High Affinity Hormone Binding to the Extracellular N-terminal Exodomain of the Follicle-stimulating Hormone Receptor Is Critically Modulated by Exoloop 3*

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The human follicle-stimulating hormone receptor (FSH-R) consists of two distinct domains of >330 amino acids, the N-terminal extracellular exodomain and membrane-associated endodomain. The exodomain alone binds hormone with high affinity, whereas the endodomain is the site of receptor activation. Coordination of these two domains is essential for successful hormone action but little is known about their functional and structural relationship. In this communication, we report that exoloop 3 of FSH-R constrains follicle-stimulating hormone binding to the exodomain. When the FSH-R exodomain was prepared by truncating its endodomain, the hormone binding affinity of the exodomain was slightly improved, compared with the wild type receptor. The binding affinity was further improved by >3-fold when the exodomain was attached to the membrane-associated domain of CD8. These results suggest that the FSH-R endodomain attenuates hormone binding at the exodomain. As a first step to test this hypothesis, the 11 amino acids except Ala388 of exoloop 3 were individually substituted with Ala. Ala substitution for Leu380 or Ile384 improved the hormone binding affinity by 4–6-fold while totally abolishing cAMP induction, indicating an inverse relationship. The Ala substitution for Lys380 or Pro382 had a similar trend but to a lesser extent. This significant improvement in the binding affinity suggests that the four residues at the N-terminal region of exoloop 3 interact with the exodomain and constrain the hormone binding in the wild type receptor. This effect is specific since substitutions for other than the 4 residues did not improve the hormone binding affinity. Computer modeling shows that the 4 residues can be positioned on one side of exoloop 3. This result and the apparent inverse relationship of hormone binding and cAMP induction suggest that these two essential functions may work against each other. Therefore, hormone binding might be compromised to preserve cAMP inducibility while maintaining a reasonably high, but below maximum, binding affinity.

The FSH1 receptor and other glycoprotein hormone (LH/CG and TSH) receptors belong to a structurally unique subfamily of G protein-coupled receptors (1). Unlike other receptor subfamilies, they comprise two equal halves, an extracellular N-terminal half (exodomain) and a membrane-associated C-terminal half (endodomain) (2–5) as shown in Fig. 1. The exodomain is ~350 amino acids long, which alone is capable of high affinity hormone binding (6–9) with hormone selectivity (10–12) but without hormone action (8, 13). Receptor activation occurs in the endodomain (14), which is structurally equivalent to the entire molecule of many other G protein-coupled receptors (15). Existing evidence suggests that glycoprotein hormones initially bind to the exodomain, and then the resulting hormone-exodomain complex interacts with the endodomain (1). This secondary interaction is responsible for signal generation (1, 14). Despite the importance of this secondary contact and the relationship between the two domains, there is little information on the subject, particularly concerning the FSH receptor. Because the exodomain of the LH/CG receptor lacking the endodomain is capable of high affinity hormone binding (6–9) the high affinity hormone binding appears to be independent of the endodomain. Contrary to this view, we recently observed that human CG binding to the LH/CG receptor is regulated by certain residues of exoloop 2 (16).

In this study, we set out to determine the effect of the endodomain on hormone binding at the exodomain of the FSH receptor. As a first step, exoloop 3 of the endodomain was examined because it is the shortest exoloop and linked to transmembrane domain 6 that is important for signal transfer (17).

EXPERIMENTAL PROCEDURES

Mutagenesis and Functional Expression of Human FSH Receptor—Each mutant human FSH receptor cDNA was prepared in a pSELECT vector using the non-PCR-based altered sites mutagenesis system (Promega), sequenced, subcloned into pcDNA3 (Invitrogen) as described (18). After subcloning pcDNA3, the mutant cDNAs were sequenced again. In one of the mutants, the codon following Asp341 was substituted with a stop codon to produce the truncated exodomain, Cys1–Asp341. The exodomain/CD8 plasmid (19) is a kind gift of Dr. A. W. Hsueh. In this hybrid, Cys1–Asp341 was fused to the transmembrane and cytoplasmic domains of CD8 via the thrombin cleavage sequence linker. Plasmids were transfected into human embryonic kidney (HEK) 293 cells by the calcium phosphate method. Stable cell lines were established in minimum essential medium containing 10% horse serum and 500 μg/ml of G-418, and then used for hormone binding and cAMP assay. All assays were carried out in duplicate and repeated 4–6 times. Means and standard variations were calculated and analyzed by Student’s t test. In addition, values for mutants were compared with the corresponding values of the wild type receptor using analysis of variance with 95% confidence to determine the statistical significance of differences as detailed in the figure legends.

125I-FSH Binding and Intracellular cAMP Assay—Human FSH (the National Hormone and Pituitary Program) was radiiodinated as described previously for radiiodination of human CG (20). Stable cells were assayed for 125I-FSH binding in the presence of increasing concentrations of nonradioactive FSH. The Kd values were determined by Scatchard plots. Truncated exodomain was solubilized in Nonidet P-40

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§ The abbreviations used are: FSH, follicle stimulating hormone; FSH-R, FSH receptor; LH, luteinizing hormone; CG, choriogonadotropin; HEK, human embryonic kidney.

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and assayed for hormone binding as described previously (16). For intracellular cAMP assay, cells were washed twice with Dulbecco's modified Eagle's medium and incubated in the medium containing isobutylmethylxanthine (0.1 mg/ml) for 15 min. Increasing concentrations of FSH were then added, and the 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 × g for 10 min at 4 °C, the supernatant was collected, dried under vacuum, and resuspended in 10 μl of the cAMP assay buffer that was provided by the manufacturer. cAMP concentrations were determined with an 125I-cAMP assay kit (Amersham Pharmacia Biotech) following the manufacturer's instruction and validated for use in our laboratory.

Computer Modeling—Computer models of exoloop 3 were built using a method similar to that developed for modeling nucleic acid loop structures (21). Crystal structure of the seven helix bundle from bacteriorhodopsin (22) was used for anchoring the loop. Using a reduced coordinates approach, random loop structures with the targeted pair of fixed ends were generated. Those loop structures with energies below a threshold were selected and subjected to a short run of energy equilibration using Metropolis Monte Carlo simulation (21), and then 1,000 cycles of energy minimization using AMBER (23).

Fig. 1. Schematic presentation of the FSH receptor. The FSH receptor consists of two halves, the 349-amino acid long extracellular N-terminal segment (exodomain) and the 339-amino acid long membrane-associated endodomain. The exodomain is thought to include nine Leu-rich repeats consisting of a helix and β sheet. The endodomain consists of three exoloops, three cytoloops, seven transmembrane domains, and the C-terminal segment.

Fig. 2. Scatchard plot of FSH binding to the wild type receptor and FSH receptor exodomain/CD8 hybrid. The wild type FSH receptor or FSH receptor exodomain/CD8 hybrid was expressed in HEK 293 cells and intact cells were assayed for 125I-FSH binding in the presence of increasing concentrations of unlabeled FSH. The results were analyzed by Scatchard plot.

Fig. 3. Ala substitution of Lys580-Pro582 of exoloop 3. Lys580, Val581, and Pro582 of exoloop 3 were individually substituted with Ala. The resulting mutant receptors were stably expressed on HEK 293 cells. The cells were assayed for 125I-FSH binding and FSH-dependent cAMP induction as described under “Experimental Procedures.” For hormone binding, counts of empty tubes (background) were ~50 cpm and nonspecific binding was ~75 cpm including background. Maximum specific binding counts per minute are normally in the range of 1,400–1,500 cpm. Nontransfected cells did not show specific binding of FSH. Each experiment was performed in duplicate, and values were determined for Kd, receptors/cell, EC50 for cAMP synthesis, and maximum cAMP level. After experiments were repeated 4–6 times, the statistical significance of each mutant data was analyzed by Student’s t test. The resulting p values are presented in the table section of the figure. ND, not detectable.
The exodomain of the FSH receptor was truncated by placing a stop codon after Asp341, and the truncated exodomain, Cys1-Asp341, was expressed in HEK 293 cells. Because the exodomain remains within the cells and is not secreted, the cells expressing it were solubilized in Nonidet P-40 and assayed for \( {\text{Kd}} \) value of slightly less than the \( {\text{Kd}} \) value of the wild type receptor solubilized in the detergent.

There are three possible differences in the truncated exodomain and the exodomain in the wild type receptor: the location, the tethering to a transmembrane domain, and the structure. The truncated exodomain is present within the cell and not attached to the membrane, whereas the exodomain of the wild type receptor is present on the cell surface and tethered to the membrane. To test whether these differences influenced the hormone binding affinity of the truncated exodomain, we used a hybrid FSH receptor in which the exodomain, Cys1-Asp341, was attached to the transmembrane and cytoplasmic domain of CD8. Cells transiently transfected with the hybrid or the wild type receptor were incubated with \( {\text{125I-FSH}} \) in the presence of increasing concentrations of unlabeled FSH. After washing the cells, the bound radioactivity was measured and analyzed for Scatchard plot (Fig. 2). The hybrid was not only expressed on the cell surface but also bound FSH on intact cells with a \( {\text{Kd}} \) value of 2.54 nM, which is \( 3 \times \) less than the \( {\text{Kd}} \) value of 8.3 nM of the wild type receptor on intact cells, indicating a \( 3 \times \) increase in the binding affinity of the hybrid. The result shows that the location and tethering to the membrane of the exodomain did not cause the difference in the binding affinities of the hybrid and the wild type receptor.

Next, we considered structural differences. The major difference between the hybrid and wild type receptor is the lack of the endodomain in the hybrid. It is possible that the endodomain may interact with the exodomain and influence FSH binding to the exodomain. Such an interaction is likely to occur at the extracellular surface of the endodomain, which is primarily comprised of exoloops. As a first step to identifying such a region, we decided to examine the shortest of the 3 exoloops, exoloop 3 of FSH Receptor 3. Exoloop 3 of the human FSH consists of 11 amino acids, Lys580, Val581, Pro582, Leu583, Thr586, Val589, Ser587, Lys588, Ala589, and Lys590. Ten of them except Ala589 were individually substituted with Ala to produce 10 substitution mutants, Lys580\( \rightarrow \)Ala, Val581\( \rightarrow \)Ala, Pro582\( \rightarrow \)Ala, Leu583\( \rightarrow \)Ala, Thr586\( \rightarrow \)Ala, Val589\( \rightarrow \)Ala, Ser587\( \rightarrow \)Ala, Lys588\( \rightarrow \)Ala, and Lys589\( \rightarrow \)Ala. In addition, Ala589 was substituted with Gly to produce the Ala589\( \rightarrow \)Gly mutant.

**Activities of Substitution Mutants**—Cells transfected with the Lys580\( \rightarrow \)Ala, Val581\( \rightarrow \)Ala, and Pro582\( \rightarrow \)Ala mutant receptor cDNAs were capable of binding \( {\text{125I-FSH}} \), indicating surface expression of the mutant receptors (Fig. 3). Their surface concentrations were 12,000–35,000 receptors/cell as compared with 77,000 receptors/cell for the wild type receptor. The \( {\text{Kd}} \) values of Lys580\( \rightarrow \)Ala and Pro582\( \rightarrow \)Ala were \( 50\% \) of the wild type receptor’s \( {\text{Kd}} \) value, indicating a \( 2 \times \) improvement in the hormone binding affinity. In contrast to the improved binding affinities, the cAMP level
produced by these two mutants was only 13–16% of the wild type value (Fig. 3C). These levels are also lower than the cAMP level of Val581Ala, which is 58% of the wild type value. These results suggest an inverse relationship between the binding affinity of and maximum cAMP level induced by Lys580Ala and Pro582Ala.

Next, Leu583Ala and Ile584Ala mutants were examined (Fig. 4). Ala substitution for Leu583 or Ile584 dramatically improved the hormone binding affinity by 4.4- or 5.7-fold, respectively. In contrast, the mutants were incapable of inducing cAMP. Interestingly, the maximum cAMP levels were not affected by the surface concentration of receptors when the receptors were transiently expressed at varying concentrations on the surface of HEK 293 cells (Table I). This is consistent with the fact that cAMP was maximally induced at the FSH concentration range of \(5–50 \text{ nM}\), which is below the FSH concentration (\(>50 \text{ nM}\)) necessary for the maximum (saturation) FSH binding of the wild type receptor (Fig. 4). Apparently,
the limiting factor(s) for cAMP induction is associated with G protein or adenyl cyclase.

Ala substitutions for the next three amino acids, Thr585 → Ala, Val586 → Ala, and Ser587 → Ala, did not significantly affect the hormone binding affinity but increased the EC50 values for cAMP induction by ~10-fold (Fig. 5). Despite the significant decrease in the affinity for cAMP induction, the mutant receptors produced FSH-induced cAMP at 22–85% of the wild type level. Substitutions for the last 3 amino acids of exoloop 3, Lys588 → Ala, Ala589 → Gly, and Lys590 → Ala, had mixed results (Fig. 6). Lys588 → Ala had no effect in the Kd value but negative effects on the EC50 value and maximum cAMP level. In the case of Ala589 → Gly, the binding affinity increased by >2-fold, whereas the potency of cAMP induction and maximum cAMP level decreased. On the other hand, Lys590 → Ala completely abolished the cAMP induction activity without affecting hormone binding.

Overall, all mutants bind hormone similar to, if not better than, the wild type receptor. In contrast to mutants’ lower or similar Kd values, the affinity and the maximum level of cAMP induction by all mutants were less than the values of the wild type. In addition, the basal cAMP level of these mutants were also below that of the wild type. One exception is the Ser587 → Ala mutant of which maximum and basal cAMP levels were similar to those of the wild type receptor. These results indicate the importance of exoloop 3 in cAMP induction and the negative effect of the substitutions on cAMP induction.

**Verification of Mutagenesis**—Our site-directed mutagenesis uses synthetic oligonucleotides containing a mutant sequence for each mutation and further, does not involve polymerase chain reaction. After mutagenesis, the mutant and flanking cDNA were cloned into the expression vector. Therefore, it is highly unlikely that a mutant cDNA might have undergone an unintended mutation(s) during the mutagenesis and subcloning. To confirm this mutation, cDNAs were reverted to the wild type cDNA, which, in turn, was used to transfect cells. All of the revertants behaved the same as the wild type receptor in surface expression, hormone binding, and cAMP induction (data not included), indicating that there was no mutations other than the intended substitutions.

**DISCUSSION**

**Relationship of Hormone Binding and cAMP Induction**—To readily compare various results of the hormone binding affinity and maximum cAMP level, each mutant’s percent values relative to the values of the wild type were generated as described in Fig. 7: the higher the percent values of Kd, the better the affinities for hormone binding. Ala substitutions for Lys588, Pro582, Leu583, and Ile584 augmented the binding affinity but attenuated the maximum cAMP level. Particularly, Leu583 → Ala samples revert to the wild type receptor.

**Table II**

| Binding | Max cAMP |
|---------|---------|
| FSH-R   | LH/CG-R |
| Kd %    | 100     |
| 100     | 100     |
| 143     | 13      |
| 58      | 95      |
| 92      | 16      |
| 128     | 0       |
| 133     | 74      |
| 131     | 83      |
| 70      | 22      |
| 101     | 83      |
| 335     | 54      |
| 106     | 37      |
| 168     | 0       |

**Fig. 7. Percent values for Kd and maximum cAMP level.** Percent Kd values for individual mutants were calculated by dividing the value for the wild type receptor with the corresponding values for each mutant. Significantly higher values are shown in solid columns and significantly lower values shown in open columns. Hatched columns represent those values that are not significantly different from the wild type value.

**Fig. 8. Computer model of exoloop 3.** A model was constructed in loop (left) and spacefill (right) structures as described under “Experimental Procedures.” Side chains of Lys580, Pro582, Leu583, and Ile584 are colored in red and the rest in gray.
Ala and Ile$^{584}$ → Ala substitutions increased the binding affinities by 4–6-fold, respectively, while completely abolishing cAMP induction. These results highlight an apparent inverse relationship between hormone binding and cAMP induction. This inverse relationship is independent of receptor concentrations and specific for the 4 residues. Therefore, they may have been compromised to preserve cAMP inducibility while maintaining a reasonably high binding affinity in the wild type receptor. If so, the Ala substitutions relieve the constraint and improve hormone binding while attenuating cAMP induction.

In contrast to the Leu$^{583}$ → Ala and Ile$^{584}$ → Ala substitutions, Lys$^{590}$ → Ala substitution abolished cAMP induction without impacting hormone binding. This suggests that the essential role of Lys$^{590}$ → Ala in cAMP induction differs from that of Leu$^{583}$ and Ile$^{584}$. On the other hand, Ala substitutions for the 4 amino acids in the Thr$^{585}$-Lys$^{588}$ sequence showed no or marginal effect on hormone binding and some effects on cAMP induction. These results suggest that the exoloop 3 residues are differentially impacted by Ala substitution and could be classified into four groups. Leu$^{583}$ → Ala and Ile$^{584}$ → Ala dramatically improved the binding affinity but abolished cAMP induction. Lys$^{586}$ → Ala and Pro$^{582}$ → Ala improved the binding affinity and attenuated the cAMP induction affinity and the maximum and basal cAMP levels. The rest of the substitutions had no significant effect on hormone binding. For example, Lys$^{590}$ → Ala abolished cAMP induction with no effect on hormone binding. Ala substitutions for Thr$^{585}$-Lys$^{588}$ had some effects on cAMP induction without significantly affecting hormone binding.

The results raise an interesting question: does exoloop 3 assume a structure more favorable for cAMP induction than hormone binding? For example, Leu$^{583}$ and Ile$^{584}$ are crucial for cAMP induction, yet along with Lys$^{586}$ and Pro$^{582}$, they appear to attenuate the hormone binding affinity. To help envision such a structure and to test whether the structure is possible, exoloop 3 was modeled (Fig. 8). Exoloop 3 was anchored to transmembrane domains 6 and 7 of bacteriorhodopsin (22). We focused on Lys$^{586}$, Pro$^{582}$, Leu$^{583}$, and Ile$^{584}$ for which Ala substitutions improved the binding affinity. These 4 residues appear on one side of exoloop 3, consistent with our experimental results.

**Differences between the FSH Receptor and LH/CG Receptor—**The FSH receptor and LH/CG receptor are closely related in their structure and function as are FSH and LH/CG. Exoloop 3 of both receptors are comprised of 11 amino acids, 8 of which are identical (Table II). Despite these similarities, the impact of certain Ala substitutions on the affinities of hormone binding and cAMP induction were significantly different. Leu$^{583}$ → Ala and Ile$^{584}$ → Ala enhanced the binding affinity by ~4–6-fold for the FSH receptor but the corresponding substitutions slightly changed the binding affinity for the LH/CG receptor (24). In addition, the same substitutions abolished cAMP induction for the FSH receptor but not for the LH/CG receptor. Conversely, Lys$^{586}$ → Ala did not impact the binding affinity of the FSH receptor, but the Ala substitution for the corresponding Asn in the LH/CG receptor enhanced the binding affinity by 3.6-fold. These results indicate the structural and functional differences of exoloop 3 of the two receptors. These differential effects seem to be specific, because other substitutions did not have them. For instance, Val$^{581}$ → Ala impacted similarly for both receptors. In addition, Lys$^{590}$ → Ala in both the FSH receptor and LH/CG receptor totally abolished cAMP induction without impacting hormone binding and surface expression.

**Conclusion—**Ala substitutional analyses indicate that exoloop 3 amino acids of the FSH receptor play crucial but differential roles in hormone binding and cAMP induction. Particularly, 4 N-terminal residues, Lys$^{580}$, Pro$^{581}$, Leu$^{583}$, and Ile$^{584}$, may be clustered at one side of exoloop 3 and are crucial for cAMP induction and constrain hormone binding to the exodomain. The mechanisms for these two essential receptor functions appear to work against each other. Therefore, hormone binding might be compromised to preserve cAMP inducibility while maintaining a reasonably high, but below maximum, binding affinity. In contrast to the 4 residues, other amino acids function differently, suggesting that the exoloop 3 residues assume a structure that differentially influences hormone binding and cAMP induction. Exoloop 3 of the FSH receptor and LH/CG receptor shares the same size and high sequence homology. However, their influences on hormone binding and cAMP induction are strikingly different, indicating the receptor specificity of exoloop 3.

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