Mutations Remote from the Human Gonadotropin-releasing Hormone (GnRH) Receptor-binding Sites Specifically IncreaseBinding Affinity for GnRH II but Not GnRH I

EVIDENCE FOR LIGAND-SELECTIVE, RECEPTOR-ACTIVE CONFORMATIONS*†

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The human gonadotropin-releasing hormone (GnRH) receptor is evolutionarily configured for high affinity binding of GnRH I ([Tyr5,Leu7,Arg8]GnRH) but at lower affinity for GnRH II ([His5,Trp7,Tyr8]GnRH). GnRH I is more potent in the activation of the Gq11 protein in the gonadotrope; however, GnRH II is more potent in the stimulation of apoptosis and antiproliferative effects through activating G1 protein-mediated signaling, implying that GnRH I and II selectively stabilize different receptor-active conformations that preferentially couple to different signaling pathways. Receptor activation involves ligand induction or conformational selection, but the molecular basis of the communication between ligand-binding sites and receptor allosteric sites remains unclear. We have sought conformational coupling between receptor-ligand intermolecular interactions and intramolecular interaction networks in the human GnRH receptor by mutating remote residues that induce differential ligand binding affinity shifts for GnRH I and II. We have demonstrated that certain Ala mutations in the intracellular segments of transmembrane domains 3 (Met132), 5 (Met247), 6 (Phe272 and Phe276), and 7 (Ile282 and Tyr335) of the human GnRH receptor allosterically increased ligand binding affinity for GnRH II but had little effect on GnRH I binding affinity. We examined the role of the three amino acids that differ in these ligands, and we found that Tyr5 in GnRH II plays a dominant role for the increased affinity of the receptor mutants for GnRH II. We propose that creation of a high affinity binding site for GnRH II accompanies receptor conformational changes, i.e. “induced fit” or “conformational selection,” mainly determined by the intermolecular interactions between Tyr5 and the receptor contact residues, which can be facilitated by disruption of particular sets of receptor-stabilizing intramolecular interactions. The findings suggest that GnRH I and II binding may selectively stabilize different receptor-active conformations and therefore different ligand-induced selective signaling described previously for these ligands.

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S The on-line version of this article (available at http://www.jbc.org) contains the homology model of the 7-TM domains.

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The human GnRH1 receptor is a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs), which regulates pituitary gonadotropin secretion through GnRH I activation of Gq11 proteins (1). Although other subtypes of GnRH receptors have been identified in various species, there is a single functional member of their receptor family in man but two or three types of endogenous ligands as follows: GnRH I and II and possibly a protease-cleaved fragment 1–5 of GnRH I (1–3). In addition, there is increasing evidence that this single human GnRH receptor (type I) is capable of coupling to different species of G proteins, such as Gq11, Ga, and Gq, mediating differential physiological functions (1, 4), as has been found for a number of other GPCRs (5). The coupling to different signaling pathways can be selectively activated by different ligands, and this is called “agonist trafficking of receptor signals” by Kenakin (6–8). This concept has been supported by a number of recent findings (9–13). Studies on the binding and signaling characteristics of the human GnRH receptor in reproductive tissue cell lines demonstrated a distinctly different pharmacology of ligand selectivity and signaling from that elucidated in pituitary gonadotropes. Specifically, the human GnRH receptor preferentially binds GnRH I and couples to Gq11 protein in pituitary cells to mediate activation of phospholipase Cβ and calcium mobilization. However, GnRH I and II and certain antagonists of the receptor-mediated Gq11 protein are potent inhibitors of proliferation in reproductive tumor cell lines through activation of Ga (14, 15). We have called the phenomenon GnRH ligand-induced selective signaling (1).

We have interpreted that GnRH ligand binding to the human GnRH receptor breaks intramolecular constraint networks that stabilize the receptor in inactive conformations, creating new sets of inter- and intramolecular contacts that stabilize the receptor in particular active conformations, determined by specific ligand receptor interactions. Ligand induction or ligand selection of receptor conformations then affects the downstream signaling selectivity. Thus, a particular receptor conformation is selective for a specific ligand and specific signaling pathway.

A number of intramolecular interaction networks that stabilize GPCRs in inactive conformations have been identified through mutation-induced constitutive activations (16–18). These include the highly conserved L3.43 (using the nomencl-
ture of Ballesteros and Weinstein in which the position of the most conserved amino acid in the TM domain N is designated as N.50 in the M1 muscarinic acetylcholine receptor (M1 mAChR) (19), the human folliculin receptor (20), the β2-adrenergic receptor (20), and the C5a receptor (21); M6.40 in rho-Phe276 (6.44) in the human GnRH receptor (27). However, there was increased receptor expression of the F272L mutant indicating a subtle conformational change of the receptor. Engineering Zn2+-binding sites among the above residues in the M1 mAChR by multiple His substitutions led to a Zn2+-dependent inhibition of ligand binding through distant allosteric effects (28). Apparently, ligand binding triggers receptor conformational changes allosterically, which disrupt receptor intramolecular constraint networks. Weakening receptor intramolecular interactions would increase receptor protein flexibility and mobility that can result in an increased affinity between ligand and receptor (29).

In the present study, we have mutated the residues of the human GnRH receptor at the equivalent positions mentioned above in other GPCRs, in which the side chains of the amino acids have been shown to functionally important intramolecular interactions to alinate that delete side chains beyond the β-carbon. These are Met132 (3.43), Phe272 (6.40), Phe276 (6.44), Ile225 (7.52), and Tyr233 (7.53). Another two highly GPCR-conserved residues in TM 5, Phe272 (6.47) and Met225 (5.54), whose function has not been fully defined yet, were also included in this study in order to understand their potential function in the human GnRH receptor binding and activation. In the human GnRH receptor model, Phe209 is modeled close to the bottom of the GnRH ligand binding pocket, whereas Met225 is modeled toward to the side chains of Met132 and Phe276. Here we show that the binding affinities of GnRH I and II at the human GnRH receptor can be differentially affected through amino acid mutations remote from the binding sites, suggesting an increased ligand-induced shift of receptor conformational equilibria, toward active conformations selective for GnRH II.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Receptor Expression—A PCR method was used to construct various Ala point mutations. Mutant receptors cloned into the pcDNA I expression vector were validated by dideoxy sequencing. Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using a Bio-Rad Gene Pulser at 230 V and 960 microfarads with 20 μl of IN3 (a non-peptide GnRH receptor antagonist) or undetectable ligand binding and IP response. The F272A mutant gave a decreased expression level to 25% that of wild type. Expression of the poorly expressed mutants was restored by the removal of the medium and addition of 10 mM formic acid. The [3H]inositol phosphates were isolated from the formic acid extracts using Dowex AG 1-X8 ion exchange resin, collected with 1 mM ammonium formate containing 0.1 mM formic acid, and quantified by liquid scintillation counting. Assays were carried out in triplicate. In most cases, receptor binding assays were conducted on the same batch of transfected cells in parallel, and therefore the receptor-binding sites were monitored.

Molecular Modeling—A computer model of the human GnRH receptor was built on the solved structure of bovine rhodopsin (34) (Protein Data Bank code 1F8B) using a similar method as described previously for the M1 mAChR (35) with the “MODELLER” (36) module within the DS modeling (version 1.1, Accelrys, San Diego). Briefly, alignment of the human GnRH receptor sequence on the rhodopsin sequence was performed using “ALIGN2D” within the MODELLER program, followed by minor manual adjustment. The most highly conserved residues, motifs, and the disulfide bridge Cys114-Cys190 were located at the same place as in the rhodopsin structure. An additional experimentally determined disulfide bond between Cys14 and Cys200 (37) was also integrated into the model. The initial models were checked with three-dimensional profile (38, 39), and the model with best score was selected and then energy-minimized using conjugate and molecular dynamics with simulated annealing employing the CHARMM force field (40). The model accommodates the experimentally determined hydrogen bonds between Asp29 (2.61)-Lys121 (3.32), Asn53 (1.50)-Asn97 (2.50)-Asp129 (7.49) and between Asp29 (3.49)-Arg28 (3.50) (1).

Materials—GnRH I ([Phe2-NH2-His5]-Tyr6-Glu7-Lys8-Arg9-Pro10-Pro11-Pro12-Phe13-Gly14) and GnRH II (His5,Trp7,Y8) were synthesized in our laboratory as described previously (32, 41). DeepVent polymerase was from New En.

RESULTS

Expression of the Human GnRH Mutant Receptors in COS-7 Cells—Ala mutations of Met132 (3.43), Phe220 (5.47), Met227 (5.54), Phe276 (6.44), and Ile232 (7.52) led to low (<5% of wild type) or undetectable ligand binding and IP response. The F272A (6.40) mutant gave a decreased expression level to 25% that of the wild type, and the Y323A (7.53) mutant gave an increased expression level to 250% that of the wild type as measured by GnRH ligand binding. Following the report by Conn and coworkers (30), we developed a modified method to raise receptor expression level by preincubation of the transfected COS-7 cells with 1 μM IN3, a membrane-permeant non-peptide GnRH receptor antagonist, followed by extensive washes of the cells with 2% MeSO, which allowed the removal of IN3 from the receptor before assays. Expression of the poorly expressed mutant receptors was rescued to 5–96% of the wild-type level (Fig. 1, top, and Table 1). Most interestingly, expression of the wild-type receptor was enhanced to 130% of the untreated wild-type level by IN3 pretreatment (Fig. 1, top). Homologous inhibition binding assay showed that there was no change in affinity for [125I]-His5-
and mutant receptors in which binding was measurable without IN3 pretreatment. The wild-type and mutant receptor transfected COS-7 cells were incubated with or without 1 μM IN3 for 48 h. The IN3 was washed off prior to binding assay. Details are given under “Experimental Procedures.” Results are representative experiments, which were repeated three times with essentially the same results. There were no significant differences in the GnRH affinity between the IN3-pretreated and the untreated cells. ○, wild type; □, wild type with IN3 pretreatment; ▽, M132A; ◐, M132A with IN3 pretreatment; ▲, F272A; ●, F272A with IN3 pretreatment.

**GnRH Ligand-selective and Receptor-active Conformations**

**FIG. 1.** Homologous competition binding of [His₅-D-Tyr₈]GnRH in wild-type and selected mutant receptors with or without IN3 pretreatment. The wild-type and mutant receptor transfected COS-7 cells were incubated with or without 1 μM IN3 for 48 h. The IN3 was washed off prior to binding assay. Details are given under “Experimental Procedures.” Results are representative experiments, which were repeated three times with essentially the same results. There were no significant differences in the GnRH affinity between the IN3-pretreated and the untreated cells. ○, wild type; □, wild type with IN3 pretreatment; ▽, M132A; ◐, M132A with IN3 pretreatment; ▲, F272A; ●, F272A with IN3 pretreatment.

**Mutations of M132A and F272A Specifically Increase Affinity of the Human GnRH Receptor for GnRH II but Not GnRH I**—The binding of GnRH I and GnRH II to the wild-type human GnRH receptor, expressed in COS-7 cells, was characterized by one-site binding isotherms (nH₁ = 0.83) with IC₅₀ at 2.75 and 29.3 nM respectively. The effect of mutations on the binding affinity of GnRH I and GnRH II is summarized in Table I. The Hill coefficients were unaltered for all mutants. Ala mutations of Met₁³², Met₂²₇, Phe²⁷², and Phe²₇₆ only had a marginal effect on their affinity for GnRH I, less than a 2-fold increase (Fig. 2a and Table I), whereas F₂²₀₀A and Y₃₆₃A gave no changes in the affinity for GnRH I (Table I). The mutations of M₂₂₇A, F₂₇₆A, I₃₂₂A, and Y₃₆₃A had 2–4-fold increases in affinity for GnRH II, whereas mutations of M₁₃₂A and F₂₇₂A strongly increased affinity for GnRH II by 12-fold (Fig. 2b). In the case of I₃₂₂A, the affinity could not be measured accurately due to the extremely low expression of the mutant receptor even after IN3 rescue. No significant change in the affinities for both GnRH I and II was observed for the F₂₂₀₀A mutant, although it had a similar reduction in receptor expression as the M₁₃₂A, M₂₂₇A, and F₂₇₆A mutants.

**Tyr₈ in GnRH II Plays a Dominant Role for the Increased Affinity of GnRH II**—There are three amino acid differences between GnRH I and GnRH II, i.e. Tyr₁, Leu⁷, and Arg⁸ in GnRH I are replaced by His₅, Trp⁷, and Tyr₈ in GnRH II. To identify which residue in GnRH II plays a key role in the affinity increase for GnRH II in the receptor mutants, we next examined the effect of the mutations on the binding affinities of GnRH analogues with single substitutions (His₅, Trp⁷, and Tyr₈) in GnRH I. Substitution of Tyr₈ in the GnRH I with His₅ led to a 2-fold increase in binding affinity of the ligand for the wild-type human GnRH receptor. Similar increases in affinity were observed for F₂₂₀₀A, F₂₇₆A, and Y₃₆₃A mutants, whereas the M₁₃₂A, M₂₂₇A, and F₂₇₂A mutants exhibited a 5–6-fold increase in affinity for the [His₅]GnRH analogue compared with GnRH I binding affinity at the wild-type GnRH receptor (Table II and Fig. 3a). In contrast, [Trp⁷]GnRH exhibited a 0.87-fold decrease in the affinity for the wild-type human GnRH receptor and a similar change for the mutant receptors (Fig. 3b). Most interestingly, substitution of Arg₈ of GnRH I with Tyr₈ led to an 82-fold reduction in the affinity for wild-type receptor compared with GnRH I. In contrast, mutations of M₁₃₂A and F₂₇₂A increased their affinity for [Tyr₈]GnRH by 33- and 35-fold, respectively (Fig. 3c). The mutations of M₂₂₇A in TM 5, F₂₇₆A in TM 6, and Y₃₆₃A in TM 7 caused a small increase in affinity about 2-fold for [Tyr₈]GnRH, but no change was observed for the mutation of F₂₂₀₀A (Table II).

**Effects of Mutations on the GnRH Ligand-elicited PhI Turnover**—GnRH I and II elicit robust PhI responses from COS-7 cells transfected with the wild-type human GnRH receptor, with EC₅₀ values of 0.7 and 6.7 nM, respectively, reflecting the 10-fold difference in the affinities for the human GnRH receptor (Table I). The maximum responses were typically six times the basal activity. The effects of the Ala mutations on the PhI responses are summarized in Table I. All of the mutant receptors that gave measurable GnRH binding were able to mediate a PhI response to GnRH I and II. However, their maximum PhI response and EC₅₀ value varied according to their expression levels. The IN3 preincubation, which increased expression level of the wild-type receptor by 130%, also increased the maximum PhI responses to the equivalent level with little effect on the EC₅₀ values, indicating that within a limited response window of the receptor system, there was a near-linear relationship between receptor-binding sites and the maximum PhI responses to GnRH II stimulation (42). Hence, the relative maximum signaling efficacy of GnRH can be calculated by normalization of the maximum PhI responses to the cell surface receptor-binding sites as the receptor population is saturated, and the affinity is not an issue (43). All mutants with reduced receptor expression levels increased GnRH signaling efficacy to PhI response by 2–8-fold. The F₂₇₂A mutant, when expressed at the wild-type level, caused 1- and 7.4-fold decreases in the EC₅₀ values in the GnRH I- and II-elicited PhI responses with the increased maximum responses at 133–150% of the wild-type levels. A 3.6-fold reduction of the EC₅₀ value was observed in the GnRH II-elicited PhI response in the mutation M₁₃₂A, even though its expression was only 22% of the wild-type level. No constitutive activation was observed in all mutant receptors.

In contrast, Ala mutation of Tyr³²³ caused 46- and 57-fold increases in the EC₅₀ values for GnRH I- and II-elicited PhI responses, respectively, but with reduced maximum responses to 8–51% of the wild-type level, even though it gave 250% expression of the wild-type level (Table I).

[Tyr₈]GnRH-stimulated PhI response was selectively examined in the F₂₇₂A mutant as its expression can be rescued to the wild-type level, thus eliminating potential effects of variation in receptor expression on the functional response.
Ligand binding and functional activity of Ala-substituted human GnRH receptors

|                  | Binding  |                |                |                | PhI response |                |                |
|------------------|----------|----------------|----------------|----------------|--------------|----------------|----------------|
|                  | $B_{\text{max}}$ | $\text{IC}_{50}$ | $\text{IC}_{50}$ | $\text{EC}_{50}$ | $E_{\text{max}}$ | $\text{IC}_{50}$ | $E_{\text{max}}$ |
| Wild-type       | 100      | 2.75 ± 0.21    | 0.70 ± 0.10    | 100            |              | 29.32 ± 3.29   | 6.71 ± 1.03    |
| Wild-type + IN3 | 130 ± 5  | 2.85 ± 0.28    | 0.81 ± 0.16    | 124 ± 5        |              | 26.94 ± 3.97   | 4.59 ± 1.52    |
| M132A + IN3     | 22 ± 3   | 1.92 ± 0.55    | 0.66 ± 0.14    | 75 ± 11        |              | 2.38 ± 1.44    | 1.84 ± 0.24    |
| F220A + IN3     | 34 ± 3   | 2.46 ± 0.58    | 3.70 ± 1.34    | 85 ± 10        |              | 23.31 ± 7.79   | 19.1 ± 5.00    |
| M227A + IN3     | 32 ± 4   | 1.87 ± 0.30    | 1.27 ± 0.21    | 88 ± 10        |              | 7.71 ± 1.40    | 5.38 ± 0.56    |
| P272A           | 25 ± 2   | 1.56 ± 0.29    | 2.27 ± 0.30    | 79 ± 4         |              | 6.05 ± 1.50    | 73 ± 5         |
| P272A + IN3     | 96 ± 14  | 1.47 ± 0.30    | 0.35 ± 0.03    | 150 ± 9        |              | 2.25 ± 0.60    | 0.91 ± 0.35    |
| P276A + IN3     | 29 ± 4   | 1.65 ± 0.32    | 1.23 ± 0.25    | 62 ± 12        |              | 12.5 ± 1.85    | 8.61 ± 1.20    |
| E222A + IN3     | 5 ± 3    | UD b           | 2.55 ± 0.49    | 44 ± 3         |              | 6.90 ± 5.58    | 13 ± 3         |
| Y323A           | 250 ± 26 | 2.88 ± 0.49    | 32.09 ± 2.31   | 51 ± 3         |              | 14.74 ± 1.73   | 380 b          |

* IN3 pretreatment.
* UD, undetected, i.e. could not be quantified accurately.

DISCUSSION

Peptide and polypeptide ligand binding at the extracellular surface of 7-TM receptors triggers conformational changes of the 7-TM domains that translate into changes in intracellular surface conformation that promotes interaction with effector proteins (17, 44, 45). The mechanisms mediating allosteric communications between the distant ligand-binding sites and intracellular effector protein recognition sites in the GPCRs have yet to be fully elucidated. An understanding of the differential effects of ligand binding on receptor conformational change and transfer to ligand-induced selective signaling (1) is even more challenging. Reciprocal interactions and cooperativity between the ligand binding and intracellular effector domains are likely to underlie receptor ligand binding and coupling specificity. This has been evidenced by ligand-selective signaling (46) and the recent finding of the effect of activation of intracellular protein kinase C by phorbol 12-myristate 13-acetate on the GnRH ligand binding selectivity of a Xenopus GnRH receptor (47, 48). Hence, long range interactions of receptor amino acids are important for both ligand binding and coupling specificity, i.e. they depend not only on the direct contact residues but also on distant residues located in other secondary structural domains of the receptor (49). Hence, delineation of the allosteric communication network between remote sites of a GPCR, which involves receptor conformational switches and ligand binding, and effector protein coupling selectivity is a fundamentally important issue in the understanding of GPCR function and in drug development.

Here we have applied Ala mutagenesis of the highly conserved GPCR residues Met132 (3.43, 74% Leu and 4% Met), Phe220 (5.47, 70% Phe), Met227 (5.54, 55% Leu, Ile, and Val and 30% Met), Phe272 (6.40, 84% Leu, Val, and Ile and 1% Phe), Phe276 (6.44, 82% Phe), Ile322 (7.52, 50% Leu and Val and 41% Ile), and Tyr323 (7.53, 92%) (50) in the human GnRH receptor to address this question. Replacement of individual amino acids by alanine, which deletes the side chain beyond the $\beta$-carbon, normally avoids steric interference (51) and has been successfully applied to the $M_1$ mAChR to define the role of amino acids in the 7-TM domains (19, 35, 52). This has enabled us to identify a set of conserved intramolecular interactions that link distant functional sites in the tertiary structure involving ligand selection of receptor conformations in the human GnRH receptor.

The GnRH ligand binding and PhI response were completely abolished by the Ala mutations of Met132, Phe220, Met227, Phe276, and Ile322, and the expression level of F272A was reduced to 25% of the wild-type level. The decrease in receptor
expression levels caused by the Ala mutations suggests that the side chains of these residues make intramolecular contacts that are crucial in stabilizing GnRH receptor folding. In the human GnRH receptor model, the side chains of Met132 and Phe272 appear to contribute to a critical packing interaction as in rhodopsin (34). This interaction may be extended into TM 7 by a stacking contact between the Phe272 and Ile322 side chains and extended into TM 5 by van der Waals contacts between the side chains of Met132, Met227, and Phe276 (Fig. 5a).

A large decrease in the receptor expression level was also observed in the mutations at the equivalent positions of Met132 (3.43) and Phe276 (6.44) of the M1 mAChR (19). These residues are highly conserved among GPCRs, and therefore the intramolecular contacts made by these residues may represent part of the conserved structural elements stabilizing the 7-TM receptors, in addition to the conserved H-bond interactions made by N1.50, D/N2.50, D3.49, W4.50, and D/N7.49 (52, 53). The conservation of the stabilizing residues has been proposed to evolve, at least in part, from requirements for protein stability and folding (54). Disruption of the intramolecular interactions made by the stabilizing residues through mutation may cause misfolding of the protein and therefore increased degradation (53). All the mutants with decreased receptor expression levels were rescued by preincubation with the membrane permeant, non-peptide GnRH receptor antagonist, IN3, acting as a pharmacological chaperone, presumably by binding and stabilizing the mutant receptors intracellularly and allowing their transport to the cell membrane. This approach has been extensively applied to rescue structurally unstable constitutively active mutants in the other GPCRs (19, 55). The rescue of many mutant receptor expressions by IN3 is also suggestive of an altered ensemble of receptor conformational states.

**TABLE II**

| Ligand binding was conducted as described under “Experimental Procedures.” Values are mean ± S.E. from three or more independent experiments. The competing radioligand was [125I-His$^5$]-GnRH. |
|---|---|---|---|
| | Binding affinity (IC$_{50},$ nM) |  |
| |  | GnRH I | His$^5$-GnRH | Trp$^7$-GnRH | Tyr$^5$-GnRH |
| Wild type | 2.75 ± 0.21 | 0.87 ± 0.26 | 5.14 ± 0.42 | 224.55 ± 38.56 |
| Wild type + IN3$^a$ | 2.85 ± 0.28 | 0.76 ± 0.20 | 4.65 ± 1.16 | 201.14 ± 33.14 |
| M132A + IN3 | 1.92 ± 0.55 | 0.34 ± 0.10 | 4.67 ± 0.78 | 6.78 ± 2.66 |
| F220A + IN3 | 2.46 ± 0.58 | 0.77 ± 0.20 | 7.41 ± 0.42 | 216.50 ± 22.23 |
| M227A + IN3 | 1.87 ± 0.30 | 0.48 ± 0.16 | 4.44 ± 0.78 | 107.39 ± 20.62 |
| F272A + IN3 | 1.47 ± 0.30 | 0.35 ± 0.08 | 5.21 ± 0.63 | 6.34 ± 0.42 |
| F276A + IN3 | 1.65 ± 0.32 | 0.58 ± 0.17 | 5.60 ± 0.68 | 113.50 ± 15.80 |
| Y323A | 2.88 ± 0.49 | 0.52 ± 0.18 | 6.24 ± 1.40 | 116.36 ± 28.65 |

$^a$ IN3 pretreatment.
The presence of the intramolecular interactions made by the side chains of Met\(^{132}\) and Phe\(^{272}\) and their surrounding residues Met\(^{227}\), Phe\(^{276}\), and Ile\(^{322}\) was also supported by the functional assay. Normalization of the maximum PhI response with the cell surface receptor number showed that all mutants, whose expression level was reduced, increased their maximum signaling capability. For instance, the Ala mutant of Ile\(^{322}\), whose receptor expression was only about 5% of the wild-type level, gave a maximum response to GnRH I and II of 38–44% of the wild-type receptor. Disruption of intramolecular interactions facilitates receptor conformational motion and hence increases ligand-induced receptor activation. If the disruption causes a significant agonist-independent shift of the receptor conformational equilibria toward an active state, this would give rise to constitutive activity (19). However, no constitutive activation of PhI turnover was observed in any of the mutant GnRH receptors, suggesting that the function of these residues in the human GnRH receptor may not be identical to that of other GPCRs, in which mutations do lead to constitutive receptor conformational changes (Fig. 6).

In contrast to the decreased expression levels of most Ala mutations in the human GnRH receptor, Y323A led to a 2.5-fold increase in receptor expression but resulted in poor coupling to G\(_{q/11}\), consistent with a previous report (56). This suggests that Tyr\(^{323}\) plays an important role in configuration of the tertiary structure of the GnRH receptor and receptor activation. The side chain of the equivalent residue of Tyr\(^{323}\) (7.53) in other GPCRs has been shown to make intramolecular contacts with a Phe residue located in helix 8 of the intracellular C-terminal tail (16). However, the human GnRH receptor is characterized by the lack of the C-terminal tail. Hence, the conformation of the human GnRH receptor in this microdomain is clearly different from other GPCRs, and its precise function requires elucidation.

The Ala mutation of Met\(^{132}\), Phe\(^{220}\), Met\(^{227}\), Phe\(^{272}\), Phe\(^{276}\), and Tyr\(^{323}\) had no or only marginal effects on receptor affinity for GnRH.
The mutations that induce an increased receptor affinity for GnRH II were remote from the GnRH-binding sites (Fig. 5b), suggesting that they facilitate ligand-induced receptor conformational changes that favor GnRH II binding, as they had only a small effect on GnRH I binding, and had no effect on GnRH antagonist binding. Characterization of the mutant receptors with GnRH analogues incorporating single GnRH II amino acid substitutions in GnRH I gave additional insight into the differential receptor binding and activation by GnRH I and II. The mutations that markedly increased affinity for GnRH II also displayed increased affinity for [Tyr8]GnRH but had little effect on [His5]GnRH and [Trp7]GnRH. Ala mutation of Met<sup>132</sup> and Phe<sup>272</sup> led to a 20–34-fold increase in affinity for [Tyr8]GnRH, but only had a 1-fold increase for [His5]GnRH and had no change for [Trp7]GnRH. The results indicate that the mutations that increased receptor affinity for GnRH II affected the intermolecular interactions between Tyr<sup>8</sup> and cognate receptor contact residues that require receptor conformational change to facilitate interaction with the Tyr<sup>8</sup> side chain. The cognate receptor contact residue for Arg<sup>8</sup> in GnRH I is the negatively charged side chain of Asp<sup>302</sup> (7.32) of extracellular loop 3 (ECL 3) of the receptor (60). Mutation of Asp<sup>302</sup> to Asn results in a 100-fold decline in binding affinity of GnRH I but no change in the lower affinity of Arg<sup>8</sup>-substituted ligands, including GnRH II that has Tyr<sup>8</sup>. Thus, Tyr<sup>8</sup> does not interact with Asp<sup>302</sup> of the human GnRH receptor, indicating that different amino acids in position 8 of the GnRH ligands select different receptor contact residues that confer GnRH I and II peptide binding selectivity. The mutant receptors displayed a similar effect on the GnRH I binding affinity for the D-6-amino acid preconfigured GnRH ligands such as [His<sup>5</sup>-D-Tyr<sup>8</sup>]GnRH (Fig. 1) and [D-Ala<sub>4</sub>]GnRH<sub>2</sub>, indicating that mutations of the receptor have no large effect on the GnRH I binding pocket, especially the interaction between Arg<sup>8</sup> of GnRH I and Asp<sup>302</sup> of ECL 3. Mutations may not affect the conformation of ECL 3 or the conformational change of the ECL 3 does not affect the interaction between Arg<sup>8</sup> and Asp<sup>302</sup>.

We suggest that Tyr<sup>8</sup> of GnRH II may make a number of hydrophobic interactions with the receptor different from the interaction between Arg<sup>8</sup> of GnRH I and Asp<sup>302</sup> of ECL 3. Mutations facilitate receptor conformational changes that may bring Tyr<sup>8</sup>-binding residues into more accessible positions or allow an improved ligand-receptor induced fit for Tyr<sup>8</sup>, which gives rise to an increased binding affinity for Tyr<sup>8</sup>-containing ligands. A schematic of how mutations remote from the ligand binding pocket might affect ligand binding affinity of GnRH I and II differentially is shown in Fig. 6.

Our identification of TM domain mutants that increase GnRH II and [Tyr8]GnRH binding affinity by an order of magnitude suggests that disruption of interactions between these TM residues allows transition to a receptor conformation with preferential binding of GnRH analogues with Tyr<sup>8</sup>. The different GnRH ligand binding, in particular the different intermolecular interactions between residue 8 and the receptor contact residues, may induce different receptor-active conformations with distinct functional abilities. The flatter dose-response curve of [Tyr8]GnRH also indicates that sequential or differential interactions of Tyr<sup>8</sup> with multiple receptor contact residues stabilize a series of different receptor conformations with distinct signaling ability. A recent study (61) has shown that even a single small organic ligand such as norepinephrine can stabilize a succession of conformational states with differential intracellular effector protein coupling via different intermolecular contacts between receptor and structural determinants of the agonist.

In summary, the differential binding of GnRH I and II to the human GnRH receptor may selectively stabilize or induce different receptor-active conformations. Distinct chemical moieties at position 8 of GnRH analogues confer different ligand induction or selection of receptor conformations, and therefore, the ligand binding affinity can be manipulated differentially by mutation of the residues located at the receptor allosteric sites. The side chains of Met<sup>132</sup> and Phe<sup>272</sup> form crucial packing interactions that control receptor conformational motion or equilibria and are involved in ligand-induced switching of different receptor conformations through selective binding affin-
ities in the human GnRH receptor, in addition to their common function in stabilization of the 7-TM receptor folding.

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