Identification of Tyr$^{290(6.58)}$ of the Human Gonadotropin-Releasing Hormone (GnRH) Receptor as a Contact Residue for Both GnRH I and GnRH II: Importance for High-Affinity Binding and Receptor Activation†

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ABSTRACT: Molecular modeling showed interactions of Tyr$^{290(6.58)}$ in transmembrane domain 6 of the GnRH receptor with Tyr^5 of GnRH I, and His^5 of GnRH II. The wild-type receptor exhibited high affinity for [Phe^5]GnRH I and [Tyr^5]GnRH II, but 127- and 177-fold decreased affinity for [Ala^5]GnRH I and [Ala^5]GnRH II, indicating that the aromatic ring in position 5 is crucial for receptor binding. The receptor mutation Y290F decreased affinity for GnRH I, [Phe^5]GnRH I, GnRH II and [Tyr^5]GnRH II, while Y290A and Y290L caused larger decreases, suggesting that both the *para*-OH and aromatic ring of Tyr$^{290(6.58)}$ are important for binding of ligands with aromatic residues in position 5. Mutating Tyr$^{290(6.58)}$ to Gln increased affinity for Tyr^5-containing GnRH analogues 3−12-fold compared with the Y290A and Y290L mutants, suggesting a hydrogen-bond between Gln of the Y290Q mutant and Tyr^5 of GnRH analogues. All mutations had small effects on affinity of GnRH analogues that lack an aromatic residue in position 5. These results support direct interactions of the Tyr$^{290(6.58)}$ side chain with Tyr^5 of GnRH I and His^5 of GnRH II. Tyr$^{290(6.58)}$ mutations, except for Y290F, caused larger decreases in GnRH potency than affinity, indicating that an aromatic ring is important for the agonist-induced receptor conformational switch.

Hypothalamic gonadotropin-releasing hormone I (GnRH I[1]) is an important regulator of the mammalian reproductive system. GnRH I is released in a pulsatile manner from the hypothalamus and binds to the GnRH receptor in the pituitary gonadotrope cells. GnRH I binding to the receptor leads to the activation of $G_{q/11}$ and downstream signaling cascades which control release of luteinizing hormone and follicle-stimulating hormone. The latter in turn regulate steroidogenesis and gametogenesis (*1*). Thus GnRH analogues are extensively applied clinically in the treatment of hormone-dependent diseases such as infertility, precocious puberty, endometriosis, uterine fibroids, benign prostate hyperplasia and breast and prostate cancers (*1*−*3*).

The GnRH receptor is a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs). There is one functional GnRH receptor type but two endogenous ligands (hypothalamic GnRH I and extra-hypothalamic GnRH II) in humans. This indicates that the single human GnRH receptor mediates all activities of both GnRH I and GnRH II, although they display differential physiological and pharmacological profiles (*4*). The distinct pharmacological and signaling profiles of GnRH I and GnRH II at the GnRH receptor are proposed to be mediated by different receptor active conformations, induced by differential ligand−receptor interactions, which we have termed GnRH ligand-induced selective signaling (LiSS) (*1, 5*). GnRH II differs from GnRH I by three amino acids in which Tyr^5, Leu^7 and Arg^8 of GnRH I are replaced by His^5, Trp^7 and Tyr^8. Delineation of differential ligand−receptor contacts made by GnRH I and GnRH II will enable identification of ligand contacts that steer LiSS and facilitate rational development of drugs with improved specificity at desired target signaling pathways. Homology and molecular docking models of GPCRs, especially those supported and refined by experimental data, can also be used for *in silico* screenings of lead compounds and structure−activity relationship studies (*6*).

The extracellular end of transmembrane domain (TM) 6 of GPCRs has been shown to play a major role in ligand binding and resultant receptor activation (*7*−*12*), but the roles of individual residues of the GnRH receptor in this segment

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[1] Abbreviations: GnRH, gonadotropin-releasing hormone; TM, transmembrane domain; GPCR, G protein-coupled receptor; H-bond, hydrogen-bond; LiSS, ligand-induced selective signaling; IP, inositol phosphates; E_{max}, maximal agonist-elicited inositol phosphate responses; MD, molecular dynamics; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PDB, Protein Data Base.

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are poorly understood as mutations of residues such as $Tyr^{283(6.51)}$, $Tyr^{284(6.52)}$ and $Trp^{291(6.59)}$ result in receptors that are inactive, probably due to abolished cell surface receptor expression, reduced ligand binding affinity or both (*13*). Molecular docking of GnRH I (*14*) in our laboratory and of [D-Trp^6]GnRH (*13*) combined with an Ala mutation of $Tyr^{290(6.58)}$ identified putative intermolecular interactions between $Tyr^{290(6.58)}$ of the receptor and Tyr^5 of GnRH I (*14*) or [D-Trp^6]GnRH (*13*). The equivalent residue at position 6.58 is also suggested to play an important role in ligand binding and/or receptor activation of other peptide GPCRs, such as the NK2 tachykinin receptor (*15*), AT_{1A} angiotensin receptor (*16*) and CXCR1 chemokine receptor (*17*). However, the ligand−receptor intermolecular contacts, predicted by molecular modeling, have not been verified experimentally. It is essential to validate the models for use in the rational design of novel functional ligands. For example, previous site-directed mutagenesis and molecular modeling data suggested that the Trp^3 side chain of [D-Trp^6]GnRH interacts with $Trp^{280(6.48)}$ of the receptor (*13, 18*). However, the Trp^3 side chain in this model has an opposite orientation to the recently available NMR structure of GnRH I (PDB code: 1YY1) (*19*). Furthermore, our detailed mutagenesis studies (*20*) show that Trp^3 of GnRH I does not interact with $Trp^{280(6.48)}$ of the GnRH receptor, but faces away toward TM 4 where it makes other intermolecular interactions (*14*). This emphasizes the importance of verifying the intermolecular interactions suggested by molecular modeling and site-directed mutagenesis of the receptor by using single amino acid modified peptides. In addition, there are few data for the human GnRH receptor binding of GnRH II (*14*) and the interaction between His^5 of GnRH II and the receptor has not been investigated. Because the $Tyr^{290(6.58)}$ side chain is proposed to interact with the Tyr^5 residue of GnRH I, which is His^5 in GnRH II, it is potentially a differential ligand−receptor interaction that may underlie receptor conformational selection and LiSS of GnRH I and GnRH II. Hence, a more detailed definition of the role of $Tyr^{290(6.58)}$ in binding of GnRH I and GnRH II would potentially elucidate intermolecular interactions that initiate LiSS. We have used GnRH analogues with substitutions of Tyr^5 or His^5 combined with site-directed mutagenesis of the $Tyr^{290(6.58)}$ residue of the receptor to provide experimental evidence that $Tyr^{290(6.58)}$ is able to interact with both Tyr^5 of GnRH I and His^5 of GnRH II. Our refined GnRH receptor molecular docking analyses, incorporating a recent NMR structure of GnRH I and docking of GnRH II, support these interactions.

EXPERIMENTAL PROCEDURES

Receptor Amino Acid Residue Numbering. Receptor residues are identified by the amino acid sequence number of the receptor followed by nomenclature of Ballesteros and Weinstein (*7*) in which the position of the most conserved amino acids in the TM domain N is designated N.50 in parentheses. This distinguishes receptor residues from that of the decapeptide GnRH analogues labeled with the sequence number only.

Materials. GnRH I (pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2) and GnRH II ([His^5,Trp^7,Tyr^8]GnRH) were purchased from Sigma-Aldrich (Dorset, U.K.) and Bachem (Bubendorf, Switzerland). Cetrorelix (Ac-D-Nap-Ala[1]-D-ClPh-Ala[2]-D-Pyr-Ala[3]-Ser[4]-Tyr[5]-D-Cit[6]-Leu[7]-Arg[8]-Pro[9]-D-Ala[10]-NH_2) and [His^5,D-Tyr^6]GnRH I were synthesized and iodinated as described previously (*21*). [Phe^5]GnRH I, [Ala^5]GnRH I, [Tyr^5]GnRH II and [Ala^5]GnRH II were purchased from EZBiolab Inc. (Westfield, IN). DeepVent polymerase was from New England BioLabs (Hertfordshire, U.K.). *Eco*RI, *Bsr*GI and *Xho*I restriction endonucleases and T4 ligase were from Promega UK Ltd. (Southampton, U.K.). D-[*myo*-^{3}H]Inositol was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, U.K.).

Site-Directed Mutagenesis and Receptor Expression. The GnRH receptor was cloned into the pcDNA1 expression vector. Mutant sequences were constructed using a PCR method and validated by dideoxy sequencing (*22*). Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using a Bio-Rad Gene Pulser at 230 V, 960 μF with 15 μg of DNA/0.4 cm cuvette (1.5×10^7 cells; 0.7 mL). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics and 2 mM glutamine for 48 h to allow receptor expression.

Ligand Binding Assays. Radioligand binding assays were performed on intact cells 48 h after transfection as described previously (*5, 14, 19*). Transfected cells in 12-well culture plates were incubated with ^{125}I-Cetrorelix (150000 cpm/0.5 mL/well) or ^{125}I-[His^5,D-Tyr^6]GnRH I (100000 cpm/0.5 mL/well) and various concentrations of unlabeled GnRH analogues in 0.1% BSA/HEPES/DMEM for 4 h at 4 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled [His^5,D-Tyr^6]GnRH I for the ^{125}I-[His^5,D-Tyr^6]GnRH I binding assays, resulting in 0.4−0.8% of the total radioactivity. For ^{125}I-Cetrorelix binding assays, the nonspecific binding was measured in the presence of 1 μM unlabeled Cetrorelix. After incubation, the cells were washed twice with ice-cold phosphate-buffered saline, pH 7.4, and solubilized in 0.5 mL of 0.1 M NaOH. Radioactivity was counted with a gamma counter.

Inositol Phosphates (IP) Assays. Measurements of GnRH-elicited IP accumulation were performed as described previously (*5, 14, 19*). Transfected COS-7 cells were seeded onto 12-well plates, and after 36 h, they were labeled overnight with 1 μCi/mL D-[*myo*-^{3}H]inositol in inositol-free DMEM containing 1% dialyzed serum. At 48 h after transfection, the cells were preincubated with 0.5 mL of buffer (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 the addition of GnRH analogues for an additional 30 min. This was shown to be within the linear period of the assay. The stimulation was terminated by the removal of the media and the addition of 0.5 mL of 10 mM formic acid. The ^{3}H-labeled IPs were isolated from the formic acid extracts using Dowex AG 1-X8 ion-exchange resin, collected with 1 M ammonium formate/0.1 M formic acid, and quantified by liquid scintillation counting.

GnRH Docking and Molecular Dynamics (MD) Simulations. The human GnRH receptor model was built by homology modeling through MODELER within DS Modeling (version 1.6, Accelrys, San Diego) as described previously (*4, 14, 19*) using the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (PDB code 2I37) (*23*) as a template. A βII′-type

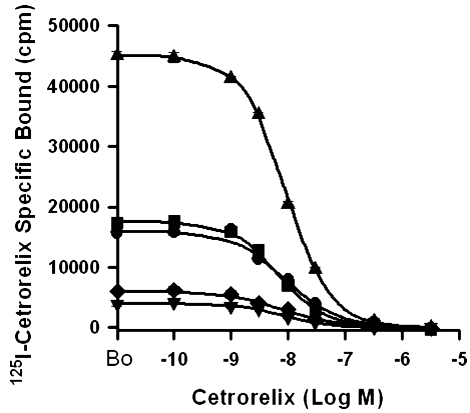

FIGURE 1: Homologous competition binding of Cetrorelix. Binding curves showing homologous competitive binding of the peptide antagonist Cetrorelix in wild-type and Tyr$^{290(6.58)}$ mutant receptors transiently expressed in COS-7 cells. Results are representative experiments, which were repeated at least three times with essentially the same results. The binding curves show little difference in receptor affinity for Cetrorelix between wild-type and mutant receptors. ●, wild-type; ▼, Y290F; ■, Y290A; ◆, Y290L; ▲, Y290Q. Points, mean ± standard error of triplicate measurements.

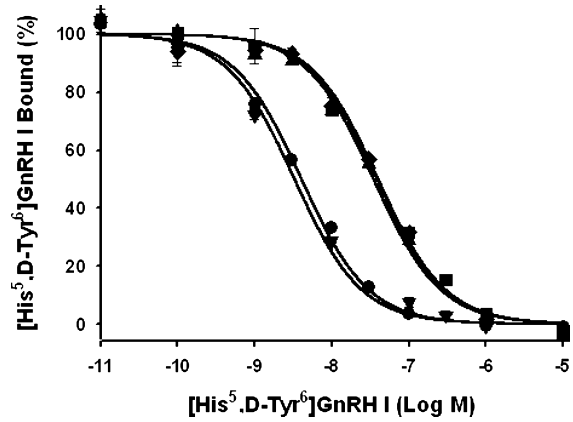

FIGURE 2: Homologous competition binding of [His^5,D-Tyr^6]GnRH I in wild-type and Tyr$^{290(6.58)}$ mutant receptors. Results are representative experiments, which were repeated at least three times with essentially the same results. ●, wild-type; ▼, Y290F; ■, Y290A; ◆, Y290L; ▲, Y290Q.

turn conformation of GnRH I (derived from an NMR structure, PDB code 1YY1) and of GnRH II was docked into the model (*1, 4, 14, 24*) according to the experimentally identified or putative contact points between GnRH and receptor, i.e. pGlu^1 with Asn$^{212(5.39)}$ (*25*), His^2 with Asp$^{98(2.61)}$/Lys$^{121(3.32)}$ (*21*), and Pro^9-Gly^{10}NH$_2$ with Arg$^{38(1.35)}$/Asn$^{102(2.65)}$ (*14, 26*). The GnRH-receptor complex was then optimized by energy-minimization and MD simulations of 150 ps by means of the CHARMM program (*27*) using a similar setup as described for the oxytocin receptor (*28*) with harmonic restraints on the receptor backbone atoms, except for extracellular loop 2 and its covalently linked N-terminal domain (*4, 19*).

Data Analysis. All experiments were performed in triplicate and repeated at least three times. Binding curves were fitted to the Hill equation or to the one-site model of the binding using Sigmaplot 9.0 (Systat Software, Inc., Point Richmond, CA) or GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA), yielding an IC_{50} value (*14, 19*). The mutant receptor expression levels (R_{exp}) were expressed relative to a wild-type control included in each transfection. IP 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 slope factor that was characteristically near 1.0. Statistical analysis was performed with Student's *t* test.

RESULTS

Homologous Binding of Radiolabeled Ligands and Receptor Expression. The IC_{50} value for binding of the peptide antagonist, Cetrorelix, to the wild-type human GnRH receptor was 5.7 ± 1.9 nM, as measured by homologous competitive binding assays using ^{125}I-Cetrorelix as a radiolabeled ligand on the intact cells (Figure 1). Mutation of Tyr$^{290(6.58)}$ of the GnRH receptor to Phe, Ala, Leu or Gln had little effect on the binding affinity of Cetrorelix, thus the B_o values measured in the absence of unlabeled ligand by this tracer reflect relative receptor expression levels (R_{exp}) on the cell surface. Mutation of Tyr$^{290(6.58)}$ of the GnRH receptor to Phe or Leu decreased receptor expression to 30% and 32% of the wild-type level, respectively (Figure 1). In contrast, mutation of Tyr$^{290(6.58)}$ to Gln markedly increased the mutant receptor expression to 228% of wild-type, while Ala mutation did not affect expression. These results indicate that mutations of Tyr$^{290(6.58)}$ have differential effects on cell surface expression of receptor, as binding was measured using intact cells, but no effect on binding affinity for the peptide antagonist, Cetrorelix. Because of high nonspecific binding of ^{125}I-Cetrorelix, we used ^{125}I-[His^5,D-Tyr^6]GnRH I as the tracer in the subsequent assays to measure receptor binding affinities for GnRH analogues. The mutants, Y290A, Y290L and Y290Q, had 8−11-fold reductions in affinity for [His^5,D-Tyr^6]GnRH I (Figure 2).

Importance of Tyr^5 of GnRH I in Receptor Binding. Previous molecular modeling studies suggested that the side chain of Tyr$^{290(6.58)}$ of the GnRH receptor may interact with Tyr^5 of GnRH I (*1, 14*). To assess the contribution of Tyr^5 to the affinity of GnRH I binding to the wild-type receptor, we examined changes in affinity of the wild-type human GnRH receptor for Tyr^5-substituted analogues of GnRH I. [Phe^5]GnRH I exhibited only a 2-fold reduction, while [Ala^5]GnRH I gave a 127-fold decrease in affinity at the wild-type receptor compared with GnRH I (Table 1). These results indicate that the aromatic ring and, to a much lesser degree, the *para*-OH of Tyr^5 of GnRH I contribute to high affinity GnRH I binding at the receptor.

Mutation of Tyr$^{290(6.58)}$ of the Human GnRH Receptor Decreased Affinity for GnRH I. To determine whether the *para*-OH group and aromatic ring of Tyr$^{290(6.58)}$ of the GnRH receptor are important for binding of GnRH I, Tyr$^{290(6.58)}$ was mutated to Phe, Ala, Leu and Gln, and the affinities of wild-type and mutant receptors for GnRH I were measured by competition binding assays. In agreement with previous reports (*5, 14, 19*), GnRH I binds to the wild-type human GnRH receptor expressed in COS-7 cells with high affinity, giving an IC_{50} value of 3.8 nM (Table 1). Mutation of Tyr$^{290(6.58)}$ to Phe, which deletes the *para*-OH group, resulted in a 4.9-fold decrease in affinity for GnRH I, while mutation of Tyr$^{290(6.58)}$ to Ala, which deletes the side chain beyond the β-carbon, led to a 332-fold reduction in affinity for GnRH I, with an IC_{50} value of 1262 nM (Table 1). Mutation of

Table 1: Binding of GnRH I and Tyr^5-Substituted Analogues to the Wild-Type and Tyr$^{290(6.58)}$ Mutant Human GnRH Receptors[a]

	GnRH I		[Phe^5]GnRH I		[Ala^5]GnRH I	
mutants	IC$_{50}$,[b] nM	fold change[c,d]	IC$_{50}$, nM	fold change	IC$_{50}$, nM	fold change
wild-type	3.8 ± 0.14	1 (1)	7.6 ± 0.4	1 (2)	481 ± 34	1 (127)
Y290F	18.6 ± 3.0	4.9 (1)	32.2 ± 4.0	4.2 (1.7)	458 ± 60	1 (25)
Y290A	1262 ± 113	332 (1)	1817 ± 111	239 (1.4)	3998 ± 729	8.3 (3.2)
Y290L	1094 ± 232	288 (1)	3799 ± 752	500 (3.5)	2682 ± 1212	5.6 (2.4)
Y290Q	382 ± 84	100 (1)	1703 ± 192	224 (4.5)	3753 ± 1221	7.8 (9.8)

[a] Receptor binding affinity (IC$_{50}$) was measured on the intact COS-7 cells, 48 h after transient transfection of the wild-type and mutant receptors, by competition binding assays using ^{125}I-[His^5,D-Tyr^6]GnRH I as a radiolabeled ligand with increasing concentrations of unlabeled GnRH analogues. [b] Values are means ± SE of three or more independent experiments. [c] Fold change relative to the wild-type receptor. [d] Fold change relative to GnRH I in parentheses.

Table 2: Binding of GnRH II and His^5-Substituted Analogues to the Wild-Type and Tyr$^{290(6.58)}$ Mutant Receptors[a]

	GnRH II		[Tyr^5]GnRH II		[Ala^5]GnRH II	
mutants	IC$_{50}$,[b] nM	fold change[c,d]	IC$_{50}$, nM	fold change	IC$_{50}$, nM	fold change
wild-type	25 ± 2	1 (1)	46 ± 4	1 (1.8)	4458 ± 122	1 (177)
Y290F	201 ± 14	8 (1)	506 ± 21	11 (2.5)	2752 ± 258	0.6 (14)
Y290A	2057 ± 291	82 (1)	2078 ± 137	45 (1)	9177 ± 1423	2.1 (4.5)
Y290L	963 ± 176	38 (1)	3207 ± 336	69 (3.3)	12800 ± 3236	2.9 (13)
Y290Q	846 ± 103	34 (1)	268 ± 19	5.8 (0.3)	8000 ± 240	1.8 (9.5)

[a] Ligand binding affinity (IC$_{50}$) was measured on the intact COS-7 cells, 48 h after transient transfection of the wild-type and mutant receptors, by competition binding assays using ^{125}I-[His^5,D-Tyr^6]GnRH I as a radiolabeled ligand with increasing concentrations of unlabeled GnRH analogues. [b] Values are means ± SE of three or more independent experiments. [c] Fold change relative to the wild-type receptor. [d] Fold change relative to GnRH II in parentheses.

Tyr$^{290(6.58)}$ of the GnRH receptor to a smaller, nonpolar residue (Leu) or a polar residue (Gln) also exhibited a marked reduction in affinity for GnRH I by 288- and 100-fold (Table 1). These results indicate that both the *para*-OH and the aromatic ring of Tyr$^{290(6.58)}$ of the GnRH receptor play an important role in the binding of GnRH I.

Effects of Mutations of Tyr$^{290(6.58)}$ of the Human GnRH Receptor on Binding Affinity for Tyr^5-Substituted Analogues of GnRH I. To determine whether the side chain of Tyr$^{290(6.58)}$ of the receptor has a role in interaction with Tyr^5 of GnRH I, we examined the effects of mutations of Tyr$^{290(6.58)}$ of the human GnRH receptor on the binding affinity for Tyr^5-substituted analogues of GnRH I. Mutation of Tyr$^{290(6.58)}$ of the GnRH receptor to Phe had similar decreases in affinity for GnRH I (4.9-fold) and [Phe^5]GnRH I (4.2-fold) affinity, but had little effect on the affinity for [Ala^5]GnRH I (Table 1). This shows that the *para*-OH of Tyr$^{290(6.58)}$ is important for binding of GnRH analogues with an aromatic residue in position 5, but not for binding of [Ala^5]GnRH I, which lacks the aromatic side-chain. Mutation of Tyr$^{290(6.58)}$ to Ala and Leu led to 288- to 332-fold reductions in affinity for GnRH I and 239- to 500-fold for [Phe^5]GnRH I, but had much smaller effects on mutant receptor binding affinity for [Ala^5]GnRH I (5.6- to 8.3-fold) (Table 1). Interestingly, the Y290Q mutant receptor, which was designed to reconstitute the hydrogen-bond (H-bond) potential of the *para*-OH group of Tyr$^{290(6.58)}$ in the absence of the aromatic ring, exhibited a 3-fold higher affinity than Y290A and Y290L mutant receptors for GnRH I (Table 1). This indicates that the Gln side chain of the Y290Q mutant receptor enhances affinity for GnRH I, but not [Phe^5]GnRH I or [Ala^5]GnRH I, and suggests that Gln may form an H-bond with the *para*-OH of Tyr^5 of GnRH I.

Effects of Mutations of Tyr$^{290(6.58)}$ on Receptor Binding Affinity for His^5-Substituted Analogues of GnRH II. Replacement of His^5 of GnRH II with Tyr^5, which is present in GnRH I, caused only a minor reduction (<2-fold) of the binding affinity at the wild-type receptor (Table 2). These results show that substitution of His^5 of GnRH II with Tyr is well tolerated. Replacement of His^5 of GnRH II with Ala led to a marked reduction (177-fold) of the peptide affinity at the wild-type receptor (Table 2). These data indicate that the aromatic ring in position 5 of GnRH II plays an important role in high affinity binding of the peptide to the receptor.

To assess the roles of Tyr$^{290(6.58)}$ of the human GnRH receptor in high affinity binding of GnRH II, we examined the effects of its mutation on receptor binding affinity for GnRH II and His^5-substituted GnRH II analogues, [Tyr^5]GnRH II and [Ala^5]GnRH II. The mutation Y290F decreased affinity for GnRH II relative to the wild-type receptor (IC$_{50}$ value 25 nM) by 8-fold, while the mutation Y290A had a much larger effect, producing an 82-fold reduction in affinity. Mutation of Tyr$^{290(6.58)}$ to Leu and Gln also decreased affinity for GnRH II (38- and 34-fold, Table 2). These results indicate that Tyr$^{290(6.58)}$ of the GnRH receptor is also important for high affinity binding of GnRH II.

Table 3: GnRH-Elicited IP Responses at the Wild-Type and Tyr$^{290(6.58)}$ Mutant Human GnRH Receptors

		GnRH I		GnRH II		[His^5,D-Tyr^6]GnRH I	
mutants	R_{exp}[a]	EC_{50},[b] nM	E_{max}, % wt	EC_{50}, nM	E_{max}, % wt	EC_{50}, nM	E_{max}, % wt
wild-type	100	0.9 ± 0.2	100	9.6 ± 2	100	0.7 ± 0.1	100
Y290F	30 ± 5	51 ± 10	94 ± 7	254 ± 27	87 ± 12	4.7 ± 0.4	107 ± 3
Y290A	102 ± 5	1724 ± 146	72 ± 6	1940 ± 175	82 ± 18	73 ± 10	88 ± 6
Y290L	32 ± 3	u.d.	<10	1674 ± 208	85 ± 10		
Y290Q	228 ± 13	227 ± 26	156 ± 7	1607 ± 162	167 ± 24		

[a] Cell surface receptor expression levels (R_{exp}) were measured on intact cells using ^{125}I-Cetrorelix, which showed no changes in affinity for Tyr$^{290(6.58)}$ mutant receptors, and were expressed relative to the wild-type control included in each transfection. [b] IP responses were determined in COS-7 cells transfected with wild-type and mutant receptors. Values are the mean ± SE of three or more independent experiments. u.d., undetectable.

Mutation of Tyr$^{290(6.58)}$ to Phe, Ala and Leu had essentially similar effects on receptor binding affinity for [Tyr^5]GnRH II as observed for GnRH II. Specifically, Y290F exhibited an 11-fold reduction for [Tyr^5]GnRH II, while Y290A and Y290L gave 45- and 69-fold reductions respectively (Table 2). This suggests that Tyr^5 can efficiently substitute for the interactions of His^5 of GnRH II with the GnRH receptor. Interestingly, the Y290Q mutant receptor exhibited a smaller reduction in affinity (5.8-fold) for [Tyr^5]GnRH II compared with wild-type. Y290Q also exhibited higher affinity for [Tyr^5]GnRH II, compared with GnRH II (3-fold), and with Y290A and Y290L mutants (8−12-fold, Table 2). These data suggest that substitution of His^5 of GnRH II with Tyr^5 may lead to the acquisition of a new H-bond interaction with the Gln side chain of the Y290Q mutant. Compared with the wild-type receptor, mutations of Tyr$^{290(6.58)}$ to Phe, Ala, Leu or Gln had only marginal effects (<3-fold) on receptor binding affinity for [Ala^5]GnRH II, which lacks an aromatic ring at position 5 of the peptide to interact with Tyr$^{290(6.58)}$ of the receptor. These results suggest that the *para*-OH and the aromatic ring of Tyr$^{290(6.58)}$ of the human GnRH receptor may make direct contacts with His^5 of GnRH II.

Effects of Mutations of Tyr$^{290(6.58)}$ on GnRH-Elicited IP Responses. Consistent with previous experiments (*14, 19*), GnRH I and GnRH II elicited robust IP responses from COS-7 cells expressing wild-type GnRH receptors with EC_{50} values of 0.9 nM and 9.6 nM (Table 3). The maximum IP responses evoked by the wild-type receptor were typically 5 times the basal activity. The effects of mutations of Tyr$^{290(6.58)}$ of the GnRH receptor on the IP responses are summarized in Table 3.

GnRH I and GnRH II elicited IP responses at the Y290F mutant receptor with increased EC_{50} values, by 57- and 26-fold relative to the wild-type respectively, and slightly decreased maxima (94% and 87% of the wild-type E_{max}). This may be caused by the decreased mutant receptor expression level (Figure 1 and Table 3) and reduced binding affinity for GnRH I (Table 1) and GnRH II (Table 2). [His^5,D-Tyr^6]GnRH I, which has no change in receptor binding affinity for the Y290F mutant (Figure 2), exhibited a 6.7-fold increase in its EC_{50} value with a slightly enhanced E_{max} relative to the wild-type (Figure 3). These data suggest that the intermolecular interaction between the *para*-OH group of Tyr$^{290(6.58)}$ and the GnRH peptides may not play a major role in the GnRH-induced receptor conformational switch associated with receptor activation.

The Y290A mutant, which had similar receptor expression as the wild-type (Figure 1 and Table 3), exhibited 1915- and 202-fold reductions in potency of GnRH I and GnRH II in stimulating IP responses, with decreased E_{max} to 72% and 82% that of the wild-type. The larger effects of the mutation of Tyr$^{290(6.58)}$ to Ala on potency than affinity for GnRH I (5.8-fold) and GnRH II (2.5-fold) indicate that the aromatic ring of Tyr$^{290(6.58)}$ of the GnRH receptor is important for GnRH I- and GnRH II-induced receptor activation. This is further supported by the studies with [His^5,D-Tyr^6]GnRH I, which had only a 10-fold decrease in affinity (Figure 2), but a 104-fold reduction in potency at the Y290A mutant receptor (Figure 3 and Table 3). Mutation of Tyr$^{290(6.58)}$ to Leu appears to be more detrimental to GnRH I than GnRH II in stimulating IP responses, giving an E_{max} less than 10% that of the wild-type for GnRH I, but 85% for GnRH II. In parallel with the increased receptor expression, Y290Q also evoked an increased E_{max} of 156% and 167% of that of the wild-type for GnRH I and GnRH II respectively.

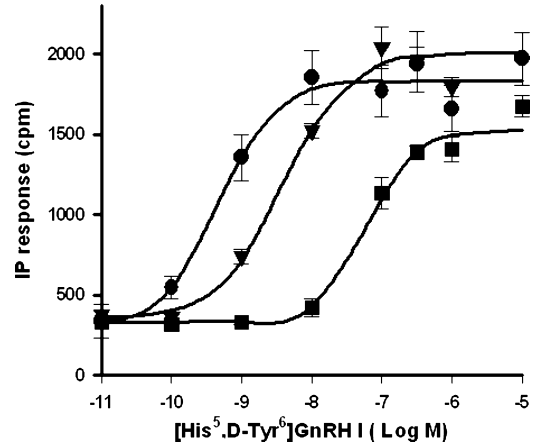

FIGURE 3: [His^5,D-Tyr^6]GnRH I elicited IP responses in human GnRH wild-type, Y290F, and Y290A mutant receptors. Results are representative experiments, which were repeated at least three times with essentially the same results. ●, wild-type; ▼, Y290F; ■, Y290A.

GnRH Docking and MD Simulations. A human GnRH receptor model was built (*19*) using the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (*23*) as a template. A βII′-turn conformation of GnRH I (derived from a recent NMR structure) and of GnRH II could be docked into the receptor model successfully, accommodating the experimentally confirmed or putative intermolecular interactions between GnRH and the receptor, followed by energy-minimization and MD simulations (*4, 14*). In the docking model of GnRH I, the side chain of Tyr$^{290(6.58)}$ of the receptor forms a T-shaped stacking interaction with Tyr^5 of GnRH I (Figure 4A). The *para*-OH group of Tyr^5 of GnRH I points away from Tyr$^{290(6.58)}$ of the receptor. The model shows no H-bond between the two *para*-OH moieties of the Tyr residues. This is consistent with the mutagenesis

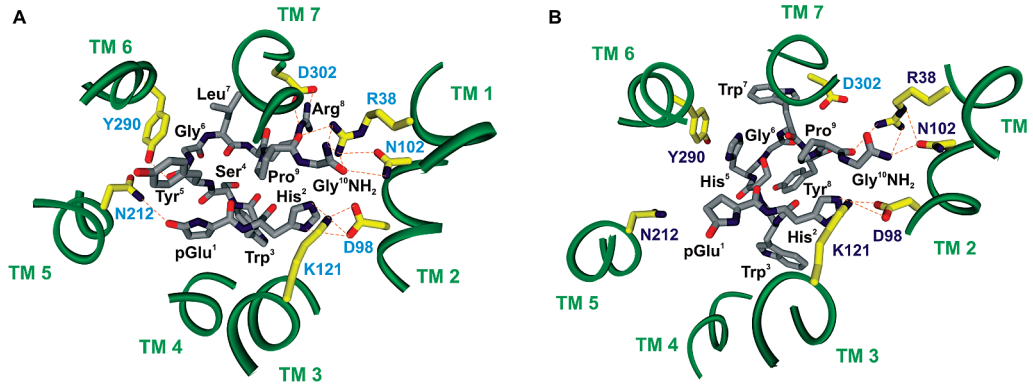

FIGURE 4: Molecular modeling of human GnRH receptor-GnRH complexes. The βII′-turn conformations of GnRH I derived from the NMR structure (PDB code 1YY1) and of GnRH II were docked into the receptor model according to the experimentally identified or putative intermolecular interactions between GnRH I/GnRH II (gray/black) and the receptor contact sites (yellow/blue), i.e. pGlu^1 interacts with Asn$^{212(5.39)}$, His^2 with Lys$^{121(3.32)}$/Asp$^{98(2.61)}$, Arg^8 (GnRH I) with Asp$^{302(7.32)}$ and Pro^9-Gly^{10}NH$_2$ with Arg$^{38(1.35)}$/Asn$^{102(2.65)}$, viewed from intracellular surface. The H-bonds are indicated by dashed lines. (A) The model of GnRH I docking showing that the side chain of Tyr$^{290(6.58)}$ in TM (green) 6 of the receptor forms a T-shaped contact with Tyr^5 of GnRH I. The *para*-OH of Tyr$^{290(6.58)}$ is able to make H-bond interactions with the aromatic ring and the backbone carbonyl oxygen of Tyr^5 of GnRH I. (B) A model of GnRH II docking showing a similar binding mode of GnRH II with the receptor as that of GnRH I. In the model, the side chain of Tyr$^{290(6.58)}$ makes an off-center parallel stacking contact with His^5 of GnRH II.

results which showed that deletion of the *para*-OH group of Tyr^5 of GnRH I via substitution of Tyr^5 with Phe had only a marginal effect (<2-fold, Table 1) on the peptide affinity toward the wild-type and Y290F mutant receptors. However, the *para*-OH of Tyr$^{290(6.58)}$ of the GnRH receptor may make an H-bond with the carbonyl backbone oxygen of Tyr^5 of GnRH I (Figure 4A), while the aromatic rings form a T-shaped stacking contact (*29*). The docking model of GnRH II suggests that the side chain of Tyr$^{290(6.58)}$ may interact with His^5 of GnRH II by a stacking contact between the aromatic rings (Figure 4B). However, there is no H-bond interaction between the *para*-OH of Tyr$^{290(6.58)}$ with the peptide backbone of GnRH II in contrast to the docked GnRH I. This may explain why mutations of Tyr$^{290(6.58)}$ had smaller effects on receptor binding affinity for GnRH II compared with GnRH I (Tables 1 and 2).

DISCUSSION

Tyr$^{290(6.58)}$ in TM 6 is highly conserved among GnRH receptors cloned from different species (*1*), implying an important role of this residue in receptor folding, ligand binding and/or receptor activation. Indeed, previous molecular modeling studies have suggested an interaction between Tyr$^{290(6.58)}$ and Tyr^5 of GnRH I (*1, 14*), but this interaction has not been validated experimentally. Here, we have investigated the interaction of Tyr$^{290(6.58)}$ with Tyr^5 of GnRH I and His^5 of GnRH II, as well as the role of Tyr$^{290(6.58)}$ in receptor expression and GnRH-induced receptor activation.

Our mutagenesis results clearly indicated that Tyr$^{290(6.58)}$ of the GnRH receptor is critical for the binding of GnRH I and GnRH II. Ala substitution of Tyr$^{290(6.58)}$ of the receptor, and of residue 5 of GnRH peptides both led to 82−332-fold reduction in affinity. These were largely nonadditive. In contrast, although [Ala^7]GnRH I, like [Ala^5]GnRH I, had reduced affinity at the wild-type receptor (85-fold, compared with the parent GnRH I) due to the loss of a contact between Leu^7 of GnRH I and a receptor residue, the mutation Y290A, which disrupts the proposed interaction between Tyr$^{290(6.58)}$ of the receptor and Tyr^5 of GnRH I, caused a further (additive) large reduction in affinity for [Ala^7]GnRH I as occurred for GnRH I (data not shown). These data are consistent with the direct interaction between Tyr$^{290(6.58)}$ of the receptor and Tyr^5/His^5 of GnRHs. Furthermore, the Y290Q mutant exhibited higher affinity (compared with Y290L and Y290A) for GnRH I (3−4-fold, $p < 0.01$) and [Tyr^5]GnRH II (8−12-fold, $p < 0.01$), but not [Phe^5]GnRH I or GnRH II, suggesting that the Gln side chain of Y290Q may make an H-bond with the *para*-OH of Tyr^5 of GnRH I and with the Tyr^5 side chain of [Tyr^5]GnRH II. The changes in affinity are consistent with the gain or disruption of an H-bond which accounts for a change in binding energy by 0.5−1.5 kcal·mol^{-1} (*30*). Molecular modeling analyses of GnRH I and [Tyr^5]GnRH II docked at the Y290Q mutant receptor showed H-bonds between the Gln^{290} side chain and the *para*-OH groups in position 5 of both peptides (data not shown). Together, these results indicate that Tyr$^{290(6.58)}$ is positioned near Tyr^5/His^5, and is important for binding of both GnRH I and GnRH II.

In our current docking model of GnRH I (Figure 4A), the side chain of Tyr$^{290(6.58)}$ makes a T-shaped stacking contact with Tyr^5 of GnRH I, with potential to form a nonclassical H-bond interaction (π-OH, where the H-bond acceptor is the aromatic ring), i.e. the Tyr−Tyr interaction is stabilized by both electrostatic and dispersive interactions (*29*). The formation of a nonclassical H-bond interaction between Tyr$^{290(6.58)}$ of the receptor and the aromatic ring of Tyr^5 of GnRH I appears to be supported by the experimental results where the mutation Y290F gave 4−11-fold ($p < 0.01$) decreases in binding affinities for GnRH analogues possessing an aromatic residue (Tyr, Phe and His) at position 5 (GnRH I, [Phe^5]GnRH I, GnRH II and [Tyr^5]GnRH II, Tables 1 and 2), but had little effect on the receptor binding affinity for [Ala^5]GnRH analogues. Mutations of Tyr$^{290(6.58)}$ to Ala, Leu and Gln caused 100−500-fold reductions in affinity for GnRH I and [Phe^5]GnRH I, but had a much smaller effect (<8.3-fold) on receptor binding affinity for [Ala^5]GnRH I which lacks the ability to make an aromatic interaction between position 5 of the peptide and Tyr$^{290(6.58)}$. These data fully support a direct interaction between the side chains of Tyr$^{290(6.58)}$ of the GnRH receptor and Tyr^5 of GnRH

I suggested by the molecular model. In our molecular docking of GnRH II (Figure 4B), the side chain of $Tyr^{290(6.58)}$ makes an off-center parallel stacking contact with His^5 of GnRH II, involving $\pi-\pi$, $\pi-OH$ and $\pi-NH$ interactions (*31, 32*). That the mutations of $Tyr^{290(6.58)}$ have much smaller effects (<3-fold) on receptor binding affinity for [Ala^5]GnRH II, which lacks the aromatic ring to interact with $Tyr^{290(6.58)}$, than the parent GnRH II supports the conclusion of a direct contact between the side chain of $Tyr^{290(6.58)}$ and His^5 of GnRH II. An interesting prediction from our molecular modeling is that, while both Tyr^5 of GnRH I and His^5 of GnRH II interact with $Tyr^{290(6.58)}$, they interact with different rotamer conformations of the $Tyr^{290(6.58)}$ side chain. This is also supported by our previous result that substitution of Tyr^5 of GnRH I with His^5 leads to a 3-fold ($p < 0.01$) increase in receptor binding affinity (*5*). This subtle difference of the intermolecular interactions between the receptor and GnRH I and GnRH II may, together with the distinct intermolecular interactions made by Arg^8 of GnRH I and Tyr^8 GnRH II (*5*), underlie LiSS and supports our hypothesis that GnRH I and GnRH II stabilize different receptor active conformations (*4, 5, 19*).

The relative expression of the $Tyr^{290(6.58)}$ mutant human GnRH receptors was calculated from the B_o values in ^{125}I-Cetrorelix competition binding assays on intact cells, as Cetrorelix showed unchanged affinity for the mutant receptors (Figure 1). The mutation Y290A had no effect on receptor expression, while Y290F and Y290L decreased, but Y290Q increased, the mutant receptor expression on cell surfaces (Figure 1 and Table 3). This also indicates that both the *para*-OH and aromatic ring are involved in the proper packing of the $Tyr^{290(6.58)}$ side chain and substitution of $Tyr^{290(6.58)}$ with Phe, Leu and Gln is able to induce subtle and differential receptor conformational changes, also supporting the above hypothesis that the subtle difference of the interactions between $Tyr^{290(6.58)}$ and Tyr^5 of GnRH I and His^5 of GnRH II may participate in ligand-induced receptor conformational selection and LiSS.

Our results, showing that mutations of $Tyr^{290(6.58)}$ had no effect on receptor affinity for Cetrorelix, indicate that it is unlikely that Tyr^5 of Cetrorelix interacts with $Tyr^{290(6.58)}$. Mutations of the GnRH receptor which affect peptide agonist binding affinity (*14, 33, 34*) often have little effect on the affinity of peptide antagonists, suggesting that the peptide antagonists, which possess bulky hydrophobic D-amino acids at the N-terminal domain and a D-amino acid at position 6 (*1*), have a different receptor binding mode from that of the native GnRH. A similar phenomenon was also observed in other peptide GPCRs (*35*). Similarly, substitution of Gly^6 in GnRH I with a D-amino acid (e.g., [D-Trp^6]GnRH) is thought to alter peptide conformations (*36*) and thus receptor binding mode, and therefore mutations of $Tyr^{290(6.58)}$ (Figure 2) and other residues (*26, 37, 38*) of the GnRH receptor, which caused large decreases in mutant receptor affinity for GnRH I, often had a much smaller effect on [His^5,D-Tyr^6]GnRH I binding affinity (Figure 2). The central residues (Tyr^5-Gly^6-Leu^7-Arg^8) of GnRH I are thought to be involved in the formation of a βII′-turn conformation of the peptide (*36*). Substitution of Gly^6 with a D-amino acid stabilizes the βII′-turn conformation and thus increases the receptor binding affinity (*1*). In addition to having this effect, the D-Tyr^6 side chain of [His^5,D-Tyr^6]GnRH I may make an intermolecular interaction with a receptor residue, thereby compensating for the loss of the interaction between Tyr^5 of GnRH I and $Tyr^{290(6.58)}$ of the receptor caused by $Tyr^{290(6.58)}$ mutations. These results also indicate that the $Tyr^{290(6.58)}$ mutations result in little disruption of the global structure of the receptor.

Measurements of GnRH-elicited functional responses showed that the Y290F mutant gave markedly decreased potencies in stimulating IP accumulation, with increased EC_{50} values for GnRH I and GnRH II by 57- and 26-fold (Table 3). This may be partially explained by the decreased receptor binding affinity and lower expression level. Nevertheless, the Y290F mutant was able to evoke an E_{max} near the wild-type receptor for both GnRH I and GnRH II, despite its expression level being less than one-third of the wild-type. This shows that the Y290F mutant was well coupled to intracellular signaling via $G_{q/11}$. In contrast, the Y290A mutant receptor, which was expressed at the wild-type level, exhibited larger decreases in potency than affinity for GnRH I (5.8-fold, $p < 0.01$) (Tables 1 and 3), GnRH II (2.5-fold, $p < 0.01$) (Tables 2 and 3) and [His^5,D-Tyr^6]GnRH I (10-fold) (Figure 2 and 3) and maximum IP responses were also reduced to less than 88% that of the wild-type. We propose that $Tyr^{290(6.58)}$ of the GnRH receptor is important for GnRH-induced receptor activation, and thus the interactions between $Tyr^{290(6.58)}$ and Tyr^5 of GnRH I and His^5 of GnRH II may be involved in triggering receptor conformational switch/selection via "ligand-induced fit" (*5*), a process that has also been observed in other GPCRs such as the β_2-adrenergic receptor (*39*). Consistent with this, we previously demonstrated that certain residues, located at the intracellular segment of the TM 6, make intramolecular interactions that stabilize the receptor in the inactive state and are broken during ligand-induced receptor conformational changes (*5, 19*). Interestingly, the mutation Y290L appears to be more detrimental to the functional responses for GnRH I compared with GnRH II. This suggests that substitution of $Tyr^{290(6.58)}$ with Leu, a smaller nonpolar amino acid lacking aromatic and H-bonding capabilities, may create a new intramolecular interaction which alters the mutant receptor conformation, supported by the decreased receptor expression level (*5*), which becomes less efficient for GnRH I to trigger receptor activation in comparison with GnRH II (Table 3). In addition, Y290L showed a 2-fold higher affinity for GnRH II in comparison with Y290A (Table 2), suggesting that the side chain of Y290L mutant interacts with His^5 of GnRH II to some extent. These data further support the prediction of our molecular models (Figure 4), which suggests that Tyr^5 of GnRH I and His^5 of GnRH II interact with different rotamer conformations of $Tyr^{290(6.58)}$ and thus influence receptor conformational selection (*40*).

In summary, we have shown that the *para*-OH group and the aromatic ring of $Tyr^{290(6.58)}$ of the human GnRH receptor are important for binding of both GnRH I and GnRH II. Our studies using molecular modeling and site-directed mutagenesis, in combination with position 5-modified GnRH I and GnRH II analogues, suggest that the side chain of $Tyr^{290(6.58)}$ of the human GnRH receptor interacts directly with Tyr^5 of GnRH I and His^5 of GnRH II, and these interactions are essential for high affinity binding. We also show that the side chain of $Tyr^{290(6.58)}$ of the human GnRH receptor is important for the switch between the inactive and activated states and may be involved in ligand-induced

receptor conformational selection. Together with the results obtained in other GPCRs (*15*−*17*), we suggest that the residues at position 6.58 may have a common role in peptide GPCR ligand binding and receptor activation.

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