Transmembrane Regions V and VI of the Human Luteinizing Hormone Receptor Are Required for Constitutive Activation by a Mutation in the Third Intracellular Loop*

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Gonadotropin receptors are members of the seven-transmembrane (TM) receptor family. Several point mutations in TM V and VI and the intracellular loop 3 (i3) have been identified in the luteinizing hormone (LH) receptor gene, leading to constitutive activation of the receptor. Because gonadotropin receptors are highly conserved, we mutated the follicle-stimulating hormone (FSH) receptor at the corresponding amino acids. However, the FSH receptor mutants showed minimal increases in basal cAMP production. Taking advantage of this difference between the two receptors, we designed chimeric receptors with or without a point mutation in the i3 to identify the region in the LH receptor important for its constitutive activation. Introduction of the point mutation into chimeric receptors containing only TM V to VI from the LH receptor led to major increases in ligand-independent cAMP production. Furthermore, a chimeric receptor with only TM V and VI derived from the LH receptor can be rendered constitutively active by the mutation in the i3 from the FSH receptor. These results suggest that interactions between TM V and VI of the FSH receptor are essential for maintaining the receptor in the more constrained state, whereas interactions between these domains of the LH receptor are permissive for constitutively activating mutations in the i3.

The seven-TM1 protein-coupled receptors probably represent one of the largest gene family in eukaryotic organisms. Members of this superfamily are functionally diverse and include receptors ranging from the cAMP receptor in slime mold to mammalian neurotransmitter and glycoprotein hormone receptors (1–5). Agonist occupancy of these plasma membrane proteins is believed to result in conformational changes of the receptors, leading to the activation of different G proteins, which in turn modulate the activity of different effector enzymes and ion channels (6). Although much is known about ligand binding to the receptors and receptor activation of G proteins, the mechanism underlying ligand activation of the receptors is still unclear. Recently, constitutive activating mutations of these membrane proteins have been found: in rhodopsin (7, 8) leading to retinitis pigmentosa and congenital night blindness, in melanocyte-stimulating hormone receptor leading to different color coats (9), in parathyroid hormone (PTH)-PTH related protein receptor leading to Jansen-type metaphyseal chondrodysplasia (10), and in Ca2+-sensing receptor leading to familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism (11, 12). Because these point mutations of the receptors lead to ligand-independent activation of G proteins, they are potentially useful to elucidate the mechanism of receptor activation.

The gonadotropin (LH and FSH) and TSH receptors within the seven-TM G protein-coupled receptor family are unique in that they have unusually large extracellular domains as the ligand binding site and share high homology in their TM regions (3). Gain-of-function mutations have also been found for genes in this subfamily. In TSH receptor, mutations in several TM regions, the i3, and the extracellular loops 2 and 3 are associated with hyperfunctioning thyroid adenoma and congenital hyperthyroidism (13–16), whereas mutations in the TM V and VI and the i3 of the LH receptor lead to familial male precocious puberty (FMPP), which is inherited in an autosomal dominant, male-limited manner (17–24). Because FSH receptor shows high homology to LH and TSH receptors, we hypothesized that constitutive activation of the FSH receptor could be generated following similar mutations. Using site-directed mutagenesis, we mutated, in the FSH receptor, several conserved amino acids whose substitution led to constitutive activation of LH or TSH receptors. To our surprise, none of these mutations significantly increased the basal activity of the FSH receptor. Taking advantage of this difference between the homologous receptors, we designed chimeric FSH/LH receptors with or without a point mutation in the i3 to identify the region of the LH receptor that is important for its constitutive activation and to elucidate differences between domains of LH and FSH receptors responsible for constitutive activation.

EXPERIMENTAL PROCEDURES

Hormones and Reagents—Purified hCG (CR129) and FSH (I-3) were supplied by the National Hormone and Pituitary Program, NIDDK, NIH. Recombinant human FSH was prepared from the conditioned medium from Chinese hamster ovary cells stably transfected with both common α and FSHβ subunit genes (25).125I-Sodium was purchased from Amersham Corp.

Construction of cDNAs for Mutant FSH Receptors and Chimeric FSH/LH Receptors with or without a Point Mutation—Wild type human LH and FSH receptor cDNAs have been cloned and characterized (26, 27). To introduce the same amino acid substitutions found in LH

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The abbreviations used are: TM, transmembrane; LH, luteinizing hormone; FSH, follicle-stimulating hormone; G protein, GTP-binding protein; PCR, polymerase chain reaction; FMPP, familial male-limited precocious puberty; TSH, thyroid-stimulating hormone; WT, wild type; i3, intracellular loop 3; hCG, human chorionic gonadotropin; PBS, phosphate-buffered saline; PTH, parathyroid hormone.
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receptor of patients with FMPP into human FSH receptor cDNA, PCR-based mutagenesis was performed using overlapping primers containing mutated sequences as described previously (19, 24). To substitute Gly for Asp at residue 567 of the FSH receptor (corresponding to amino acid position 564 in the LH receptor), PCR was performed with deep VENT8 DNA polymerase (New England Bio Lab, Beverly, MA) using pCMEmFSHR as the template (19). The PCR fragment containing the mutated sequence was used to replace the corresponding region in pCMEmFSHRWT. In the same manner, three other mutant cDNAs (D581G, D581Y, and C584R) were constructed.

Different chimeric receptors were also constructed by PCR-based mutagenesis. The junctional sequences for each chimeric construct are shown in Table I. All of the cDNAs were subcloned into the expression vector pCDNA3 (Invitrogen, San Diego, CA) and purified twice using cesium chloride gradient ultracentrifugation, followed by phenol/chloroform extraction. DNA concentration and plasmid purity were estimated by reading optical density at 260/280 nm and confirmed using ethidium bromide staining following agarose gel electrophoresis. When PCR was used to generate plasmids, at least two clones derived from different PCR were prepared for each construct and used for expression studies.

Transfection of Cells and Analysis of Signal Transduction—293 cells derived from human embryonic kidney fibroblast were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (Life Technologies, Inc.) supplemented with 5% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Before transfection, 2 × 10⁶ cells were seeded in 10-cm dishes (Nunc, Naperville, IL). When cells were 70–80% confluent, the medium was replaced with Dulbecco's modified Eagle's medium (Life Technologies, Inc.), and transient transfection was performed using up to 12 μg of expression vector with or without cDNA inserts by the calcium phosphate precipitation method (29). After 12–16 h of incubation with the calcium phosphate-DNA precipitates, cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) to remove excess precipitates. To monitor transfection efficiency, 0.5 μg of RSV-beta-gal plasmid (30) was routinely included in the transfection mixture, and β-galactosidase activity in cell lysate was measured as described previously (31).

Forty-eight hours after transfection, cells were washed twice with PBS, harvested from culture dishes, and centrifuged at 400 × g for 5 min. Cell pellets were then resuspended in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 0.1% bovine serum albumin. 200,000 cells in 300 μl were placed on 24-well tissue culture plates (Costar, Cambridge, MA) and pre-incubated at 37°C for 30 min in the presence of 0.25 mM 3-isobutyl-1-methylxanthine (Sigma) before treatment with or without hormones for 1 h. To estimate basal cAMP production by cells transfected with increasing amounts of plasmid DNA, 293 cells (300,000 cells/well) were seeded in 12-well culture plates (Costar, Cambridge, MA) 1 day before transfection. Forty-eight hours after transfection with increasing amounts of plasmids (0.1–2 μg/well), cells were washed once with PBS and incubated for 90 min at 37°C in the presence of 0.25 mM 3-isobutyl-1-methyl xanthine (Sigma). At the end of incubation, cells and medium in each well were frozen and thawed once and then collected and boiled at 95°C for 3 min to inactivate phosphodiesterase activity. Total cAMP in each well was measured in triplicates by specific radioimmunoassay (32). All experiments were repeated at least three times using cells from independent transfections.

Ligand Binding Analysis—Purified FSH (I-3) and hCG (CR129) were iodinated by the lactoperoxidase method (33) and characterized by radioligand receptor assay using recombinant human gonadotropin receptors stably expressed in 293 cell line. Specific activity and maximal binding of the receptors were 100,000–150,000 cpm/ng and 40–50% for ¹²⁵I-hCG and 200,000–500,000 cpm/ng and 20–30% for ¹²⁵I-FSH, respectively. To estimate the ligand binding on cell surface, cells were washed twice with PBS and collected in PBS and centrifuged at 400 × g for 5 min. Pellets were resuspended in 0.1% bovine serum albumin/ PBS (assay buffer). For saturation analysis, 200,000 cells/300 μl were incubated with increasing amounts of ¹²⁵I-hCG or ¹²⁵I-FSH at room temperature for 18–22 h in the presence of or the absence of excess amount of unlabeled hCG (Pregnyl, 100 IU/tube) or unlabeled recombinant human FSH (2.5 IU/tube). At the end of incubation, cells were centrifuged, and radioactivities in the pellets were determined in a γ-counter. For displacement analysis, cells were incubated with increasing amounts of unlabeled hCG (0.1–100 ng/tube) or unlabeled purified FSH (0.1–50 ng/tube) in the presence of ¹²⁵I-hCG or ¹²⁵I-FSH (1 ng/tube). Data from both saturation and displacement analyses were used to derive equilibrium constant (Kd) values based on Scatchard plots (34). For all studies, CAMP production was normalized based on the number of expressed receptors derived from Scatchard plot analysis.

RESULTS

Point Mutations in the FSH Receptor Comparable with Those Found in LH Receptors of FMPP Patients Did Not Lead to Constitutive Activation—Earlier studies demonstrated that most point mutations found in patients with FMPP are present in the TM V to VI region of the LH receptor gene (Fig. 1, bold circles). When comparing the homology of LH and FSH receptors (Fig. 1), all the mutated amino acids were found to be conserved between these two receptors. To test the possibility that mutations found in LH receptor could also induce constitutive activation of the FSH receptor, we constructed mutant FSH receptors using PCR-based mutagenesis. The wild type (WT) and four different mutant FSH receptor (D567G, D581G, D581Y, and C584R) cDNAs were transiently transfected into 293 cells for analysis of FSH receptor ligand binding and cAMP responses.

As shown in Fig. 2B and Table I, Scatchard plot analysis indicated that the introduction of these mutations led to an increase in the affinity of the mutant receptors to ¹²⁵I-FSH. The total FSH binding sites on the cell surface were similar or
slightly decreased in the mutant receptors as compared with the WT receptor. The ratio between the WT and mutant receptors are 1.00 for WT, 0.81 ± 0.10 for D567G, 0.93 ± 0.15 for D581G, 1.12 ± 0.28 for D581Y, and 0.47 ± 0.04 for C584R (mean ± S.D., n = 3). We normalized the cAMP production by all receptors based on their expression levels from at least three separate experiments. As shown in Fig. 2A, FSH treatment stimulated major increases in cAMP production by these receptors. In contrast to the constitutive activation of LH receptor mutants (17, 19), there was no change in basal cAMP production per ng FSH bound by all four mutant FSH receptors as compared with cells expressing the WT receptor. When the basal levels of cAMP production in WT receptor was set at 100%, the mutant receptors were 103 ± 10% for D567G, 95 ± 15% for D581G, 107 ± 17% for D581Y, and 106 ± 21% for C584R (p > 0.05, mean ± S.D., n = 3). These basal levels were comparable with cells transfected with the vector plasmid without receptor cDNA insert.

**Mutant Receptors with the TM Region and Carboxyl Termi-**

nus or TM V to Carboxyl Terminus of FSH Receptor Replaced

**TABLE I**

| Binding to 125I-FSH (Kd) (mean ± S.D., n = 3) |
|-----------------------------------------------|
| Without mutation | D564G mutation |
|------------------|----------------|
| FSHR WT | 1.00 ± 0.13 | 1.05 ± 0.18 |
| FSHR D567G | 0.15 ± 0.02 | 0.25 ± 0.07 |
| FSHR D581G | 0.21 ± 0.05 | 0.32 ± 0.07 |
| FSHR D581Y | 0.32 ± 0.07 | 0.48 ± 0.19 |
| FSHR C584R | 1.00 ± 0.10 | 1.15 ± 0.22 |
| FL (V-VI)F  | 0.02 ± 0.05 | 0.13 ± 0.03 |
to the carboxyl terminus from the LH receptor (Fig. 3C) and incorporated the D567G mutation in this receptor. As shown in Fig. 3D and Table I, the receptor FL(V-C) showed similar affinity to 125I-FSH as compared with the wild type FSH receptor. However, introduction of the D567G mutation increased ligand binding affinity (Table I). Furthermore, the chimeric receptor, FL(V-C), was expressed at comparable level with that of the wild type FSH receptor, but the D567G mutant showed lower expression (61 ± 7% as compared with that of FL(V-C); n = 3). As shown in Fig. 3C, both FL(V-C) and its D567G mutant responded to FSH treatment with substantial increases in cAMP production. In addition, the incorporation of the D567G mutation into FL(V-C) led to constitutive activation of the receptor with 7.8 ± 0.7-fold increases in basal cAMP production as compared with FL(V-C) (n = 3).

A Mutant Receptor with TM V to TM VI from the LH Receptor Shown Constitutive Activation—We constructed a chimeric receptor containing only the TM V to VI (including the i3) from the LH receptor (FL(V-VI); Fig. 4A). As shown in Fig. 4B, the chimeric receptor had similar $K_d$ values to the WT FSH receptor, whereas incorporation of the D567G mutation increased its ligand binding affinity (Table I). The expression levels of FL(V-VI) were comparable with the WT FSH receptor, but the mutated receptor has lower expression (WT FSH receptor, 100%; FL(V-VI), 90 ± 8%; FL(V-VI)D567G, 27 ± 4%; n = 3). As shown in Fig. 4A, both FL(V-VI) and its D567G mutant responded to FSH treatment with increases in cAMP production. Again, the D567G mutant receptor showed constitutive activation as demonstrated by 10.5 ± 0.7-fold increases in basal cAMP production as compared with FL(V-VI) (p < 0.01, n = 6).

A Mutant Receptor with TM V to TM VI from FSH Receptor Did Not Show Constitutive Activation—To examine the role of TM V to VI region of the FSH receptor in receptor activation, we introduced this region into the LH receptor to construct LF(V-VI). We further mutated the conserved Asp to Gly at the
corresponding residue 564 (Fig. 5A). As shown in Fig. 5B, both chimeric receptors with or without the point mutation showed similar affinity to $^{125}$I-hCG, and these values were comparable with the WT LH receptor (Table I). The expression level of chimeric receptor, LF(V-VI)L, was comparable with that of WT LH receptor but lower when the point mutation was introduced (WT LH receptor, 100%; LF(V-VI)L, 105 ± 5%; LF(V-VI)L/D564G, 66 ± 6%; n = 3). As shown in Fig. 5A, LF(V-VI)L with or without the point mutation responded to hCG treatment with major increases in cAMP production. However, basal cAMP production did not show differences between these two receptors ($p > 0.05$, $n = 3$).

A Mutant Receptor Containing the i3 of the LH Receptor Was Not Constitutively Activated—Because the D567G mutation is present in the i3, we further tested if the mutated i3 of LH receptor alone was capable of activating the chimeric receptor. We constructed FL(i3)F and its D567G mutant (Fig. 6A). As shown in Fig. 6B, the chimeric receptor, FL(i3)F, showed comparable ligand binding affinity and cell surface expression to WT FSH receptor. When mutated, an increased affinity (Table I) and decreased receptor expression (28 ± 3% of FL(i3)F; $n = 3$) were observed. FSH treatment for both receptors increased cAMP production. However, the basal levels of both receptors were not different from cells transfected with vector alone ($p > 0.05$, $n = 3$) (Fig. 6A).

Mutant Chimeric Receptors Containing Either TM V to i3 or i3 to TM VI of the LH Receptor Showed Small Increases in Basal cAMP Production—As shown in Fig. 6C, we constructed chimeric receptors and their D567G mutants in which either TMV to i3 or i3 to TM VI of the FSH receptor was replaced with the corresponding LH receptor regions. Both chimeric receptors, FL(V-i3)F and FL(i3-VI)F, showed similar affinity and expression on cell surface comparable with WT FSH receptor. In contrast, chimeric receptors with the D567G mutation showed increased affinity (Fig. 6, D and F) and decreased cell surface expression as compared with their counterparts without the point mutation (30 ± 5% for FL(V-i3)/FD567G; 50 ± 10% for FL(i3-VI)/FD567G; $n = 3$). As shown in Fig. 6C (C and E), FSH treatment of these chimeric receptors increased cAMP production. Basal level of FL(V-i3)/FD567G showed a moderate increase in cAMP production (3.2 ± 0.3-fold, $n = 3$), whereas FL(i3-VI)/FD567G showed minimal constitutive activity (1.9 ± 0.1-fold; $n = 3$).
Minimal constitutive activation of mutant chimeric receptors containing i3 with or without TM V or TM VI from the LH receptor and the remaining parts from the FSH receptor. Mutagenesis and gene transfer experiments were performed to construct chimeric receptors and their D567G mutants. A, for FL(i3)F, the i3 of the FSH receptor is replaced by the corresponding LH receptor sequence. Basal and FSH-stimulated cAMP production by FL(i3)F and its D567G mutant were determined as described in the legend to Fig. 2. B, Scatchard plot analysis of chimeric FL(i3)F receptors showing an increase in binding affinity after introduction of the point mutation. C, for FL(V-i3)F, the TM V to i3 of the FSH receptor is replaced by the corresponding LH receptor sequence. Basal and FSH-stimulated cAMP production by transfected 293 cells were determined as described in the legend to Fig. 2. D, Scatchard plot analysis of chimeric FL(V-i3)F receptors showing an increase in binding affinity after introduction of the point mutation. E, for FL(i3-VI)F, the i3 to TM VI of the FSH receptor is replaced by the corresponding LH receptor sequence. Basal and FSH-stimulated cAMP production by FL(i3-VI)F and its D567G mutant were determined as described in the legend to Fig. 2. F, Scatchard plot analysis of chimeric FL(V-i3)F receptors showing an increase in binding affinity after introduction of the point mutation.
APoint Mutation in the Chimeric Receptor Containing TM V and VI of the LH Receptor but i3 from the FSH Receptor Led to Constitutive Activation—We further tested the hypothesis that a point mutation in the i3 of the FSH receptor may alter interactions between TM V and VI from the LH receptor but the flanking i3 and remaining parts from the FSH receptor. Basal and FSH-stimulated cAMP production by transfected 293 cells were determined as described in the legend to Fig. 2. B, Scatchard plot analysis of chimeric FL(V/VI)F receptor and its D567G mutant showing an increase in binding affinity after introduction of the point mutation.

Fig. 7. Constitutive activation of the mutant receptor FL(V/VI)FD567G with only TM V and TM VI from the LH receptor but the i3 and the remaining parts from the FSH receptor. A, mutagenesis and gene transfer experiments were performed to construct a chimeric receptor, FL(V/VI)F, and its D567G mutant with only the TM V and VI from the LH receptor but the flanking i3 and remaining parts from the FSH receptor. Basal and FSH-stimulated cAMP production by transfected 293 cells were determined as described in the legend to Fig. 7A. A Point Mutation in the Chimeric Receptor Containing TM V and VI of the LH Receptor but i3 from the FSH Receptor Led to Constitutive Activation—We further tested the hypothesis that a point mutation in the i3 of the FSH receptor may alter interactions between TM V and TM VI of the LH receptor. We constructed a chimeric receptor, FL(V/VI)F, in which TM V and VI regions are from LH receptor but the i3 and remaining regions from the FSH receptor (Fig. 7A). A point mutation D567G was also introduced into the i3 of the FSH receptor sequence. As shown in Fig. 7B, both chimeric receptors with or without the point mutation were expressed on the cell surface at similar levels and comparable with that of wild type FSH receptor (100% for WT FSH receptor; 100 ± 10% for FL(V/VI)F; 110 ± 10% for FL(V/VI)FD567G; mean ± S.D., n = 3). However, the D567G mutant showed higher affinity to 125I-FSH than FL(V/VI)F (Table I). A, as shown in Fig. 7A, FSH treatment stimulated cAMP production in both FL(V/VI)F and its D567G mutant. Of interest, basal cAMP production showed an increase after the introduction of the point mutation (3.6 ± 0.2-fold as compared with FL(V/VI)F; mean ± S.D., n = 3).

Transfection with Increasing Plasmid Concentrations Confirmed Constitutive Activation of FL(V-VI)F and FL(V/VI)F but Not FL(i3)F and WT FSH Receptors Containing the D567G Mutation—To confirm the findings of constitutive activation of the D567G mutation for FL(V-VI)F and FL(V/VI)F, we transfected 293 cells with increasing amounts of plasmids (0.1–2 µg/well) containing these constructs or those for mutated FL(i3)F and WT FSH receptors. Basal cAMP production for each construct was determined as described under “Experimental Procedures.” As shown in Fig. 8, only FL(V-VI)F and FL(V/VI)F with the D567G mutation showed dose-dependent increases in basal cAMP production, demonstrating these two receptors are indeed constitutively activated.

DISCUSSION

The present findings demonstrated that the introduction of single amino acid mutations in the i3 and TM VI of the human FSH receptor, unlike similar changes in the LH receptor (17, 19), did not lead to constitutive receptor activation. Studies using chimeric receptors further indicated that the extracellular region was important for ligand binding but not involved in receptor activation, whereas the TM V to VI region of the LH receptor was essential for constitutive activation. Although the i3 of adrenergic receptors has been shown to directly interact with the Gs protein (35–37), a chimeric gonadotropin receptor with the mutated i3 derived from the LH receptor and the remaining sequences derived from FSH receptor did not show constitutive activation. Instead, FSH receptor with the flanking TM V and VI regions replaced by the homologous sequences from the LH receptor, when combined with a mutation in the i3, showed ligand-independent increases in basal cAMP production. These data suggest that interactions between specific TM domains and not only the specific site of the point mutation in the i3 are responsible for constitutive activation of the LH receptor.

The chimeric receptor approach was originally used to study functional domains in the adrenergic receptors (38), and the i3 of several G protein-coupled receptors has been shown to be important for interaction with the Gs protein (38–42). The present use of the chimeric gonadotropin receptors to study receptor activation offers unique advantages. First, gonadotropin receptors, like TSH, PTH (10), and calcitonin (43) receptors, contain a large extracellular ligand binding domain. The ligand binding and signal transduction regions in gonadotropin receptors are interchangeable (44), thus allowing the genera-
tion of chimeric receptors with alterations in the signal-transducing TM domains without perturbation of ligand binding. In contrast, generation of chimeric receptors from receptors with short extracellular domains (e.g., adrenergic receptors) invariably leads to alterations in both signal transduction and ligand binding properties (38–42). Second, because both LH and FSH receptors are coupled to Gα protein, cAMP production can be used as a convenient end point to check if the chimeric receptors are functional. Third, chimeric receptor analysis of loss-of-function mutation is generally more difficult because construction of hybrid receptors sometimes disrupts optimal electrostatic interactions among different receptor regions. In contrast, the present analysis of gain-of-function mutations are more informative. Finally, the unique differential susceptibility of the homologous human LH and FSH receptors to constitutively activating mutations allows analysis of this phenomenon by chimeric receptors. Our results indicate that the chimeric gonadotropin receptors constructed here are efficiently synthesized, transported to the plasma membrane, recognized by appropriate ligands based on their extracellular sequence, and stimulated by specific ligands with increases in cAMP production. Following a point mutation of selected chimeric receptors, constitutive activation could be found.

The concept of constitutive or ligand-independent activation of G protein-coupled receptors was discovered in α1-adrenergic receptor following introduction of point mutations in the i3 (45). Recently, multiple naturally occurring point mutations have been found in different disease states (46–48). These include mutations of rhodopsin (7–8), melanocyt-stimulating hormone receptor (9), PTH-PTH related protein receptor (10), and Ca2+-sensing receptor (11–12). For LH and TSH receptors, activating mutations are also present in patients with familial male-limited precocious puberty and nonautoimmune hyperthyroidism, respectively (17–25). Of interest, several wild type receptors, including 5-hydroxytryptamineGC and dopamine D1B receptors, have been found to be constitutively activated in their native state (49, 50).

A recent study identified a heterozygous D567G mutation of the human FSH receptor in a hypophysecomized man showing normal testis volume after testosterone supplementation (51). Although a 50% increase in basal cAMP production was found in COS-7 cells transfected with the mutant cDNA, no ligand binding or normalization of cAMP data was performed. The magnitude of observed increases in basal cAMP was minor as compared with similar mutations in LH receptor (6–10-fold) (17, 19) or the present chimeric receptors (Fig. 4, −10-fold).

Constitutive activation of seven-TM receptor have been explained by allosteric ternary complex models in which the receptor exists in equilibrium between two conformational states. The inactive state is stabilized by negative antagonists, whereas the activated state is stabilized by agonists and interacts with G proteins (52, 53). For the α1b adrenergic receptor, systematic replacement with all possible amino acids of Ala293 in the i3 leads to varying degrees of receptor activation (54). For rhodopsin, a critical interhelical electrostatic interaction appears to explain constitutive activation of the mutation associated with retinitis pigmentosa and congenital night blindness. A point mutation in the TM VII of rhodopsin prevents the coherent attachment of the chromophore retinal and leads to constitutive activation of rhodopsin signaling and retinal degeneration. Retinal acts as an endogenous antagonist to stabilize the inactive conformation of this protein and a salt bridge between Lys296 in TM VII and Glu132 in TM III maintains the protein in a constrained state. Mutations at these sites or the neighboring Gly290 in TM I that break this salt bridge lead to constitutive receptor activation (7, 8).

The observation that mutations at multiple sites within the region extending from TM V through TM VI of the LH receptor can lead to constitutive activation suggests that these mutations may alter interactions between the i3 of the receptor and G protein by changing the position of TM V and TM VI relative to each other or to other TM domains. In the FSH receptor, movement of the TM V and TM VI may be more tightly constrained by stronger intramolecular interactions. A possible model explaining our findings is shown in Fig. 9. We propose that movement of TM V and TM VI relative to each other would likely alter the conformation of i3. As shown in Fig. 9A, interactions between TM V and TM VI as well as interactions between the amino and carboxyl-terminal ends of i3 maintain the LH receptor in an inactive state. These interactions can be overcome by agonist binding or by mutation of critical amino acids (Fig. 9B). In the FSH receptor the number of stabilizing interactions between TM V and TM VI is greater such that mutation of the amino acids in i3 (or TM VI) is not sufficient to alter the position of TM V relative to TM VI (Fig. 9, C and D). Thus, we might expect a chimeric receptor consisting of TM V and TM VI from the LH receptor and the remaining sequence from the FSH receptor would be more susceptible to a mutation in the i3 loop of the FSH receptor (Fig. 9, E and F). Due to the high homology between TM VI of LH and FSH receptors (83%;

![Fig. 9. Models depicting differences between LH and FSH receptors in their susceptibility to constitutive activation.](http://www.jbc.org/)

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**Fig. 9. Models depicting differences between LH and FSH receptors in their susceptibility to constitutive activation.** We hypothesize that differences in interactions between TM V and VI of gonadotropin receptors lead to their different susceptibility to constitutive activation by a point mutation in the i3. Stronger interactions (shown as black bars) between TM V and VI of the FSH receptor restrain it in an inactive state, whereas weaker interactions (shown as one pair of black bars) between TM V and VI of the LH receptor allow easy activation of the receptor following the i3 mutation. For the chimeric receptor, FL(V/V)F, with TM V and VI from the FSH receptor but the remaining parts (including i3) from the FSH receptor, a point mutation in i3 disrupts the weak interactions between the two TM domains of LH receptor.

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[Image of Fig. 9]
Constitutive Activation of Chimeric Gonadotropin Receptors

Fig. 1), the FL(V-33)F mutant behaves more like the LH receptor mutant compared with the mutant for more constrained FL(i-3-VII)F (Fig. 6). This model is consistent with a recent report showing that reciprocal point mutations among the α₁-, adrenergic receptor subtypes in TM V and VI are responsible for constitutive activation (55).

Studies using short peptides mimicking the i3 sequences of adrenergic receptors (35, 56–58) showed that these peptides may form a charged surface of an amphipathic α helix, which is important for the activation of G proteins. These peptides could compete for receptor binding to the G proteins or bind to the receptor itself, thus impeding receptor interaction with the G proteins. However, the exact nature of interactions between the receptors and G proteins is not clear. Although mutations in the TM V, i3, and TM VI of LH receptor lead to constitutive receptor activation in patients with FMPP, constitutive activating mutations can also arise from TM II (59). Similar activating mutations have been found in TM II and intracellular loop 1 of the melanocyte-stimulating hormone receptor (9), in TM III and VII of the TSH receptor, and in intracellular loop 1 of the PTH receptor (10). These findings suggest that intramolecular interactions between domains other than TM V and VI may be involved in maintaining the receptor in an inactive state. Moreover, it is possible that some constitutively activating mutations may not alter the tertiary structure of the receptor but exert a direct effect on the G protein.

Characterization of ligand binding properties of the present chimeric receptors indicated that all the constitutively activated receptors with a point mutation showed a higher binding affinity to the appropriate ligand as compared with their counterpart receptors without the point mutation. These findings are consistent with reports indicating high affinity for constitutively activated adrenergic receptors (45, 51, 52, 60, 61), suggesting conformational changes of receptor following introduction of the point mutation. Of interest, FSH receptors of the part receptors without the point mutation. These findings suggested that intramolecular interactions between domains other than TM V and VI may be involved in maintaining the receptor in an inactive state. Moreover, it is possible that some constitutively activating mutations may not alter the tertiary structure of the receptor but exert a direct effect on the G protein.

In conclusion, using chimeric receptors with or without a point mutation, we demonstrated that the TM V to VI region of human LH receptor is important for its constitutive activation and the corresponding domains of human FSH receptor might be more constrained and keep it in an inactive state. Future studies using chimeric gonadotropin receptors could provide useful information regarding the structural-functional relationship of seven-TM receptors in signal transduction.

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