Addition of Catfish Gonadotropin-Releasing Hormone (GnRH) Receptor Intracellular Carboxyl-Terminal Tail to Rat GnRH Receptor Alters Receptor Expression and Regulation

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Mammalian GnRH receptor (GnRHR) is unique among G protein-coupled seven-transmembrane segment receptors due to the absence of an intracellular C-terminal tail frequently important for internalization and/or desensitization of other G protein-coupled receptors. The recent cloning of nonmammalian (*i.e.* catfish, goldfish, frog, and chicken) GnRHRs shows that these contain an intracellular C terminus. Addition of the 51-amino acid intracellular C terminus from catfish GnRHR (cfGnRHR) to rat GnRHR (rGnRHR) did not affect rGnRHR binding affinity but elevated receptor expression by about 5-fold. Truncation of the added C terminus impaired the elevated receptor-binding sites by 3- to 8-fold, depending on the truncation site. In addition, introducing the C terminus to rGnRHR altered the pattern of receptor regulation from biphasic down-regulation and recovery to monophasic down-regulation. The extent of down-regulation was also enhanced. The alteration in receptor regulation due to the addition of a C terminus was reversed by truncation of the added C terminus. Furthermore, addition of the cfGnRHR C terminus to rGnRHR significantly augmented the inositol phospholipid (IP) response of transfected cells to Buserelin, but this did not result from the elevation of receptor-binding sites. Addition of the C terminus did not affect Buserelin-stimulated cAMP and PRL release. GH_3 cells transfected with wild-type cfGnRHR did not show measurable Buserelin binding or significant stimulation of IP, cAMP, or PRL in response to Buserelin (10^{-13}-10^{-9} M). GH_3 cells transfected with C terminus-truncated cfGnRHR showed no IP response to Buserelin (10^{-13}-10^{-7} M). These results suggest that addition of the cfGnRHR intracellular C terminus to rGnRHR has a significant impact on rGnRHR expression and regulation and efficiency of differential receptor coupling to G proteins. (Molecular Endocrinology 12: 161–171, 1998)

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INTRODUCTION

Pituitary gonadotropes respond to GnRH with the synthesis and release of gonadotropins (LH and FSH), development of desensitization, and regulation of GnRH receptors (GnRHR). The first step in GnRH action is its recognition by the specific high-affinity GnRHR at the surface of gonadotrope cells (1). The mammalian GnRHR has been cloned from at least six species (2); the amino acid sequences from these sources are substantially homologous and contain seven putative transmembrane domains and many of the conserved residues and sequences, characteristic of other members of the rhodopsin-like G protein-coupled receptor (GPCR) family (2), consistent with a role for multiple G proteins in GnRH action (3, 4). GnRHR is coupled to $G_{q/11}\alpha$ in αT3–1 gonadotrope cells (5, 6). In GGH_3 cells (GH_3 cells expressing rat GnRHR), GnRHR is coupled to $G_{q/11}\alpha$, resulting in

activation of phospholipase C and inositol phospholipid (IP) turnover (7, 8). In addition, GnRHR appears to be coupled to adenylate cyclase-mediated PRL release through $G_s\alpha$ in GGH_3 cells (9, 10), further emphasizing the promiscuity of GnRHR as a function of the availability of G protein in the microenvironment of the target cells (11). More recent studies using G protein knockout mice and confocal microscopy showed that GnRHR in the primary pituitary cell is coupled to $G_{q/11}\alpha$ (7, 12, 13).

Mammalian GnRHR has several unique features that distinguish it from other GPCRs. Most striking is the absence of the intracellular carboxyl-terminal tail (2, 14). The intracellular C terminus of many GPCRs has been shown to be functionally important for G protein coupling (15–17), agonist-induced receptor internalization (17–25), and/or Ser/Thr phosphorylation-mediated desensitization (17, 26–30). The intracellular C terminus of most GPCRs also contains a highly conserved Cys that may be palmitoylated and form a fourth intracellular loop (31–34). However, the function of the intracellular C terminus appears to be different among GPCRs. For example, in some GPCRs agonist-stimulated Ser/Thr phosphorylation of the C terminus has been implicated in receptor desensitization (26–28), while the C terminus of others is involved in agonist-stimulated internalization, but not in desensitization (21, 25, 35). Truncation of the CCK-A and β-adrenergic receptor did not result in altered internalization (20, 36), and truncation of the LH and FSH receptor did not affect desensitization (37, 38).

Recently, a GnRHR cDNA was cloned from a teleost, the African catfish, with only 38% amino acid sequence identity with mammalian GnRHR (39). Catfish GnRHR (cfGnRHR) expressed in HEK 293 cells was shown to mediate the native cfGnRH-stimulated phosphatidylinositol hydrolysis and production of cAMP (39, 40), suggesting G protein coupling for cfGnRHR similar to that observed in the mammalian GnRHR. Another recent report of cloning of goldfish, frog, and chicken GnRHR cDNAs showed that these nonmammalian GnRHRs have a high overall homology (58–67%) with each other, but only 42–47% homology with mammalian GnRHR (41). The surprising feature of these nonmammalian GnRHRs is that they all contain an intracellular C terminus with phosphorylation consensus sites and Cys residues. The presence of intracellular C terminus in nonmammalian GnRHRs and in other GPCRs raises the question of the evolutionary significance and physiological implication of the absence of the intracellular C-tail in mammalian GnRHR.

To elucidate the structural determinants and structure/function evolution of GnRHR, a chimeric receptor was constructed by addition of cfGnRHR intracellular C terminus to rat GnRHR (rGnRHR). The chimera was truncated in some instances to create mutant receptors containing different lengths of the intracellular C terminus. The wild-type (wt) and mutant receptor cDNAs were transiently expressed in GH_3 cells, and the receptor binding, homologous regulation, and receptor-mediated signal transduction pathways were examined.

RESULTS

Addition of an Intracellular C Terminus Does Not Affect rGnRHR Binding Affinity but Significantly Elevates the Number of rGnRHR Binding Sites

A chimeric receptor (rGnRHR-Ctail, Fig. 1) was constructed by addition of cfGnRHR intracellular C terminus to rGnRHR. The intracellular C terminus of cfGnRHR contains 51 amino acids, including two consensus phosphorylation sites for protein kinase C and two Cys residues (Fig. 1). Thus, the chimeric rGnRHR-Ctail is comprised of the 327 amino acids of wt rGnRHR and 51 amino acid of cfGnRHR, forming a chimera of 378 amino acids with a presumptive intracellular C terminus of 53 amino acids (Fig. 1).

wt rGnRHR, wt cfGnRHR, and chimeric rGnRHR-Ctail were transiently expressed in GH_3 cells. To compare the receptor expression and binding characteristics of wt rGnRHR with cfGnRHR and chimeric rGnRHR-Ctail, receptor binding assays were performed using a metabolically stable agonist of GnRH, [^{125}I]Buserelin. Scatchard analysis of the binding of [^{125}I]Buserelin (Fig. 2) showed that rGnRHR and rGnRHR-Ctail have similar binding affinity for Buserelin, with dissociation constant (K_d) values of 1.52 nM (rGnRHR) and 1.46 nM (rGnRHR-Ctail), while wt cfGnRHR showed no measurable binding of Buserelin. In contrast to the K_d, the number of binding sites of chimeric rGnRHR-Ctail receptor was about 5-fold higher than that of wt rGnRHR, with B_{max} of 17,087 sites per cell

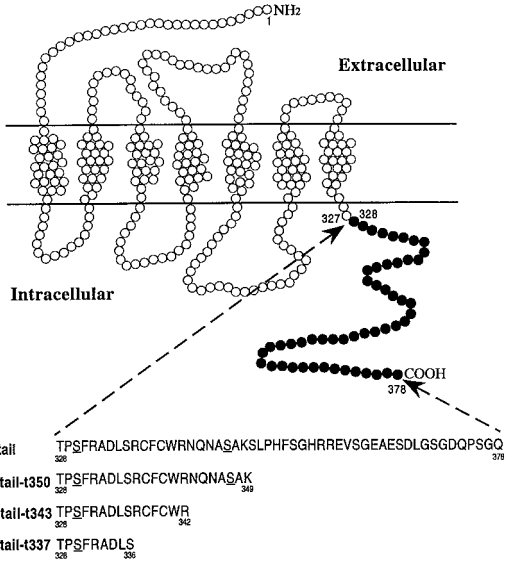

Fig. 1. Schematic Model of the rGnRHR Showing Addition of the Intracellular Carboxyl-Terminal Tail of cfGnRHR

The putative structure of rGnRHR is shown by *open circles*; the portion of intracellular carboxyl-terminal tail of the cfGnRHR is represented by *solid circles*. The amino acid sequences of the cfGnRHR intracellular carboxyl terminus and three truncated C termini are indicated. Two consensus phosphorylation sites for protein kinase C are *underlined*.

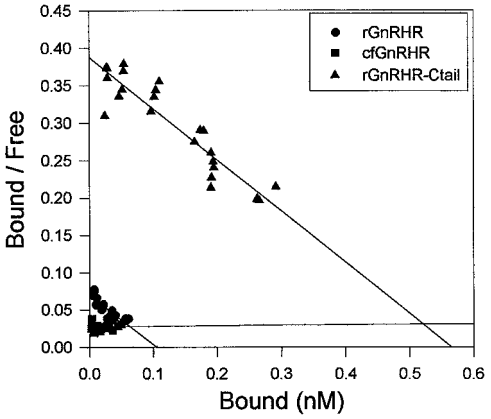

Fig. 2. Scatchard Plots for Binding of [^{125}I]Buserelin to GH_3 Cells Expressed wt rGnRHR, wt cfGnRHR, or Chimeric rGnRHR-Ctail

Seventy two hours after transfection of GH_3 cells, the cell suspension (10^6 cells) was incubated with increasing concentrations of [^{125}I]Buserelin, as indicated, for 3 h at 4 C. Cell-associated specific activity was measured (see *Materials and Methods*).

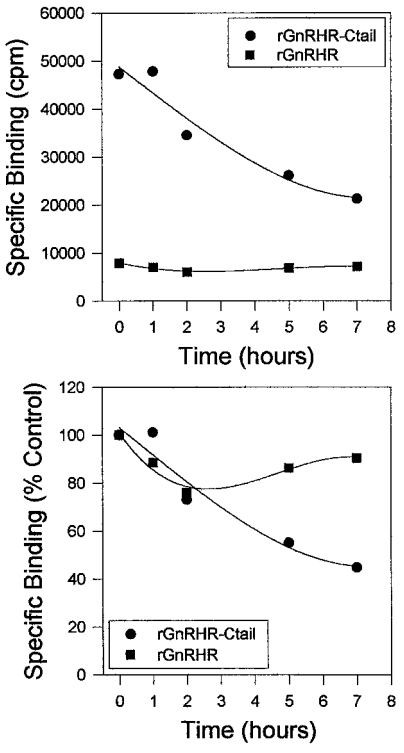

Fig. 3. Homologous Regulation of the GnRHR in GH_3 Cells Expressed wt rGnRHR or Chimeric rGnRHR-Ctail

Seventy two hours after transfection of GH_3 cells, cells were incubated with 10 nM GnRH for the indicated times. The GnRH was removed, and the binding of [^{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 min (*upper panel*) and in the percentage of control at initial incubation time (*lower panel*). Each experiment was repeated at least three times, with similar results.

(assuming similar transfection efficiency) for wt rGnRHR and B_{max} of 92,046 sites per cell (assuming similar transfection efficiency) for rGnRHR-Ctail, indicating a significantly increased receptor expression at the cell surface due to the addition of the intracellular C terminus of cfGnRHR. RT-PCR showed no difference between the mRNA levels for wt rGnRHR and rGnRHR-Ctail (data not shown).

Addition of an Intracellular C Terminus Changes the Pattern of Homologous Regulation of rGnRHR and Enhances the Extent of Down-Regulation

To study the homologous regulation of wt and chimeric receptor, GH_3 cells were transiently transfected with either wt rGnRHR or rGnRHR-Ctail and incubated with 10 nM GnRH for the indicated times (Fig. 3). Consistent with results of the binding study (Fig. 2), the number of binding sites of rGnRHR-Ctail was about 5-fold higher than that of wt rGnRH (Fig. 3, *upper panel*). In addition, the receptor-binding assay showed that the receptor number of wt rGnRHR and chimeric rGnRHR-Ctail was regulated differentially (Fig. 3, *lower panel*). The wt rGnRHR number was regulated in a biphasic fashion. The receptor was initially down-regulated, reaching its nadir at 2 h, with approximately 25% reduction of specific binding compared with control cells at initial time point (zero hour). The wt rGnRHR number recovered thereafter (2–7 h) but did not overshoot the control value, with 10% reduction of specific binding at 7 h compared with the control. In contrast, the rGnRHR-Ctail receptor number was regulated in a monophasic fashion during the incubation period. The receptor was progressively down-regulated during 7 h of incubation. After 2 h, both wt and rGnRHR-Ctail receptors were similarly down-regulated, with a 25% decrease in specific binding compared with that of control at the initial time. Instead of recovery of wt GnRHR after 2 h incubation, the rGnRHR-Ctail remained down-regulated, with a 55% decrease in specific binding at 7 h compared with that observed at the initial time. These results indicate that addition of an intracellular C terminus to rGnRHR changes the pattern of homologous regulation of rGnRHR from a biphasically down- and up-regulated pattern to a monophasic down-regulated pattern without recovery.

Addition of an Intracellular C-Terminus Augments rGnRHR-Mediated Inositol Phosphate Production Which Is Uncoupled from the Increase in Receptor Binding Sites

A dose-response study of Buserelin-stimulated IP production is shown in Fig. 4. Two hours of stimulation with Buserelin resulted in a significant, dose-depen-

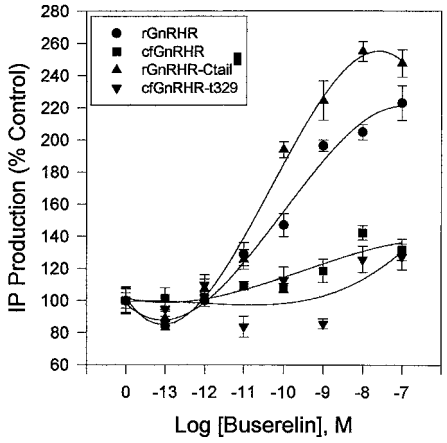

Fig. 4. Dose-Response of Buserelin-Stimulated IP Production in Transfected GH_3 Cells

Forty eight hours after transfection of GH_3 cells with wt rGnRHR, wt cfGnRHR, chimeric rGnRHR-Ctail, or truncated cfGnRHR (cfGnRHR-t329), the cells were preloaded with 4 μCi/ml [^{3}H]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. The data shown are the means of triplicate determinations, represented by the percentage of control (treated with medium alone). Error bars show the SEM. Each experiment was repeated at least three times with similar results.

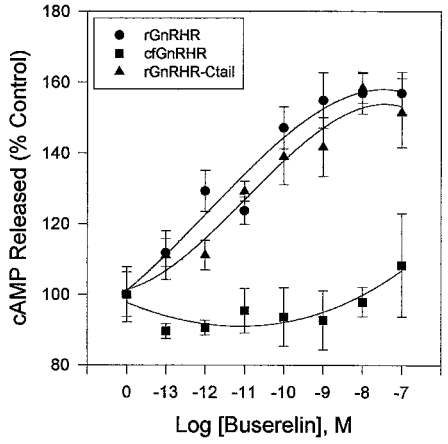

Fig. 5. Dose-Response of Buserelin-Stimulated cAMP Release in Transfected GH_3 Cells

Forty eight hours after transfection of GH_3 cells with wt rGnRHR, wt cfGnRHR, or chimeric rGnRHR-Ctail, the cells were incubated with the indicated concentrations of Buserelin and 0.2 mM MIX for 24 h. The samples were heated at 95 C for 5 min with 1 mM theophylline, and their cAMP contents were determined by RIA. The data shown are the means of triplicate determinations, represented by the percentage of control (treated with medium alone). Error bars show the SEM. Each experiment was repeated at least three times with similar results.

dent response in IP production from GH_3 cells expressing wt rGnRHR and chimeric rGnRHR-Ctail. The response of IP production from GH_3 cells expressing chimeric receptor was higher than that observed for GH_3 cells expressing wt receptor, with EC_{50} of 8.25×10^{-11} M for rGnRHR-Ctail and EC_{50} of 1.56×10^{-10} M for wt rGnRHR. However, this difference (~2-fold in EC_{50}) in IP production between wt rGnRHR and rGnRHR-Ctail was not proportional to the 5-fold increase in receptor binding sites of rGnRHR-Ctail compared with wt rGnRHR. Two hours of treatment with 10^{-13}-10^{-9} M Buserelin did not stimulate IP production from GH_3 cells transfected with wt cfGnRHR. However, a significant increase in IP production was observed at higher doses (10^{-8}-10^{-7} M) of Buserelin. There was no measurable elevation in IP production from GH_3 cells transfected with C terminus-truncated cfGnRHR (cfGnRH-t329) at 10^{-13}-10^{-7} M Buserelin treatment.

Addition of an Intracellular C Terminus Does Not Affect rGnRHR-Mediated cAMP Production and PRL Release

Incubation with 10^{-13}-10^{-7} M Buserelin for 24 h stimulated cAMP release in a dose-dependent manner in GH_3 cells expressing either wt rGnRHR or chimeric rGnRHR-Ctail (Fig. 5). However, there was no significant difference in Buserelin-stimulated cAMP release between GH_3 cells expressing wt rGnRHR and GH_3 cells expressing chimeric rGnRHR-Ctail. Similarly, a 24-h Buserelin treatment stimulated PRL release in a dose-dependent manner in GH_3 cells expressing wt and chimeric receptors (Fig. 6), and there was no significant difference between the response of wt receptor and chimeric receptor. No significant elevation of responses of cAMP release or PRL release above basal levels was observed from GH_3 cells transfected with cfGnRHR at 10^{-13}-10^{-8} M of Buserelin treatment.

Truncation of a Portion of the Added C Terminus in rGnRHR Reduces the Number of Receptor Binding Sites and Attenuates Homologous Down-Regulation

To construct rGnRHR containing different lengths of the C terminus, the chimeric rGnRHR-Ctail was truncated at either residue Arg^{337}, Asn^{343}, or Ser^{350}, respectively, in the added intracellular C terminus. This resulted in a rGnRHR construct containing an 11-amino acid C terminus (rGnRHR-Ctail-t337), a 17-amino acid C terminus (rGnRHR-Ctail-t343), or a 24-amino acid C terminus (rGnRHR-Ctail-t350) (Fig. 1).

The rGnRHR-Ctail and three truncated rGnRHR-Ctail were transiently expressed in GH_3 cells. The GH_3 cells were then continuously incubated with 10 nM GnRH for the indicated times (Fig. 7), and receptor binding to [^{125}I]Buserelin was assessed. Compared with the rGnRHR-Ctail, the three truncated receptors show 3- to 8-fold reduced specific binding for [^{125}I]Buserelin at the initial time point of incubation (Fig. 7, *upper panel*), with 3-fold reduction for rGnRHR-Ctail-t350 (longest tail), 4-fold reduction for rGn-

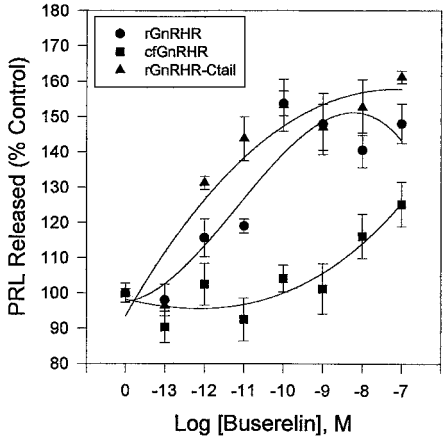

Fig. 6. Dose Response of Buserelin-Stimulated PRL Release in Transfected GH_3 Cells

Forty eight hours after transfection of GH_3 cells with wt rGnRHR, wt cfGnRHR, or chimeric rGnRHR-Ctail, the cells were incubated with the indicated concentrations of Buserelin for 24 h. The medium was collected, and PRL release was measured by RIA. The data shown are the means of triplicate determinations, represented by the percentage of control (treated with medium alone). Error bars show the SEM. Each experiment was repeated at least three times with similar results.

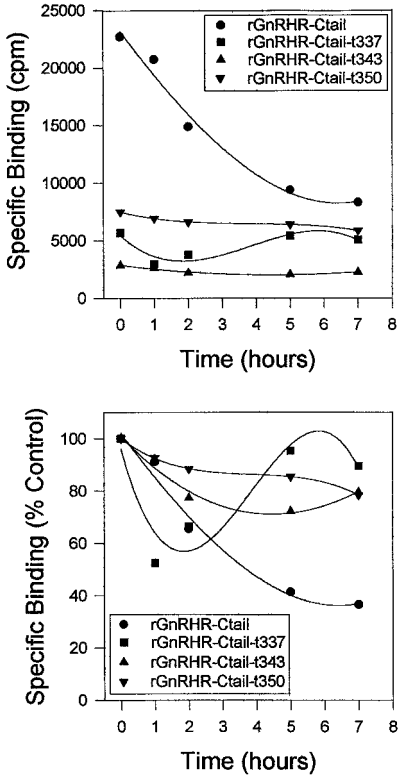

Fig. 7. Homologous Regulation of the GnRHR in GH_3 Cells Expressed rGnRHR-Ctail and Three C Terminus Truncated Receptors

Seventy two hours after transfection of GH_3 cells, cells were incubated with 10 nM GnRH for the indicated times. The GnRH was removed and the binding of [^{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 min (*upper panel*) and in the percentage of control at initial incubation time (*lower panel*). Each experiment was repeated at least three times, with similar results.

RHR-Ctail-t337 (shortest tail), and 8-fold reduction for rGnRHR-Ctail-t343 (medium length of tail). These results indicate that truncation of the C terminus of rGnRHR-Ctail reduced the number of receptor binding sites; however, this reduction was not directly related to the length of C terminus. In addition, truncation of the C terminus of rGnRHR-Ctail also changed the pattern of homologous regulation of rGnRHR (Fig. 7, *lower panel*). Similar to the biphasic pattern of homologous regulation of wt rGnRHR, the specific binding of rGnRHR-Ctail-t337 was reduced by 47% after a 1-h incubation with GnRH. The rGnRHR-Ctail-t337 receptor number recovered thereafter (2–7 h), but did not overshoot the control value, with 5% and 10% reduction of specific binding at 5 h and 7 h, respectively, compared with the control at the initial time. The specific binding of rGnRHR-Ctail-t343 was gradually reduced over 1–5 h in the presence of GnRH, with a 28% reduction at the 5-h time point. The specific binding of rGnRHR-Ctail-t343 slightly recovered at the 7-h time point. The specific binding of rGnRHR-Ctail-t350 was modestly down-regulated during 1–7 h incubation of GnRH, with a 21% reduction at the 7-h time point.

DISCUSSION

Mammalian GnRHR is unique among GPCRs and distinct from nonmammalian GnRHRs, since the former lack an intracellular C-terminal tail. In the present study, addition of the C terminus did not affect receptor-binding affinity, but significantly elevated the receptor expression at the cell surface. Truncation of the added C terminus impaired the elevated receptor binding. Addition of the C terminus altered the pattern of receptor regulation from biphasic down- and up-regulation to monophasic down-regulation alone and significantly enhanced the extent of down-regulation. This alteration in receptor regulation was reversible by truncation of the added C terminus. Addition of the C terminus significantly augmented the IP response to Buserelin, but this augmentation was not coupled to the elevation of receptor- binding sites. Addition of the C terminus did not affect the Buserelin-stimulated cAMP and PRL release. GH_3 cells transfected with wild-type cfGnRHR did not show measurable binding to Buserelin and significant IP, cAMP, and PRL responses to Buserelin (10^{-13}-10^{-9} M). GH_3 cells transfected with C terminus-truncated cfGnRHR showed no IP response to Buserelin (10^{-13}-10^{-7} M). These results suggest that introduction of cfGnRHR intracel-

lular C terminus to rGnRHR has a significant impact on rGnRHR expression and regulation and efficiency of receptor coupling to G protein in GH_3 cells. The results imply that due to the absence of the C terminus, mammalian GnRHR might have evolved distinct receptor expression levels and patterns of receptor regulation needed to adapt to physiological requirements.

The role of the intracellular C-terminal tail of GPCRs on the receptor cell surface expression is unclear, as truncation of C terminus of different GPCRs results in varied levels of receptor expression. In some GPCRs, the truncation of C terminus did not affect receptor number at the cell surface (19, 21, 37), whereas in some other GPCRs, the C terminus-truncated receptor showed either reduced (17, 24, 27, 35) or increased binding sites (30) but no difference in binding affinity compared with wt receptor. However, the effect of the truncation of the C-terminal tail on the number of receptor-binding sites was dependent on the site where the truncation occurred. Studies in a number of GPCRs showed that truncation of the distal portion of the C-terminal tail, which usually includes the Ser/Thr-enriched region, did not significantly alter the receptor-binding capacity, while truncation of a large portion or the entire C-terminal tail typically impaired or abolished the receptor expression at the cell surface due to the intracellular localization of truncated receptor (17, 24, 35). These results suggest that part of the C-terminal tail is involved in the trafficking and routing of the receptor to the plasma membrane.

Since mammalian GnRHR normally lacks the intracellular C-terminal tail, it is a useful model with which to examine the impact of extension of a C terminus on receptor expression and function. The present results show that addition of a C-terminal tail significantly enhances the rGnRHR expression at the cell surface. This enhancement can be reversed by truncation of a portion of the added C-terminal tail; however, the mechanism involved in this action of the added C terminus remains unknown. The absence of an intracellular tail in mammalian GnRHR is likely to be accompanied by structural accommodations in other parts of the receptor, forming the intact receptor conformation required for correct expression and function. The RT-PCR showed that addition of the nucleotide sequence encoding the C-terminal tail did not affect mRNA levels transcribed from the mutant plasmid construct. Therefore, the structural determinants in the added C terminus may contribute to the changes in receptor conformation that favor more efficient receptor-membrane interaction and receptor insertion into the membrane. The reduction in receptor binding sites after truncation of added C-tail may be explained by the increase in intracellular localization of truncated receptor as that demonstrated in other C tail-truncated GPCRs (17, 24, 35). A recent study shows that truncation of the cytoplasmic tail of the LH receptor results in an increase in the relative number of mobile LH receptors on the cell surface (42), supporting the role of an intracellular tail on the receptor movement and localization in the plasma membrane. In addition, it was reported that the capacity of high-affinity cfGnRHR sites (1, 678 fmol/mg protein) is much higher compared with those reported in rats (43). Whether the presence of a C terminus in cfGnRHR contributes to this difference in receptor binding capacity remains an open question.

Mammalian GnRHR was shown to undergo biphasic homologous regulation by physiological concentrations of GnRH (44). Initially, down-regulation of receptors is observed (0.5–4 h posttreatment) followed by an increase in the number of GnRHRs (9 h posttreatment). In the present study, GH_3 cells transiently expressing wt rGnRHR also showed a biphasic pattern of regulation of GnRHR. This regulation by GnRHR is similar to that reported for primary pituitary cells (44) and is also similar to previous results from GH_3 cells stably expressing rGnRHR [GGH_3 cells (45)]. The ability of the GnRHR to be homologously regulated in GH_3 cells suggests that GnRHR regulation does not require cell-specific components and may not involve regulation at the transcriptional level, as the expression of the GnRHR in GH_3 cells is driven by a cytomegalovirus promoter.

Introduction of the intracellular tail of cfGnRHR altered the pattern of homologous regulation of rGnRHR and markedly enhanced the extent of homologous down-regulation of GnRHR. These results suggest that structural changes in the receptor due to addition of C terminus had a significant impact on receptor regulation. Conversely, truncation of the added C terminus to rGnRHR impaired receptor regulation, indicating that the role of the C terminus is reversible. Notably, truncation at position 350 or 343 (which deletes 28 and 35 residues, respectively, of the added C terminus) markedly impaired the extent of down-regulation but did not significantly alter the pattern of receptor regulation. Truncation at position 337 of rGnRHR-Ctail, which deletes six additional residues including the Cys-Phe-Cys motif (two potential palmitoylation sites) from rGnRHR-Ctail-t343, not only impaired the extent of regulation but also altered the pattern of regulation, from a monophasic down-regulation pattern back to a biphasic down- and up-regulation as shown for wt rGnRHR. These results indicate that the Cys-X-Cys motif may contribute to the change of receptor regulation pattern. Similarly, two putative palmitoylation sites, Cys-X-Cys, in the C terminus of TRH receptor, appear to be involved in the agonist-induced internalization (23).

The mechanism of homologous regulation of GnRHR is unclear. It is evident that down-regulation of GnRHR occurs, in part, by physical internalization of agonist-occupied receptors (46), and up-regulation of GnRHR requires calcium mobilization and protein synthesis (44, 47, 48). The initial down-regulation of GnRHR is temporally associated with desensitization of gonadotropes to GnRH (46). Regulation of the β-adrenergic receptor (βAR) involves G protein, phosphorylation of receptor by protein kinase A (PKA), and a

decline in mRNA stability resulting from elevated cAMP levels as well as a second signal transduction pathway activated by the agonist (49). In β_2AR, mutation of the consensus sequence for phosphorylation by PKA in the third intracellular loop abolished cAMP-induced receptor phosphorylation and significantly delayed the rate and reduced the extent of down-regulation of receptor numbers by cAMP (50). It was suggested that phosphorylation of β_2AR enhances the rate of down-regulation by shortening the receptor half-life in the membrane. However, whether agonist-stimulated phosphorylation of the sites in the C terminus by PKA is involved in receptor down-regulation is unknown. Mutation of four Ser and Thr residues in the C terminus in β_2AR (51) or mutation of Tyr residue in NPLIY motif in the junction between the C terminus and the transmembrane segment of β_2AR (52) abolished agonist-stimulated receptor phosphorylation and internalization, but did not affect long-term down-regulation. However, mutation of two Tyr residues in the middle of the C terminus of β_2AR dramatically decreased the agonist-stimulated down-regulation of the receptor, but did not affect sequestration of the receptor (53). These results suggest that the C terminus is involved in receptor regulation, and differential structural determinants in the C terminus are implicated in receptor regulation and internalization. In the present study, addition of a C terminus, which contains 10 Ser and Thr residues, may introduce extra phosphorylation sites into the receptor, leading to increased receptor phosphorylation and enhanced receptor down-regulation. On the other hand, the potential conformational change in the receptor due to the addition of the C terminus may result in decline in receptor stability in the membrane and contribute to the enhanced down-regulation. In nonmammalian vertebrate, GnRH-stimulated homologous receptor down-regulation has been demonstrated (54). However, the time course of GnRHR regulation has not been examined in nonmammalian species, and whether biphasic receptor regulation is also present in nonmammalian GnRHR is unknown. The mechanism for alteration in the pattern of receptor regulation due to the addition of a C terminus remains to be investigated.

The intracellular C-terminal tail has been implicated in agonist-stimulated internalization and/or rapid desensitization in most GPCRs examined (2). Truncation of the intracellular C-terminal tail or mutations of potential phosphorylation sites in the intracellular tail attenuates or abolishes agonist-induced receptor internalization and/or delays the onset of rapid desensitization (17–30). While mammalian GnRHR lacks an intracellular C terminus, rapid desensitization ($<$15 min) is evident in the primary pituitary cells continuously exposed to GnRH (55); agonist-stimulated internalization of GnRHR has been observed to occur within 10–15 min (48). Results from these studies indicate that mammalian GnRHR can internalize and undergo rapid desensitization without the presence of an intracellular C terminus, suggesting that different mechanism from that used by other GPCRs may be used by the GnRHR system. In the present study, we did not examine whether the introduction of an intracellular C terminus can affect rGnRHR internalization. In β_2AR, since mutations of the C terminus abolished agonist-stimulated receptor phosphorylation and internalization but did not affect long-term down-regulation, it was suggested that receptor internalization is dissociated from a slowly evolving down-regulation process (51). However, because of the difference in a C terminus and consequent difference in the mechanism of internalization between GnRHR and other GPCRs, we cannot exclude the possibility that addition of the C terminus alters receptor internalization, which contributes, in part, to the alteration in receptor regulation.

In GGH_3 cells, GnRHR is coupled to $G_{q/11}\alpha$, resulting in activation of phospholipase C and IP turnover (7, 8); the GnRHR also appears to be coupled to adenylate cyclase-mediated PRL release through $G_s\alpha$ (9, 10). In the present study, GH_3 cells transiently transfected with rGnRHR or with chimeric rGnRHR-Ctail showed a significant and dose-dependent increase in IP production and cAMP and PRL release after Buserelin stimulation. These results suggest a similar G protein-coupling pattern for rGnRHR transiently expressed in GH_3 cells as established for continuous GGH_3 cell lines; addition of C terminus to rGnRHR did not appear to affect the pattern of coupling of this receptor to G protein ($G_{q/11}$ and G_s). The intracellular C terminus has been shown to be involved in G protein coupling in several GPCRs (15–17). However, truncation of the C terminus of a number of GPCRs caused an attenuation of receptor internalization without affecting G protein coupling (20, 21, 23, 25), suggesting that the C terminus may not contribute to the receptor conformation required for the sites for G protein coupling. In addition, GH_3 cells expressing rGnRHR-Ctail receptor showed a significantly higher increase in Buserelin-stimulated IP production (2-fold in EC_{50}) compared with that from GH_3 cells expressing wt rGnRHR. This elevation in IP production may result from the increase in receptor-binding sites due to the addition of a C terminus. However, the elevation in IP production was not proportional to the increase (5-fold) of receptor-binding sites caused by addition of the C terminus. Furthermore, GH_3 cells expressing rGnRHR-Ctail and expressing wt rGnRHR show an indistinguishable response of cAMP and PRL release to Buserelin stimulation. These results suggest that the conformational change of the receptor due to the addition of a C terminus preferentially impairs the efficiency of receptor coupling to G protein. In addition, the enhanced receptor down-regulation due to the addition of a C terminus could also be responsible for the decreased signal transduction. The differential effects of the addition of a C terminus on receptor-mediated IP production and cAMP release suggests differential re-

quirements for receptor conformation for coupling to G_s and $G_{q/11}$.

MATERIALS AND METHODS

Materials

rGnRHR cDNA in pcDNA1 was generously provided by Dr. W. W. Chin (56). The African catfish GnRHR was prepared as described (39). The expression vector pcDNA3.1 was purchased from Invitrogen (San Diego, CA). Natural sequence GnRH was provided by the National Pituitary Agency. Buserelin (D-tert-butyl-Ser^6-des-Gly^{10}-Pro^9-ethylamide-GnRH) was a kind gift from Hoechst-Roussel Phamaceuticals (Somerville, NJ). Myo-[^{3}H] inositol was purchased from Dupont (New England Nuclear, Boston, MA). 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.

Methods

Generation of Mutant Receptor Constructs wt rGnRHR cDNA in pcDNA1 was subcloned into pcDNA3.1 at *Bam*HI and *Xho*I restriction enzyme sites. Chimeric receptor (rGnRHR-Ctail) containing wt rGnRHR and intracellular C terminus of cfGnRHR was constructed by overlap extension PCR, a procedure used to join DNA fragments that contain an overlap region (57). To construct the chimera, the fragments originating from each receptor were amplified in separate reactions, each containing one receptor as template. rGnRHR sequence, including 5′-untranslated region and complete coding region but not stop codon, was amplified from the wt rGnRHR cDNA in pcDNA3.1, using a 20-mer vector primer (T7) corresponding to sequence within the T7 polymerase promoter of pcDNA3.1 vector and a 42-mer primer that is the reverse complement of 5′-CCA CTT ATA TAT GGG TAT TTC TCT TTG/ACG CCA TCG TTC CGT. This primer is comprised of 27 bases from the rGnRHR template (*underlined*) and a 15-base adaptor from the 5′-sequence for cfGnRHR intracellular C terminus. The sequence for the intracellular C terminus of cfGnRHR was amplified from wt cfGnRHR cDNA in pcDNA3, using a 18-mer pcDNA3.1/BGH reverse primer (BGH-rev) complementary to sequence within the BGH polyadenylation signal of pcDNA3.1 vector and a 34-mer primer, 5′-GGG TAT TTC TCT TTG/ACG CCA TCG TTC CGT GCC G. This primer is comprised of 19 bases from the 5′-sequence for cfGnRHR intracellular C terminus and a 15-base adaptor (*underlined*) from rGnRHR template. The two chimeric primers used in each reaction were complementary (overlap region) for 30 bases, with the junction (indicated as a *slash*) between rGnRHR and cfGnRHR sequence. The result of the two PCR reactions was the amplification of one fragment of the rGnRHR sequence with a 15-base cfGnRHR sequence end, and one fragment of cfGnRH sequence for intracellular C terminus with a 15-base rGnRHR 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, T7 and BGH-rev. The third PCR reaction produced a full-length chimeric receptor cDNA, presumably by the formation of heteroduplexes between complementary ends of the two templates. The junction of chimeric receptor is between the last amino acid (Leu^{327}) of rGnRHR and the first residue (Thr^{329}) of cfGnRHR intracellular C terminus, forming the sequence -Phe^{325}-Ser^{326}-Leu^{327}/Thr^{328}-Pro^{329}-Ser^{330}-.

A truncated cfGnRHR mutant (cfGnRHR-t329) was created by substitution of the codon for the first residue (Thr^{329}) of wt cfGnRHR intracellular C terminus with a stop codon (TAA) using the overlap extension PCR as described above. Briefly, two fragments were amplified separately from the same template (wt cfGnRHR) using primer set, T7 and a 35-mer primer 5′-CG GAA CGA TGG TTA AAA GAA GCC GTA TAT TAC TGG, and BGH-rev and a 24-mer primer 5′-C GGC TTC TTT TAA CCA TCG TTC CG, respectively. The sequence *underlined* in the primers corresponds to or is complementary to the introduced stop codon (TAA). The two fragments were then used as templates in a third PCR reaction with primer set, T7 and BGH-rev. The third PCR reaction produced a full-length cfGnRHR with stop codon after amino acid Phe^{328}, yielding a truncated cfGnRHR that lacks intracellular C terminus. The chimeric rGnRHR-Ctail was further truncated to create rGnRHR with different lengths of intracellular C terminus; three truncated rGnRHR-Ctail, designated as rGnRHR-Ctail-t337, rGnRHR-Ctail-t343, and rGnRHR-Ctail-t350, were made by substitution of stop codon (TAA) for the residue Arg^{337}, Asn^{343}, and Ser^{350} in the C terminus, respectively, using the overlap extension PCR as described above. The internal primers are 5′-GAC TTG TCC TAA TGT TTC TGT TGG AG and 5′-ACA GAA ACA TTA GGA CAA GTC GGC ACG for rGnRHR-Ctail-t337, 5′-TGT TGG AGG TAA CAA AAT GCT TCA GCC and 5′-AGC ATT TTG TTA CCT CCA ACA GAA ACA TC for rGnRHR-Ctail-t343, and 5′-TCA GCC AAA TAA CTG CCA CAC TTC TCT G and 5′-GTG TGG CAG TTA TTT GGC TGA AGC ATT TTG for rGnRHR-Ctail-t350.

All mutant receptor cDNAs (chimeric and truncated receptor cDNAs) were flanked by the restriction sites present in the polylinker of pcDNA3.1 vector. The cDNAs were thus digested with *Bam*HI and *Xho*I and subcloned into the same sites of pcDNA3.1 vector. The identity of all mutant constructs and the correctness of all PCR-derived coding 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 containing wt or mutant receptor cDNAs 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 GH_3 Cells Wt and mutant receptors were transiently expressed in GH_3 cells (45). GH_3 cells were maintained in growth medium [DMEM containing 10% FCS (Hyclone Laboratories, Logan, UT) and 20 μg/ml gentamicin (Gemini Bioproducts, Calabasas, CA)] in a humidified atmosphere (37 C) containing 5% CO_2. Cells (10^5 per well) were seeded in 24-well plates (Costar, Cambridge, MA). Twenty four hours after plating, the cells were transfected with 0.8 μg plasmid DNA/well using 2 μl lipofectamine in 0.25 ml OPTI-MEM. Five hours later, 0.25 ml 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 48 h before functional assays (IP production; cAMP and PRL release) were done. For receptor binding, the same transfection procedure was followed except that 20 μg plasmid DNA and 50 μl lipofectamine were used to transfect the cells in 75-cm^2 flasks (Costar) when they are 60–80% confluent. For studies of down-regulation of GnRHR, the same transfection procedure was followed except that 2 μg plasmid DNA/well and 5 μl lipofectamine in 1 ml OPTI-MEM were used to transfect cells (5×10^5/well) seeded in six-well plates (Costar), when they were 60–80% confluent.

Quantification of IPs Forty eight hours after the start of transfection, the cells transfected with wt or mutant receptor DNAs were washed with DMEM-0.1% BSA and incubated in 0.5 ml DMEM (without inositol) containing 4 μCi/ml [^{3}H]inositol for 18 h at 37 C. After the preloading period, cells were washed twice in DMEM (inositol free) containing 5 mM LiCl and stimulated with Buserelin at indicated doses in 0.5 ml DMEM-LiCl for 2 h at 37 C. The treatment solution was

removed, and 1 ml 0.1 M formic acid was added to each well. The cells were frozen and then thawed to disrupt cell membranes. IP accumulation was determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy, as previously described (58).

Quantification of cAMP Forty eight hours after the start of transfection, the cells transfected with wt or mutant receptor DNAs were washed with DMEM containing 0.1% BSA (Irvine Scientific, Santa Ana, CA) and 20 μg/ml gentamicin. The cells were then stimulated for 24 h with Buserelin (10^{-13}-10^{-7} M) in DMEM-0.1% BSA-20 μg/ml gentamicin containing 0.2 mM methylisobutylxanthine (MIX) to prevent degradation of cAMP. After stimulation, the medium from each well was collected in tubes containing sufficient theophylline for a final concentration of 1 mM. The samples were heated (95 C) for 5 min to destroy phosphodiesterases. RIA of cAMP was performed by a modification of the method of Steiner *et al.* (59), with the addition of the acetylation step described by Harper and Brooker (60). cAMP antiserum C-1B [prepared in our laboratory (61)] was used at a titer of 1:5100. This antiserum showed less than 0.1% cross-reaction with cGMP, 2′,3′-cAMP, 5′-cAMP, 3′-cAMP, ADP, GDP, ATP, CTP, MIX, or theophylline.

Quantification of PRL Release Forty eight hours after the start of transfection, the cells transfected with wt or mutant receptor DNAs were washed twice with DMEM containing 0.1% BSA and 20 μg/ml gentamicin (DMEM-BSA-Gentamicin). The cells were then incubated with different doses of Buserelin in a 1 ml volume of DMEM-BSA-Gentamicin at 37 C for 24 h. The medium was collected, and the PRL release in medium was measured by RIA, using materials obtained from the Hormone Distribution Program of the National Pituitary Agency, NIDDK. PRL was radioiodinated by standard procedures (62). Intra- and interassay variances were 5% and 7%, respectively.

Receptor Binding and Down-Regulation Intact cell binding was assessed in a range of concentrations of [^{125}I]Buserelin, prepared as previously reported (63), in DMEM-0.1% BSA. Seventy two hours after the start of transfection, the cells transfected with wt or mutant receptor DNAs were scraped and resuspended in warm DMEM-BSA. Cells then were pelleted and washed twice with ice-cold DMEM-BSA. One hundred microliters of the cell suspension (1×10^6 cells) were added to each tube, and the assay was allowed to come to equilibrium (3 h) at 4 C at a final volume of 150 μl. Binding was terminated by overlayering each sample on 2 ml DMEM-0.3 M sucrose at 4 C and centrifuging at 2,000 $\times$ *g* for 10 min at 4 C in Sorvall SM-24 rotor. The supernate was aspirated. The cell pellet was resuspended in 1 ml PBS, and radioactivity was determined using a 10-channel γ-counter (Packard Instruments, Meriden, CT). For studies of down-regulation of the GnRHR, 72 h after start of the transfection, cells were washed twice with DMEM-BSA, treated with 10 nM GnRH (a desensitizing dose) or medium alone for the indicated times, and washed three times (4 ml/well) at 23 C with DMEM-BSA to remove excess GnRH. The medium was decanted and replaced with 2 ml [^{125}I]Buserelin/well at a concentration of 0.4 μCi/ml. Binding was assessed after 30 min (23 C). Nonspecific binding was determined in the presence of 10 μM 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-BSA. Cells were then collected by scraping in 1 ml DMEM-BSA containing 2.5 mM EGTA (4 C) twice. The cell lysate was layered over 2 ml 0.3 M sucrose in DMEM, and the cell pellet was collected and its radioactivity was counted as described above.

Data Analysis

Data shown are the mean of triplicate assay wells and are presented as the mean $\pm$ SEM of replicates in each experiment. The SEM was typically less than 10% of the mean. The data were analyzed by Student's *t* test, $P < 0.05$ being considered significant. Each experiment was repeated three or more times to ensure the reproducibility of the findings.

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