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Functional Importance of Transmembrane Helix 6 Trp^{279} and Exoloop 3 Val^{299} of Rat Gonadotropin-Releasing Hormone Receptor

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ABSTRACT

Previous studies have established that the interaction of gonadotropin-releasing hormone (GnRH) with its receptor (GnRHR) would require partial entry of the N- and C-terminal regions of ligand into the transmembrane core. The functional significance of the conserved aromatic residue Trp^{279} present in the transmembrane helix 6, and Val^{299} located in exoloop 3 of the rat GnRHR was investigated by mutagenesis followed by expression in Chinese hamster ovary-K1 cells. Compared with wild-type, substitution of Trp^{279} with Ser or Arg resulted in a marked reduction or total abolition, respectively, of ligand binding and, in both cases, abrogation of GnRH-induced inositol phosphate production. A total absence of functionality was observed when Val^{299} was simply replaced with Ala. Mention should be made that an expression of all mutated and wild-type receptor proteins was observed. Interestingly, the double mutant [Trp^{279}Arg/Val^{299}Ala]GnRHR restored B_{max} to wild type (504 ± 43 versus 541 ± 41 fmol/mg protein), but with a diminished affinity (4.95 ± 1.05 versus 0.94 ± 0.35 nM), and GnRH failed to induce inositol phosphate. No influence of the mutations was seen on internalization of the receptor. The three-dimensional model of GnRH binding to the rat GnRHR was built predicting that Trp^{279} is buried at 20 Å in the transmembrane core of the receptor, directly in contact with Trp^3 of GnRH. In contrast, Val^{299} is located in a region that cannot be precisely defined at the extracellular end of transmembrane helix 7. Although models cannot provide any clue concerning the observed interactivity between the two distal residues, altogether these data reveal the functional importance of both GnRHR Trp^{279} and Val^{299} and suggest that Trp^{279}, interacting with GnRH Trp^3, represents the bottom of the binding pocket.

The effects of gonadotropin-releasing hormone (GnRH), a decapeptidic neurohormone secreted by the hypothalamus, are mediated by a specific receptor present on the cell surface of anterior pituitary gonadotropes (Counis, 1999). The cloning of cDNAs from various species and the amino acid sequence analysis have confirmed that the GnRH receptor (GnRHR) possesses the typical structural features of members of the G-protein-coupled receptor (GPCR) superfamily, which include seven membrane-spanning helices connected with three intracellular and three extracellular loops (Stojilkovic et al., 1994). However, the GnRHR exhibits unique structural features, such as the replacement of the conserved DRY sequence in the second intracellular loop by DRS, the reciprocal exchange of the highly conserved Asp in transmembrane helix (TMH) 2 and Asn in TMH7, and, most striking, the lack of a cytoplasmic C-terminal tail, which accounts for the slow internalization postactivation and, together with a relatively short N-terminal sequence, its reduced size (327 amino acids in rat and mouse; 328 in human, porcine, bovine, and ovine).

Due to the central role of GnRH in reproductive physiology and the large range of clinical applications for both agonist and antagonist analogs (Conn and Crowley, 1994), an in-depth knowledge of the GnRHR characteristics and ligand interaction is required. Site-directed mutagenesis has allowed the identification of several amino acid residues of the GnRHR that are functionally important (for binding and/or transduction, or internalization). In particular, evidence has been obtained that the binding site of GnRHR, like other GPCRs for peptide ligands, involves the extracellular domains as well as the transmembrane domains of the receptor. Specifically, His^2 of GnRH has the potential to interact with

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2); GnRHR, GnRH receptor; TMH, transmembrane helix; GnRH-Ag, [His^5-DTyr^6]GnRH; GPCR, G protein-coupled receptor; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyrotropin-stimulating hormone; EGFP, enhanced green fluorescent protein; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; ConA, concanavalin A; IP, inositol phosphate.

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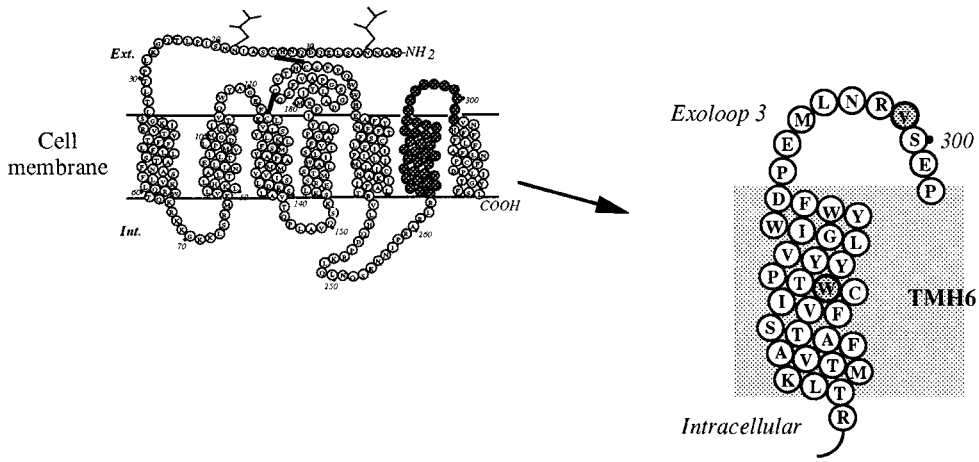
Lys[121] present near the top of TMH3 (Zhou et al., 1995), Arg[8] of GnRH with Glu[301] (mouse, rat), or Asp[302] (human, porcine, ovine, bovine) of exoloop 3 (Flanagan et al., 1994) and the C-terminal glycinamide with Asn[102] located at the apex of TMH2 (Davidson et al., 1996). Altogether, these data are consistent with earlier studies related to the GnRH structure-activity demonstrating that the N- and C-terminal region are the most important for binding and receptor activation, whereas the central region (residues 5, 6, and 7) are most involved in conferring flexibility to the molecule (Karten and Rivier, 1986) and indicate that GnRH would interact with its receptor in a hairpin structure, with both the N and C tails partially penetrating the TMH bundle of its receptor.

The transmembrane core of the GnRHR, like other GPCRs, is highly hydrophobic and contains conserved aromatic residues on helices 4, 5, 6, and 7, some of which have been shown to be critical determinants for ligand-receptor interaction in a large range of GPCRs (Trumpp-Kallmeyer et al., 1992). An implication of aromatic amino acids, and particularly Trp and Tyr, in the recognition site of GnRHR has been suggested earlier by Keinan and Hazum (1985) using a specific chemical approach. Consistently, aromatic residues Tyr[284] (human), present in TMH6, and Tyr[322] (mouse), present in TMH7, have been recently shown to be functionally essential in GnRH binding and/or signal transduction and internalization (Arora et al., 1996; Layman et al., 1998). The comparison of the primary sequence of a large number of GPCRs (Probst et al., 1992), including all known mammalian and nonmammalian GnRHR, has emphasized the highly conserved position of the Trp in both TMH4 and TMH6. Nevertheless, whereas the TMH4 Trp displayed total conservation among GPCRs, TMH6 Trp appeared essentially absent in odorant receptors and, most interestingly, in all members of the glycoprotein hormones receptor family (see Fig. 1), the ligand of which consists of large, complex hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH), or thyrotropin-stimulating hormone (TSH)], which do not seem to enter the TMH core. Thus, we hypothesized that the TMH6 Trp residue could be of specific importance in GnRHR functionality.

In this study, we have constructed a model for the GnRHR and docking of GnRH, and analyzed the functional influence of mutating the conserved TMH6 Trp present at position 279 of the rat GnRHR through heterologous expression in Chinese hamster ovary (CHO)-K1 cells. We found that Trp[279] is precisely at the base of the binding pocket and appears crucial for receptor activity. In addition, a Val[299] residue located in the third exoloop on the border of TMH6 appears important for GnRH interaction and is remarkable in that it seems to have an influence on ligand binding in interdependence with Trp[279] by an as yet unknown mechanism. The three-dimensional model together with the mutational analyses are compatible with a tight interaction of Trp[3] of GnRH with Trp[279], strengthening the idea that the N terminus of the

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                        270         280         290
rGnRHR          RLRTLKMTVA FATSFVICWT PYYVLGIWYW FDP
mGnRHR          RLRTLKMTVA FATSFVVCWT PYYVLGIWYW FDP
hGnRHR          RLKTLKMTVA FATSFTVCWT PYYVLGIWYW FDP
o/bGnRHR        RLRTLKMTVA FATSFTVCWT PYYVLGIWYW FDP
pGnRHR          RLRTLKMTVA FAASFTVCWT PYLVLGIWYW FDP
cfGnRHR         RMKTLKMTII IVASFTVCWT PYYLLGIWYW FDP

hVaso/Ocyt-R    ...T..MT.. .......CW. P......W.. ...
hRhodopsin-R    ......M... F......CW. PY........ ...
hß2-Adren-R     .(K)LK.... F.T....CW. P.....I... ...
hm1-Muscar-R    .........A ....F...WT PY........ ...

hLH/FSH-R       .......... ..T F.-C.. P.....I... ...
hTSH-R          .......... ..T F.-C.. P......... ...
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Fig. 1. Alignment of TMH6 regions of rat (r), mouse (m), human (h), ovine (o), bovine (b), porcine (p), and catfish (cf). GnRHR primary sequence with representative corresponding sequences of various GPCRs including vasotocin/ocytocin, rhodopsin, β_2-adrenergic, m1-muscarinic, LH, FSH, and TSH receptors. Conserved residues (W and P) are bold, and the putative TMH6 region is underlined. On top of the figure is the entire schematic representation of the rat GnRHR with the TMH6 and exoloop 3 (shaded), which are enlarged in the inset.

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neurohormone extends deep into the transmembrane helix bundle.

Materials and Methods

Reagents and Peptides. Native GnRH and [DTrp^6] analog were obtained from Sigma (Saint-Quentin-Fallavier, France). [His^5-DTyr^6]GnRH was kindly provided by Dr. R. P. Millar (Cape Town, South Africa, and Edinburgh, UK). LipofectAMINE and OPTI-MEM were purchased from Life Technologies (Gaithersburg, MD). Cell culture-related products were purchased from Sigma. Restriction and DNA-modifying enzymes and oligonucleotide primers for site-directed mutagenesis were obtained from Eurogentec (Angers, France). The polymerase chain reaction (PCR) in vitro mutagenesis kit was obtained from Takara (Amersham/Takara, Les Ulis, France). Vent$_R$ DNA polymerase was provided by New England BioLabs (Beverly, MA) and the Expand High Fidelity PCR System by Boehringer Mannheim (Meulan, France). Dowex AG 1-X8 resin, formate form, 200 to 400 mesh, was obtained from Bio-Rad (Ivry-sur-Seine, France), and myo-[2-^{3}H]inositol (16.3 Ci/mmol) was obtained from Amersham. The expression vectors pGEM-T, pMSGCAT, and pEGFP-N3 were obtained, respectively from Promega (Lyon, France), Pharmacia Biotech (Orsay, France), and Clontech (Paris, France). Lab Tek chamber slides were obtained from Nunc (Poly-Labo, Strasbourg, France), Vectashield was obtained from Biosys (Compiègne, France); and Alexa 594 Concanavalin A (ConA) was obtained from Molecular Probes (Interchim, Paris, France).

Construction of Wild-Type and Mutant GnRH Receptors. A 1.038-kb rat GnRHR cDNA inserted at the *Sma*I site of pUC19 (Moumni et al., 1994) served as a template for creating site-directed mutations using an in vitro mutagenesis kit. The sequences of the 22-mer mutagenic primers for Trp^{279} were 5′-GTCATCTGC[<u>C</u>GG/T<u>C</u>G/T<u>T</u>G]GACTCCCTAC-3′; and at the underlined base, codon TGG for Trp was altered to CGG for Arg, TCG for Ser, and TTG for Leu. These mutations were performed using separate primers. For Val^{299}, the 21-mer mutagenic primer was 5′-GTTAAACAGGG<u>C</u>GT-CAGAGCC-3′; and at the underlined base, codon GTG for Val was replaced with GCG for Ala. *Nhe*I/*Xho*I restriction sites were created by PCR with Vent$_R$ DNA polymerase to subclone into pGEM-T.

After subcloning, the sequence of the wild-type and mutated receptor cDNAs was confirmed by automated DNA sequencing (Li-Cor, MWG-Biotech, Lincoln, NE). Plasmids were *Nhe*I/*Xho*I digested, and the insert was ligated into pMSGCAT previously digested by the same restriction enzymes. The same strategy was used for the construction of double mutant receptor.

Preparation of ^{125}I-Labeled GnRH Agonist. The [His^5-DTyr^6]GnRH (GnRH-Ag) was radioiodinated using a modified oxidative reaction catalyzed by chloramine-T (Millar et al., 1995) and purified by chromatography on a Sephadex G25 column in 0.01 M acetic acid. The specific radioactivity of the tracer was 100 μCi/μg as determined from self-displacement binding assays (Clayton et al., 1979) using rat pituitary homogenates (Noël et al., 1987).

Transient Transfection of GnRHR in CHO-K1 Cells. CHO-K1 cells routinely maintained at 37°C in Ham's F12 medium supplemented with 10% newborn calf serum containing 100 μg/ml gentaline were seeded in six-well plates at a density of 9×10^4 cells per well. At 60 to 70% confluence, the cells were transfected in 1.2 ml of serum-free OPTI-MEM per well with 1.2 μg wild-type or mutant plasmid DNA and 24 μg of LipofectAMINE. Five hours later, the medium was changed and cells were cultured for 60 h to allow optimal expression of receptors before ligand binding and functional assays.

Agonist Binding to Transfected Cells. Saturation and competition binding assays for wild-type and mutant receptor were carried out on cells or pituitary extracts (Noël et al., 1987). Binding reactions were performed at 25°C for 75 min in Ham's F12 medium containing 0.1% BSA. For saturation experiments, assays contained 0.2 to 3 nM ^{125}I-GnRH-Ag. Nonspecific binding was determined using either 10^{-6} M [His^5-DTyr^6]GnRH or [D-Trp^6]GnRH, which both gave identical values. For competition experiments, duplicates contained 0.5 nM ^{125}I-GnRH-Ag in the absence or in the presence of increasing concentrations of unlabeled peptide. Binding was stopped by medium removal after placing the plates on ice followed by two washings with ice-cold PBS. Cells were then treated with 0.2 M NaOH-0.1% SDS solution, and radioactivity was measured using a gamma counter (Wallach-EG&G, Evry, France). All assay points were performed in duplicate, and independent experiments were repeated at least three times.

Internalization Assays. Transfected CHO-K1 cells were washed twice with binding medium before the addition of 0.5 nM ^{125}I-GnRH-Ag. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled GnRH agonist. After incubation at 25°C for the indicated periods, the cells were washed twice with ice-cold PBS and incubated with 1 ml of 50 mM acetic acid-150 mM NaCl, pH 2.8, for 12 min to remove surface-bound tracer (Arora et al., 1995). The acid-released radioactivity was collected to determine the receptor-bound radioactivity, and the internalized radioactivity was quantified after solubilizing the cells in NaOH-SDS. Internalized radioligand at each time point was expressed as the percentage of the total bound (acid-resistant + acid-released).

Inositol Phosphate (IP) Production. Forty-eight hours after transfection, CHO-K1 cells were labeled by incubation in an inositol and serum-free medium containing 20 mM LiCl and 6 μCi/ml myo-[2-^{3}H]inositol. After an optimal 16- to 18-h labeling, cells were washed twice with Hank's medium containing 0.1% BSA and 20 mM LiCl and incubated in the same solution in the absence or in the presence of 10^{-7} M GnRH for 1 h. Reaction was stopped by the addition of ice-cold perchloric acid (5% v/v final concentration) followed by scraping and incubation at 4°C for 5 min to allow extraction of IPs. After neutralization with 0.5 M KOH, the supernatant was applied to a Dowex AG 1-X8 column for the separation of total IPs with 1 M ammonium formate-0.1 M formic acid (Millar et al., 1995). Radioactivity was measured by liquid scintillation in a beta counter (Wallach-EG&G).

Preparation, Expression, and Confocal Imaging of Enhanced Green Fluorescent Protein (EGFP)-Tagged GnRHR. To assess the expression of mutated receptors that exhibit no biological activity (binding and IP production), wild-type and mutant receptors were fused at their C terminus with the codon-optimized

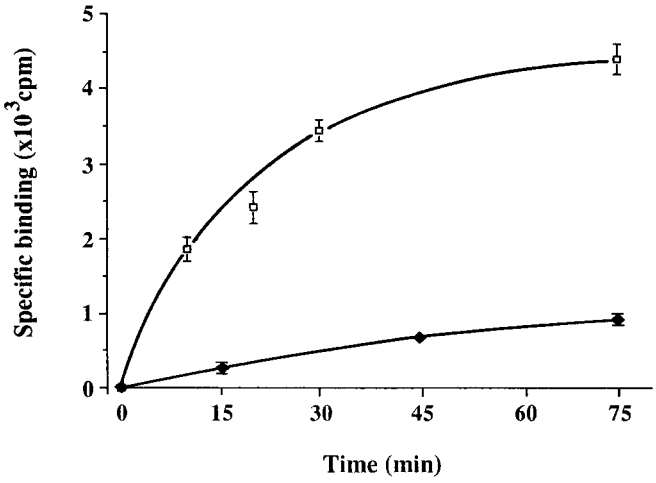

Fig. 2. Time course of receptor binding and internalization in CHO-K1 cells transfected with wild-type GnRHR. Cells were incubated with ^{125}I-GnRH-Ag in the absence and in the presence of 10^{-6} M [D-Trp^6]GnRH to determine total specific binding (□), whereas an additional acetic acid treatment was performed after binding to measure internalization (◆) according to the methods described in *Materials and Methods*. Values expressed as specific binding (in 10^3 cpm) are the mean ± S.E. of at least three independent experiments. No specific binding was observed in control, nontransfected CHO cells.

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EGFP derived from *Aqueorea victoria* according to the procedure recently described for the murine GnRHR (Nelson et al., 1999), with appropriate adaptations. Briefly, wild-type and mutated GnRHR sequences were PCR-generated from recombinant pMSGCAT using Expand High Fidelity PCR system and the following pair of specific primers: 1) 5′-ACCAAGGAATTCCGTCCTTGGAGA-3′, which includes an *Eco*RI restriction site (underlined), and 2) 5′-TGGTTGGGTAGTCTCCGGTACCCAAAGAG-3′, which eliminates the stop codon and substitutes a *Kpn*I site (underlined) at its 3′ end to create an in-frame restriction site. The PCR products were digested with *Eco*RI and *Kpn*I and ligated into pEGFP-N3 cut with the same enzymes. The resulting fusion protein comprised the wild-type or mutated GnRHR, a spacer sequence of 10 amino acids (GTAGPGSIAT), and EGFP. After the sequence was confirmed, the constructions were transfected into CHO-K1 cells as described above except that cells were plated in Lab Tek chamber slides. Cells were then stained with 10 μg/ml ice-cold Alexa 594 ConA in PBS, rinsed three times with cold PBS, and fixed with 4% paraformaldehyde for 20 min. After another three washes with PBS, the slides were mounted with Vectashield. Images were acquired using a confocal laser scanning microscope (Leica TCS-SP, Leica Microsystems, Heidelberg, Germany) in an epifluorescence mode (lens ×100, zoom 2; step, 1 μM). The 488- and 568-nm wavelengths of an argon ion laser were used to excite the sample, the wavelengths were separated with a spectral system using the fluorescein isothiocyanate-tetramethylrhodamine isothiocyanate standard module. Images were saved on CD-ROM, exported to Adobe Photoshop for image processing, and printed on a Hewlett Packard color printer.

Molecular Modeling. A three-dimensional model of the complex between GnRH and its rat receptor has been defined using the experimental data. The three-dimensional model of the rat GnRHR

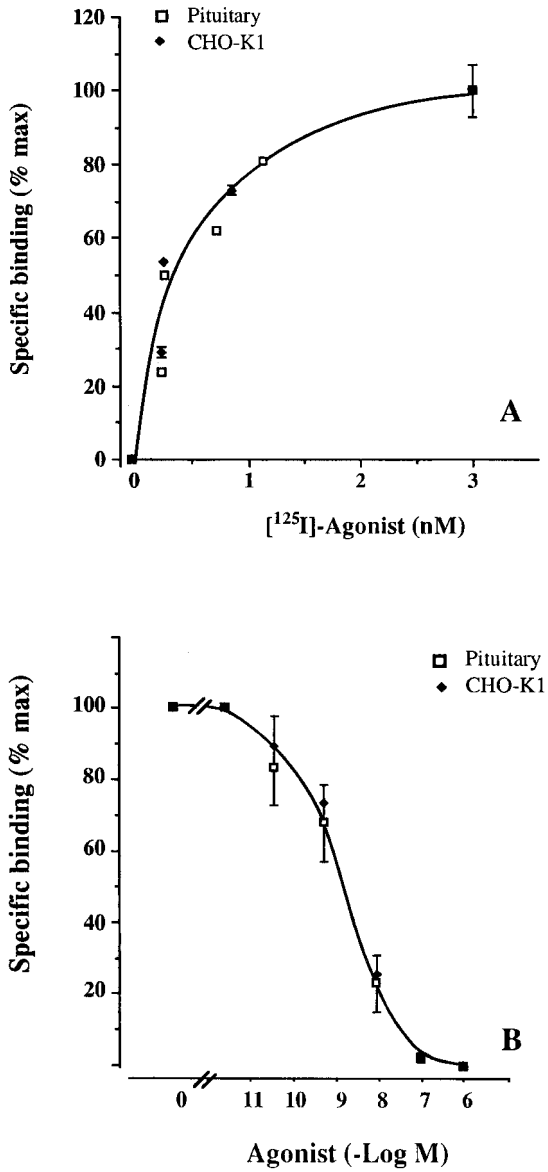

Fig. 3. Saturation (A) and competition (B) binding for the GnRHR in CHO-K1 cells (□) and anterior pituitary homogenate (♦). Incubation was performed in duplicate or triplicate (A) in the presence of 0.2 to 3 nM ^{125}I-GnRH-Ag and (B) in the presence of 0.5 nM ^{125}I-GnRH-Ag supplemented with unlabeled peptide to the indicated final concentrations. Data expressed as the percentage of maximum binding are the mean ± S.E. of three independent experiments.

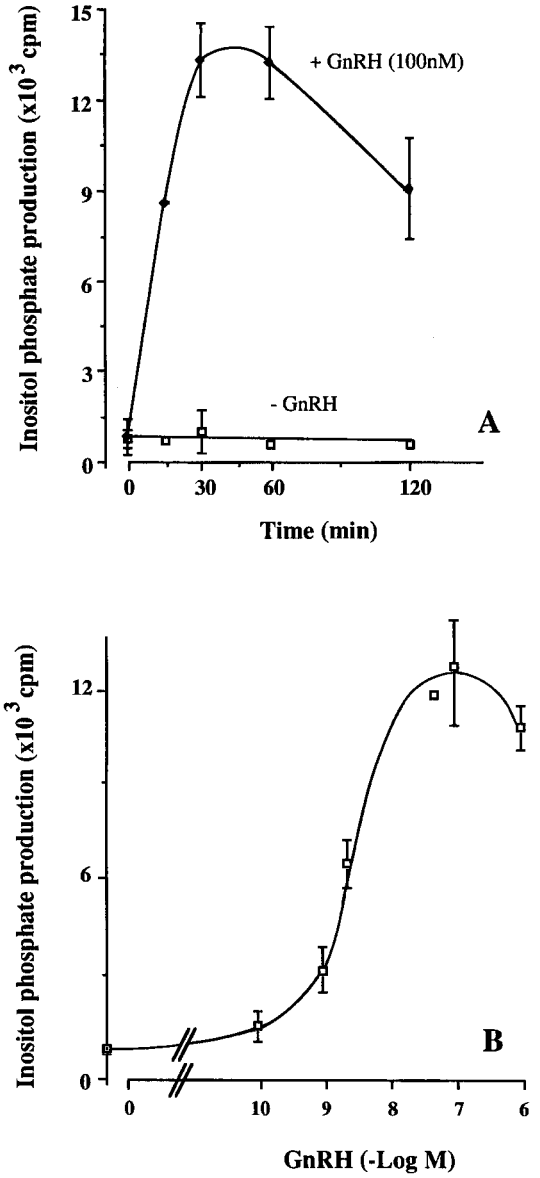

Fig. 4. Time and concentration dependence of GnRH effects on phosphoinositol hydrolysis in CHO-K1 cells transfected with the GnRHR. Cells were labeled for 18 h with myo-[2-^{3}H]inositol and further incubated in the presence of 20 mM LiCl either (A) for varying periods of time in the presence (♦) or absence (□) of 100 nM GnRH or (B) for 1 h in the presence of grading concentrations of GnRH. Total IP was determined as described in *Materials and Methods,* and data are expressed as the mean ± S.E. of three independent experiments.

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hosting GnRH was constructed using the procedure already extensively described and validated in a previous publication for the rat V1A vasopressin receptor (Mouillac et al., 1995). Briefly, the transmembrane domain of the GnRHR was constructed using the three-dimensional model first developed based on the bacteriorhodopsin experimental structure (Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992) and refined on the bovine rhodopsin footprint (Schertler et al., 1993). The extracellular regions of the receptor were then built using the LoopSearch facility in Sybyl (Tripos), and the disulfide bridge connecting Cys^{114} and Cys^{195} (Cook and Eidne, 1997; Davidson et al., 1997) was added. The whole receptor structure (side chains only) was then energy minimized to relax the structure and to remove unfavorable steric constraints. GnRH was manually docked to optimize the complementarity between the host and the ligand and to account for existing experimental data. The conformation of the ligand and of the receptor side chains has been manually controlled to optimize the electrostatic and steric interactions with the receptor walls. The ligand-receptor complex has then been minimized without constraints (Tripos force-field, Gasteiger-Hückel charges, convergence criteria, 0.05 Kcal/mol). Among the infinite number of putative receptor-bound conformations based on interaction energies, one conformer fitting best with experimental data has been selected.

Results

Characterization of GnRH Receptors Expressed in CHO-K1 Cells. The wild-type GnRHR transiently expressed in CHO-K1 cells was characterized by assessing its ability to bind ^{125}I-GnRH-Ag, to be internalized, and to induce IP production in response to GnRH.

Figure 2 illustrates the time-dependent increase in ^{125}I-GnRH-Ag specifically bound to wild-type receptors expressed in the CHO cells. Binding attained a plateau within 60 to 75 min. The residual radioactivity retained after acid treatment, which represents internalized radiolabeled receptors, progressively increased with time. Internalized GnRHR was 21.4 ± 1.5% of the total radioactivity bound after 75 min.

Figure 3 shows the saturation binding of ^{125}I-GnRH-Ag (Fig. 3A), and the competitive displacement of the labeled agonist by increasing concentrations of cold agonist (Fig. 3B), for both wild-type receptor heterologously expressed in CHO cells and native receptor present in rat pituitary membrane extracts. In both Figs. 3A and 3B, the curves superimposed, implying that there was no significant difference in either the K_d or IC_{50} between the pituitary and the GnRHR-expressing CHO cells (K_d = 1.50 ± 0.27 and 0.94 ± 0.35 nM, respectively; IC_{50} = 3.5 ± 0.7 and 4.5 ± 0.2 nM, respectively). No specific radioactivity was bound by nontransfected CHO-K1 cells (not shown).

The time- and concentration-dependent IP responses of CHO cells expressing wild-type receptors are shown in Fig. 4, A and B, respectively. Under the experimental conditions used (including the presence of 20 mM LiCl), a maximal IP production of ~12-fold the basal level was observed after 1 h with 100 nM GnRH, and the EC_{50} observed was 2.1 ± 0.2 nM. A negative control consisting of cells transfected with the vector alone did not exhibit any IP production in response to GnRH whatever the concentration used (not shown).

Characterization of Mutant Receptors. Substitution mutations were created using a PCR-based mutagenesis method. As shown in Table 1, replacement of Trp^{279} by the nonaromatic, ambivalent amino acid, Ser, induced a 5.7-fold decrease in B_{max} (94 ± 19 versus 541 ± 41 fmol/mg protein) and a 3.1-fold decrease in affinity (K_d = 2.98 ± 0.89 versus 0.94 ± 0.35 nM), whereas replacement by a positively charged amino acid, Arg, completely abolished the binding. In both cases, no IP production was observed in response to GnRH. We also substituted Trp^{279} by Leu; however, the construction generated only minute levels of GnRHR mRNA compared with wild type, thus precluding its use in this study. In contrast, Northern blot analysis of GnRHR mRNA expressed in all other recombinant cells revealed transcript levels and length comparable with the wild-type receptor (not shown), indicating that an absence of binding did not result from a default at the pretranslational level.

Table 1 also shows that the substitution of exoloop 3 Val^{299} with Ala (which differs from Val by the lack of a γ-branched side chain) resulted in a complete loss of both binding and IP production. Surprisingly, whereas each mutation Trp^{279}Arg or Val^{299}Ala caused a complete loss of activity on individual expression, the double mutation [Trp^{279}Arg/Val^{299}Ala] restored B_{max}, which appeared almost similar to that of wild-type receptor (504 ± 43 versus 541 ± 41 fmol/mg protein). However, the affinity of the double mutant (K_d = 4.95 ± 1.05 nM) was reduced 5.3-fold compared with wild type. Moreover, when exposed to 100 nM GnRH, the double mutant receptor did not induce IP production. Thus, the two modifications introduced into the GnRHR sequence, while capable of restoring binding capacity, still dramatically impaired the ability of the receptor to transduce signal and generate phosphoinositol hydrolysis. As shown in Table 1, the cotransfection of individually inactive mutants Trp^{279}Arg and Val^{299}Ala (at equimolar concentrations) did not restore bind-

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TABLE 1

Characteristics of GnRH binding and IP production in CHO-K1 cells transfected with wild-type GnRHR or GnRHR mutated on Trp^{279} and/or Val^{299}

Receptor binding assays were performed using ^{125}I-GnRH-Ag. K_d and B_{max} were calculated from saturation experiments. The half-maximum stimulation (EC_{50}) for total IPs was determined from concentration-dependence incubations. ND, no sufficient binding to allow determination. Basal, absence of GnRH-induced IP production. Values are the mean ± S.E. of at least three individual experiments.

GnRHR Construct	Receptor Binding		Total IP Production EC_{50}
	K_d	B_{max}	
	nM ± S.E.M.	*fmol/mg protein ± S.E.M.*	*nM ± S.E.M.*
Wild-type	0.94 ± 0.35	541 ± 41	2.10 ± 0.20
W279S	2.98 ± 0.89	94 ± 19	Basal
W279R	ND	ND	Basal
V299A	ND	ND	Basal
[W279R/V299A]	4.95 ± 1.05	504 ± 43	Basal
[W279R]+[V299A]	ND	ND	Basal

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ing as observed with the double mutation on the same GnRHR molecule.

A possible influence of mutation on ligand-induced internalization was examined by measuring the ^{125}I-GnRH-Ag retained by the cells after an acid wash treatment to remove surface-bound ligand. A direct comparison between the wild-type and mutant receptors was made by expressing the percentage of radioligand internalized versus total binding. The data (Fig. 5) show that the internalization rate of mutant receptors was in each case not significantly different from the wild-type. Thus, Trp^{279} and Val^{299} would not be involved in receptor internalization but rather in binding and transduction.

Expression of the Wild-Type and Mutated GnRHR Protein. Figure 6 shows images obtained by confocal analysis of the wild-type GnRHR, Trp^{279}Arg and Val^{299}Ala mutants rendered intrinsically fluorescent with EGFP. In each case, a clear expression of the fusion protein was attested by the presence of the green fluorescence in the cells. The distribution and intensity of the fluorescence appeared very similar whether the cells expressed a mutant or wild-type receptor. Images obtained by expression of the [Trp^{279}Arg/Val^{299}Ala] or Trp^{279}Ser fusion proteins were also very similar to those visible on the figure and therefore not illustrated. In contrast, control, CHO cells transfected with EGFP alone resulted in a totally different image, in particular the fluorescence was present in the nucleus (not shown). Alexa 594 ConA, a red fluorescent derivative of ConA that binds to plasma membrane carbohydrate moieties, accordingly labeled the periphery of cells and allowed the demonstration of a colocalization with at least a portion of the green fluorescence of EGFP resulting in yellow color. Comparing at least 10 different cells in each case, no significant difference was detected between mutant and wild-type GnRHRs, or between the different GnRHR mutants for the intensity or distribution of the green fluorescence and yellow coloration.

Molecular Modeling. The overall architecture of the GnRHR three-dimensional model that has been derived from the experimental structure of rhodopsin (Fig. 7) is very similar to that described previously for other peptide GPCRs (Baldwin, 1993; Mouillac et al., 1995) and also for GnRHR (Zhou et al., 1994). Obviously, some differences might exist, more particularly in the loop conformations because the topography of these regions cannot be accurately determined with the homology modeling approach. In the model, Asp^{318} in TMH7 is adjacent in space to Asn^{87} in TMH2, in agreement with experimental data (Zhou et al., 1994). The preferred docking mode for GnRH was the following: PyroGlu^{1} of GnRH is buried in the central receptor cleft in the neighborhood of TMH5; it is putatively involved in hydrogen bonding with Asn^{211} and/or Thr^{214}; His^{2} points toward Lys^{121} in TMH3 in agreement with experimental data (Zhou et al., 1995); Trp^{3} interacts with a cluster of aromatic residues in TMH6 and TMH7: Trp^{279}, Tyr^{282}, Phe^{307}, and Phe^{310}; Tyr^{5} is in the neighborhood of Phe^{308} in TMH7; and the sequence Gly-Leu-Arg-Pro-Gly-NH$_2$ lies along TMH2, TMH3, and TMH7 bringing Arg^{8} side chain in contact with Glu^{301} and Gly^{10} carboxamide in the vicinity of Asn^{102}, in agreement again with experimental data (Flanagan et al., 1994; Davidson et al., 1996). This binding mode is necessarily speculative. However, it accounts qualitatively well for most known experimental data.

Discussion

In this work, we have expressed the rat GnRHR in CHO-K1 cells, with functional properties that reflect those of the native receptor in pituitary or other expression systems. In particular, the GnRHR displayed a K_d of 0.94 ± 0.35 nM with the recently developed (Flanagan et al., 1998) ^{125}I-labeled [His^{5}-DTyr^{6}]GnRH, in agreement with the 1.50 ± 0.27 nM measured in rat pituitary with the same agonist. Moreover, GnRH elicited a dose-dependent IP production with an EC$_{50}$ of 2.1 ± 0.2 nM and an internalization rate again comparable with those measured in pituitary cells (Huckle and Conn, 1987; Arora et al., 1995). This expression system was thus used to examine the effects of mutations on GnRHR functionality.

Our initial, specific interest was to examine the role of Trp^{279} on GnRHR function. This amino acid is present in the TMH6 of a large range of GPCRs (Probst et al., 1992) and was reported, in some cases, to be actively involved in ligand binding (Nakayama and Khorana, 1991; Yamano et al., 1995). Indeed our present observations that substitution of Trp^{279} with Ser or Arg resulted in a marked reduction or total abolition, respectively, of ligand binding and, in all cases, abrogation of GnRH-induced IP production, whereas internalization was unaffected, emphasizes the critical importance of this amino acid.

The finding that Val^{299}Ala could compensate the effects of Trp^{279}Arg mutation on binding when both were introduced into the GnRHR was totally unexpected and surprising as well. Such an investigation took advantage of an early exploration of exoloop 3, which demonstrated the total suppression of both binding and IP production after mutation of this residue, as reported herein. Val is present in all known mammalian GnRHRs with the unique exception of human, where it is replaced by a Leu, and in the nonmammalian catfish, where it is replaced by an Ile. All of these residues are characterized by the presence of a short side chain, ab-

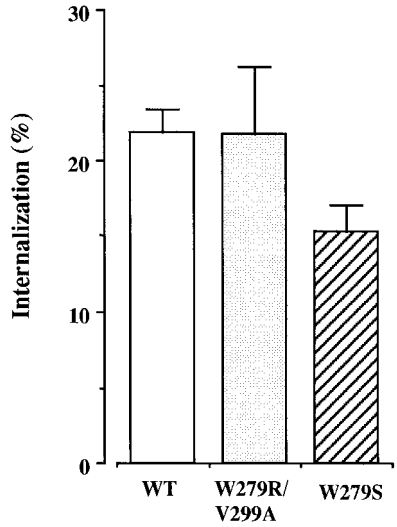

Fig. 5. Agonist-induced internalization in CHO-K1 cells transfected with mutated GnRHR. Only the W279S and W279R/V299A mutants, which were capable of binding GnRH, could be examined, in parallel with the wild-type GnRHR. Internalization was measured after a 75-min incubation with ^{125}I-GnRH-Ag. Values are expressed as percentage of total specific binding and are the mean ± S.E. of three independent experiments.

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sent in Ala, suggesting that this motif is crucial for GnRHR functionality. Exoloop 3 of δ-opioid receptor also contains Val residues that are functionally critical (Valiquette et al., 1996).

That the mutation of Trp^{279} or Val^{299} both resulted in a loss of binding raises the question of whether these alterations affect ligand-receptor interactions or interfere with synthesis and/or insertion of the receptor into the cell membrane (Arora et al., 1999). Northern analysis indicated that mRNA of mutant and wild-type receptors were expressed at comparable levels in all cases. However, for a more in-depth answer to this question, in the absence of a specific antisera the incorporation of a flag sequence was necessary. The construction of fusion proteins, which made the wild-type and mutant GnRHR intrinsically fluorescent using the EGFP, provided direct evidence for their expression in CHO cells. A large percentage of the fusion protein expressed was localized in the cytoplasm, nevertheless an obvious presence at the membrane was observed, in each case, by colocalization with a membrane marker. Only the GnRHR, coupled to EGFP by the C terminus with an intercalated spacer, has been shown to retain biological activity (Lin et al., 1998; Nelson et al., 1999). Accordingly, we observed that the binding characteristics of cells expressing the wild-type GnRHR-EGFP was totally preserved compared with the normal GnRHR, whereas the Trp^{279}Arg as well as the Val^{299}Ala fusion proteins were still incapable of ligand binding (data not shown), as was found for the corresponding unlabeled mutants. Because the wild-type GnRHR as well as Trp^{279}Arg or Val^{299}Ala mutants were expressed in an undistinguishable manner and only the wild type was biologically active whereas the mutants were not, the differences could result from mutation-induced intrinsic modifications. Consistently, no loss of expression was noted in the angiotensin-A1 receptor or rhodopsin mutated on the positionally equivalent TMH6 Trp (Nakayama and Khorana, 1991; Yamano et al., 1995).

Compared with the double [Val^{299}Ala/Trp^{279}Arg]GnRHR mutant, the coexpression of Val^{299}Ala and Trp^{279}Arg mutants did not generate any binding activity, suggesting an interdependence of the two loci. Nevertheless, whatever the potentiality to retain major ligand binding and normal internalization, the double mutant appears unable to induce phosphoinositol hydrolysis after GnRH stimulation, suggesting that agonist binding leads, in this receptor, to an antagonist activity. Unfortunately, the ability of the receptor to activate G-protein proved to be impossible to investigate using nonhydrolyzable GTP, because neither GTPγS nor GppNHp decreased binding even for the wild-type GnRHR (data not shown), a situation that has been attributed, when present, to the low intrinsic GTP turnover rate of the Gαq family of proteins (Burns et al., 1997).

To attempt to interpret our experimental data, we have defined a three-dimensional model of the GnRHR according to an established procedure (Hibert et al., 1991; Ballesteros and Weinstein, 1992; Baldwin, 1993; Mouillac et al., 1995). This crude model happens to be in agreement with most data available on GPCR in general and on the GnRHR in particular. Thus, making the assumption that the helix bundle is similar in the GnRHR and in rhodopsin, the model derived by homology brings Asn^{87} and Asp^{318} in relative positions that allow their interaction (Zhou et al., 1994). It is also possible to create the Cys^{114}/Cys^{195} disulfide bond (Cook and Eidne, 1997; Davidson et al., 1997). The binding mode of GnRH

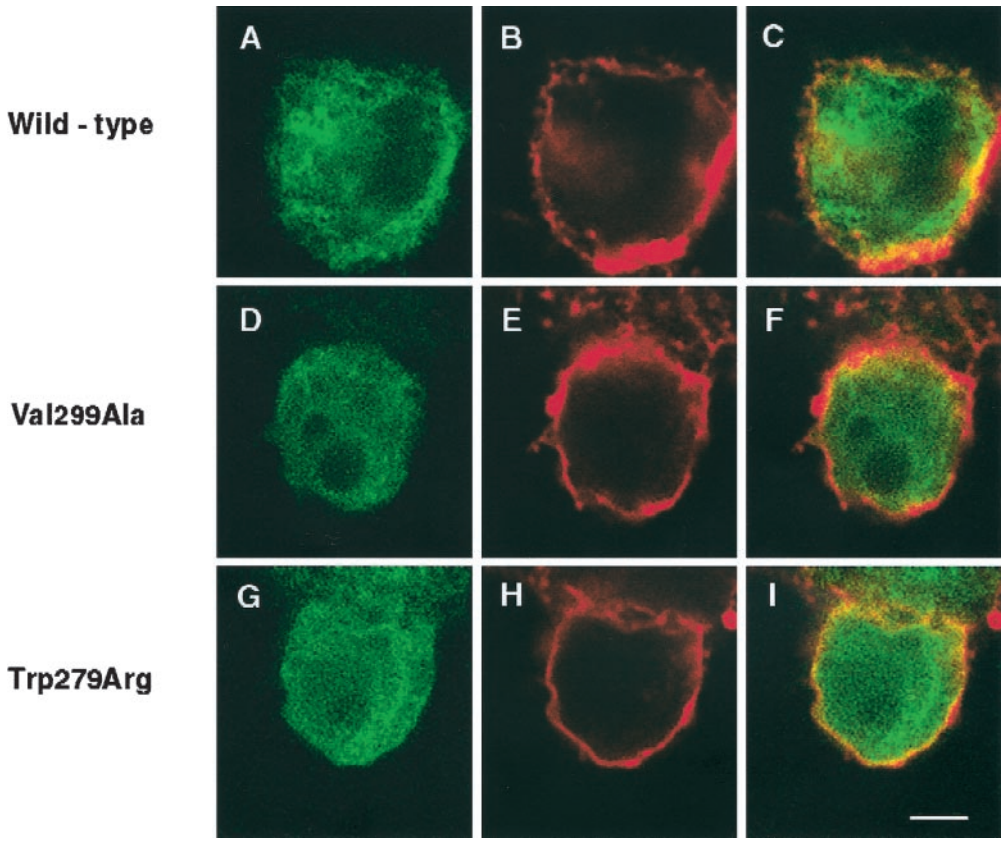

Fig. 6. Images obtained by dual channel confocal laser scanning microscope analysis of CHO-K1 cells expressing the wild-type GnRHR, [Trp^{279}Arg]GnRHR, and [Val^{299}Ala]GnRHR fused to EGFP. A, D, and G, green fluorescent signals from each GnRHR-EGFP fusion protein (identified on the left of the photograph mounting). B, E, and H, red signals from Alexa 594 ConA. C, F, and I, combination of the red and green fluorescent images acquired for each receptor form, colocalization resulting in the yellow color. Scale bar in I is 4.3 μm for each image. Overall magnification is 2500× for all panels.

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cannot be predicted by modeling studies based on energy calculations because a quasi-infinite number of solutions can be found within a reasonable energy window. We tried to orient the docking in two ways: first in using the cyclic backbone of bound vasopressin as a template to fold GnRH (Mouillac et al., 1995); second, in taking conformations of GnRH derived from the NMR study of a cyclic antagonist (Baniak et al., 1987) or from calculations (Gupta et al., 1993). None of these led to a complex with the receptor model, which agreed with the laws of physics and experimental data. Then we tried to find whether there exists at least one energetically acceptable conformation of GnRH that could fit with the set of mutagenesis data already available.

Such a conformer was indeed characterized and is displayed in Fig. 7. In this model, the PyroGlu^1 residue of GnRH lies at the base of the central receptor cleft near TMH5. This location corresponds to the domain occupied by the ionone ring of retinal (Schertler et al., 1993) and the small biogenic amines (Hibert et al., 1991). In agreement with previous studies (Zhou et al., 1995), His^2 is properly located to form hydrogen bonds with Lys^{121}, which corresponds to the pivotal Asp in TMH3 in cationic neurotransmitter receptors. The sequence Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 goes from the base of the central cleft to the external surface of the receptor along TMH2, TMH3, and TMH7. It allows the Arg^8 side chain to make contact with Glu^{301} and brings Gly^{10}-carboxamide in the vicinity of Asn^{102}, in agreement again with experimental data (Flanagan et al., 1994; Davidson et al., 1996). Interestingly, Trp^3 is in contact with a cluster of aromatic residues: Trp^{279} and Tyr^{282} in TMH6, Phe^{307} and Phe^{310} in TMH7. This is coherent with the fact that substitution of Trp^3 with nonaromatic residues leads to GnRH analogs with a very low potency, whereas some activity is still present in Tyr^3 (0.1%), Phe^3 (0.5%), and pentamethyl-Phe^3 (30–70%) analogs, suggesting that the indole moiety of Trp^3 is located in an aromatic environment (Yanaihara et al., 1973; Sandow et al., 1978). The role of Trp^3 in receptor activation is further demonstrated by the antagonist activity of [Leu^3]GnRH (Vilchez-Martinez et al., 1975). The present study thus indicates that Trp^{279} may play a crucial role for interaction with GnRH Trp^3 and receptor activation.

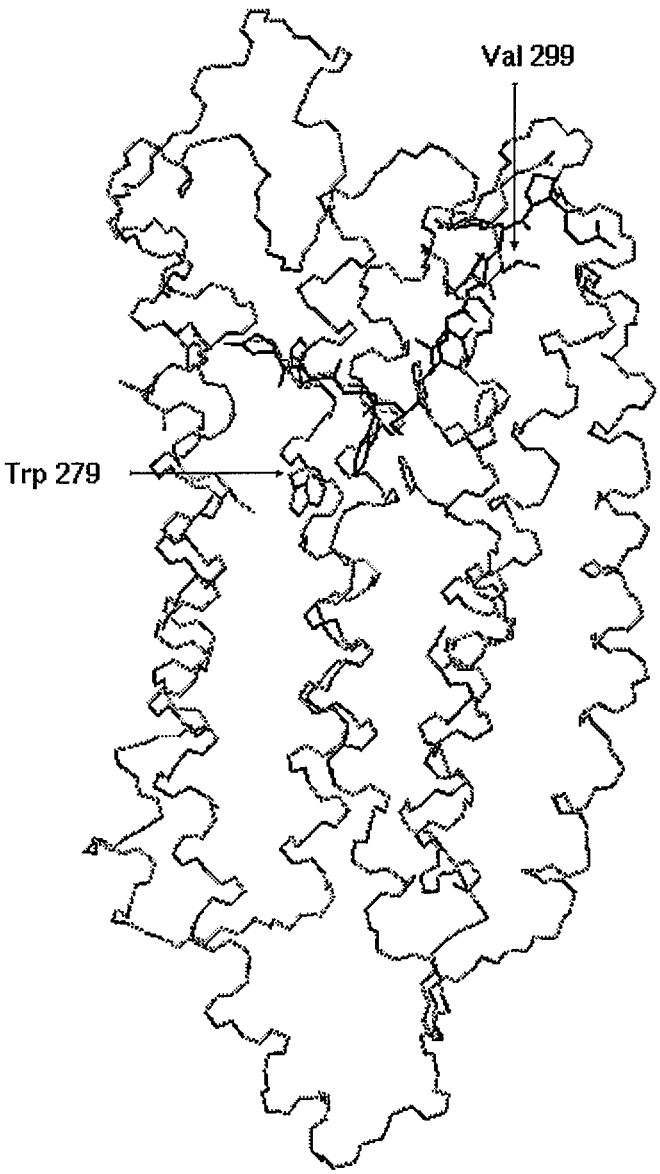

Fig. 7. Three-dimensional model of GnRH bound to the rat GnRHR. GnRH is in black; the receptor backbone and some key binding residues are in gray.

Our findings agree with the proposed concepts that: 1) a cluster of aromatic residues in the TMH6 represents a preferential area of interaction for most ligands (Javitch et al., 1998), and 2) aromatic-aromatic interaction is crucial in maintaining receptor structure and driving conformational changes in GPCRs (Trumpp-Kallmeyer et al., 1992). However, the putative contribution of Val^{299} is less clear. In the model, Val^{299} is not in the binding pocket. In fact, its position cannot be accurately predicted because it is on the border of the transmembrane region. If one assumes that the helical structure of TMH7 starts before Val^{299}, then this residue would be at the top of the hydrophobic face of TMH7. Its side chain would point toward the phospholipid bilayer and could even participate in receptor homo- or heterodimerization or aggregation. This might explain the dramatic effect observed on mutation of this residue. In contrast, if the helical structure starts further in the sequence, Val^{299} would be located in exoloop 3 in the neighborhood of Glu^{301} (close to Arg^8 and Gly^{10} on GnRH) in a position that cannot be precisely predicted. It would then be difficult to understand the importance of the contribution of this residue to receptor folding, binding, and function. Nevertheless, in both cases, Val^{299} would be about 28 Å away from Trp^{279}, excluding any possibility of direct interaction between these two residues. Thus the restored binding of the double mutant cannot be explained in this way.

In conclusion, our results show that the conserved TMH6 Trp^{279} residue plays an important role in the GnRHR in ligand binding and signal transduction, but not internalization, most likely by direct interaction with the aromatic radical of GnRH Trp^3. Such an interaction implies penetration of the ligand N-terminal region in the transmembrane core by about 20 Å away from the surface of the membrane. However, we do not know the real role and mechanisms by which Val^{299}, located in exoloop 3 at the top border of TMH7, operates to alter GnRHR functionality, especially in relation with Trp^{279} of TMH6. Further studies are in progress to examine these questions.

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References

Arora KK, Cheng ZY and Catt KJ (1996) Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. *Mol Endocrinol* **10:**979–986.

Arora KK, Chung HO and Catt KJ (1999) Influence of a species-specific extracellular amino acid on expression and function of the human gonadotropin-releasing hormone receptor. *Mol Endocrinol* **13:**890–896.

Arora KK, Sakai A and Catt KJ (1995) Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. *J Biol Chem* **270:**22820–22826.

Baldwin JM (1993) The probable arrangement of the helices in G protein-coupled receptors. *EMBO J* **12:**1693–1703.

Ballesteros J and Weinstein H (1992) Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. *Biophys J* **62:**107–109.

Baniak EL, Rivier JE, Struthers RS, Hagler AT and Gierasch LM (1987) Nuclear magnetic resonance analysis and conformational characterization of a cyclic decapeptide antagonist of gonadotropin-releasing hormone. *Biochemistry* **26:**2642–2656.

Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E and Emeson RB (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature (Lond)* **387:**303–308.

Clayton RN, Shakespear RA, Duncan JA, Marshall JC, with appendix by Munson PJ and Rodbard D (1979) Radioiodinated nondegradable gonadotropin-releasing hormone analogs: New probes for the investigation of pituitary gonadotropin-releasing hormone receptors. *Endocrinology* **105:**1369–1381.

Conn PM and Crowley WF (1994) Gonadotropin-releasing hormone and its analogs. *Annu Rev Med* **45:**391–405.

Cook JV and Eidne KA (1997) An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation. *Endocrinology* **138:**2800–2806.

Counis R (1999) Gonadotropin biosynthesis, in *Encyclopedia of Reproduction* (Neill J and Knobil E eds) pp 507–520, Academic Press, New York.

Davidson JS, Assefa D, Pawson A, Davies P, Hapgood J, Becker I, Flanagan C, Roeske R and Millar RP (1997) Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: Localization of attachment site to Cys residue in N-terminal segment. *Biochemistry* **36:**12881–12889.

Davidson JS, McArdle CA, Davies P, Elario R, Flanagan CA and Millar RP (1996) Asn[102] of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide. *J Biol Chem* **271:**15510–15514.

Flanagan CA, Becker II, Davidson JS, Wakefield IK, Zhou W, Sealfon SC and Millar RP (1994) Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone. *J Biol Chem* **269:**22636–22641.

Flanagan CA, Fromme BJ, Davidson JS and Millar RP (1998) A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors. *Endocrinology* **139:**4115–4119.

Gupta HM, Talwar GP and Salunke DM (1993) A novel computer modeling approach to the structures of small bioactive peptides: The structure of gonadotropin-releasing hormone. *Proteins* **16:**48–56.

Hibert MF, Trumpp-Kallmeyer S, Bruinvels A and Hoflack J (1991) Three-dimensional models of neurotransmitter G-binding protein-coupled receptors. *Mol Pharmacol* **40:**8–15.

Huckle WR and Conn PM (1987) The relationship between gonadotropin-releasing hormone-stimulated luteinizing hormone release and inositol phosphate production: Studies with calcium antagonists and protein kinase C activators. *Endocrinology* **120:**160–169.

Javitch JA, Ballesteros JA, Weinstein H and Chen J (1998) A cluster of aromatic residues in the sixth membrane-spanning segment of the dopamine D2 receptor is accessible in the binding-site crevice. *Biochemistry* **37:**998–1006.

Karten MJ and Rivier J (1986) Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspective. *Endocrinol Rev* **7:**44–46.

Keinan D and Hazum E (1985) Mapping of gonadotropin-releasing hormone receptor binding site. *Biochemistry* **24:**7728–7732.

Layman L, Cohen D, Jin M, Xie J, Li Z, Reindollar R, Bolbolan S, Bick D, Sherins R, Duck L, Musgrove L, Sellers J and Neill J (1998) Mutations in gonadotropin-releasing hormone receptor gene cause hypogonadotropic hypogonadism. *Nat Genet* **18:**14–15.

Lin X, Cornea A, Janovick JA and Conn PM (1998) Visualization of GnRH receptor with green fluorescent protein (Abstract). 80th Annual Meeting of the Endocrine Society, June 24–27, New Orleans, Louisiana, P3-430.

Millar RP, Davidson J, Flanagan C and Wakefield I (1995) Ligand binding and second-messenger assay for cloned Gq/G11-coupled neuropeptide receptors: The GnRH receptor. *Methods Neurosci* **25:**145–162.

Mouillac B, Chini B, Balestre MN, Elands J, Trumpp-Kallmeyer S, Hoflack J, Hibert M, Jard S and Barberis C (1995) The binding site of neuropeptide vasopressin V1a receptor: Evidence for a major localization within transmembrane regions. *J Biol Chem* **270:**25771–25777.

Moumni M, Kottler M-L and Counis R (1994) Nucleotide sequence analysis of mRNAs predicts that rat pituitary and gonadal gonadotropin-releasing hormone receptor proteins have identical primary structure. *Biochem Biophys Res Commun* **200:**1359–1366.

Nakayama TA and Khorana HG (1991) Mapping of the amino acids in membrane-embedded helices that interact with the retinal chromophore in bovine rhodopsin. *J Biol Chem* **266:**4269–4275.

Nelson S, Horvat RD, Malvey J, Roess DA, Barisas BG and Clay CM (1999) Characterization of an intrinsically fluorescent gonadotropin-releasing hormone receptor and effects of ligand binding on receptor lateral diffusion. *Endocrinology* **140:**950–957.

Noël N, Bérault A, Théoleyre M and Jutisz M (1987) Pituitary GnRH receptor content and LH release from rat pituitary cells in culture: Effect of castration in the male rat. *Neuroendocrinol Lett* **9:**231–236.

Probst WC, Snyder LA, Schuster DI, Brosius J and Sealfon SC (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol* **11:**1–20.

Sandow J, Konig W, Geiger R, Uhman R and Von Rechenberg W (1978) Structure-activity relationships in the LH-RH molecule, in *Control of Ovulation* (Crighton DB, Haynes NB, Foxcroft GR and Lamming GE eds) pp 47–70, Buttersworth, London.

Schertler GF, Villa C and Henderson R (1993) Projection structure of rhodopsin. *Nature (Lond)* **362:**770–772.

Stojilkovic SS, Reinhart J and Catt KJ (1994) Gonadotropin-releasing hormone receptors: Structure and signal transduction pathways. *Endocr Rev* **15:**462–499.

Trumpp-Kallmeyer S, Hoflack J, Bruinvels A and Hibert M (1992) Modeling of G-protein-coupled receptors: Application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. *J Med Chem* **35:**3448–3462.

Valiquette M, Vu HK, Yue SY, Wahlestedt C and Walker P (1996) Involvement of Trp-284, Val-296, and Val-297 of the human δ-opioid receptor in binding of δ-selective ligands. *J Biol Chem* **271:**18789–18796.

Vilchez-Martinez LA, Coy DH, Coy EJ, Schally AV and Arimura A (1975) Anti-luteinizing hormone (LH)-releasing activity of several analogues of LH-releasing hormone. *Fertil Steril* **26:**554–559.

Yamano Y, Ohyama K, Kikyo M, Sano T, Nakagomi Y, Inoue Y, Nakamura N, Morishima I, Guo D-F, Hamakubo T and Inagami T (1995) Mutagenesis and the molecular modeling of the rat angiotensin II receptor (AT1). *J Biol Chem* **270:**14024–14030.

Yanaihara N, Hashimoto T, Yanaihara C, Tsuji K, Kenmochi Y, Ashizawa F, Kaneko T, Oka H, Arimura A and Schally AV (1973) Synthesis and biological evaluation of analogs of luteinizing hormone-releasing hormone (LH-RH). *Biochem Biophys Res Commun* **52:**64–73.

Zhou W, Flanagan C, Ballesteros JA, Konvicka K, Davidson JS, Weinstein H, Millar RP and Sealfon SC (1994) A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor. *Mol Pharmacol* **45:**165–170.

Zhou W, Rodic V, Kitanovic S, Flanagan CA, Chi L, Weinstein H, Maayani S, Millar RP and Sealfon SC (1995) A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites. *J Biol Chem* **270:**18853–18857.

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