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Combined Modification of Intracellular and Extracellular Loci on Human Gonadotropin-Releasing Hormone Receptor Provides a Mechanism for Enhanced Expression

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The mammalian gonadotropin-releasing hormone (GnRH) receptor (GnRH-R) has been a therapeutic target for human and animal medicine. This receptor is a unique G-protein-coupled receptor that lacks the intracellular C-terminal domain commonly associated with this family. Development of highthrough put screens for agents active in humans has been hampered by low expression levels of the hGnRH-R in cellular models. Two sites have attracted the interest of laboratories studying regulation of expression. The chimeric addition of the C-terminal tail from catfish GnRH-R (cfGnRH-R) to the rat GnRH-R significantly augmented receptor expression in GH_3 cells. In addition, rodent GnRH-R contains 327 amino acids, but cow, sheep, and human GnRH-R (hGnRH-R) contain 328 residues, the "additional" residue being a Lys 191. Deletion of Lys 191 (del 191) from the hGnRH-R resulted in increased receptor expression levels and decreased internalization rates in both COS-7 and HEK 293 cells. In this study, the combined effect of the addition of the C-tail from cfGnRH-R and deletion of the Lys 191 from the hGnRH-R was compared to expression of the wild-type (WT) or either alteration alone in a transient expression system using primate cells. The altered receptor (hGnRH-R[del 191]-C-tail) showed significantly increased receptor expression at the cell surface compared with the WT or either modification alone. The inositol phosphate response to stimulation was also significantly elevated in response to GnRH agonist. After treatment with a GnRH agonist, the altered receptors showed a slower internalization rate. The homologous steady-state regulation of the WT and the altered receptors was similar, although the response of the altered receptors was significantly decreased. These results suggest that the conformational change in the receptor as a result of the deletion of Lys 191 and the addition of the C-terminus tail substantially increased the steady-state receptor expression and decreased internalization and homologous regulation. Because the effects on expression are greater than additive, it appears that these alterations exert their effects by differing means. These techniques for expression of the hGnRH-R in transfected mammalian cells provide the basis for a therapeutic screen for GnRH analogs, agonists, and antagonists of the hGnRH.

Key Words: Gonadotropin-releasing hormone receptor; chimeric receptor; enhanced receptor expression.

Introduction

Gonadotropin-releasing hormone (GnRH) is synthesized in the hypothalamus and stimulates the synthesis and release of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), acting through a specific receptor (GnRH-R) on the plasma membrane. This receptor has been a significant target for theraputic intervention in several human and animal diseases *(1)*. Mammalian GnRH-Rs exhibit more than 85% amino acid identity among seven species that have been cloned *(2,3)*. Sequence analysis of these receptors is consistent with the seven-transmembrane domain motif, characteristic of the G-protein-coupled receptor (GPCR) family. GnRH-R regulates levels of multiple messenger molecules in the gonadotrope, an observation that may explain how the activation of the GnRH-R by the releasing hormone regulates multiple cellular events in a coordinate fashion *(4)*. Unlike other GPCRs, the mammalian GnRH-R has several altered features, including the reciprocal exchange of the conserved Asp and Asn residues in transmembrane domains II and VII, and replacement of Tyr with Ser in the Asp-Arg-Tyr motif located in the junction of transmembrane domain III and the second intracellular loop *(2,5)*. In addition, a striking feature of the mammalian GnRH-R is the absence of the

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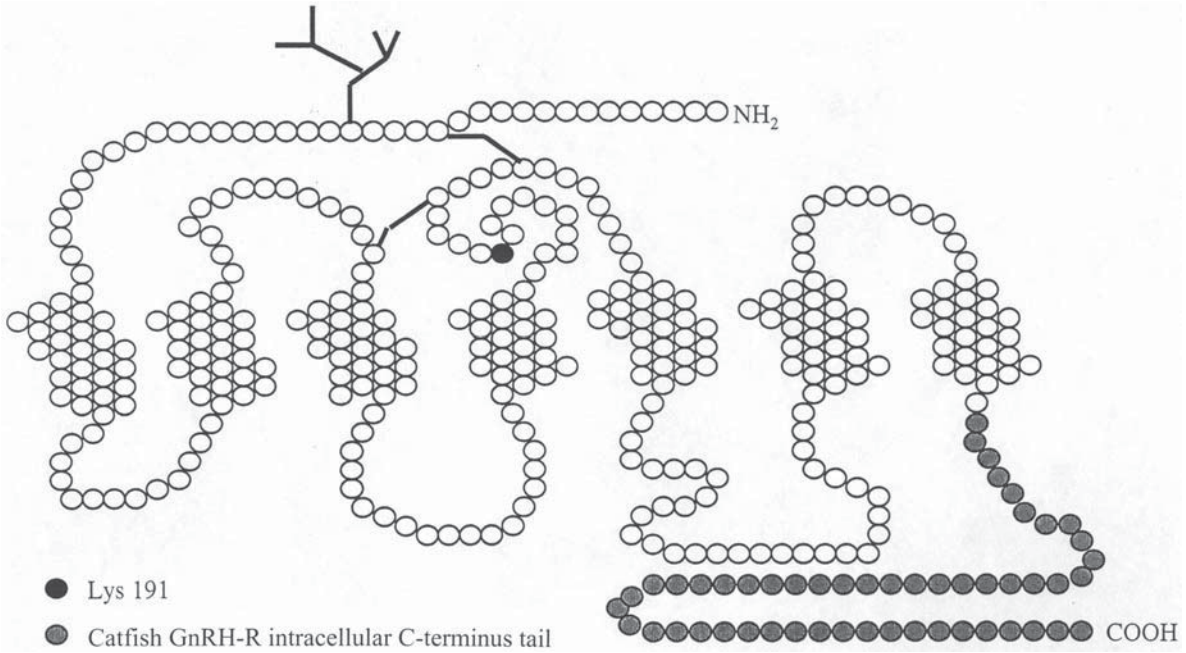

Fig. 1. Schematic representation of the hGnRH-R. Open circles represent the putative structure of the hGnRH-R, and gray circles represent the 51 amino acids, corresponding to the intracellular C-terminus tail from cfGnRH-R. The black circle represents the Lys 191. The deletion of Lys 191 and the addition of the C-tail were prepared by overlap extension PCR (*see* Materials and Methods).

intracellular carboxyl-terminal tail characteristic of other members of the GPCR family, making the GnRH-R one of the smallest receptors with the seven-transmembrane segment motif. Interestingly, however, piscine and avian GnRH-Rs contain such an intracellular C-terminus *(2)*.

The function of the intracellular C-terminal tail appears to differ among receptors; in catfish, the GnRH-R C-terminal tail is responsible for rapid desensitization and enhanced internalization kinetics *(6)*. In chicken, truncation of the cytoplasmic tail results in a decrease in the internalization rate to a level equivalent to the human GnRH-R (hGnRH-R) *(7)*. In addition, the chimeric addition of the C-terminal tail from catfish GnRH-R (cfGnRH-R) to the rat GnRH-R significantly augmented receptor expression at the cell surface in GH_3 *(8)*.

The rat and mouse GnRH-R contain 327 amino acids; however, cow, sheep, and human GnRH-R contain 328 residues. Using consensus numbering, an «additional» amino acid (Lys 191) is present in the second extracellular loop domain *(2)*. Deletion of Lys 191 from the hGnRH-R resulted in increased receptor expression levels in both COS-1 and HEK 293 cells. However, the major functional difference between the hGnRH-R wild-type (WT) and the deletion of Lys 191 was the internalization kinetics. The rate of internalization of the deletion of Lys 191 human receptor was significantly reduced *(9)*.

In this study, we assessed the results of simultaneous addition of the C-terminal tail from cfGnRH-R and deletion of Lys 191 on the level of expression and releasing hormone regulation of hGnRH-R, transiently expressed in (primate) COS-7 cells.

Results

Expression of WT and Altered hGnRH Receptors in COS-7 Cells

Deletion of Lys 191 (del 191) and the addition of cfGnRH-R intracellular C-terminus to hGnRH-R (Fig. 1) were achieved by overlap extension polymerase chain reaction (PCR) *(10)*. The chimeric hGnRH-R-C-tail construct comprises the 328 amino acids from WT hGnRH-R and 51 amino acids from the C-terminus of cfGnRH-R (Fig. 1).

WT hGnRH-R, hGnRH-R(del 191), hGnRH-R-C-tail, and hGnRH-R(del 191)-C-tail were transiently transfected in COS-7 cells. To compare their expression and binding characteristics, receptor binding assays were performed using a metabolically stable agonist of GnRH ([^{125}I]Buserelin). As shown in Fig. 2, cells expressing hGnRH-R(del 191), hGnRH-R-C-tail, or hGnRH-R(del 191)-C-tail bound more than 7-, 5-, and 20-fold as much [^{125}I]Buserelin, respectively, than the WT receptor. Scatchard analysis of the binding data (Fig. 3) showed that the dissociation constant (K_d) for hGnRH-R(del 191), hGnRH-R-C-tail, and hGnRH-R(del 191)-C-tail were 21, 34, and 15 n*M*, respectively (Table 1). The WT hGnRH-R is expressed at very low levels and the K_d is not measurable. These results indicate that either the deletion of Lys 191 or the addition of the intracellular carboxyl-tail to the hGnRH-R significantly increases the expression level of the hGnRH-R construct. In combination, these changes have a greater than additive effect on the expression level of the hGnRH-R.

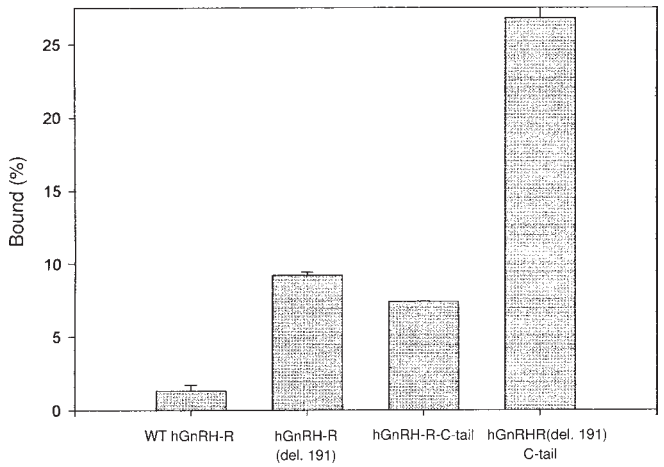

Fig. 2. Binding of [^{125}I]Buserelin to WT and altered hGnRH-R. The effects of either deletion of Lys 191 or addition of an intracellular C-terminus tail or both modifications are shown. COS-7 cells were transiently transfected with WT receptor, hGnRH-R(del 191), hGnRH-R-C-tail, and hGnRH-R(del 191)-C-tail. A total of 100 μL of the cell suspension (10^6 cells) was added to each tube, and the assay was allowed to reach equilibrium (3 h) at 4°C at a final volume of 150 μL. Error bars show the SEM. Each experiment was repeated at least three times with similar results.

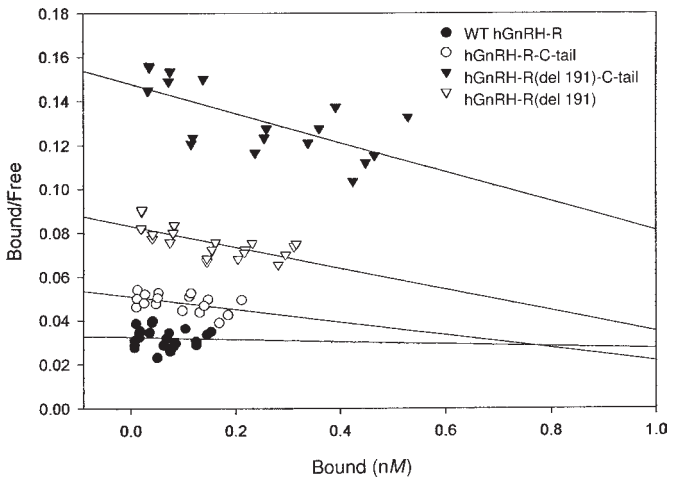

Fig. 3. Scatchard plots for binding of [^{125}I]Buserelin to WT and altered human GnRH receptors. Seventy-two hours after transfection of COS-7 cells, the cell suspension (10^6 cells) was incubated with increasing concentrations of [^{125}I]Buserelin, as indicated for 3 h at 4°C (*see* Materials and Methods).

Deletion of Lys 191 and Addition of an Intracellular C-Terminus Increase Accumulation of hGnRH-R-Mediated Inositol Phosphate

After plates were washed twice with Dulbecco's modified Eagle's medium (DMEM)/0.1% bovine serum albumin (BSA)/20 μg/mL of gentamicin to remove serum and unattached cells, cellular inositol phospholipids were labeled in DMEM (inositol free) supplemented with [^{3}H]myo-inositol (4 μCi/mL) for 18 h. Figure 4 presents the dose response of Buserelin-stimulated inositol phosphate (IP) production. Two hours of stimulation with Buserelin resulted in dose-dependent IP production from COS-7 cells expressing the WT hGnRH-R, hGnRH-R(del 191), hGnRH-R-C-tail, or hGnRH-R(del 191)-C-tail. The response of IP production from COS-7 cells expressing the hGnRH-R(del 191), hGnRH-R-C-tail, or hGnRH-R(del 191)-C-tail was higher than observed for cells expressing the WT receptor.

Comparison of Internalization Rates Among WT and Altered hGnRH Receptors

The effect of these modifications on ligand-induced internalization was evaluated by measuring the kinetics of [^{125}I]Buserelin (0.25 μCi/mL) uptake over a period of 60 min. To compare the rate of internalization of WT hGnRH-R and the other constructs, an acid wash protocol was used *(6)*. Internalization rate data are given in Fig. 5 (top). Cells expressing hGnRH(del 191), hGnRH-R-C-tail, and hGnRH-R(del 191)-C-tail internalized [^{125}I]Buserelin at slower rates. When hGnRH-R(del 191) was combined with the addition of the intracellular C-terminal tail, the rate of internalization was slower than in the cells expressing either the WT or any other altered receptors (Fig. 5, bottom).

Pattern of Homologous Regulation Among WT and Altered hGnRH Receptors

Consistent with results of the Scatchard analysis binding study (Fig. 3), the number of binding sites of hGnRH-R(del 191)-C-tail was higher than that of WT hGnRH-R (Fig. 6, top). Receptor numbers in the WT and hGnRH-R(del 191), hGnRH-R-C-tail, or hGnRH-R(del 191)-C-tail were initially downregulated in response to 10 μM Buserelin for 2 h at or to 65, 39, 34, or 43%, respectively (Fig. 6, bottom), compared with control cells at time zero. The WT and altered receptor levels recovered thereafter (2–5 h) but did not exceed the control value, as is the case for the other nonprimate, mammalian GnRH-R. To distinguish whether or not homologous regulation of the receptor is dependent on protein or RNA synthesis, cells were incubated for 0, 1, 2, and 5 h with 1 μg/mL of either actinomycin D or cycloheximide plus Buserelin. These experiments showed that homologous regulation of the receptor is not modified by actinomycin D or cycloheximide treatment (data not shown).

Discussion

This study shows, for the first time, that the combined deletion of Lys 191 and addition of the cfGnRH-R intracellular carboxyl-terminal tail to the hGnRH-R significantly increases human receptor expression at the cell surface compared to WT or either modification alone. The IP response to stimulation with a GnRH agonist was significantly elevated for either hGnRH-R(del 191) or hGnRH-R-C-tail, and was higher still in cells expressing hGnRH-R(del 191)-C-tail; such results are consistent with the binding results. After treatment with a GnRH agonist,

Table 1
Binding Characteristics of WT and Altered hGnRH Receptors[a]

	K_d (n*M*)	B_{max} (n*M*)	Sites/cell
WT hGnRH-R	Undetectable	Undetectable	Undetectable
hGnRH-R(del 191)	21 ± 1	1.72 ± 0.08	103,597 ± 4818
hGnRH-R-C-tail	34 ± 2	1.73 ± 0.1	104,198 ± 6023
hGnRH-R(del 191)-C-tail	15 ± 2	2.2 ± 0.2	134,313 ± 10239

[a]Seventy-two hours after transfection of COS-7 cells, the cell suspension (10^6 cells) was incubated with increasing concentrations of [^{125}I]Buserelin, as indicated for 3 h at 4 C.

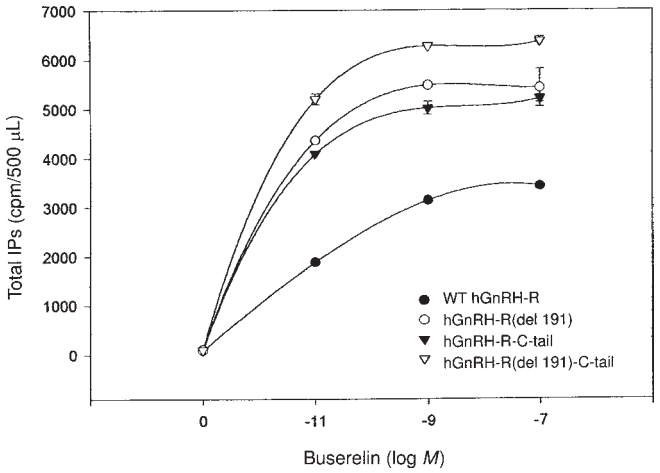

Fig. 4. Buserelin-stimulated IP production in cells expressing the WT and altered human GnRH receptors. Forty-eight hours after transfection of COS-7 cells with WT and altered hGnRH receptors, the cells were preloaded with 4 µCi/mL of [^{3}H]myo-inositol for 18 h. The cells were treated with the indicated concentrations of Buserelin for 2 h. Total IP production was determined by ion-exchange chromatography. Error bars show the SEM. Each experiment was repeated at least three times with similar results.

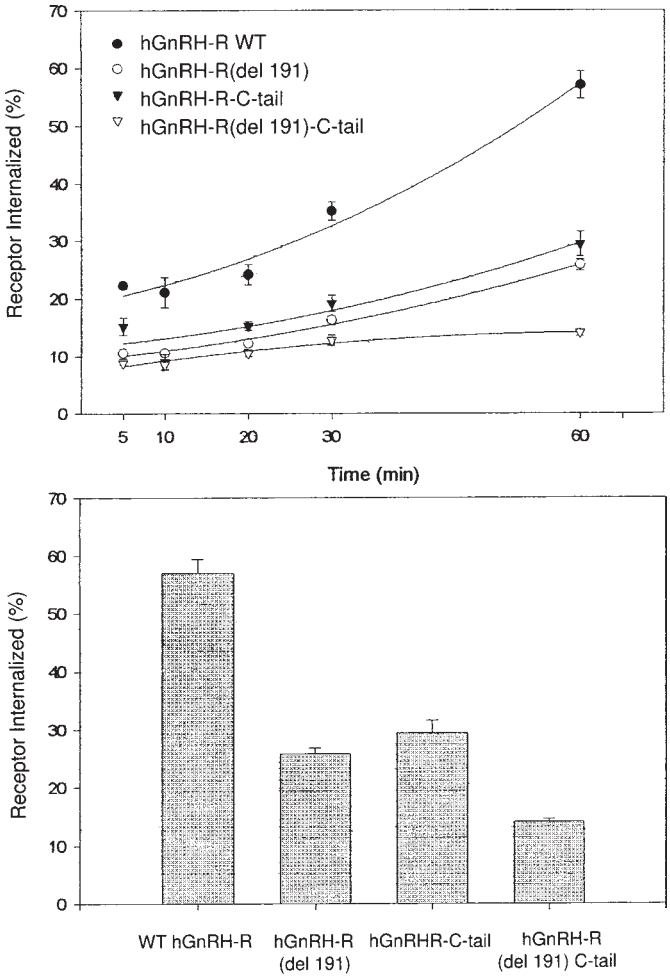

Fig. 5. Internalization of the WT and altered human GnRH receptors. Seventy-two hours after transfection, the cells were incubated with [^{125}I]Buserelin (0.25 µCi/mL) for the indicated times. To determine the surface-bound iodinated ligand, the acid wash was collected and counted (*see* Materials and Methods). The effect of time course internalization (top) and percentage internalization at 60 min (bottom) are shown. Error bars show the SEM. Each experiment was repeated at least three times with similar results.

the receptors were internalized in a time-dependent manner, but hGnRH-R(del 191) and hGnRH-R-C-tail internalized slowly, an effect that was even more pronounced in hGnRH-R(del 191)-C-tail. By contrast, the WT human receptor showed a comparatively rapid internalization rate. All the altered hGnRH-Rs showed a significantly enhanced extent of downregulation compared with the WT receptor. These results suggest that the deletion of Lys 191 and the introduction of cfGnRH-R intracellular C-tail to hGnRH-R have significant effects on receptor expression and regulation. These results also suggest that the combination of these mutations seems to exert greater than additive effects.

The role of the Lys 191 residue in the hGnRH-R is not clear, but substitutions by other residues had no effect on receptor expression or function. In addition, the insertion of a Lys residue at position 191 in the mouse receptor did not have a deleterious effect on its expression *(9)*. Furthermore, mutation of the Phe 272 (to Leu) in the hGnRH-R increased receptor expression and apparently indicates a requirement for an aromatic or large lipophilic residue at this position for WT expression, whereas mutations to residues with acidic, basic, hydrophilic, or small hydrophobic side chains decrease expression *(11)*. In the present study, COS-7 cells

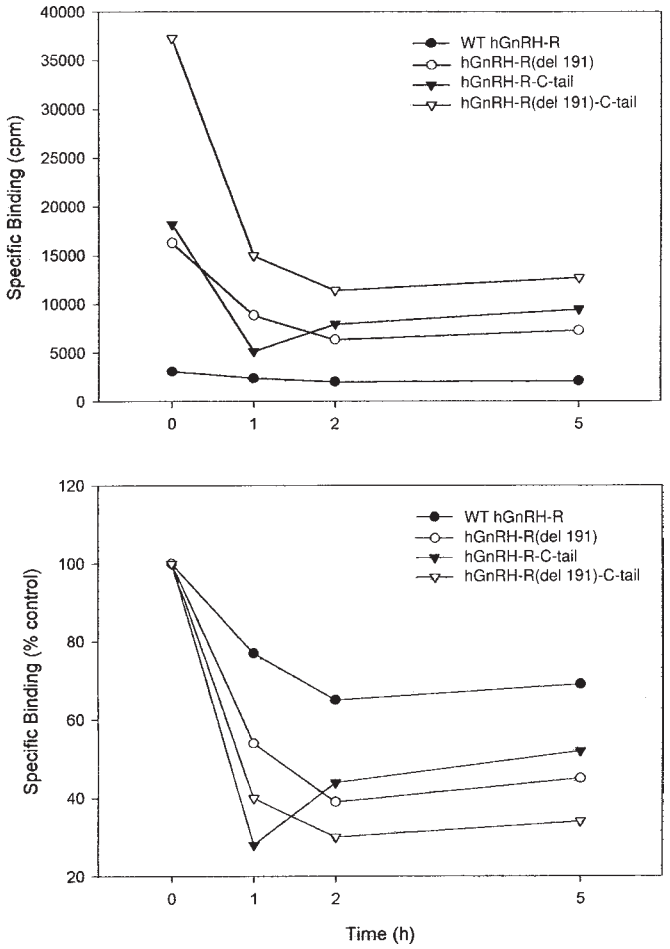

Fig. 6. Homologous regulation of the WT and altered human GnRH receptors. Seventy-two hours after transfection of COS-7 cells, the cells were incubated with 10 n*M* GnRH for the indicated times. The GnRH was removed and the binding [^{125}I]Buserelin was assessed as described in Materials and Methods. Data shown are the mean of triplicate treatments, represented by specific binding in counts per minute (top) and in the percentage of control at time zero incubation (bottom). Each experiment was repeated at least three times with similar results.

transiently transfected with hGnRH-R(del 191) showed a significant increase in receptor expression and a decrease in internalization kinetics; however, the structural basis for this effect is not clear, but it may indicate that deletion of Lys 191 stabilizes the receptor protein.

In most of the GPCRs, the intracellular C-terminal domain contains a Cys that has been shown to be palmitoylated in several receptors *(12–15)*. This domain also contains sites involved in phosphorylation-mediated regulation and desensitization of several GPCRs *(16,17)*. Truncation or mutation of specific C-terminal domains has been reported to diminish agonist-mediated internalization of the angiotensin receptor *(18,19)*, and to diminish desensitization of the LH receptor *(20)* and the α-1β-adrenergic receptor *(21)*. On the other hand, the truncation of the α-adrenergic receptor was reported not to affect sequestration *(22)*, and truncation of the FSH receptor was found not to alter desensitization *(23)*.

The function of the intracellular C-terminal tail appears to be different even among GnRH-Rs. In cfGnRH-R, the C-terminal tail is responsible for enhanced internalization kinetics owing to a C-tail phosphorylation *(24)* and rapid desensitization. The introduction of the cytoplasmic C-terminal tail of the rat thyrotropin-releasing hormone receptor into the C-terminus of the rat GnRH-R increased the internalization rates and demonstrates that the mammalian GnRH-R does not undergo agonist-dependent phosphorylation. This correlates with the inability of the receptor to undergo rapid desensitization, and these features are associated with the lack of a cytoplasmic C-terminal tail *(6,25)*. In chicken GnRH-R, the truncation of the cytoplasmic tail resulted in a decreased internalization rate equivalent to the hGnRH-R *(7)*. In addition, the chimeric addition of the C-terminal tail from cfGnRH-R to the rat GnRH-R significantly augmented receptor expression at the cell surface in GH_3 cells, and its presence altered the pattern of receptor regulation from biphasic (down- and upregulation) to monophasic downregulation alone and significantly enhanced the extent of downregulation *(8)*. Furthermore, the internalization rate of the chimeric rat GnRH-R-C-tail-GFP expressed in GH_3 cells showed that the receptor is recycled to the plasma membrane rather than from newly synthesized receptors *(26)*. In the present study, the addition of the cfGnRH-R intracellular C-tail resulted in an increase in receptor expression as well as a decrease in the internalization rate, indicating that introduction of the intracellular C-tail into the hGnRH-R has a significant impact on receptor expression and regulation.

Deletion of Lys 191 combined with the addition of cfGnRH-R-C-tail resulted in a significantly enhanced increase in receptor expression compared to either deletion of Lys 191 or addition of the C-tail alone. In addition, internalization kinetics were significantly decreased in the hGnRH-R(del 191)-C-tail construct. Homologous regulation of the WT and the altered receptors was similar; however, in COS-7 cells expressing the hGnRH-R(del 191)-C-tail construct, the effect was significantly decreased. This process appears to be independent of either protein or RNA synthesis, because cycloheximide and actinomycin D did not inhibit the homologous regulation of both WT and altered receptors. This suggests that the addition of the cfGnRH-R C-terminal tail and the deletion of Lys 191 may utilize different mechanisms to evoke more efficient expression.

The present results suggest that the conformational change of the receptor owing to the deletion of Lys 191 and the addition of the C-terminal tail substantially increases receptor expression and decreases internalization rates and homologous regulation. The absence of the C-terminal tail in mammalian GnRH-R and its presence in nonmammalian vertebrate GnRH-R may be associated with altered physiologic regulation seen in mammals and nonmammals *(8)*.

The absence of the cytoplasmic tail as well as the presence of Lys 191 may have been selected to prevent receptor overexpression and rapid internalization of hGnRH-R. We believe that this is the first demonstration of synergistic actions resulting from the modification of intracellular and extracellular loci. Practically, the enhanced expression of the hGnRH-R owing to the deletion of Lys 191 and addition of C-tail in transfected mammalian cells could be of value for developing screens for therapeutic GnRH analogs.

Materials and Methods

Materials

Natural sequence GnRH was provided by the National Pituitary Agency. A GnRH agonist, Buserelin (D-*tert*-butyl-Ser^6-des-Gly^{10}-Pro^9-ethylamide-GnRH), was a kind gift from Hoechst-Roussel Pharmaceuticals (Somerville, NJ). DMEM, OPTI-MEM, lipofectamine, and PCR reagents were purchased from Life Technologies (Grand Island, NY). Restriction enzymes, modified enzymes, and competent cells for subcloning were purchased from Promega (Madison, WI). Other reagents were of the highest degree of purity available from commercial sources.

Vector Construction

WT hGnRH-R cDNA in pcDNA3 was subcloned into pcDNA3.1 at KpnI and XhoI restriction enzymes sites. The deletion of Lys 191 of hGnRH-R was constructed by overlap extension PCR *(10)*, and this construct was used as a template for creating the chimeric receptor. Chimeric receptor (hGnRH-R-C-tail) containing hGnRH-R and the intracellular C-terminus of cfGnRH-R was constructed by overlap extension PCR. The hGnRH-R sequence, including the 5'-untranslated region and complete coding region (without the stop codon), was amplified from the WT hGnRH-R cDNA in pcDNA3.1. The sequence for the intracellular C-terminus of cfGnRH-R was amplified from WT cfGnRH-R cDNA. The result of the two PCR reactions was the amplification of one fragment of the hGnRH-R sequence with a 15-base cfGnRH-R sequence end, and one fragment of cfGnRH-R sequence for the intracellular C-terminus with a 15-base hGnRH-R sequence end, yielding 30 bases of overlap region between two fragments. The two fragments were gel purified and used as templates in a third PCR reaction with only the two outer primers. The junction of chimeric receptor is between the last amino acid (Leu 328) of hGnRH-R and the first residue (Thr 329) of cfGnRH-R intracellular C-terminus.

All cDNAs were digested with KpnI and XhoI. The identity of all constructs and the correctness of all PCR-derived sequences were verified by Dye Terminator Cycle Sequencing according to the manufacturer's instructions (Perkin-Elmer, Foster City, CA).

For transfection, large-scale plasmid DNAs were prepared by double-banded CsCl gradient centrifugation. The purity and identity of plasmid DNAs were further verified by restriction enzyme analysis.

Transient Transfection of COS-7 Cells

WT hGnRH and altered receptors were transiently expressed in COS-7 cells. COS-7 cells were maintained in growth medium DMEM containing 10% fetal calf serum (FCS) (Life Technologies, Grand Island, NY) and 20 μg/mL of gentamicin (Gemini Bioproducts, Calabasas, CA) in a humidified atmosphere (37°C) containing 5% CO_2 cells (10^5 cells/well) were seeded in 24-well plates (Costar, Cambridge, MA). Twenty-four hours after plating, the cells were transfected with 0.8 μg of cDNA/well using 2 μL of lipofectamine in 0.25 mL of OPTI-MEM. Five hours later, 0.25 mL of DMEM containing 20% FCS was added to each well. Twenty-four hours after the start of transfection, the medium was replaced with fresh growth medium, and the cells were allowed to grow for another 24 h before treatment, and IP production was measured. For receptor binding, the same transfection procedure was followed except that 20 μg of plasmid DNA and 50 μL of lipofectamine were used to transfect the cells in 75-cm^2 flasks (Costar) when they were 60–80% confluent.

Measurement of IP Accumulation

For quantification of IP production, 48 h after the start of transfection, COS-7 cells transiently transfected with WT or altered receptor DNAs were washed twice with DMEM/0.1% BSA, and cellular inositol lipids were labeled in DMEM (inositol free) supplemented with 4 μCi/mL of [^{3}H]myo-inositol for 18 h at 37°C. After preloading, the cells were washed twice in DMEM (inositol free) containing 5 m*M* LiCl and stimulated with Buserelin at the indicated doses in 0.5 mL of DMEM/LiCl for 2 h. The treatments were removed and 1 mL of 0.1 *M* formic acid was added to each well. The cells were frozen and thawed to disrupt the cell membranes. IP accumulation was determined by Dowex Anion exchange chromatography and liquid scintillation spectroscopy as previously described *(27)*.

Receptor Binding

Intact cell binding was assessed over a range of concentrations of [^{125}I]Buserelin, prepared as reported previously *(28)*, in DMEM/0.1% BSA. Seventy-two hours after the start of transfection, COS-7 cells were scraped and resuspended in warm DMEM/0.1% BSA. Cells were then pelleted and washed twice with ice-cold DMEM/0.1% BSA. A total of 100 μL of the cell suspension (10^6 cells) was added to each tube, and the assay was allowed to reach equilibrium (3 h) at 4°C at a final volume of 150 μL. Binding was determined by overlayering each sample on 2 mL of DMEM/0.3 *M* sucrose at 4°C and centrifugations at 2000*g* for 10 min at 4°C in a Sorvall SM-24 rotor. The supernate was then aspirated. The cell pellet was resuspended in 1 mL of phosphate-buffered saline (PBS), and

radioactivity was determined using a Packard 10-channel gamma counter (Downers Grove, IL).

For studies of receptor downregulation and recovery, 72 h after transfection, cells in six-well plates were washed twice with warm DMEM/0.1% BSA, then treated with 10 n*M* GnRH or medium alone at 37°C for the indicated times. The cells were washed three times with warm DMEM/0.1% BSA to remove GnRH. The medium was replaced with 2 mL of [^{125}I]Buserelin (0.4 μCi/mL). Binding was assessed after 30 min at room temperature. Nonspecific binding was determined in the presence of 10 μ*M* unlabeled GnRH. Binding was terminated by decanting the radioligand-containing medium and placing the cells on ice. Cells were washed twice with ice-cold DMEM/0.1% BSA. Cells were then collected by scraping in 1 mL of DMEM/0.1% BSA/2.5 m*M* EGTA twice. The cell lysate was layered over 2 mL of 0.3 *M* sucrose in DMEM, and the cell pellet was collected and its radioactivity counted as described above.

For internalization studies, COS-7 cells were transiently transfected using the same method as described above, except that 200,000 cells were plated per well in a 12-well plate. Approximately 72 h after transfection, the cells were washed twice with warm DMEM/0.1% BSA. The cells were incubated with [^{125}I]Buserelin (0.25 μCi/mL) for the indicated times. At the appropriate time, the iodinated ligand was removed, and the plate was placed on ice. The cells were washed twice with ice-cold PBS, and 500 μL of acid solution (50 m*M* acetic acid, 150 m*M* NaCl, pH 2.8) was added to each well and incubated for 12 min on ice. To determine the surface-bound iodinated ligand, the acid wash was collected and counted in a Packard 10-channel gamma counter. To determine the internalized radioligand/receptor complex, cells were solubilized in 500 μL of 0.1% Triton-X 100/PBS, collected, and counted. Nonspecific binding for all time points and cDNAs were determined using the same procedure but in the presence of 10 μ*M* unlabeled agonist. Nonspecific binding was substracted from the surface-bound and internalized radioligand, and the internalized radioligand was expressed as the percentage internalized of the total bound at each time point.

Data Analyses

Data shown are the means of triplicate assay wells and are presented as the mean ± SEM of replicates in each experiment. The SEM was typically <10% of the mean. The data were analyzed by one-way analysis of variance followed by Duncan multiple range test, with $p < 0.05$ considered significant. Each experiment was repeated three or more times.

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