Orientation of Follicle-stimulating Hormone (FSH) Subunits Complexed with the FSH Receptor

β SUBUNIT TOWARD THE N TERMINUS OF EXODOMAIN AND α SUBUNIT TO EXOLOOP 3

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Follicle-stimulating hormone (FSH) comprises an α subunit and a β subunit, whereas the FSH receptor consists of two halves with distinct functions: the N-terminal extracellular exodomain and C-terminal membrane-associated endodomain. FSH initially binds to exodomain, and the resulting FSH/exodomain complex modulates the endodomain and generates signal. However, it has been difficult to determine which subunit of FSH contacts the exodomain or endodomain and in what orientation FSH interacts with them. To address these crucial issues, the receptor was Ala-scanned and the hormone subunits were probed with photoaffinity labeling with receptor peptides corresponding to the N-terminal region of the exodomain and exoloop 3 of the endodomain. Our results show that both regions of the receptors are important for hormone binding and signal generation. In addition, the FSH β subunit is specifically labeled with the N-terminal peptide, whereas the α subunit is labeled with the exoloop 3 peptide. These contrasting results show that the FSH β subunit is close to the N-terminal region and that the α subunit is projected toward exoloop 3 in the endodomain. The results raise the fundamental question whether the α subunit, common among the glycoprotein hormones, plays a major role in generating the hormone signal common to all glycoprotein hormones.

In contrast to the tightly held hormone structure, the FSH receptor (FSHR), a G protein-coupled receptor, has two distinct domains as shown in Fig. 1B. The extracellular N-terminal exodomain comprises ~350 amino acids, and the membrane-associated C-terminal endodomain with a similar number of amino acids consists of seven transmembrane helices, three exoloops, three cytoloops, and the C-terminal cytoplasmic tail (6–8). The exodomain binds the hormone with high affinity (9–16) and selectivity (17), whereas the hormone signal is generated in the endodomain (18–22). FSH initially interacts with the exodomain, and the resulting FSH/exodomain complex modulates the endodomain to generate hormone signal. Important amino acids have been identified for the interaction of the hormone and receptor (23, 24). However, the orientation of FSH α and β in the ternary complex of the hormone, exodomain, and endodomain has been a major enigma and difficult to determine.

The bulk of the exodomain comprises 8–9 Leu-rich repeats (LRR) (7, 25–28), which are flanked by the short upstream N-terminal region and the downstream hinge region. LRRs are thought to form a one-third of a doughnut structure (26–28). FSH appears to interact with LRRs (23, 29) and the N-terminal and hinge regions (19, 30). However, it is unclear which subunit of FSH contacts the exodomain or endodomain and in what orientation FSH interacts with them, although the concave C-terminal side of FSH appears to interact with the receptor. For example, FSH may interact horizontally or vertically with LRRs (Fig. 1, C and D) and in two directions. These are crucial pieces of information for understanding the interactions among the hormone, exodomain, and endodomain and the mechanisms of signal generation. In addition, the information will facilitate the design of agonists and antagonists and development of new therapeutics. Because of the importance, the interactions of glycoprotein hormones with their receptors have been modeled (3–5, 23, 28) based on the crystal structure of the LRRs of ribonuclease inhibitor complexed with its ligand (31). However, the evidence has been elusive.

In a step to resolve this issue, we set out to distinguish the interactions of the FSH subunits with the N-terminal region of the exodomain and exoloop 3 in the endodomain. Our results show the interaction of FSHβ with the N-terminal region of the exodomain and the α tip of FSHα with exoloop 3 (Fig. 1E).
hormone in 8 M urea for 30 min. Rabbit anti-FSHα serum, rabbit anti-FSHβ serum, and monoclonal anti-FSHR 106.105 antibody were kindly provided by Dr. James Dias. Anti-rabbit IgG conjugated with peroxidase was purchased from Pierce. Peptide mimics including wild type peptides corresponding to the Ser9-Lys40 sequence (FSHR940) and exoloop 3 and a photoactivable peptide containing benzoyl phenylalanine (Bpa) in place of Phe13 (FSHR13-40F13Bpa) were synthesized by Genemed Synthesis (San Francisco, CA) and purified on a Vydac C18 high pressure liquid chromatography column using solvent gradient from 100% of 0.1% trifluoroacetic acid in water to 20% of 0.1% trifluoroacetic acid in water and 80% 1-propanol.

**Mutagenesis and Functional Expression of FSH Receptors—Mutant FSHR cDNAs** were prepared in the pSELECT vector using the Altered Sites mutagenesis system (Promega), sequenced on a Beckman CEQ 2000XL capillary sequencer, subcloned into pcDNA3 (Invitrogen) as described previously (32), and sequenced again to verify mutation sequences. This procedure does not involve polymerase chain reaction and therefore does not have its infidelity problems. Wild type and mutant receptor constructs were transfected into HEK 293 cells by the calcium phosphate method as described previously (32). Stable cell lines were established in minimum essential medium containing 10% horse serum, and monoclonal anti-FSHR 106.105 antibody were dated for use in our laboratory. cDNAs were prepared in the pSELECT vector using the Altered Sites mutagenesis system (Promega), sequenced on a Beckman CEQ 2000XL capillary sequencer, subcloned into pcDNA3 (Invitrogen) as described previously (32), and sequenced again to verify mutation sequences. This procedure does not involve polymerase chain reaction and therefore does not have its infidelity problems. Wild type and mutant receptor constructs were transfected into HEK 293 cells by the calcium phosphate method as described previously (32). Stable cell lines were established in minimum essential medium containing 10% horse serum, and monoclonal anti-FSHR 106.105 antibody were dated for use in our laboratory.

**125I-FSH Binding and Intracellular cAMP Assay—Stable** cells were assayed for 125I-FSH binding in the presence of 100,000 cpm of 125I-FSH (33) and increasing concentrations of unlabeled FSH. The $K_i$ values were determined by Scatchard plots. For intracellular cAMP assay, cells were washed twice with Dulbecco’s modified Eagle’s medium and incubated in the medium containing 0.1 $\mu$g/ml isobutylmethylxanthine for 15 min. Increasing concentrations of FSH were then added, and incubation was continued for 45 min at 37°C. After removing the medium, the cells were rinsed once with fresh medium without isobutylmethylxanthine, lysed in 70% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000 x g for 10 min at 4°C, the supernatant was collected, dried under vacuum, and resuspended in 10 $\mu$l of cAMP assay buffer (Amersham Biosciences). cAMP concentrations were determined with an 125I-cAMP assay kit (Amer sham Biosciences) following the manufacturer’s instructions and validated for use in our laboratory. cDNAs were prepared in the pSELECT vector using the Altered Sites mutagenesis system (Promega), sequenced on a Beckman CEQ 2000XL capillary sequencer, subcloned into pcDNA3 (Invitrogen) as described previously (32), and sequenced again to verify mutation sequences. This procedure does not involve polymerase chain reaction and therefore does not have its infidelity problems. Wild type and mutant receptor constructs were transfected into HEK 293 cells by the calcium phosphate method as described previously (32). Stable cell lines were established in minimum essential medium containing 10% horse serum, and monoclonal anti-FSHR 106.105 antibody were dated for use in our laboratory.

**125I-FSH Binding to Solubilized FSHR—**Transfected cells were washed twice with ice-cold 150 mM NaCl, 20 mM HEPES, pH 7.4 (buffer A). Cells were scraped on ice, collected in buffer A containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 10 mM EDTA), and pelleted by centrifugation at 10,000 x g for 15 min. The supernatant (100,000 cpm of 125I-FSH and 6.5 $\mu$l of 0.9% NaCl and 10 mM Na2HPO4 at pH 7.4 containing increasing concentrations of unlabeled FSH. After incubation for 12 h...
at 4 °C, the solution was thoroughly mixed with 250 μl of buffer A containing bovine γ-globulin (5 μg/ml) and 750 μl of buffer A containing 20% polyethylene glycol 8000. After incubation for 10 min at 4 °C, samples were pelleted at 1,300 × g for 30 min and supernatants removed. Pellets were resuspended in 1.5 ml of buffer A containing 20% polyethylene glycol 8000, centrifuged, and counted for radioactivity. Monoclonal anti-FSHR antibodies were radioiodinated and used for binding to nonbinding mutant FSHRs expressed on the intact cell surface as described previously (34).

**Derivatization and Radiiodination of Peptides**—In the dark, 30 μg of receptor peptides in 40 μl of 0.1 M sodium phosphate, pH 7.5, was mixed with 1 nCi of Na-125I in 10 μl of 0.1 M NaOH and 7 μl of chloramine-T (1 mg/ml) in 10 mM NaHPO4, pH 7.4. After 20 s, 7 μl of sodium metabisulfite (2.5 mg/ml) in 10 mM NaHPO4, pH 7.4, was added.
introduced to terminate radioiodination. Radioiodinated peptides were mixed with 60 μL of 16% sucrose solution in PBS and fractionated on Sephadex Superfine G-10 column (0.6 x 15 cm) using PBS. Peptides were derivatized with 4-azidobenzoyl glycine (ABG) and radioiodinated as described previously (35).

Photoaffinity Labeling of FSH—The following solutions were sequentially introduced to siliconized glass tubes: 20 μL of 0.9% NaCl and 10 mM Na2HPO4, pH 7.4, in PBS, 10 μL of FSH in PBS, and 10 μL of 125I-FSHR9–40F13Bpa in PBS. Competitive inhibition experiments were carried out as described for the photoaffinity-labeling experiments with the exception that 10 μL instead of 20 μL of PBS was introduced to each tube and the mixture was incubated with 10 μL of increasing concentrations of nonradioactive receptor peptides. The mixtures were incubated at 37 °C for 90 min in the dark, irradiated with a Mineralight R-52 UV lamp for 3 min as described previously (36), and solubilized in 2% SDS, 100 mM dithiothreitol, and 8 M urea. The samples were electrophoresed on 8–12% polyacrylamide gradient gels. Gels were dried on filter paper and exposed to an imaging screen overnight, which was

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**Figure 4. Ala substitutions for Thr21–Glu33.** A–F, residues from Thr21 to Glu33 of the FSH receptor were individually substituted with Ala, and the resulting mutant receptors were expressed in HEK 293 cells and assayed as described in the legend to Fig. 3.
Deglycosylation—The FSH α and β subunits co-migrate on SDS-PAGE. To separate them on the gel, FSH was photoaffinity-labeled and deglycosylated with PNGase F. Enzymatic cleavage was done by incubation of the labeled FSH complex with 20 or 50 units of PNGase F (New England BioLabs) in 40 μl for 18 h at 37 °C. The samples were solubilized in SDS under the reducing condition and electrophoresed on 15% gel containing 9 M urea.

Immunoblot of FSH Subunits—Separated proteins were blotted onto 0.2-μm nitrocellulose membrane as described previously (37). Membranes were treated for 1 h with 5% blocking buffer (25 mM Tris-HCl, 1.4 mM NaCl, 0.2% sodium azide, 1% Nonidet P-40, pH 7.4) and incubated with polyclonal anti-FSH and anti-FSHR α and β antibodies (dilution 1:2000 and 1:3500 each in blocking buffer) for 1 h at room temperature. Membranes were washed three times (5 min each) with the blocking buffer and incubated with anti-rabbit peroxidase-conjugated IgG (dilution 1:5000 in blocking buffer) for 1 h at room temperature. Membranes were washed three times (5 min each) with the blocking buffer and twice (5 min each) with 25 mM Tris-HCl, pH 7.4. Membranes were incubated in staining solution (0.05% 3,3′-diaminobenzidine, 0.02% CoCl2, 0.03% H2O2) until bands became visible.

RESULTS

Activities of the N-terminal Region—In a first step to check the importance of the N-terminal region of human FSHR, each amino acid of the SNRFLCQESKTEIPSDLPRNAIE sequence was individually substituted with Ala. This sequence is highly conserved among species, implicating its importance. In contrast, the FSHR sequence is highly conserved among species, implicating its importance.

Comparison of Ala substitution mutations. In a first step to check the importance of the N-terminal region of human FSHR, each amino acid of the SNRFLCQESKTEIPSDLPRNAIE sequence was individually substituted with Ala. This sequence is highly conserved among species, implicating its importance. In contrast, the FSHR sequence is highly conserved among species, implicating its importance.

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Fig. 5. Hormone binding in solution and anti-FSHR antibody binding to intact cells. A, cells individually transfected with the C15A, P24A, D26A, or L27A mutant receptor were solubilized in Nonidet P-40 and assayed for 125I-FSH binding as described under “Experimental Procedures.” B, intact cells were also probed with 125I-labeled monoclonal anti-FSHR 106.105 antibody for the surface expression of the nonbinding mutants.

Ala Substitutions

Fig. 6. Comparison of Ala substitution mutations. To easily compare the activities of the wild type and mutant receptors, the ratios of Kd, wild type mutant, Ec50 wild type mutant, and maximum cAMP mutant wild type were presented in a bar graph.
washed three times and counted for the bound radioactivity.

concentrations of unlabeled FSHR peptides, FSHR9 and is capable of reacting with unreactive acids (41, 42, 44). To determine whether the resulting peptide moiety of the Bpa group can be activated with UV at substituted with Bpa for photoaffinity labeling (41). The ketone

bar graph measured, and the percentage of the labeled FSH band in a gel lane was calculated based on the total intensity of a gel lane and presented in the

ABC

Fig. 7. Photoaffinity labeling of FSH with photoactivatable FSHR<sup>9–40</sup>F13Bpa. The FSH receptor peptide corresponding to the sequence Ser<sup>9</sup>Lys<sup>40</sup> (FSHR9–40) was synthesized with a Tyr at the N terminus for radioiodination and Bpa at the position of Phe<sup>13</sup> for photoaffinity labeling (A). The peptide was radioiodinated, and the resulting 125I-FSHR9–40F13Bpa was incubated with FSH and irradiated with UV. B, the sample was irradiated with UV for increasing time periods from 0 to 150 s, solubilized in SDS under the reducing condition, and electrophoresed on polyacrylamide gel. After drying gels, they were exposed to a phosphorimaging screen and scanned on a PhosphorImager. The peptide appeared as the lower band, and the FSH α and FSH β subunits comigrated and appeared in the upper band. The intensity of each band in a gel lane was measured, and the percentage of the labeled FSH band in a gel lane was calculated based on the total intensity of a gel lane and presented in the bar graph above the autoradiograph. C, increasing amounts of 125I-FSHR9–40F13Bpa from 0 to 3.7 μM were incubated with a constant amount (0.1 μM) of FSH and photolyzed for 60 s. The samples were processed as described above. D, increasing amounts of FSH from 0 to 0.2 μM were incubated with a constant amount (3.1 μM) of 125I-FSHR9–40F13Bpa. E, FSH (80 nM) was incubated with 3 μM 125I-FSHR9–40F13Bpa in the presence of increasing concentrations of unlabeled FSHR peptides, FSHR9–40, FSHR<sup>9–40</sup>F13Bpa, exoloop 1 peptide (FSHR<sup>exo</sup>1), exoloop 2 peptide (FSHR<sup>exo</sup>2), and exoloop 3 peptide (FSHR<sup>exo</sup>3). The samples were irradiated and processed as described in B.

abc

Fig. 8. Photoaffinity labeling of denatured FSH and other glycoproteins. A, increasing concentrations of 125I-FSHR<sup>9–40</sup>F13Bpa were incubated with 80 nM denatured FSH, irradiated with UV, and processed as described in the legend to Fig. 7. FSH was denatured by boiling in 8 M urea for 30 min. B, a constant amount of 125I-FSHR<sup>9–40</sup>F13Bpa was incubated with increasing concentrations of denatured FSH, treated with UV, and processed. C, a constant amount of 125I-FSHR<sup>9–40</sup>F13Bpa was incubated with 5 nM each of FSH, phospholipase A (PLA), urokinase, growth hormone (GH), LH, or TSH, treated with UV, and processed as described in the legend to Fig. 7. D, cells stably expressing FSHR were incubated with 125I-FSH and washed as described in Fig. 3 but in the presence of increasing concentrations of FSHR<sup>9–40</sup> or FSHR<sup>9–40F13Bpa</sup>. Cells were washed three times and counted for the bound radioactivity.

ably, S9A, V12A, F13A, S18A, and I32A reduced the EC<sub>50</sub> value by 2–3-fold while maintaining or slightly enhancing the maximum cAMP induction level. These results suggested the importance of this region of the receptor in hormone binding and cAMP induction and raised a question as to whether this region directly interacts with the hormone or indirectly impacts the global structure of the receptor.

Photoaffinity Labeling of FSH—To examine the two general possibilities, a peptide mimic corresponding to the receptor sequence of SNRVFLCQESKVTEIPSDELPRNAIELRFVLTK<sup>40</sup> was synthesized, FSHR<sup>9–40</sup> (Fig. 7A). A Tyr residue was attached to the N terminus for radioiodination, and the N terminus was acetylated while the C terminus amidated. Phe<sup>13</sup> was substituted with Bpa for photoaffinity labeling (41). The ketone moiety of the Bpa group can be activated with UV at >350 nm and is capable of reacting with unreactive α-CH bonds of amino acids (41, 42, 44). To determine whether the resulting peptide 125I-FSHR<sup>9–40F13Bpa</sup> could bind and photoaffinity-label FSH, it was incubated with FSH and irradiated with UV for increasing time periods. Samples were solubilized in SDS under reducing conditions and then electrophoresed. The autoradiographic phosphorimaging of the gel (Fig. 7B) revealed labeling of the FSH band. The autoradiograph suggests that the two subunits of human FSH comigrated. The band was not labeled when the sample was not irradiated with UV, indicating the requirement for UV irradiation. The extent of the labeling was dependent on the irradiation time, reaching maximum labeling after 30-s irradiation. The result shows that the labeling is saturable. The hormone was labeled next with increasing concentrations of 125I-FSHR<sup>9–40F13Bpa</sup> while maintaining FSH at a constant concentration (Fig. 7C). Conversely, increasing concentrations of FSH were labeled with a constant concentration of 125I-FSHR<sup>9–40F13Bpa</sup> (Fig. 7D). If the labeling was specific, the concentrations should reach a plateau under both conditions. Indeed, the labeling plateaued under both conditions, indicating saturable and specific labeling of a substantial portion (>50%) of FSH. Furthermore, the labeling should be inhibited by nonradioactive peptide and unmodified wild type peptide.
Therefore, FSH was incubated with 125I-FSHRexoloop 3 in the presence of increasing concentrations of wild type peptide and nonradioactive FSHRexoloop 3 (Fig. 7E). The peptides inhibited the photoaffinity labeling in a dose-dependent manner and eventually blocked the labeling. Peptides corresponding to exoloops 1–3, FSHRexoloop 1, FSHRexoloop 2, and FSHRexoloop 3 were also tested. FSHRexoloop 1 inhibited the labeling as FSHRexoloop 2 did. In contrast, FSHRexoloop 3 inhibited the labeling with a 30-fold less potency. On the other hand, FSHRexoloop 3 failed to block the labeling.

Labeling Specificity—Although the photoaffinity labeling was specific for FSH, our data do not show the biological specificity of the affinity labeling. To address this concern, a constant amount of denatured FSH was incubated with increasing concentrations of 125I-FSHRexoloop 3 and treated with UV. Denatured FSH was not labeled at all despite high concentrations of the peptide (Fig. 8A). Denatured FSH was not labeled when increasing concentrations of denatured FSH were incubated with a constant amount of 125I-FSHRexoloop 3 and treated with UV (Fig. 8B). When FSH was denatured by boiling in 8 M urea for 30 min, it did not bind to FSHR and induce cAMP production. To test whether the denatured FSH remained in solution, the mixture of radioactively labeled FSH and unlabeled FSH was denatured and varying volumes of the mixture were transferred to other tubes and the radioactivity was counted. The transfer was quantitative with 99–100% efficiency, indicating that denatured FSH was present in the photoaffinity-labeling tube. These results indicate the specificity of the affinity labeling for biologically active FSH. To determine the labeling specificity, luteinizing hormone (LH), thyroid-stimulating hormone (TSH), growth hormone, phospholipase A, and urokinase were subjected to photoaffinity labeling with 125I-FSHRexoloop 3 (Fig. 8C). None of them was labeled. If 125I-FSHRexoloop 3 specifically binds to FSH and labels it as indicated by the results, the peptide is expected to inhibit the in vivo binding of FSH to the receptor on intact cells. Indeed, FSHRexoloop 3 inhibited 125I-FSH binding to the receptor (Fig. 8D).

Labeling of FSH β Subunit—Because the two subunits of purified human FSH appeared to comigrate on SDS-PAGE, it was unclear which of the subunits was labeled. To resolve the subunits, FSH was deglycosylated with PNGase F and electrophoresed (Fig. 9A, lane 2). The two subunits were clearly separated into two distinct bands. Because the β subunit is larger than the α subunit, the upper band is probably the β subunit. To conclusively determine the identity of the upper band, deglycosylated FSH was electrophoresed, the gel was blotted on nitrocellulose membrane, and the membrane was probed with anti-FSHα and anti-FSHβ antibodies. Anti-FSHα antibody conspicuously labeled the lower band, whereas the anti-FSHβ antibody recognized primarily the upper band and faintly the lower band (Fig. 9, A and B). These results show that the lower band represents the FSHα subunit, whereas the upper band is the FSHβ subunit, indicating that FSHβ was labeled. To compare this labeling of FSHβ with the N-terminal peptide, the exoloop 3 peptide, FSHRexoloop 3, was used for labeling FSH. The peptide was derivatized with a UV-activatable reagent, ABG, and radioiodinated. The resulting 125I-ABG-FSHRexoloop 3 was incubated with FSH, treated with UV, deglycosylated with PNGase F, solubilized, and electrophoresed (E3). E3 was compared with FSH labeled with 125I-FSHRexoloop 3 (N).

The labeling of FSHβ by 125I-FSHRexoloop 3 contrasts the labeling of the FSHα subunit by the FSHR exoloop 3 peptide. If this contrasting labeling is specific and reflects the true interaction between FSH and the receptor, some of the peptides representing parts of the α subunit sequence might inhibit the labeling. Particularly, if some of the α peptides block the labeling of the α subunit but not the β subunit, the result would support the selective labeling results and show labeling specificity. Four α peptides, α1–15, α26–46, α61–75, and α81–92 were tested, and none of them inhibited the labeling of the FSHβ subunit by 125I-ABG-FSHRexoloop 3 (Fig. 10A). In contrast, the labeling of the FSHα subunit by 125I-ABG-FSHRexoloop 3 was blocked by α26–46 and somewhat by α61–75 (Fig. 10B). α1–15 and α81–92 Peptides failed to block the labeling. These results support the differential labeling and its specificity and validity.

**DISCUSSION**

Our Ala-scanning results indicate that the Ser9-Glu33 sequence of the FSH receptor is important for surface expression, hormone binding, and signal generation. The photoaffinity-labeling results show that FSHRexoloop 3 photoaffinity labels FSH but not LH, TSH, growth hormone, phospholipase A, and urokinase. The labeling is saturable and dependent on the concentrations of FSH and derivatized 125I-FSHRexoloop 3. UV activation, and UV exposure time. 125I-FSHRexoloop 3 photoaffinity labels bioactive FSH but not denatured hormone, and the labeling is blocked by nonderivatized wild type peptide and nonradioactive FSHRexoloop 3. The labeling specificity is further underscored by the fact that it labeled the β subunit but not the α subunit. These results suggest that the N-terminal region of the FSH receptor is in close proximity to FSH, probably interacting with the hormone. This conclusion is consistent with the previous reports that some residues of the region...
are important for hormone binding and receptor trafficking (45–47) and that the similar region of the LH receptor interacts with human chorionic gonadotropin (34, 48).

In contrast to the labeling of the β subunit by the N-terminal peptide, the exoloop 3 peptide labeled the α subunit. The significance of these contrasting results is 2-fold. First, it supports the validity and specificity of the photoaffinity labeling. Second, it provides the crucial information on the overall arrangement of the ternary complex involving the exodomain, FSH, and endodomain. The results suggest that the β subunit is near the N-terminal region of the exodomain, whereas the α subunit is close to the exoloops of the endodomain. These are consistent with the previous reports that only the αβ dimer is capable of high affinity binding to receptors and inducing biological responses (49, 50). Interestingly, all glycoprotein hormones utilize similar if not identical signal pathways consisting of adenylyl cyclase and phospholipase Cβ. Therefore, the α subunit has been implicated in the signal generation (49).

Based on these results, it is now possible to project the hormone interacting with both the exodomain and endodomain. Furthermore, they suggest that the hormone is probably in a vertically tilted position with respect to LRRs of the exodomain and the endodomain. To help visualize the arrangement and facilitate modeling of the ternary complex, one of several possible models is presented in Fig. 1E. The model suggests that parts of the α subunit might interact with LRRs and, conversely, that some parts of the β subunit may be close to the exoloops. Such interactions could be probed by strategically attaching a photoactivable group at appropriate positions of the hormone subunits and/or using a reagent that can reach farther than ABG and Bpa. These two reagents can reach 10 and 7 Å, respectively. Other additional information will also be necessary to more precisely define the ternary structure. For example, the crystal structure of FSH shown in the model does not likely represent its structure in the ternary complex, because the gonadotropin undergoes conformational changes, particularly the interaction between the two subunits upon the initial interaction with the receptor (51). These conclusions are consistent with observations that the original quaternary structure of unbound hormone dimers is not essential for hormone action (1, 52).

The results that Ala substitution for some N-terminal residues improved hormone binding, cAMP induction or both suggest an interesting possibility that this region is involved in modulating not only hormone binding but also signal generation. The most dramatic improvement is seen in the S18A substitution, which improved the EC\textsubscript{50} value of cAMP induction by 3-fold as compared with the wild type value. Additionally, the maximum level of cAMP production only slightly increased. These observations indicate that the affinity and maximum level of cAMP induction are distinctly regulated. They suggest that FSH activates FSHR\textsubscript{S18A} more effectively than the wild type receptor does, which in turn results in better activation of the G protein. It will be interesting to see whether the number of activated G protein molecules is the limiting factor. The improved EC\textsubscript{50} is not related to the hormone binding affinity because the binding affinity of the mutant is somewhat less than the wild type affinity. These novel observations suggest an intriguing possibility that FSHR\textsubscript{S18A} is more sensitive to hormone binding and is capable of activating the G protein with higher affinity without significantly impacting the level of activation. Because the exodomain is likely to modulate the endodomain to generate hormone signals at the exoloops (18–20, 38, 43), a simple possibility that is the affinity of the modulation at the interface between the exodomain and exoloops is improved in FSHR\textsubscript{S18A}. Several other Ala substitutions, S9A, V12A, and F13A, also showed similar yet less dramatic results.

In conclusion, the evidence is presented that in the ternary exodomain/FSH/endodomain complex, FSH is vertically oriented with the β subunit close to the N-terminal region and the α tip projecting toward the exoloop 3.

REFERENCES

1. Jackson, A. M., Berger, P., Piazzi, M., Klein, C., Heuse, A. J., and Boime, I. (1999) Mol. Endocrinol. 13, 2175–2184
2. Narayan, P., Gray, J., and Puett, D. (2002) Mol. Endocrinol. 16, 2733–2745
3. Fox, K. M., Daa, J. A., and Van Roey, P. (2001) Mol. Endocrinol. 15, 378–389
4. Lapthorn, J. P., Harris, D. C., Littlejohn, A., Lushbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) Nature 369, 455–461
5. Wu, H., Lushbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) Structure 2, 545–558
6. Sprengel, R., Braun, T., Nikolics, K., Segaloff, D. L., and Seeburg, P. H. (1990) Mol. Endocrinol. 4, 525–530
7. McFarland, K., Sprengel, R., Phillips, H., Kohler, M., Rosenblit, N., Nikolics, K., Segaloff, D. L., and Sprengel, R. (1989) Science 245, 484–489
8. Lososfél, M., Mihrani, M., Atger, M., Salesse, R., Thi, M., Jolivet, J., Guiochon-Mantel, A., Sar, S., Jallal, B., Garnier, J., and Milgrom, E. (1989) Science 245, 525–528
9. Tsai-Morris, C. H., Buczko, E., Wang, W., and Dufau, M. L. (1990) J. Biol. Chem. 265, 19385–19388
10. Xia, X. B., Wang, H., and Segaloff, D. L. (1990) J. Biol. Chem. 265, 21411–21414
11. Ji, L. and Ji, T. H. (1991) Endocrinology 128, 2648–2650
12. Seetharamaiah, G. S., Kurosky, A., Desai, R. K., Dallas, J. S., and Prabhakar, B. S. (1994) Endocrinology 134, 549–554
13. Davis, D., Liu, X., and Segaloff, D. (1995) Mol. Endocrinol. 9, 159–170
14. Babu, P. S., Jiang, L., Sairam, M. R., Touyz, R. M., and Sairam, M. R. (1999) Mol. Cell. Biol. Res. Commun. 2, 21–27
15. Schmidt, A., MacCell, R., Lindau-Shepard, B., Buckler, D. R., and Daa, J. A. (2001) J. Biol. Chem. 276, 23373–23381
16. Angeli, M., Fanelli, P., and Segaloff, D. L. (2002) Endocr. Rev. 23, 141–174
17. Vischer, H. F., Gramman, J. C., Noordam, M. J., Mosselman, S., and Bogeard, J. (2003) J. Biol. Chem. 278, 15505–15513
18. Ji, T. H., Murdock, W. J., and Ji, I. (1995) Endocrinology 137, 187–194
19. Zeng, H., Phang, T., Song, Y. S., Ji, I. I., and Ji, T. H. (2001) J. Biol. Chem. 276, 3451–3458
20. Nishi, S., Nakabayashi, K., Kubilka, B., and Huse, A. J. (2002) J. Biol. Chem. 277, 3938–3964
21. Tao, Y. X., Miarchi, D., and Segaloff, D. L. (2002) Mol. Endocrinol. 16, 1881–1892
22. Angeli, M., Fanelli, P., and Puett, D. (2002) J. Biol. Chem. 17, 17
23. Daa, J. A., Lindau-Shepard, B., Hauer, C., and Auger, I. (1998) Biol. Reprod. 58, 1331–1336
24. Ouza, Y., Kudo, M., Kaipia, A., Kubilka, B., and Huse, A. J. (1997) Mol. Endocrinol. 11, 1659–1668
25. Koo, Y. B., Ji, I., Slaughter, R. G., and Ji, T. H. (1991) Endocrinology 128, 2297–2308
26. Jiang, X., Dreno, M., Buckler, D., Cheng, S., Yihier, A., Wu, H., Hendrickson, W., and Tayar, N. (1995) Structure 3, 1341–1353
27. Couture, L., Nahariosa, H., Grebret, D., Bemy, J. J., Aja-Atvyre, E., Bozon, V., Haertle, T., and Salesse, R. (1996) J. Mol. Endocrinol. 14, 15–25
28. Bhowmick, N., Huang, J., Puett, D., Isaacs, N. W., and Lapthorn, A. J. (1996) Mol. Endocrinol. 10, 1147–1159

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Orientation of FSH Subunits

29. Song, Y. S., Ji, I., Beauchamp, J., Isaacs, N. W., and Ji, T. H. (2001) J. Biol. Chem. 276, 3426–3435
30. Nakabayashi, K., Kudo, M., Kobilka, B., and Hsueh, A. J. (2000) J. Biol. Chem. 275, 30264–30271
31. Kobe, B., and Deisenhofer, J. (1995) Nature 374, 183–186
32. Ji, I., and Ji, T. H. (1993) J. Biol. Chem. 268, 20851–20854
33. Ji, I., and Ji, T. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5465–5469
34. Hong, S., Phang, T., Ji, I., and Ji, T. H. (1995) J. Biol. Chem. 270, 18355–18360
35. Sohn, J., Ryu, K. S., Sievert, G., Jeoung, M., Ji, I., and Ji, T. H. (2002) J. Biol. Chem. 277, 50165–50175
36. Ji, I., and Ji, T. H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7167–7170
37. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
38. Ji, T. H., Grossmann, M., and Ji, I. (1998) J. Biol. Chem. 273, 17299–17302
39. Rozell, T., Wang, H., Liu, X., and Segaloff, D. (1995) Mol. Endocrinol. 9, 1727–1736
40. Hong, S., Ryu, K.-S., Oh, M.-O., Ji, I., and Ji, T. H. (1997) J. Biol. Chem. 272, 4166–4171
41. Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., and DeGrado, W. F. (1986) J. Biol. Chem. 261, 10695–10700
42. Bayley, H. (1963) Photogenerated Reagents in Biochemistry and Molecular Biology, Elsevier, New York
43. Dufau, M. L. (1998) Annu. Rev. Physiol. 60, 461–496
44. Dorman, G., and Prestwich, G. D. (1994) Biochemistry 33, 5661–5673
45. Bradbury, F., Kawate, N., Foster, C., and Menon, K. (1997) J. Biol. Chem. 272, 5921–5926
46. Nechamen, C. A., and Dias, J. A. (2000) Mol. Cell. Endocrinol. 166, 101–110
47. Nechamen, C. A., and Dias, J. A. (2003) Mol. Cell. Endocrinol. 201, 123–131
48. Phang, T., Kundu, G., Hong, S., Ji, I., and Ji, T. H. (1998) J. Biol. Chem. 273, 13841–13847
49. Pierce, J. G., and Parson, T. S. (1981) Annu. Rev. Biochem. 50, 465–495
50. Roche, F., and Ryan, R. (1985) in Luteinizing Hormone Action and Receptors (Aceolli, A., ed) pp. 17–56, CRC Press, Inc., Boca Raton, FL
51. Ji, I., Pan, Y.-N., Lee, Y.-M., Phang, T., and Ji, T. H. (1995) Endocrine 3, 907–911
52. Garcia-Campayo, V., Kumar, T. R., and Beimel, I. (2002) Endocrinology 143, 3773–3778