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

STÉPHANIE CHAUVIN, ANNETTE BÉRAULT, YANNICK LERRANT, MARCEL HIBERT, and RAYMOND COUNIS

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 interaction 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 GnRHR 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 DRX, 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^{5} of GnRH has the potential to interact with

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}-oTyr^{3}]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.
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 glycaminide 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 (Trumpf-Kallmeyer et al., 1992). An implication of aromatic amino acids, and particularly Trp and Tyr, in the recognition site of GnRH 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 GnRH, 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

![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, β2-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.](image-url)
neurohormone extends deep into the transmembrane helix bundle.

**Materials and Methods**

**Reagents and Peptides.** Native GnRH and [dTrp³] analog were obtained from Sigma (Saint-Quentin-Fallavier, France). [His⁵-dTyr²] 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 (Amerham/Takara, Les Ulis, France). Ventg 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-[2-3H]inositol (163 Ci/mmol) was obtained from Amersham. The expression vectors pGEM-T, pMSGCAT, and pEGFP-N3 were obtained, respectively, from Promega (Lyons, France), Pharmacia Biotech (Orsay, France), and Clontech (Paris, France). Lab Tek chamber slides were obtained from Nunc (Poly-Lab, Strasbourg, France), Vectashield was obtained from Bioys (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 GnRH cDNA inserted at the Smal 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 Trp⁹ were 5'-GTCATCCTGCAG/GCG/TGG/GCT/CACGTCCTAC-3' (GdTyr⁶GnRH or [D-Trp⁶]GnRH); 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 Val⁹⁹, the 21-mer mutagenic primer was 5'-GTTAAACAGGGCAG/GTACGAGCC/GTCACGTT-3'; and at the underlined base, codon GTG for Val was replaced with GCG for Ala. NheI/XhoI restriction site regions were created by PCR with Ventg 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, South Africa, and Edinburgh, UK). LipofectAMINE and OPTI-MEM 2.038-kb rat GnRHR cDNA inserted at the SmaI site of pMSGCAT previously digested by the NheI and XhoI enzymes 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 [²⁵³T]labeled GnRH Agonist.** The [His⁵-dTyr²]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 (Noel 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 gentamicin were seeded in six-well plates at a density of 9 × 10⁴ 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 (Noel 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 [¹²⁵I]GnRH-Ag. Nonspecific binding was determined using either 10⁻⁶ M [His⁵-dTyr²][¹²⁵I]GnRH or [d-Trp³][¹²⁵I]GnRH, which both gave identical values. For competition experiments, duplicates contained 0.5 nM [¹²⁵I]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 [¹²⁵I]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 measured using a gamma counter 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-[2-3H]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

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**Fig. 2.** Time course of receptor binding and internalization in CHO-K1 cells transfected with wild-type GnRHR. Cells were incubated with [¹²⁵I]-GnRH-Ag in the absence and in the presence of 10⁻⁶ M [d-Trp³]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 10⁶ cpm) are the mean ± S.E. of at least three independent experiments. No specific binding was observed in control, nontransfected CHO cells.
EGFP derived from *Aequorea 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'-ACCAAGGAAATTCCGTCCTGGAGA-3', which includes an EcoRI restriction site (underlined), and 2) 5'-TGTTGGG-TAGTCTCCGGTACCCAAAGAG-3', which eliminates the stop codon and substitutes a KpnI site (underlined) at its 3' end to create an in-frame restriction site. The PCR products were digested with EcoRI and KpnI 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

![Graph A](image1.png)

**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 

![Graph B](image2.png)

**Fig. 4.** Time and concentration dependence of GnRH effects on phosphoinositide hydrolysis in CHO-K1 cells transfected with the GnRHR. Cells were labeled for 18 h with myo-[2-3H]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.
hosting GnRH was constructed using the procedure already extensively described and validated in a previous publication for the rat V1a vasopressin receptor (Moullac et al., 1995). Briefly, the transmembrane domain of the GnRH receptor was constructed using the three-dimensional model first developed based on the bacteriorhodopsin experimental structure (Hibert et al., 1991; Trumpf-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 Cys114 and Cys195 (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 GnRH 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 defect 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 phosphoinositid 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-

| TABLE 1 |
| Characteristics of GnRH binding and IP production in CHO-K1 cells transfected with wild-type GnRH or GnRH 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 |
| | $K_d$ nM ± S.E.M. | $B_{max}$ fmol/mg protein ± S.E.M. | EC$_{50}$ 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 |
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 $^{125}\text{I}-\text{GnRH-Ag}$ 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, Trp279 and Val299 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, Trp279Arg and Val299Ala mutants rendered intrinsically fluorescent with EGFP. In each case, a clear expression of the fusion protein was attributed 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 [Trp279Arg/Val299Ala] or Trp279Ser 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 topology of these regions cannot be accurately determined with the homology modeling approach. In the model, Asp318 in TMH7 is adjacent in space to Asn7 in TMH2, in agreement with experimental data (Zhou et al., 1994). The preferred docking mode for GnRH was the following: PyroGlu3 of GnRH is buried in the central receptor cleft in the neighborhood of TMH5; it is putatively involved in hydrogen bonding with Asn211 and/or Thr214; His5 points toward Lys121 in TMH3 in agreement with experimental data (Zhou et al., 1995); Trp3 interacts with a cluster of aromatic residues in TMH6 and TMH7; Trp279, Tyr282, Phe307, and Phe310, Tyr5 is in the neighborhood of Phe308 in TMH7; and the sequence Gly-Leu-Arg-Pro-Gly-NH2 lies along TMH2, TMH3, and TMH7 bringing Arg8 side chain in contact with Glu501 and Gly10 carboxamide in the vicinity of Asn102, 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 [His5-DTyr6]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 EC50 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 Trp279 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 Trp279 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 Val299Ala could compensate the effects of Trp279Arg 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.](image-url)
sent in Ala, suggesting that this motif is crucial for GnRHR functionality. Exolooop 3 of δ-opioid receptor also contains Val residues that are functionally critical (Valiquette et al., 1996).

That the mutation of Trp279 or Val299 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 antiserum 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 Trp279Arg as well as the Val299Ala 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 Trp279Arg or Val299Ala 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 [Val299Ala/Trp279Arg]GnRHR mutant, the coexpression of Val299Ala and Trp279Arg 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 non-hydrolyzable GTP, because neither GTPγS nor GppNHz 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 Asn87 and Asp318 in relative positions that allow their interaction (Zhou et al., 1994). It is also possible to create the Cys114/Cys195 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, Trp279Arg/GnRHR, and [Val299Ala]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.]
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 PyroGlu1 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), His2 is properly located to form hydrogen bonds with Lys121, which corresponds to the pivotal Asp in TMH3 in cationic neurotransmitter receptors. The sequence Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 goes from the base of the central cleft to the external surface of the receptor along TMH2, TMH3, and TMH7. It allows the Arg2 side chain to make contact with Glu301 and brings Gly10-carboxamide in the vicinity of Asn102, in agreement again with experimental data (Flanagan et al., 1994; Davidson et al., 1996). Interestingly, Trp3 is in contact with a cluster of aromatic residues: Trp279 and Tyr282 in TMH6, Phe307 and Phe310 in TMH7. This is coherent with the fact that substitution of Trp3 with nonaromatic residues leads to GnRH analogs with a very low potency, whereas some activity is still present in Tyr3 (0.1%), Phe3 (0.5%), and pentamethyl-Phe3 (30–70%) analogs, suggesting that the indole moiety of Trp3 is located in an aromatic environment (Yanaihara et al., 1973; Sandow et al., 1978). The role of Trp3 in receptor activation is further demonstrated by the antagonist activity of [Leu3]GnRH (Vilchez-Martinez et al., 1975). The present study thus indicates that Trp279 may play a crucial role for interaction with GnRH Trp3 and receptor activation.

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 Val299 is less clear. In the model, Val299 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 Val299, 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, Val299 would be located in exoloop 3 in the neighborhood of Glu301 (close to Arg3 and Gly10 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, Val299 would be about 28 Å away from Trp279, 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 Trp279 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 Trp3. 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 Val299, located in exoloop 3 at the top border of TMH7, operates to alter GnRHR functionality, especially in relation with Trp279 of TMH6. Further studies are in progress to examine these questions.

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.
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