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# Chimeras of the Rat and Human FSH Receptors (FSHRs) Identify Residues that Permit or Suppress Transmembrane 6 Mutation-Induced Constitutive Activation of the FSHR via Rearrangements of Hydrophobic Interactions Between Helices 6 and 7

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**Although a large number of naturally occurring activating mutations of the human LH receptor (hLHR) and human TSH receptor (hTSHR) have been identified, only one activating mutation of the human FSH receptor (hFSHR) has been found. Furthermore, mutations of several residues within the i3/transmembrane domain (TM) 6 region of the hFSHR that were done based upon known constitutively activating mutations of the human LHR were found to have no effect on hFSHR signaling. One of the hFSHR mutations examined in this context was the substitution of a highly conserved aspartate (D581) in TM6 with glycine. We show herein that although the basal activity of the rat FSHR (rFSHR) is similar to the hFSHR, mutation of the comparable residue (D580) in the rFSHR causes marked constitutive activation. Taking advantage of the high degree of amino acid identity between the rat and human FSHRs, we have used chimeras and point substitutions to determine the precise residues that suppress or permit constitutive activity by the D580/581G mutation. Thus, the simultaneous substitution of M576 in TM6 and H615 in TM7 of the hFSHR with the cognate rFSHR residues (threonine and tyrosine, respectively) now renders the hFSHR(D581G) mutant constitutively active. Conversely, the substitution of Y614 of the rFSHR with the cognate hFSHR residue (histidine) fully suppresses the constitutive activity of the rFSHR (D580G) mutant. Computer models of the human and rat FSHRs and mutants thereof were created based upon the crystal structure of rhodopsin. These models suggest that differences in hydrophobic interactions between TMs 6 and 7 of the rat and human FSHRs may account for the ability of TM6 of the rat, but not human, FSHR to adopt an active conformation as a result of the D580/581G mutation. (*Molecular Endocrinology* 16: 1881–1892, 2002)**

THE FSH RECEPTOR (FSHR) is a member of the superfamily of G protein-coupled receptors (GPCRs). The C-terminal half of the FSHR is composed of the seven transmembrane (TM) regions prototypical of all GPCRs and contains highly conserved residues found in receptors related to rhodopsin and the $\beta$-adrenergic receptor. The N-terminal half is represented by an ectodomain composed largely of leucine-rich repeats that has been shown to be responsible for the high-affinity binding of FSH to the receptor (1). The FSHR is most closely related to two other glycoprotein hormone receptors, the LH receptor (LHR) and the TSH receptor (TSHR). These GPCRs also contain hormone-binding ectodomains composed of leucine-rich repeats (2). Recent studies suggest that the glycoprotein hormone receptors make up one of three subgroups of mammalian leucine-rich repeat-containing GPCRs (LGRs) (2, 3).

In response to the binding of FSH, the FSHR activates Gs, thereby increasing intracellular levels of cAMP. Under certain conditions, *i.e.* relatively high receptor density and high hormone concentrations, FSH can also be observed to stimulate the production of inositol phosphates (4). Whereas the physiological significance of the cAMP pathway in the actions of FSH is well established, it is still not clear what physiological role, if any, the stimulation of the inositol phosphate pathway may have.

The revised ternary model for GPCR activation predicts that a given receptor exists in the plasma membrane in equilibrium between inactive and active states (5). The activation of receptor by agonist is thought to occur as a result of the higher binding affinity of agonist for the active *vs.* the inactive conformation, thereby shifting the equilibrium toward the active state. Studies suggest that there may be multiple active conformations, some of which may be of

Abbreviations: FSHR, FSH receptor; GPCR, G protein-coupled receptor; h, human; LGR, leucine-rich repeat-containing GPCR; LHR, LH receptor; r, rat; TM, transmembrane; TSHR, TSH receptor.

an intermediate nature (5–10). Activation of G proteins by the GPCR may occur not only as a result of agonist binding, but also as a result of constitutively activating mutations. These mutations result in the stabilization of the GPCR in an active or partially active form. Under these circumstances, an increase in second messenger production is observed in cells expressing the constitutively active mutant even in the absence of any agonist. Investigations of mutation-induced constitutively active forms of GPCRs have proven to be a valuable approach toward understanding the mechanism of activation of GPCRs.

To date, only one naturally occurring constitutively active mutant of the human (h) FSHR, D567G, has been reported (11). In contrast, numerous naturally occurring constitutively active mutants of the hLHR and hTSHR have been described (see Refs. 12 and 13 for recent reviews). This disparity in the frequency of naturally occurring activating mutations between the glycoprotein hormone receptors may be attributed to the physiological ramifications of such mutations. For example, activating mutations of the hLHR are associated with gonadotropin-independent precocious puberty in boys, and activating mutations of the hTSHR have been found to be associated with nonautoimmune hyperthyroidism and with thyroid adenomas. The physiological consequences of an activating hFSHR would be predicted to be less clear in that activation of both the hLHR and hFSHR are required for the onset of puberty in females and for spermatogenesis in males. Therefore, the constitutive activation of only the hFSHR may be insufficient to cause a noticeable change in these physiological processes. Due to the observation that activating mutations of the hTSHR can cause thyroid adenomas, a possible link between activating hFSHR mutations and ovarian tumors has been examined, but no activating hFSHR mutations have been identified thus far from ovarian tumors (14, 15). The one naturally occurring activating mutation of the hFSHR that has been described was identified in the unusual circumstances of a hypophysectomized man in whom normal spermatogenesis was observed when testosterone only (as opposed to testosterone and FSH) was administered (11).

It has also been suggested that the hFSHR may be less susceptible to constitutive activation than the hLHR and hTSHR. This hypothesis derives from observations that certain activating mutations of the hLHR, when introduced into the homologous residues, do not cause activation of the hFSHR (16). One of these mutations corresponds to an activating D578G substitution in TM6 of the hLHR. In contrast to the hLHR (17), substitution of the comparable residue was shown to have no effect on the basal activity of the hFSHR (16). In the context of other studies, though, we observed that the same substitution caused constitutive activation in the rat (r) FSHR. The difference in the ability of the human *vs.* the rFSHRs to be stabilized in a constitutively active conformation by this mutation in TM6 seems rather remarkable given the high degree of amino acid identity between the two species of receptor. Using chimeras and point substitutions exchanging residues between the two receptors, we sought to determine the specific residues within the rFSHR that permitted constitutive activation by the D580/581G[1] substitution in TM6 and what interactions in the hFSHR suppressed constitutive activation by the comparable substitution. Our results identify two residues, one in TM6 and one in TM7, that determine whether or not the D580/581G mutation stabilizes an active conformation of the FSHR. Computer modeling of the human and rat FSHRs suggests a role for hydrophobic interactions between TMs 6 and 7 in determining the potential activation of the FSHR by the D580/581G mutation.

## RESULTS AND DISCUSSION

### Constitutive Activity of the Rat, but Not the Human, FSHR by a D580/581G Mutation

As shown in Fig. 1, the conserved aspartate in TM6 that is the focus of this study is residue 581 in the hFSHR and 580 in the rFSHR. HEK 293 cells were transiently transfected with varying concentrations of plasmids encoding the wild-type rFSHR or wild-type hFSHR. As shown in Fig. 2A, the hFSHR is expressed at much lower levels than the rFSHR. The introduction of a D580/581G mutation further decreased the cell surface expression of both the rat and human FSHRs (Fig. 2A). Although the cell surface expression of the hFSHR and the D580/581G mutants thereof were decreased significantly as compared with the wild-type rFSHR, they remained detectable, thereby permitting the analysis of their functional properties. When basal cAMP was determined over a wide range of receptor levels, the constitutive activity of rFSHR(D580G) was readily apparent (Fig. 2B). An expanded view of the basal levels of cAMP in cells expressing relatively low levels of receptor, as seen with the hFSHR, is shown in Fig. 2C. These data show that even at very low receptor densities, the constitutive activity of the rFSHR (D580G) remains evident. Confirming earlier studies by Hsueh and colleagues (16), these data also show that substitution of D581 in TM6 of the hFSHR with glycine does not cause constitutive activation of the hFSHR. For all further experiments, the cells were deliberately transfected with submaximal concentrations of plasmids encoding the rFSHR and rFSHR(D580G) constructs to match the receptor levels with cells expressing hFSHR and hFSHR(D581G). Data compiled from a number of experiments comparing the wild-type and D580/581G forms of the rat and human FSHR are shown in Table 1. These data demonstrate that the identical D580/581G substitution in the rat *vs.* human

[1] D580/581G is used herein to designate the mutation of the conserved aspartate that corresponds to D580 in the rFSHR and D581 in the hFSHR.

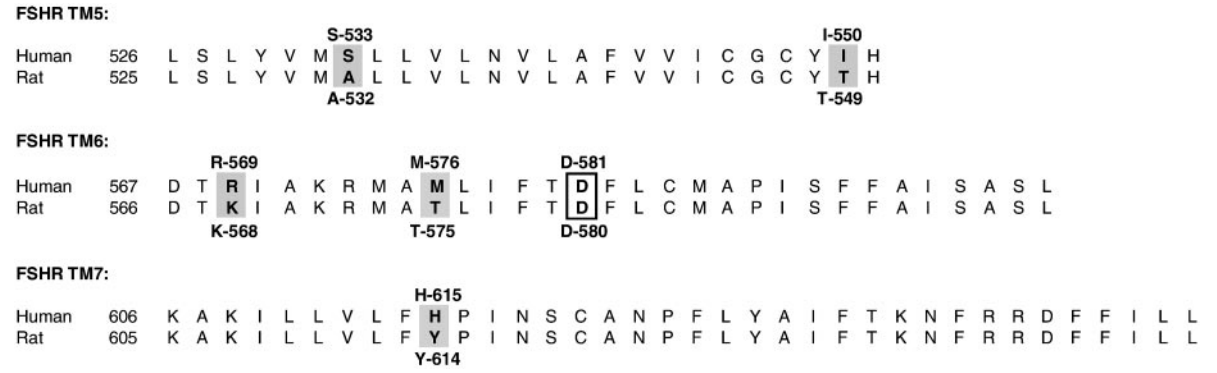


**Fig. 1.** Sequence Alignments of TM Domains 5, 6, and 7 of the Rat and Human FSHRs

The residues that are in *bold and shaded* are those that are nonidentical between the rat and human FSHRs. The conserved aspartate in TM6 that was mutated to glycine as a test for constitutive activation is noted by the *boxed area*. The assignment of the TM regions was based on the assignments of the TM domains of rhodopsin (19) and conform to the alignment of the hTSHR with rhodopsin as predicted by Ballesteros *et al*. (24).

FSHR has distinctly different effects with regard to stabilizing the receptor in a constitutively active state.

### Effects of the TM Domains *vs.* the Ectodomains on the Ability of the D580/581G Mutation to Cause Constitutive Activation

The following sets of experiments were performed to determine the molecular interactions in the rat and the human FSHRs that permit or suppress, respectively, constitutive activation of the receptor by the D580/581G substitution. First, we sought to determine whether the receptor ectodomain has any effect on the ability of the FSHR to be constitutively activated by this TM6 mutation. Toward this end, chimeric receptors were constructed in which the ectodomain of one FSHR species was grafted onto the serpentine region of the other FSHR species. In addition, chimeric receptors were prepared in which the ectodomain of one FSHR species was grafted onto the serpentine region of the other species of FSHR containing the TM6 D580/581G mutation. As shown in Fig. 3, the hFSHR containing the D580/581G mutation was not constitutively active even when its serpentine region was fused to the ectodomain of the rFSHR. Similarly, the rFSHR containing the D580/581G mutation remained constitutively active even when its serpentine region was fused to the ectodomain of the hFSHR. Therefore, the rFSHR ectodomain does not permit constitutive activation of the D580/581G substitution in the hFSHR serpentine domain. Nor does the hFSHR ectodomain suppress constitutive activation of the D580/581G substitution in the rFSHR serpentine domain. These data conclusively demonstrate that the FSHR ectodomain has no influence on whether or not constitutive activity is observed by the D580/581G mutation. Although this finding was not entirely unexpected, there have been a few instances where the integrity of the ectodomain was required to observe constitutive activity induced by a mutation within the serpentine region of a GPCR (18).

### Point Replacements to Identify TM Residues That Permit or Suppress Constitutive Activity by the D580/581G Mutation

The data presented thus far suggest that residues permitting constitutive activation of the rFSHR(D580G) and suppressing constitutive activation of the hFSHR(D581G) must be within the serpentine regions of each receptor. Technically, the serpentine portion of the FSHR is composed of the seven TM domains as well as three interconnecting extracellular loops, three interconnecting intracellular loops, and the cytoplasmic tail. However, because G580/581 is predicted to be in the midportion of TM6, interactions with other transmembrane residues are more likely to be responsible for this effect. Of the seven TM domains, TMs 1, 2, and 4 were not examined given their more distal location relative to TM6 as predicted by the crystal structure of rhodopsin (19). TM3 was also not examined because the amino acid sequences of the rat and human TM3 FSHR sequences are identical. Therefore, we focused on the potential roles of TM domains 5, 6, and 7 in permitting or suppressing constitutive activation by the D580/581G mutation. The amino acid sequences for the rat and human FSHR sequences in these TM domains are shown in Fig. 1. In one set of experiments the hFSHR(D581G) mutant, which is not constitutively active, was used as a template, and selective mutations within TM domains 5, 6 and/or 7 that inserted one or more rat FSHR residues in place of hFSHR residues were introduced. It was then ascertained whether any of these substitutions now permitted constitutive activation of hFSHR(D581G). Conversely, in another set of experiments, the rFSHR(D580G) mutant, which is constitutively active, was used as a template, and mutations were made that substituted one or more rat receptor residues in TM domains 5, 6 and/or 7 with human receptor residues. In this case, it was then determined whether any of these substitutions suppressed the constitutive activation of the rFSHR(D580G) mutant.

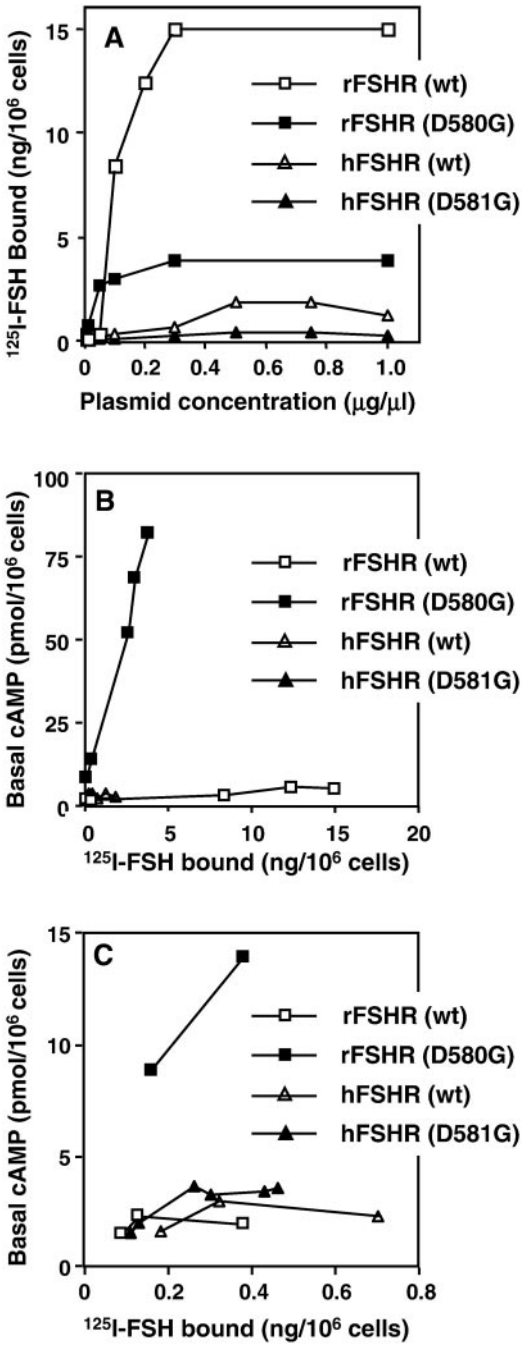


**Fig. 2.** Constitutive Activity of rFSHR(D580G) as Compared with hFSHR(D581G)

293 Cells were transiently transfected with varying concentrations of FSHR plasmids. Cell surface binding of $^{125}$I-FSH and basal levels of cAMP were then determined. A, Cell surface $^{125}$I-FSH binding as a function of plasmid concentration is depicted. The plasmid concentration noted is that added to the transfection cocktail. Under these conditions, a 1 $\mu$g/$\mu$l plasmid concentration results in 4 $\mu$g plasmid added per 35-mm well. B, Basal cAMP as a function of the full range of receptor numbers obtained as in panel A are shown. C, A magnification of the data from panel B is shown to depict the data at the lower levels of receptor expression as observed for the rFSHR and rFSHR(D580G). Results of one experiment representative of three independent experiments are shown.

**Table 1.** Constitutive Activity of rFSHR(D580G), But Not hFSHR(D581G)

| Receptor | Cell Surface Binding (ng/10$^6$ cells) | Basal cAMP (pmol/10$^6$ cells) |
|---|---|---|
| hFSHR (wt) | 1.06 ± 0.15 | 2.55 ± 0.43 |
| hFSHR(D581G) | 0.86 ± 0.15 | 3.87 ± 0.94 (1.5-fold increase over wt) |
| rFSHR (wt) | 2.73 ± 0.53 | 4.09 ± 0.89 |
| rFSHR(D580G) | 2.53 ± 0.51 | 40.0 ± 8.4 (9.8-fold increase over wt) |

293 cells were transiently transfected with the FSHR constructs listed and assayed within the same experiment for cell surface $^{125}$I-hFSH binding as well as basal cAMP accumulation. Data shown are the mean ± SEM of nine experiments.

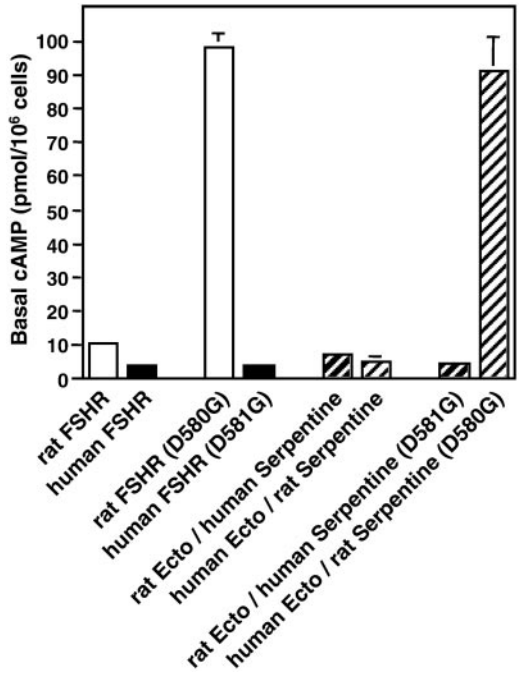


**Fig. 3.** Chimeras of the Rat and Human FSHRs Demonstrate That the Serpentine Regions Confer the Ability to Suppress or Permit Constitutive Activation by the Mutation in TM6

Chimeras of the rat and human FSHRs were constructed in which the ectodomain (Ecto) of one receptor was fused to the serpentine region of the other receptor. 293 cells transiently expressing comparable numbers of cell surface receptors were assayed for basal cAMP levels. Data shown are the mean ± SEM of triplicate determinations within one experiment representative of three independent experiments. The absence of an error bar indicates that the SEM was too small to be observed graphically.

There are only two divergent amino acids in the predicted TM5 regions of the rat and human FSHRs (Fig. 1). In the context of the wild-type hFSHR, the replacement of these two residues individually with the respective rFSHR residues had no effect on basal cAMP levels (Fig. 4). When done in the context of the

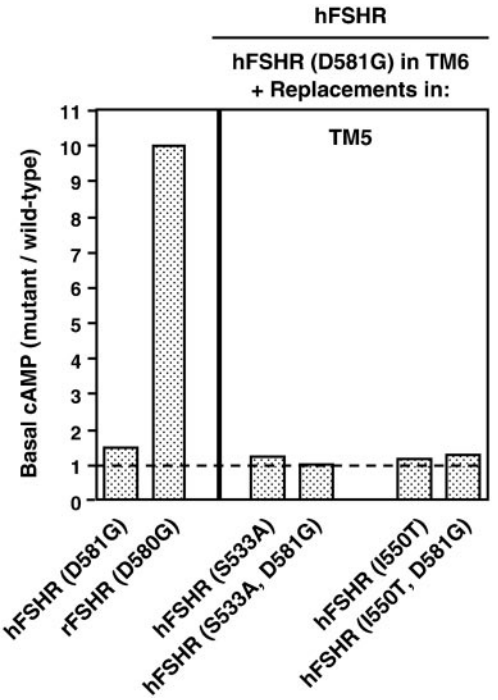


**Fig. 4.** Substitution of TM5 of the hFSHR with Residues from the rFSHR Does Not Permit Constitutive Activation of the hFSHR Containing the TM6 D581G Mutation

The two divergent amino acids in TM5 of the hFSHR (see Fig. 1) were individually replaced with the corresponding residues of the rFSHR in the context of the wild-type hFSHR and the hFSHR(D581G) mutant. 293 cells transiently expressing comparable numbers of cell surface wild-type and mutant FSHRs were assayed for basal cAMP levels. Basal cAMP levels are expressed as the ratio of the cAMP in cells expressing the mutant receptor relative to those expressing the wild-type receptor of a given species. Therefore, a value of 1.0 (shown by the *dotted line*) would be obtained if the mutant receptor behaved identically as the wild-type receptor of the same species. The basal levels of cAMP in rFSHR(D580G) and hFSHR(D581G) also expressed relative to the wild-type form of each receptor (calculated from the data shown in Table 1) are included for comparison. Data shown are the mean ± SEM of four to six experiments. The absence of an error bar indicates that the SEM was too small to be observed graphically.

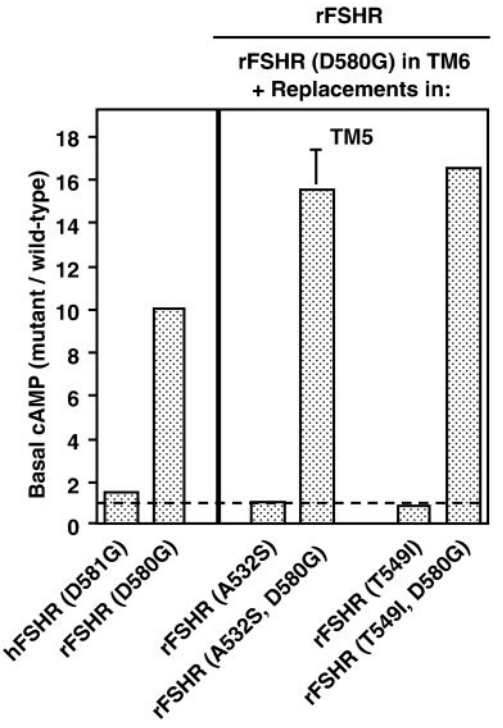


**Fig. 5.** Substitution of TM5 of the rFSHR with Residues from the hFSHR Does Not Suppress Constitutive Activation of the rFSHR Containing the TM6 D580G Mutation

The two divergent amino acids in TM5 of the rFSHR (see Fig. 1) were individually replaced with the corresponding residues of the hFSHR in the context of the wild-type rFSHR and the rFSHR (D580G) mutant. 293 cells transiently expressing comparable numbers of cell surface wild-type and mutant FSHRs were assayed for basal and FSH-stimulated cAMP. Basal cAMP levels are expressed as the ratio of the cAMP in cells expressing the mutant receptor relative to those expressing the wild-type receptor of a given species. Therefore, a value of 1.0 (shown by the *dotted line*) would be obtained if the mutant receptor behaved identically as the wild-type receptor of the same species. The basal levels of cAMP in rFSHR(D580G) and hFSHR(D581G) also expressed relative to the wild-type form of each receptor (calculated from the data shown in Table 1) are included for comparison. Data shown are the mean ± SEM of four to eight experiments. The absence of an error bar indicates that the SEM was too small to be observed graphically.

hFSHR(D581G) mutant, the replacement of each of these hFSHR TM5 residues with rFSHR residues also had no effect on basal cAMP levels. Unfortunately, it was not possible to examine the combined effects of S533A and I550T substitutions on hFSHR(D581G) because the triply substituted receptor was not expressed at the cell surface.

The converse experiment was then performed in which individual substitutions of the TM5 residues of the wild-type rFSHR were replaced with hFSHR. As shown in Fig. 5, these substitutions, when made individually, did not suppress the constitutive activation caused by the D580/581G substitution in the rFSHR. It was not possible to study the effects of both TM5 substitutions simultaneously in the context of the rFSHR(D580G) mutant because of the lack of cell surface expression of the triply substituted mutant. Taken altogether, the data shown in Figs. 4 and 5 suggest that the residues in TM5 are not involved (as examined individually) in suppressing the constitutive activity of the D580/581G mutation in the hFSHR or permitting the constitutive activity of the D580/581G mutation in the rFSHR.

Our attention then turned to TM domains 6 and 7. TM6 has two divergent amino acids between the rat and human FSHR sequences (Fig. 1). However, because R569 of the hFSHR is represented by K568 of the rFSHR, thus conserving the positive charge, we chose to focus initially on the M576 of the hFSHR *vs.* T575 of the rFSHR.

Within TM7 there is only one divergent residue between the two receptors, H615 in the hFSHR *vs.* Y614 in the rFSHR. First, we asked whether substitutions of TM6 or 7 of the hFSHR with rFSHR residues permit constitutive activation of hFSHR(D581G). As shown in Fig. 6, individual M576T or H615Y substitutions in the wild-type hFSHR were without effect. Each of these substitutions, when placed in the context of the hFSHR(D581G) mutant, caused slight (statistically insignificant) increases in basal levels of cAMP. However, when both TM6 and TM7 of hFSHR(D581G) were converted to rFSHR sequences by the combined mutations of M576T and H615Y, a substantial (>8-fold) constitutive activation of hFSHR(D581G) was observed (Fig. 6). These results show that whereas the D580/581G mutation in TM6 of the hFSHR does not cause constitutive activation, the simultaneous substitutions of M576 in TM6 and H615 in TM7 with the corresponding rFSHR residues threonine and tyrosine, respectively, permits constitutive activation. These data suggest that M576 and H615 residues in the hFSHR constrain the hFSHR from adopting an active conformation in response to the D580/581G mutation and/or that the rFSHR T575 and Y614 residues stabilize the D580/581G mutant of the hFSHR in an active conformation.

The converse experiment was then performed in which we asked whether substitutions of TMs 6 or 7 of the rFSHR with hFSHR residues suppress the constitutive activation of the rFSHR(D580G) mutant. Based upon the above results with the hFSHR (Fig. 6), we had predicted that neither a T575M nor a Y614H mutation alone would suppress the constitutive activity of rFSHR(D580G), but that together they would cause suppression. As shown in Fig. 7, this was not observed. Rather, the substitution of T575 in TM6 of the rFSHR with methionine enhances the constitutive activity of rFSHR(D580G). However, the substitution of Y614 in TM7 of the rFSHR with histidine fully suppresses the constitutive activity of rFSHR(D580G). The simultaneous mutations of rFSHR T575M and Y614H had a similar suppressive effect on rFSHR(D580G) as did Y614H alone. These data demonstrate that the conversion of TM7 of the rFSHR to the hFSHR sequence is sufficient to prevent the ability of rFSHR(D580G) to assume a constitutively active conformation.

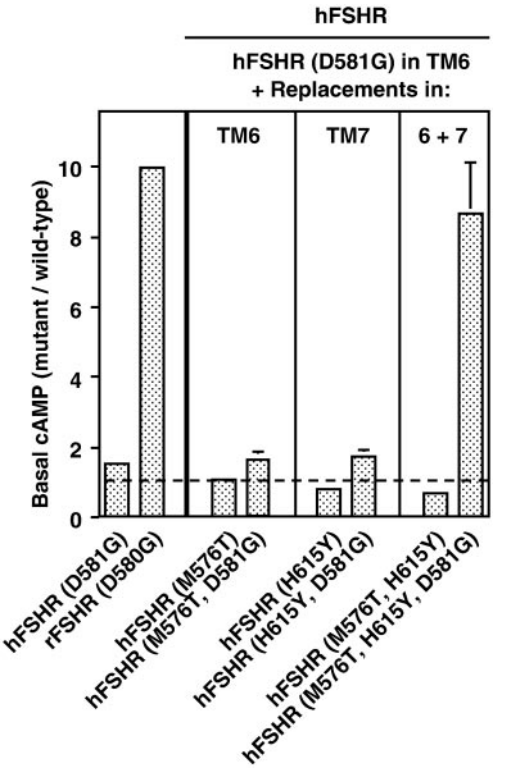


**Fig. 6.** Substitution of TM6 and TM7 of the hFSHR with Residues from the rFSHR Permits Constitutive Activation of the hFSHR Containing the TM6 D581G Mutation

The divergent amino acids in TM6 and in TM7 of the hFSHR (see Fig. 1) were individually or simultaneously replaced with the corresponding residues of the rFSHR in the context of the wild-type hFSHR and the hFSHR(D581G) mutant. 293 cells transiently expressing comparable numbers of cell surface wild-type and mutant FSHRs were assayed for basal cAMP. Basal cAMP levels are expressed as the ratio of the cAMP in cells expressing the mutant receptor relative to those expressing the wild-type receptor of a given species. Therefore, a value of 1.0 (shown by the *dotted line*) would be obtained if the mutant receptor behaved identically as the wild-type receptor of the same species. The basal levels of cAMP in rFSHR(D580G) and hFSHR(D581G) also expressed relative to the wild-type form of each receptor (calculated from the data shown in Table 1) are included for comparison. Data shown are the mean ± SEM of three to ten experiments. The absence of an *error bar* indicates that the SEM was too small to be observed graphically.

## Computer Modeling of the Human and Rat FSHRs

The mutagenesis data presented show that the D580/581G mutation in TM6 confers constitutive activation in the rat, but not the human, FSHR. The combined M576T (TM6) plus H615Y (TM7) substitutions in the hFSHR (replacing human FSHR residues with rat), however, fully permit constitutive activation of the hFSHR by the D580/581G mutation. Neither the M576T nor the H615Y substitutions alone, though, had a permissive effect on hFSHR(D581G). In a similar, but not completely reciprocal manner, the substitution of Y614H (TM7) in the rFSHR (replacing a rat FSHR residue with human) fully suppresses the constitutive activation of the rFSHR(D580G). In this case, the switch of only TM7 from rat to human was sufficient to suppress constitutive activity of the D580/581G mutation. Interestingly, the switch of TM6 from rat to human enhanced the constitutive activity of rFSHR(D580G). These data strongly suggest that interhelical interactions between TMs 6 and 7 confer the ability to permit (rFSHR) or suppress (hFSHR) constitutive activation by the TM6 D580/581G mutation.

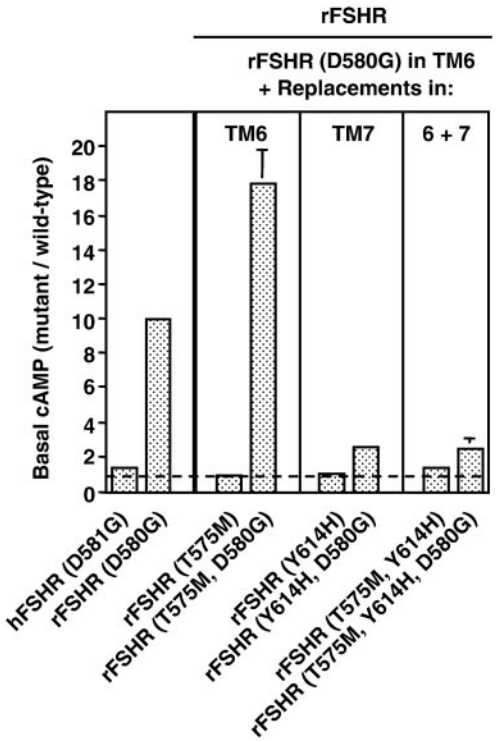


**Fig. 7.** Substitution of TM7 of the rFSHR with Residues from the hFSHR Suppresses Constitutive Activation of the rFSHR Containing the TM6 D580G Mutation

The divergent amino acids in TM6 and in TM7 of the rFSHR (see Fig. 1) were individually or simultaneously replaced with the corresponding residues of the hFSHR in the context of the wild-type rFSHR and the rFSHR(D580G) mutant. 293 cells transiently expressing comparable numbers of cell surface wild-type and mutant FSHRs were assayed for basal cAMP. Basal cAMP levels are expressed as the ratio of the cAMP in cells expressing the mutant receptor relative to those expressing the wild-type receptor of a given species. Therefore, a value of 1.0 (shown by the *dotted line*) would be obtained if the mutant receptor behaved identically as the wild-type receptor of the same species. The basal levels of cAMP in rFSHR(D580G) and hFSHR(D581G) also expressed relative to the wild-type form of each receptor (calculated from the data shown in Table 1) are included for comparison. Data shown are the mean ± SEM of 4–13 experiments. The absence of an error bar indicates that the SEM was too small to be observed graphically.

The aspartate residue in TM6 of the FSHR that is the site of the D580/581G substitution is conserved among the glycoprotein hormone receptors and LGRs, but is not conserved among other rhodopsin-like GPCRs. The functional importance of this aspartate is underscored by the findings that several activating mutations of the hLHR and hTSHR result from different substitutions of this residue (12, 20, 21). The most common naturally occurring activating mutation of the hLHR is actually a substitution of this aspartate to glycine (22). As shown in the models of TMs 6 and 7 of the human and rat FSHRs and D580/581G mutants thereof (Fig. 8), this aspartate lies midway in TM6 below a proline that is conserved in all rhodopsin-like GPCRs (23). This conserved proline is thought to induce a kink in the $\alpha$-helix and provide a hinge-like movement to TM6 (24). A growing body of evidence suggests that activation of GPCRs involves a movement of TM6 (reviewed in Refs. 5, 24 and 25). It has been proposed that the basis for this movement may, in fact, be dictated by the extent of bending of the TM6 Pro-kink, such that activation leads to a straightening of TM6 about this kink (24, 26, 27). The insertion of a glycine in the $\alpha$-helix of TM6, as in the D580/581G mutation of the FSHR, would be predicted to cause increased flexibility and possibly an additional kink in the $\alpha$-helix at this glycine position (24, 28). In addition, the absence of a side chain in glycine would also create a void in the region of the receptor previously occupied by the side chain of aspartate. Therefore, one would predict that other helices might change their packing relative to TM6 to fill that space. Presumably, the increased flexibility of TM6 and readjustments of neighboring residues ultimately allow the receptor to assume an active conformation.

To further understand the molecular basis for the ability of the rat, but not human, FSHR to be rendered constitutively active by the TM6 D580/581G mutation, we developed computer models of the hFSHR, rFSHR, and mutants thereof based upon the crystal structure of rhodopsin (19). Because homology modeling by definition holds the TM helices constant relative to the ground state of rhodopsin, it is not possible by this approach to predict the actual structural rearrangements of TM6 resulting from the increased flexibility of the helix induced by the D580/581G mutation. Nor can one visualize any changes in the packing of the helices as a result of the void created by the aspartate-to-glycine substitution. The models can, however, predict the changes in the composition and orientation of the side chains of the residues. In this context, the models provide a useful framework to examine the ramifications of the T575 (rFSHR) *vs.* M576 (hFSHR) in TM6 and Y614 (rFSHR) *vs.* H615 (hFSHR) in TM7 in the permission (rFSHR) *vs.* the suppression (hFSHR) of constitutive activation by the D580/581G mutation in TM6.

As shown in Fig. 8, M576 of the hFSHR and T575 of the rFSHR are located on the lower portion (*i.e.* the region extending toward the cytoplasmic space) of TM6. H615 of the hFSHR and Y614 of the rFSHR are situated in the upper portion (*i.e.* the region extending toward the extracellular space) of TM7. An examination of the interaction patterns involved with these residues suggests that they may influence hydrophobic interactions between TMs 6 and 7 (Fig. 8 and, in more detail, Fig. 9). Thus, in the hFSHR, one would predict a hydrophobic interaction between the upper portions of TMs 6 and 7 mediated by I588 in TM6 and V612 in TM7. The side chain of the nearby H615 would not interfere with this interaction. In contrast, though, in the rFSHR, both the steric and polar imposition of the tyrosine side chain between the TM6 isoleucine

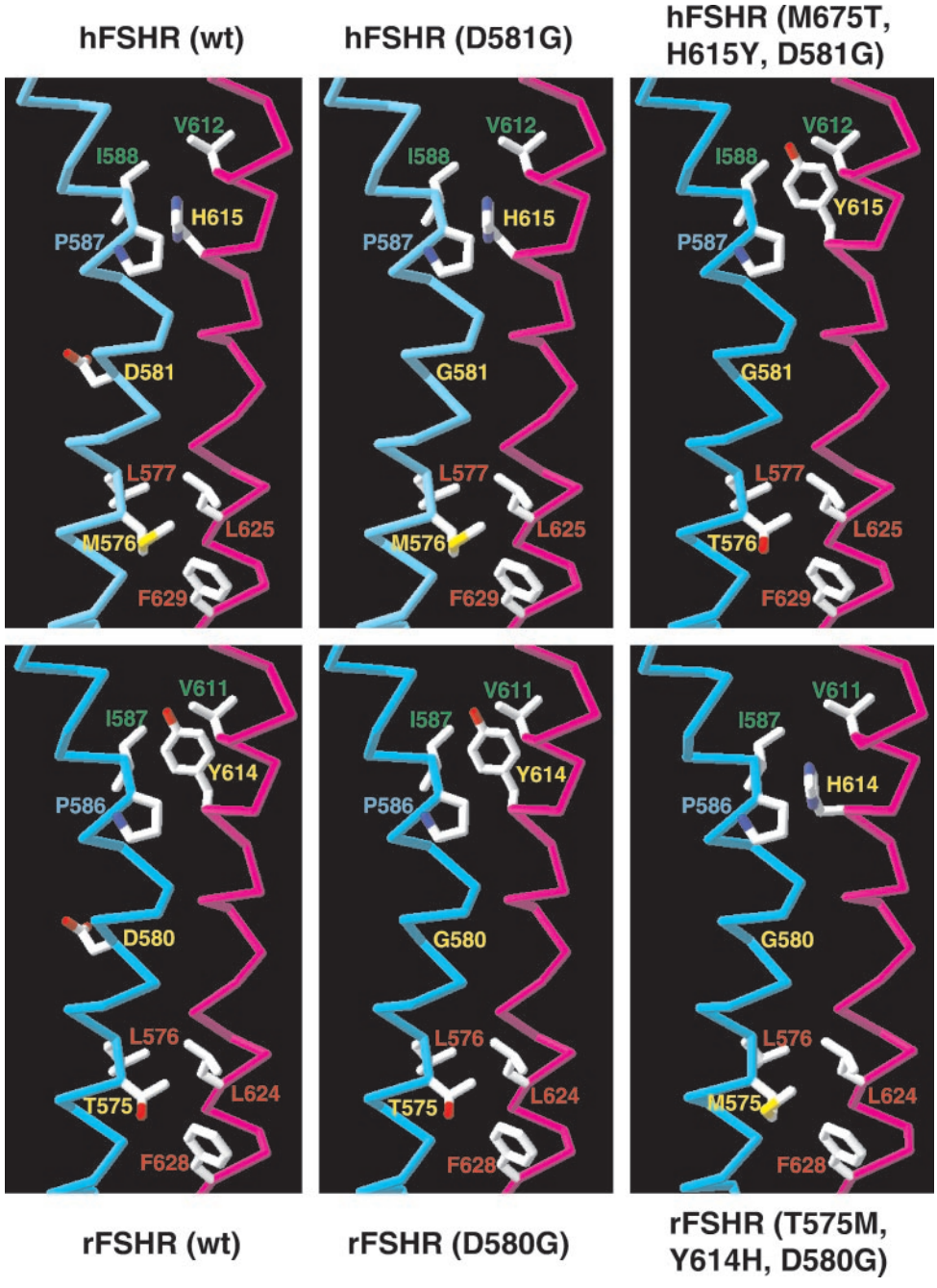


**Fig. 8.** TM Domains 6 and 7 of the Rat and Human FSHRs and D580/581G Mutants Thereof

Structural models of the rat and human FSHRs and mutants thereof were constructed based upon the crystal structure of rhodopsin as described in *Materials and Methods*. In this figure, only TMs 6 and 7 are visualized, where TM6 is shown in *blue* and TM7 in *violet*. The *upper portions* of the helices extend toward the extracellular space and the *lower portions* extend toward the cyptoplasmic space. The residues that were the sites for mutagenesis are labeled in *yellow*. The proline in TM6 is labeled in *blue*. The interacting hydrophobic residues in the *upper* portion of TMs 6 and 7 that may be affected by rat *vs.* human FSHR sequences at 614/615 are labeled in *green*. The interacting hydrophobic residues in the *lower portion* of TMs 6 and 7 that may be affected by rat *vs.* human FSHR sequences at 575/576 are labeled in *red*.

and TM7 valine would weaken that hydrophobic interaction. This suggests that the interaction between I588 and V612 of the hFSHR constrains the mobility of the upper portions of TMs 6 and 7 and prevents them from assuming an active conformation in response to the D580/581G mutation. Replacing the Y614 of the rFSHR with histidine is entirely sufficient to completely suppress the constitutive activity of rFSHR(D580G), emphasizing the strength of the interaction between this isoleucine in TM6 with valine in TM7. Interestingly,

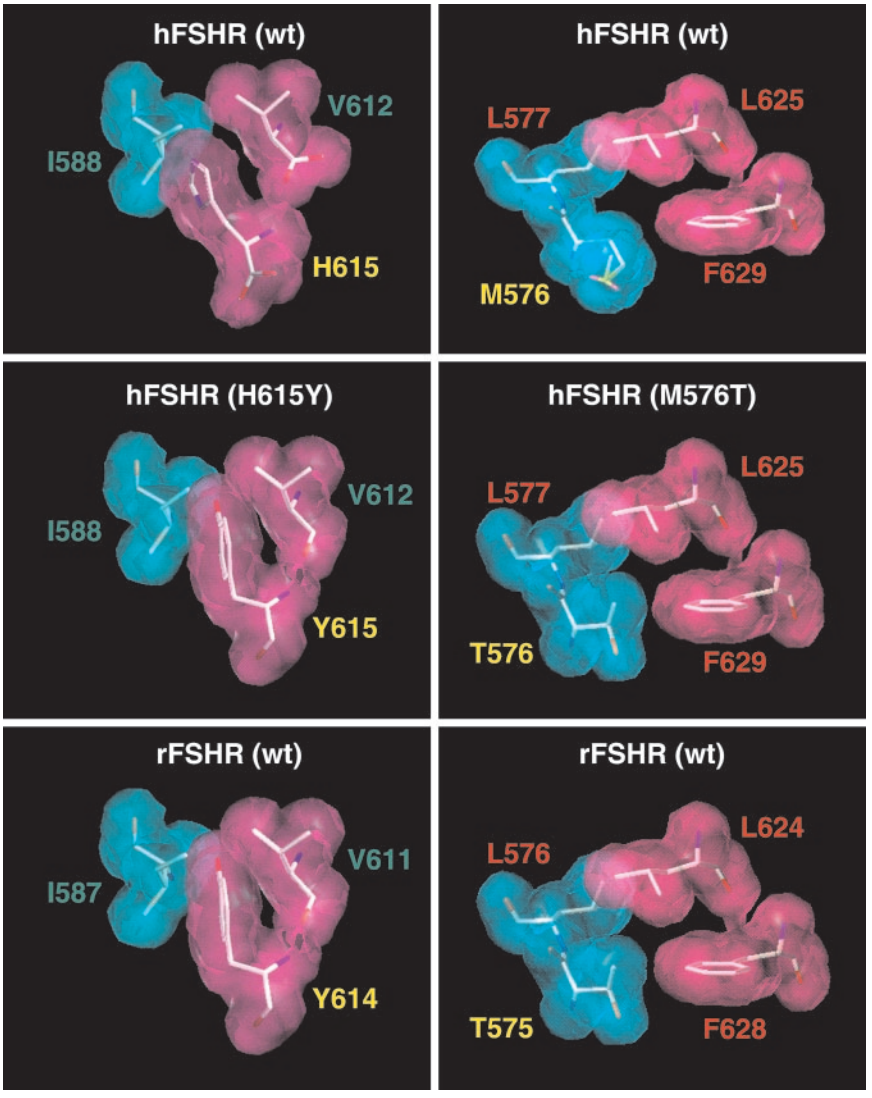


**Fig. 9.** The Divergent Residues of the Rat *vs*. Human FSHR TM6 and TM7 Predict Different Hydrophobic Interactions Between these Two Helices

A more detailed view of the hydrophobic patches between TMs 6 and 7 that are postulated to be different between the rat and human FSHRs is shown. The *colored shadings* depict the predicted space occupied by the residue, where *blue* represents a residue in TM6 and *purple* represents a residue in TM7. The residues that were the sites for mutagenesis are labeled in *yellow*. The interacting hydrophobic residues in the *upper portion* of TMs 6 and 7 that may be affected by rat *vs.* human FSHR sequences at 614/615 are labeled in *green*. The interacting hydrophobic residues in the *lower portion* of TMs 6 and 7 that may be affected by rat *vs.* human FSHR sequences at 575/576 are labeled in *red*.

examination of the TM sequences of the human and rat LHRs and hTSHR with the human and rat FSHRs reveals that of these glycoprotein hormone receptors, only the hFSHR has a histidine residue at position 615 (per the numbering of the hFSHR) in TM7. Similar to the rFSHR, the other glycoprotein hormone receptors have a tyrosine at that position. All the other glycoprotein hormone receptors have been shown to be constitutively active in response to the TM6 aspartate-to-glycine mutation (12, 20, 21). These observations further support the hypothesis that in the absence of a residue (tyrosine) that would disrupt the I588/V612 interaction, the strong hydrophobic nature of this interaction would constrain the D580/581G mutant from assuming an active conformation.

Although a histidine at position 614/615 in TM7 (as in the hFSHR) prevents the acquisition of the active conformation of the D580/581G mutant presumably due to hydrophobic interactions of I588 (TM6) and V612 (TM7), the disruption of this interaction by a tyrosine at position 614/615 is not sufficient for achieving constitutive activation (cf. Figs. 6 and 7). In this case, the simultaneous substitutions of the hFSHR with both H615Y and M576T are required to induce constitutive activation by D580/581G. The models predict that M576 of the hFSHR lies in the vicinity of a

hydrophobic patch formed by L577 (TM6), F629 (TM7), and L625 (TM7) (Figs. 8 and 9). M576 would not contribute to this hydrophobic patch because its side chain is predicted to project toward the lipid bilayer. In the rFSHR the cognate residue is T575. In this case, the carboxyl group of the threonine is predicted to project between helices 6 and 7. Consequently, the hydrophobic patch formed between the cyptoplasmic regions of TMs 6 and 7 would now be stabilized by four, rather than three, residues. In agreement with this analysis, the rFSHR, rLHR, hLHR, and hTSHR have the hydrophobic residues threonine, isoleucine, or valine at the position corresponding to M576 in the hFSHR. Whereas these other glycoprotein hormone receptors are constitutively active in response to the TM6 aspartate-to-glycine mutation (12, 20, 21), the hFSHR is not. Therefore, we hypothesize that stronger hydrophobic interactions between the cyptoplasmic regions of TMs 6 and 7, together with a disruption of a hydrophobic interaction between the upper portions of TMs 6 and 7, is required for the ability of the FSHR to assume an active conformation in response to the D580/581G mutation.

In summary, our studies with the human and rat FSHRs suggest that hydrophobic interhelical interactions between TMs 6 and 7 play a critical role in stabilizing the inactive and active conformations of the FSHR.[2] Combined with predictions based upon computer models of the FSHR, our data further propose a mechanistic basis for the resistance of the hFSHR to a TM6 aspartate-to-glycine substitution that is frequently observed in naturally occurring mutations of the hLHR and hTSHR.

## MATERIALS AND METHODS

### Hormones and Supplies

Pregnant mare serum gonadotropin (PMSG) and highly purified FSH were purchased from Dr. A. F. Parlow and the National Hormone and Pituitary Program of the NIH/NIDDK. hFSH was iodinated following the procedure described for the iodination of hCG (29). $^{125}$I-cAMP and cell culture media were obtained from the Iodination Core and the Media and Cell Production Core, respectively, of the Diabetes and Endocrinology Research Center of the University of Iowa. Tissue culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD) and Corning, Inc., plasticwares were obtained from Fisher Scientific (Pittsburgh, PA).

[2] In an earlier study by Kudo *et al.* (16), chimeras between the hLHR and the hFSHR were examined to determine why a mutation in the third intracellular loop of the hLHR caused constitutive activation whereas the comparable mutation in the hFSHR did not. The results of these studies suggested that interactions between TMs 5 and 6 were required for maintaining the mutated hFSHR in the inactive state and the mutated hLHR in the active state. The apparent discrepancies between their study and ours may be due to the different locations of the activating mutations (*i.e.* the third intracellular loop in their study *vs.* TM6 in our study).

### Plasmids and Cells

The wild-type hFSHR cDNA was kindly provided by Ares Advanced Technology (Ares-Serono Group, Randolph, MA) and was subcloned into pcDNA 3.1 (Invitrogen, San Diego, CA). Mutagenesis was performed using the PCR overlap extension method (30, 31). The entire region amplified by PCR, as well as the sites of ligation, were sequenced to ensure the fidelity of the mutant cDNA. DNA sequencing was performed by the DNA Core of the Diabetes and Endocrinology Research Center of the University of Iowa.

HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA; CRL 1573) and were maintained at 5% $CO_2$ in a culture medium consisting of high-glucose DMEM containing 50 $\mu$g/ml gentamicin, 10 mM HEPES, and 10% newborn calf serum. Cells were plated onto 35-mm wells that had been precoated for 1 h with 0.1% gelatin in calcium and magnesium-free PBS, pH 7.4. Cells were transiently transfected when they were 50–70% confluent following the protocol of Chen and Okayama (32) except that the overnight precipitation was performed in a 5% $CO_2$ atmosphere. The cells were then washed with Waymouth's MB752/1 media modified to contain 50 $\mu$g/ml gentamicin and 1 mg/ml BSA, after which fresh growth media were added. The cells were used for experiments 24 h later.

### Binding Assays to Intact Cells Expressing the hFSHR

HEK 293 cells were plated onto gelatin-coated 35-mm wells and transiently transfected as described above. On the day of the experiment, cells were washed two times with warm Waymouth's MB752/1 containing 50 $\mu$g/ml gentamicin and 1 mg/ml BSA. To determine the maximal binding capacity, the cells were then incubated 1 h at 37 C in the same media containing a saturating concentration of $^{125}$I-hFSH (500 ng/ml final concentration) with or without an excess of unlabeled PMSG (480 IU/ml final concentration). The assay was finished by washing the cells three times with cold Hanks' balanced salt solution modified to contain 50 $\mu$g/ml gentamicin and 1 mg/ml BSA. The cells were then solubilized in 100 $\mu$l of 0.5 N NaOH, transferred to plastic test tubes with cotton swabs, and counted in a $\gamma$-counter. Under these assay conditions, the percentage of nonspecific binding was $\leq$ 30% of the total counts per minute bound.

### Measurement of cAMP Production

293 cells were plated on gelatin-coated 35-mm wells and transfected as described above. In each experiment that cAMP production was measured, parallel plates were used for binding assays to determine the levels of cell surface receptors (see above). For the determination of basal cAMP, cells were washed twice with warm Waymouth's MB752/1 media containing 50 $\mu$g/ml gentamicin and 1 mg/ml BSA and placed in 1 ml of the same medium containing 0.5 mM isobutylmethylxanthine. The incubation was continued for 75 min at 37 C. The cells were then placed on ice, the media were aspirated, and intracellular cAMP was extracted by the addition of 0.5 N perchloric acid containing 180 $\mu$g/ml theophylline and then measured by RIA. All determinations were performed in triplicate.

### Homology-Based Modeling of the TM Regions of the Human and Rat FSHRs

Three-dimensional models of the TM regions of the human and rat FSHR receptors were built using the crystal structure of bovine rhodopsin (19) as a template (Protein Data Bank entry no. 1F88). The modeling was performed using the SYBYL program package, version 6.7 (Tripos Associates, Inc., St. Louis, MO), on a UNIX workstation $O_2$ (Silicon Graph-

ics Inc., Mountain View, CA). To facilitate homology modeling based upon rhodopsin, the rhodopsin coordinates were modified to exclude the amino-terminal extension (Met1-Pro34), the carboxyl-terminal extension (Cys322-Ala348), and the HET groups (*i.e.* mercury ion, $\alpha$-D-mannose, *N*-acetyl-D-glucosamine, retinal, and zinc). The FSHR alignments to rhodopsin were based upon a recently published alignment of the helices of the hTSHR to rhodopsin by Ballesteros *et al*. (24). As such, the FSHR sequences were manually docked to the rhodopsin sequence using the most conserved residue in each helix [Asn I:18, Asp II:14, Arg III:25, Trp VI:11, Tyr V:22, Pro VI.18, and Pro VII:18 per the numbering system of Baldwin *et al*. (23)] as seeds. Each of the other residues in a given helix (including helix 8) of rhodopsin was then replaced by the corresponding residue of the human or rat FSHR sequence. The length of the TM regions was kept unchanged from the rhodopsin crystal structure, again in keeping with alignment of the hTSHR helices with rhodopsin as described by Ballesteros *et al*. (24). The predicted structures of the FSHR extracellular and intracellular loops were generated using the LOOP SEARCH tool on SYBYL. Energy minimizations were then performed using 100 cycles of the Kollman ALL-Atom force field, with the default distance-dependent dielectric constant of 1.0 and the Powell conjugate gradient algorithm having a convergence criterion of 0.05 kcal/(mol A). The resulting structures were inspected visually.

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