Activation of the Luteinizing Hormone Receptor Following Substitution of Ser-277 with Selective Hydrophobic Residues in the Ectodomain Hinge Region*

Koji Nakabayashi, Masataka Kudo, Brian Kobilka‡, and Aaron J. W. Hsueh§

From the Division of Reproductive Biology, Department of Gynecology and Obstetrics and the ‡Department of Medicine, Stanford University School of Medicine, Stanford, California 94305-5317

Glycoprotein hormone receptors are G protein-coupled receptors with ligand-binding ectodomains consisting of leucine-rich repeats. The ectodomain is connected by a conserved cysteine-rich hinge region to the seven transmembrane (TM) region. Gain-of-function mutants of luteinizing hormone (LH) and thyroid-stimulating hormone receptors found in patients allowed identification of residues important for receptor activation. Based on structurally constitutively active mutations at Ser-281 in the hinge region of the thyroid-stimulating hormone receptor, we mutated the conserved serine in the LH (S277I) and follicle-stimulating hormone receptors (S273I) and observed increased basal cAMP production and ligand affinity by mutant receptors. For the LH receptor, conversion of Ser-277 to all natural amino acids led to varying degrees of receptor activation. Hydropathy index analysis indicated that substitution of neutral serine with selective nonpolar hydrophobic residues (Leu>Val>Met>Ile) confers constitutive receptor activation whereas serine deletion or substitution with charged Arg, Lys, or Asp led to defective receptor expression. Furthermore, mutation of the angular proline near Ser-273 to flexible Gly also led to receptor activation. The findings suggest the ectodomain of glycoprotein hormone receptors constrain the TM region. Point mutations in the hinge region of these proteins, or ligand binding to these receptors, could cause conformational changes in the TM region that result in Gα activation.

The large G protein-coupled receptor (GPCR) superfamily of proteins plays an important role in virtually every physiological process and represents the largest group of genes in nature, accounting for 1–2% of the entire human genome (1). These seven transmembrane (TM) proteins are capable of transducing messages as different as photons, organic odorants, nucleotides, nucleosides, peptides, lipids, and proteins. After ligand binding, GPCRs interact with a specific subset of heterotrimeric G-proteins that can in the activated forms inhibit or activate various effector enzymes and/or ion channels (2).

One subfamily of GPCRs has a large amino-terminal ectodomain containing leucine-rich repeats connected to the seven TM region. The leucine-rich repeat-containing G protein-coupled receptors (LGRs) consist of the classic gonadotropin and thyrotropin (TSH) receptors (3) together with the recently identified mammalian orphan receptors, LGR4 and LGR5/Fex/HG38 (4–6). Constitutively activated TSH and luteinizing hormone (LH) receptors have been identified in patients with nonimmune hyperthyroidism (7) and male-limited precocious puberty (8–10), respectively. These gain-of-function receptor mutants not only allow the understanding of the etiology of different pathological states but also provide the unique opportunity to investigate the molecular mechanisms by which GPCRs are activated.

The allosteric ternary complex model proposes the isomerization of GPCRs from an inactive to an active state capable of coupling to the G proteins (11). This isomerization involves conformational changes that may occur spontaneously or be induced by agonists or appropriate mutations, which abrogate the normal constraining function of the receptor, allowing it to ‘relax’ into the active conformation. Although the majority of gain-of-function mutations in LH and TSH receptors, like most other GPCRs, are present in the TM region (9, 12, 13), recent studies in patients with toxic thyroid nodules and congenital hyperthyroidism indicate that point mutations of a serine residue in the ectodomain of the TSH receptor lead to constitutive receptor activation (14–16). These findings are consistent with the hypothesis that the ectodomain of the glycoprotein hormone receptors could constrain the TM region.

Because the key serine residue in the hinge region between leucine-rich repeats and the TM region of the TSH receptor is conserved in the homologous LH and follicle stimulating hormone (FSH) receptors, we performed mutagenesis analysis to test the constitutive activation of LH and FSH receptors following serine substitutions. Taking advantage of the observation that the human LH receptor is more constrained than the TSH receptor, we further substituted Ser-277 of this receptor with all natural amino acids and demonstrated the importance of a nonpolar hydrophobic residue in this position for receptor activation. In addition, substitution of an angular proline immediately adjacent to Ser-277 with a flexible glycine but not a more rigid alanine also led to receptor activation.

EXPERIMENTAL PROCEDURES

Hormones and Reagents—Purified human CG (hCG, CR-129) was supplied by the National Hormone and Pituitary Program (NIDDK, National Institutes of Health, Bethesda, MD) and human recombinant FSH (Org32489) was from Organon (Oss, The Netherlands). Anti-FLAG M1 monoclonal antibody and FLAG peptide were purchased from...
**RESULTS**

**Substitution of the Conserved Serine Residue in the Hinge Region of Both LH and FSH Receptors Led to Constitutive Increases in Basal cAMP Production**—In addition to multiple gain-of-function mutations found in the TM regions of LH or TSH receptors, recent studies further indicated that replacement of Ser-281 in the human TSH receptor with isoleucine, threonine, or asparagine is associated with constitutive activation of the receptor, thereby leading to nonimmune hyperthyroidism (14–16). As shown in Fig. 1A, this serine is situated in a stretch of residues (YPSHCCAFXXN) conserved in all three glycoprotein hormone receptors. This conserved region is present in the hinge between the nine leucine-rich repeats (LRR) found in the ectodomain and the seven TM domain. Key cysteine residues are also shown.

![Fig. 1. Conservation of the hinge region between the ectodomain leucine-rich repeats and the seven TM domain in the three glycoprotein hormone receptors.](image)

**Fig. 1. Conservation of the hinge region between the ectodomain leucine-rich repeats and the seven TM domain in the three glycoprotein hormone receptors.** A, conservation of key residues in the hinge region of LH, FSH, and TSH receptors is shown in bold letters. Ser-281 of the TSH receptor (TSHR), together with the conserved serine in the LH and FSH receptors (LHR and FSHR), are boxed. Gain-of-function mutations found in the TSH receptor were found in patients with toxic thyroid nodules and congenital hyperthyroidism (14–16). Numbering of amino acid residues for each receptor is shown in parentheses. B, proposed secondary structure of the human LH receptor based on a model proposed for the TSH receptor (56). The hinge region is situated between the leucine-rich repeats (LRR) found in the ectodomain and the seven TM domain. Key cysteine residues are also shown.

A 125I-sodium iodine and myo-[3H]inositol were purchased from Amersham Pharmacia Biotec. Sigma. 125I-sodium iodine and myo-[3H]inositol were purchased from Amersham Pharmacia Biotec.

**Construction of Mutant Receptor cDNAs**—Polymerase chain reaction-based mutagenesis was performed to generate mutant LH and FSH receptor cDNAs as described previously (10) using cDNA encoding human LH receptor (17) or human FSH receptor (10). Polymerase chain reaction was performed with Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA) in accordance with manufacturer’s instructions. All cDNAs were subcloned into the expression vector pcDNA3 (Invitrogen Corp., Carlsbad, CA) and the plasmids were purified using the Maxi plasmid preparation kit (Qiagen, Inc., Valencia, CA). Fidelity of the polymerase chain reaction products was confirmed by sequencing on both strands of the final constructs before use in expression studies.

**Transfection of Cells and Analysis of Signal Transduction**—Human 293T cells derived from embryonic kidney fibroblasts were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Before transfection, cells (2 × 10^6/culture) were seeded in 10-cm dishes (Nalge, Nunc International, Naperville, IL). When cells were 70–80% confluent, transient transfection was performed with 10 μg of plasmid using the calcium phosphate precipitation method (18) following replacement of culture medium. Each well was transfected separately with different amounts of plasmid. To monitor transfection efficiency, 0.5 μg of pRL-SV40 (Promega, Madison, WI) was frequently included in the transfection mixture, and luciferase activity was measured in the cell lysate.

**Rous sarcoma virus-lacZ** transfections of 293T cells were performed as described previously (21) in accordance with manufacturer’s instructions. The Rous sarcoma virus-lacZ plasmid (21) was routinely included in the transfection mixture, and the plasmids were purified using the Maxi plasmid preparation kit (Qiagen, Inc., Valencia, CA). Fidelity of the polymerase chain reaction products was confirmed by sequencing on both strands of the final constructs before use in expression studies.

**Ligand Binding Analysis**—Human CG (CR-129) and recombinant human FSH were iodinated by the lactoperoxidase method (23) and the plasmids were purified using the Maxi plasmid preparation kit (Qiagen, Inc., Valencia, CA). Fidelity of the polymerase chain reaction products was confirmed by sequencing on both strands of the final constructs before use in expression studies.

**RESULTS**

**Substitution of the Conserved Serine Residue in the Hinge Region of Both LH and FSH Receptors Led to Constitutive Increases in Basal cAMP Production**—In addition to multiple
We further monitored cAMP production in cells transfected with plasmids encoding the wild-type FSH receptor or the FSH receptor hinge mutant. As shown in Fig. 2D, basal cAMP production was increased in cells that expressed increasing amounts of the S273I FSH receptor mutant but not the wild-type receptor. Also, treatment with increasing doses of FSH increased cAMP production in cells expressing the mutant FSH receptor. Scatchard plot analysis showed increased ligand affinity for the mutant FSH receptor S273I as compared with the wild-type FSH receptor. Bars indicate mean ± S.D.

**Fig. 2. Constitutive elevation of basal cAMP production and increased ligand affinity of mutant LH and FSH receptors following serine to isoleucine substitution in the hinge region.** A, basal cAMP production in cells transfected with increasing amounts of plasmids expressing wild-type LH receptor (LHR WT) or the S277I mutant. The constitutively activated LH receptor mutant (D564G) with a single amino acid alteration in the intracellular loop 3 is used for comparison. Results are expressed as cAMP production per culture containing cells transfected with different amounts of the expression plasmid. B, dose-dependent stimulation of cAMP production by hCG in cells expressing wild-type or mutant (S277I or D564G) LH receptors. Production of cAMP is normalized based on LH receptor content of the same cell preparation. C, Scatchard plot analysis showing increased ligand affinity of mutant LH receptors as compared with the wild-type receptor. D, basal cAMP production in cells transfected with increasing amounts of the plasmid expressing wild-type FSH receptor (FSHRWT) or the S273I mutant. Results are expressed as cAMP production per culture. E, dose-dependent stimulation of cAMP production by FSH in cells expressing wild-type (WT) or mutant (S273I) FSH receptors. Production of cAMP is normalized based on FSH receptor content of the same cell preparation. F, Scatchard plot analysis showing increased ligand affinity for the mutant FSH receptor S273I as compared with the wild-type FSH receptor. Bars indicate mean ± S.D.
further stimulated cAMP mediated by either the wild-type or mutant FSH receptor (Fig. 2E). Similar to the LH receptor mutants, Scatchard plot analysis indicated that the mutant FSH receptor exhibited increased affinity to its ligand as reflected by a decrease in the $K_d$ value (Fig. 2F, $K_d$ values: wild-type receptor, 666 pm; S273I mutant, 71 pm).

**Substitution of Ser-277 in the LH Receptor by All Natural Amino Acids Led to Different Degrees of Constitutive Activation:** Comparison with Hydropathy Index of Individual Residues—To elucidate the molecular basis of the constitutive activation of the LH receptor at residue 277, random mutagenesis was performed to change Ser-277 to each of all natural amino acids or to delete Ser-277. The deletion of Ser-277 or its substitution into charged Arg, Lys, or Asp resulted in defective expression of these mutants as reflected by negligible $125^I$-hCG binding and the lack of cAMP production in response to hCG by transfected cells. However, the remaining 16 mutants showed varying degrees of constitutive activation based on basal cAMP production by cells transfected with expression plasmids encoding each mutant construct (Fig. 3A). To correct for varying levels of receptor expression, the cAMP results were normalized based on cell surface $125^I$-hCG binding. When challenged with a saturating dose (100 ng/ml) of hCG, all but two highly constitutively activated mutant receptors (S277V and S277I) showed further increases in cAMP production (Fig. 3B). With the exception of the four most active mutants (S277L, S277V, S277M, and S277I), these mutant receptors showed maximal cAMP production within 2- to 3-fold of the levels found for the wild-type LH receptor. As shown in Table I, most mutant receptors also showed increased binding affinity to the hCG ligand as reflected by decreases in their $K_d$ values.

We plotted the degree of constitutive activation of different LH receptor mutants in relation to the hydropathy index (24) of the individual amino acid residue that was replaced. As shown in Fig. 3C, four mutants with a substitution of Ser-277 by residues with the highest hydropathy values (Leu, Val, Met, Ile) also showed the highest constitutive activity. We further subgrouped the receptor mutants into six categories based on a quantitative structure-activity relationship (QSAR) analysis (25) of the substituted residue. The variation in individual amino acid residue is described by three principal properties, z1, z2, and z3, derived from a principal component analysis of a matrix of 29 physicochemical variables for all amino acids.

<table>
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<th>$K_d$</th>
<th>Maximal binding</th>
<th>cAMP production</th>
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<tr>
<td>$nm$</td>
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<tr>
<td>LHR D578Y</td>
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*Significantly different from the wild-type receptor in $K_d$ values ($p < 0.05$).
DISCUSSION

Pathological studies in patients provided unique opportunities to investigate the molecular mechanisms of glycoprotein hormone receptor activation. Based on findings of gain-of-function mutants found in the hinge region of TSH receptors in hyperthyroid patients, we have established the importance of this region in the maintenance of an inactive conformation in all three glycoprotein hormone receptors for Gs coupling but not for IP turnover. For the LH receptor, substitution of Ser-277 with a subgroup of hydrophobic residues confers a constitutively active phenotype together with higher agonist-binding affinity. In addition, replacement of the neighboring Pro-276 to a flexible glycine also led to receptor activation. These findings highlight the importance of the hinge region in the maintenance of an inactive protein conformation in this subgroup of leucine-rich repeat-containing G protein-coupled receptors. It is likely that agonist binding, like hinge region mutations, could lead to changes in receptor conformation important for Gs activation.

The observed constitutive activity of various gain-of-function mutants of the LH receptor is closely related to their increased binding affinity to the labeled hCG ligand. Increased agonist-binding affinity is a common observation for constitutively activated GPCRs (29–31). Because Ser-277 is not situated within the leucine-rich repeats of the LH receptor that are postulated to be important for ligand binding, the observed enhancement in agonist binding likely reflects a conformational shift of the mutant receptor toward a more active state. Furthermore, substitution of an angular proline residue adjacent to Ser-277 of the LH receptor to a flexible glycine also led to constitutive receptor activation and higher agonist affinity, further confirming the importance of the conformation of the hinge region in the activation of the glycoprotein hormone receptors. It is also interesting to note that observed increases in basal cAMP production mediated by different LH and FSH receptor mutants were not accompanied by increases in PI turnover, consistent with an earlier report demonstrating that the activation of adenylate cyclase and phospholipase C by these receptors is mediated through different receptor conformational states (28).

Mutagenesis of Ser-277 to all possible natural amino acid residues further indicated that the most active gain-of-function mutants...
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A, serine in the hinge region of the LH receptor led to constitutive activation of function mutations have been found in patients for both TSH and LH receptors, few cases of constitutively activated mutations were identified for the FSH receptor. Indeed, studies using chimeric LH/FSH receptors have indicated that interactions between TM V and VI of the FSH receptor maintain this receptor in a more constrained state (10). As shown in Fig. 2, the hinge region mutation (S273I) in the FSH receptor does lead to constitutive activation; however, the basal cAMP production of the FSH receptor mutant is lower than that of the comparable mutation in the homologous LH receptor.

Although LH receptor mutants with Ser-277 substituted by Leu, Val, Met, or Ile residues showed the highest constitutive activity, it is interesting to note that only the Ser to Ile mutation of the TSH receptor was found in patients with toxic thyroid nodules. This is probably because of the fact that only one nucleotide alteration is involved for the Ser to Ile codon switch whereas substitution to the remaining three residues involves two nucleotide changes. Based on studies on LH receptor mutants, it is possible that additional gain-of-function mutations with substitution of these hydrophobic residues could be identified in the future in hyperthyroid patients. Although LH receptor mutations found in patients with male-limited precocious puberty have only been localized to the TM region, it is also of interest to screen for gain-of-function mutations in the hinge region of the LH receptor gene.

The GPCRs usually exist in an inactive state in the absence of ligands whereas ligand binding converts the receptor into an active conformation capable of coupling to the G protein. In the “allosteric ternary complex model,” this isomerization of receptor proteins involves conformational changes that may occur spontaneously or be induced by agonists or appropriate mutations that abrogate the normal constraining function of the receptor, allowing it to relax into the active conformation (34). It appears that at least three regions are important to maintain the LH receptor in an inactive state. Earlier studies analyzed two prominent LH receptor point mutations in greater detail. The Asp-578 side chain in the TM VI serves as a properly positioned hydrogen bond acceptor that is important for stabilizing the inactive state of the LH receptor. A bulky aromatic side chain at this position rather than the negative charge destabilizes the inactive receptor conformation (27). In addition, studies on the point mutant in Asp-546 of intracellular loop 3 indicated that a negative charge at this position might interact with an unidentified cationic residue to maintain receptor constraint (35, 36). The present study documents the amino-terminal hinge region as a third region important in maintaining all three glycoprotein hormone receptors in an inactive conformation. Most constitutively active mutants for GPCRs have been found in the TM domains or connecting loop sequences. To our knowledge, gain-of-function mutations in the amino-terminal sequences have only been observed in glycoprotein hormone receptors and calcium-sensing receptors (37).

Unlike the majority of GPCRs, the mammalian LGR subfamily of proteins, including glycoprotein hormone receptors and the orphan LGR4 and LGR5, all have large ectodomain-containing leucine-rich repeats presumably important for ligand binding. Of interest, a conserved hinge region between the leucine-rich repeats and the TM domain is also found in the completely sequenced genome of Caenorhabditis elegans. Although the nematode LGR showed constitutive activity, its TM region is mainly responsible for its activation (38). The conserved serine found in the hinge region of mammalian glycoprotein hormone receptors (VP5HCCAF) is substituted by a histidine in the worm receptor; however, similar substitution in the LH receptor did not lead to increases in basal cAMP production (Fig. 3A). It is interesting to note that the hinge region of two other known LGRs from lower species (fly LGR, HSEFHCCAF; sea anemone LGR, NGEFLCCEF) (39, 40), and the two mammalian orphan LGRs (LGR4, YAYQC- CAF; LGR5, YAYQCCAF) (4) are not conserved despite the preservation of two downstream cysteine residues. Future

FIG. 4. Mutation of the conserved proline immediately before serine in the hinge region of the LH receptor led to constitutive receptor activation and increased ligand affinity. A, basal and hCG (100 ng/ml)-stimulated cAMP production by the wild-type LH receptor (LHR WT) and two mutant LH receptors (P276G and P276A) was determined and normalized based on 125I-hCG binding to the same cell preparation. The S277I mutant was included for comparison. Bars indicate mean ± S.D. B, Scatchard plot analysis showing increased affinity of the mutant LH receptors as compared with the wild-type receptor.

belong to the same subgroup of amino acids with similar properties based on QSAR analysis (25). In this analysis, the variation in individual amino acid residue was described by three principal properties, z1, z2, and z3, derived from a principal components analysis of a matrix of 29 physicochemical variables for all amino acids. z1 is mainly related to hydrophilicity, z2 is additionally influenced by size and some hydrophobicity/hydrophilicity scales, and z3 contains information from the analysis of pK, pI, and 1H NMR values. A similar approach has been used to analyze the role of an aspartate residue in the amino-terminal sequence of the alpha1A-adrenergic receptor (32).

Among the three glycoprotein hormone receptors, we selected the LH receptor for extensive mutagenesis analysis based on its unique activation property. As reported earlier (33), the unliganded TSH receptor is less constrained than its homologs and more susceptible to activation by a wide spectrum of mutations. Following overexpression in transfected mammalian cells, the wild-type TSH (but not LH) receptor showed constitutive activity, thus rendering it difficult to analyze minimally active TSH receptor mutants. In contrast, the FSH receptor appears to be highly constrained. Although gain-of-function mutations have been found in patients for both TSH and LH receptors, few cases of constitutively activated mutations were identified for the FSH receptor. Indeed, studies using chimeric LH/FSH receptors have indicated that interactions between TM V and VI of the FSH receptor maintain this
studies are needed to investigate the importance of the hinge region for the activation of these LGRs.

At least three different but nonexclusive models could explain the activation of glycoprotein hormone receptors. First, based on studies on hinge mutants of gonadotropin receptors (present study) and the TSH receptor (15), one can propose that the large ectodomain of these proteins directly constrains the TM region, whereas ligand binding or hinge mutations relax the receptor (Fig. 6, R to R*). An earlier study proposed that the ectodomain of the LH receptor makes several contacts with the TM domain (41). Ligand binding to the curved portion of the U- or J-shaped ectodomain of the receptor leads to steric influences on the distances between the “arms” of the ectodomain of the receptor. Although the exact contact sites between the ectodomain and the TM region are still unclear, key residues in exoloops 2 and 3 have been shown to be involved in the modulation of ligand binding by the ectodomain of the LH and FSH receptor, respectively (42, 43). It is likely that ligand binding to the leucine-rich repeats in the ectodomain leads to conformational alterations in the hinge region, followed by the relaxation of the TM bundles leading to Gs activation.

Second, in the “two-step activation” model (44), agonist binding to the ectodomain brings the hormone close to the TM region. After binding mediated by the hormone-specific β-subunit, the common α-subunit directly interacts with the TM domain (45–47), leading to signal transduction (Fig. 6, AR*). This model is supported by the findings that a mutant LH receptor containing mainly the TM region can mediate hCG stimulation of signal transduction at extremely high ligand concentrations (48, 49). Modifications at key residues of the α-subunit of hCG (50, 51) and the first extracellular loop of the LH receptor also suggest direct counterionic interactions between the TM region and the receptor. Although this model does not take into account the hinge region in receptor activation, hinge mutations could independently cause conformational changes in the receptor resembling those induced by the α-subunit of hCG and the receptor. This model is supported by the findings that a mutant LH receptor containing mainly the TM region can mediate hCG stimulation of signal transduction at extremely high ligand concentrations (48, 49).

For several LH hinge mutants with the highest constitutive activity (Fig. 3), hCG treatment did not lead to further increases in cAMP production. For the more constrained FSH receptor, point mutation in the hinge region is less effective in inducing constitutive activation, thus allowing further increases in cAMP production induced by FSH. Thus, ligand interaction with the TM region could be important in eliciting and maintaining full signal transduction by the FSH receptor.

Finally, several studies have suggested the importance of...
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receptor dimerization or oligomerization in the signal transduction of different GPCRs (52), including the LH receptor (53–55). Ligand binding could confer a conformation that allows the receptors to form dimers. For the LH receptor hinge mutants, one could speculate that substitution of the conserved serine by hydrophobic residues also facilitates receptor dimerization. Future studies on the hinge region of the glycoprotein hormone receptors could elucidate their ligand signaling mechanisms and allow the design of small molecular weight molecules capable of modifying the conformation of these constrained proteins.

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REFERENCES

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