The Role of Asp? in Maintaining the Inactive Conformation of the Human Lutropin/Choriogonadotropin Receptor*

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Shinji Kosugi$, Toru Mori‡, and Andrew Shenker¶

From the §Department of Laboratory Medicine, Kyoto University School of Medicine, Kyoto 606-01, Japan, ¶Metabolic Diseases Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892, and ‡Division of Endocrinology, Department of Pediatrics, Northwestern University Medical School and Children's Memorial Hospital, Chicago, Illinois 60614

A constitutively activating mutation encoding Asp?→Gly in transmembrane helix 6 of the lutropin/choriogonadotropin receptor (LHR) is the most common cause of gonadotropin-independent, male-limited precocious puberty. This mutant LHR produces a 4.5-fold increase in basal cAMP when expressed in COS-7 cells. To better understand the normal role of Asp? in the LHR, we studied the effect of seven other amino acid substitutions at this position. No agonist binding or response was detected with the Asp?→Pro mutant. Agonist binding affinity was unaffected by the other substitutions and estimated receptor concentrations ranged from 11 to 184% of wild type. Substitution of Asp? with Asn, a similarly sized, uncharged residue, did not produce agonist-independent activation. In contrast, replacement with Glu, Ser, or Leu caused 4.9–5.5-fold stimulation of basal cAMP. Substitution with Tyr (8.5-fold) or Phe (7.5-fold) had a greater activating effect. Only the Tyr, Phe, and Leu mutants showed constitutive activation of the inositol phosphate pathway. Our data suggest that it is the ability of the Asp? side chain to serve as a properly positioned hydrogen bond acceptor, rather than its negative charge, that is important for stabilizing the inactive state of the LHR. A bulky aromatic side chain at position 578 may further destabilize the inactive receptor conformation.

The lutropin receptor (LHR) is a member of the family of G protein-coupled receptors (GPCRs) and its structure is predicted to consist of a large extracellular domain connected to a bundle of seven membrane-spanning α-helices (1, 2). Hormone binding to the extracellular domain triggers a conformational change in the transmembrane bundle that leads to G protein activation. We (3–5) and others (6–11) have described mutations of the LHR gene that promote agonist-independent receptor activation in familial and sporadic cases of gonadotropin-independent, male-limited precocious puberty (testotoxicosis). The Asp?→Gly mutation is the most common cause of testotoxicosis (3, 9). An Asp residue is found at this position in transmembrane helix 6 (TM 6) of all mammalian glycoprotein hormone receptors and of partially homologous invertebrate GPCRs (2, 12, 13), suggesting that it may play an evolutionarily conserved function in this group of receptors. According to the GPCR model developed by Baldwin (14) the side chain of Asp? is predicted to face toward the internal hydrophilic cleft, in position to form an electrostatic or hydrogen bond with one or more residues in another helix (3, 15).

Inactive receptors are thought to exist in a constrained conformation that is destabilized by the binding of agonist (16–18). The resulting conformational change allows cytoplasmic domains of the receptor, including portions of the third intracellular loop, to interact productively with G proteins. Some activating amino acid substitutions may mimic agonist occupancy by increasing the proportion of receptors that are in the active conformation (17). Characterization of such substitutions may provide insight into the nature of the inactive state and the normal mechanism of receptor activation by agonist.

Although many activating GPCR mutations have now been described, the molecular basis of the activating effects has only been explored in a few cases. In rhodopsin, loss or weakening of an electrostatic bond between TM 3 and TM 7 (Lys196) causes constitutive activation (19, 20), and the degree of activation is also inversely correlated with the size of the side chain at position 296 (19). For the α1B-adrenergic receptor, substitution of an Ala residue at the junction of the third intracellular loop and TM 6 with any one of 19 other amino acids is constitutively activating, but there is no obvious relationship between the level of activity and the size, charge, or hydrophobicity of the substituent (16).

To better understand the normal role of position 578 in maintaining the inactive receptor conformation of the LHR, we used site-directed mutagenesis to substitute 7 other amino acids with varying chemical properties for the wild-type (WT) Asp in the human LHR. The mutant receptors were transiently expressed in COS-7 cells, and human chorionic gonadotropin (hCG) binding, cAMP, and inositol phosphate production were measured in intact transfected cells.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis of the LHR—Human LHR cDNA (1) was inserted into the EcoRI site of the M13mp18 vector, and oligonucleotide-mediated site-directed mutagenesis was used to generate clones encoding the desired mutation (T7GEN kit; US Biochemical, Cleveland,
oh). Residue numbers were determined by counting from the methionine start site (1). WT and mutant clones were inserted into the EcoRI site of the SV-40-driven pSG5 vector (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing of the final construct, and plasmid DNA was purified by CsCl gradient ultracentrifugation.

Transfection and Assays—COS-7 cells (~10^5 cells) were transfected by electroporation (Bio-Rad) with 25 μg of purified plasmid DNA containing a mutant or WT LHR sequence. When smaller amounts of LHR DNA were used, the total amount of DNA per cuvette was kept constant by adding pSG5 vector DNA. After electroporation, each batch of transfected cells was divided into aliquots for binding, cAMP, and inositol phosphate assays. Cells intended for binding assays were suspended in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, and transferred to 6-well plates (~5 × 10^5 cells/well). Cells for cAMP and inositol phosphate assays were suspended in inositol-free medium supplemented with 10% fetal calf serum and 2.5 μCi/ml myo-[2-3H]inositol (DuPont NEN, Boston, MA) and were transferred to 24-well plates (~10^5 cells/well). 48 h after transfection, cells were washed with assay buffer (Hanks’ balanced salt solution containing 0.5% (w/v) crystalline bovine serum albumin and 20 mM HEPES-NaOH, pH 7.4).

125I-hCG binding was measured by incubating cells for 16 h at 4°C in 1 ml of assay buffer containing approximately 300,000 cpm of 125I-hCG (CR-127, 14,900 IU/mg, National Hormone and Pituitary Program; labeled to about 40 μCi/μg by Hazelton Washington, Vienna, VA) and 0–10^-7 M unlabeled hCG. cAMP and inositol phosphate production were measured concurrently by incubating cells for 1 h at 37°C in 0.2 ml of assay buffer containing 10 mM LiCl, 0.5 mM IBMX (3-isobutyl-1-methylxanthine), and 0–1000 ng/ml hCG. Perchloric acid was added to each well, samples were centrifuged, aliquots of supernatant were neutralized with KOH and HEPES, and total cAMP in each aliquot was determined by 125I radioimmunoassay (Eiken, Tokyo, Japan). Total inositol phosphates were measured using Dowex AG1-X8 anion exchange column chromatography (Bio-Rad). All assays were performed at least in triplicate, on at least three separate occasions with different batches of cells, and always included control cells transfected with WT LHR DNA. COS-7 cells transfected with pSG5 vector alone were not stimulated by hCG and did not exhibit specific 125I-hCG binding. The program LIGAND (21) was used to calculate Kd and Bmax values for hCG binding. Kd and EC50 values were log-transformed, averaged, and recomputed to calculate the geometric mean. The 95% confidence limits of Kd and EC50 were obtained by log transformation, calculating the mean ± 1.96 S.D., and reconversion. cAMP and inositol phosphate data are expressed as fold increase over basal in cells transfected with WT human LHR DNA (mean ± S.E., n ≥ 3 experiments). The density of live cells when the assays were performed varied ~10% between wells transfected with WT LHR and those transfected with mutant constructs.

RESULTS AND DISCUSSION

For electroporation of COS-7 cells 25 μg of human LHR DNA is routinely used (3, 4). To examine the effect of receptor density on cAMP and inositol phosphate responses, we tested different amounts of WT DNA; 25, 5, 1, and 0.2 μg/cuvette. Vector DNA was added to keep the total DNA amount constant (25 μg/cuvette). Receptor density estimated by 125I-hCG binding (Bmax) increased with increasing amounts of LHR DNA used for transfection, but there was no effect on hCG affinity (Fig. 1). The maximal agonist-stimulated cAMP production was proportional to estimated receptor density. At the lowest density (Bmax = 8%), hCG caused only a 1.5-fold increase in cAMP. The EC50 of the hCG-stimulated cAMP response (4 ng/ml) was not affected by receptor density. Cells transfected with WT human LHR exhibit increased production of inositol phosphates in response to high concentrations of hCG (4) and this response was also dependent on the density of cell surface receptors (Fig. 1B). Agonist-induced inositol phosphate production was barely detectable in cells with Bmax ≤ 25% of control.

Cells transfected with LHR DNA encoding Asp578→Pro (D578P) did not exhibit high affinity binding of 125I-hCG and hCG-induced cAMP or inositol phosphate production (Table 1 and Fig. 2C). This mutant LHR may never reach the cell surface or may exist in a conformation that is unable to bind hCG.

None of the other six amino acid substitutions at position 578 had a significant effect on the equilibrium dissociation constant (Kd) of LHR for the agonist hCG (Table I). This is consistent with data demonstrating that glycoprotein hormone binding

**Fig. 1. Binding and response of COS-7 cells transfected with various amounts of WT human LHR DNA.** A shows cAMP production, and B shows inositol phosphate production. Data are mean ± S.E. of at least three independent experiments. Basal levels of cAMP and inositol phosphates (IP) did not vary with the amount of LHR DNA used for transfection, and are equivalent to those in cells transfected with pSG5 vector alone. For the WT LHR (25 μg of DNA), Bmax was 5.3 ± 1.0 × 10^4 receptors/cell, basal cAMP was 1.7 ± 0.29 pmol/10^6 cells, and basal IP was 14.8 ± 29 pmol/10^6 cells.
occur primarily to the large N-terminal extracellular domain (2). The estimated surface concentrations of the mutant receptors, expressed as a percentage of the WT receptor (Table I). These data are consistent with earlier results obtained with the rat LHR (22).

Another amino acid residue that is capable of participating in a hydrogen bond is Ser. As shown in Fig. 2B, basal cAMP production by the Ser mutant (D578S) was increased 4.9-fold. Because D578S showed much higher expression than WT (Bmax = 184% of WT) we also transfected COS-7 cells with 10-fold less of the D578S DNA construct (2.5 μg) using our results with the WT LHR (Fig. 1) as a guide. This transfectant had a Bmax that was 65% of WT, but still exhibited a significantly elevated basal cAMP level (1.8-fold) (Table I, Fig. 2B). These results imply that a Ser residue at position 578 is unable to fully stabilize the inactive receptor conformation. This may be due to the fact that the Ser side chain is shorter than that of Asp or Asn. If one assumes that a Ser residue at position 578 is unable to fully stabilize the inactive receptor conformation, this may be due to the fact that the Ser side chain is shorter than that of Asp or Asn.

### Table I

Summary of activities of mutants involving Asp578 in human LHR

| Mutant | hCG binding | cAMP production | IP production |
|--------|--------------|-----------------|--------------|
|        | Kd | Bmax | +1 μg/ml hCG | EC50 | Basal | +1 μg/ml hCG |
| WT     | 4.1 (2.9–5.9) 100% WT | 1 | 13.36 ± 0.92 | 4.3 (2.9–4.6) | 1 | 4.71 ± 0.24 |
| D578G  | 2.7 (1.3–5.7) 56 ± 10 | 1.15 ± 0.11 | 11.19 ± 1.33 | 3.6 (2.5–5.2) | 0.97 ± 0.04 | 3.86 ± 0.45 |
| D578N  | 4.8 (4.1–5.7) 165 ± 28 | 5.05 ± 0.36 | 17.15 ± 1.72 | 6.3 (3.9–10.0) | 1.04 ± 0.05 | 4.14 ± 0.27 |
| D578E  | 3.6 (2.8–4.9) 50 ± 6 | 5.55 ± 0.48 | 6.41 ± 0.56 | ND | 1.40 ± 0.06 | 1.59 ± 0.06 |
| D578L  | 5.4 (1.1–11.7) 11 ± 4 | 4.93 ± 0.55 | 12.34 ± 1.51 | 2.2 (1.7–2.8) | 1.15 ± 0.03 | 6.42 ± 0.85 |
| D578S  | 4.8 (3.4–6.6) 184 ± 4 | 1.82 ± 0.02 | 5.93 ± 1.02 | 7.5 (4.8–11.0) | 0.98 ± 0.01 | 2.78 ± 0.03 |
| D578Y  | 1.9 (1.1–3.3) 65 ± 5 | 8.53 ± 0.71 | 13.07 ± 1.13 | 4.2 (2.9–6.1) | 1.93 ± 0.05 | 7.57 ± 0.02 |
| D578Y3* | 1.4 (0.9–2.0) 135 ± 5 | 7.33 ± 0.05 | 11.02 ± 0.07 | 2.0 (1.6–2.5) | 1.60 ± 0.02 | 3.32 ± 0.04 |
| D578P  | 2.4 (1.3–6.4) 65 ± 6 | 7.57 ± 0.39 | 12.38 ± 0.40 | 2.2 (1.8–2.7) | 1.47 ± 0.01 | 4.92 ± 0.29 |
| Non-detectable |

*Geometric mean (95% confidence limit) of at least three experiments.

Mean ± S.E. of at least three experiments.

WT Bmax averaged 5.3 ± 1.0 × 10^4 receptors per cell.

WT basal cAMP level averaged 1.74 ± 0.29 pmol/10^5 cells.

WT basal IP level averaged 148 ± 29 cpm/10^5 cells.

Non-detectable.

Parentheses after the mutant name, if shown, are the amount of DNA (μg per cuvette) used for transfection; otherwise 25 μg was used.

**Fig. 2.** Cyclic AMP production in COS-7 cells transfected with mutant and WT human LHR DNA. Data are mean ± S.E. of at least three independent experiments. Transfections were performed with 25 μg of DNA, except as noted for D578S(2.5) and D578Y(3). The WT basal cAMP was 1.74 ± 0.29 pmol/10^5 cells.
tions when judged on a “per receptor” basis.

The hydrophobic side chain of Leu is only slightly larger than that of Asp or Asn, but it lacks the ability to form a hydrogen bond. Although the density of mutant Leu receptors (D578L) estimated by $B_{max}$ was only 11% of WT (Table I), it was found to cause 5.6-fold stimulation of basal cAMP accumulation. Unlike the other mutant receptors, which showed maximal hCG-stimulated cAMP levels similar to that of WT (Fig. 2, A, B, and D), D578L was virtually unresponsive to agonist (Fig. 2C). This may be related to the markedly decreased concentration of D578L receptors on the surface (COS-7 cells with a similarly low concentration of WT receptors exhibit minimal response to agonist; see Fig. 1), or it may be due to an intrinsic difference in this mutant receptor (e.g. a conformation that is already maximally activated and/or inaccessible to agonist).

We (5) and others (9) recently identified a naturally occurring LHR mutation encoding the substitution of Asp578 with Tyr (D578Y) in three boys with unusually early and severe presentations of testotoxicosis. COS-7 cells expressing the Tyr mutant receptor exhibited an 8.5-fold increase in cAMP production in the absence of agonist (Fig. 2D), an effect that is significantly greater than that produced by any of the other Asp578 substitutions. To verify that this strong activation was an intrinsic property of the mutant receptor and not due at least in part to its relative overexpression (B$_{max}$ = 135% of WT), we also transfected COS-7 cells with only 3 mg of DNA, except as noted for D578S(2.5) and D578Y(3). The WT basal inositol phosphate was 148 ± 29 cpm/10^5 cells.

To investigate whether the bulky aromatic side chain of Tyr was responsible for the remarkably high level of basal activation, we made another mutant receptor with Phe at position 578 (D578F). This mutant receptor was found to be just as strongly activated (7.6-fold increase in basal cAMP) as D578Y (Fig. 1D).

Taken together, our data suggest that it is the ability of the Asp^578 side chain to serve as a properly positioned hydrogen bond acceptor, rather than its negative charge, that is normally important for stabilizing the inactive state of the LHR. We hypothesize that a hydrogen bond between Asp578 and its partner(s) is critical for maintaining the inactive conformation, and that loss or weakening of this bond increases the proportion of receptor molecules that become activated in the absence of agonist. Hydrogen bonds formed by the Asn carboxamide side chain can be as strong as those formed by the Asp carboxylate side chain (23). That the inactive state of the LHR is dependent on the geometry of the hydrogen bond formed by Asp^578 and its partner(s) is indicated by the fact that replacement of the Asp side chain with smaller (Ser) or larger (Glu, Tyr) polar side chains causes destabilization of the inactive conformation. It is important to note that substitutions at position 578 do not fully activate the LHR, and that mutations of different residues in TM6, TM5, and TM2 are also capable of promoting receptor activation (4–11). Bonds formed by Asp^578 may be part of a larger interhelical network involved in maintaining the inactive state.

Yyr and Phe are the most activating substitutions tested. In addition to loss of a hydrogen bond, introduction of a bulky aromatic side chain at position 578 may further destabilize the inactive receptor conformation by disrupting the packing of adjacent transmembrane helices. This contrasts with the data obtained on Lys^296 in TM7 of rhodopsin, where substitutions with smaller, “cavity-creating” residues were found to be especially activating (19).

Cells transfected with WT LHR not only produce cAMP in response to agonist, but have also been shown to exhibit increased production of inositol phosphates in response to high concentrations of agonist (4, 24) (Table I). The coupling of the LHR to this secondary signaling pathway is less efficient, and is more dependent on receptor density. Of the six substitutions that cause constitutive activation of the cAMP pathway, only the Leu, Tyr, and Phe mutants also cause constitutive activation of the inositol phosphate pathway, and the degree of stimulation (1.4–1.9-fold over WT basal) is less dramatic (Table I and Fig. 3). This may be due to differences in coupling effi-

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**FIG. 3.** Inositol phosphate production in COS-7 cells transfected with mutant and WT human LHR DNA. Data are mean ± S.E. of at least three independent experiments. Transfections were performed with 25 μg of DNA, except as noted for D578S(2.5) and D578Y(3). The WT basal inositol phosphate was 148 ± 29 cpm/10^5 cells.
ciency or to the fact that different receptor conformations are involved in activating the two pathways (25, 26).

In the human thyrotropin receptor (TSHR) the residue that corresponds to Asp<sup>578</sup> is Asp<sup>633</sup>. Two naturally occurring mutations of Asp<sup>633</sup> have been found in hyperfunctioning thyroid adenomas and shown to cause constitutive activation (27, 28). In contrast to our data on the LHR, the Asp<sup>633</sup>→Tyr TSHR mutant did not appear to possess a more strongly activating phenotype than Asp<sup>633</sup>→Glu or other TSHR mutations, nor did it cause constitutive activation of the inositol phosphate pathway (28). Despite extensive sequence similarity between these two receptors, the TSHR has been shown to differ significantly from the LHR in its level of spontaneous basal activity (29), and it may be that interhelical packing is less constrained in the TSHR than in the LHR.

Certain Asp and Glu residues have been shown to play key functional roles in bacteriorhodopsin (23, 30), sensory rhodopsin (31), rhodopsin (19, 32), and other GPCRs (14, 18, 22, 33). Changes in protonation can influence the equilibrium between conformational states. Replacement of Asp or Glu with similarly sized but uncharged residues (Asn and Gln, respectively), has often been used to test the importance of a potentially negatively charged side chain on receptor function. “ Genetic neutralization” of different ionizable residues has been shown to facilitate (19, 31, 32), impair (22, 33), or have no effect on (19, 31, 34) conformational signaling. In the case of the LHR and many other GPCRs, for example, it appears that a negative charge on the highly conserved Asp in TM 2 is needed to facilitate the conformational change to an active state (18, 22, 33). In contrast, substitution of Asp<sup>578</sup> in the LHR with Asn results in a receptor that functions exactly like the WT receptor (Fig. 2A). This suggests that a negative charge at position 578 is not necessary for stabilizing the inactive state, nor is it needed for the transition to the agonist-activated state.

In summary, the ability of the Asp<sup>578</sup> side chain to serve as a properly positioned interhelical hydrogen bond acceptor, rather than its negative charge, appears important for stabilizing the inactive state of the LHR. Studies are underway to identify those residues that may normally interact with Asp<sup>578</sup>. In addition to loss of a hydrogen bond, introduction of a bulky aromatic side chain at position 578 may further destabilize the inactive receptor conformation by disrupting the packing of adjacent transmembrane helices.

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