The human follicle-stimulating hormone (FSH) receptor consists of two distinct domains of 330 amino acids, the N-terminal extracellular exodomain and membrane-associated endodomain including three exoloops and seven transmembrane helices. The exodomain binds the hormone with high affinity, and the resulting hormone/exodomain complex modulates the endodomain where receptor activation occurs. It has been an enigma whether the hormone interacts with the endodomain. In a step to address the question, exoloop 3 of 580KVPLITVSK590 was examined by Ala scan, multiple substitution, assays for hormone binding, cAMP and inositol phosphate (IP) induction, and photoaffinity labeling. We present the evidence for the interaction of FSH and exoloop 3. A peptide mimic of exoloop 3 specifically and saturably photoaffinity-labels FSH α but not FSH β. This is in contrast to photoaffinity labeling of FSH β by the peptide mimic of the N-terminal region of the receptor. Leu583 and Ile584 are crucial for the interaction of FSH and exoloop 3. Substitutions of these two residues enhanced the hormone binding affinity. This is due to the loss of the original side chains but not the introduction of new side chains. The Leu583 and Ile584 side chains appear to project in opposite directions. Ile584 appears to be so specific and to require flexibility and stereo specificity so that no other amino acids can fit into its place. Leu583 is less specific. The improvement in hormone binding by substitutions was offset by the severe impairment of signal generation of cAMP and/or inositol phosphate. For example, the Phe or Tyr substitution of Leu583 improved the hormone binding and cAMP induction but impaired IP induction. On the other hand, the substitutions for Ile584 and Lys580 abolished the cAMP and IP induction. Our results raise a logical question whether Leu583, Ile584, and Lys580 interact with the exodomain and/or the hormone. The answers will provide new insights into the mechanisms of hormone binding and signal generation.

The FSH receptor (FSHR) and other glycoprotein hormone (luteinizing hormone/chorionic gonadotropin and thyroid-stimulating hormone) receptors belong to a structurally unique subfamily of G protein-coupled receptors. Unlike other receptor subfamilies, they comprise two equal halves, an N-terminal extracellular half (exodomain) and a C-terminal membrane-associated half (endodomain) (14–4). The endodomain is —350 amino acids long and alone is capable of high affinity hormone binding (5–8) with hormone selectivity (9–11) but without hormone action (7, 12). Receptor activation occurs in the endodomain (13), which is structurally equivalent to the entire molecule of many other G protein-coupled receptors (14). Glycoprotein hormones initially bind to the exodomain, and then the resulting hormone/exodomain complex modulates the endodomain (13), which activates adenylyl cyclase (AC) to generate cAMP and phospholipase Cβ (PLCβ) to produce inositol phosphate and diacylglycerol. Therefore, the ternary interactions among the hormone, exodomain, and endodomain are crucial for successful signal generation. However, there is little information on the subject, particularly concerning the FSH receptor. Since the exodomain lacking the endodomain is capable of high affinity hormone binding (5–8, 15), the high affinity hormone binding appears to be independent of the endodomain. Contrary to this view, it has been reported that FSH and human chorionic gonadotropin binding to their cognate receptors is regulated by certain residues of exoloops 2 and 3 of the endodomain (15, 16). Furthermore, the hinge region of the exodomain interacts with exoloop 2 and modulates cAMP induction (17–19). These results suggest that the exodomain interacts with the exoloops and modulates them for signal generation. Yet it is unclear whether the hormone complexed with the exodomain also contacts the exoloops. In this study, we set out to investigate whether exoloops interact with the hormone at all. In a step toward this goal, we examined exoloop 3 of FSHR for its involvement in the activation of the two effectors and interaction with FSH. It is the shortest of the three exoloops, consisting of 11 amino acids, and has been implicated in the cAMP signal generation (15, 20, 21). Our observations show, for the first time, the interaction of exoloop 3 with FSH, in particular the FSH α subunit, the mode of this interaction, and its role in the AC and PLCβ activation.

EXPERIMENTAL PROCEDURES

Mutagenesis and Functional Expression of Human FSH Receptor—Each mutant human FSHR cDNA was prepared in a pSELECT vector using the nonPCR-based Altered Sites mutagenesis system (Promega), sequenced on a Beckman CEQ 2000XL capillary sequencer, and then cloned into pcDNA3 (Invitrogen) as described (22). After subcloning into pcDNA3, the mutant cDNAs were sequenced again. 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 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.

235F FSH Binding and Intracellular cAMP Assays—Human FSH (the National Hormone and Pituitary Program) was radioiodinated as de-
scribed previously for radioiodination of human chorionic gonadotropin (23). Denatured FSH was prepared by boiling in 8 M urea for 30 min. Stable cells were assayed for 125I-FSH binding in the presence of increasing concentrations of nonradioactive FSH. The $K_d$ values were determined by Scatchard plots. Truncated exodomain was solubilized in Nonidet P-40 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 10 μl of the cAMP assay buffer, which was provided by the manufacturer. cAMP concentrations were determined with an 125I-cAMP assay kit (Amersham Biosciences) following the manufacturer’s instructions and validated for use in our laboratory. Exoloop 3 of FSHR was modeled based on the crystal structure of rhodopsin as a template (24).

**Inositol Phosphate Assay**—Stable cells were plated in 12-well plates and grown in inositol-free Dulbecco’s modified Eagle’s medium (Atlanta Biologicals) supplemented with 8% heat-inactivated horse serum and 2 μCi/ml [3H]inositol (PerkinElmer Life Sciences) for 48 h to 40–50% confluency. After removing the medium, the cells were incubated in 1 ml of fresh wash buffer consisting of Dulbecco’s modified Eagle’s medium without inositol and 15 mM HEPES (pH 7.3) for 1 h at 37 °C. This medium was removed, and 0.3 ml of wash buffer containing 20 mM LiCl was added and incubated for 15 min at 37 °C. After the cells were stimulated with increasing concentrations of hormone for 30 min at 37 °C, the incubation was terminated by the removal of medium and the addition of 0.25 ml of 0.6 N HCl. The combined washes were treated with 0.9 ml of a mixture of chloroform:methanol (2:1), vortexed, and centrifuged at 1000 × g for 5 min at room temperature. The top aqueous layer, which was free of phospholipids, was removed, and the remaining chloroform layer was treated with 0.2 ml of methanol:water (1:1), vortexed, and centrifuged, as above. This aqueous layer was added to the previous aqueous layer, and the samples were dried in a vacuum concentrator. The dried samples were redissolved in 0.5 ml of 50 mM

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**Fig. 1.** Sequence alignment of exoloop 3. The exoloop 3 sequences of FSHR, luteinizing hormone receptor, and thyroid-stimulating hormone receptor were aligned among species. Identical residues are presented as dashed lines. LHR, luteinizing hormone receptor; TSHR, thyroid-stimulating hormone receptor.

**Fig. 2.** Effects of Ala substitutions on IP production, $K_d$, and cAMP induction. As shown in A, the exoloop 3 amino acids, $^{580}$KVPLTVSKAK$^{590}$, were individually substituted with Ala except the A588G substitution, and the mutant receptors were assayed for IP, IP$_1$, IP$_2$, and IP$_3$ as described under “Experimental Procedures.” As shown in B, the ratios of $K_d$wt/mut (blank bar), maximum cAMPmut/wt (gray bar), and IP$_3$mut/wt (black bar) of the mutants were presented in bars. The ratios above 1.0 indicate that the binding affinity of the mutants is better than the wild type affinity, and the maximum cAMP and IP levels of the mutants are higher than those of the wild type.

**Fig. 3.** Computer modeling of FSHR exoloop 3. FSHR exoloop 3 was modeled. A, stick model. As shown in B, all of the exoloop 3 residues except Val$^{581}$ and Pro$^{582}$, which are crucial for activation of PLCβ and production of IP, are presented in gold. C, Leu$^{583}$, Ile$^{584}$, and Lys$^{590}$, which are crucial for activation of AC and cAMP induction, are presented in blue. D, Leu$^{583}$ and Ile$^{584}$, which are crucial for hormone binding, are presented in red.
FIG. 4. Multiple substitutions of Leu<sup>583</sup>. Leu<sup>583</sup> was substituted with a panel of amino acids with various side chains. The mutant receptors were expressed in HEK 293 cells and assayed for <sup>125</sup>I-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 CPM are normally in the range of 1,400–500 CPM. Nontransfected cells did not show specific binding of FSH. Each experiment was performed in duplicate, and values were determined for K<sub>d</sub>, receptors/cell, EC<sub>50</sub> for cAMP synthesis, and maximum cAMP level. After experiments were repeated 6–10 times, the means and standard deviations were calculated. NS indicates not significant.
Table I

| IP 
| Leu 
| Tyr |
|---|---|---|---|---|---|---|
| L(wt) | 1,660 \(\pm\) 240 | CPM | Tyre  | 440 \(\pm\) 17 | Phe  | NS |
| Ala  | NS |  | Glu   | NS | Arg  | NS |
| del  | NS |  |  |  |  |  |

**Dermivatization and Radiiodination of Peptide**—A peptide mimic corresponding to the exoloop 3 sequence of 580KVPLITVSKAK590 (FSHR wt) was synthesized, to which a Tyr residue was attached to the C terminus for radiiodination. The N terminus of the peptide was acetylated, and the C terminus was amidated. NHS-ABG was synthesized as described previously (23) and freshly dissolved in dimethyl sulfoxide to a concentration of 50 mM and NHS-ABG was freshly dissolved in 0.1 \(\mu\)M sodium phosphate (pH 7.5) to a concentration of 20 mM. These reagent solutions were immediately used to derivatize receptor peptides. In the dark, 10 \(\mu\)l of NHS-ABG was added to 30 \(\mu\)l of receptor peptides in 40 \(\mu\)l of 0.1 \(\mu\)M sodium phosphate (pH 7.5). The mixture was incubated with NHS-ABG for 30 min at 25°C. The following were added to the derivatization mixture: 1 \(\mu\)Ci of Na\(^{125}\)Iodine in 10 \(\mu\)l of 0.1 \(\mu\)M NaOH and 7 \(\mu\)l of chloramine-T (1 mg/ml) in 10 mM Na\(_2\)HPO\(_4\), pH 7.4. After 20 s, 7 \(\mu\)l of sodium metabisulfite (2.5 mg/ml) in 10 mM Na\(_2\)HPO\(_4\), pH 7.4, was introduced to terminate radiiodination. Derivatized and radiiodinated ABG-125I-FSHR wt solution was mixed with 60 \(\mu\)l of 16% sucrose solution in PBS and fractionated on a Sephadex G-10 column (0.6 x 15 cm) using PBS. The following solutions were sequentially washed with 4.5 ml of H\(_2\)O and 4.5 ml of 60 mM ammonium formate and 5 mM sodium tetraborate—IP1, IP2, and IP3, which are conserved among species except L583A, I584A, and K590A, did not produce cAMP. Therefore, the activation of PLC\(\beta\) is more sensitive to Ala substitution than is the activation of AC and hormone binding. The results also show different mechanisms, in particular the sites, of the PLC\(\beta\) activation, AC activation, and hormone binding. We cannot, however, unequivocally dismiss the possibility that the lack of the IP induction was due to the limitation of the detectable IPs. To visualize the difference, exoloop 3 was computer-modeled (Fig. 3A). The results showed the contrasting topography of the sensitive residues for the signal generation and hormone binding. The residues crucial for the PLC\(\beta\) signal cover most of exoloop 3 except the N-terminal region (Fig. 3B). On the other hand, the residues sensitive to the AC signal are confined in the middle and C terminus of the exoloop (Fig. 3C). Leu583 and Ile584 are most sensitive to hormone binding and are located near the middle of the exoloop (Fig. 3D). Their side chains protrude in opposite directions. The sensitive residues appear to be accessible from one side of the exoloop, suggesting the intriguing possibility that they might be modulated from the side of the exoloop by the exodomain and/or the hormone. Particularly, Leu583 and Ile584 are sensitive to all of the three functions: hormone binding, PLC\(\beta\) activation, and AC activation. In addition to Leu583 and Ile584, Lys580 is important to the activation of PLC\(\beta\) and AC.

**Multiple Mutational Analysis and Specificity of Leu583, Ile584, Lys580, and Pro582** —The Ala substitution for Leu583 or Ile584 enhanced the hormone binding affinity by 2-3-fold but impaired signal generation for IP and cAMP. These two residues have large hydrophobic side chains, which are exposed on the surface according to the computer model (Fig. 3). Hydrophobic side chains are generally incompatible with surface exposure, especially to water molecules. However, surface exposure provides a hydrophobic contact site. To examine the glycoprotein hormone receptor family except 587SKA589 near the C terminus. The previous Ala scan has demonstrated that exoloop 3 constrains the hormone binding at the exodomain and plays a crucial role in cAMP induction (15). However, little is known about the mechanism or its role in IP induction. To address these questions, the Ala substitutions of individual residues, except for the A589G substitution, were stably expressed on HEK 293 cells. The cells were assayed for inositol phosphates, IP1, IP2, IP3, and IP4. Most of the mutants receptors, except the V581A, P582A substitutions, were incapable of inducing IP production in response to FSH (Fig. 2A). In contrast, V581A was capable of producing noticeable levels of IP1 and IP2, and an insignificant level of IP3. P582A produced a detectable level of IP1 but not IP2 and IP3. These results raise the question of whether the non-responding mutant receptors were expressed on the cell surface in this study, although they were in the previous study (15). Therefore, the cells stably transfected with the mutants were assayed for 125I-FSH binding as well as FSH-dependent CAMP production.

**Distinct Effects of Ala Substitutions on Hormone Binding, IP Induction, and cAMP Induction**—For easy comparison of the data, the ratios of \(K_d^{wt/mut}\) and \(K_d^{mut/wt}\), maximum IP \(t^{wt/mut}\), and maximum cAMP \(t^{mut/wt}\) were calculated (Fig. 2B). The results show that all of the cells bound the hormone, indicating the surface expression of the mutant receptors. Of interest is that the L583A and I584A mutations improved the hormone binding affinity by 2-3-fold. This is in striking contrast to the loss of IP induction by most of the mutants except the V581A and P582A mutants. On the other hand, the mutational effect is less severe on the activation of adenyl cyclase to produce cAMP. For example, most of the mutants were capable of producing some cAMP, although less than the wild type. The three mutants, L583A, I584A, and K590A, did not produce cAMP. Therefore, the activation of PLC\(\beta\) is more sensitive to Ala substitution than is the activation of AC and hormone binding. The results also show different mechanisms, in particular the sites, of the PLC\(\beta\) activation, AC activation, and hormone binding. We cannot, however, unequivocally dismiss the possibility that the lack of the IP induction was due to the limitation of the detectable IPs. To visualize the difference, exoloop 3 was computer-modeled (Fig. 3A). The results showed the contrasting topography of the sensitive residues for the signal generation and hormone binding. The residues crucial for the PLC\(\beta\) signal cover most of exoloop 3 except the N-terminal region (Fig. 3B). On the other hand, the residues sensitive to the AC signal are confined in the middle and C terminus of the exoloop (Fig. 3C). Leu583 and Ile584 are most sensitive to hormone binding and are located near the middle of the exoloop (Fig. 3D). Their side chains protrude in opposite directions. The sensitive residues appear to be accessible from one side of the exoloop, suggesting the intriguing possibility that they might be modulated from the side of the exoloop by the exodomain and/or the hormone. Particularly, Leu583 and Ile584 are sensitive to all of the three functions: hormone binding, PLC\(\beta\) activation, and AC activation. In addition to Leu583 and Ile584, Lys580 is important to the activation of PLC\(\beta\) and AC.

**Multiple Mutational Analysis and Specificity of Leu583, Ile584, Lys580, and Pro582**—The Ala substitution for Leu583 or Ile584 enhanced the hormone binding affinity by 2-3-fold but impaired signal generation for IP and cAMP. These two residues have large hydrophobic side chains, which are exposed on the surface according to the computer model (Fig. 3). Hydrophobic side chains are generally incompatible with surface exposure, especially to water molecules. However, surface exposure provides a hydrophobic contact site. To examine the
roles of the side chains, Leu\textsuperscript{583} and Ile\textsuperscript{584} were substituted with a panel of amino acids with various side chains: negative or positive, hydroxyl, neutral, ring or aliphatic groups. In addition, the residues were deleted in deletion mutants, which is helpful in assessing the effect of removing the original side chain without introducing a new side chain.

The first question raised was whether the improved binding affinity upon the Ala substitution was due to the introduction of the Ala side chain or loss of the original side chains. As shown in Fig. 4, A, B, D, and E, all of the substitutions of Leu\textsuperscript{583} decreased the \( K_d \) values, thus improving the binding affinity. Even when Leu\textsuperscript{583} was deleted, the affinity improved by more than 4-fold, suggesting that the loss of the Leu\textsuperscript{583} side chain contributes to the improved binding affinity. In contrast to the

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**Fig. 5. Multiple substitutions of Ile\textsuperscript{584}.** Ile\textsuperscript{584} was substituted with a panel of amino acids with various side chains, and the mutant receptors were expressed and assayed as described in the legend for Fig. 4. NS indicates not significant.
improvement in hormone binding, most of the mutants did not 
duce noticeable amounts of cAMP (Fig. 4C). Exceptions were 
the L583F and L583Y mutants that induced significant 
amounts of cAMP with reasonable EC50 values. Besides the 
L583F and L583Y mutants, the L583Q mutant induced a mar-
ginal level of cAMP (Fig. 4F). These results suggest the need for 
a specific group such as the Leu side chain or a ring group for 
the AC activation. When the Tyr, Phe, Ala, Glu, Arg, and 
deletion mutants were assayed for IPt production, only the 
L583Y mutant produced a small amount of IPt (Table I). This 
result suggests a similarity in the interactions to activate AC 
and PLCβ.
All substitutions for and deletion of Ile$^{584}$ decreased the $K_d$ values by up to 5-fold (Fig. 5). In contrast to this improved hormone binding, none of the mutants induced cAMP. Therefore, the deletion of Ile$^{584}$, not the introduction of new side chains, was likely responsible for the improved binding affinity. Furthermore, Ile$^{584}$ appears to be crucial for cAMP induction (Fig. 5C). To further test these hypotheses, the adjacent Pro$^{582}$ was substituted with various amino acids (Fig. 6). The mutational effect on hormone binding was diverse and less dramatic. Some mutations decreased the $K_d$ value, whereas others increased it. Several mutants were capable of inducing cAMP, whereas several others failed to induce the second messenger. Clearly, Pro$^{582}$ appears to play a role different from those of Leu$^{583}$ and Ile$^{584}$. In addition to Pro$^{582}$, Lys$^{590}$ was examined with multiple substitutions. Lys$^{590}$ is located at the far end of exoloop 3, at the boundary with the transmembrane 7. The $K_d$
values of the substituents varied widely from 1.6 to 50 nM as compared with the wild type value of 4 nM, whereas none of the mutants induced significant amounts of cAMP (Fig. 7). Therefore, Lys$^{590}$ is also essential for activation of AC. In addition, the mutants with Cys, Phe, Leu, Tyr, Ala, and Arg substitutions for Lys$^{590}$ were assayed for the production of IP$_1$, IP$_2$, IP$_3$, and IP$_4$. None of the mutants induced significant amounts of any IP species (data not included).

**Photoaffinity Labeling of FSH with Exoloop 3 Peptide—**To test the possible interaction of exoloop 3 with the hormone, 125I-ABG-FSHR$^{exo3}$ was incubated with FSH and irradiated with UV for increasing time periods. Samples were solubilized in SDS under the reducing conditions and electrophoresed as described under “Experimental Procedures.” After electrophoresis, the gel was dried and autoradiographed using phosphorimaging device. 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.

The two subunits of the human FSH preparation comigrate on SDS-PAGE. The band was not labeled when the sample was not irradiated with UV, suggesting the requirement for UV irradiation. The extent of the labeling was dependent on the irradiation time, reaching the maximum after 60 s of irradiation. The results show that the labeling is saturable. To determine the nature of the labeling, increasing concentrations of the hormone were labeled with a constant amount of 125I-ABG-FSHR$^{exo3}$ (Fig. 8B). Conversely, increasing concentrations of 125I-ABG-FSHR$^{exo3}$ were used to label a constant amount of FSH (Fig. 8C). The labeling plateaued under both conditions, indicating saturable labeling. To examine the relationship of the labeling with other exoloops and receptor peptide, FSH was incubated with 125I-ABG-FSHR$^{exo3}$ in the presence of increasing concentrations of unlabeled FSH peptides corresponding to exoloops 1, 2, and 3 as well as the N-terminal sequence Ser$^9$–Lys$^{40}$, FSHR$^{9–40}$, which is known to interact with FSH. Increasing concentrations of the peptides inhibited the photoaffinity labeling in a dose-dependent manner and eventually blocked the labeling with varying affinity (Fig. 9), suggesting a specificity. FSHR$^{590}$ is the most potent inhibitor, suggesting the possibility of its strong interaction with the hormone. Furthermore, 125I-ABG-FSHR$^{exo3}$ failed to label denatured FSH that does not bind to the receptor, despite high concentrations of the peptide (Fig. 10A), suggesting the specificity of the affinity labeling for biologically active FSH. FSH was denatured by boiling in 8 M urea for 30 min. To test whether the denatured FSH remained in solution, the mixture of radioactively labeled FSH and unlabeled FSH was denatured, varying volumes of the mixture were transferred to other tubes, and the radioactivity was counted. The transfer was quantitative with a 99–100% efficiency, indicating that denatured FSH was present in the photoaffinity labeling tube. 125I-ABG-FSHR$^{exo3}$ did not label urokinase, nor did it label phospholipases A, C, and D (Fig. 10B). In addition, it failed to noticeably label human growth hormone (Fig. 10B). The exoloop 3 peptide inhibited 125I-FSH binding to the receptor on intact cells in a dose-dependent manner. These results show that the binding to and labeling of FSH by the peptide were specific to bioactive FSH.

Since the α and β subunits of human FSH comigrate on SDS-PAGE, it is unclear which of the subunits was labeled. To determine the identity of the labeled subunit(s), FSH was labeled with 125I-ABG-FSHR$^{exo3}$, deglycosylated with PNGase F, and electrophoresed. The labeled band corresponded to the α subunit (Fig. 10B). Deglycosylated human FSH separates into two bands on SDS-PAGE, the higher molecular weight β subunit in the upper band and the smaller α subunit in the lower band, which was verified by monoclonal antisubunit antibodies.

**DISCUSSION**

Our results show that the exoloop 3 is crucially, yet differently, involved in hormone binding and induction of cAMP and IP. They show that FSHR exoloop 3 contacts the α subunit of FSH as part of the ternary complex consisting of FSH, the exodomain, and endodomain. Particularly, Leu$^{583}$ and Ile$^{584}$ are more important than other amino acids and project from one side of exoloop 3 in opposite directions. Interestingly, the substitutions of the two residues significantly improved the hormone binding, which is due to the loss of the original side chains rather than the introduction of the side chains from the substitutions. For example, all substitutions of Ile$^{584}$ with Tyr, Pro, Ala, Cys, Ser, Gln, Asp, Glu, and Arg enhanced the hormone binding affinity, as did the deletion of Ile$^{584}$ as shown by the $K_d$ values in Fig. 11A. To analyze the nature of the effect, the side chain hydrophobicity (27, 28) of substituting amino acids was plotted against the $K_d$ values. The plot showed...
two distinct groups, one consisting of ICAYQPS with a hydrophobicity/Kd coefficient of −1.03 and the other of PSEDR with a hydrophobic coefficient of 0.19 (Table II). The first group had a negative hydrophobic effect on the binding affinity, whereas the second had a positive hydrophobic effect, suggesting a complex, specific microenvironment and interaction of the Ile584 side chain. The interaction appears to be partly hydrophobic and may involve other specificity such as stereospecificity.

Substitutions of Pro582 show diverse results, independent of the hydrophobicity of the side chain. On the other hand, substitutions of Lys590 showed two distinct groups (Fig. 11B): the first group of FYQEDR with the most severe hydrophobicity/Kd coefficient of −8.1 and the second group of LCAPSEDR with a coefficient of −0.87 (Table II). In the first group, ring groups such as the phenyl and phenolic side chains of Phe and Tyr, respectively, severely impaired the binding affinity, considerably more than any substitutions of Leu583 and Ile584 did. The second group also negatively impacted the binding affinity, but the effects were mild. A striking difference of the two groups is the substitutions with Phe and Leu. The Kd value of FSHR<sup>K590F</sup> was 50 nM in contrast to 7.2 nM for FSHR<sup>K590L</sup>, raising a question of whether the side chain flexibility and geometry play a role. The deletion of Lys<sup>590</sup>, thus, likely relieves the constraint and improves the Kd value as shown by the deletion mutant.

All of the substitutions, except L583Y, diversely impaired the cAMP induction (Fig. 11C). This adverse impact was seen the least on the substitutions of Pro582. Among the various Leu583 mutants, only L583F and L583Y were capable of inducing cAMP production. This suggests that a hydrophobic side chain larger than a methyl group is necessary at the position, regardless of whether the side chain is aliphatic or aromatic. This is in contrast to the adverse effect of a ring group at Lys<sup>590</sup> on the hormone binding, clearly indicating the requirements for distinct groups at Leu<sup>583</sup> and Lys<sup>590</sup>. All other substitutions
of Leu<sup>583</sup> lead to insignificant cAMP induction. In addition, every substitution of Ile<sup>584</sup> and Lys<sup>590</sup> abolished cAMP, showing the irreplaceable nature of Ile<sup>584</sup> and Lys<sup>590</sup>. In particular, the fact that the K590R substitution failed to induce cAMP production raises the question of whether the positive charge of Lys<sup>590</sup> is necessary for cAMP induction. The substitutions of Lys<sup>590</sup> with Ala, Cys, Phe, Leu, Tyr, and Arg abrogated IP induction, suggesting the irreplaceable role of Lys<sup>590</sup> for both IP and cAMP induction. This is in contrast to the differential effects on cAMP and IP induction by Ala substitutions for the exoloop 3 amino acids, Lys<sup>580</sup>–Lys<sup>590</sup>.

In conclusion, our observations in this study show the interaction of the FSHR exoloop 3 with FSH, specifically the \( \alpha <sub>H9252</sub> \) subunit. This is consistent with the decade-long view that the common \( \alpha <sub>H9252</sub> \) subunit of the glycoprotein hormones is likely to induce the common hormone action, including cAMP induction (26). Leu<sup>583</sup> and Ile<sup>584</sup> are crucial for this interaction, in different ways. For example, Ile<sup>584</sup> appears to be so specific, requiring some flexibility and stereospecificity so that no other amino acids fit into its place. On the other hand, Leu<sup>583</sup> is less specific. Substitutions of these residues often enhanced the hormone binding affinity. However, these improvements in the hormone binding were offset by severe impairment of signal generations for cAMP and/or IP. For example, the Phe or Tyr substitution of Leu<sup>583</sup> improved the hormone binding and cAMP induction but impaired IP induction. On the other hand, substitutions for Ile<sup>584</sup> and Lys<sup>590</sup> abolished the cAMP and IP induction. These conflicting effects on the hormone binding and signal generation likely have constrained any further improvements in receptor functions. Our results open the next logical question of
whether Leu\textsuperscript{583}, Ile\textsuperscript{584}, and Lys\textsuperscript{590} interact with the exodomain and/or the hormone. The answers will provide new insights into the mechanisms of hormone binding and signal generation.

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