Overlapping, Nonidentical Binding Sites of Different Classes of Nonpeptide Antagonists for the Human Gonadotropin-Releasing Hormone Receptor

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Peptide agonists and antagonists of the human gonadotropin-releasing hormone receptor (GnRH-R) are widely used to treat a range of reproductive hormone related diseases. Recently, nonpeptide, orally available GnRH-R antagonists have emerged from several chemical classes. To understand how a relatively large peptide-binding pocket can recognize numerous nonpeptide ligands, we undertook a systematic mapping of GnRH-R residues involved in the binding of three nonpeptide antagonists. A region composed of the extracellular portions of transmembrane helices 6 and 7, extracellular loop 3, and the N-terminal domain significantly contributed to nonpeptide antagonist binding. However, each molecule was affected by a different subset of residues in these regions, indicating that each appears to occupy distinct, partially overlapping subregions within the more extensive peptide-binding pocket. Moreover, the resulting receptor interaction maps provide a basis to begin to reconcile structure−activity relationships between various nonpeptide and peptide series and facilitate the design of improved therapeutic agents.

Introduction

Gonadotropin releasing hormone (GnRH,[a] also known as leutenizing hormone releasing hormone, or LH-RH) is a 10-residue peptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) produced by the hypothalamus that regulates the reproductive axis by binding to and activating its G protein-coupled receptor, GnRH-R, in the pituitary. Modulation of GnRH signaling has several clinical applications, including treatments for infertility, prostate cancer, benign prostate hyperplasia, endometriosis, uterine fibroids, and hirsutism.[1−7] Chronic treatment with GnRH agonists (e.g., leuprolide for prostate cancer) causes down-regulation of the receptor,[3,8] eventually leading to castrate levels of gonadal hormones. A drawback of this strategy is an initial stimulation of gonadotropin secretion, or "flare", due to the initial agonism, which can lead to an exacerbation of symptoms.[8,9] Recently, androgen ablation via antagonism of GnRH-R has emerged as a promising therapeutic strategy, because GnRH-R antagonists do not cause the hormonal flare.[7] Current anti-GnRH-R therapies use peptides, or modified peptides, which carry the liability of needing to be administered parenterally due to a lack of oral bioavailability. Recently, orally active, nonpeptide antagonists of GnRH-R have begun to emerge as a potentially important new class of therapeutic agents.[10]

Multiple chemical classes of nonpeptide GnRH antagonists have been described in the literature ranging from relatively simple furan derivatives[11] to analogues of macrolide antibiotics.[12] All appear to compete with peptide analogues for a common binding site raising the question: How can such diverse nonpeptide chemical structures bind with high affinity to a common peptide receptor? While receptor residues involved in binding peptide ligands have been extensively described,[13−15] relatively little is known about the interaction of nonpeptide ligands with GnRH-R.

To begin to understand how a relatively large peptide-binding pocket can recognize such a wide range of nonpeptide ligands, we undertook a systematic mutagenesis study of the ligand-binding pocket of the GnRH-R. Here, we describe the production of 76 GnRH-R mutants, probing those proteins with different classes of nonpeptide antagonists and agonist peptides, and a comparison of the binding sites of the different molecules. The resulting map of receptor interactions can be used to understand the differences in binding between peptide agonists and nonpeptide antagonists, between different classes of nonpeptide antagonists, and in reconciling the SAR within and across chemical series. This information also provides data to allow more accurate modeling of the receptor and receptor/ligand interactions, improving the likelihood of designing nonpeptide antagonists with desired affinities and "drug-like" characteristics.

Experimental Procedures

Nomenclature. GnRH peptide variants are indicated by the standard three-letter code for a residue, superscripted by its sequence position in the peptide (e.g., His^5 GnRH indicates that the tyrosine at position 5 in mammalian GnRH is replaced by histidine). Residues from GnRH-R are referred to by their standard one-letter code, their position in the human primary sequence, and their reference position in the GPCR nomenclature of Ballesteros and Weinstein superscripted[16] (e.g., $D302^{(7.32)}$). GnRH-R mutants are named as above with the mutant residue indicated after the identity and position of the residue in the wild-type protein (e.g., $D302^{(7.32)}N$). Note that residues and mutants in the N-terminal domain are only referred to by their primary sequence position (e.g., M24 and M24I).

Nonpeptide Antagonists. The compounds displayed in Figure 1 were all made in-house by previously described methods.[17−19]

Mutagenesis. The gene for human GnRH-R has been cloned and expressed as described previously.[20,21] Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The cDNA for GnRH-R mutants was cloned into the pcDNA3.1(+) expression vector for transient transfections in mammalian cell lines. The complete coding regions of all mutant receptor genes were verified by DNA sequencing analysis (ABI

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[a] Abbreviations: ECL, extracellular loop; ICL, intracellular loop; GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; GPCR, G protein-coupled receptor; SAR, structure−activity relationship; TM, transmembrane helix.

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Prism 377 DNA sequencer, Applied Biosystems, Inc., Foster City, CA).

Cell Culture and Transient Transfections. All cell culture media and solutions were purchased from Cellgro (Fisher Scientific, Tustin, CA). COS-7 cells were obtained from American Type Cell Culture (Manassas, VA) and were maintained in Dulbeccos's modified Eagle's medium (DMEM, MediaTech, Inc., Herndon, VA) containing 10% fetal bovine serum, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin. COS-7 cells were seeded in 500 cm^2 tissue culture plates and grown to confluency prior to cell transfection. Cells (5×10^7) were transfected with 50 μg of the appropriate GnRH-R DNA construct by electroporation in a BTX ElectroCell Manipulator ECM 600 (Fisher Scientific, Pittsburgh, PA) using the following settings: 1000 μF capacitance, 48 Ω resistance, and 300 V/cm charging voltage. Transfected cells were cultured for 36−48 h prior to membrane preparation.

Membrane Preparation. Transiently transfected COS-7 cells were harvested, washed, and resuspended in membrane buffer (20 mM HEPES, pH 7.2, 6 mM $MgCl_2$, and 1 mM EDTA). Cells were centrifuged at 1500 rpm, and the cell pellets were resuspended in a small volume of membrane buffer. Cells were lysed by release of pressure at 900 psi for 30 min at 4 °C in a nitrogen chamber. The homogenate was centrifuged at 1000 rpm for 10 min at 4 °C to remove nuclei and cellular debris. Membranes were collected from the supernatant by centrifugation at 16 500 rpm for 45 min at 4 °C. Finally, the membranes were resuspended in membrane buffer at a concentration of 1 mg/mL; aliquots were frozen in liquid nitrogen and stored at −80 °C until used.

Binding Assays and Data Analysis. Competitive ligand-binding assays were performed in 96-well filter plates (Multi-screen 1.2 μm glass-fiber plates, Millipore, Bedford, MA). COS-7 membranes (5−20 μg) expressing the GnRH-R of interest were used for each assay point. Cell membranes in each assay were incubated with 0.3 nM [^{125}I-His^5, D-Tyr^6]GnRH^{22} and varying concentrations of nonpeptide or unlabeled peptide. The final volume of each reaction was adjusted to 100 μL per well with assay buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, and 0.1% bovine serum albumin). Membranes were captured onto a 96-well plate by vacuum filtration after a 2-h incubation at room temperature, followed by washing twice with phosphate-buffered saline. The filter plates were dried overnight in a drying oven at 37 °C, and 100 μL of scintillation fluid (Scint20, Packard Instruments, Downers Grove, IL) was added to each well prior to counting in a TopCount NXT (Packard Instruments, Downers Grove, IL). Experiments were performed using 12 points per experiment. IC_{50} values were calculated using the "one-site competition" nonlinear regression analysis of Prism (GraphPad, Version 4.01, San Diego, CA). Each experiment was performed at least three times. It is important to note that the peptides (GnRH and [His^5, D-Tyr^6]GnRH) and the nonpeptide molecules tested were each able to completely inhibit the binding of both ^{3}H-**1** and [^{125}I-His^5, D-Tyr^6]GnRH (data not shown), consistent with an orthosteric interaction of these nonpeptides and peptides at the human GnRH-R.[23]

Molecular Modeling. A preliminary model of the human GnRH-R structure was prepared based on the coordinates of bovine rhodopsin (PDB_ID: 1F88).[24] Sequences were aligned as shown in Figure 2A based on highly conserved residues in GPCR helical regions,[25] and a schematic of the receptor sequence is displayed in Figure 2B. In general, helical regions, ECL1, and portions of ECL2 and ECL3 were modeled as indicated by the shaded portions of the sequence alignment in Figure 2A. ECL2 residues were aligned based on the conserved C114$^{(3.25)}$−C196$^{(5.23)}$ disulfide between TM3 and ECL2 and extending outward through the β-sheet found in rhodopsin. The amino-terminus (residues 1−33), ICL2, ICL3, the C-terminal tail (residues 325−328), portions of ECL2 (residues 177−185), and ECL3 (residues 294−298) were not included in the model due to lack of clear corresponding sequences in rhodsopsin. Ends of peptide segments were acetylated or amidated to preserve charge neutrality. An initial structure was formed by replacing side chains in rhodopsin with the corresponding GnRH-R residues as indicated. For refinement, conserved side chains and backbone atoms were held fixed (with the exception of residues 199−206 in ECL2) and the remaining side chains and loop regions relaxed by partial energy minimization. The resulting structure was then further relaxed using 5 ps of simulated annealing from 400 to 200 K followed by conjugate gradients minimization to a maximum derivative of less than 1 kcal/Å. For all molecular mechanics simulations, the CFF91 force field was employed using a distance-dependent dielectric constant, a nonbonded cutoff of 12 Å, and no explicit solvent using the molecular mechanics package Discover (Accelerys, San Diego, CA). The quality of the final model was evaluated using ProEval (Chemical Computing Group, Montreal, Quebec, Canada). Molecular graphics visualization was performed using DSViewerPro (Accelerys, San Diego, CA).

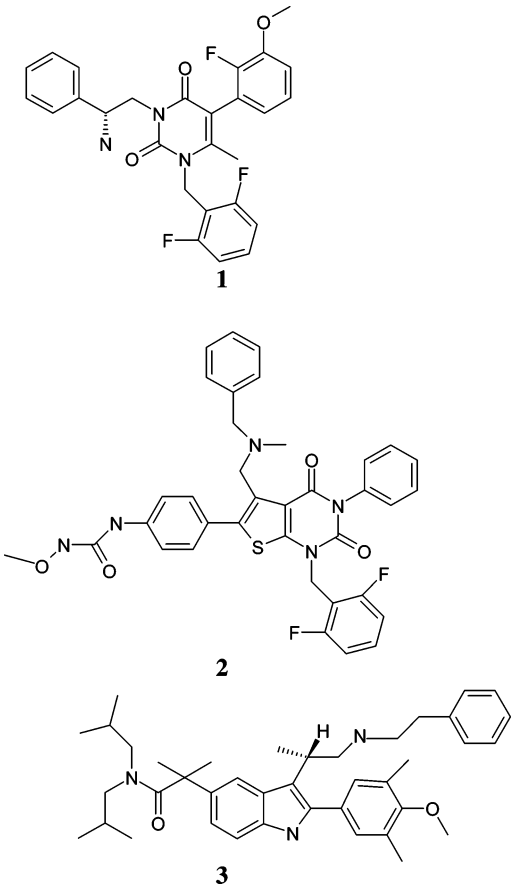

Figure 1. Chemical structures of nonpeptide GnRH-R antagonists used in the present studies.

Results

Three nonpeptide, small molecule GnRH-R antagonists were chosen to probe the binding site(s) of GnRH-R and are shown in Figure 1. **1** (NBI-42902) contains a uracil-based core,[23] **2** (TAK-013)[18] is a thienopyrimidinedione described by Sasaki et al., and **3** is similar to indole-based antagonists described by Simeone et al.[19] The IC_{50} values of these compounds for competition of a radiolabeled GnRH analogue (described above) binding to GnRH-R are presented in Table 1.

It has been shown previously that mutation at position 272 (F272$^{(6.40)}$L), increases receptor cell-surface expression of human GnRH-R.[26] In the COS-7 transient-transfection system employed in this study, the F272$^{(6.40)}$L mutant expresses better than the native wild-type receptor by approximately 3-fold (data not shown). The pharmacological profile of the wild-type human GnRH-R and the F272$^{(6.40)}$L mutant receptor are virtually identical, indicating that peptide and nonpeptide binding were

Table 1. Wild-type Human and F272$^{(6.40)}$L GnRH-R Interact with Nonpeptides and Peptides Similarly[a]

	F272$^{(6.40)}$L		human WT	
	IC_{50} (nM)	SEM	IC_{50} (nM)	SEM
1	1.5	0.7	0.9	0.7
2	0.8	0.5	2.5	2.3
3	16.9	6.4	17.7	7.3
[His^5, D-Tyr^6]GnRH	2.4	1.0	4.1	3.1
native GnRH	4.4	1.6	8.1	5.3

[a] IC_{50} and SEM values versus F272$^{(6.40)}$L and human wild-type GnRH-R for nonpeptides and peptides. COS-7 cells were transiently transfected with GnRH-R (wild-type or F272$^{(6.40)}$L) systems. Competition-binding assays were performed as described in Experimental Procedures. IC_{50} values are an average of at least three experiments.

unaffected by the mutation (Table 1). We therefore incorporated this substitution into all subsequent GnRH receptor mutants.

Figure 2B shows a schematic of GnRH-R, indicating the residues examined in this study. Each selected residue was evaluated with reference to the following characteristics: steric bulk, hydrogen-bonding capacity, charge, and evolutionary conservation across mammalian GnRH receptors. Typically, a strategy was employed that included generating more than one mutation at each selected position. In total, 35 positions in the receptor were evaluated using 76 different mutant receptors.

To assist in the interpretation of the mutagenesis data, we generated a side chain substitution model for GnRH-R based on the crystal structure of bovine rhodopsin.[24] To generate the model, the human GnRH-R sequence was aligned to the sequence of rhodopsin as shown in Figure 2A, and regions corresponding to the conserved TM helices, ECL1, and portions of ECL2 and ECL3 were modeled and partially refined using simulated annealing as described. Despite a minimal refinement protocol, the sequence of GnRH-R was well-accommodated by the tertiary structure of rhodopsin. There were few residual steric clashes. Several residual contacts were located at helical proline substitutions, suggesting that subtle, local adjustments may be required. ECL2 also contained a number of residual unfavorable contacts (T190$^{(4.76)}$, K191$^{(4.77)}$, V192$^{(4.78)}$, V197$^{(5.24)}$, H199$^{(5.26)}$, and S201$^{(5.28)}$), suggesting that this region may not be structurally homologous to rhodopsin, as aligned in Figure 2A. However, in the helical regions homologous to the retinal binding site, only one unfavorable contact was not fully resolved during refinement, suggesting that this region is well-packed. Thus, the present structure provides a reasonable model of the helical regions implicated in ligand binding, though the level of resolution should be viewed at the residue level, rather than as detailed interatomic interactions.

The resulting model is illustrated in Figure 2C, which shows the location of mutated residues in the context of the structure. As will be discussed in detail below, mutation of residues that line a pocket between the TM helices resulted in changes in binding for the various ligands and thus define a common orthosteric binding site. Residues outside this region (e.g., E111$^{(3.22)}$, L112$^{(3.23)}$, K115$^{(3.25)}$, and D293$^{(6.61)}$) show little or no effect on binding. ECL2 in rhodopsin is positioned within the helical bundle contacting portions of the retinal cofactor and as a cap over the retinal bindng site. It is held in place by both a highly conserved disulfide bond to TM3 as well as by the tertiary structure of the N-terminus "above" it. This disulfide is conserved in GnRH-R, and we therefore modeled the adjacent region of ECL2 similarly and mutated several residues in this region.

Figure 3 shows examples of competition binding curves obtained for the compounds and the [His^5, D-Tyr^6]GnRH peptide versus the F272$^{(6.40)}$L receptor (i.e., wild-type for this study) and a representative mutant receptor (S118$^{(3.29)}$N). Six mutants were found that did not bind the iodinated [His^5, D-Tyr^6]-GnRH: N102$^{(2.65)}$D, K115$^{(3.26)}$E, S118$^{(3.29)}$D, K121$^{(3.32)}$E, S124$^{(3.35)}$D, and Y283$^{(6.51)}$L. Table 2 presents the competition binding data for the compounds and peptides examined. The observation that very few of the mutants affect the binding of the radioligand peptide permits a straightforward comparison of IC_{50} values. Mutations that significantly decrease the binding of the radioligand peptide (and the implications thereof) are described within the text.

A convenient parameter used to estimate the relative effect of a mutation on nonpeptide or peptide binding is the fold change in affinity compared to that for the native sequence. Fold change is defined as the binding of the indicated ligand for a mutant receptor compared to the F272$^{(6.40)}$L receptor: [IC_{50}(mutant)/IC_{50}(F272$^{(6.40)}$L)]. Table 3 presents the fold change data for residues that had at least one mutant that affected the binding of at least one compound (or peptide) greater than 8-fold.

Figure 4 presents maps of residues that alter the binding of the indicated molecule greater than 8-fold in the context of the model for the proposed three-dimensional structure of GnRH-R. It is important to note that 8-fold is an arbitrary cutoff selected to identify major effects on binding, and more subtle effects of other mutations cannot be excluded. Our results indicated that the N-terminal domain, TM3, TM6, TM7, and ECL3 play essential roles in peptide and nonpeptide binding. Inspection of Figure 4 shows that, while the regions important for binding are proximal, each molecule shows a different pattern of residues that are critical for receptor binding. Below, we discuss the differences between the nonpeptide antagonists and peptides, and the role of specific residues in discrete regions and their interactions with peptides and nonpeptides.

N-Terminal Domain. This region is responsible for a large portion of the rat/human GnRH-R specificity differences observed in several nonpeptide antagonists.[21] Two residues in the N-terminal domain were examined and shown to affect the binding of two compounds. Mutations at M24 result in a near-complete loss of binding of **1** (~3000-fold) and drastic (~100−300-fold) interference with the binding of **2**. Interestingly, **3** and the peptides are unaffected by mutations at this residue. This suggests that M24 is required for maintaining receptor interactions with features that are similar in both affected molecules, indicating a potential interaction with either the difluorophenyl ring, the phenyl ring on the "right-hand side" of each molecule, or a common feature within the molecules' cores. It is important to note that M24 may or may not interact directly with the compounds and could be critical for the formation of a structural element required for high-affinity binding of these molecules and that the residues used to replace M24 (alanine, isoleucine, and threonine) may be unable to form this substructure. The current compounds cannot be used to distinguish between these explanations. There are also smaller effects observed at the nearby residue N27. The N27E mutation affects the binding of **2** to the greatest extent (10-fold) and moderately affects the binding of **1** (5.0-fold), although the N27A mutation causes little effect to the binding of any of the compounds or peptides in this study (Tables 2 and 3).

Transmembrane Domains 1 and 2. Few mutations were produced in these regions for this study. Neither of the mutants in TM1 produced meaningful differences, and the N102$^{(2.65)}$D receptor did not bind to the iodinated peptide ligand (see above). N102$^{(2.65)}$ has been proposed to interact with the glycinamide

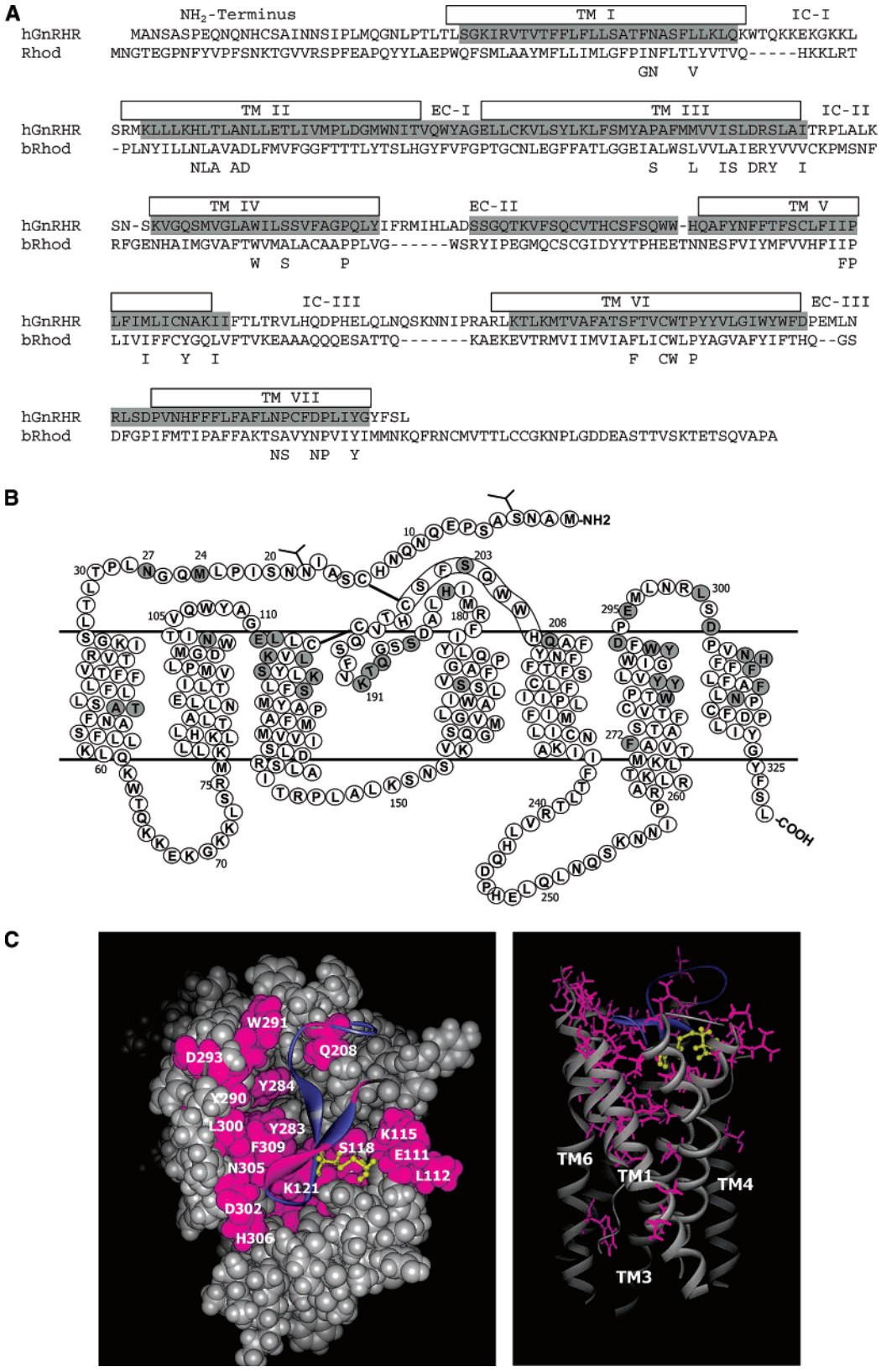

Figure 2. Schematic representation of the primary and predicted secondary and tertiary structures of human GnRH-R. (A) Alignment of the human GnRH-R sequence to bovine rhodopsin. Alignment of helical regions is based on highly conserved residues present throughout the class A GPCR family as previously described.[25] These signature residues are shown beneath the rhodopsin sequence. ECL2 was aligned centered on the position of the conserved disulfide-bonded cysteine, C196$^{(5.23)}$, and extended outward to the ends of the β-sheets present in rhodopsin. (B) Predicted secondary structure of GnRH-R. Predicted α-helical regions are based on the crystallographically determined structure of rhodopsin[24] and are indicated with a 3−4 repeating pattern. F272$^{(6.40)}$ (changed to leucine in these experiments) is located at the intracellular edge of TM6. (C) Homology model of GnRH-R based on rhodopsin. The conserved disulfide between TM3 (C114$^{(3.25)}$) and ECL2 (C196$^{(5.23)}$) is shown in yellow. Left, space filling representation viewed from the extracellular surface toward the plane of the membrane. Residues mutated in this study are indicated and shown in magenta. ECL2 is shown as a ribbon. Right, ribbon representation viewed in the plane of the membrane. Side chains for residues mutated in this study are shown as lines (magenta). ECL2 is indicated as a blue ribbon.

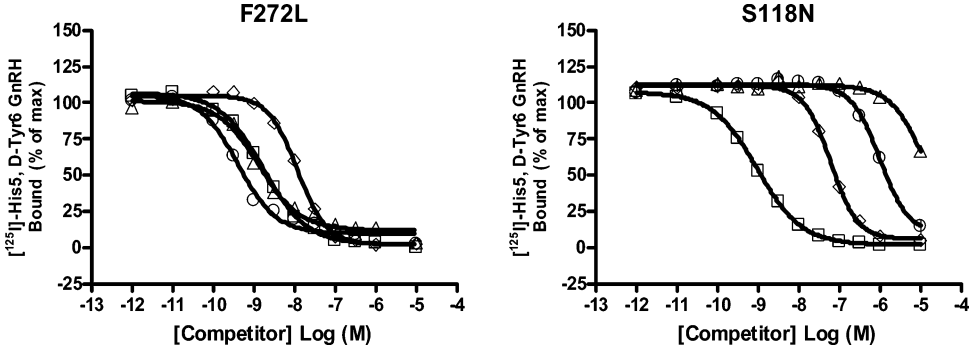

Figure 3. Competition binding data of wild-type and mutant GnRH-R. Comparison of the competition binding of [His^5, D-Tyr^6]GnRH peptide and nonpeptide antagonists to F272$^{(6.40)}$L and S118$^{(3.29)}$N/F272$^{(6.40)}$L GnRH-Rs. (□) [His^5, D-Tyr^6]GnRH; (△) **1**; (○) **2**; and (◇) **3**.

at position 10 of native GnRH peptide,[27] and changes at this position might be expected to cause significant problems with ligand binding, though the complete lack of iodinated peptide ligand binding in this mutant makes interpretation difficult.

Transmembrane Domain 3. The extracellular side of TM3 of GnRH-R has many residues that dramatically affect the binding of nonpeptides and peptides. This region has also been observed to be important for ligand binding in other receptor families, notably biogenic amine receptors.[28] A few mutants in this region cannot be assayed, while others cause significant changes in the binding of **1** and **2** (Tables 2 and 3). Interestingly, none of the mutants in this region affected the binding of **3**, suggesting that this molecule does not interact with this region of the receptor. In addition, the K121$^{(3.32)}$A mutant receptor also drastically affects the binding of **1** and **2** (>500-fold). The interaction between K121$^{(3.32)}$ and the compounds is likely not a charge-based interaction because none of the compounds tested possess a negative charge that might interact with a lysine side chain, though there is the possibility that K121$^{(3.32)}$ could form a cation$-\pi$ interaction with one of the aromatic rings common to both **1** and **2**.[29] K121$^{(3.32)}$ (along with D98$^{(2.61)}$) has been suggested to be involved in binding His^2 (and/or pGlu^1) of the native GnRH peptide.[30−32] Both agonist peptides' binding are affected by the K121$^{(3.32)}$A mutation: 61.2-fold for the native GnRH peptide and 28.9-fold for [His^5, D-Tyr^6]GnRH. This is consistent with a lack of agonist activation observed with several mutants at this position.[30] The same authors observed that peptide antagonist binding is not significantly affected by either the K121$^{(3.32)}$R or K121$^{(3.32)}$Q mutations. The same mutant receptors have not been studied here, but the binding of two (but not all) of the nonpeptide antagonists is clearly altered by the K121$^{(3.32)}$A mutation, indicating there could be differences between the modes in which peptide and nonpeptide antagonists interact with the receptor. It should be noted that the difference in IC_{50} for [His^5, D-Tyr^6]GnRH between the mutant and the wild-type receptors can make interpretation of the data for this mutant problematic, because straightforward comparison of compound$-IC_{50}$ is most robust if the dissociation constant and concentration of the iodinated peptide are similar between the mutant and wild-type receptors. However, the large differences in effects observed between **1** and **2** and those from **3** suggest that qualitatively the differences are significant.

There are mutants at two other residues, K115$^{(3.26)}$ and S124$^{(3.35)}$, where mutations either abolish binding of the iodinated peptide ligand (K115$^{(3.26)}$E and S124$^{(3.35)}$D) or cause very little effect in binding of any of the peptides and nonpeptides described here (K115$^{(3.26)}$Q and S124$^{(3.35)}$A). For K115$^{(3.26)}$E, the likely explanation is that the replacement by a differently charged amino acid may affect the folding of the receptor, perhaps via a charge repulsion with E111$^{(3.22)}$, while the mutation to glutamine permits the receptor to fold into a wild-type conformation. Similarly, the side chain of S124$^{(3.35)}$ is predicted to be in proximity to that of E90$^{(2.53)}$, such that the S124$^{(3.35)}$D mutant may cause a charge repulsion and misfolding, whereas the S124$^{(3.35)}$A mutation will support folding, though this residue bears no impact on ligand binding.

Transmembrane Domain 4. The mutation S168$^{(4.72)}$R has been reported as a nonfunctional mutant and has been identified in patients with hypogonadotropic hypogonadism.[33] Although the mechanism of how this mutant causes the disease is unknown; it is not rescued by a peptidomimetic antagonist that assists misfolded GnRH receptors.[34] Changes to hydrophobic or smaller residues (S168$^{(4.72)}$I and S168$^{(4.72)}$A) did not affect the binding of any of the nonpeptide antagonists or of the peptides tested, suggesting a minimal role for this serine in ligand binding.

Extracellular Loop 2 and Transmembrane Domain 5. When chimeric receptors are used, the N-terminal extension and ECL2 (linked by a disulfide between positions 14 and 200) together recover a considerable fraction of the rat/human affinity differences observed in certain nonpeptide antagonists, although replacement of only ECL2 produced diminished effects.[21] Site-directed mutagenesis studies indicated that S203$^{(5.30)}$ and Q208$^{(5.35)}$ contribute to nonpeptide antagonist binding and rat/human selectivity,[21] although the Q208$^{(5.35)}$A mutant showed little effect on receptor expression or GnRH peptide affinity.[31] Here, we adopted a more extensive site-directed mutagenesis strategy to explore different residues in these regions.

In general, mutations within several potentially hydrogen-bonded and/or charged residues in ECL2 produced very little effect in compounds' affinities (Table 2). None of the mutations in this region affected the binding of **3**, reinforcing that this molecule has binding modes for the receptor that are different than the other more similar compounds (Figure 4). Replacements at Q208$^{(5.35)}$ produced modest effects similar to those observed earlier.[21] The Q208$^{(5.35)}$D mutant affected the affinity of **1** and **2** (28.7- and 11.1-fold, respectively). For both **1** and **2**, the observed effects were consistently greater with the Q208$^{(5.35)}$D compared to the Q208$^{(5.35)}$E mutation, which suggests a steric requirement for the residue side chain, perhaps interacting with a less mobile part of the nonpeptide that is unable to accommodate the subtle E → D change. Substitution of S203$^{(5.30)}$ by charged residues (S203$^{(5.30)}$K and S203$^{(5.30)}$E) had no effect on ligand binding, while introduction of the corresponding rat residue (S203$^{(5.30)}$P) resulted in a 5.3-fold loss of affinity for **1**. These results suggest that the effect of S203$^{(5.30)}$ on **1** binding is not direct but due to a conformational change introduced by the proline. These results are consistent with competition binding data of similar nonpeptide antagonists versus S203$^{(5.30)}$ mutant receptors.[21] However, it is interesting to note that in the

Table 2. Nonpeptide Antagonist and Peptide Agonist Affinities for Mutant GnRH-R[a]

	1		2		3		[His^5, D-Tyr^6]GnRH		native GnRH			1		2		3		[His^5, D-Tyr^6]GnRH		native GnRH	
	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM		IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM	IC$_{50}$ (nM)	SEM
F272L	1.5	0.7	0.8	0.5	16.9	6.4	2.4	1.0	4.4	1.6	**S203K**	1.0	0.2	0.5	0.1	14.6	6.2	1.7	0.4	3.1	1.0
M24A	5030	200	240	130	17.9	15.2	1.2	0.5	5.9	2.6	**S203P**	8.0	2.7	1.4	0.4	41.4	13.7	2.7	0.6	5.4	1.8
M24I	4200	3300	108	69	23.2	12.6	1.3	0.6	2.2	0.6	**S203Q**	1.4	0.3	0.5	0.1	15.7	8.1	1.7	0.3	2.9	0.8
M24T	5000	700	193	105	57.1	14.9	3.3	0.1	4.9	0.3	**Q208D**	42.7	29.5	9.7	7.6	16.2	9.9	5.3	1.2	10.1	4.6
N27A	2.5	0.9	0.7	0.1	8.8	1.7	2.9	1.2	5.8	3.2	**Q208E**	9.2	2.2	1.3	0.3	6.0	1.4	3.8	1.2	15.0	3.3
N27E	7.5	2.4	8.5	2.0	10.8	2.8	2.8	1.4	3.8	0.9	**W280F**	5.5	0.9	2.3	0.6	139	28	8.5	10.9	10.3	4.9
A50T	1.2	0.2	0.6	0.0	17.8	7.8	2.0	0.3	3.6	1.1	**Y283F**	13.9	1.6	0.5	0.1	108	11	50.6	9.0	316	180
T51A	1.2	0.2	0.6	0.0	15.8	5.2	2.1	0.2	3.1	0.5	**Y283L**	does not bind [^{125}I-His^5 D-Tyr^6]GnRH									
N102D	does not bind [^{125}I-His^5 D-Tyr^6]GnRH										**Y284F**	2.3	0.9	0.9	0.4	28.8	10.5	0.5	0.2	2.8	1.1
E111A	0.8	0.4	0.4	0.1	5.4	3.3	2.7	0.2	4.6	1.8	**Y284L**	123	40	12.1	3.5	277	48	13.2	10.2	186	81
E111K	0.7	0.0	0.8	0.4	13.6	4.5	3.3	0.2	10.5	1.3	**Y290F**	3.2	0.5	0.6	0.0	39.6	16.8	1.8	0.1	48.8	18.5
E111Q	1.4	0.2	0.6	0.2	11.5	8.2	2.1	0.1	3.3	0.7	**Y290L**	182	57	0.5	0.2	202	98	7.9	2.2	951	650
L112A	1.5	0.5	0.6	0.1	22.4	11.0	1.8	0.5	2.5	0.6	**Y290Q**	262	124	1.1	0.2	229	80	9.7	2.8	192	109
L112F	1.8	1.0	0.6	0.3	29.5	21.8	1.8	0.7	2.7	1.1	**W291F**	3.2	0.3	0.9	0.0	6.8	5.3	4.6	0.5	60.1	27.5
L112K	1.2	0.5	1.9	2.3	24.2	11.8	3.2	2.7	3.5	1.5	**D293A**	3.4	0.8	0.6	0.1	24.5	4.1	5.2	0.3	5.7	0.2
K115E	does not bind [^{125}I-His^5 D-Tyr^6]GnRH										**D293K**	5.7	1.9	2.0	0.1	56.1	32.0	5.2	1.1	4.8	0.4
K115Q	2.7	0.5	1.2	0.6	27.0	8.0	2.2	0.3	8.2	0.5	**D293N**	3.7	1.2	0.8	0.3	32.6	10.3	3.8	1.7	4.7	2.1
L117A	1.2	0.2	0.4	0.1	4.8	1.5	3.3	0.7	4.1	0.6	**D293E**	3.0	0.7	1.2	0.2	27.2	4.6	4.1	0.2	6.1	1.0
S118A	1.3	0.6	0.6	0.1	46.8	6.4	1.6	0.4	3.3	0.1	**E295A**	1.1	0.0	0.5	0.0	11.2	6.1	1.7	0.1	1.9	0.4
S118D	does not bind [^{125}I-His^5 D-Tyr^6]GnRH										**E295K**	1.4	0.2	0.5	0.0	9.6	3.7	2.1	0.1	3.1	0.4
S118N	5200	1600	1070	400	63.7	7.9	1.2	0.3	1.1	0.2	**E295Q**	1.6	0.1	0.6	0.2	15.2	1.5	1.6	0.1	2.4	0.2
K121A	980	260	470	33	82.3	22.7	69.2	56.9	267	122	**L300A**	24.2	4.6	1.9	0.4	87.9	7.6	3.7	0.8	18.2	3.9
K121E	does not bind [^{125}I-His^5 D-Tyr^6]GnRH										**L300K**	80	40	5.2	3.3	277	97	13.2	3.6	35.3	16.0
S124A	3.1	0.4	0.9	0.0	21.3	1.4	1.3	0.2	3.9	1.7	**L300V**	8.9	3.1	1.8	0.6	164	28	1.3	0.5	6.5	3.4
S124D	does not bind [^{125}I-His^5 D-Tyr^6]GnRH										**D302A**	1.8	1.5	192	81	11.0	2.4	15.2	7.8	42.2	14.3
S168A	1.1	0.2	0.6	0.3	8.9	1.5	3.2	1.4	4.0	1.3	**D302N**	2.6	1.7	115	27	27.3	5.1	7.6	3.3	16.5	2.9
S168I	1.4	0.5	0.8	0.7	15.2	4.0	2.5	0.6	3.8	0.8	**D302Q**	1.7	0.2	148	36	20.6	2.1	13.9	4.1	26.5	7.9
H182A	0.5	0.1	0.3	0.1	3.7	0.8	5.2	1.1	6.6	2.9	**N305A**	1.0	0.5	0.4	0.1	3.7	2.5	1.3	0.4	12.7	1.7
H182E	0.6	0.1	0.5	0.4	7.6	1.8	4.4	0.8	3.6	0.5	**N305D**	1.4	0.2	4.3	0.7	46.5	3.1	9.5	0.1	41.1	9.1
S186D	1.1	0.1	0.5	0.1	11.3	4.6	2.1	0.6	3.1	0.7	**N305K**	1.6	0.0	3.0	0.9	3.7	0.3	6.4	1.3	12.4	1.4
S186K	1.7	0.7	0.8	0.3	13.1	5.3	2.4	0.7	4.1	1.7	**H306A**	1.4	0.5	23.4	5.4	23.3	7.8	15.3	2.9	50.6	27.4
Q189A	1.1	0.3	0.7	0.5	10.0	1.6	2.7	1.5	2.4	0.8	**H306E**	2.7	0.7	94.9	19.1	48.8	12.7	11.2	8.9	52.8	29.1
Q189E	1.3	0.6	0.6	0.1	15.7	9.9	7.3	1.1	16.7	3.5	**H306K**	11.4	1.2	242	48	101	37	18.9	5.0	66.2	26.4
Q189K	1.1	0.2	0.7	0.5	15.8	6.4	1.9	0.7	2.4	0.5	**F309L**	2.8	0.8	6.3	1.3	60.1	18.1	7.8	9.6	37.7	10.9
Q189P	1.2	0.3	0.5	0.1	10.8	3.5	1.7	0.6	1.9	0.3	**F309Q**	6.6	2.9	10.0	2.1	222	44	9.6	3.2	65.1	22.7
T190K	1.5	0.6	0.9	0.4	19.8	10.6	3.0	1.3	3.7	1.3	**F313L**	15.5	5.8	24.4	7.1	623	85	3.8	1.4	5.6	1.8
K191E	1.1	0.2	0.5	0.1	11.0	2.0	3.3	1.3	3.7	1.0	**N315A**	2.4	0.6	1.2	0.1	47.1	17.1	0.6	0.1	0.8	0.2
S203E	1.4	0.5	0.5	0.2	15.1	7.4	1.9	0.6	2.9	0.2	**N315D**	1.4	0.2	0.7	0.0	49.3	8.9	1.0	0.3	1.5	0.2

[a] IC$_{50}$ and SEM values versus mutant GnRH-R for nonpeptides and peptides. COS-7 cells were transiently transfected with mutant GnRH-R systems. Competition-binding assays were performed as described in Experimental Procedures. IC$_{50}$ values are an average of at least three experiments. All mutants listed are in the F272$^{(6.40)}$L background. The membranes prepared from the N102$^{(2.65)}$D, K115$^{(3.26)}$E, S118$^{(3.29)}$D, K121$^{(3.32)}$E, S124$^{(3.35)}$D, and Y283$^{(6.51)}$L mutants did not bind the iodinated [His^5, D-Tyr^6]GnRH peptide, thus, could not be assayed. The values for the F272$^{(6.40)}$L receptor are listed at the top of the table for reference.

Table 3. Nonpeptide Antagonists and Peptide Agonists Are Each Affected by Different Subsets of Mutations[a]

		fold change				
mutant	location[16]	1	2	3	His^5, D-Tyr^6 GnRH	native GNRH
M24A	**N-term**	**3380**	**282**	1.1	0.5	1.4
M24I	**N-term**	**2850**	**128**	1.4	0.5	0.5
M24T	**N-term**	**3340**	**228**	3.4	1.4	1.1
N27A	**N-term**	1.7	0.8	0.5	1.2	1.3
N27E	**N-term**	5.0	**10.0**	0.6	1.2	0.9
S118A	**3.29**	0.9	0.7	2.8	0.7	0.7
S118N	**3.29**	**3460**	**1260**	3.8	0.5	0.2
K121A	**3.32**	**659**	**555**	4.9	**28.9**	**61.2**
Q208D	**5.35**	**28.7**	**11.4**	1.0	2.2	2.3
Q208E	**5.35**	6.2	1.5	0.4	1.6	3.4
W280F	**6.48**	3.7	2.8	**8.2**	3.5	2.4
Y283F	**6.51**	9.3	0.6	6.4	**21.1**	**72.4**
Y284F	**6.52**	1.6	1.1	1.7	0.2	0.6
Y284L	**6.52**	**82.9**	**14.2**	**16.4**	5.5	**42.7**
Y290F	**6.58**	2.2	0.7	2.3	0.8	**11.2**
Y290L	**6.58**	**123**	0.5	**12.0**	3.3	**218**
Y290Q	**6.58**	**176**	1.4	**13.6**	4.1	**44.1**
W291F	**6.59**	2.1	1.0	0.4	1.9	**13.8**
L300A	**6.68/ECL3**	**16.3**	2.2	5.2	1.6	4.2
L300K	**6.68/ECL3**	**53.7**	6.2	**16.4**	5.5	**8.1**
L300V	**6.68/ECL3**	6.0	2.1	**9.7**	0.5	1.5
D302A	**7.32/ECL3**	1.2	**226**	0.7	6.4	**9.7**
D302N	**7.32/ECL3**	1.8	**136**	1.6	3.2	3.8
D302Q	**7.32/ECL3**	1.1	**175**	1.2	5.8	6.1
N305A	**7.35**	0.7	0.5	0.2	0.5	2.9
N305D	**7.35**	1.0	5.1	2.8	4.0	**9.4**
N305K	**7.35**	1.1	3.5	0.2	2.7	2.8
H306A	**7.36**	0.9	**27.7**	1.4	6.4	**11.6**
H306E	**7.36**	1.8	**112**	2.9	4.7	**12.1**
H306K	**7.36**	7.6	**287**	6.0	7.9	**15.2**
F309L	**7.39**	1.9	7.5	3.6	3.3	**8.7**
F309Q	**7.39**	4.5	**11.8**	**13.1**	4.0	**14.9**
F313L	**7.39**	**10.4**	**28.9**	**36.9**	1.6	1.3

[a] Location refers to the position of the residue in the schematic in Figure 2. "N-term" refers to the extracellular N-terminal extension preceding the transmembrane region. Fold-change values for nonpeptide compounds and peptides versus mutant GnRH-Rs. Fold changes are defined as [IC_{50}(mutant)/IC_{50}(F272$^{(6.40)}$L)]. Values >8-fold are indicated in bold face type.

homology model to the rhodopsin structure, S203$^{(5.30)}$ and Q208$^{(5.35)}$ are immediately adjacent (Figure 2C) suggesting that together they may influence the overall conformation and/or orientation of ECL2.

Transmembrane Domain 6. TM6 contains a large number of aromatic residues that are critical to the binding of the GnRH peptide, and several mutants in this region considerably affect the binding of nonpeptides as well. Alanine mutants of Y283$^{(6.51)}$, Y284$^{(6.52)}$, and W291$^{(6.59)}$ [35] and other replacements at the equivalent position of W280$^{(6.48)}$ in rat GnRH-R have either greatly diminished or undetectable activity in peptide binding.[36] In this study, the mutants made in this region were generally conservative changes and designed to be sensitive to subtle differences in the binding of peptides and nonpeptides. W280$^{(6.48)}$ has been proposed to interact with Trp^3 in the GnRH peptide. The affinities of the peptides are not greatly altered by the W280$^{(6.48)}$F mutation, apparently maintaining the aryl−aryl interaction with Trp^3. The W280$^{(6.48)}$F mutant had small effects on the binding of the nonpeptides, affecting the affinity of **3** the most (though still <10-fold). W280$^{(6.48)}$ is the deepest residue in the binding pocket identified as affecting the binding of this panel of ligands (Figure 4C), and its effect on **3** is consistent with the view that this compound binds more deeply within the TM bundle than the other molecules tested.

As shown in Figure 4, Y283$^{(6.51)}$ is prominent within the center of the ligand binding pocket. The Y283$^{(6.51)}$L receptor was unable to be assayed, while the Y283$^{(6.51)}$F receptor produced moderate changes in the binding of **1** and **3** (9.3- and 6.4-fold, respectively), although the binding of both peptides tested was considerably affected ([His^5, D-Tyr^6]GnRH, 21.1-fold; native GnRH, 72.4-fold).

Y284$^{(6.52)}$ forms part of the wall of the binding pocket. The Y284$^{(6.52)}$F mutation produced no significant changes, while the Y284$^{(6.52)}$L mutant caused considerable changes in affinity to all the nonpeptides and the native GnRH peptide (though not to the [His^5, D-Tyr^6] peptide). The marked difference in binding between the two peptides suggests Y284$^{(6.52)}$ is near positions 5 or 6 of the native GnRH peptide, though this does not agree with published models of GnRH bound to GnRH-R.[32] There are also substantial effects on the binding of the nonpeptides, and the most drastic changes were observed for **1** (>80-fold).

Y290$^{(6.58)}$ forms part of the upper wall of the binding pocket adjacent to L300$^{(6.68)}$ (Figure 4). Similar to Y284$^{(6.52)}$, substituting a phenylalanine for Y290$^{(6.58)}$ produced very small effects in the binding of the nonpeptides, but the peptides were again differentially affected. Y290$^{(6.58)}$ has been proposed to interact with the Tyr^5 of the native GnRH peptide.[35] Consistent with this hypothesis, the native peptide is greatly affected by mutations at this site (Y290$^{(6.58)}$F, 11.2-fold; Y290$^{(6.58)}$L, 218-fold), while the [His^5, D-Tyr^6] peptide is barely affected (0.8- and 3.3-fold, respectively). These are the largest relative differences in binding between the two peptides observed for any of the mutant receptors characterized here. Y290$^{(6.58)}$ mutants also selectively affect **1**. Y290$^{(6.58)}$L and Y290$^{(6.58)}$Q cause >100-fold changes in the binding of **1**, while the effect is more moderate against **3** (12.0−13.6-fold), and no effect is observed against **2**. The lack of effects seen for both Y284$^{(6.52)}$F and Y290$^{(6.58)}$F and the large changes seen with other substitutions suggest this region may form an aryl-stacking interaction with the nonpeptide antagonists.

Extracellular Loop 3 and Transmembrane Domain 7. Another region that has been implicated in the binding of GnRH peptide to its receptor is ECL3.[32] Residue D302$^{(7.32)}$ has been shown to interact with Arg^8 of the native GnRH peptide, though this interaction is either not present or not required in some conformationally constrained GnRH peptide analogues.[37,38] Mutation of D302$^{(7.32)}$ to either neutral (D302$^{(7.32)}$A) or amide-bearing amino acids (D302$^{(7.32)}$N and D302$^{(7.32)}$Q) moderately affects the binding of both peptides examined, and the alanine substitution is the most deleterious to each peptide's binding. The largest effects observed with mutants of D302$^{(7.32)}$ are with **2**. Mutations at this residue interfere with the binding of **2** by >100-fold. Neither of the other nonpeptide antagonists are affected by mutations at this residue.

Mutations at H306$^{(7.36)}$ affect the nonpeptide set similarly to D302$^{(7.32)}$. The effects are pronounced regardless of the replacement (H306$^{(7.36)}$A, H306$^{(7.36)}$E, and H306$^{(7.36)}$K) and only affect **2**. Interestingly, oppositely charged mutants (H306$^{(7.36)}$E and H306$^{(7.36)}$K) both result in similarly diminished affinities for **2** (both >100-fold), while the H306$^{(7.36)}$A mutant has the smallest affect (27.7-fold). This suggests this interaction is not specifically charge-based but possibly steric, hydrogen bond-mediated, or a dipolar effect. The location of D302$^{(7.32)}$ and H306$^{(7.36)}$ immediately adjacent to each other is consistent with their parallel effects on ligand binding (Figure 4B,D).

Mutations at L300$^{(6.68)}$ also produced several changes to nonpeptide binding. **1** was the most affected by mutations at this residue (6.0−53.7-fold), while **2** was largely indifferent to mutations at L300$^{(6.68)}$ (2.1−6.2-fold). The L300$^{(6.68)}$K mutation causes the largest changes in affinity for each class of compound, but there are differing effects with the L300$^{(6.68)}$A and L300$^{(6.68)}$V

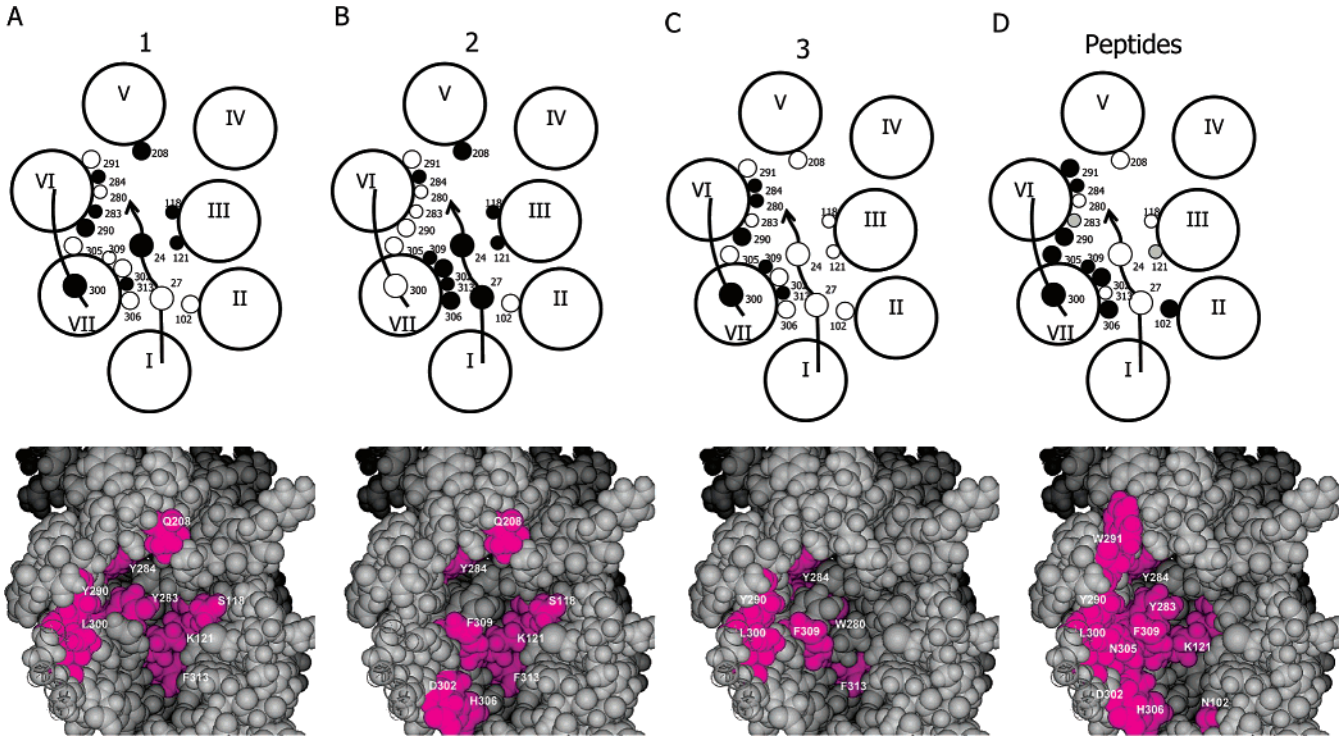

Figure 4. Comparison of the binding motifs of nonpeptide antagonists and peptide agonists of GnRH-R. Each section shows a schematic of the receptor illustrating the location of residues that affect the binding (colored in black) of the indicated molecule. The top panels present a projection of GnRH-R based on the structure of rhodopsin.[24] Circles indicate residue positions and the diameter of a circle corresponds roughly to the residue's proximity to the extracellular environment. ECLs 1 and 2 are omitted, and the N-terminal extension is truncated for clarity. The position of the N-terminal extension is approximated based on the presence of the disulfide linking C14 and C200$^{(5.27)}$. (A) **1** (M24, S118$^{(3.29)}$; K121$^{(3.32)}$; Q208$^{(5.35)}$; Y283$^{(6.51)}$; Y284$^{(6.52)}$; Y290$^{(6.58)}$; L300$^{(6.68)}$; F313$^{(7.43)}$); (B) **2** (M24, N27; S118$^{(3.29)}$; K121$^{(3.32)}$; Q208$^{(5.35)}$; Y284$^{(6.52)}$; D302$^{(7.32)}$; H306$^{(7.36)}$; F309$^{(7.39)}$; F313$^{(7.43)}$); (C) **3** (W280$^{(6.48)}$; Y284$^{(6.52)}$; Y290$^{(6.58)}$; L300$^{(6.68)}$; F309$^{(7.39)}$; F313$^{(7.43)}$); and (D) GnRH peptides. In panel D, residues interacting with native GnRH are shaded black (N102$^{(2.65)}$; Y284$^{(6.52)}$; Y290$^{(6.58)}$; W291$^{(6.59)}$; L300$^{(6.68)}$; D302$^{(7.32)}$; N305$^{(7.35)}$; H306$^{(7.36)}$; F309$^{(7.39)}$); residues interacting with both native GnRH and [His^5, D-Tyr^6]GnRH are shaded gray (K121$^{(3.32)}$; Y283$^{(6.51)}$). The lower panel shows a space-filling model of GnRH-R based on an alignment of its primary sequence with that of bovine rhodopsin.[25,32] Residues shaded in the top panel are highlighted in magenta. Extracellular loops and the N-terminal domain have been omitted to best show the binding cavity for the nonpeptides and peptides.

mutations. **3** is moderately more affected by L300$^{(6.68)}$V than L300$^{(6.68)}$A, while **1** is more affected by L300$^{(6.68)}$V than L300$^{(6.68)}$A. These differences make determination of specific molecular interactions with L300$^{(6.68)}$ difficult to assess. The deleterious impact of the L300$^{(6.68)}$K mutation against all the compounds and peptides suggests that replacing this hydrophobic residue for a positively charged one may cause a significant structural rearrangement of this region. However, the significant variation between certain compounds (i.e., **1** is ~50-fold and **2** is ~6-fold different for binding to the L300$^{(6.68)}$K receptor) implies the compounds interact with this area dissimilarly.

Two aromatic residues, F309$^{(7.39)}$ and F313$^{(7.43)}$, were also investigated. F309$^{(7.39)}$ is in the center of the binding pocket in a aromatic stacking relationship with the side chains of Y283$^{(6.51)}$, while F313$^{(7.43)}$ forms part of the deepest floor of the pocket (Figure 4). None of the substitutions affected the binding of [His^5, D-Tyr^6]GnRH, but changes at F309$^{(7.39)}$ moderately affected (8.7−14.9-fold) the binding of the native peptide. All three nonpeptide antagonists were affected by the F313$^{(7.43)}$L mutation. **3** was affected to the greatest extent (36.9-fold), and F313$^{(7.43)}$L was the mutation that most affected the binding of that molecule overall, reinforcing the observation that **3** binds deeply within the receptor pocket. The F309$^{(7.39)}$L mutation was reasonably well-tolerated by the nonpeptide antagonists, and F309$^{(7.39)}$Q caused similar, moderate affects to the binding of **2** and **3** (11.8- and 13.1-fold, respectively).

Other charged and potentially hydrogen-bonding residues in this region were examined including D293$^{(6.61)}$, E295$^{(6.63)}$, N305$^{(7.35)}$, and N315$^{(7.45)}$. None of the mutations (11 different replacements) at these positions produced significant effects in the binding of nonpeptides, although the N305$^{(7.35)}$D mutation has a moderate effect on the affinity of the native GnRH peptide (9.4-fold). The relative lack of effects of N305$^{(7.35)}$ substitutions such as N305$^{(7.35)}$D or N305$^{(7.35)}$K is inconsistent with the proposed receptor model, which places this residue in a wall of the binding pocket between L300$^{(6.68)}$ and F309$^{(7.39)}$ (Figure 4D), both of which have been shown to be a critical for binding of multiple ligands.

Discussion

Site-directed mutagenesis and nonpeptide SAR have been used to probe receptor structure and its effects on the binding of nonpeptide antagonists and peptides. We examined the competition binding of three classes of nonpeptides to compare their interactions with those of GnRH peptides and with one another. Maps of the residues that show an effect on nonpeptide antagonist and peptide agonist binding are presented in Figure 4. This figure illustrates the similarities and differences between each class of molecules binding to GnRH-R. The results demonstrate that the diverse set of ligand structures examined here do not employ the same binding motifs when interacting with GnRH-R; however, all ligands bind within a common overall pocket.

Specific Peptide Interaction Sites. Two peptides were chosen in addition to the nonpeptides shown in Figure 1: mammalian GnRH and [His^5, D-Tyr^6]GnRH. Interestingly, few of the 70 mutants (that could be assayed) presented in this study significantly adversely affect the binding of the high affinity

[His^5, D-Tyr^6]GnRH peptide, while several more affect the binding of the native GnRH (which has Tyr at position 5 and Gly at position 6). Only mutations at two residues, K121$^{(3.32)}$ and Y283$^{(6.51)}$, impact the binding of [His^5, D-Tyr^6]GnRH greater than 8-fold. The presence of a more conformationally constrained D-Tyr at position 6 may preform the peptide structure and promote binding that is less sensitive to changes in local receptor structure. This has previously been proposed to explain the different requirements for Arg^8 to D302$^{(7.32)}$ interactions in peptides with and without a D-amino acid substitution at position 6.[38]

Specific Nonpeptide Interaction Sites. There were 23 mutations over 14 residue positions that produced large (>8-fold change) effects in binding affinity for the nonpeptide antagonists. Interestingly, only two mutations affected the binding of all the compounds tested: Y284$^{(6.52)}$L and F313$^{(7.43)}$L. Y284$^{(6.52)}$L affects the binding of all the classes of nonpeptides (each >10-fold) as well as cause significant effects on the binding of the native GnRH peptide (42.7-fold). However, the [His^5, D-Tyr^6] peptide is less affected by the mutation (5.5-fold). It is noteworthy that the Y284$^{(6.52)}$F mutation causes no changes in the binding of any of the molecules or peptides tested, suggesting that an aryl group at this position is required for proper receptor−ligand interaction. Other mutations (Y284$^{(6.52)}$A and Y284$^{(6.52)}$C) at this residue have been reported to cause decreased or undetectable GnRH-R activity.[35,39,40] F313$^{(7.43)}$L significantly affects all classes of nonpeptides but does not alter the binding of the peptides. This residue has been shown to be a determinant for human/dog species selectivity for a class of nonpeptide antagonists.[41] Another interesting mutation, F309$^{(7.39)}$Q, affects **2** and **3** to a similar effect (more than **1**), suggesting an overlapping binding mode for at least part of these two antagonists.

It is critical to note that many of these residues essential for nonpeptide binding only partially correlate with those residues identified as important in agonist peptide binding. Eight of the 14 sites identified with a >8-fold effect on the binding affinity of at least one compound have an important effect on the binding of native GnRH peptide, while two other mutants (W291$^{(6.59)}$F and N305$^{(7.35)}$D) affect the native peptide but none of the compounds tested. These results indicate that the binding site-(s) for the nonpeptides tested here only partially overlap the native peptide-binding site (Figure 4). It has also been demonstrated that agonist and antagonist peptides interact with different areas of the receptor, and that there are mutations that discriminate between the two classes of peptides,[31,32,35,42] including several residues that were examined here (W280$^{(6.48)}$, Y290$^{(6.58)}$, and D302$^{(7.32)}$). However, the nonpeptide molecules tested here also have different profiles for mutations at those positions, indicating that they do not bind in the same manner as one another nor in the same manner as the antagonist peptides.

1 and **2** share certain chemical similarities that make comparison of the residues that interact with each noteworthy. As might be expected, several residues including M24, N27, S118$^{(3.29)}$, F309$^{(7.39)}$, and F313$^{(7.43)}$ affect the two compounds comparably, suggesting that parts of the molecules that are similar (difluorophenyl ring and "right-hand" phenyls) might be the areas that interact with those residues. However, a more detailed examination of multiple compounds within each series will be required to identify the specific nature of these interactions.

Interestingly, there are also sets of residues that are more "specific" for each compound. Figure 4 shows that for **1** these residues cluster on TM6 and ECL3, while for **2**, these residues cluster on one face of TM7. Two polar residues specific to **2** are D302$^{(7.32)}$ and H306$^{(7.36)}$, which are located adjacent to one another at the ECL3−TM7 interface (Figure 4B). D302$^{(7.32)}$ has been postulated as the site of interaction of the basic amine in **2**[43,44] as well as other antagonists similar to **1**.[45] However, while all the classes of nonpeptides in this study have basic amines, mutation of D302$^{(7.32)}$ only effects binding of **2**. Therefore, the differing effects observed with mutants of D302$^{(7.32)}$ demonstrate that only the amine in **2** could potentially interact with this residue. However, there is also the possibility that *none* of the basic amines in any of the nonpeptides (including the one in **2**) interact with this residue. Recently, Millar et al.[32] cited unpublished data on a compound similar to **2** that had a 5-fold change in affinity to the D302$^{(7.32)}$N receptor. The observed effects for **2** are much larger (>100-fold) and suggest a unique part of **2** interacts with this residue. Such a distinctive functional group is the methoxyphenyl−urea,[18] which could potentially form a hydrogen-bonding interaction with D302$^{(7.32)}$. H306$^{(7.36)}$ (also **2**-specific) is potentially a half-helical turn from D302$^{(7.32)}$, and their side chains are adjacent in the proposed model (Figure 4B), raising the intriguing possibility that these two residues may together be involved in a hydrogen-bonding network with **2** that cannot be mimicked by the other molecules.

Examining the structures in Figure 1 and the binding modes in Figure 4 together, we hypothesize that common parts of **1** and **2** bind deep within the TM bundle of GnRH-R (i.e., near F313$^{(7.43)}$) and that the specificity determinants for each molecule lie closer to the extracellular side of the receptor. This would place the amino-phenyl "left-hand side" of **1** near TM6 and ECL3, and the unique methoxyurea "left-hand side" of **2** near one face of TM7. It is less clear how to orient the binding of **3**. Further studies with more closely related compounds and GnRH-R mutants could be used reciprocally to understand the structure−activity relationships between antagonist and receptor structure.

The Impact of Extracellular Loop 2. One of the striking results in Table 2 is the lack of effects caused by the mutation of residues in ECL2 upon the binding of the peptides or nonpeptide antagonists. In the rhodopsin crystal structure, this region forms an extracellular "cap" for the binding of the retinal cofactor,[24,46] and this region is involved in binding dopamine D2 receptor ligands.[47] We sought to further explore the effect of this region on antagonist binding by mutating several residues that had the capacity of making charged-based and/or hydrogen bonds. None of these mutations (which included several charge-changes) produced very significant changes in the affinities of either the peptides or the nonpeptide antagonists, despite being located adjacent to other residues that influence binding and if one assumes a rhodopsin-like structure for ECL2 in GnRH-R (represented as a ribbon in Figure 2C). One hypothesis is that the region selected for mutation (between residues 182 and 191) points toward solvent, and a different part of the loop (perhaps residues 195−199, which in GnRH-R corresponds to a β-sheet in rhodopsin) may form interactions with ligands. However, several of the residues chosen for mutation in GnRH-R (e.g., T190$^{(4.76)}$) correspond to residues in rhodopsin (in the alignment in Figure 2A) that would be predicted to come within 5 Å of the retinal side chain and could be expected to play a part in receptor function. In fact, if a rhodopsin-like structure for ECL2 in GnRH-R is assumed, then many of the residues identified here as critical for ligand interactions above become occluded (Figure 2C). This forces the conclusion that, with a ligand bound, ECL2 of GnRH-R cannot be structurally homologous to

rhodopsin, despite the conserved disulfide, which constrains proximity to TM3. In addition, several antibodies against ECL2 in other GPCRs have been generated which act as agonists, and it is difficult to understand how they can recognize this region which is mostly buried in the rhodopsin fold.[24,48,49] Further, isomerization of the highly conserved TM3−ECL2 disulfide bond in the 5-HT4 receptor has been associated with receptor activation.[50] Finally, mutations in this region of GnRH-R (V197$^{(5.24)}$A, W205$^{(5.32)}$H) can together convert a peptide antagonist into an agonist.[20] Further investigation is warranted to study the role of ECL2 in the binding of both agonists and antagonists to this receptor.

Similar to some other receptors that have been investigated, the data presented above indicates that GnRH-R possesses distinct but orthosteric binding sites for peptide agonists and nonpeptide antagonists.[28] More importantly, however, a comparison of different classes of nonpeptide antagonists indicates that there are overlapping, but nonidentical, binding sites for these different ligand classes. This information, coupled with computational modeling of compound overlaps, can be used to guide future detailed modeling of the receptor−ligand complex with more precision. The three-dimensional map of the complex can then be used to design libraries of molecules that have the capacity to take advantage of various contact points, leading to composite compounds with even greater affinity and desired characteristics. It will also be interesting to further probe the hypotheses described here with individual SAR series and GnRH-R mutant panels to achieve a more fine-grained understanding of molecular interactions. In addition, this work lays the foundation for future experiments that will explore the correlations between different antagonist binding motifs and changes in downstream receptor signaling inhibition and ultimately therapeutic efficacy.

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