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Structural Determinants for Ligand-Receptor Conformational Selection in a Peptide G Protein-coupled Receptor*

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G protein coupled receptors (GPCRs) modulate the majority of physiological processes through specific intermolecular interactions with structurally diverse ligands and activation of differential intracellular signaling. A key issue yet to be resolved is how GPCRs developed selectivity and diversity of ligand binding and intracellular signaling during evolution. We have explored the structural basis of selectivity of naturally occurring gonadotropin-releasing hormones (GnRHs) from different species in the single functional human GnRH receptor. We found that the highly variable amino acids in position 8 of the naturally occurring isoforms of GnRH play a discriminating role in selecting receptor conformational states. The human GnRH receptor has a higher affinity for the cognate GnRH I but a lower affinity for GnRH II and GnRHs from other species possessing substitutions for Arg^{8}. The latter were partial agonists in the human GnRH receptor. Mutation of Asn$^{7.45}$ in transmembrane domain (TM) 7 had no effect on GnRH I affinity but specifically increased affinity for other GnRHs and converted them to full agonists. Using molecular modeling and site-directed mutagenesis, we demonstrated that the highly conserved Asn$^{7.45}$ makes intramolecular interactions with a highly conserved Cys$^{6.47}$ in TM 6, suggesting that disruption of this intramolecular interaction induces a receptor conformational change which allosterically alters ligand specific binding sites and changes ligand selectivity and signaling efficacy. These results reveal GnRH ligand and receptor structural elements for conformational selection, and support co-evolution of GnRH ligand and receptor conformations.

G protein-coupled receptors (GPCRs)[2] constitute the largest family of signaling molecules in the mammalian genome. Over 800 GPCRs have been identified in the human genome (1). GPCRs bind a variety of structurally diverse ligands ranging from photons, ions, bioamines, lipids, nucleotides, and peptides to large polypeptide hormones at the extracellular surface. They activate a number of different intracellular effector proteins including G proteins or non-G proteins which participate in the majority of physiological processes. About 50% of current clinical drugs target GPCRs, and these receptors thus remain a major avenue for future drug development.

The 7-TM GPCRs are presumed to have evolved from a common ancestor (2), and are thought to share important structural and functional characteristics (3–5), but have undergone specialization to mirror the nature of their cognate ligands. It is not clear, however, how GPCRs developed ligand selectivity to cognate ligands during evolution. We hypothesized that GPCR binding specificity is not only determined by ligand contact residues but also by receptor conformations specified by receptor intramolecular interactions. There is also increasing evidence that ligands can selectively stabilize different receptor active conformations thereby mediating ligand-induced selective signaling (LiSS) (6–9). Selection of signaling by analogues clearly has potential for future drug development with novel activities and reduced side-effects. Hence, delineation of receptor allosteric communication networks which couple selective ligand structural elements to specific receptor conformational changes is fundamental to understanding LiSS (10).

Although only one functional member of the GnRH receptor and two isoforms of GnRH ligands (GnRH I and GnRH II) (Fig. 1*A*) exist in humans, coexistence of multiple types of GnRH ligands and receptors was identified in the majority of chordate and vertebrate species (6). The human GnRH receptor has high affinity for GnRH I (Fig. 1, *A* and *B*) but a 10-fold lower affinity for GnRH II, which differs by three amino acids (Fig. 1*A*). In contrast, the marmoset and macaque type II GnRH receptors have a high affinity for GnRH II but a much lower affinity for GnRH I (11). The human type II GnRH receptor has been silenced by stop codons and frameshift deletions (6, 12), suggesting that the single subtype of the human GnRH receptor mediates actions of both ligands. The ligand binding sites identified in the human GnRH receptor for the conserved N- and C-terminal amino acids of GnRHs (Fig. 1*C*) are almost fully conserved among all GnRH receptors (6). This implies that the evolutionarily variable residues in position 5, 7, and 8 of the jawed vertebrate GnRHs confer ligand binding and functional selectivity (6, 13). We have recently revealed that mutations in the single subtype of the human GnRH receptor remote from

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[2] The abbreviations used are: GPCRs, G protein-coupled receptors; TM, transmembrane domain; GnRH, gonadotropin-releasing hormone; LiSS, ligand-induced selective signaling; PhI, phosphoinositide; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; mAChR, muscarinic acetylcholine receptor; PDB, Protein Data Bank.

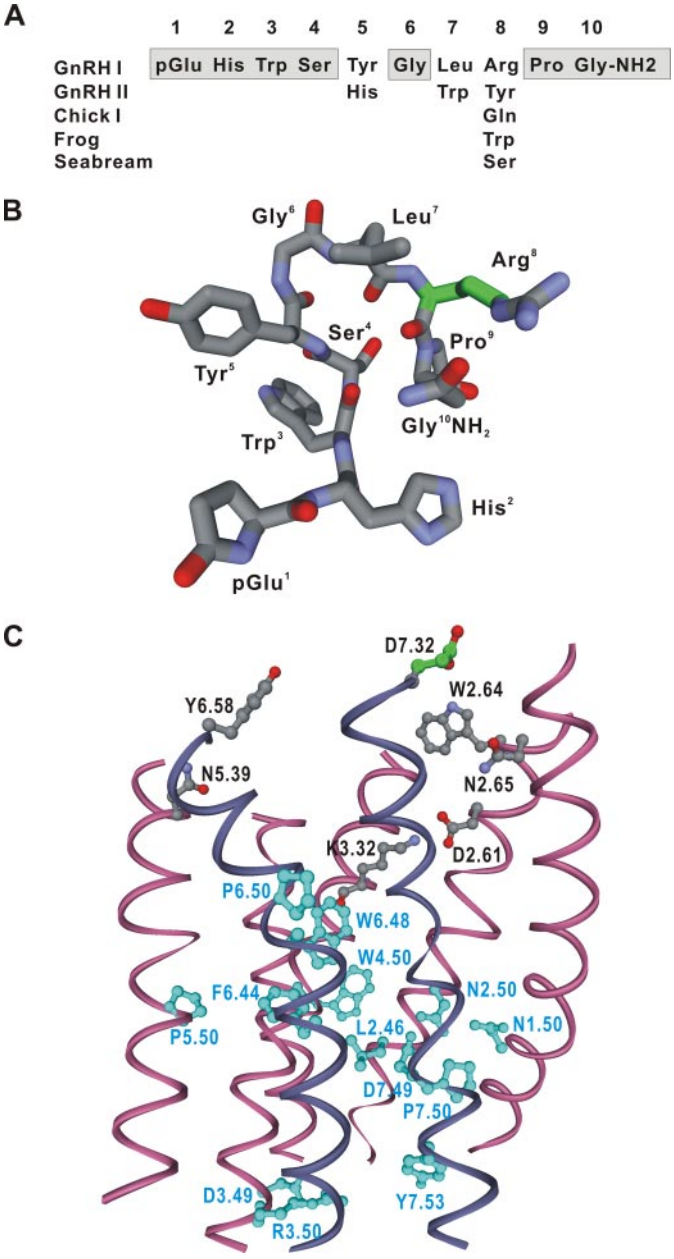

FIGURE 1. **Structures of GnRHs and the human GnRH receptor.** *A*, primary structures of GnRH I, GnRH II, and GnRHs from other species with Arg^8 substitution. The N-terminal amino acids ($pGlu^1$-His^2-Trp^3-Ser^4) and C-terminal amino acids (Pro^9-Gly^{10}-NH_2) of the decapeptide ligands are highly conserved over 600 million years of the chordate evolution and are important for ligand binding (see below) and receptor activation (6). *B*, an NMR structure of GnRH I (PDB code: 1YY1) showing a βII′ conformation. *C*, a homology model of the 7-TM domains of the human GnRH receptor in the inactive state. The ligand binding residues for GnRH I are labeled in *black*. $pGlu^1$ is proposed to interact with $Asn^{5.39}$; His^2 with $Asp^{2.61}$/$Lys^{3.32}$; Tyr^5 with $Tyr^{6.58}$; Arg^8 with $Asp^{7.32}$; and $Gly^{10}NH_2$ with $Asn^{2.65}$. These interactions can all be satisfactorily accommodated when GnRH in the βII′ conformation is docked to the receptor (not shown for clarity) (6, 10). There is no intermolecular interaction between Tyr^8 of GnRH II and $Asp^{7.32}$ (*green*) that interacts with Arg^8 of GnRH I (*green*) (41). The most highly conserved (80–100%) residues in the 7 TM domains among rhodopsin-like family of GPCRs are colored *blue*.

ligand binding sites have differential effects on the binding affinity of the two endogenous ligands (10), implying differential ligand-receptor conformational selections. Here we present studies supporting the hypothesis that changes in the GnRH receptor conformation occurred coincident with amino acid changes of GnRH ligands, which modify ligand structure/conformation, *i.e.* there was a reciprocal structural/conformational selection between ligands and receptors during evolution.

Our preliminary screening mutagenesis of putative TM interacting residues revealed candidates which appeared functionally important for ligand binding and receptor activation for further studies. Here we report that the highly conserved $Asn^{7.45}$ in TM 7 makes intramolecular interactions with a highly conserved $Cys^{6.47}$ in TM 6 which plays an important role in control of receptor conformational states of the human GnRH receptor, involved in binding selectivity and signaling efficacy of GnRH analogues, which differ by only one amino acid in position 8.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Receptor Expression—A PCR method was used to construct mutant receptors of N7.45A, C6.47A, and C6.47Y. The mutant receptor DNAs cloned into the pcDNA I expression vector were validated by di-deoxy 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 μg of DNA/0.4-cm cuvette (1×10^7 cells; 0.7 ml). After transfection, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics, and 2 mM glutamine (complete DMEM) in the absence or presence of 1 μM IN3 (a membrane-permeant non-peptide GnRH receptor antagonist). Cells were washed four times, each wash lasted for 30 min, with 2% Me_2SO, 0.1% BSA/Hepes/DMEM at 37 °C after a 48-h incubation. The cells were then incubated with complete DMEM overnight (~18 h), and were washed again as above prior to assaying. This allowed complete removal of IN3 from pretreated cells before assaying (10).

Ligand Binding—Radioligand binding assays were performed on intact cells 72 h after transfection (10). Transfected cells in 12-well culture plates were washed as above and then incubated with ^{125}I-[His^5,D-Tyr^6]GnRH (100,000 cpm/0.5 ml/well) and various concentrations of unlabeled GnRH ligands in 0.1% BSA/Hepes/DMEM for 4 h at 4 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled GnRH I. After incubation, the cells were rapidly washed with 1 ml ice-cold phosphate-buffered saline (pH 7.4) twice and solubilized in 0.5 ml of 0.1 M NaOH. Radioactivity was counted by γ-spectrometry. All experiments were performed in triplicate and repeated at least three times.

PhI Hydrolysis—Assays for ligand stimulation of inositol phosphate production were previously described (10). Transfected COS-7 cells were seeded onto 12-well plates in the absence or presence of 1 μM concentration of IN3. After 48 h, IN3 was washed off as above and labeled overnight with 1 μCi/ml myo-D-[^{3}H]inositol in inositol-free DMEM containing 1% dialyzed fetal calf serum. Before conducting PhI assay, the cells were washed again as above. Cells were then preincubated with 0.5 ml of buffer A (140 mM NaCl, 20 mM HEPES, 8 mM glucose, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml BSA) containing 10 mM LiCl at 37 °C for 30 min, followed by addition of GnRHs for an additional 30 min. The stimulation was terminated by removal of the medium and addition 0.5 ml of 10 mM

formic acid. The [^{3}H]inositol phosphates were isolated from the formic acid extracts using Dowex AG 1-X8 ion exchange resin and collected with 1 M ammonium formate containing 0.1 M formic acid and quantified by liquid scintillation counting.

Comparative Modeling of the GnRH Receptor and Molecular Dynamics (MD) Simulations—Initial homology models of the human GnRH receptor in the inactive or active state were built on the crystal structures of bovine rhodopsin (14, 15) (PDB codes 1U19 and 2I37) using a similar method as previously described (10, 16, 17), with the "MODELLER" module within DS Modeling (version 1.6, Accelrys, San Diego, CA). Although there are concerns in regard to the use of the rhodopsin structure as a template to model other GPCRs due to low sequence similarities among the rhodopsin-like family of GPCRs, sequence analysis suggested that GPCRs share a similar arrangement of the 7-TM domains. This is also because of the presence of a few, but significantly conserved residues and motifs in each of the 7-TM domains (4, 18, 19). The amino acids possessing 80–100% conservation are Asn$^{1.50}$, Leu$^{2.46}$, Asp/Asn$^{2.50}$, Cys$^{3.25}$, Glu/Asp$^{3.49}$, Arg$^{3.50}$, Trp$^{4.50}$, Phe$^{6.44}$, Trp/Phe$^{6.48}$, Pro$^{6.50}$, Pro$^{7.50}$, and Tyr$^{7.53}$ (4, 18) (Fig. 1*C*). Molecular modeling of GPCRs has recently been extensively reviewed by Fanelli and De Benedetti (18) in which they suggest that comparative modeling of the 7-TM bundle of GPCRs using rhodopsin structure as a template is likely to produce reliable results. The use of rhodopsin to comparatively model the mammalian GnRH receptor has been validated by the authors (6, 10) and also by independent groups using extensive site-directed mutagenesis studies and peptide (20, 21) and non-peptide docking supported by 76 mutations (22). The MODELLER-generated models with the highest values of the three-dimensional profile score, computed by means of the module of "verify protein" in the DS modeling, were selected for further refinement. The models incorporating all previously experimentally identified amino acid interactions were subjected to *in vacuo* energy minimization and MD simulations by means of the CHARMM program (23), using a setup similar to that described for the modeling of the closely-related oxytocin receptor (24). Harmonic restraints of 2.5 kcal/mol/Å^2 on the receptor backbone atoms except for the second extracellular loop and the experimentally identified disulfide-bonded N-terminal domain (25) were applied to allow small conformational changes of the receptor during the MD simulations without loss of the overall receptor topology (26). Minimizations were carried out by using 1500 steps of steepest descent followed by a conjugate gradient minimization, until the root-mean-square gradients was less than 0.001 kcal/mol/Å. A distance-dependent dielectric term ($\epsilon = 4r$) and a 12 Å non-bonded cut-off distance were chosen. The system was heated to 300 K with 5 K rise, every 100 steps per 6000 steps, by randomly assigning velocities from the Gaussian distribution. After heating, the system was allowed to equilibrate for 34 ps. Finally, a production phase was carried out involving a 100 ps simulation using an NVT ensemble at 300 K, with a time steps of 1 fs. The models were minimized as above and used for the comparative analysis.

Materials—GnRH I and GnRH II were purchased from Sigma and Bachem. [His^5,D-Tyr^6]GnRH, [His^5]GnRH, [Trp^7]GnRH, [Tyr^8]GnRH, chicken GnRH I ([Gln^8]GnRH), frog GnRH ([Trp^8]GnRH), and seabream GnRH ([Ser^8]GnRH) were synthesized in our laboratory as previously described (10). IN3, (2*S*)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxoethyl]-2-(3,5-dimethylphenyl)-*1H*-indol-3-yl]-*N*-(2-pyridin-4-ylethyl) propan-1-amine was obtained from MERCK (10).

Data Analysis—Binding curves were fitted to the Hill equation or to the one-site model using Sigmaplot 9.0 (SPSS) yielding an IC_{50} value. The receptor expression levels were calculated as percentage of the wild-type control included in each transfection. PhI dose-response curves were fitted to a four-parameter logistic function, yielding a basal activity, a maximum response (E_{max}), an EC_{50} value and a slope factor.

RESULTS

Mutation of Asn$^{7.45}$ to Ala Induces a Receptor Conformational Instability That Is Rescued by a Membrane-permeant Non-peptide GnRH Antagonist—Mutation of Asn$^{7.45}$ to Ala completely abolished receptor expression on cell surfaces when transiently transfected into COS-7 cells, as measured by ligand binding assays with a hydrophilic peptide agonist ^{125}I-[His^5,D-Tyr^6]GnRH on intact cells (Fig. 2*A*, *inset*). The mutant receptor also gave undetectable PhI responses.[3] To investigate if a cell membrane-permeant non-peptide GnRH antagonist, IN3, could rescue the mutant receptor by chaperoning it to the cell surface, the wild-type and mutant receptor transfected COS-7 cells were preincubated with 1 μM IN3 for 48 h. After washing out the IN3 as described previously (10), the expression level of the mutant receptor was measured by radioligand binding giving 40% of the wild-type level (Fig. 2*A*, *inset*). The action of the chaperone ligand IN3 on receptor expression was observed not only in the mutant GnRH receptor but also in the wild-type receptor (Fig. 2*A*, *inset*). Pretreatment of the receptor-transfected COS-7 cells with IN3 had no effect on GnRH ligand binding affinity in the wild-type receptor after washout (Fig. 2*A*). This result suggests that the membrane-permeant non-peptide GnRH antagonist IN3 can bind with the newly synthesized receptor inside of cells, and alter receptor conformations from an unstable to a more stable state.

Effect of Mutation of Asn$^{7.45}$ to Ala on Receptor Binding Affinity of GnRH Analogues—The mutation N7.45A had little effect on binding affinity of GnRH I (Fig. 2*B*) and [His^5,D-Tyr^6]GnRH, which we conventionally use as a radioligand (Fig. 2*A*), but increased affinity of the mutant receptor for GnRH II by 8-fold, as compared with the affinity of the wild-type receptor for GnRH II (Fig. 2*B* and Table 1). There are three amino acid differences between GnRH I and GnRH II in which Tyr^5, Leu^7, and Arg^8 of GnRH I are replaced by His^5, Trp^7, and Tyr^8 in GnRH II (Fig. 1*A*). We examined the effect of mutation of Asn$^{7.45}$ to Ala on the binding affinity of GnRH I analogues with single amino acid substitutions (His^5, Trp^7, or Tyr^8). The mutation N7.45A had no significant effect on the receptor binding affinity for [His^5]GnRH and [Trp^7]GnRH, but increased receptor binding affinity for [Tyr^8]GnRH by 14-fold (Fig. 2*C* and Table 1).

[3] Z. L. Lu, unpublished observation.

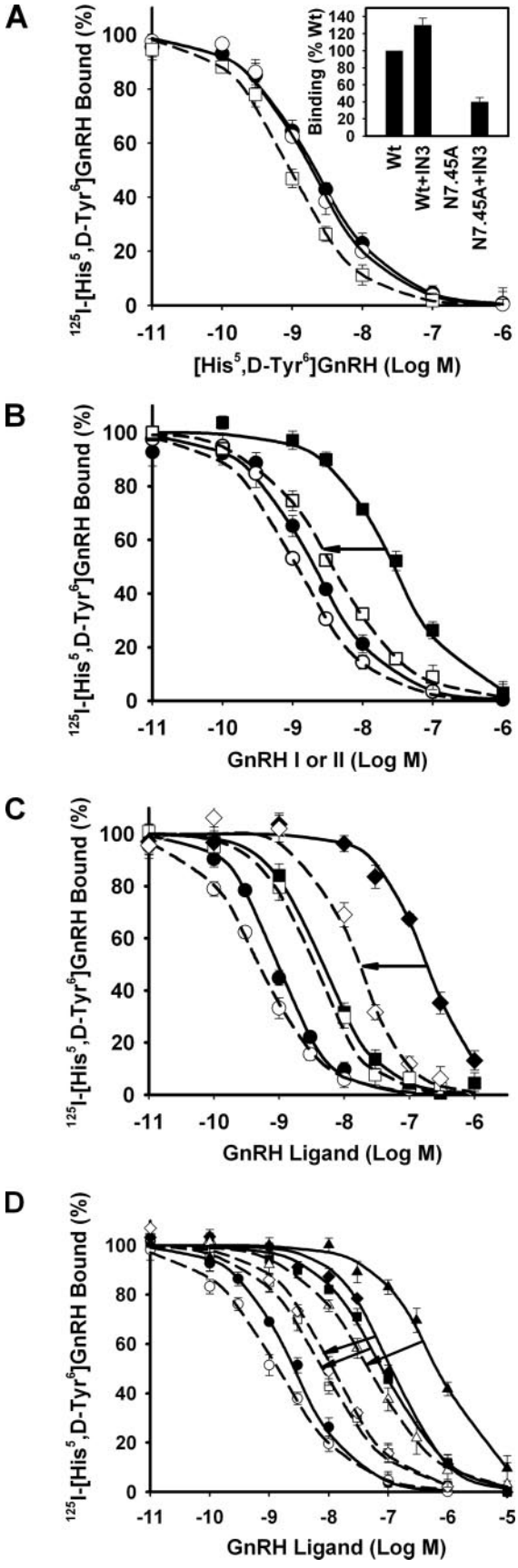

FIGURE 2. **Competitive binding of GnRH analogues at wild-type and N7.45A mutant receptors.** The wild-type and N7.45A mutant receptor transfected COS-7 cells were preincubated with or without 1 μM IN3 for 48 h. The IN3 was washed off prior to binding assays. *A*, homologous binding of [His^5,D-Tyr^6]GnRH, mutation of Asn$^{7.45}$ to Ala led to an undetectable GnRH ligand binding, which was rescued by IN3 preincubation of the transfected cells (*inset*). There was no difference in the GnRH binding affinity between the IN3 pretreated and the untreated cells of wild-type receptors. ●, wild-type; ○, wild-type with IN3 pretreatment; □, N7.45A with IN3 pretreatment. *B*, competitive binding of GnRH I (● and ○) and GnRH II (■ and □) at the wild-type and N7.45A mutant receptors. *C*, competitive binding of GnRH I analogues with single amino acid substitution of GnRH II in GnRH I at position 5, 7, or 8 at the wild-type and N7.45A mutant receptors, [His^5]GnRH (● and ○); [Trp^7]GnRH (■ and □); [Tyr^8]GnRH (♦ and ◇). *D*, binding of GnRHs from different species with Arg^8-substitution. GnRH I (● and ○); chicken GnRH I ([Gln^8]GnRH) (■ and □); frog GnRH ([Trp^8]GnRH) (♦ and ◇); seabream GnRH ([Ser^8]GnRH) (▲ and △). - - - - - -, wild-type; - - - -, N7.45A. *Arrows* indicate shift in affinity of the mutant receptor for Arg^8-substituted GnRHs at the N7.45A mutant receptor.

The binding of GnRH I, which contains Arg^8, to the wild-type human GnRH receptor was characterized by one-site binding isotherms (n_H = 0.86) with an IC_{50} at 2.6 nM. The binding affinities of the wild-type human GnRH receptor for GnRHs from other species which only have one amino acid difference in position 8, including chicken GnRH I ([Gln^8]GnRH), frog GnRH ([Trp^8]GnRH), and seabream GnRH ([Ser^8]GnRH) (Fig. 1*A*) were much lower than GnRH I with IC_{50} values at 80 nM, 111 nM, and 684 nM (Fig. 2*D* and Table 1). Interestingly, mutation of Asn$^{7.45}$ to Ala also increased the mutant receptor affinity for chicken GnRH I ([Gln^8]GnRH), frog GnRH ([Trp^8]GnRH), and seabream GnRH ([Ser^8]GnRH) by 10-fold as was seen for [Tyr^8]GnRH (Fig. 2*D* and Table 1).

Effect of Mutation of Asn$^{7.45}$ to Ala on the GnRH Ligand-elicited PhI Turnover—When the N7.45A mutant receptor expression was rescued, GnRHs were able to elicit a functional response. Interestingly, GnRH I and GnRH II elicited a maximum phosphoinositide (PhI) response in the N7.45A mutant receptor of 110–122% that of the wild-type receptor (Fig. 3*A*) even though its expression was only 40% of the wild-type level, suggesting increased signaling efficacy. In parallel with the increased GnRH II binding affinity, the mutant receptor was also more potent in eliciting PhI response, leading to a 3-fold decrease in the EC_{50} value as compared with the wild-type receptor (Fig. 3*A* and Table 2).

Activation of GnRH receptors from different species by their cognate ligands can selectively couple to different members of the G protein family such as $G_{q/11}$, G_s, and $G_{i/o}$. The human GnRH receptor preferentially couples to $G_{q/11}$, although coupling to G_s and $G_{i/o}$ was reported in certain cell types (6). GnRH I elicits a robust PhI response from COS-7 cells transfected with the human GnRH receptor, giving a maximum response five times the basal activity and an EC_{50} value of 0.2 nM (Fig. 3, *A* and *B*). Chicken GnRH I ([Gln^8]GnRH), frog GnRH I ([Trp^8]GnRH), and seabream GnRH I ([Ser^8]GnRH) were able to activate $G_{q/11}$-mediated PhI turnover in the human GnRH receptor with increased EC_{50} values (Table 2). However, all of them acted as partial agonists giving reduced E_{max} responses at 44–83% of that elicited by GnRH I (Fig. 3*B* and Table 2). Most interestingly, all of them became full agonists in the N7.45A mutant receptor, even though the expression is only 40% of the wild-type, yielding E_{max} responses equal to or greater than that elicited by GnRH I in the wild-type receptor with little or only a small effect on the signaling potency (EC_{50} value) (Fig. 3*B* and Table 2).

TABLE 1

The binding of GnRH analogues to wild-type and mutant human GnRH receptors

Ligand binding were conducted as described under "Experimental Procedures." Values are mean ± S.E. from three or more independent experiments. The competing radioligand was ^{125}I-[His^5,D-Tyr^6]GnRH.

	Binding affinity (IC_{50})					
	GnRH I	GnRH II	[Tyr^8]GnRH	[Gln^8]GnRH	[Trp^8]GnRH	[Ser^8]GnRH
	nM	nM	nM	nM	nM	nM
Wild-type	2.6 ± 0.2	29 ± 2	222 ± 13	80 ± 6	111 ± 8	684 ± 43
Wild-type + IN3[a]	2.5 ± 0.3	28 ± 3	211 ± 23	94 ± 10	107 ± 5	621 ± 30
N7.45A[a]	1.5 ± 0.2	3.7 ± 0.5	16 ± 3	8.1 ± 2.2	12 ± 1	48 ± 3
C6.47A[a]	3.2 ± 0.1	9.6 ± 0.7				
C6.47Y[a]	2.8 ± 0.3	5.2 ± 0.6				

[a] With IN3 pretreatment.

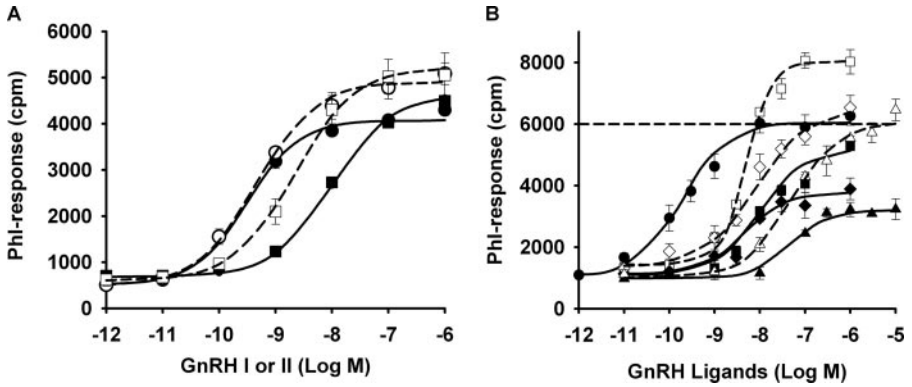

FIGURE 3. **GnRHs elicited PhI responses at wild-type and N7. 45A mutant receptors.** *A*, GnRH I (● and ○) and GnRH II (■ and □) stimulated PhI responses. *B*, PhI responses elicited by GnRHs from other species with Arg^8-substitution, ●, GnRH I; ■ and □, chicken GnRH I ([Gln^8]GnRH); ◆ and ◇, frog GnRH ([Trp^8]GnRH); ▲ and △, seabream GnRH ([Ser^8]GnRH). - - - - - -, wild-type; - - - -, N7.45A.

Identification of the Asn$^{7.45}$-interacting Residue in the Human GnRH Receptor—In the refined GnRH receptor homology model in the inactive state, built on the crystal structure of bovine rhodopsin in the dark state (14), Asn$^{7.45}$ faces toward Cys$^{6.47}$. Mutation of Cys$^{6.47}$ to Ala or to Tyr (a naturally occurring mutant in human infertility) led to undetectable ligand binding and PhI responses. This phenotype is similar to that of N7.45A. The mutant receptor expression of C6.47A and C6.47Y at the cell surface was rescued by IN3 pretreatment, giving 38 and 18% that of the wild-type (Fig. 4*B*, *inset*). As with N7.45A, the mutations C6.47A and C6.47Y led to 3–5-fold increases in GnRH II binding affinity as compared with the wild-type receptor (Fig. 4*A* and Table 1). Both mutant receptors showed an increased signaling efficacy for GnRH II, yielding E_{max} responses greater than that of the wild-type with decreased EC_{50} values (Fig. 4*B* and Table 2).

DISCUSSION

GPCRs for peptide ligands are frequently present as a variety of subtypes encoded by distinct genes. The presence of multiple isoforms of peptide ligands which preferentially bind to the cognate receptor subtypes implies co-evolution of peptide ligands and receptors. The conventional wisdom in regard to ligand binding and effector coupling selectivity is that receptor subtypes incorporate changes in binding site residues for ligand selectivity and changes in intracellular domains for coupling selectivity. Our studies here have shown an important role of receptor conformations in determining ligand binding selectivity and signaling efficacy in the human GnRH receptor.

Mutation of Asn$^{7.45}$ to Ala led to undetectable ligand binding and PhI responses, which were rescued by a membrane-permeant, non-peptide GnRH antagonist, IN3 (Fig. 2, *A* and *B*), suggesting that the side chain of Asn$^{7.45}$ makes intramolecular interactions which are important for receptor folding. Disruption of the intramolecular interactions appears to cause receptor conformational changes which can be modulated by the pharmacological chaperon, facilitating mutant receptor trafficking to the cell surface and indicating a ligand influence on receptor conformations. The pharmacological chaperoning effects of IN3 have been extensively studied by Conn and co-workers (27–30) in which IN3 has been shown to increase protein expression levels of mutant receptors on the cell membranes and to facilitate trafficking of the misfolded mutant receptors from endoplasmic reticulum (ER) to the cell surfaces. Membrane-permeant antagonists have also been extensively used to rescue receptor expression of structurally unstable mutants on cell surfaces in other GPCRs (31–39).

The mutation N7.45A had no or only a marginal effect on the receptor binding affinity for GnRH I, [His^5]GnRH and [Trp^7]GnRH, but markedly increased receptor binding affinity for GnRH II and [Tyr^8]GnRH (Fig. 2, *B* and *C*). This is consistent with our previous suggestion that Tyr^8 in GnRH II is involved in receptor conformational selection (10). An allosteric effect of Asn$^{7.45}$ mutation on ligand binding affinity was also observed in the M_1 muscarinic acetylcholine receptor (mAChR), which increased receptor binding affinity not only for agonists, but also for certain antagonists (16).

The conformation of the wild-type human GnRH receptor has apparently evolved for high affinity binding to mammalian GnRH I, which contains Arg^8 and therefore has a lower affinity for the second endogenous ligand, GnRH II, possessing Tyr^8 (Fig. 2*B*) and GnRHs from other species, which only have one amino acid difference in position 8, including chicken GnRH I ([Gln^8]GnRH), frog GnRH ([Trp^8]GnRH), and seabream GnRH ([Ser^8]GnRH) (Fig. 2*D*). Most interestingly, mutation of Asn$^{7.45}$ to Ala also markedly increased the

TABLE 2

Receptor expression and functional responses of the wild-type and mutant GnRH receptors

Measurements of the receptor expression levels on cell surfaces (B_{max}) using radioligand binding assay on whole cells, and PhI responses were conducted as described under "Experimental Procedures." The B_{max} and the maximum PhI responses (E_{max}) were expressed relative to a wild-type control in each transfection. Values are mean ± S.E. from three or more independent experiments.

		PhI responses									
	B_{max}	GnRH I		GnRH II		[Gln^8]GnRH		[Trp^8]GnRH		[Ser^8]GnRH	
		EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
	%	nM	%	nM	%	nM	%	nM	%	nM	%
Wild-type	100	0.2 ± 0.1	100	6.9 ± 1.4	100	10 ± 2	83 ± 9	4.6 ± 0.6	53 ± 6	48 ± 6	44 ± 5
N7.45A[a]	40 ± 4	0.2 ± 0.1	110 ± 13	2.2 ± 0.4	122 ± 7	5.1 ± 1.7	132 ± 12	8.5 ± 0.3	104 ± 5	50 ± 4	102 ± 4
C6.47A[a]	38 ± 3	0.1 ± 0.1	122 ± 8	1.8 ± 0.9	131 ± 9						
C6.47Y[a]	18 ± 3	0.2 ± 0.1	102 ± 7	2.2 ± 1.2	115 ± 6						

[a] With IN3 pretreatment.

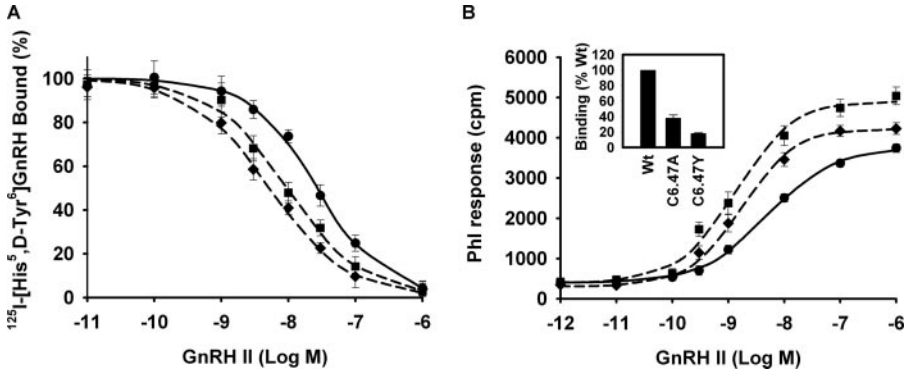

FIGURE 4. **Binding and PhI assays of GnRH II at wild-type, C6.47A and C6.47Y mutant receptors.** *A*, competitive binding showing an increased affinity of the mutant receptors for GnRH II. *B*, PhI assay of GnRH II. ●, wild-type; ■, C6.47A; ◆, C6.47Y. *Inset* shows the mutant receptor expressions relative to the wild-type level.

mutant receptor affinity for chicken GnRH I, frog GnRH, and seabream GnRH, as that of GnRH II and [Tyr^8]GnRH. These results indicate an important role of receptor conformations in determining ligand binding selectivity developed during evolution, which can be manipulated by mutation-induced receptor conformational changes without alteration of the side chains of the ligand binding sites. These results support our proposal of co-evolution of ligand-receptor conformations. A single amino acid in position 8 of GnRHs acts as a structural determinant for receptor binding selectivity. We propose that ligands might exert a directive role in the evolution of receptor structure including primary and tertiary structures accounting for the origin of receptor specificity and diversity, consistent with the proposal that neuropeptide genes arose before the corresponding receptor genes and that their receptors might have evolved as targets for extant peptide ligands (40).

Chicken GnRH I ([Gln^8]GnRH), frog GnRH I ([Trp^8]GnRH), and seabream GnRH I ([Ser^8]GnRH), whose side chains at position 8 appear to be able to make H-bonds with receptor contact residues, act as partial agonists in the wild-type human GnRH receptor (Fig. 3*B*). Most interestingly, all of them became full agonists in the N7.45A mutant receptor. We propose that GnRHs from different species that differ by only one amino acid in position 8 can selectively stabilize different receptor active conformations with different signaling efficacy. We envisage this occurs through common and differential receptor intramolecular and receptor-ligand intermolecular interactions. Arg^8 of GnRH I (Fig. 1*B*) has been shown to interact with Asp$^{7.32}$ (Fig. 1*C*), but this is not the case for Tyr^8 of GnRH II (41). There is increasing evidence that different ligands can induce different receptor conformations with different signaling capability (42, 43) and in such a way, some partial agonists and even some inverse agonists become full agonists in activating different signaling pathways (44–46). Interestingly, agonists differing by only a single hydroxyl group can lead to differential signaling in a *Drosophila* octopamine/tyramine receptor (47). Apparently, high affinity ligand binding can be achieved not only by optimization of ligand binding sites, but also by inducing ligand-specific receptor intramolecular contacts that stabilize each binding partner (48), hence creating a ligand-specific receptor conformation which can be facilitated by weakening the receptor constraint networks. Recent studies have clearly shown that partial agonists stabilize a receptor conformation differing from that of full agonists in other GPCRs (49–51).

The marked loss of receptor binding (undetectable) without loss of binding affinity (determined after rescue with IN3) and the increases of signaling efficacy of the mutation of Asn$^{7.45}$ to Ala indicate that the side chain of Asn$^{7.45}$ makes intramolecular interactions, forming part of the receptor constraint network. To identify the residues interacting with Asn$^{7.45}$, we mutated residues (Glu$^{2.53}$, Ser$^{3.35}$, Cys$^{6.47}$, Trp$^{6.48}$ (52), Thr$^{6.49}$, and Asp$^{7.49}$), which potentially interact with Asn$^{7.45}$, predicted by molecular modeling. Only Cys$^{6.47}$ mutants gave a similar phenotype of unchanged binding affinity for GnRH I but increased binding affinity for GnRH II (Fig. 4) to the N7.45A mutant. Our molecular modeling has shown that the side chain of Asn$^{7.45}$ can make an intramolecular interaction with Cys$^{6.47}$ in the inactive state of the receptor (Fig. 5, *A* and *B*). The model was validated by accommodation of all experimentally identified receptor intramolecular interactions (10, 53–56) and the experimentally identified GnRH ligand-receptor intermolecular interactions (Fig. 1*C*) (6, 10). We therefore propose that the residues of Asn$^{7.45}$ and Cys$^{7.49}$ form part of the intramolecular constraint network involved in the stabilization of different

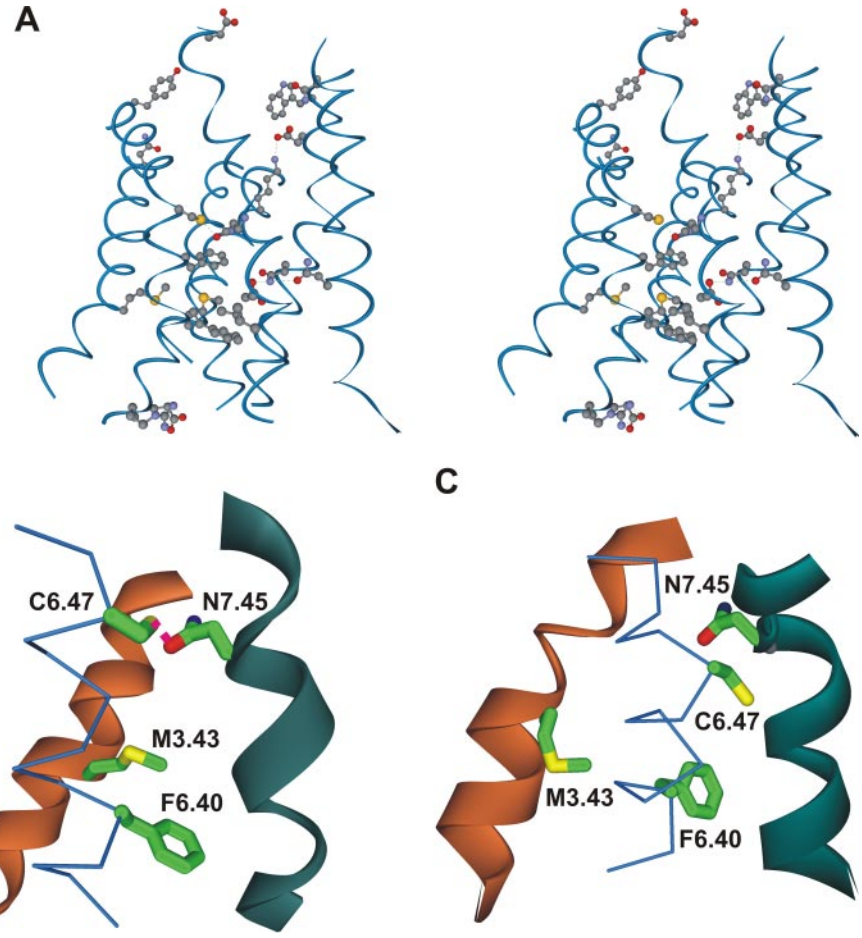

FIGURE 5. **Homology models of human GnRH receptor in inactive and active conformations.** *A*, stereo view of the 7-TM domains of the human GnRH receptor. The model was derived from the crystal structure of bovine rhodopsin in the inactive state (see "Experimental Procedures"). The model reveals the hydrogen bond interaction between $Cys^{6.47}$ and $Asn^{7.45}$. The previously experimentally identified hydrogen bonds (*green dash lines*) between $Asp^{2.61}$ and $Lys^{3.32}$ (56), between $Asp^{3.49}$ and $Arg^{3.50}$ (55), and between $Asn^{1.50}$, $Asn^{2.50}$, and $Asp^{7.49}$ (53, 54) and the hydrophobic interactions between $Met^{3.43}$ and $Phe^{6.40}$ (and surrounding residues $Met^{5.54}$, $Phe^{6.44}$, and $Ile^{7.32}$) (10) among the 7-TM domains, which validate our GnRH receptor model, were also shown. The GnRH receptor binding residues $Asp^{2.61}$, $Trp^{2.64}$, $Asn^{2.65}$, $Lys^{3.32}$, $Asn^{5.39}$, $Tyr^{6.58}$,and $Asp^{7.32}$ (see Fig. 1*C*) are also included. *B*, intramolecular interactions between $Cys^{6.47}$ and $Asn^{7.45}$. The side chains of $Cys^{6.47}$ and $Asn^{7.45}$ form part of allosteric intramolecular communication networks that confer GnRH ligand binding selectivity and signaling efficacy. Two previously identified residues ($Met^{3.43}$ and $Phe^{6.40}$) whose mutations have no effect on GnRH I binding affinity, but specifically increase affinity for GnRH II and [Tyr^8]GnRH are also shown (10). *C*, a GnRH receptor model in the active conformation, in which there are no intramolecular interactions between $Cys^{6.47}$ and $Asn^{7.45}$ and between $Met^{3.43}$ and $Phe^{6.40}$. TM 3 is shown in *orange*, TM 6 in *blue*, and TM 7 in *olive green* in *B* and *C*.

receptor conformations which have preferential engagement with partial and full agonists. Partial agonists may only break part of the intramolecular constraint network. Mutations of $Cys^{6.47}$ in the β_2 adrenergic receptor (57) and $Asn^{7.45}$ in the histamine H_1 receptor (58) lead to constitutive activation of the receptors, indicating an important role of this residue in the receptor conformational switch. We have built a model of the human GnRH receptor in an active conformational state using the crystal structure of a photoactivated deprotonated intermediate of bovine rhodopsin (15) as a template. In the model, the intramolecular interactions between $Cys^{6.47}$ and $Asn^{7.45}$, and between $Met^{3.43}$ and $Phe^{6.40}$, which we identified previously (10), are disrupted due to a motion of the middle section of TM 3, as seen as a disorder of the helix (Fig. 5*C*), and a slight outward movement of TM 6 followed by a small clockwise rotation (viewed from the extracellular surface) of the intramolecular segment by using $Pro^{6.50}$ as a hinge (59) (Fig. 5*C*). Consistent with the mutagenesis results, our molecular modeling also indicates that these intramolecular interactions are involved in receptor conformational transition. Interestingly, no constitutive activity in any mutations of the human GnRH receptor was observed, unlike the β_2 (57) and H_1 (58) receptors. This indicates that weakening the intramolecular interactions in the human GnRH receptor is not sufficient to obtain an active conformation, but rather modifies receptor conformational states which are, at least, allosterically involved in ligand binding selectivity and signaling efficacy. We propose that GnRH ligand-induction of new receptor intra- and intermolecular interactions might be a fundamental component for GnRH receptor activation, rather than a ligand-induced disruption of the receptor intramolecular constraint networks, which we proposed as a mechanism of the M_1 mAChR activation (16). This may provide an explanation for the distinct pharmacological profiles of GnRH analogues in stimulating pituitary gonadotropin and inhibiting cancer cell proliferation (6). Of the endogenous ligands GnRH I is more potent in stimulating gonadotropins (6) but GnRH II has greater antiproliferative potency (60). Interestingly, the presence of $Asp^{7.49}$, located one helix below $Asn^{7.45}$ in the GnRH receptor because of a reciprocal exchange of the highly conserved $Asp^{2.50}$-$Asn^{7.49}$ pair in other GPCRs prevents the GnRH receptor from coupling to phospholiphase D via small G proteins (61), supporting our conclusion that residues within this region play an important role in the stabilization of different receptor conformations, and account for ligand binding and signaling selectivity.

In conclusion, our molecular modeling and mutagenesis studies have indicated that the side chains of the highly conserved $Asn^{7.45}$ and $Cys^{6.47}$ make intramolecular contacts in the inactive state (Fig. 5, *A* and *B*) which form part of the receptor allosteric network, coupling to specific structural elements of the GnRH analogues. This may underlie different receptor activation mechanisms, creating different receptor active conformations with potential ligand selective signaling described for these ligands. The identification of structural elements for

ligand and receptor conformational selection could have implications for the development of novel ligands that selectively activate one signaling pathway, bypassing others, and hence with improved pharmacological specificity and profiles. Our studies also support our proposal that ligand binding selectivity is determined not only by ligand binding residues, but also by receptor conformation. The conformation of GPCRs has been specialized during evolution by forming a complex receptor intramolecular interaction network. This accounts for selective binding of the cognate ligands and G proteins. The highly conserved amino acids appear to form part of the allosteric network which might serve as constraints for receptor inactive states. Mutation of a residue within the allosteric network can alter receptor binding selectivity of ligands and G proteins through subtle receptor conformational changes, which might be one of the mechanisms of development of ligand binding and signaling selectivity and diversity of GPCRs during evolution.

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Structural Determinants for Ligand-Receptor Conformational Selection in a Peptide G Protein-coupled Receptor

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