The gonadotropin-releasing hormone (GnRH) receptor belongs to the superfamily of heptahelical G protein coupled receptors, most of which have a highly conserved DRYXXV/IXXPL sequence in the second intracellular (2i) loop that has been implicated in G protein coupling. The predicted 2i loop of the GnRH receptor contains serine rather than tyrosine in the DRY sequence but retains the conserved hydrophobic Leu residue, which is required for G protein coupling and internalization of muscarinic receptors. The present study examined the effects of mutating the unique Ser140 to the conserved Tyr, and the conserved Leu147 to Ala or Asp, on agonist binding, internalization, and signal transduction. The S140Y mutant showed a 100% increase in agonist binding affinity, and its internalization was increased by 60% above that of the wild-type receptor. The binding characteristics of the Leu147 mutants were indistinguishable from those of the wild-type receptor, but their internalization was reduced by about 50%. The L147A and L147D mutants also showed significant impairment of GnRH-stimulated inositol phosphate production. These findings demonstrate that substitution of Ser140 by Tyr does not affect G protein coupling but significantly increases receptor affinity and internalization rate. In contrast, replacement of a conserved aliphatic residue (Leu147) impairs both G protein coupling and agonist-induced receptor internalization.

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), acts via its specific receptors in the anterior pituitary gland to regulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone, and mobilizing Ca2+ and activating protein kinase C to initiate a variety of cellular responses. Receptor activation is followed by extensive internalization and down-regulation of the receptor via endocytosis and processing. The molecular mechanisms of GnRH receptor activation have yet to be clarified.

Mutagenesis and chimeric studies have suggested that the intracellular regions of the G protein-coupled receptors, in particular the second and third intracellular loops and sometimes the cytoplasmic tail, interact with G proteins and mediate signal transduction. Most of these structural and functional studies have been performed on β-adrenergic and muscarinic receptors, and data on these properties of the peptide hormone receptors are more limited. In addition, recent reports have suggested that the domains/residues involved in G protein activation and receptor internalization processes are multi-site in nature and that some of these regions may either be similar or overlapping. In the muscarinic receptor, a conserved leucine residue in the second intracellular loop has been implicated in both signal transduction and receptor sequestration. This hydrophobic amino acid is also conserved (as Leu147) in the GnRH receptor and is present at an equivalent position in the 2i loop. In studies on the structure-function relationships of the GnRH receptors, we examined the roles of the conserved Leu147 residue and the unique Ser140 residue, which is Tyr or Trp in most other GPCRs, in agonist-induced signal transduction and receptor internalization. To this end, these residues were replaced with other amino acids by site-directed mutagenesis, and the expressed receptors were analyzed for ligand binding, GnRH-induced inositol phosphate formation and agonist-induced internalization. The results show that replacement of Leu147 with either Asp or Ala impairs both G protein coupling and receptor internalization. However, replacement of Ser140 by Tyr did not affect the signaling response but increased ligand binding and receptor internalization.
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1. Introduction

The GnRH receptor is a G protein-coupled receptor (GPCR) that plays a crucial role in the regulation of reproductive functions. Its structure and function have been extensively studied, and recent advancements have shed light on the mechanisms underlying its activity. In this chapter, we will discuss the structure and function of the GnRH receptor, focusing on its putative structure and the functional consequences of specific mutations.

2. Experimental Procedures

Recombinant DNA Procedures—Unless otherwise specified, standard molecular biological protocols described by Sambrook et al. (21) were used.

Construction of Vectors Expressing Wild-type and Mutant GnRH Receptors—The 1220 base pairs of the cDNA encoding the GnRH receptor, previously cloned into pcDNA8/BST at Not I and Not I restriction sites, were digested with Xba I, and the gel-purified Xba I-Xba I fragment was subcloned into pcDNA8/Amp at the Xba I site. Site-directed mutations were constructed according to the method of Kunkel et al. (22).

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Receptor Expression—Wild-type and mutant GnRH receptors were transiently expressed in COS-7 cells. 5 × 10⁴ cells/well were seeded in 24-well plates (Costar) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units/ml of penicillin and 100 μg/ml streptomycin (Pen-Strep) at 37°C and 5% CO₂. At 60–70% confluency, the cells were transfected with 1 μg of wild-type or mutant plasmid DNA/well using 0.5 ml of serum-free OPTI-MEM medium and 6–8 μg of the polycomplexation lipid, lipofectamine. 6 h later, the medium was replaced with fresh Dulbecco's modified Eagle's medium, and the cells were allowed to grow for 48 h before ligand binding and functional assays were done in parallel.

Receptor Binding and Internalization Assays—48 h post-transfection, cells were washed once with the binding medium (M199 containing 25 mM HEPES and 0.1% bovine serum albumin) before the addition of 125I-labeled GnRH agonist at a final concentration of 2 nM. Nonspecific binding was determined under the same conditions using 1000-fold excess of unlabeled GnRH agonist. Cells were allowed to bind the radioligand for time intervals ranging from 2 min to 1 h. The appropriate times, cells were transferred onto ice and rapidly washed twice with ice-cold phosphate-buffered saline (pH 7.4). Total binding was determined by γ-spectrometry after solubilizing the cells in 0.2 mM NaOH and 1% SDS (NaOH/SDS).

For assays of GnRH-induced internalization, cells were allowed to bind the radioligand for appropriate times, followed by two rapid washes with ice-cold phosphate-buffered saline. The extracellular receptor-associated ligand was then removed by washing once with 1 ml of acid solution (50 mM acetic acid and 150 mM NaCl (pH 2.8)) for 12 min. The acid wash was collected to determine the surface-bound radioactivity, and the internalized radioactivity was quantitated after solubilizing the cells in NaOH (pH 12.8). The radioactivity is expressed as a percent of the total binding at that time point. For displacement of 125I-labeled GnRH agonist binding with native GnRH and GnRH agonist or antagonist analogs, cells were incubated with radioligand in the absence or presence of increasing doses of displacing ligands for 1 h at 37°C. IC₅₀ values for the three ligands were estimated using the FLEXIt program (23). All time points were performed in triplicate, in at least three separate experiments. Binding parameters were determined by the LIGAND program (24).

Binding to COS-7 Cell Membranes—For these experiments, cells were plated and transfected in 100-mm Petri dishes. 48 h post-transfection, the cells were washed twice with ice-cold 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and scraped into 1 ml of the same solution. Cells were lysed by 15–20 cycles of freezing and thawing, and crude membranes were prepared by centrifuging the samples at 16,000 × g. The pellet was washed in the same medium, and the protein content was measured by the BCA method of Pierce. Radioligand binding assays were performed in 0.25 ml of binding buffer (20 mM HEPES, 2 mM EGTA, 5 mM MgCl₂, and 0.1% bovine serum albumin (pH 7.5)) containing 125I-labeled GnRH-Ag at a final concentration of 1–5 nM. 25–20 μg of membrane preparation, and the indicated concentrations of GTPγS. Since preliminary experiments indicated that the effect of GTPγS is temperature dependent and is more evident at 37°C than at 25°C, all experiments were conducted at 37°C for 30 min. After incubation, binding was terminated by rapid filtration and washing three times with ice-cold phosphate-buffered saline, and the bound radioactivity was measured by γ-spectrometry.

Insoluble Phosphate Production—24 h after transfection, COS-7 cells were labeled with [3H]inositol for 24 h in inositol-free DMEM medium containing 20 μCi/ml [3H]inositol, 2.5% fetal calf serum, and 1% Pen-Strep. After one wash with inositol-free M199, the cells were reincubated in 4 ml medium containing 30 μM LICI for 30 min and were then stimulated with 50 nM GnRH for 15 min. The reactions were terminated by the addition of ice-cold perchloric acid to a final concentration of 5%. The insoluble phosphates were extracted and separated by HPLC using a SynChropak Q100 SAX column (Thomson Instrument Co., Springfield, VA) run with a gradient of ammonium phosphate (pH 3.4) as described previously (25, 26). All incubations were performed in duplicate, and the mean insolubles ranging from 2 min to 1 h. The appropriate mutant receptors was expressed as a percentage of that measured in cells expressing wild-type receptors in the same experiment.
RESULTS

Characteristics of GnRH Binding, Internalization, and Signal Generation of GnRH Receptors Expressed in COS-7 Cells—

The functional properties of the wild-type and mutant GnRH receptors expressed transiently in COS-7 cells were analyzed by measurement of agonist-induced internalization and inositol phosphate formation. COS-7 cells expressing wild-type receptors specifically bound $^{125}$I-GnRH-Ag in a time- and temperature-dependent manner. At room temperature (24 °C) and 37 °C, radioligand binding was rapid and reached a steady state level within 30 to 45 min (see Fig. 2, panels A and B). Although the total binding achieved at 24 °C was higher than seen at 37 °C, the acid-resistant radioactivity, which represents the radiolabel bound to internalized receptors, increased relatively slowly. At 37 °C, approximately 20% of the bound tracer was internalized after 30 min, and this increased to 26% by 60 min (panel B), whereas at 24 °C, these values were 11 and 15%, respectively (panel A). All subsequent internalization studies were performed at 37 °C.

The signal transduction efficiency of the wild-type and mutant GnRH receptors was determined by assaying GnRH-induced inositol phosphate production in receptor-transfected COS-7 cells. The inositol phosphate responses of cells expressing wild-type receptors to a maximally effective concentration of GnRH are shown in Fig. 3. The metabolic products of inositol 1,4,5-trisphosphate (Ins(1,3,4)P$_3$, InsP$_2$, and InsP$_3$) were increased severalfold during the 15-min period of stimulation (see panel A for unstimulated cells and panel B for stimulated cells). Under the experimental conditions used here, i.e. in the presence of 10 mM LiCl, InsP$_2$ was the major accumulated product and showed a 19 ± 5-fold increase after 15 min of stimulation, and InsP$_3$ showed a 7 ± 2-fold increase over the basal level ($n = 3$). The basal levels of inositol monophosphates were higher and showed relatively smaller (2.4-fold) increases than InsP$_2$ and InsP$_3$ during agonist stimulation.

Binding Parameters of Wild-type and Mutant GnRH Receptors Expressed in COS-7 Cells—When transfected into COS-7 cells and screened by $^{125}$I-GnRH-Ag binding, the mutant receptors (S140Y, L147A, and L147D) were expressed at almost the same level as the wild-type receptor. As shown in Table I, transient expression yields were 675–925 fmol/mg protein for the wild-type or mutant receptors. Scatchard analysis of the binding data yielded linear plots, reflecting a single class of high-affinity GnRH-binding sites, with similar dissociation constants for the wild-type and Leu$^{147}$ mutant receptors. However, the S140Y mutant had substantially increased binding affinity, with 2-fold higher $K_d$ than the wild-type receptor. This was reflected in its 2.5–3-fold higher specific binding than the wild-type receptor, with no effect on its expression levels.

The ability of guanine nucleotides to cause a significant decrease in agonist binding affinity is a characteristic feature of most of the G protein-coupled receptors. GnRH agonist binding to wild-type and S140Y mutant receptors was analyzed in the presence of the GTP analog GTP-S. As expected, agonist binding to the wild-type GnRH receptors was inhibited by GTP-S in a dose-dependent manner, with half-maximal displacement occurring in a concentration range of 1–10 nM and...
maximum reduction of about 55% at 100-1000 nM (see Fig. 4). Scatchard analysis showed that the reduction in binding was a consequence of decreased receptor affinity rather than a change in receptor number. GnRH-Ag binding to S140Y receptors was also reduced in the presence of GTPγS, and the inhibitory effect was essentially the same as for the wild-type receptor (Fig. 4). This finding suggests that the higher binding affinity of this mutant, reflected by its increased binding of the agonist ligand, is independent of its interaction with G proteins.

The ability of GnRH receptor ligands to compete for the binding of 125I-GnRH-Ag to COS-7 cells expressing the wild-type and mutant receptors was also determined. Radioligand binding to the wild-type receptor was competitively displaced in a dose-dependent manner by the unlabeled agonist (IC50 = 1.5 nM) as well as by native GnRH (IC50 = 23 nM) and an antagonist (IC50 = 4.0 nM). Essentially similar patterns of inhibition and IC50 values were obtained for the mutant receptors (not shown).

Effect of 2i Loop Mutations on GnRH Receptor Internalization—As demonstrated above, the Leu147 mutants showed no change in the specific binding of 125I-GnRH-Ag, whereas the S140Y mutant showed significantly higher agonist binding. To evaluate the effects of these substitutions on the internalization kinetics of the mutant receptors, the uptake of labeled GnRH-Ag bound to cells expressing wild-type or mutant receptors was measured over 60 min at 37°C. A direct comparison between the wild-type and mutant receptors, made by plotting the percent of bound tracer internalized with increasing time of incubation, showed that cells expressing mutant receptors internalized the labeled agonist at markedly different rates (Fig. 5, panel A). The Leu147 to Ala or Asp mutants showed much slower internalization, and the amount of tracer sequestered after 60 min was only 54% of that of the wild-type receptor (Fig. 5, panel B). On the other hand, the S140Y mutant showed accelerated internalization kinetics, and the sequestration of tracer was 63% higher than that of the wild-type receptor (Fig. 5, panel B).

Effect of 2i Loop Mutations on GnRH Receptor Signal Transduction—The GnRH-induced InsP3 responses mediated by each of the mutant receptors were qualitatively similar to those of the wild-type receptor (see Fig. 6, panels A and B). Therefore, combined InsP2 and InsP3 responses were used to compare the InsP responses as percentages of those for the wild-type receptor. The InsP2/InsP3 responses mediated by the S140Y' receptor were similar to those of the wild-type receptor. However, the replacement of Leu147 by Ala or Asp reduced the InsP2/InsP3 responses to 46 and 23% of those for the wild-type receptor, respectively (Fig. 6, panel C). Since the levels of receptor expression of these mutants were similar to those of the wild-type receptor, these differences must reflect reductions in the abilities of the mutant receptors to induce inositol phosphate production.

**DISCUSSION**

The functional roles of the unique Ser140 and the conserved Leu147 residues in the second intracellular loop of the GnRH receptor were analyzed in mutant receptors in terms of their involvement in agonist-induced receptor internalization and signal transduction. Our findings indicate that both residues influence internalization of the GnRH receptor, but do so in different ways. Thus, the S140Y mutation increased ligand-induced internalization of the receptor by 60%, while substitu-
critical for G protein coupling of the GnRH receptor and subsequent phospholipase C activation. However, the Ser140 to Tyr mutation reduced the $K_d$ by 2-fold, a change that was reflected by the increase in its radioligand binding (Table I). The ability of a single residue mutation in intracellular loops remote from the binding pockets to increase ligand binding affinity has also been observed in studies on the $\alpha_2$-adrenergic receptor (28).

In the present study, the effects of Leu147 mutations on agonist-induced internalization and G protein coupling of a peptide hormone receptor were found to resemble those reported for the muscarinic acetylcholine receptor (19, 20). A comparison of the amino acid sequence in the second intracellular loop of members of the GPCR superfamily indicates that all receptors have a conserved Leu or another hydrophobic residue at this position, even among distantly related members of the G protein-coupled receptor superfamily. In the angiotensin II receptor, replacement of the conserved Tyr residue in the DRY sequence by GGA indicated that these residues are critical for G protein coupling of the angiotensin II receptor (28). Studies with synthetic peptides (30, 31) and receptor antibodies (32), as well as mutagenesis studies on the muscarinic acetylcholine receptor (19, 20), have shown that the NH$_2$- and COOH-terminal regions of the 3i loop, and often the amino-terminal region of the cytoplasmic tail, are important for coupling to G proteins (33-35). In the TSH receptor, the 1i loop and the carboxyl-terminal regions of the 2i and 3i loops are involved in signal transduction (36), suggesting that the presence and location of regions involved in G protein coupling may vary among individual receptors. Such regions in the primary structure of GPCRs are usually non-contiguous and thus are multisite. In the absence of a cytoplasmic tail, specific regions of the intracellular loops of the GnRH receptor must be responsible for its interaction with G proteins. Further mutagenesis studies combined with the application of physical methods should aid in determining the regions/residues that are directly involved, and those which act indirectly by stabilizing a specific receptor conformation. The present results using mutagenesis clearly demonstrate the importance of the conserved Leu in signal transduction by the GnRH receptor.

In previous studies, the highly conserved "DRY" sequence in the amino-terminal region of the 2i loop has been shown to be involved in signal transduction. Single residue mutations of the highly conserved Asp in this sequence of $\beta_2$-adrenergic, muscarinic, and rhodopsin receptors have shown that the NH$_2$- and COOH-terminal regions of the 3i loop, and often the amino-terminal region of the cytoplasmic tail, are important for coupling to G proteins (33-35). In the TSH receptor, the 1i loop and the carboxyl-terminal regions of the 2i and 3i loops are involved in signal transduction (36), suggesting that the presence and location of regions involved in G protein coupling may vary among individual receptors. Such regions in the primary structure of GPCRs are usually non-contiguous and thus are multisite. In the absence of a cytoplasmic tail, specific regions of the intracellular loops of the GnRH receptor must be responsible for its interaction with G proteins. Further mutagenesis studies combined with the application of physical methods should aid in determining the regions/residues that are directly involved, and those which act indirectly by stabilizing a specific receptor conformation. The present results using mutagenesis clearly demonstrate the importance of the conserved Leu in signal transduction by the GnRH receptor.

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dues are important for G protein coupling (42). However, mutation of Ser140 to Tyr (converting the unique DRS sequence in the GnRH receptor to the conserved DRY sequence) indicates that this residue is not critical for efficient signal transduction. These results are consistent with a preliminary report (43) that replacement of Ser by Ala at position 140 of the GnRH receptor did not affect inositol phosphate production. Our studies also demonstrated that receptors bearing a point mutation of Ser140 to Tyr were more rapidly internalized than the native receptors, in the absence of a change in second messenger production. Interestingly, the internalization kinetics of the Ser140 to Ala mutant receptor were similar to those of the wild-type receptor, with sequestration of approximately 27% of the bound ligand over a period of 1 h (data not shown). These findings suggest that the polar group of the Ser residue is not an important factor in controlling GnRH receptor internalization. They also demonstrate the ability of an aromatic amino acid such as Tyr, located within a motif that is highly conserved even among distantly related GPCRs, to promote receptor internalization. However, the possibility that the unique Ser140 of the GnRH receptor may be involved in receptor regulation or desensitization awaits future studies.

The impaired ability of the L147D and L147A mutants to mediate inositol phosphate production and to undergo agonist-induced internalization might appear to support the idea that internalization of the hormone-receptor complex is causally related to receptor activation and signal transduction. However, recent studies on angiotensin II (44) and insulin-like growth factor-I (45) receptors, involving mutational studies in regions in the COOH-terminal tail with multiple Ser and Thr residues that are potential phosphorylation sites are critical for the internalization of these receptors, with sequestration of approximately 27% of the bound ligand over a period of 1 h, compared to 26% of the radioligand bound to the GnRH receptor at this time (Figs. 2 and 5). Furthermore, the GnRH receptor does not possess multiple Ser and Thr residues in any of its intracellular loops, suggesting that other regional signal motifs are involved in this process. In this regard it is noteworthy that sequences resembling the conserved NPXXY motif that is present in the seventh transmembrane domains of most GPCRs tend to form tight β-turns and have been implicated as recognition signals for high efficiency ligand-induced receptor internalization (51). For example, Tyr in the NPXXY motif was shown to be important for internalization of the β2-adrenergic receptor (52). However, this sequence was not found to be involved in the internalization of the receptors for gastrin-releasing peptide (53) or angiotensin II (54). The present data suggest that Leu147 is required for normal internalization of the GnRH receptor. However, it appears that additional receptor subdomains are required to regulate the endocytosis mechanism. The GnRH receptor deviates from the NPXXY sequence in that it has a DPXXY sequence in the equivalent position. The role of this sequence in receptor internalization remains to be evaluated.

Our observations on the effects of the Ser140 to Tyr mutation on receptor internalization are relevant to the reported importance of aromatic amino acids in maintaining efficient endocytosis. McGraw et al. (55) showed that substitution of a Tyr for a Ser within the cytoplasmic domain of the transferrin receptor can reconstitute internalization activity in an internalization-defective mutant. Also, it was shown that a single amino acid change in the cytoplasmic domain of the influenza virus hemagglutinin, substituting Tyr for Cys at position 543, caused its more rapid internalization than the wild-type hemagglutinin, while replacement with Ser or Phe had no such effect (56). It is possible that Tyr may be a part of a recognition feature comprised of two to four residues, as is the case for the motifs recognized by the oligosaccharyltransferase responsible for glycosylation of asparagines, or by individual protein kinases including CAMP-dependent protein kinase, Ser/Thr kinase, and Tyr kinase. In addition to such motifs, there may be topological/conformational requirements conferred by interactions with cytoplasmic loops and/or other proteins.

In conclusion, we have analyzed the functional significance of two residues, a unique Ser140 and a conserved Leu147, in the second intracellular loop of the GnRH receptor. Leu147 appears to play an important role in receptor-G protein coupling and in agonist-induced internalization of the receptor. In contrast, Ser140 is not required for G protein coupling and stimulation of inositol phosphate production, but influences the binding affinity and internalization of the receptor.

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Effects of Second Intracellular Loop Mutations on Signal Transduction and Internalization of the Gonadotropin-releasing Hormone Receptor

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