Role of the Transmembrane Domain 4/Extracellular Loop 2 Junction of the Human Gonadotropin-releasing Hormone Receptor in Ligand Binding and Receptor Conformational Selection*

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Recent crystal structures of G protein-coupled receptors (GPCRs) show the remarkable structural diversity of extracellular loop 2 (ECL2), implying its potential role in ligand binding and ligand-induced receptor conformational selectivity. Here we have applied molecular modeling and mutagenesis studies to the TM4/ECL2 junction (residues Pro174(4.59)–Met180(4.66)) of the human gonadotropin-releasing hormone (GnRH) receptor, which uniquely has one functional type of receptor but two endogenous ligands in humans. We suggest that the above residues assume an α-helical extension of TM4 in which the side chains of Gln174(4.60) and Phe178(4.64) face toward the central ligand binding pocket to make H-bond and aromatic contacts with pGlu1 and Trp3 of both GnRH I and GnRH II, respectively. The interaction between the side chains of Phe178(4.64) of the receptor and Trp3 of the GnRHs was supported by reciprocal mutations of the interacting residues. Interestingly, alanine mutations of Leu175(4.61), Ile177(4.63), and Met180(4.66) decreased mutant receptor affinity for GnRH I but, in contrast, increased affinity for GnRH II. This suggests that these residues make intramolecular or intermolecular contacts with residues of transmembrane (TM) domain 3, TM5, or the phospholipid bilayer, which couple the ligand structure to specific receptor conformational switches. The marked decrease in signaling efficacy of I177A and F178A also indicates that Ile177(4.63) and Phe178(4.64) are important in stabilizing receptor-active conformations. These findings suggest that the TM4/ECL2 junction is crucial for peptide ligand binding and, consequently, for ligand-induced receptor conformational selection.

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† The abbreviations used are: GPCR, G protein-coupled receptor; TM, transmembrane domain; ECL, extracellular loop; GnRH, gonadotropin-releasing hormone; IP, inositol phosphate; AR, adrenergic receptor; PDB, Protein Data Bank; MD, molecular dynamics.

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tide ligands are larger than many biogenic amines, the binding pocket for GnRH is located toward the extracellular side of the receptor rather than buried further down the TM domains. It is likely to involve interactions with the extracellular loops as well as the TM domains. Interestingly, the molecular model of GnRH I docking to the human GnRH receptor suggests a potential interaction between Trp3 of the ligand with a region at the extracellular end of TM4 leading into ECL2 (12).

From the recently obtained crystal structures of the β2-adrenergic receptor (β2-AR) (13), β1-AR (14), A2A adenosine receptor (15), D2 dopamine receptor (16), and CXCR4 receptor (17), it has been shown that the ECL2 region flanked by TM4 and TM5 is highly variable compared with the better known structure of rhodopsin (1). In rhodopsin the ECL2 has two short anti-parallel β-sheets that form a stable cap over the covalently bound ligand 11-cis-retinal. Conversely, the ECL2 regions in β1-AR and β2-AR form an α-helix that is constrained by two disulfide bonds and is more solvent-exposed to allow diffusion of ligands into the binding pocket (Fig. 1A). In contrast, the ECL2 region in the A2A adenosine receptor lacks any prominent secondary structure, with random coils and low electron density in the middle portion of the loop indicating a more mobile structure. Functionally, ECL2 is implicated in ligand binding (18, 19), regulating whether ligands are agonists/antagonists (20, 21), acting as a binding site gate-keeper (22, 23), and constraining the receptor in the inactive conformation by acting as an activation damper (24, 25). Therefore, alanine-scanning mutagenesis of residues Pro173(A.50)–Met180(A.66) (receptor residues are identified by the amino acid sequence number followed in parentheses by Ballesteros and Weinstein numbering, where the position of the most conserved amino acid in the TM domain, X, is designated X.50) was employed to better understand the role of this TM4/ECL2 junction of the GnRH receptor in terms of structure, ligand binding, and function.

Our studies have demonstrated that Phe178(A.64) is a contact site in the human GnRH receptor for GnRH I and GnRH II binding, presumably through aromatic π interactions with Trp3 of GnRH. Molecular modeling indicates that the residues surrounding Phe178(A.64) form an extended α-helical structure from the extracellular end of TM4 into ECL2, with mutagenesis studies suggesting that certain residues play a role in ligand-dependent receptor conformational selection. This may lead to different ligand-induced selective signaling as suggested in previous studies (8, 9).

**EXPERIMENTAL PROCEDURES**

**Materials**—GnRH I (pGlu1-His2-Trp3-Ser4-Tyr5-Gly6-Leu7-Lys8-Gly9-NH2) and GnRH II ([His5-Trp7, Tyr9]GnRH) were purchased from Sigma and Bachem (Bubendorf, Switzerland). Teverelix (Ac-D-Nal1-D-Cpa2-D-Pal3-Ser4-Tyr5-D-Hci6-D-Hci7-OH) and [His5-Trp7, Tyr9]GnRH I (derived from an NMR structure (PDB code 1YY1)) was docked into the active state model according to the previously experimentally identified contact points between GnRH and its receptor (pGlu1 with Asn212(5.39), His2 with Asp98(2.61)/Lys121(3.32), Tyr5/His5 with Tyr290(5.68), and Gly167NH2 with Arg38(1.35)/Asn102(2.65) (2, 3, 10, 12)). The above prepared molecules were inserted into the membrane bilayer. The membrane thickness, centered at Z = 0, was set to 30 Å. The N terminus and loops were built ab initio using MODELLER and/or LOOPER (a molecular mechanics-based algorithm (28)). The MD simulations were performed in implicit membrane using the generalized Born with simple switching (GBSW) method implemented using CHARMM (29). The empty and GnRH I-occupied GnRH receptor structures were first energy-minimized and then subjected to 1-ns MD simulations using a setting similar to that described previously (8) at a temperature of 300 K with time steps of 0.002 ps and SHAKE constraints for all bond lengths involving hydrogen atoms. Harmonic restraints of 5 kcal/mol/Å2 on the receptor backbone atoms of the 7-TM domains were applied to allow small conformational changes but preserve the helical structure of the TM domains.

**Site-directed Mutagenesis and Receptor Expression**—The human GnRH receptor was cloned previously into the pcDNA1 expression vector. Mutant sequences were constructed using a polymerase chain reaction method (30). Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using the Bio-Rad Gene Pulser (Bio-Rad Laboratories) at 230 V, 960 microfarads, with 15 μg of DNA/0.4 cm cuvette (1.5 × 105 cells; 0.7 ml). After transfection, cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin (10, 000 units/ml)/streptomycin (10, 000 μg/ml), and 2 mM glutamine (complete DMEM) in the absence or presence of 1 μM NBI-42902 (a membrane-permeant, nonpeptide GnRH receptor antagonist (26)) at 37 °C in a humidified 5% CO2 atmosphere for 48 h to allow receptor expression prior to binding or functional assays. Cells were washed four times, with each wash lasting for 30 min, with 2% Me2SO and 0.1% BSA/HEPES/DMEM at 37 °C after 28 h of incubation. The cells were further incubated with complete DMEM overnight (~16 h) and then washed again as above before assays were performed (9).

**Ligand Binding Assays**—Radioligand binding assays (31) were performed on intact cells 48 h after transfection. After washing the transfected cells in 12-well culture plates (as...
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Inositol Phosphate (IP) Accumulation Assays—Assays for ligand stimulation of IP production were carried out as described previously (31, 33) using multiwell filtration plates (34). Briefly, transfected cells were seeded onto 24-well plates in the absence or presence of 1 μM NBI-42902. After 28 h, cells were washed as described above and labeled overnight with 1 μCi/ml [myo-3H]inositol in inositol-free DMEM containing 1% dialyzed fetal calf serum. Before the IP assay was conducted, the medium was removed, and cells were washed again as above. Cells were then preincubated with 0.5 ml of buffer (140 mM NaCl, 20 mM HEPES, 8 mM glucose, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mg/ml BSA containing 10 mM LiCl) at 37 °C for 30 min followed by the addition of GnRH peptides for an additional 60 min. This was shown to be within the linear period of the assay. The stimulation was terminated by the removal of the medium and the addition of 200 μl of 10 mM formic acid. The 3H-labeled IPs were isolated from the formic acid. The 3H-labeled IPs were isolated from the formic acid. Radioactivity was counted by γ-spectrometry. All experiments were performed in triplicate and repeated at least three times.

RESULTS

Comparative Modeling of the Human GnRH Receptor and MD Simulations—Initial models of the human GnRH receptor in the inactive and active conformations were built based on the crystal structures of β₂-AR in its inverse agonist binding conformation and opsin in its G protein-interacting conformation. The crystal structures of bovine rhodopsin, the β₂-ARs, and A₂a adenosine receptor reveal the conformational diversity of ECLs, especially ECL2 (35), but all of the above GPCR structures predict ECL2 as part of the ligand binding pocket (36, 37). A βII'-turn formation of GnRHI (derived from a recent NMR structure) was docked into the active state model according to previous experimentally determined interactions (2, 10, 12). In our current GnRH receptor models, the 7-TM domains were constructed based on β₂-AR and opsin, whereas the ECLs and intracellular loops were modeled ab initio by means of a molecular mechanics-based algorithm implemented in LOOPER (28), which often finds near native loop conformations. Because of the length limitation (maximum 25 amino acids) of the LOOPER program and the fact that ECL2 is pinned to the extracellular end of TM3 by a highly conserved disulfide bond (Cys¹¹⁴–Cys¹⁹⁶), the ECL2 of GnRH receptor was built as N (Gly¹⁷²–Cys¹⁹⁶, ECL2a) and C-terminal segments (Cys¹⁹⁶–Ala²⁰⁹, ECL2b) by LOOPER. The models were then subjected to energy minimization and MD simulations in the membrane environments. Our molecular modeling shows that the residues at the N terminus of ECL2 (Gly¹⁷²–Ala²⁰⁹) of the human GnRH receptor form a helical extension from TM4 (Fig. 1B), which leaves ECL2 with a trajectory similar to that of β-ARs. It is noteworthy that Phe¹⁷⁸ faces toward the binding pocket in the current model, but it pointed away from the binding pocket in the rhodopsin-based homology models prior to refinements. To validate our modeling predictions, Ala-scan-ning mutagenesis studies were applied to the residues from Pro⁴⁵⁹ to Met¹⁸⁰⁶ of the human GnRH receptor.

Expression of Human GnRH Receptors in COS-7 Cells—When [¹²⁵I]-[His⁶, D-Tyr⁶]GnRH I, a traditional, labeled peptide agonist in the laboratory, was used for receptor binding assays, we found that Ala mutation of Phe¹⁷⁸ displayed little binding. Because mutations of the GnRH receptor often have less effect on the mutant receptor binding affinity for peptide antagonists (10, 12), [¹²⁵I]-Teverelix, a water-soluble peptide antagonist, was applied to validate the mutant receptor binding. Surprisingly, the mutant F178A displayed a markedly increased receptor cell surface expression level as compared with the wild type (see below). Hence, we used [¹²⁵I]-Teverelix as a labeled ligand for all receptor binding assays. This preliminary result indicated that Ala mutation of Phe¹⁷⁸ led to a markedly decreased receptor binding affinity for peptide agonist but not a decreased receptor expression. In view of the fact that the Ala mutation of Phe¹⁷⁸ caused a large effect on [His⁶, D-Tyr⁶]GnRH I binding affinity, we further mutated this residue to Trp and Leu to examine the role of the aromatic ring in GnRH binding.

Homologous competition binding experiments of [¹²⁵I]-Teverelix on intact cells transiently transfected with wild-type and mutant receptors demonstrated little or only a marginal shift in affinity for any of the mutants compared with the pIC₅₀ of the wild-type receptor, 8.34 ± 0.11 (4.6 nM, Table 1). The mutants P173A, L175A, Y176A, and R179A gave 3–6-fold increases in
the IC$_{50}$ values. Therefore, in most cases, the $B_0$ values of the specific binding measured in the absence of competitive ligands reflect the relative expression levels ($R_{exp}$) of receptors on the cell surface. Overall there were reductions in cell surface expression of the mutant receptors except for the F178A mutant, which displayed approximately a 3-fold increase in cell surface expression (Table 1). The cell surface expression levels of the mutants with greatly reduced $R_{exp}$ (less than one-fourth of the wild type: L175A, F178L, and R179A mutant receptors) were rescued by a non-peptide antagonist, NBI-42902 (26). We propose that this cell-permeant small molecule may also act as a pharmacological chaperone to stabilize the receptor structure, increasing trafficking through the ER, preventing degradation of misfolded receptors in a fashion similar to that of IN3 (8, 9) and other non-peptide GnRH antagonists (38). After rescue, the cell surface expression levels of the mutant receptors were about 3-fold higher relative to their non-rescued mutants (Table 1). Interestingly, cell surface expression of wild-type receptors was also enhanced 164% by NBI-42902 pretreatment, whereas their affinity for Teverelix was unaffected (Table 1).

**Effect of Mutations of the Human GnRH Receptor TM4/ECL2 Function on GnRH Affinity**—Competition binding studies of receptors with Ala mutations of residues from Pro$^{173}$(4.59) to Met$^{180}$(4.66) were performed to investigate any effects on the binding affinity of GnRH I and GnRH II (Table 1). Consistent with previous studies (8–10, 12), GnRH I binds to wild-type receptors expressed in COS-7 cells with high affinity (3.4 nM), which is unaffected by pretreatment with NBI-42902 (Table 1). Ala mutations of residues Leu$^{175}$(4.61), Tyr$^{176}$(4.62), Ile$^{177}$(4.63), and Arg$^{179}$(4.65) of the human GnRH receptor caused a minor (2–3 fold) reduction, whereas mutation of Phe$^{178}$(4.64) to Ala led to a 2738-fold reduction in receptor affinity for GnRH I. Ala mutation of Pro$^{173}$(4.59), Gln$^{174}$(4.60), and Met$^{180}$(4.66) led to a moderate (5–10 fold) reduction in mutant receptor binding affinity for GnRH I.

As described previously (8–10, 12), GnRH II has lower affinity for the human GnRH receptor than GnRH I (52.5 compared with 3.4 nM). The affinity of GnRH II for wild-type receptors was also unaffected by pretreatment with NBI-42902 (Table 1). In contrast to GnRH I, Ala mutations of the residues from Pro$^{173}$(4.59) to Met$^{180}$(4.66) gave differential effects on the receptor binding affinity for GnRH II. Ala mutations of Pro$^{173}$(4.59), Gln$^{174}$(4.60), Tyr$^{176}$(4.62), Phe$^{178}$(4.64), and Arg$^{179}$(4.65) gave a phenotypic effect similar to that of GnRH I on the receptor affinity for GnRH II. However, mutations of Leu$^{175}$(4.61), Ile$^{177}$(4.63), and Met$^{180}$(4.66) led to 2–10 fold increases in affinity for GnRH II but 2–10 fold reductions for GnRH I (Table 1 and Fig. 2). The above results suggest differential functions of these residues in ligand binding and ligand-induced receptor conformational selections.

**Mutation of Phe$^{178}$(4.64) of the Human GnRH Receptor Decreases Affinity for GnRH I and II**—The affinities of GnRH I and GnRH II for the wild-type and Phe$^{178}$(4.64) mutant receptors were determined by competition binding experiments (Fig. 3, A and B). Mutation of Phe$^{178}$(4.64) to Ala, which deletes the side chain beyond the β-carbon, brought about a 2738-fold decrease...
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A reduction in affinity for GnRH I. Mutation of Phe178(4.64) to Leu, which is also lipophilic but lacks the aromatic group, was a more tolerated mutation with only a 57-fold decrease in affinity for GnRH I, whereas mutation of Phe178(4.64) to the bulkier aromatic residue Trp gave only a 6-fold decrease in affinity, indicating that mutations of Phe178(4.64) to Leu and Trp retain part of the original receptor-ligand interactions (Fig. 3A and Table 1). Similar patterns were seen with GnRH II. However, mutations of Phe178(4.64) to Ala, Leu, or Trp increased the IC50 values by 506-, 86-, or 2-fold, respectively (Fig. 3B and Table 1). These results suggest that the aromatic ring of Phe178(4.64) is important for GnRH I and II binding to the receptor.

**Importance of Trp3 in GnRH for Receptor Binding**—Previous molecular modeling and docking of GnRH to the receptor (12) suggests a potential interaction between Trp3 of GnRH and the extracellular end of TM4/ECL2. To determine the role of Trp3 of GnRH in binding with the receptor, Trp3-substituted analogues of GnRH I were examined in competition binding experiments. [His3]GnRH I and [Ala3]GnRH I were unable to displace 125I-Teverelix (data not shown), suggesting that they are low affinity ligands. However, [Phe3]GnRH I could bind to the wild-type receptor but had a 146-fold reduced affinity compared with GnRH I (with IC50 values of 499 nM versus 3.4 nM; Fig. 3C and Table 2). Pretreatment with NBI-42902 had little effect on the affinity of [Phe3]GnRH I (Table 2). To determine whether Phe178(4.64) of the receptor interacts with Trp3 of GnRH I, as suggested by the molecular model (Fig. 4A), the affinity of [Phe3]GnRH I for the Phe178(4.64) mutant receptors was determined (Table 2). Similar to the results with GnRH I, mutation of Phe178(4.64) to Ala caused the largest shift in affinity for [Phe3]GnRH I with a 61-fold increase in the IC50 value to 30500 nM, whereas mutation of Phe178(4.64) to Leu led to a 9-fold reduction in the affinity for [Phe3]GnRH I. Most interestingly, mutation of Phe178(4.64) to Trp produced effectively no shift in affinity for [Phe3]GnRH I, with an IC50 of 672 nM, compared with the IC50 of 499 nM for the wild-type receptor. This demonstrates a partial rescue of binding with reciprocal mutations between Phe and Trp in the receptor and ligand.

**Effects of Mutations of the Human GnRH Receptor TM4/ECL2 Junction on GnRH-elicited IP Responses**—GnRH I and GnRH II elicited robust IP responses from COS-7 cells expressing wild-type GnRH receptors with EC50 values of 3.4 and 20.1 nM (Table 3). The maximal IP responses (E max) for all experiments with wild-type receptors were typically eight times the basal activity. The effects of alanine mutations of residues Pro173(4.59) ➔ Met180(4.66) on the IP responses are summarized in Table 3. The changes in potency of the mutant receptors in mediating IP responses for both GnRH I and GnRH II are closely related to the changes of the mutant receptor binding affinities for the ligands (Fig. 5A). In parallel with the decreased mutant receptor binding affinities, all of the mutants gave

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**TABLE 2**

| Mutant | pIC50 (nM) | pEC50 (nM) | E max |
|--------|------------|------------|-------|
| WT     | 6.45 ± 0.31 (354) | 7.68 ± 0.23 (20.8) | 98 ± 9 |
| F178A  | 4.52 ± 0.08 (30500) | 4.94 ± 0.15 (11400) | 338 ± 174 |
| F178L  | 5.33 ± 0.08 (4680) | 5.59 ± 0.06 (4060) | 178 ± 61 |
| F178W  | 6.17 ± 0.08 (672) | 6.21 ± 0.17 (621) | 209 ± 62 |

* Pretreated with NBI-42902.

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**FIGURE 3.** Competition binding of GnRH peptides to wild-type and Phe178(4.64) mutant receptors. COS-7 cells transfected with wild-type or mutant receptors were preincubated with (F178L) or without (F178A and F178W) NBI-42902 for 48 h and then washed prior to the binding assay. Details are given under “Experimental Procedures.” Normalized competitive binding of GnRH I (A), GnRH II (B), and [Phe3]GnRH I (C) at the wild-type , F174A, F178L, and F178W receptors. Results are representative experiments repeated at least three times with essentially the same results. Points are mean ± S.E. of triplicate measurements.
decreased potency in GnRH I-stimulated IP responses (Tables 1 and 3). A similar change in affinity and potency of GnRH II was observed for most of the mutants, except for L175A, I177A, and M180A, which displayed 2–3-fold increases in receptor binding affinity for GnRH II, but gave little change (L175A), or a 2-fold (M180A) and 5-fold (I177A) decrease in GnRH II potency (Table 3).

It has been shown that there is a near linear relationship between the GnRH receptor binding sites and the maximum IP responses (9, 39). Hence, the relative maximum signaling effi-

TABLE 3
GnRH-elicited IP responses at wild-type and mutant human GnRH receptors

| Mutant       | pEC_{50} | E_{\text{max}} | % WT | pEC_{50} | E_{\text{max}} | % WT |
|--------------|----------|----------------|------|----------|----------------|------|
| WT           | 8.77 ± 0.11 (1.7) | 0.11 (1.7) | 100  | 7.70 ± 0.07 (20.1) | 0.07 (20.1) | 100  |
| WT + NBI-42902 | 8.74 ± 0.08 (1.8) | 0.12 (4.8) | 86 ± 10 | 7.69 ± 0.04 (20.4) | 0.04 (20.4) | 86 ± 10 |
| P173A        | 7.39 ± 0.17 (42.1) | 0.09 (42.1) | 79 ± 8 | 6.10 ± 0.12 (787) | 0.12 (787) | 79 ± 8 |
| Q174A        | 7.56 ± 0.18 (27.7) | 0.10 (27.7) | 81 ± 4 | 6.33 ± 0.12 (464) | 0.12 (464) | 81 ± 4 |
| L175A + NBI-42902 | 8.41 ± 0.06 (3.9) | 0.09 (3.9) | 60 ± 8 | 7.66 ± 0.04 (22.1) | 0.04 (22.1) | 60 ± 8 |
| Y176A        | 7.82 ± 0.11 (15.0) | 0.08 (15.0) | 37 ± 3 | 6.81 ± 0.01 (153) | 0.01 (153) | 37 ± 3 |
| L177A        | 8.50 ± 0.34 (3.1) | 0.03 (3.1) | 17 ± 4 | 7.02 ± 0.12 (94.6) | 0.12 (94.6) | 17 ± 4 |
| F178A        | 6.41 ± 0.10 (386) | 0.07 (386) | 105 ± 23 | 4.71 ± 0.24 (19400) | 0.24 (19400) | 105 ± 23 |
| F178L + NBI-42902 | 6.86 ± 0.11 (139) | 0.09 (139) | 82 ± 18 | 5.44 ± 0.16 (3640) | 0.16 (3640) | 82 ± 18 |
| F178W        | 8.30 ± 0.26 (5.1) | 0.06 (5.1) | 46 ± 8 | 6.86 ± 0.25 (138) | 0.25 (138) | 46 ± 8 |
| R179A + NBI-42902 | 8.53 ± 0.15 (3.0) | 0.10 (3.0) | 59 ± 4 | 7.05 ± 0.17 (90.2) | 0.17 (90.2) | 59 ± 4 |
| M180A        | 7.79 ± 0.08 (16.4) | 0.05 (16.4) | 82 ± 2 | 7.35 ± 0.14 (45.1) | 0.14 (45.1) | 82 ± 2 |

* Pretreated with NBI-42902.
cacy of GnRH can be calculated by normalization of the maximum IP responses to the cell surface receptor binding sites as the receptor population is saturated, and the affinity is not an issue (9, 40). Most mutants, except for I177A and F178A, had little or only a marginal effect on the signaling efficacy of GnRH I (Fig. 5B) and GnRH II. The above results indicate that conformational changes of this region are involved in GnRH-induced receptor activation. The mutant, I177A, was expressed at the cell surface to 77% of wild-type level but gave reduced $E_{\text{max}}$ to 17% that of the wild-type for both GnRH I and GnRH II. The differential effects of Ala mutation of Ile$^{177(4.63)}$ on GnRH affinity and potency, combined with the reduced efficacy of this mutant, indicate that Ile$^{177(4.63)}$ is important for the stabilization of the GnRH-induced receptor-active conformational states and may also act as a determinant of ligand-induced receptor conformational selection.

**Effects of Mutations of Phe$^{178(4.64)}$ on GnRH-elicited IP Responses**—In parallel with the decreased receptor binding affinity, the mutations of Phe$^{178(4.66)}$ also gave decreased potency for both GnRH I and GnRH II (Fig. 5A, insert). The effects of the mutation of Phe$^{178}$ to Ala, Leu, and Trp on the IP responses are shown in Fig. 6. Similar to the outcome on affinity, mutation of Phe$^{178}$ to Ala had the largest effect on potency, with 228- and 962-fold increases in EC$_{50}$ in response to GnRH I (Fig. 6A) and GnRH II (Fig. 6B). Mutation of Phe$^{178}$ to Leu gave intermediate decreases of potency with 82- and 181-fold increases in the EC$_{50}$ values, whereas mutation of Phe$^{178}$ to Trp had only a small effect on potency with 3- and 7-fold increases in the EC$_{50}$ values. Despite the mutant F178A having an increased expression of 324% compared with the wild-type receptors, the maximum responses of GnRH I at this mutant were similar to the wild-type receptors with $E_{\text{max}}$ values of 105%, implying a potential decrease in signaling efficacy (Fig. 5B). However, mutations of Phe$^{178(4.64)}$ to Leu and Trp had little or only a marginal effect on the GnRH I-induced signaling efficacy (Fig. 5B). A similar phenotype was also observed for GnRH II, derived from the calculated $E_{\text{max}}$, which could not be measured accurately because of the solubility limit of the maximum GnRH II concentration (10$^{-5}$ M) to be used for F178A and F178L (Fig. 6B and Table 3).

[Phe$^{3}$]GnRH I-elicited IP responses at the Phe$^{178(4.64)}$ mutant receptors followed a similar pattern to that of GnRH I (Fig. 5C and Table 2). However, although the affinity of [Phe$^{3}$]GnRH I was similar at both the wild-type and F178W mutant receptors, the EC$_{50}$ value of [Phe$^{3}$]GnRH I at the F178W mutant was 8-fold higher than the wild-type receptor. Interestingly, [Phe$^{3}$]GnRH I-stimulated IP responses at F178W mutant receptors (reciprocal mutation between Trp$^{3}$ of GnRH I and Phe$^{178(4.66)}$ of the receptor) gave higher maximal IP responses than that of the wild-type receptor (Fig. 6C), suggesting the reciprocal mutations between the ligand and the receptor can rescue the maximum functional response. Consistent with the previous report (41), [Phe$^{3}$]GnRH I is a partial agonist at wild-type receptors with decreased $E_{\text{max}}$, but the reciprocal mutations of Phe and Trp between the ligand and the receptor converts this partial agonist to a full or nearly full agonist.

**DISCUSSION**

The recently available crystal structures of GPCRs show significant structural diversity of ECL2, which connects TM4 and TM5 and is pinned onto the extracellular end of TM3 (1, 13, 15–17, 42). The roles of the extracellular residues for GPCRs remain poorly defined compared with that of residues in the TMs. Our previous docking models of GnRH I suggest an interaction of Trp$^{3}$ of GnRH I with the extracellular end of TM4 bordering ECL2 (12). The aim of this study was to apply molecular modeling and mutagenesis to the eight residues, Pro$^{173(4.59)}$–Met$^{180(4.66)}$, of the N-terminal region of ECL2 of the human GnRH receptor to define the functional role of the...
targeted residues within the receptor structure. Our molecular modeling suggests that the above residues assume an α-helical extension of TM4, which positions the side chains of Gln\(^{174}\)(4.60) and Phe\(^{178}\)(4.64) toward the central ligand binding pocket while leaving the subsequent ECL2 away from the receptor TM core (Fig. 1B). A similar helical extension was also observed in the crystal structure of the CXCR4 receptor (Fig. 1C) (17).

In the rhodopsin-like family of GPCRs (43), TM4 appears as an outlier of the helical bundle, making extensive contacts with the phospholipid bilayer. Two mutations, L175A and R179A, had a destabilized effect on the receptor structure, reflected by the strongly decreased receptor cell surface expression level to less than 80% of the wild-type level that could be rescued by NBI-42902. In the models, the side chain of Leu\(^{175}\)(4.61) interacted with the side chains of Tyr\(^{119}\)(3.30) and Leu\(^{122}\)(3.33) of TM3. However, Arg\(^{179}\)(4.65) located one helix above Leu\(^{175}\)(4.61) appears to face toward the phospholipid bilayer and may interact with the negatively charged phospholipid head group. In addition, Arg\(^{179}\)(4.65) interacts intramolecularly with the negatively charged Asp\(^{180}\) of ECL2, in which the Ala mutation also gave a reduction in the mutant receptor cell surface expression level similar to that of Arg\(^{179}\)(4.65). Interestingly, Ala mutation of Phe\(^{178}\)(4.64) created a stabilized mutant receptor reflected by a 3-fold increase in cell surface expression level relative to the wild type, whereas mutation of Phe\(^{178}\)(4.64) to Leu destabilized the receptor, giving a 4-fold reduction in cell surface expression. This suggests that the side chain of Phe\(^{178}\)(4.64) may also play a role in the configuration of the receptor structure.

One of the main findings of this study was the identification of a residue, Phe\(^{178}\)(4.64), that is crucial for binding of both GnRH I and GnRH II. Mutation of Phe\(^{178}\)(4.64) to Ala led to a 2737- and 511-fold reduction in the affinity for GnRH I and GnRH II, respectively. Mutation of Phe\(^{178}\)(4.64) to a non-aromatic Leu also reduced receptor binding affinity for GnRH I and GnRH II by 57–86-fold, whereas mutation to Trp gave only a minor reduction in affinities (2–6 fold; Table 1). The much smaller effect of mutation to Leu rather than Ala, which deletes the side chain beyond the β-carbon, indicates that the non-polar side chain of Leu retains some interactions with GnRH peptides, whereas substitution of Phe with aromatic Trp is well tolerated. The minor reduction of F178W in affinity for GnRH I suggests that substitution of Phe\(^{178}\)(4.64) with a bulkier Trp may have steric detrimental effect on agonist-induced receptor conformational changes. This is more obvious from the functional assay results whereby F178W gave a reduced \(E_{\text{max}}\) for both GnRH I and GnRH II to 39–46% that of the wild type (Table 3 and Fig. 6, A and B). These results indicate the importance of Phe\(^{178}\)(4.64) of the GnRH receptor in high affinity ligand binding and receptor activation.

Our new molecular modeling studies revealed that residues Pro\(^{173}\)(4.59)–Met\(^{180}\)(4.66) assume an α-helical extension of TM4 and that Phe\(^{178}\)(4.64) of the receptor may interact with Trp\(^{3}\) of GnRH, consistent with our previous prediction (12). The involvement of Trp\(^{3}\) of GnRH in binding the receptor was confirmed using Trp\(^{3}\)-substituted peptides. [Ala\(^{3}\)]GnRH I and [His\(^{3}\)]GnRH I were unable to bind to the receptor, whereas [Phe\(^{3}\)]GnRH I exhibited a 146-fold reduction in affinity compared with the parent peptide. This is consistent with the finding from a previous structure-activity report that substitution of Trp\(^{3}\) of GnRH with non-aromatic amino acids gives rise to very low activity, whereas some activity is present with Phe substitution and the activity can be substantially increased in [pentamethyl-Phe\(^{3}\)]GnRH I (41). This indicates that Trp\(^{3}\) of GnRH makes a crucial aromatic contact with the receptor, which facilitates other intermolecular interactions between the ligand and receptor.

Trp\(^{3}\) of GnRH analogues were proposed to interact with Trp\(^{280}\)(6.48) of the human GnRH receptor or the equivalent residue of the receptors from different species (44, 45). However, the above proposed interaction not only contradicts the NMR structure of GnRH I (PDB code 1YY1), which was docked into our GnRH receptor model (Fig. 4), but also is inconsistent with our mutational results of Trp\(^{280}\)(6.48), as none of the mutations has significant effect on either ligand binding or receptor activation (46). Here, we have shown that Trp\(^{3}\) of GnRH makes an aromatic contact with Phe\(^{178}\)(4.64). The reduction in affinities for GnRH I seen when Phe\(^{178}\)(4.64) was mutated to Ala or Leu was also observed with [Phe\(^{3}\)]GnRH I but to lesser extent, with affinity reductions of only 61- and 9-fold for [Phe\(^{3}\)]GnRH I compared with 2737- and 57-fold with GnRH I. We propose thatke that side chain of Phe\(^{178}\)(4.64) makes extensive contacts with Trp\(^{3}\) of GnRH but less so for [Phe\(^{3}\)]GnRH I, which possesses a smaller aromatic residue at position 3, reflected by a 146-fold decreased affinity of [Phe\(^{3}\)]GnRH I compared with the wild-type receptor. The much smaller effect of the mutations of Phe\(^{178}\)(4.64) to Ala and Leu on mutant receptor binding affinity for [Phe\(^{3}\)]GnRH I supports a direct contact between Trp\(^{3}\) of GnRH and Phe\(^{178}\)(4.64). A similar phenotype was observed in the mutation of other ligand-receptor-interacting residues (10, 12). When the mutations in the receptor and ligand were reciprocal (Phe\(^{178}\)(4.64)Trp in the receptor and [Phe\(^{3}\)]GnRH I), their affinity was similar to [Phe\(^{3}\)]GnRH I binding to the wild-type receptor. More interestingly, the functional response was partially rescued by the reciprocal mutations between the receptor and ligand, as the maximum IP responses of [Phe\(^{3}\)]GnRH I in the F178W mutant exceeded that of [Phe\(^{3}\)]GnRH I in the wild-type receptor (Fig. 6C). Taken together, these results support our molecular modeling prediction that the side chain of Phe\(^{178}\)(4.64) makes a direct contact with Trp\(^{3}\) of GnRH peptide agonists. This hydrophobic interaction is also important for GnRH-induced receptor activation, as F178A significantly reduced the maximum efficacy of GnRH I (Fig. 5B). However, this residue is not important for the binding of peptide antagonists, as mutations of Phe\(^{178}\)(4.64) had little effect on the mutant receptor binding affinity of Teverelix, which was used as the radiolabeled ligand in our competition binding assays. Interestingly, a cation-π interaction between Trp\(^{186}\)(4.64) of the sphingosine 1-phosphate receptor and the ammonium head group of sphingosine 1-phosphate was proposed (47).

The extracellular portion of TM4 from 4.59 to 4.80 exhibits high structural divergence, implying its importance in receptor activation.

3 R. Forfar and Z. L. Lu, unpublished observation.
functional selectivity. Consistent with other GPCRs (48), mutation of Pro\textsuperscript{173(4.59)} affected ligand affinity for both peptide agonists and antagonist by 5–8-fold, although this residue faces away from the central ligand binding pocket toward the phospholipid bilayer, close to the side of TM5. We suggest that Pro\textsuperscript{173(4.59)} may be involved as a ligand-induced receptor conformational switch (see below). Ala mutation of Gln\textsuperscript{174(4.60)}, next to Pro\textsuperscript{173(4.59)}, specifically decreased the affinity for peptide agonists GnRH I and GnRH II by 8–9-fold but not for the antagonist Teverelix. Our molecular modeling studies suggest that the side chain of Gln\textsuperscript{174(4.60)} makes an H-bond contact with pGlu\textsuperscript{1} of GnRH I and GnRH II (Fig. 4A).

Our other important finding is that, in addition to playing a role in ligand binding, the TM4/ECL2 junction of the GnRH receptor is likely to be involved in ligand binding selectivity via ligand-induced receptor conformational selectivity, a concept we proposed previously (8, 9). Our Ala scanning mutagenesis supported our modeling prediction that the residues Pro\textsuperscript{173(4.59)}–Met\textsuperscript{180(4.66)} form an extended α-helix of TM4. Interestingly, Ala mutations of Ile\textsuperscript{177(4.63)} and Met\textsuperscript{180(4.60)} located on the same face as Pro\textsuperscript{173(5.59)} in the boundary of TM5, and of Leu\textsuperscript{175(4.61)}, facing toward TM3, led to 2–10 fold reductions in affinity for GnRH I but 2–3 fold increases in affinity for GnRH II (Fig. 2). The mutation-induced differential effects on the affinities of GnRH I and GnRH II have led us to propose that the binding of GnRH I and GnRH II stabilizes different receptor-active conformations (8, 9). In our GnRH receptor models, Leu\textsuperscript{175(4.61)} and Ile\textsuperscript{177(4.63)} may make intramolecular contacts (Leu\textsuperscript{175(4.61)} with Tyr\textsuperscript{119(3.30)} and Leu\textsuperscript{122(3.33)} of TM3 and Ile\textsuperscript{177(4.63)} with Phe\textsuperscript{210(5.37)} and Tyr\textsuperscript{211(5.38)} of TM5), whereas Met\textsuperscript{180(4.60)} may make contacts with the phospholipid bilayer. The latter can also regulate receptor conformations (49). Ala mutation of Ile\textsuperscript{177(4.63)} markedly reduced the signaling efficacy of GnRH I (Fig. 5B). The GnRH II-induced IP responses at the I177A mutant receptor also demonstrated a 5-fold decrease in potency compared with wild-type receptors, yet there was a 3-fold increase in affinity in addition to the decreased $E_{\text{max}}$ (Table 3). This indicates that Ile\textsuperscript{177(4.63)} is important in stabilizing receptor-active conformational states. Binding of GnRH I and GnRH II appears to be involved in the differential rearrangements of the above contacts, bringing on distinct receptor-active conformations. Mutation of these key residues to Ala allows the receptor to adopt new conformations that selectively affect (increase or decrease) receptor binding affinity for GnRH I and GnRH II. We therefore propose that the TM4/ECL2 junction of the GnRH receptor may act as a ligand-dependent receptor conformational selector, which is coupled to helix movements and/or ligand-specific conformational switches (37, 50, 51). The discovery of structural elements for ligand and receptor conformational selection could have implications for the development of novel ligands that selectively activate one signaling pathway and bypass others, allowing improved pharmacological specificity and profiles.

In summary, we have identified that the side chain of Phe\textsuperscript{178(4.64)} of the GnRH receptor is important for high affinity binding of both GnRH I and GnRH II. Site-directed mutagenesis of the receptor and substitution of GnRH at position 3 indicates that the aromatic ring of Phe\textsuperscript{178(4.64)} of the GnRH receptor interacts directly with Trp\textsuperscript{3} of GnRH. The molecular docking also reveals an H-bond interaction between Gln\textsuperscript{174(4.60)} of the receptor and pGlu\textsuperscript{1} of GnRH peptides, confirmed by the mutagenesis results. We propose that residues Pro\textsuperscript{173(4.59)}–Met\textsuperscript{180(4.66)} of the GnRH receptor form an extended α-helix. The side chains of Leu\textsuperscript{175(4.61)}, Ile\textsuperscript{177(4.63)} and Met\textsuperscript{180(4.66)} make contact with other TM domains or the phospholipid bilayer, which couple ligand structure to specific receptor conformational switches in the human GnRH receptor, thereby accounting for the ligand-induced selective signaling of GnRH analogues described previously (3).

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