

# Identification of a Novel Ligand Binding Residue Arg<sup>38(1.35)</sup> in the Human Gonadotropin-Releasing Hormone Receptor

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## ABSTRACT

Delineation of peptide ligand binding sites is of fundamental importance in rational drug design and in understanding ligand-induced receptor activation. Molecular modeling and ligand docking to previously experimentally identified binding sites revealed a putative novel interaction between the C terminus of gonadotropin-releasing hormone (GnRH) and Arg<sup>38(1.35)</sup>, located at the extracellular end of transmembrane domain 1 of the human GnRH receptor. Mutation of Arg<sup>38(1.35)</sup> to alanine resulted in 989- and 1268-fold reduction in affinity for GnRH I and GnRH II, respectively, the two endogenous ligands. Conservative mutation of Arg<sup>38(1.35)</sup> to lysine had less effect, giving reduced affinities of GnRH I and GnRH II by 24- and 54-fold, respectively. To test whether Arg<sup>38(1.35)</sup> interacts with the C-terminal Gly<sup>10</sup>-NH<sub>2</sub> of GnRH, binding of GnRH analogs with substitution of the C-terminal glycine with ethylamide

([Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRH) was studied with wild-type and Arg<sup>38(1.35)</sup> mutant receptors. Mutation of Arg<sup>38(1.35)</sup> to lysine or alanine had much smaller effect on receptor affinity for [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRH analogs and no effect on binding affinity of peptide antagonist cetrorelix. In parallel with the decreased affinity, the mutants also gave a decreased potency to GnRH-elicited inositol phosphate (IP) responses. The mutant receptors had effects on [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRH-elicited IP responses similar to that of the parent GnRHs. These findings indicate that Arg<sup>38(1.35)</sup> of the GnRH receptor is essential for high-affinity binding of GnRH agonists and stabilizing the receptor active conformation. The mutagenesis results support the prediction of molecular modeling that Arg<sup>38(1.35)</sup> interacts with the C-terminal glycine and probably forms hydrogen bonds with the backbone carbonyl of Pro<sup>9</sup> and Gly<sup>10</sup>-NH<sub>2</sub>.

The gonadotropin-releasing hormone (GnRH) receptor is a member of the rhodopsin-like family of 7-transmembrane domain (7-TM) G-protein-coupled receptors (GPCRs). It mediates the regulation of GnRH to the reproductive hormonal cascades. In humans, there is a single functional type of the receptor (type I receptor) and two types of endogenous ligand, GnRH I and GnRH II, although some vertebrate species are known to contain as many as three types of functional receptors (Millar et al., 2004). GnRH analogs are extensively used clinically in the treatment of disorders such as reproductive cancers, precocious puberty, and endometriosis (Casper, 1991). For well over a decade, G<sub>q/11</sub> has been known to be the predominant G-protein coupled to the mammalian GnRH receptors in various cellular environments (Kaiser et al., 1997; Ruf et al., 2003). Binding of agonist to the GnRH

receptor triggers gonadotropin secretion from the pituitary after its coupling to G<sub>q/11</sub> protein, which activates phospholipase C- $\beta$  to stimulate turnover of inositol phosphates (IP), leading to the release of Ca<sup>2+</sup> from intracellular stores and activation of protein kinase C by diacylglycerol. There are also reports that the human GnRH receptor is capable of activating other G-protein species such as G<sub>s</sub> (Ulloa-Aguirre et al., 1998; Liu et al., 2002) and G<sub>i/o</sub> (Gründker et al., 2001; Krsmanovic et al., 2003) mediating differential physiological and pharmacological effects of GnRH analogs, such as anti-proliferative effects of GnRH analogs in cancer cells (Gründker et al., 2001; Maudsley et al., 2004).

These findings give rise to the potential for development of signal-selective GnRH analogs, which preferentially activate one signaling pathway, bypassing others, via ligand-induced selective receptor active conformations (Lu et al., 2005, 2007; Millar et al., 2007). The binding of various agonists to GnRH receptor may break intramolecular constraint networks that stabilize the receptor in inactive conformations, creating new sets of inter- and intramolecular contacts that stabilize the

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**ABBREVIATIONS:** GnRH, gonadotropin-releasing hormone; IP, inositol phosphate; GPCR, G-protein-coupled receptor; TM, transmembrane domain; wt, wild type; IN3, (2S)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl) propan-1-amine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; MD, molecular dynamics.

receptor in particular active conformations that affect the downstream signaling selectivity. This concept is supported by our recent finding that GnRH I is more potent than GnRH II in stimulating IP responses, but the reverse is true in stimulating antiproliferative effects (Millar et al., 2007). Consistent with this, mutations of GnRH receptor at loci remote from the ligand binding sites specifically increase binding affinity for GnRH II and GnRHs from other species that possess Arg<sup>8</sup> substitution, but not GnRH I (Lu et al., 2005, 2007), indicating that GnRH I and GnRH II stabilize different receptor active conformations. To fully understand this phenomenon and to assist in the development of novel signal-selective GnRH analogs directed at different therapeutic end points, structural characterization of the ligand binding pocket of signal-selective GnRH analogs is essential.

Using these previously identified contact points between GnRH and the receptor, we have performed ligand docking experiments with our model constructed previously of the GnRH receptor (Lu et al., 2007; Millar et al., 2007). The resultant model suggested that Arg<sup>38(1.35)</sup> of the human GnRH receptor (receptor residues are identified by sequence number of amino acids of the receptor followed by the nomenclature of Ballesteros and Weinstein, in which the position of the most conserved amino acids in the TM domain N is designated as N.50 in parentheses. This distinguishes receptor residues from GnRH peptide residues labeled with sequence number only) is positioned near to the C terminus of GnRH and therefore may interact directly with the peptide. This residue is located at the extracellular end of TM 1 and is completely conserved among all known GnRH receptors (Fig. 1). Using site-directed mutagenesis studies we demonstrate that Arg<sup>38(1.35)</sup> of the GnRH receptor is important for the binding of both endogenous ligands GnRH I and GnRH II but less so for [Pro<sup>9</sup>-NHet]GnRH derivatives of GnRHs and is not important for the binding of the peptide antagonist, cetrorelix, which possesses D-Ala<sup>10</sup>-NH<sub>2</sub>. These data suggest that Arg<sup>38(1.35)</sup> of the GnRH receptor interacts directly with the C termini of GnRH I and GnRH II, which is important for high-affinity binding and consequent receptor activation.

## Materials and Methods

**Materials.** GnRH I (pGlu<sup>1</sup>-His<sup>2</sup>-Trp<sup>3</sup>-Ser<sup>4</sup>-Tyr<sup>5</sup>-Gly<sup>6</sup>-Leu<sup>7</sup>-Arg<sup>8</sup>-Pro<sup>9</sup>-Gly<sup>10</sup>-NH<sub>2</sub>) and GnRH II ([His<sup>5</sup>,Trp<sup>7</sup>,Tyr<sup>8</sup>]GnRH) were purchased from Sigma (St. Louis, MO) and Bachem (Bubendorf, Switzerland). Cetrorelix (Ac-D-Nap-Ala<sup>1</sup>-D-CIPh-Ala<sup>2</sup>-D-Pyr-Ala<sup>3</sup>-Ser<sup>4</sup>-Tyr<sup>5</sup>-D-Cit<sup>6</sup>-Leu<sup>7</sup>-Arg<sup>8</sup>-Pro<sup>9</sup>-D-Ala<sup>10</sup>-NH<sub>2</sub>), [Pro<sup>9</sup>-NHet]GnRH I, and [Pro<sup>9</sup>-NHet]GnRH II were synthesized as described previously (Mamputha et al., 2007). DeepVent polymerase was from New England Biolabs (Hertfordshire, UK). EcoRI, BsrGI, and XhoI restriction endonucleases and T4 ligase were from Promega (Madison, WI). D-[myo-<sup>3</sup>H]Inositol was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). IN3 ([2S]-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl)propan-1-amine] (Janovick et al., 2002) was obtained from Merck (Whitehouse Station, NJ).

**GnRH Docking and Molecular Dynamics Simulations.** A model of the human GnRH receptor was built by comparative modeling through MODELLER within DS Modeling (version 1.6; Accelrys, San Diego, CA) as described previously (Lu et al., 2007; Millar et al., 2007) using the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (Protein Data Base code 2I37) (Salom et al., 2006) as a template. A  $\beta$ II'-type turn conformation of GnRH I (derived from an NMR structure, Protein

Data Base code 1YY1) and of GnRH II was docked into the model according to the previously experimentally identified contact points between GnRH and receptor (pGlu<sup>1</sup> with Asn<sup>212(5.39)</sup>, His<sup>2</sup> with Asp<sup>98(2.61)</sup>/Lys<sup>121(3.32)</sup>, and Tyr<sup>5</sup>/His<sup>5</sup> with Tyr<sup>290(6.58)</sup>, and Gly<sup>10</sup>NH<sub>2</sub> with Asn<sup>102(2.65)</sup>; Millar et al., 2004, 2007; Coetsee et al., 2007; Mamputha et al., 2007; Fig. 2). The GnRH receptor complex was then optimized by energy minimization and molecular dynamics (MD) simulations of 150 ps by the means of the CHARMM program (Brooks et al., 1983) using a similar setup as described for the oxytocin receptor (Favre et al., 2005) with harmonic restraints on the receptor backbone atoms, except for extracellular loop 2 and its covalently linked N-terminal domain (Millar et al., 2007).

**Site-Directed Mutagenesis and Receptor Expression.** The GnRH receptor was cloned into the pcDNA1 expression vector. Mutant sequences were constructed using a polymerase chain reaction method (Lu et al., 1997). Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA) at 230 V, 960  $\mu$ F, with 15  $\mu$ g of DNA/0.4 cm cuvette ( $1.5 \times 10^7$  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  $\mu$ M IN3 (a membrane-permeant, nonpeptide GnRH receptor antagonist) (Lu et al., 2005, 2007). Cells were washed four times, each wash lasted for 30 min, with 2% Me<sub>2</sub>SO, 0.1% BSA/HEPES/DMEM at 37°C after 48 h of incubation. The cells were then continued to be

|               |                            |
|---------------|----------------------------|
| Human I       | SGKIRVTVTFFFLFLLSATFNASFL  |
| M. radiata I  | SGKIRVTVTFFFLFLLSATFNASFL  |
| Marmoset I    | SGKIRVTVTFFFLFLLSSTTFNASFL |
| Dog I         | SGKIRVTVTFFFLFLLSSTIFNASFL |
| Horse I       | SGKIRVTVTFFFLFLLSSTTFNASFL |
| Pig I         | SPNIRVTVTFFFLFLLSATFNASFL  |
| Sheep I       | SGKIRVTVTFFFLFLLSSTIFNTSFL |
| Mouse I       | SGKIRVTVTFFFLFLLSATFNASFL  |
| Rat I         | SGKIRVTVTFFFLFLLSATFNASFL  |
| Possum I      | SGKIRVMVTFFFLFLVSTAFNASFL  |
| Chicken I     | AAKRVVAVTAVFLFLAACSNVAVLC  |
| Bullfrog II   | AAKRVGVGTCCFFLIASCSNVAVLC  |
| Rana Dy. II   | AAKRVGVGTCCFFLIASCSNVAVLC  |
| Xenopus I     | AAKRVGVGTCCFFLIASCSNVAVLC  |
| Fugu Iia      | AAQCFVATTLVLFVFAAVSNVAVLI  |
| Goldfish I    | AAHFRVATLVLFVFAAISNLSVLI   |
| Fugu II       | AAQCFVATTLVLFVFAACSNNALLA  |
| Cichlid I     | AAQCFVATTLVLFVFAACSNNALLV  |
| Medaka II     | AAQCFVATTLVLFVFAACSNNALLT  |
| R. Trout      | AAQCFVATTLVLFVFAACSNNALLV  |
| Goldfish Ia   | AAQCFVATTLVLFVFAACSNNALLI  |
| Catfish I     | AAKRVVAVTAVFLFLAACSNVAVLI  |
| Catfish II    | AAQCFVATTLVLFVFAACSNNALLI  |
| Japanese Eel  | AAQCFVATTLVLFVFAACSNNALLI  |
| Gn. Moneky II | AAKRVGVGTIVLFVSSAGGNLAVLW  |
| Rh. Moneky II | AAKRVGVGTIVLFVSSAGGNLAVLW  |
| Marmoset II   | AAKRVGVGTIVLFVSSAGGNLAVLW  |
| T. Natans     | AAKRVVITFVFLFISSACFNIALW   |
| Bullfrog III  | AAKRVVITFVFLFISSACFNIALW   |
| Rana Dy. III  | AAKRVVITFVFLFISSACFNIALW   |
| Xenopus II    | AAKRVVITFVFLFISSACFNIALW   |
| Bullfrog I    | AAKRVVITFVFLFISSACFNIALW   |
| Rana Dy. I    | AAKRVVITFVFLFISSACFNIALW   |
| Medaka I      | AAKRVVITFVFLFISSACFNIALW   |
| Fugu Ia       | AAKRVVITFVFLFISSACFNIALW   |
| M. SeaBass    | AAKRVVITFVFLFISSACFNIALW   |
| S. SeaBass    | AAKRVVITFVFLFISSACFNIALW   |
| Amberjack     | AAKRVVITFVFLFISSACFNIALW   |
| Fugu I        | AAKRVVITFVFLFISSACFNIALW   |
|               | : . * * : : * *            |

**Fig. 1.** ClustalW alignment of sequences of TM 1 from vertebrate GnRH type I, II, and III receptors. Arg<sup>38(1.35)</sup> in human GnRH receptor and the corresponding residue in other GnRH receptors from different species is shown by the black box. \*, identical or conserved residues in all sequences in the alignment; :, conserved substitutions; ., semiconserved substitutions.

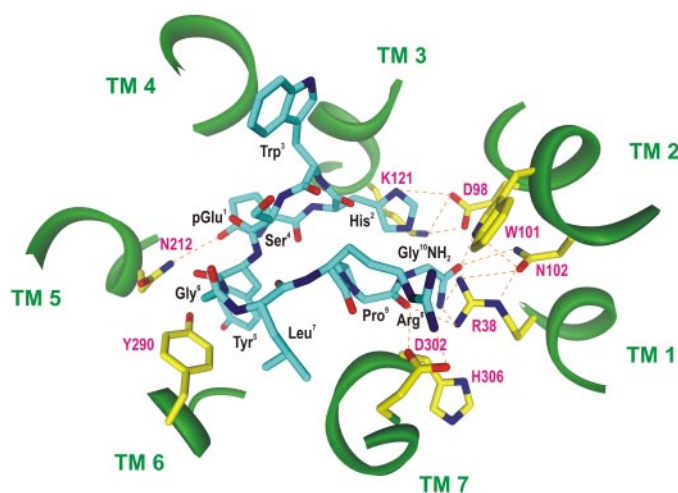


incubated with complete DMEM overnight (~18 h) and were washed again as above before assays.

**Ligand Binding Assays.** Radioligand binding assays were performed on intact cells 72 h after transfection (Lu et al., 2005, 2007). Transfected cells in 12-well culture plates were washed as above and then incubated with [<sup>125</sup>I]cetrorelix (Hoffmann et al., 2000) at  $1 \times 10^5$  cpm/well and various concentrations of unlabeled GnRH ligands in 0.1% BSA/HEPES/DMEM for 4 h at 4°C. After incubation, 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 by  $\gamma$  spectrometry. All experiments were performed in triplicate and repeated at least three times.

**IP Accumulation Assays.** Assays for ligand stimulation of IP production were carried out as described previously (Lu et al., 2005, 2007). Transfected cells were seeded onto 12-well plates in the absence or presence of  $1 \mu\text{M}$  concentration of IN3. After 48 h, cells were washed as above and labeled overnight with  $1 \mu\text{Ci/ml}$  D-[myo-<sup>3</sup>H]-inositol in inositol-free DMEM containing 1% dialyzed fetal calf serum. Before conducting IP assay, the medium was removed, and cells were washed again as above. Cells were then preincubated with 0.5 ml of buffer composed of 140 mM NaCl, 20 mM HEPES, 8 mM glucose, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 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 30 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 10 mM formic acid. The <sup>3</sup>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.

**Data Analysis.** Binding curves were fitted to the Hill equation or to the one-site model of the binding using SigmaPlot 9.0 (Systat Software, Inc., San Jose, CA) or Prism 4.0 (GraphPad Software Inc., San Diego, CA), yielding an IC<sub>50</sub> value. The maximum receptor binding sites ( $B_{\text{max}}$ ) were expressed relative to a wild-type control included in each transfection. IP dose-response curves were fitted to a sigmoidal dose-response model, yielding a basal activity, a maximum response ( $E_{\text{max}}$ ), and an EC<sub>50</sub> value.



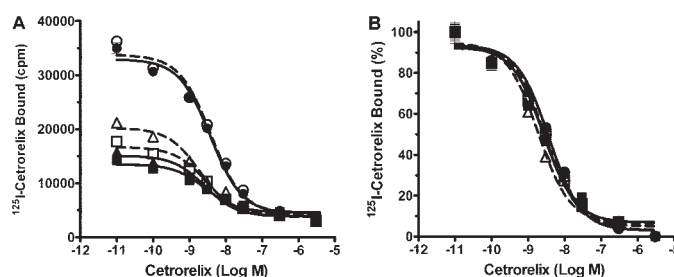
**Fig. 2.** Molecular model of human receptor-GnRH I complex. A  $\beta\text{II}'$  conformation of GnRH I derived from the NMR structure (1YY1) was docked into the receptor model according to the experimentally identified intermolecular interactions between GnRH I (cyan/black) and the receptor contact sites (yellow/magenta); pGlu<sup>1</sup> interacts with Asn<sup>212(5.39)</sup>, His<sup>2</sup> with Lys<sup>121(3.32)</sup>/Asp<sup>98(2.61)</sup>, Tyr<sup>5</sup> with Tyr<sup>290(6.58)</sup> and Arg<sup>8</sup> with Asp<sup>302(7.32)</sup>, and Pro<sup>9</sup>-Gly<sup>10</sup>NH<sub>2</sub> with Trp<sup>101(2.64)</sup>/Asn<sup>102(2.65)</sup>. The H-bonds are indicated by broken lines. The model shows that the side chain of Arg<sup>38(1.35)</sup> in TM 1 (green) of the receptor is capable of making H-bonds with the carbonyl oxygen of Pro<sup>9</sup> and Gly<sup>10</sup>NH<sub>2</sub> of GnRH, in addition to the intramolecular interactions with Trp<sup>101(2.64)</sup> and Asn<sup>102(2.65)</sup> in TM 2 and Asp<sup>302(7.32)</sup> and His<sup>306(7.36)</sup> in TM 7.

## Results

**GnRH Docking and MD Simulations.** We performed GnRH docking experiments using our model constructed previously of the human GnRH receptor (Lu et al., 2007; Millar et al., 2007), which was built on the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (Salom et al., 2006). A  $\beta\text{II}'$  conformation of GnRH I and GnRH II was satisfactorily docked into the model according to the previously experimentally identified intermolecular interactions between GnRH and receptor followed by energy minimization and MD simulations (Favre et al., 2005; Millar et al., 2007) (Fig. 2). In our model, Arg<sup>38(1.35)</sup> of the GnRH receptor is located in close proximity (~2–3 Å) to Pro<sup>9</sup> and Gly<sup>10</sup>-NH<sub>2</sub> of GnRH and may therefore bind directly to the ligand. We have validated the proposed interactions by examining whether mutations of Arg<sup>38(1.35)</sup> of the receptor affects binding of native GnRHs and their analogs possessing C-terminal modification.

**Expression of Human GnRH Receptors in COS-7 Cells.** Wild-type and mutant GnRH receptor constructs were transiently transfected into COS-7 cells, and the binding affinity of cetrorelix to each was measured by homologous competition assay. Alanine and lysine mutations of Arg<sup>38(1.35)</sup> of the receptor decreased  $B_{\text{max}}$  values to 25 and 30% of wild type, respectively (Fig. 3A). Expression levels of mutant receptors were moderately increased by preincubation of the transfected COS-7 cells with  $1 \mu\text{M}$  concentration of the membrane-permeant, nonpeptide GnRH antagonist, IN3, measured after extensive washes of the cells with 2% Me<sub>2</sub>SO, which allowed the removal of IN3 from the cells (Lu et al., 2005, 2007). The receptor expression of the poorly expressed mutant receptors were rescued typically between 15 and 30% by IN3 pretreatment. No changes in affinity for cetrorelix between IN3 pretreated and untreated cells were observed for wild-type or mutant receptors. In addition, neither mutation had any significant effect on the affinity of the receptor for cetrorelix (Fig. 3B).

**Differential Effects of Mutations of Arg<sup>38(1.35)</sup> of the Receptor on Binding Affinities for GnRH I, GnRH II, and Their Pro<sup>9</sup>-NH<sub>2</sub> Analogs.** To examine the roles of the side chain of Arg<sup>38(1.35)</sup> of the GnRH receptor in ligand binding, we examined the effects of mutation of Arg<sup>38(1.35)</sup> to alanine and lysine on receptor binding affinity for GnRH I



**Fig. 3.** Homologous inhibition binding of cetrorelix. A, binding curves showing homologous competitive binding of peptide antagonist cetrorelix in wild-type and mutant receptors in IN3 pretreated (broken line) and nonpretreated (solid line) cells. Details are given under *Materials and Methods*. Results are representative experiments, which were repeated at least three times with essentially the same results. B, normalized binding curves, which show no significant differences in receptor affinity for cetrorelix between the IN3-pretreated and the untreated cells in wild-type and mutant receptors. ●, wild type; ○, wild type with IN3 pretreatment; ■, R38A; □, R38A with IN3 pretreatment; ▲, R38K; △, R38K with IN3 pretreatment. Points, mean  $\pm$  S.E. of triplicate measurements.

and GnRH II and their Pro<sup>9</sup>-NHet analogs (Fig. 4). Binding curves for each analog are shown in Fig. 5, and the IC<sub>50</sub> values are summarized in Table 1. The Hill coefficients were unaltered for all mutants. The mutation R38A had a much greater effect on ligand binding affinity than that of the R38K mutant. GnRH I and GnRH II exhibited IC<sub>50</sub> values of 3.97 and 13.4 nM, respectively, to wild-type human GnRH receptor. GnRH I exhibited a 989-fold reduction in affinity toward the R38A mutant and a 24-fold reduction in affinity toward the R38K mutant relative to wild-type receptor. Similar to GnRH I, the R38A and R38K mutants gave 1268- and 54-fold reductions in affinity toward GnRH II, respectively. Both mutations of the receptor, however, had much less of an effect on binding affinity of the Pro<sup>9</sup>-NHet analogs. The R38A and R38K mutants only had 35- and 5-fold reductions, respectively, in affinity for GnRH I analog with substitution of the C-terminal glycine by an ethylamine group. The mutations R38A and R38K had a similar effect on GnRH II analog with the same substitution of GnRH I, giving reduced affinities by 40- and 11-fold.

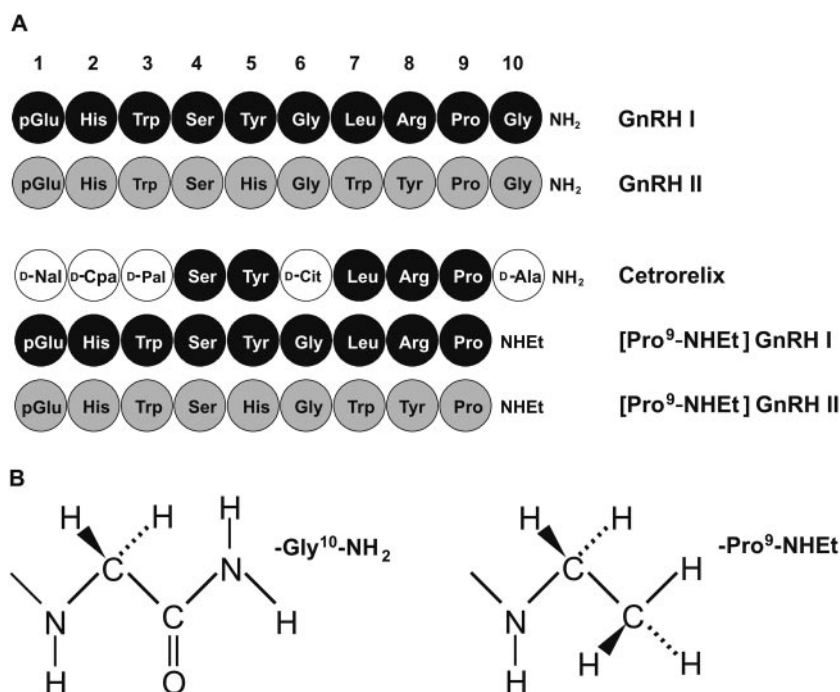
**Effect of Mutations on GnRH Ligand-Induced IP Turnover.** GnRH I, GnRH II, [Pro<sup>9</sup>-NHet]GnRH I, and [Pro<sup>9</sup>-NHet]GnRH II elicited robust IP responses from COS-7 cells transfected with the wild-type human GnRH receptor. The  $E_{\max}$  for all experiments were typically greater than 5 times the basal activity. The effects of mutation of Arg<sup>38(1.35)</sup> of the receptor to alanine and lysine on the IP responses are shown in Fig. 6. The EC<sub>50</sub> and  $E_{\max}$  values are summarized in Table 2. The mutation R38A had a much greater effect on IP response than that of the R38K mutant. GnRH I and GnRH II exhibited EC<sub>50</sub> values of 0.87 and 26 nM, respectively, in COS-7 cells transfected with wild-type GnRH receptor. Mutation of Arg<sup>38(1.35)</sup> of the receptor to alanine and lysine led to 6520- and 146-fold reductions, respectively, in potency of GnRH I (EC<sub>50</sub>) in stimulating IP responses. There was also a reduction in potency of GnRH II in the mutations of Arg<sup>38(1.35)</sup> to alanine (656-fold reduction)

and lysine (76-fold reduction). It is interesting that mutations of Arg<sup>38(1.35)</sup> of the receptor to alanine and lysine had similar reductions (<3-fold differences) on potency of [Pro<sup>9</sup>-NHet]GnRH I (2405-fold for R38A and 77-fold for R38K) and [Pro<sup>9</sup>-NHet]GnRH II (374-fold for R38A and 65-fold for R38K) as to GnRH I and GnRH II, although the mutants have significantly differential effects on binding affinity for GnRHs and their Pro<sup>9</sup>-NHet analogs, the latter exhibited a much smaller decrease toward the mutations. The  $E_{\max}$  values for all peptide agonists were reduced by ~50% relative to wild type with the R38A mutant and were little affected by the mutation R38K (Table 2). No increase in basal activity was observed in both mutants R38A and R38K.

## Discussion

GPCRs recognize and bind a variety of structurally diverse ligands and modulate the majority of physiological processes, and thus are major drug targets. Molecular modeling and site-directed mutagenesis studies have been extensively applied to delineate ligand binding sites in the GnRH receptors (Sealfon et al., 1997; Millar et al., 2004, 2007) and other GPCRs (Ballesteros et al., 2001; Lu et al., 2002). Previous studies have shown that pGlu<sup>1</sup> of GnRH I interacts with Asn<sup>212(5.39)</sup> (Hoffmann et al., 2000; Hövelmann et al., 2002; Söderhäll et al., 2005); His<sup>2</sup> interacts with Asp<sup>98(2.61)</sup> and Lys<sup>121(3.32)</sup> (Zhou et al., 1995; Flanagan et al., 2000); Tyr<sup>5</sup> interacts with Tyr<sup>290(6.58)</sup> (Coetsee et al., 2007); Arg<sup>8</sup> of GnRH I interacts with Asp<sup>302(7.32)</sup> (Flanagan et al., 1994; Fromme et al., 2001); and Gly<sup>10</sup>NH<sub>2</sub> with Asn<sup>102(2.65)</sup> (Davidson et al., 1996; Hoffmann et al., 2000).

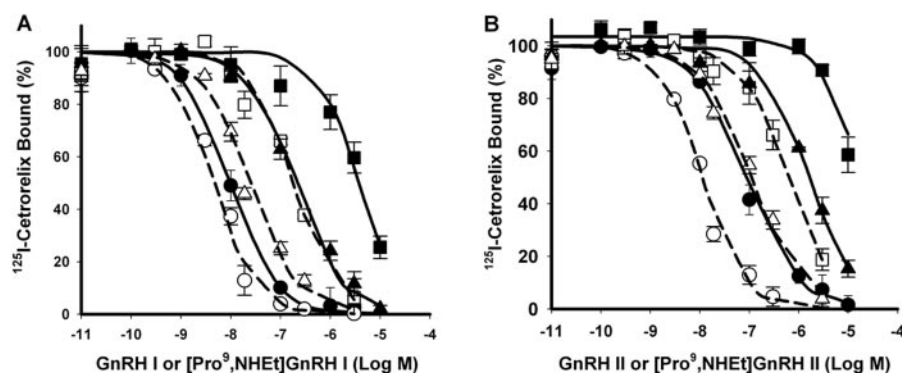
We have successfully docked a  $\beta$ II'-type turn conformation of GnRH derived from a three-dimensional structure of GnRH based on a recent NMR report into the experimentally identified ligand binding sites of the receptor model (Fig. 2). This reveals that Arg<sup>38(1.35)</sup> of the GnRH receptor may act as a potential binding site for GnRH. Arg<sup>38(1.35)</sup> of the GnRH



**Fig. 4.** A, primary structures of GnRH peptides used in this study. B, the chemical structures of the C termini of GnRH I, GnRH II, and their Pro<sup>9</sup>-NHet derivatives.

receptor is completely conserved in all vertebrate type I, II, and III GnRH receptors (Fig. 1), implying its functional importance in receptor folding, ligand binding, or activation. Mutation of Arg<sup>38(1.35)</sup> of the GnRH receptor to alanine or lysine markedly reduced receptor binding affinities for GnRH I and GnRH II. The mutation R38A led to 989- and 1268-fold reduction in affinity for both GnRH I and GnRH II compared with wild-type receptor (Table 1 and Fig. 5). Conservative mutation of Arg<sup>38(1.35)</sup> to lysine had a lesser effect on the receptor binding affinities for both GnRH I and GnRH II, giving 24- and 54-fold reductions, respectively (Table 1 and

Fig. 5). The much smaller effect of mutation to lysine than alanine, which deletes the side chain beyond  $\beta$ -carbon, suggests that the side chain of Arg<sup>38(1.35)</sup> of the GnRH receptor makes multiple contacts with GnRH by forming hydrogen-bond networks and Van der Waals contacts (Fig. 2). Substitution of Arg<sup>38(1.35)</sup> of the receptor with lysine seems to maintain part of the GnRH receptor-ligand interactions. Mutations of Arg<sup>38(1.35)</sup> of the receptor to alanine and lysine had much less effect on receptor binding affinity for Pro<sup>9</sup>-NHEt analogs, with affinity reductions for [Pro<sup>9</sup>-NHEt]GnRH I of 35- and 5-fold, and for [Pro<sup>9</sup>-NHEt]GnRH



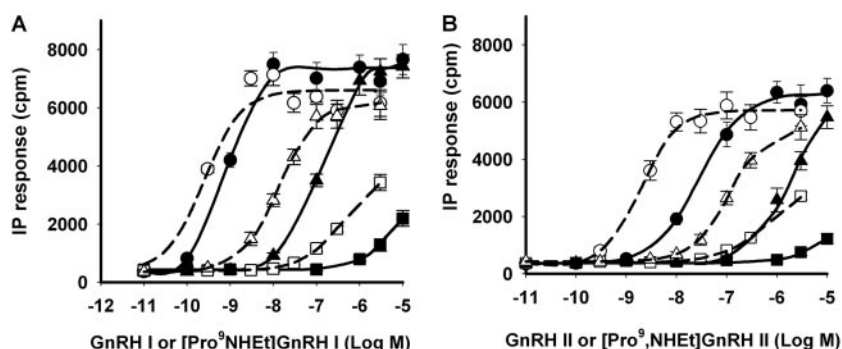
**Fig. 5.** Binding of GnRH peptides. A, inhibition curves of GnRH I (solid line) and [Pro<sup>9</sup>-NHEt]GnRH I (broken line) in human GnRH wild-type and R38A and R38K mutant receptors. B, inhibition curves of GnRH II (solid line) and [Pro<sup>9</sup>-NHEt]GnRH II (broken line) in human GnRH wild-type and R38A and R38K mutant receptors. The radiolabeled ligand used in the assays is [<sup>125</sup>I]cetrorelix. ●, wild type with GnRH I/GnRH II; ○, wild type with Pro<sup>9</sup>-NHEt analogs; ■, R38A with GnRH I/GnRH II; □, R38A with Pro<sup>9</sup>-NHEt analogs; ▲, R38K with GnRH I/GnRH II; △, R38K with Pro<sup>9</sup>-NHEt analogs.

TABLE 1

Binding of GnRH peptides to human wild-type and mutant receptors

The IC<sub>50</sub> values were measured by inhibition of [<sup>125</sup>I]cetrorelix binding by increasing concentrations of unlabeled GnRH peptides and are expressed as the mean  $\pm$  S.E. of three or more experiments performed in triplicates. Fold increases in IC<sub>50</sub> values of R38A and R38K mutants are expressed relative to those measured for the wild-type receptor.

| Peptide                         | Wild-Type<br>IC <sub>50</sub><br><br>nM | R38A                   |                | R38K                   |                |
|---------------------------------|---|------------------------|----------------|------------------------|----------------|
|                                 |   | IC <sub>50</sub><br>nM | -Fold Increase | IC <sub>50</sub><br>nM | -Fold Increase |
| GnRH I                          | 3.97 $\pm$ 0.25                         | 3530 $\pm$ 233         | 989            | 95.2 $\pm$ 8.0         | 24             |
| [Pro <sup>9</sup> -NHEt]GnRH I  | 2.34 $\pm$ 0.08                         | 82 $\pm$ 11            | 35             | 11.9 $\pm$ 3.0         | 5              |
| GnRH II                         | 13.4 $\pm$ 1.1                          | 17000 $\pm$ 1681       | 1268           | 721 $\pm$ 33.3         | 54             |
| [Pro <sup>9</sup> -NHEt]GnRH II | 6.43 $\pm$ 0.22                         | 255 $\pm$ 35           | 40             | 72.4 $\pm$ 20.2        | 11             |



**Fig. 6.** GnRH peptide stimulated increases of total IP in COS-7 cells transiently expressing the wild-type and mutant receptors. A, GnRH I- (solid line) and [Pro<sup>9</sup>-NHEt]GnRH I- (broken line) elicited IP responses in human GnRH wild-type, R38A, and R38K mutant receptors. B, GnRH II- (solid line) and [Pro<sup>9</sup>-NHEt]GnRH II- (broken line) elicited IP responses in wild-type, R38A, and R38K mutant receptors. ●, wild-type with GnRH I/GnRH II, ○, wild-type with Pro<sup>9</sup>-NHEt analogs; ■, R38A with GnRH I/GnRH II; □, R38A with Pro<sup>9</sup>-NHEt analogs; ▲, R38K with GnRH I/GnRH II; △, R38K with Pro<sup>9</sup>-NHEt analogs.

TABLE 2

Effect of mutations of Arg<sup>38(1.35)</sup> of the receptor to alanine and lysine on the GnRH peptide-stimulated IP responses

EC<sub>50</sub> and E<sub>max</sub> values are presented as mean  $\pm$  S.E. of three or more independent experiments. The fold-change was calculated as the ratio of the EC<sub>50</sub> values in the mutant and wild-type receptors. E<sub>max</sub> is expressed relative to a wild-type control included in each transfection.

|                                 | Wild-Type              |                          | R38A                   |                                    |                          | R38K                   |                                    |                          |
|---------------------------------|------------------------|--------------------------|------------------------|------------------------------------|--------------------------|------------------------|------------------------------------|--------------------------|
|                                 | EC <sub>50</sub><br>nM | E <sub>max</sub><br>% wt | EC <sub>50</sub><br>nM | EC <sub>50</sub><br>-Fold Increase | E <sub>max</sub><br>% wt | EC <sub>50</sub><br>nM | EC <sub>50</sub><br>-Fold Increase | E <sub>max</sub><br>% wt |
| GnRH I                          | 0.87 $\pm$ 0.06        | 100                      | 5672 $\pm$ 302         | 6520                               | 42 $\pm$ 4               | 129 $\pm$ 9            | 146                                | 100 $\pm$ 9              |
| [Pro <sup>9</sup> -NHEt]GnRH I  | 0.18 $\pm$ 0.01        | 100                      | 433 $\pm$ 21           | 2405                               | 56 $\pm$ 3               | 14 $\pm$ 2             | 77                                 | 91 $\pm$ 6               |
| GnRH II                         | 26 $\pm$ 3             | 100                      | 17070 $\pm$ 690        | 656                                | 41 $\pm$ 5               | 1978 $\pm$ 334         | 76                                 | 104 $\pm$ 7              |
| [Pro <sup>9</sup> -NHEt]GnRH II | 1.9 $\pm$ 0.15         | 100                      | 711 $\pm$ 62           | 374                                | 58 $\pm$ 3               | 125 $\pm$ 12           | 65                                 | 96 $\pm$ 4               |



II, of 40- and 11-fold (Table 1 and Fig. 5). These data suggest that the side chain of Arg<sup>38(1.35)</sup> of the receptor is crucial for high-affinity binding of GnRH I and GnRH II, which contain a C-terminal glycine moiety, but is relatively less important for binding of GnRH analogs with glycine substitution by an ethylamide group. The much smaller effect of the mutations on ligand binding affinity for [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRH analogs than the parent GnRHs indicates that the ethylamide moiety of [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRHs may make hydrophobic contacts with some other sites in the receptor, which compensate for the loss of interactions between glycine of GnRHs and Arg<sup>38(1.35)</sup> of the receptor. Similarly there was no reduction of binding affinity of the mutants for the peptide antagonist cetrorelix, which possesses D-Ala<sup>10</sup>-NH<sub>2</sub>. Consistent with the molecular modeling, these results suggest that the side chain of Arg<sup>38(1.35)</sup> in the GnRH receptors make direct contacts with the glycine moiety of GnRH I and GnRH II. Our molecular docking shows that Arg<sup>38(1.35)</sup> of the human GnRH receptor, whose side chain is positioned above the C-terminal end of GnRH with potential Van der Waal's contacts along the side chain, may form an additional H-bond with the backbone carbonyl oxygen of Pro<sup>9</sup>. This may explain why mutations of Arg<sup>38(1.35)</sup> of the receptor had small effects on receptor binding affinity for [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRH analogs. Ligand docking experiments performed with GnRH II on the human GnRH receptor model also suggested that GnRH II is likely to interact with Arg<sup>38(1.35)</sup> of the receptor in a similar manner as GnRH I, which is supported by the mutagenesis studies.

Studies on other peptide GPCRs have also shown that the extracellular end of TM 1 is important for high affinity binding of peptide agonists (Silvente-Poirot et al., 1998; Anders et al., 1999; Wesley et al., 2002; Hawtin et al., 2005; Marco et al., 2007). Mutation of the residue Glu<sup>1.35</sup> of the V<sub>1a</sub> vasopressin receptor (which is positionally equivalent to Arg<sup>38(1.35)</sup> and is totally conserved among vasopressin and oxytocin receptors) to alanine leads to a 1700-fold decrease in affinity for peptide agonist vasopressin but has no effect on peptide antagonist binding affinity (Hawtin et al., 2005). The equivalent residue Arg<sup>1.35</sup> in the cholecystokinin-2 receptor has also been shown to be important for peptide ligand binding (Silvente-Poirot et al., 1998; Marco et al., 2007). Direct evidence on the role of the extracellular end of TM 1 in peptide agonist binding was obtained via the covalent linking experiment in which a photoreactive tritiated analog of sulfated cholecystokinin octapeptide was covalently attached to the exofacial sequences of TM 1 (Anders et al., 1999). Together with our studies, we propose that the extracellular end of TM 1 of peptide GPCRs may play a common role for peptide agonist binding.

In parallel with the reduced receptor binding affinity and expression levels, the receptor mutants R38A and R38K also gave markedly decreased potencies in mediating IP responses with increased EC<sub>50</sub> values for GnRH I by 6520- and 146-fold, for GnRH II by 656- and 76-fold (Table 2 and Fig. 6). The mutations had similar effect on IP responses elicited by Pro<sup>9</sup>-NH<sub>2</sub>Et analogs as that of the parent GnRHs (less than 3-fold differences; Table 2 and Fig. 6), although they had much less effect on the binding affinity of [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRHs than GnRHs (Table 1 and Fig. 5). Mutation of Arg<sup>38(1.35)</sup> of the receptor to alanine also resulted in approximately 50%

reduction in maximum IP responses for both GnRH and [Pro<sup>9</sup>-NH<sub>2</sub>Et]GnRH analogs, whereas the receptor mutation R38K had no significant effect on E<sub>max</sub> (Table 2 and Fig. 6). These results indicate that Arg<sup>38(1.35)</sup> of the receptor plays an important role in stabilizing the receptor active conformation through forming a new set of inter- and intramolecular interactions (Hulme et al., 1999). This is in agreement with a previous report suggesting that the N- and C-terminal domains are important in receptor binding and activation (Sealfon et al., 1997). The reduction in receptor expression levels caused by mutations of Arg<sup>38(1.35)</sup> to alanine and lysine, which were moderately increased by IN3 preincubation, suggests that the side chain of Arg<sup>38(1.35)</sup> may form intramolecular interactions that stabilize receptor folding (Lu et al., 1997). When these interactions are disrupted, incorrect folding of the receptor protein increases, resulting in increased degradation (Lu and Hulme, 1999). The side chain of Glu<sup>1.35</sup> in the human V<sub>2</sub> receptor (equivalent to Arg<sup>38(1.35)</sup> of the GnRH receptor) has been shown to make intramolecular contacts with Gln<sup>2.61</sup> and Lys<sup>2.65</sup> in TM 2, but this is not the case in the murine V<sub>2</sub> receptor, in which Glu<sup>1.35</sup> is proposed to interact with Arg<sup>7.32</sup> in TM 7 (Oksche et al., 2002). Apparently the interactions depend on the local environments. The extracellular ends of TM 1 and TM 7 are also shown in proximity in opioid receptors (Xu et al., 2005). In our molecular model, the side chain of Arg<sup>38(1.35)</sup> of the GnRH receptor is located in a close proximity with the residues Asp<sup>98(2.61)</sup>, Trp<sup>101(2.64)</sup>, and Asn<sup>102(2.65)</sup> in TM 2, and Asp<sup>302(7.32)</sup> and His<sup>306(7.36)</sup> in TM 7, and is able to make a H-bond network. However, no constitutive activation was observed in both receptor mutants R38A and R38K. This is consistent with our previous proposal that the GnRH receptor might be strongly constrained in the inactive state because none of the mutations of the equivalent residues of other GPCRs whose mutation leads to constitutive activity (Lu et al., 2002; Smit et al., 2007) gives rise to constitutive activity in the GnRH receptor (Lu et al., 2005, 2007). We therefore propose that Arg<sup>38(1.35)</sup> of the GnRH receptor may participate with other residues as a ligand-dependent receptor activation switch.

In summary, we have shown that the side chain of Arg<sup>38(1.35)</sup> of the GnRH receptor may act as a direct binding site for both endogenous ligands GnRH I and GnRH II. Molecular modeling and site-direct mutagenesis studies in combination with ligand modification suggest that Arg<sup>38(1.35)</sup> of the GnRH receptor interacts directly with the backbone carbonyl oxygen of Pro<sup>9</sup> and C-terminal glycine in both GnRH I and GnRH II. The reduced receptor expression levels and signaling potency given by mutation of Arg<sup>38(1.35)</sup> to alanine or lysine suggest that Arg<sup>38(1.35)</sup> may make intramolecular interactions, which stabilize the receptor in the ground state but are broken by ligand binding, creating a new set of inter- and intramolecular interactions that stabilize receptor active conformations.

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