follicle-stimulating hormone receptor (hFSHR) belongs to family I of G protein-coupled receptors. FSHR extracellular domain (ECD) is predicted to have 8–9 αβ or leucine-rich repeat motif elements. The objective of this study was to identify elements of the FSHR ECD involved in ligand binding. Preincubation of recombinant hFSHR ECD with rabbit antisera raised against synthetic peptides of hFSHR ECD primary sequence abolished follitropin binding primarily in the region of amino acids 150–254. Accessibility of hFSHR ECD after hormone binding, captured by monoclonal antibodies against either ECD or FSH, was decreased for the region of amino acids 150–220 but additionally for amino acids 15–100. Thus, when hFSH bound first, accessibility of antibody binding was decreased to a much larger extent than if antibody was bound first. This suggestion of a conformational change upon binding was examined further. Circular dichroism spectra were recorded for purified single chain hFSH, hFSHR ECD, and hFSHR ECD-single chain hFSH complex. A spectral change indicated a small but consistent conformational change in the ECD-FSH complex after hormone binding. Taken together, these data demonstrate that FSH binding requires elements within the leucine-rich repeat motifs that form a central region of hFSHR ECD, and a conformational change occurs upon hormone binding.

Follicle stimulating hormone (FSH), together with luteinizing hormone (LH), thyroid stimulating hormone, and chorionic gonadotropin, belongs to the group of pituitary or placental glycoprotein hormones (GPH). Members of this group belong to the cysteine knot fold family. A common α-subunit and a hormone-specific β-subunit, each sharing the same fold, form the functional heterodimer. FSH plays a central role in the regulation of mammalian reproduction. In females, it is essential for ovarian and follicular development and maturation, whereas in males it regulates spermatogenesis (1, 2).

FSH binds specifically to follicle-stimulating hormone receptors (FSHR) on granulosa cells in the ovary or Sertoli cells in the testis. Binding of FSH to its receptor induces signal transduction by stimulation of adenylate cyclase, which leads to a specific cell response through protein kinase A-dependent pathways (3).

The FSHR belongs to the family of G protein-coupled receptors and consists of an uncharacteristically large extracellular domain (ECD) and a seven-pass helical transmembrane domain (reviewed by Simoni et al. (1)). Sequence analysis suggests that the ECD of the glycoprotein hormone receptors (GPHR) contains 8–9 imperfect leucine-rich repeats (LRR) like the porcine ribonuclease inhibitor (RI), a member of the LRR family. Crystal structure analysis showed that RI consists of 15 LRR, which have a β-α hairpin unit structure, where the units are aligned parallel to a common axis (4). The resulting structure resembles a horseshoe with β-strands forming the inner circumference, whereas the α-helices form the outer circumference. This kind of structure shows high flexibility in structure, and binding of RNase A results in a conformational change of the RI (4, 8, 9).

Based on this structure, theoretical models have been proposed for the GPHR, where the hormone presumably mainly interacts with the inner circumference of the receptor LRR motifs (5–7). Using charge inversion mutagenesis, Bhownik et al. (10, 11) could show the involvement of several residues between aa 40 and 206 in hormone binding, located in the concave surface of the LRR model of the LHR. More recently, mutational analysis of the LRR of the FSHR and LHR indicated that several regions within this domain of the receptor are involved in binding (12, 13). Furthermore, antibody inhibition combined with peptide binding studies gave rise to evidence that the most N-terminal part of the FSHR, aa 9–30, is also important in the interaction of receptor and hormone (14–16).

The specificity of binding, however, seems to be located differently in the LHR and FSHR. Fragments of the LHR ECD as short as 206 aa are able to bind LH with little reduction in affinity, and chimera studies demonstrate the importance of aa 1–164 for the specificity of the LHR, whereas aa 1–257 are needed for specific FSH binding, with the main part of the specificity being located after LRR5, beyond aa 165 (17, 18). Altogether, these data indicate that the binding domain of
FSHR is composed of multiple regions of the ECD, but exactly which elements of the receptor interact and whether the FSHR exhibits the same flexibility as the RI and changes upon ligand binding has not yet been determined.

It has been difficult to address this issue because the full-length receptor is located within the membrane and, thus, might be sterically restricted and less accessible to antibodies or peptides, and no biophysical data have been provided yet for the receptor-hormone interaction. We reasoned that using solely the ECD might provide a suitable solution for this problem. Over the past several years there have been several attempts to express the ECD of the GPCR to analyze issues like glycosylation (19, 20) or trafficking (21). However, expression in *Escherichia coli* or mammalian or insect cells yielded ECD trapped in the cell, with none or just a little secretion of the recombinant protein (19, 21–24).

This study used a recombinant form of the hFSHR ECD, secreted from insect cells, that was able to bind FSH with high affinity. This form of the hFSHR ECD allowed analysis of hormone binding independently of a cell system, providing a highly accessible protein. The objective of this study was to utilize this model system to locate the specific elements of the FSHR interacting with the hormone and to collect biophysical data of the interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**

Iodination grade human pituitary LH and human pituitary TSH were from the National Pituitary Hormone Program. Chemicals were purchased from Sigma, Pierce, and Bio-Rad.

**Expression of hFSHR ECD in Hi 5 Insect Cells**

The expression vector used was kindly provided by Ares Advanced Technologies (Randolph, MA). Briefly, plasmid pACgpg67-FSHR-ECD contains the hFSHR ECD cDNA (aa 1–349) under the control of the polyhedrin promoter. The secretion of the ECD is facilitated by the signal sequence of the baculovirus envelope surface glycoprotein from *Autographa californica* (25). This creates a fusion protein with three additional amino acids beyond the consensus cleavage site at the N-terminal end, resulting in a secreted protein with the N terminus ADQCHHR. Recombinant baculovirus was amplified in Sf9 cells (Invitrogen) were infected with recombinant virus at a multiplicity of infection of 5–10, and Hi 5 cells were infected for expression using a titer of 5–10 multiplicity of infection (as above). Purification of expressed schFSH from media was performed by affinity chromatography using a 46.3H6.B7 column as previously described (29). Purified schFSH was dialyzed against 0.1 M phosphate buffer, pH 7.2, and stored either frozen or lyophilized.

**Polyacrylamide Gel Electrophoresis and Western Blot Analysis**

To analyze the expression of hFSHR ECD, media was collected as described above, and aliquots were boiled in Laemmli sample buffer. The samples were separated on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were stained either by Coomassie blue or silver staining (Bio-Rad) or electroblotted onto Immunoblot-P membranes (Millipore, Bedford, MA) using a Transblot semi-dry electrophoresis transfer apparatus (Bio-Rad) and 48 mm Tris, 39 mm glycine, 20% methanol, 1.3 mm SDS, pH 9.2, transfer buffer. The membranes were blocked overnight in TBST (10 mm Tris, 145 mm NaCl, pH 7.2, 0.5% Tween 20) containing 5% nonfat milk at 4 °C. The following day, blots were probed with 5 μg of protein A-purified anti-FSHR mAb 106.105 (27) diluted in TBST containing 5% nonfat milk for 2 h at room temperature. Blots were washed (4× for 10 min) in TBST and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-SOURCE International, Camarillo, CA) diluted 1:10,000 in TBST + 5% nonfat milk for 1 h at room temperature. Blots were washed (4× for 10 min), and signals were developed using ECL Western blot detection kit (Amerham Pharmacia Biotech) or Pierce Super Signal kit.

**Polyclonal Anti-peptide Antibodies**

Some of the antibodies used in this study have been described in other studies (30). Briefly, rabbits were immunized with synthetic peptides encompassing nearly the entire FSHR ECD primary sequence. Antisera were tested in Western blot and in ELISA assays for their specificity of reaction with both immunizing peptides and hFSHR ECD. Antibody testing positive (see Table I) were precipitated with ammonium sulfate according to the protocol of Harlow and Lane (31) and dialyzed against PBS with 0.2% sodium azide.

**Solid-phase Radio Receptor Assay**

To analyze the binding characteristics of secreted hFSHR ECD, a new solid-phase radio receptor assay format was developed. Nunc® tubes (Nalge Nunc International, Rochester, NY) were coated with 1 μg of mAb 106.105 in 200 μl of coating buffer (50 mm Tris, pH 9.5) at 4 °C overnight. The tubes were washed once with 1 ml of wash buffer (phosphate-buffered saline with 0.02% azide and 0.05% Tween 20; all the following washes were done in this wash buffer) and blocked with 500 μl of 10% nonfat milk, 2% bovine serum albumin in coating buffer for 3 h at 37 °C. After the tubes were washed (3× 1 ml of wash buffer), 200 μl of insect media containing hFSHR ECD diluted in binding buffer (phosphate-buffered saline, 0.127% EDTA, 0.25% bovine serum albumin, 0.05% Tween 20) was added and incubated for 2 h at 37 °C.

To analyze the binding affinity of hFSHR ECD, the tubes were washed three times, and radiolabeled purified pituitary human FSH (125I-FSH, 150,000 cpm) as well as unlabeled pituitary FSH in increasing concentrations was added and incubated for 2 h at 37 °C. The specificity of binding was analyzed in competitive displacement assays utilizing unlabeled purified pituitary LH, TSH, and FSH. Counts bound were measured in a γ-counter and plotted using the PRISM3 program. Affinity constants were calculated based on the ED<sub>50</sub>, from data sets with limiting amounts of captured hFSHR ECD using National Institutes of Health radioimmunoassay software.

To assess the effect of binding of the anti-peptide antibodies on 125I-FSH binding to ECD, captured hFSHR ECD was incubated with different dilutions of antibodies (dilutions ranging from 1:3 to 1:100) for 2 h at 37 °C followed by washing and incubating with 125I-FSH as described above. Radioactivity counts were compared with the addition of normal rabbit serum.
**ELISA**

The ELISA format was used not only to determine the relative concentration of hFSHR ECD in media but also to examine the effects of FSH-hFSHR-ECD complex formation on the accessibility of hFSHR ECD epitopes for the anti-peptide antibodies. Two complementary approaches were used for protein footprinting in which either hFSHR ECD (see the next paragraph, "hFSHR ECD-capture ELISA") or hormone (see "FSH-capture ELISA," below) was captured by specific monoclonal antibodies.

**hFSHR ECD-capture ELISA**—Immunol 4HBX 96-well plates (DYNEX Technologies Inc., Alexandria, VA) were coated with 500 ng of mAb 106.105 in 100 µl of coating buffer at 4 °C overnight. Plates were washed thoroughly (4–6 times) with wash buffer (phosphate-buffered saline with 0.02% azide and 0.05% Tween 20; all the following washes are done with this buffer) between all of the following steps. Plates were blocked for 2 h at 37 °C with 200 µl of 5% nonfat milk in coating buffer, and hFSHR ECD from 100 µl of insect media was captured on the antibody for 1 h at 37 °C (Hi 5 cell-conditioned media was used as control). To detect hFSHR ECD captured, plates were incubated with polyclonal anti-peptide antibody W970 (1:1000 in 100 µl of binding buffer; anti-hFSHR 150–183) for 1 h at 37 °C followed by a 1-h incubation with alkaline phosphatase-labeled goat anti-rabbit IgG (1:4000 in 100 µl of binding buffer, BIO-RAD) and hFSHR ECD (0.5–0.8 µM in 0.01 M phosphate buffer) and hFSHR ECD (0.5–0.8 µM in 0.01 M phosphate buffer) individually as well as for a mixture containing 3.4–5.8 µM schFSH and 0.5–0.8 µM hFSHR ECD, thereby containing exactly the same concentration of each protein as in the individually measured samples. schFSH was thus used in a 4–12-fold molar excess to ECD in separate experiments. Before obtaining the spectra, the individual proteins as well as a mixture of both were incubated at room temperature or 37 °C for 0.5–2 h.

Individual spectra were analyzed using the JASCO software to subtract the background and smooth the spectra. The computer program SELCON 3 was used to calculate the secondary structure estimates for schFSH and hFSHR ECD utilizing two data bases containing 37 and 42 proteins, respectively, enabling an analysis in the wavelength range from 185 to 260 nm (32–35). For comparison of the spectra (in molar ellipticity) of the hFSHR ECD-schFSH complex versus theoretically calculated ECD + FSH spectra, the individual spectra of FSH and ECD were added after background subtraction and smoothed, and the molar ellipticity (mean residue ellipticity (#)) was calculated. This sum of the individual spectra, representing a mixture of noninteracting schFSH and hFSHR ECD, was compared with the spectra experimentally determined for the hFSHR ECD-schFSH complex.

**RESULTS**

**Expression and Purification of hFSHR ECD**—To analyze domains of the hFSHR ECD involved in hormone binding in a cell-free system, we utilized an expression system that afforded high amounts of secreted soluble ECD. Hi 5 cells were infected with a plasmid encoding aa 1–349 of ECD under the control of the polyhedrin promoter to ensure high expression. Using the signal sequence of the envelope protein gp67, the recombinant protein was effectively secreted into media (Fig. 1A) with only 10–15% of ECD left in the cells (data not shown). On reducing SDS-polyacrylamide gel, the recombinant ECD appeared as a single protein with an approximate molecular mass of 50 kDa. Thus, it seemed to be glycosylated in the chosen system, as the estimated molecular mass of the protein alone would be 40 kDa. Purification of hFSHR ECD from
4 liters of conditioned media using an mAb 106.105 affinity column routinely yielded hundreds of micrograms of pure protein (Fig. 1B). When elution was done with buffer of pH 2.2, a partial loss of binding activity of the purified ECD could be observed. The addition of 30% glycerol to the elution buffer was found to stabilize the biological activity of purified ECD. The ECD expressed in this system was therefore found to be suitable for studying the binding of FSH to its cognate receptor in a cell-free system.

Characterization of Soluble hFSHR ECD—To analyze the binding characteristics of hFSHR ECD, a new solid-phase radio receptor assay was developed using mAb 106.105 as capture antibody. This antibody, which recognizes human FSHR aa 300–315, did not interfere with the time-dependent hFSH binding when used to capture soluble hFSHR ECD (Fig. 2), indicating that this region is not directly involved in hormone binding.

Because the hFSHR ECD lacks the entire C-terminal half of the receptor, it was important to determine whether the specificity among gonadotropin hormones was retained. To examine this, captured ECD was incubated with $^{125}$I-hFSH and increasing amounts of each hormone preparation of pituitary (pit) FSH, LH, and TSH.

For this purpose, ECD was captured from insect cell media and incubated with the specific anti-peptide antibodies. Binding of FSH was analyzed by adding trace amounts of $^{125}$I-hFSH. A schematic model of this experiment is presented in Fig. 4A, and a representative result is illustrated in Figs. 4, B and C. There is an obvious inhibition of binding by antibodies against peptides in the region aa 150–254, whereas binding is not affected by antibodies against aa 1–100 and 265–296, with one exception. For some areas, two antisera were available, and both were used in this assay to verify the results. As can be seen in Fig. 4B, antibody W971 inhibited hFSH binding, whereas W963 did not, although both antisera were prepared against the same epitope. These results suggest a high affinity binding site of FSH between aa 150 and 254 of the hFSHR ECD, whereas there is no steric hindrance by antibodies binding to the N-terminal part of the ECD, indicating that these peptide regions are not important for high affinity hormone binding.

Protein Footprinting by Epitope Mapping of the ECD and ECD-FSH Complexes—In the second part of the study an epitope mapping approach was used to determine epitopes on the ECD that participate in forming the ECD-FSH complex. For this purpose a sandwich ELISA was designed in which ECD was captured by mAb 106.105, and the accessibility of hormone-occupied ECD epitopes was analyzed. Fig. 5A shows a schematic model of the experimental design. A representative result of these experiments is shown in Fig. 5B. Only the specific signals are shown, varying in intensity between the different antibodies due to a high variability in background between rabbit sera, which has been subtracted. The captured unoccupied hFSHR ECD could be detected with all anti-peptide antibodies (open bars). This signal intensity was compared with ECD that was occupied by schFSH, suggesting a steric hindrance of this region after the hormone bound. The residual detection signal that can be seen after hormone binding in Fig. 5B (closed bars) may have been due to free ECD that did not bind FSH but that was captured by mAb 106.105. To focus the analysis only on ECD that is bound to FSH, a new ELISA was developed that was complementary to the approach described before. In this assay, schFSH was captured by a monoclonal antibody mAb 46.3H6.B7, which is specific for the $\beta$-subunit. This mAb was chosen from a panel of antibodies because it does not interfere with binding of ECD to captured FSH. Purified schFSH was captured by mAb 46.3H6.B7, and ECD from insect media was bound, followed by challenging the complex with anti-peptide antibodies (Fig. 6). Only antibodies against aa 1–15 and 220–296 were able to detect ECD when complexed with schFSH, confirming the data determined before (Fig. 6, B and C). These observations suggest
that after hormone binding, epitopes in areas 15–100 and 150–220 are involved in hormone-receptor contacts because they are no longer accessible to the anti-peptide antibodies.

Circular Dichroism—The data described above show two different aspects of the ECD-FSH interaction. Hormone binding could be inhibited by binding of antibodies against aa 150–254, suggesting a high affinity binding site in this area, whereas antibodies against the N-terminal part of the ECD did not interfere with hormone binding. Yet when the hormone was bound and the ECD-FSH complex was formed first, the accessibility of a much larger region, namely aa 15–100 and 150–220, was reduced or even abolished. This suggests a conformational change had taken place after the hormone was bound to the hFSHR ECD. Presumably, hormone binding to region aa 150–254 leads to a change in the conformation of the ECD, now recruiting N-terminal parts of the receptor, rendering these regions unavailable for antibody binding.

To test the hypothesis that a conformational change occurs upon hormone binding, the biophysical technique of circular dichroism (CD) was applied. The CD spectra of the purified hFSHR ECD as well as purified schFSH were taken with a JASCO 720 spectropolarimeter (Fig. 7A). Spectra were analyzed with the program SELCON 3 using two data bases containing 37 and 42 proteins, respectively, thus restricting the wavelength range to 185–260 nm (32–35).

Analysis of the CD for purified schFSH spectra yielded a calculated distribution of 7.2% α-helix, 34.9% β-sheet, 18.7% turn, and 35.2% other (Table II) compared with an actual average of 5.2% α-helix, 50.1% β-sheet, and 44.7% random structure as determined in the hFSH crystal structure (36). For the purified hFSHR ECD, the SELCON 3 analysis resulted in an estimate of 22.7% α-helix, 24.1% β-sheet, 20.5% turns, and 34.5% other structures. All percentages are mean values; see Table II for S.D.

Protein spectra obtained separately were added using the JASCO 720 software, and the spectrum was converted to molar ellipticity (Fig. 7B). This sum of spectra represents the theoretical spectrum of a protein mixture where hFSHR ECD and schFSH are not interacting. To analyze a potential conformational change due to hormone binding, the spectrum of a mixture of ECD and FSH containing the same concentrations as in each single spectrum was measured after a short incubation period at room temperature or 37 °C. The theoretically calculated spectrum should have been identical with the spectrum of the mixture if no change in secondary structure occurred. Fig. 7B shows the comparison of the average spectra of three independent experiments measured compared with the average of the theoretically calculated spectra. There is a small but reproducible difference in the spectra, indicating a small change in secondary structure due to complex formation. The change in secondary structure, though, is relatively small and not significant based on SELCON 3 estimates (not shown). Nevertheless, the observed difference is repeatable, indicating a very small secondary structure change upon hormone binding.

DISCUSSION

Expression of Secreted hFSHR ECD—The results of this study show that soluble hFSHR ECD can be effectively expressed and secreted using an insect cell system. The secreted protein is capable of binding FSH with high affinity. Former studies show that the gonadotropin receptor ECD can be expressed in E. coli (23, 37) or mammalian cells (19, 21, 38, 39), but the ECD is trapped in cells and sometimes even is not functional in hormone binding (24, 37). The baculovirus system has also been widely used to express the ECD of GPHRs, but secretion was not detectable or extremely low (20). Progress has been made by changing the strong polyhedrin promoter to the weaker P10 promoter or using an insect signal sequence. This results in lower expression levels of recombinant ECD, which seems to affect the way the protein is processed posttranslationally, resulting in a more mature protein that carries more complex carbohydrates. This mature ECD could be detected in low levels in the media and was able to bind hormone (24, 40, 41). Another approach used the native hormone receptor signal sequence and co-expressed the hormone subunits together with the ECD (22). Enhanced expression levels for FSHR were observed when co-expressed with either βFSH or αβFSH and biologically active ECD was secreted into the media, although the majority still was localized intracellularly when expressed alone.

The results of the study reported here show for the first time that high expression levels and less complex carbohydrates are not incompatible with ECD secretion. Use of the polyhedrin...
promoter resulted in high expression levels of ECD, and by fusion to the signal sequence of insect cell envelope glycoproteins gp67, high secretion of functional ECD could be achieved. The resulting protein is most probably glycosylated (50 kDa in size compared with 40 kDa calculated molecular mass), and due to the high expression levels, it most likely contains just high mannose forms. The produced hFSHR ECD binds FSH with high affinity and is therefore a valuable model to study ECD-hormone interaction in a cell-free system.

Protein Footprinting by Antibody Inhibition and Epitope Mapping—The current antibody inhibition study revealed a high affinity binding site for FSH located centrally in the hFSHR ECD (approximately aa 150–250). Hormone binding to ECD could be inhibited by binding of antibodies against aa 150–254, suggesting that elements required for binding are located in this area, whereas antibodies against the N-terminal part of the ECD did not interfere with hormone binding. Remarkably, exactly this region has been indicated by receptor chimera studies to be most important in FSH specificity, whereas LH binding seems to be more localized in the N-terminal part of the ECD (17). Mutagenesis of the proposed LRR of the receptors (13) indicated that for the FSH receptor, there are several regions involved in hormone binding, but mutations of leucines in LRR7 and -8 (aa 177 and 179 and aa 202 and 204) most severely impaired binding. Using charge reversal mutagenesis, several residues in the LHR ECD were identified that are involved in hormone binding of the LHR, but the most severe effects were seen in mutations between aa 132 and 206 (10, 11). Whether the region involved in FSH binding is spread further between aa 100 and aa 150 could not be studied but cannot be excluded with our data.

Interestingly, when analyzing full-length receptor expressed in insect cells, Liu et al. (30) could show, using some of the antisera described in this work, that region aa 265–296 seems to be involved in hormone binding; thus, antibody X179 inhibited 125I-FSH binding. Indeed, scanning alanine mutagenesis of the hFSHR 265–296 region failed to identify side chains critical for hFSH-hFSHR interaction (42). However, the present studies indicate that this region is not involved in FSH binding. Inhibition of FSH binding by anti-peptide antibodies to the 265–296 region may be explained by the close proximity of the ECD to the membrane or transmembrane domain. The inhibitory effect seen in that study may be due to binding of antibody X179 to the ECD of full-length receptor, which keeps the ECD, tethered to the transmembrane domain, in a sterically hindered position for FSH binding. Another possible explanation might be a greater conformational flexibility of the secreted ECD compared with the full-length receptor ECD. Interestingly, the monoclonal antibody used to immobilize ECD, directed against aa 300–315, also inhibits FSH binding to membrane-bound FSHR (21). Yet, as presented herein, FSH can bind effectively to hFSHR ECD immobilized with this mAb. This is reminiscent of the apparent conundrum discussed above. All things considered, it may be that the 265–315 epitope (region) is highly flexible. Thus, although the present study as well as two other studies from our group (27, 42) show that the region aa 265–315 seems not to be directly involved in FSH binding, 125I-FSH binding to membrane-bound FSHR can be blocked by mAb 106.105. This again suggests a greater conformational flexibility of ECD as soluble receptor than in membrane-bound form.

The second part of our study revealed another interesting aspect of hormone binding to hFSHR ECD. After hormone binding, the accessibility of a much larger region than involved before, namely aa 15–100 and 150–220, was reduced or even abolished. The involvement of aa 15–100 of the hFSHR ECD is in agreement with several studies analyzing the more N-terminal part of the receptor. This area and the cysteines herein seem to be involved in hormone binding, as revealed by other studies. Deletion mutants of aa 9–30 failed to bind FSH, and alanine-scanning mutants revealed that mutants of aa 12–14, 22–25, and 26–30 were not able to bind hormone (16). Indeed, one of the antisera against hFSHR peptide 15–44 inhibited binding of hFSH to the receptor. That the other antiserum against the 15–44 peptide did not inhibit binding is likely to be

FIG. 5. Decrease in detection of ECD after FSH binding as determined by ELISA. A, schematic diagram of the experiment. hECD was captured from insect media using mAb 106.105, incubated with either buffer or schFSH, and detected by different antibodies. Gray in the schematic representation of the ECD peptide sequence indicates no effect on antibody binding. B, signal intensity for different antisera detecting ECD or ECD + schFSH in a capture ELISA. The data shown represent one of three separate experiments, in which the data points have been determined in duplicate. C, differences in absorbance of ECD complexed with schFSH compared with unoccupied ECD signal represented as percent of control for the experiment shown in B. The FSH binding clearly decreased the accessibility for antibodies covering the regions 15–100 and 100–220, suggesting that this region is involved in hormone contact after hormone binding.
due to differences in epitope specificity between the two rabbits.

The data obtained in this study suggest a conformational change taking place after the hormone has bound to a site centrally located of the ECD, in region of aa 150–254. This leads to a change in the conformation of the ECD, now involving also the more N-terminal parts of the receptor, which are thus no longer available for antibody binding.

There are several models of FSH binding and signal transduction in the literature. The first model of hormone binding and receptor activation proposed (17) presented two possible ways of receptor activation. The first possibility assumes a so-called “tethered” model in which hormone binding and signal transduction are determined by two different parts of the receptor. After hormone binding, the bound hormone in this model is positioned and interacts with the activation site in the transmembrane domain of the receptor, leading to signal transduction. According to the second model, the hormone binds to the ECD, which then undergoes a conformational change including the transmembrane domain, thereby activating signal transduction. The data presented herein support this model and are further confirmed by circular dichroism analysis of the ECD-FSH complex (see below).

Circular Dichroism—This study presents the first circular dichroism data ever collected for a free GPCR ECD and schFSH. While these studies were in progress, a report was published that described CD analysis of a human chorionic gonadotropin-LHR-ECD complex (43).

The analysis of the spectrum for schFSH revealed a distribution of 7.2% α-helix, 34.9% β-sheet, 18.7% turn, and 35.2% other. These values are in relatively good agreement with the values detected in the hFSH crystal structure (36), with an average of 5.2% α-helix, 50.1% β-sheet, and 44.7% random coil. The relatively low estimate of β-sheet in this study may be due to the SELCON 3 program used to analyze the data. Comparing different analysis software, Greenfield (44) determined a very good overall performance of the SELCON software (an earlier version of SELCON 3), but she also found that it has a tendency to poorly estimate β-sheets in proteins containing high amounts of β-sheets, as FSH does. An additional consideration is the fact that the calculations for the secondary structure based on the CD data were divided in four components, compared with three components for the crystal structure. Comparison of CD spectra obtained for schFSH with pituitary FSH and insect-expressed wild type FSH (data not shown) revealed no difference in the spectra-predicted secondary structure for these forms of hFSH. Thus, the short tether does not appear to adversely affect formation of the heterodimer. Importantly, these data provide assurance that the conformation of the schFSH faithfully replicates that of the native hFSH.

Sequence analysis of the ECD of the GPHR identified LRR-like motifs as seen in many proteins involved in protein-protein interaction. The structure of the porcine ribonuclease inhibitor (RI) (4, 8, 9), a member of the LRR protein family, consists of 15 LRR repeats of the GPHR are a little shorter than in the RI (7). Additionally, the shape of the CD spectrum of ECD can give information about the tertiary structure (Ref. 45, reviewed by Greenfield (44)). It is suggested that for α- and

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**Fig. 6. Accessibility of ECD after binding to captured schFSH as determined by ELISA.** A. Schematic diagram of the experiment. schFSH was captured by βFSH-specific mAb 46.3H6.B7, and binding of ECD was analyzed by different polyclonal antisera. ECD regions detectable by antisera are indicated in gray in the schematic representation of the ECD peptide sequence. B. ECD bound to captured schFSH was clearly detectable with antisera against 1–15 and 220–296, whereas antibodies against aa 15–100 and 150–220 could not (or only weakly) detect the ECD. These data are completely consistent with the data shown in Fig. 5. The data shown represent one out of three separate experiments, in which data points have been determined in duplicate. C, representation of the data shown in B as percentage signal intensity, with the signal of antibody X803 set as 100%.
b-containing proteins, the ratio of intensity of bands at 208 and 222 nm distinguishes proteins with intermixed α-helices and β-sheets from proteins with separate α-helix and β-sheet regions, the latter showing a larger band at 208 nm. The ECD spectrum showing a slightly more intense band at 208 nm may give rise to the conclusion that the ECD contains separate α-helical and β-sheet regions, further supporting the RI-like structure.

Circular dichroism is a very powerful technique in analyzing conformational changes in proteins, and it has been used in a wide range of settings to analyze the influence of pH and salts, detergent, mutations, and ligand binding on the secondary structure of proteins, including glycoprotein hormones and receptor ECDs (46–51).

To analyze a potential secondary structure change due to hormone binding in the FSHR ECD, the protein spectra collected separately were mathematically added using the JASCO J720 software. After conversion to molar ellipticity, this theoretical spectrum was compared with the spectrum experimentally obtained from the mixture. There is a slight but repeatable difference in the spectra, indicating a small conformational change due to complex formation. Whether just the ECD or also the hormone is changing its secondary structure upon binding cannot be concluded from our data. Purohit et al. (46), analyzing structural changes in human chorionic gonadotropin mutants missing the α52Asn carbohydrate,
postulated a model in which the hormone makes a first contact through its β-subunit. This binding produces a conformational change in the β-subunit that is relayed first to the α-subunit and then passed to the receptor, leading to subsequent events in the signal transduction cascade. In this model the two subunits act as mediators between the two “arms” of the horseshoe-shaped ECD. The crystal structure of hFSH revealed that there is some flexibility in the structure (36), which might be important for interaction with the receptor and could point to the fact that the hormone also changes upon binding. But considering that the conformational change of the hFSHR ECD-schFSH complex tends to increase the α-helix of the structure content, although this is not significant as based on SELCON 3 analysis, this is unlikely to be caused by the β-sheet dominated structure of the hormone. The small change in secondary structure for the complex may possibly suggest that a larger change in tertiary structure might occur, explaining the immunochemical data.

Comparison of the crystal structure of the RI and RI-RNase A complex revealed an unusual conformational change of the RI, with continuous accumulation of small shifts along the whole protein chain, resulting in changes in the curvature and the twist of the horseshoe (8, 9) rather than showing an obvious hinge region. To accommodate RNase A, which does not change conformation upon binding, the cavity in the RI structure is enlarged, with an increase in the distance between the N- and C-terminal repeats. The proposed conformational change in the hFSHR ECD might also be seen as “wrapping” or “expanding” of the ECD arms around the hormone as described by Moyle et al. (6). Also, binding of hFSH could lead to a bending of the whole structure of the ECD and, thereby, to a change in epitope structure.

Interestingly, the conformation seen in the RNase-bound RI resembles the conformation of free RI obtained from a different crystallization solution (8, 9). This may reflect the overall flexibility of the RI structure but might also lead to the hypothesis of different conformers existing for the RI protein. Based on the RI model, one might hypothesize that the hFSHR ECD exhibits the same flexibility that also exists in different conformers where binding of FSH leads to stabilizing the active conformer of the receptor.

Taken together, the results reported here support an ECD structure containing high amounts of α-helix, supporting RI-based models. Furthermore, they strongly suggest a model where a first hormone contact is made in a relatively small region inside the ECD, leading to a conformational change in ECD, hormone, or both, finally resulting in signal transduction. This conformational change is strongly indicated by the immunological data, whereas the change of secondary structure observed by CD is relatively small. This may indicate that the conformational change is rather due to tertiary structure changes than changes in secondary structure.

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