

# Pivotal Role for the Cytoplasmic Carboxyl-Terminal Tail of a Nonmammalian Gonadotropin-Releasing Hormone Receptor in Cell Surface Expression, Ligand Binding, and Receptor Phosphorylation and Internalization

MARION BLOMENRÖHR, ANDERS HEDING, ROBIN SELLAR, ROB LEURS, JAN BOGERD, KARIN A. EIDNE, and GARY B. WILLARS

*Department of Experimental Zoology, Utrecht University, Utrecht, The Netherlands.*

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## ABSTRACT

The gonadotropin-releasing hormone receptor (GnRH-R) of the African catfish couples to phospholipase C and belongs to the large family of G protein-coupled receptors. We recently demonstrated that removal of the carboxyl-terminal tail (S331–Q379) from the catfish GnRH-R results in a loss of agonist binding; the current study sought to define more precisely the role of this region in receptor function. Progressive truncations of the carboxyl-terminal tail decreased cell surface expression detected by either enzyme-linked immunosorbent assay or agonist-binding. The two most truncated receptors (stop331 and stop337) showed no binding but were detected at the cell surface by enzyme-linked immunosorbent assay. All receptors able to bind agonist were also able to activate phospholipase C. The catfish GnRH-R was phosphorylated after agonist-oc-

cupation and use of truncated mutants showed this phosphorylation to be within the carboxyl-terminal tail. Furthermore, studies with S356A, S363A and SS356,363AA mutant receptors demonstrated that Ser363 is a major site of agonist-induced phosphorylation. The absence of this phospho-acceptor site markedly impaired agonist-mediated receptor internalization. In addition, both, Ser363 and the last 12 residues of the tail (not containing Ser363) were shown to be important for  $\beta$ -arrestin-dependent internalization. These observations are relevant to the regulatory function of the carboxyl-terminal tail of G protein-coupled receptors in general and are particularly intriguing given the absence of this region in mammalian GnRH-Rs.

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that acts on cells of the anterior pituitary to stimulate the release of both follicle-stimulating hormone and luteinizing hormone. These hormones play a major regulatory role in gonadal steroidogenesis and gamete maturation and GnRH is therefore a central player in the control of vertebrate reproduction (Fink, 1988). The receptor for GnRH (GnRH-R) is a member of the G protein-coupled receptor (GPCR) family and the binding of GnRH activates phospholipase C (PLC), resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate. The resulting generation of both inositol 1,4,5-trisphosphate and diacylglycerol is able to mobilize  $\text{Ca}^{2+}$  from intracellular stores and activate protein

kinase C, respectively (Berridge, 1993), thereby mediating many of the biological effects of GnRH.

GPCRs are characterized structurally by an extracellular amino terminus and an intracellular carboxyl terminus linked by seven transmembrane-spanning helices, which themselves are joined by three extracellular loops and three intracellular loops. In general, the extracellular domains and/or transmembrane regions are involved in ligand-recognition, whereas cytoplasmic regions present sites for interactions with not only G proteins but also other proteins (Wess, 1997; Ji et al., 1998). The full extent of such interactions and their functional consequences has yet to be established, but there clearly exists the potential for regulation of both receptor trafficking and agonist-receptor-effector coupling. In this context, it is interesting that the mammalian GnRH-Rs are unique among the GPCR family, because they completely lack an intracellular carboxyl-terminal tail (Sealfon et al.,

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M.B. and A.H. contributed equally to this work.

**ABBREVIATIONS:** GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; GPCR, G protein-coupled receptor; PLC, phospholipase C; wt, wild type; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; cGnRH-II, chicken GnRH-II; IP, inositol phosphate; PAGE, polyacrylamide gel electrophoresis;  $R_{s,ss}$ , steady-state number of receptors at cell surface; GRK, G protein-coupled receptor kinase.

1997). In many receptors, this region has indeed been demonstrated to impart regulatory features. In particular, this region is important for agonist-induced phosphorylation, which may both uncouple the receptor from its cognate G protein and target the receptor for internalization (Ferguson et al., 1996; Lefkowitz, 1998). Accordingly, our recent studies have indicated that the tail-less mammalian GnRH-R is resistant to rapid desensitization and the receptor also has slow internalization kinetics (Heding et al., 1998).

In contrast to the mammalian versions of the GnRH-R, but in common with other GPCRs, the cloned nonmammalian GnRH-Rs all have a carboxyl-terminal tail (Sealfon et al., 1997; Tensen et al., 1997; Illing et al., 1999). We have demonstrated recently that in contrast to the mammalian GnRH-R, the GnRH-R of the African catfish (*Clarias gariepinus*) is susceptible to rapid desensitization and has enhanced internalization kinetics (Heding et al., 1998). We have also demonstrated that removal of the carboxyl-terminal tail from the catfish GnRH-R results in a loss of GnRH binding (Blumenröhr et al., 1997). Moreover, addition of the carboxyl-terminal tail of the catfish GnRH-R to the tailless rat GnRH-R results in an increase in the level of cell-surface expression (Lin et al., 1998). Taken together, these data suggest that the carboxyl-terminal tail of this nonmammalian GnRH-R plays a pivotal role in its function, and this receptor provides a model system in which to explore the relationship between structural features of GPCRs and their functional correlates.

The aim of the current study was, therefore, to characterize the function of the carboxyl-terminal tail of the catfish GnRH-R in relation to cell-surface expression, ligand binding, and agonist-induced receptor phosphorylation and internalization. A truncation and point mutation strategy was adopted to identify the key residues that underlie the functional characteristics imparted by the carboxyl-terminal tail.

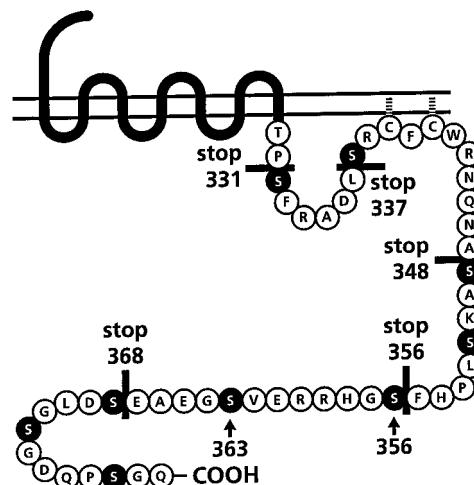
## Materials and Methods

**Mutant Catfish GnRH Receptor Constructs.** Mutations in the catfish GnRH-R cDNA insert (Tensen et al., 1997) were introduced using the pALTER-1 in vitro mutagenesis system (Promega, Madison, WI) according to the manufacturer's instructions. Ser331, Ser337, Ser348, Ser356, and Ser368 were replaced by a stop codon, generating truncated catfish GnRH-Rs designated stop331, stop337, stop348, stop356, and stop368, respectively (Fig. 1). Furthermore, Ser356 and Ser363 were mutated to alanine residues, generating single or double mutant constructs, designated S356A, S363A and SS356,363AA. Mutant receptor constructs were confirmed by sequence analysis and subcloned in pcDNA3 (Invitrogen, San Diego, CA) for expression studies. For phosphorylation studies, a stretch of nucleotides coding for a nine amino-acid epitope (YPYDVPDYA) derived from the influenza virus hemagglutinin protein (HA-tag) was inserted after the initiating methionine codon of wild-type (wt) or mutant receptor constructs using a PCR-based procedure. The entire polymerase chain reaction-amplified portion of the HA-tagged receptor constructs was sequenced to confirm the absence of random mutations.

**Cell Culture and Transfection.** HEK 293T cells and COS-7 cells were cultured as described previously (Blumenröhr et al., 1997; Heding et al., 1998) and transiently transfected with wt or mutant catfish GnRH-R cDNA using the SuperFect transfection method (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To prevent cells from detaching, 24-well plates were coated with poly-D-lysine (Sigma, St. Louis, MO) before seeding cells.

**Enzyme-Linked Immunosorbent Assay (ELISA) Detection.** HEK 293T cells in 60-mm plates were transfected with various concentrations of wt catfish GnRH-R cDNA (1–10  $\mu$ g). In addition, HEK 293T cells were transfected with 5  $\mu$ g of wt receptor cDNA (positive control) or mutant receptor cDNA or pcDNA3 vector only (negative control). After 24 h, cells were transferred into 24-well plates ( $5 \times 10^5$  cells/well) and after an additional 24 h, fixed using 4% paraformaldehyde in phosphate-buffered saline at room temperature for 30 min. Samples were then blocked with 1% nonfat dried milk in 0.1 M NaHCO<sub>3</sub> at room temperature for 4 h and subsequently incubated with an antiserum raised against the amino terminus of the catfish GnRH-R (Blumenröhr et al., 1997) overnight at 4°C (diluted 1:500 in Tris-buffered saline containing 0.1% bovine serum albumin). After exposure to peroxidase-conjugated goat anti-rat IgG [1:1000 in 0.1 M NaHCO<sub>3</sub>/1% nonfat dried milk (Sigma)] at room temperature for 2 h, peroxidase was visualized using TMB liquid substrate system (Sigma) for 30 min. Absorbance values (at 450 nm) of the negative control were subtracted and the values then expressed as a percentage of the positive control. All constructs are tested in triplicate in three separate experiments.

**Receptor Binding Assay.** Chicken GnRH-II (cGnRH-II; [His<sup>5</sup>, Trp<sup>7</sup>, Tyr<sup>8</sup>]-GnRH; American Peptide Company, Sunnyvale, CA) was iodinated using either the chloramine-T method and subsequent purification by C18 column chromatography (Blumenröhr et al., 1997) or the glucose oxidase/lactoperoxidase method and subsequent Sephadex G-25 column chromatography (Heding et al., 1998). The specific activity of the radioligand was 111  $\mu$ Ci/mmol. Initial binding assays were carried out as described previously (Heding et al., 1998) on cell membranes from receptor-expressing cells. These cells were also used for ELISA detection. Because the relative levels of binding of different receptors as tested on whole cells were similar to those in membrane fractions, further studies were performed as follows. HEK 293T cells transfected for ELISA detection were also seeded in 24-well plates ( $5 \times 10^5$  cells/well) for binding studies. Forty-eight hours after transfection, cells were washed with assay medium [HEPES-modified Dulbecco's modified Eagle's medium (DMEM) with 0.1% bovine serum albumin, pH 7.4] before being incubated with approximately 1 nM <sup>125</sup>I-labeled cGnRH-II in 0.5 ml of assay medium at 4°C for 2 h in the presence or absence of 1  $\mu$ M unlabeled cGnRH-II. The concentration of <sup>125</sup>I-cGnRH-II used approximated its  $K_d$  value at the wt catfish GnRH-R (2 nM, see Results). After two washes with ice-cold phosphate-buffered saline,



**Fig. 1.** Schematic representation of the carboxyl-terminal domain of the African catfish GnRH-R. The GnRH-R was truncated by insertion of a stop codon at positions 331 (stop331), 337 (stop337), 348 (stop348), 356 (stop356), and 368 (stop368) by site-directed mutagenesis as described under *Experimental Procedures*. Solid circles indicate the presence of a serine residue. The assumed palmitoylation and membrane anchorage of Cys339 and Cys341 are indicated by hatched bars.

extracellular receptor-associated ligand was removed by washing with 1 ml of acid solution (50 mM acetic acid and 150 mM NaCl, pH 2.8) for 12 min. The amount of radioactivity present in the collected acid wash was determined. Internalized radioactivity was determined after solubilizing the cells in 0.2 M NaOH/1% SDS. All binding studies were carried out in triplicate in at least three independent experiments. Specific binding was calculated by subtracting nonspecific binding (in the presence of 1  $\mu$ M unlabeled cGnRH-II) from total binding.

**Total Inositol Phosphates (IPs).** Total IPs were extracted and separated as described previously (Tensen et al., 1997). Briefly, 24 h after transfection, cells were transferred to 24-well plates ( $5 \times 10^5$  cells/well in 0.5 ml of inositol-free DMEM containing 10% dialysed fetal calf serum) and incubated for 24 h with [ $^3$ H]inositol (1  $\mu$ Ci/ml; Amersham, Little Chalfont, England). Medium was removed, cells were washed and preincubated for 10 min with assay medium (HEPES-modified DMEM containing 10 mM LiCl) followed by addition of 1  $\mu$ M cGnRH-II at 37°C for 60 min. The assay medium was then aspirated and cells were frozen with liquid nitrogen. After a methanol/chloroform extraction, the aqueous phase was transferred to tubes containing Dowex (AG 1 $\times$ 8) anion-exchange resin (Sigma). Total IPs were then eluted, and the amount of radioactivity was counted. Assays were performed in triplicate in three separate experiments.

**Receptor Phosphorylation.** This was carried out by modification of a method described previously (Tobin and Nahorski, 1993). Cells were removed from 25-cm<sup>2</sup> flasks 24 h after transfection and replated in 6-well multidishes (one flask of cells/well). After a further 24 h, cells were washed with Krebs/HEPES buffer (10 mM HEPES, 4.2 mM NaHCO<sub>3</sub>, 11.7 mM glucose, 1.2 mM MgSO<sub>4</sub>, 4.7 mM KCl, 118 mM NaCl, and 1.3 mM CaCl<sub>2</sub>, pH 7.4) and incubated at 37°C for 1 h in 1 ml of buffer per well containing 50  $\mu$ Ci of [ $^{32}$ P]orthophosphate. Cells were then either untreated or challenged with 1  $\mu$ M cGnRH-II for 5 min by adding the agonist directly to the wells. Buffer was then aspirated and 0.5 ml of ice-cold solubilization buffer was added (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100  $\mu$ g/ml iodoacetamide, and 100  $\mu$ g/ml benzamidine). After 30 min on ice, the solubilization buffer was removed and centrifuged at 10,000g for 3 min. The primary antibody (0.6  $\mu$ g of a rabbit polyclonal IgG raised against the HA epitope-tag; Santa-Cruz Biotechnology, Santa Cruz, CA) was then added to 0.4 ml of the cleared supernatant. After 60 min on ice, immune complexes were separated by incubation at 4°C for 15 to 30 min with protein A-Sepharose beads (150  $\mu$ l of 30 mg/ml) under constant agitation. Beads were harvested by centrifugation at 10,000g for 10 s and washed twice with 1 ml of ice-cold 100 mM Tris-base, 1.5 M NaCl, 0.5% Tween-20, pH 7.4, followed by two washes with 1 ml of ice-cold 10 mM Tris, 10 mM EDTA, pH 7.4. Samples were then extracted into 20  $\mu$ l of sample buffer (100 mM Tris  $\cdot$  HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 200 mM dithiothreitol) by standing in boiling water for 5 min. Proteins were then resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE). The gels were dried and subjected to autoradiography.

**Immunoprecipitation/Western Blot.** To examine the phosphorylation state of receptors, we had to ensure that the immunoprecipitations, as described above, were effective. To demonstrate this and to obtain an indication of the relative efficiencies of immunoprecipitation for the different receptor constructs, we carried out immunoprecipitation followed by Western blotting. Moreover, to confirm the identity of phosphorylated protein, mock-transfected cells were used as receptor-negative control cells in the same experiments. Immunoprecipitations were performed exactly as described above with the exception that cells were not labeled with [ $^{32}$ P]orthophosphate; instead, 1.2 mM KH<sub>2</sub>PO<sub>4</sub> was added to the Krebs/HEPES buffer. After resolution by 8% SDS-PAGE, proteins were transferred to nitrocellulose. The blot was blocked overnight in 20 mM Tris, 500 mM NaCl, 0.05% Tween-20, and 5% dried milk at 4°C and then probed with 1  $\mu$ g/ml of a mouse monoclonal antibody

raised against the HA epitope-tag (clone 12CA5; Boehringer-Mannheim, Sussex, UK). This antibody was visualized using a peroxidase conjugated anti-mouse IgG and enhanced chemiluminescence reagents (Amersham International plc, Buckinghamshire, UK).

**Receptor Internalization Assay.** Internalization assays were performed as described for binding assays on whole HEK 293T cells (Heding et al., 1998), except that radioligand incubations were performed using time intervals ranging from 5 min to 2 h at 37°C. Nonspecific binding for each time point was determined under the same conditions in the presence of 1  $\mu$ M unlabeled cGnRH-II. After subtraction of nonspecific binding, the amount of surface-bound radioactivity was expressed as a percentage of the total binding at that time interval. All time points were performed in duplicate in at least three independent experiments. To determine the effect of  $\beta$ -arrestin on internalization, 10-cm dishes containing COS-7 cells were transiently transfected with 5  $\mu$ g of receptor cDNA together with 5  $\mu$ g of  $\beta$ -arrestin cDNA in pcDNA3 or 5  $\mu$ g of pcDNA3 vector.

**Statistical Analysis.** All data are presented as mean  $\pm$  S.E. of three independent experiments. Statistical analysis was performed using one-way analysis of variance and, where  $p < .05$ , followed by the Bonferroni test. A  $p < .05$  was considered significant.

## Results

Figure 1 shows a schematic representation of the African catfish GnRH-R depicting the amino acid sequence of the intracellular carboxyl-terminal tail (T329-Q379). To study the functional relevance of the carboxyl-terminal tail, five different, progressively truncated catfish GnRH-R constructs (stop331, stop337, stop348, stop356, and stop368) were generated. Because we knew from previous work that HEK 293T cells transiently transfected with the stop331 mutant catfish GnRH-R show very little binding of  $^{125}$ I-cGnRH-II (Blomenröhr et al., 1997), we developed an ELISA to quantitatively measure catfish GnRH-R protein expressed at the cell surface. A polyclonal antibody raised against the extracellular amino terminus of the catfish GnRH-R specifically recognized the catfish GnRH-R. Thus, cells transfected with pcDNA3 vector or with rat GnRH-R cDNA gave similar absorbance values [ $0.062 \pm 0.006$  ( $n = 3$ ) and  $0.067 \pm 0.006$  ( $n = 3$ ), respectively], whereas cells transfected with catfish GnRH-R cDNA gave absorbance values up to 5.3 times higher (Fig. 2A). Moreover, increasing amounts of transfected catfish GnRH-R cDNA yielded increasing absorbance values with a maximum of  $0.328 \pm 0.008$  ( $n = 3$ ) at 5  $\mu$ g of DNA/60-mm dish (Fig. 2A). These ELISA results reflected the data obtained in binding studies using 1 nM  $^{125}$ I-cGnRH-II (Fig. 2B), because maximal specific binding of  $^{125}$ I-cGnRH-II [ $11182 \pm 1349$  cpm ( $n = 3$ )] was also achieved at 5  $\mu$ g of DNA/60-mm dish. We therefore used the ELISA detection method to quantify mutant receptor expression levels at the surface of transfected HEK 293T cells.

Progressively larger truncations of the carboxyl-terminal domain of the catfish GnRH-R resulted in progressively decreasing levels of receptors detected at the cell surface, ranging from  $81.7 \pm 9.8\%$  to  $41.0 \pm 2.9\%$  of wt catfish GnRH-R levels (Fig. 3, solid bars). The results of binding studies on membrane fractions largely resembled the ELISA data: the shorter the carboxyl-terminal tail, the lower the specific binding of  $^{125}$ I-cGnRH-II (Fig. 3, hatched bars). However, there was a greater loss of binding than truncated receptor expression determined by ELISA. Indeed, the stop331 and stop337 mutant receptors, although showing cell surface expression (as measured by ELISA) of about 40% of wt catfish



GnRH-R levels, showed hardly any detectable binding of  $^{125}\text{I}$ -cGnRH-II (Fig. 3). Binding to whole cells rather than membranes gave similar results with respect to relative levels of binding of different receptors (data not shown). Binding of  $^{125}\text{I}$ -cGnRH-II to its receptor was saturable with a  $B_{\text{max}}$  value of  $9.87 \pm 0.88$  pmol/mg membrane protein and a  $K_d$  value of  $-8.67 \pm 0.15$  ( $\log_{10}$  M, 2.16 nM;  $n = 3$ ).

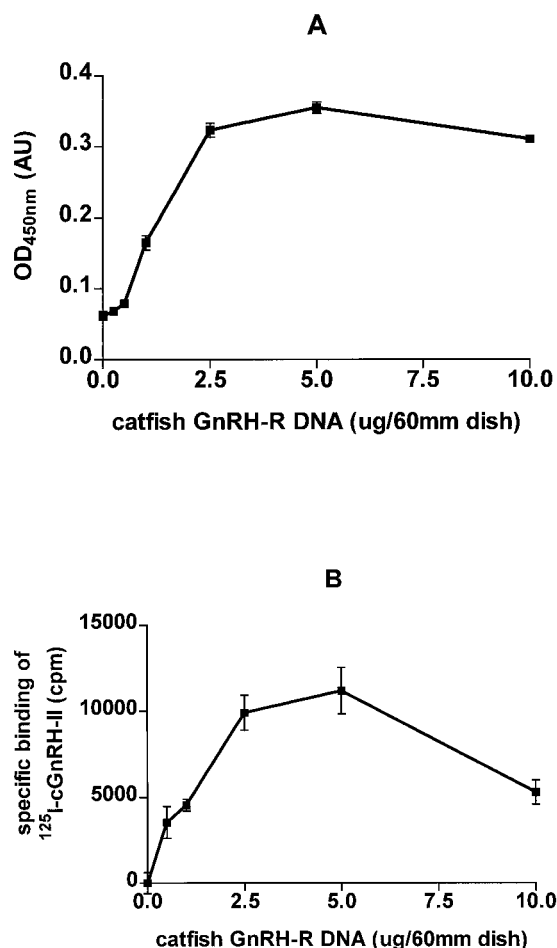
In addition to the expression studies, we also investigated the function of truncated receptor constructs by measuring their ability to activate PLC. After 24-h labeling with  $[\text{H}]$ -inositol and 1-h stimulation with  $1 \mu\text{M}$  cGnRH-II, the cellular levels of total IPs were determined. Basal IP levels for the wt and mutated receptor constructs were similar ( $284 \pm 34$  dpm). Stimulation of the wt GnRH-R resulted in an accumulation of IPs to  $940 \pm 232\%$  of basal. With the exception of the stop331 and stop337 mutant receptors, stimulation of all other truncated receptors resulted in an accumulation of  $[\text{H}]$ IPs against a  $\text{Li}^+$ -block of inositol monophosphatase activity (Fig. 3, open bars). The relative levels of  $[\text{H}]$ IP accumulation largely paralleled the binding levels.

To determine if the wt and mutant catfish GnRH-Rs were phosphorylated after agonist occupation, we inserted an HA-tag into the catfish GnRH-R. The insertion of this epitope

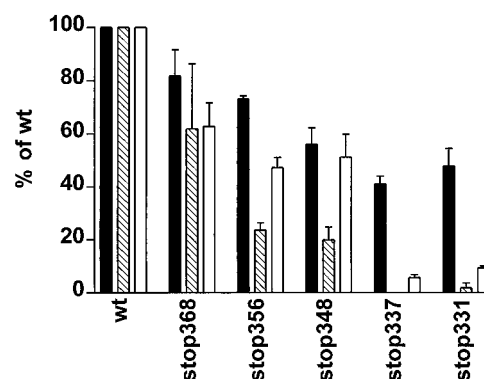
allowed immunoprecipitation of the receptors using a commercially available antibody recognizing the HA epitope. We already had experience in receptor immunoprecipitation using an antibody raised against this epitope; given the commercial availability of this antibody, we chose to use this method for our immunoprecipitations. The antibody raised against the HA epitope-tag also recognized the receptors in our ELISA assay (data not shown). Moreover, the HA-tagged catfish GnRH-R showed binding and signal transduction characteristics similar to those of untagged wt catfish GnRH-Rs (data not shown).

Immunoprecipitation followed by Western blotting demonstrated that all of the truncated catfish GnRH-Rs could be immunoprecipitated using the anti-HA polyclonal antibody (Fig. 4A). Bands were detected at  $\sim 82$  kDa for the wt catfish GnRH-R,  $\sim 81$  kDa for stop368,  $\sim 79$  kDa for stop356, and  $\sim 77$  kDa for stop348. Additional bands were also observed for all receptors at higher molecular masses. It is unclear whether these bands represent receptor dimers/multimers or aggregates that form either naturally or during the preparative process (despite strongly reducing conditions). There were also bands of lower molecular mass, which probably represent unprocessed (particularly unglycosylated) receptors. Agonist challenge did not, however, alter the distribution of immunoreactivity between the bands, nor did it affect the ability of the anti-HA antibody to immunoprecipitate the receptors (Fig. 4A). Moreover, extracts of mock-transfected HEK 293T cells did not show these immunoreactive bands (Fig. 4A), identifying them as receptor-specific bands.

In phosphorylation studies, we were able to demonstrate that a 5-min challenge with  $1 \mu\text{M}$  cGnRH-II resulted in the phosphorylation of the wt catfish GnRH-R and stop368 but not stop356 or stop348 (Fig. 4B). The phosphorylations were observed at  $\sim 82$  kDa and  $\sim 81$  kDa for wt catfish GnRH-R and stop368, respectively, suggesting that these are the



**Fig. 2.** ELISA measurement and specific binding of wt catfish GnRH-Rs. Intact HEK 293T cells transiently transfected with increasing amounts of wt catfish GnRH-R cDNA (0–10  $\mu\text{g}$ ) were subjected to (A) ELISA measurements using an antibody raised against the amino-terminal part of the catfish GnRH-R and (B) to binding of  $^{125}\text{I}$ -labeled cGnRH-II as described under *Experimental Procedures*. Results shown are the mean  $\pm$  S.E. of triplicate observations from a single representative experiment.



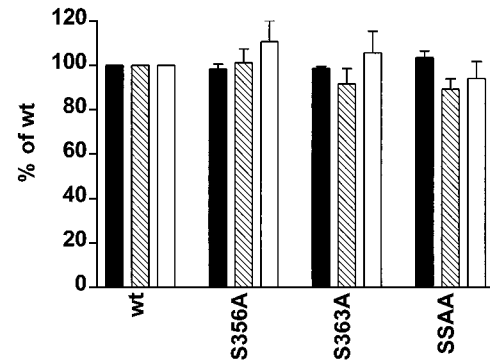
**Fig. 3.** Expression, specific binding, and agonist-mediated  $[\text{H}]$ IP production of wt and truncated catfish GnRH-Rs. Cell-surface expression of wt and truncated catfish GnRH-R constructs, transiently transfected in HEK 293T cells, was measured in an ELISA using an antibody raised against the amino-terminal part of the catfish GnRH-R. The binding of  $^{125}\text{I}$ -labeled cGnRH-II to cell membranes prepared from HEK 293T cells transiently expressing wt or truncated catfish GnRH-R constructs was performed in the presence or absence of  $10^{-6}$  M unlabeled cGnRH-II. Agonist-induced accumulation of  $[\text{H}]$ IPs was determined in HEK 293T cells transiently expressing wt or truncated catfish GnRH-R constructs after 1 h stimulation with  $1 \mu\text{M}$  cGnRH-II as described under *Experimental Procedures*. Expression (■), specific binding (▨), and  $[\text{H}]$ IP production (□) data obtained for each of the receptor constructs are expressed as a percentage of wt catfish GnRH-R levels in the respective assay. Results shown are the mean  $\pm$  S.E. of three independent experiments.

forms of the receptor inserted into the plasma membrane and accessible to a kinase after agonist binding. Again, extracts of mock-transfected cells did not contain phosphorylated proteins.

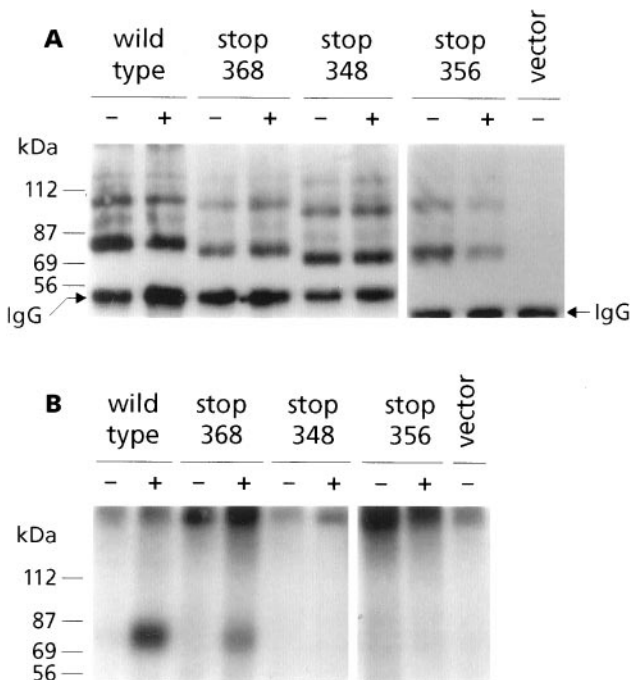
These data indicated that phosphorylation is likely to occur between residue 355 and 368 of the carboxyl-terminal tail. In this region there are two candidate phosphorylation sites, namely Ser356 and Ser363. To identify which of these potential sites are phosphorylated after agonist challenge of the catfish GnRH-R, we created and transfected S356A, S363A, and SS356,363AA mutant catfish GnRH-R constructs (see Fig. 1). All of these mutant receptors (S356A, S363A, and SS356,363AA) were expressed at the cell surface, bound  $^{125}$ I-cGnRH-II, and increased [ $^3$ H]IP accumulation after agonist challenge to similar extents as the wt catfish GnRH-R ( $p > .05$ ; Fig. 5). Immunoprecipitation followed by Western blotting again demonstrated that we were able to immunoprecipitate these expressed receptor constructs with the anti-HA antibody and that their immunoprecipitation was not affected by agonist treatment (1  $\mu$ M cGnRH-II for 5 min; Fig. 6A, data not shown). Bands were present at  $\sim 82$  kDa for all of these receptors, although bands of higher molecular mass were again present. Studies demonstrated that a band at  $\sim 82$  kDa was phosphorylated after agonist challenge (1  $\mu$ M cGnRH-II for 5 min) of S356A (Fig. 6B) although no such

phosphorylation of either S363A (Fig. 6B) or SS356,363AA (data not shown) occurred.

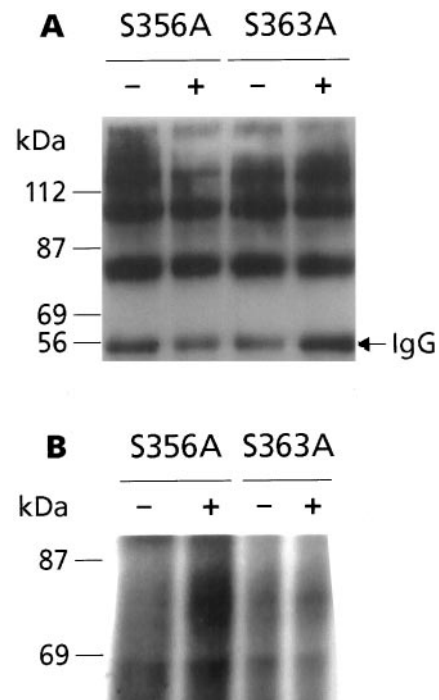
We also investigated the importance of the carboxyl-terminal tail, the role of agonist-induced phosphorylation of Ser363, and the involvement of  $\beta$ -arrestin in the process of agonist-induced receptor internalization. Using a two-compartment model described by Koenig and Edwardson (1997),



**Fig. 5.** Expression, specific binding, and IP production of wt and Ser-mutant catfish GnRH-Rs. Cell-surface expression of wt and Ser-mutant catfish GnRH-R constructs, transiently transfected in HEK 293T cells, was measured in an ELISA using an antibody raised against the amino-terminal part of the catfish GnRH-R.  $^{125}$ I-labeled cGnRH-II binding to intact HEK 293T cells transiently expressing wt or Ser-mutant catfish GnRH-R constructs was performed in the presence or absence of  $10^{-6}$  M unlabeled cGnRH-II. GnRH-induced total IP accumulation in HEK 293T cells transiently expressing wt or Ser-mutant catfish GnRH-R constructs after 1-h stimulation with 1  $\mu$ M cGnRH-II as described under *Experimental Procedures*. Expression (■), specific binding (▨), and IP production (□) data obtained for each of the receptor constructs are expressed as a percentage of wt catfish GnRH-R levels in the respective assay. Results shown are the mean  $\pm$  S.E. of three independent experiments.



**Fig. 4.** Agonist-induced phosphorylation of wt and truncated catfish GnRH-Rs. Immunoprecipitation followed by Western blotting of HA-tagged wt and mutant catfish GnRH-Rs was carried out to demonstrate that all of the receptor constructs could be immunoprecipitated using this protocol and that agonist stimulation did not affect the efficiency of the immunoprecipitation. The immunoprecipitation was performed on solubilized cells, which were either untreated (–) or challenged with 1  $\mu$ M cGnRH-II for 5 min (+). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose and probed with a different HA-antibody to that used for immunoprecipitation (A). Using an identical protocol for immunoprecipitation as in (A), experiments were performed in which HA-tagged wt or mutant catfish GnRH-Rs were immunoprecipitated from solubilized cells that had been labeled with [ $^{32}$ P]orthophosphate and either untreated (–) or challenged with 1  $\mu$ M cGnRH-II for 5 min (+). Proteins were resolved by SDS-PAGE, the gels dried and exposed to film (B).



**Fig. 6.** Agonist-induced phosphorylation of wt and Ser-mutant catfish GnRH-Rs. (A) Immunoprecipitation followed by Western blotting of HA-tagged wt and Ser-mutant catfish GnRH-Rs was carried out as described in Fig. 4. (B) Subsequently, phosphorylation studies were performed to determine which of the receptors were phosphorylated after agonist challenge. Cells were either untreated (–) or treated with 1  $\mu$ M cGnRH-II for 5 min (+).

we calculated the steady-state proportion of receptors at the cell surface ( $R_{s,ss}$ ) in the presence of agonist.  $R_{s,ss}$  values represent means  $\pm$  S.E. of three individual experiments. The wt catfish GnRH-R internalized up to about 60% in HEK 293T cells over a 2-h period (Fig. 7). We have previously published similar results in HEK 293 cells (Heding et al., 1998). All truncated receptor constructs tested were internalized significantly less than the wt catfish GnRH-R, resulting in a greater proportion of receptors at the cell surface at steady state [ $R_{s,ss}$  values of  $39.88 \pm 1.39\%$ ,  $63.65 \pm 1.93\%$ ,  $58.40 \pm 1.71\%$ , and  $49.68 \pm 2.80\%$  for wt, stop368 ( $p < .001$ ), stop356 ( $p < .001$ ), and stop348 ( $p < .05$ ), respectively,  $n = 3$ ; Fig. 7A]. Interestingly, the receptor construct with the shortest tail did not exhibit the most attenuated rate and extent of internalization. The stop368 mutant receptor had a significantly lower proportion of internalized receptors than the stop348 mutant receptor ( $p < .05$ ). The stop356 mutant receptor, on the other hand, was not significantly different from either stop368 or stop348 mutant receptors ( $p > .05$ ). Moreover, we demonstrated that the S356A mutant receptor construct had an  $R_{s,ss}$  value indistinguishable from the  $R_{s,ss}$  value of the wt catfish GnRH-R ( $43.23 \pm 1.69\%$  and  $42.47 \pm 1.01\%$ , respectively;  $p > .05$ ,  $n = 3$ ), whereas the S363A

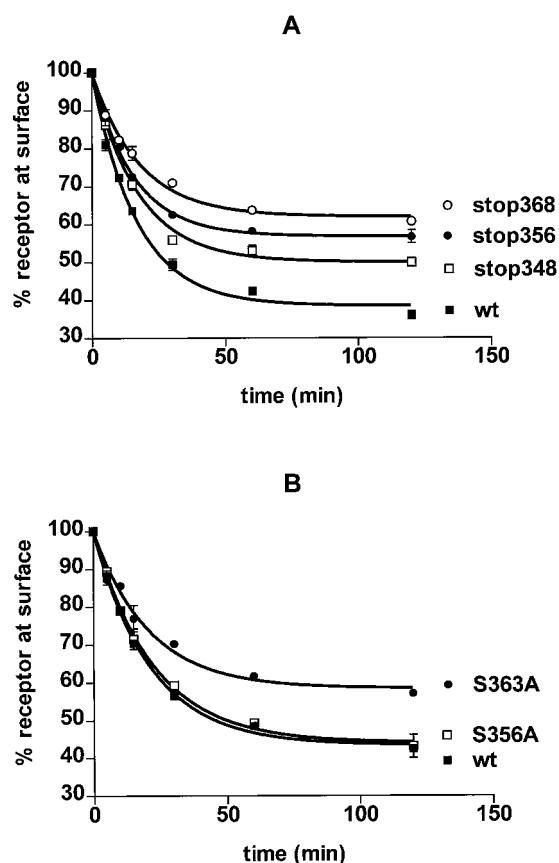
receptor construct ( $56.38 \pm 1.95\%$ ) differed significantly from wt and S356A mutant catfish GnRH-Rs ( $p < .01$ ;  $n = 3$ ; Fig. 7B) but was similar to the truncated receptor constructs ( $p > .05$ ).

We next wanted to investigate the relationship between the phosphorylation site and the last 12 amino acid residues in the process of endocytosis of the catfish GnRH-R. To this end, the effect of different endogenous levels of  $\beta$ -arrestin on catfish GnRH-R internalization and the importance of Ser363 and the last 12 amino acid residues in this event were evaluated by performing experiments in COS-7 cells, which express about 70% less total  $\beta$ -arrestin/mg of protein than HEK 293 cells (Menard et al., 1997). Under conditions of low endogenous  $\beta$ -arrestin expression, the wt catfish GnRH-R had a dramatically decreased extent of internalization in COS-7 cells compared with HEK 293T cells, resulting in a significantly greater portion of receptors at the cell surface at steady state [ $R_{s,ss}$  of  $70.47 \pm 1.73\%$  (Fig. 8A) and  $R_{s,ss}$  of  $39.88 \pm 1.39\%$  (Fig. 7A), respectively;  $p < .01$ ;  $n = 3$ ]. However, this impaired internalization under conditions of low endogenous  $\beta$ -arrestin expression could be largely recovered by coexpression of  $\beta$ -arrestin, resulting in a decreased  $R_{s,ss}$  value of  $45.83 \pm 2.78\%$  (Fig. 8A). Internalization of the stop368 mutant receptor, on the contrary, was only poorly affected by coexpression of  $\beta$ -arrestin in COS-7 cells ( $R_{s,ss}$  of  $69.77 \pm 3.07\%$  without  $\beta$ -arrestin coexpression and  $R_{s,ss}$  of  $60.83 \pm 2.22\%$  with  $\beta$ -arrestin coexpression;  $n = 3$ ; Fig. 8B). Internalization of the S363A mutant receptor, like the stop368 mutant receptor, was also largely unaffected by coexpression of  $\beta$ -arrestin in COS-7 cells ( $R_{s,ss}$  of  $61.62 \pm 5.05\%$  without  $\beta$ -arrestin coexpression and  $R_{s,ss}$  of  $55.12 \pm 2.01\%$  with  $\beta$ -arrestin coexpression;  $n = 3$ ; Fig. 8C).

## Discussion

The GnRH receptor is essential in the control of reproduction in both mammalian and nonmammalian species; the absence of an intracellular carboxyl-terminal tail in the mammalian GnRH-R is intriguing given the functional importance of this region in other GPCRs. The aim of this study was to gain insight into the physiological role of the carboxyl-terminal tail using a nonmammalian GnRH-R by comparing the wt catfish GnRH-R with five truncated versions (with decreasing lengths of the carboxyl-terminal tail) and three mutant receptors in which serine residues of the carboxyl-terminal tail were mutated to alanine residues.

Progressive truncation of the carboxyl-terminal tail reduced levels of receptor expression as determined by either ELISA or agonist binding. This is consistent with the observation that addition of the catfish GnRH-R carboxyl-terminal tail to the naturally tailless rat GnRH-R increases its expression (Lin et al., 1998). However, the progressive truncation of the catfish GnRH-R resulted in a proportionally greater loss of agonist binding than cell-surface expression detected by ELISA. In stop331 and stop337 receptors, there was little or no binding of agonist despite cell-surface expression (detected by ELISA) of approximately 40% of wt receptors. Recognition of these most severely truncated receptors by antibody but not agonist suggests that these receptors are transported and inserted into the plasma membrane (although in reduced amounts), but have a disturbed ligand-binding site because of the absence of the carboxyl-terminal tail or altered G



**Fig. 7.** Agonist-induced internalization of catfish GnRH-R constructs. HEK 293T cells transiently expressing wt or truncated (A) or Ser-mutant (B) catfish GnRH-R constructs were incubated with 1 nM <sup>125</sup>I-labeled cGnRH-II for the indicated time. Surface-bound radioactivity was then determined as the radioactivity that could be removed by acid wash. Internalized radioactivity was determined after solubilization of the cells as described under *Experimental Procedures*. A curve has been fitted to the data points as described by Koenig and Edwardson (1997). The results shown are the mean  $\pm$  S.E. of triplicate observations from a single representative experiment.

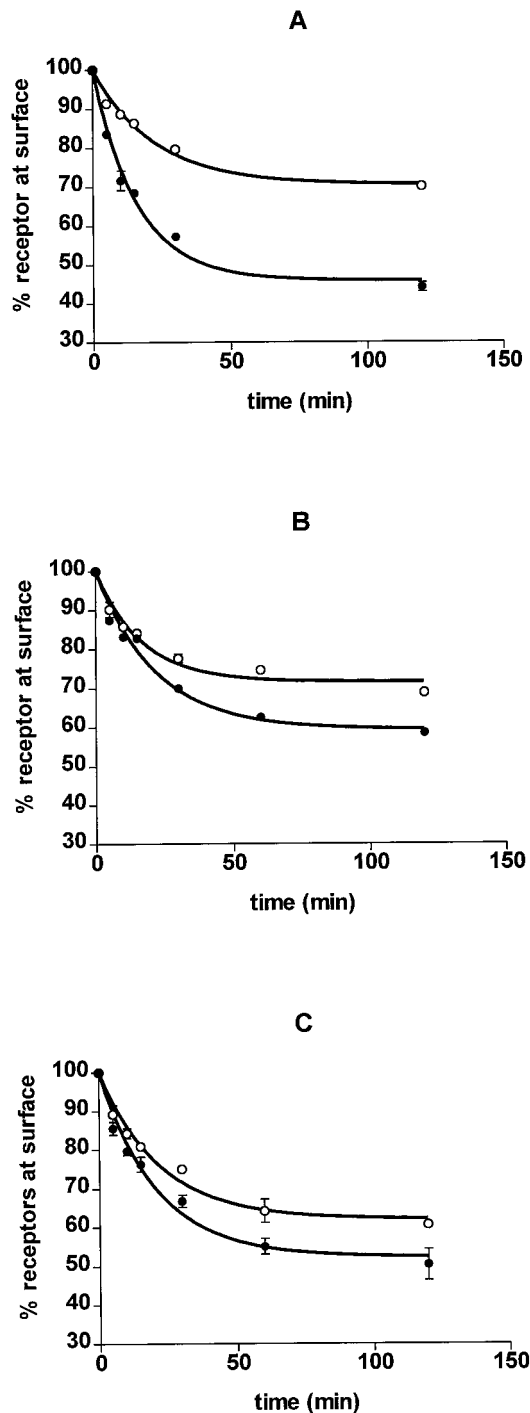
protein binding. This could relate to the loss of cysteine residues, because palmitoylation of these sites within the carboxyl-terminal tail of other GPCRs allows anchoring to the plasma membrane (Ovchinnikov et al., 1988; O'Dowd et

al., 1989; Ng et al., 1993). Furthermore, mutants of the luteinizing hormone receptor (Kawate et al., 1997) and  $V_2$  vasopressin receptor (Sadeghi et al., 1997), which lack palmitoylation sites, show decreased binding levels compared with their wt counterparts. Based on the dramatic loss of agonist binding for the stop331 and stop337 mutant catfish GnRH-Rs, we suggest that Cys339 and/or Cys341 may be palmitoylated, thereby anchoring the receptor to the plasma membrane and allowing formation of a functional ligand-binding site. Future studies will test this hypothesis.

Measurement of the accumulation of inositol phosphates demonstrated both that progressive truncation of the catfish GnRH-R reduced agonist-activation of PLC and that only receptors that bound agonist could transduce a signal. Furthermore, stop368, stop356, and stop348 mutants couple to G proteins despite their lack of the last 12, 24, and 32 amino acids, respectively, of the carboxyl-terminal tail.

Agonist-dependent phosphorylation is a general phenomenon among the GPCR family, including receptors coupling preferentially to the activation of PLC (Tobin, 1997). According to the ternary complex model, agonist-occupied GPCRs isomerize from an inactive to an active conformation. This is likely to involve the conserved NP(X)<sub>1,2</sub>Y motif in transmembrane 7 and seems to enable coupling to G proteins (Gether and Kobilka, 1998). Moreover, this conformational change increases the effectiveness of GPCRs as substrates for phosphorylation by GPCR kinases (GRKs) (Ferguson et al., 1996). This model is supported by a mutant  $\beta_2$ -adrenoceptor in which the tyrosine of the NP(X)<sub>1,2</sub>Y motif has been replaced by alanine. This mutant has markedly reduced agonist-dependent phosphorylation; however, this reduction can be reversed by overexpression of GRK2 (Ferguson et al., 1995). For several GPCRs, including the  $\beta_2$ -adrenoceptor, the luteinizing hormone receptor, the  $\alpha$ -factor receptor, and the C5a anaphylatoxin receptor, serine and threonine residues in distal portions of the carboxyl-terminal tail are sites for GRK(s) phosphorylation after receptor activation (Hausdorff et al., 1990; Wang et al., 1996; Böhm et al., 1997; Koenig and Edwardson, 1997; Naik et al., 1997; Hicke et al., 1998). In other receptors, phosphorylation sites are located in different regions of the receptor, most notably the first and third intracellular loops (Nakamura et al., 1998). Here, we show that the catfish GnRH-R is rapidly phosphorylated after exposure to agonist and that Ser363 within the carboxyl-terminal tail may be the major site for this phosphorylation. Mammalian GnRH-Rs lack this potential phospho-acceptor site, because the carboxyl-terminal domain is completely absent and an understanding of the role of such sites may be key to understanding the physiological relevance of the lack of a carboxyl-terminal tail within the mammalian GnRH-Rs.

Agonist-induced phosphorylation of GPCRs seems to be involved in regulating several aspects of their function, including desensitization, internalization, and the switching of coupling between different G proteins (Ferguson et al., 1996; Daaka et al., 1997; Lefkowitz, 1998). In the current study, we have examined the potential effects on agonist-induced internalization and, in particular, whether agonist-induced phosphorylation of Ser363 is important for sequestration of the catfish GnRH-R. Compared with the catfish GnRH-R, the S363A mutant (which is not phosphorylated), but not the S356A mutant (which is phosphorylated), had a significantly greater proportion of cell surface receptors ( $R_{s,ss}$ ) in the pres-



**Fig. 8.** Effect of  $\beta$ -arrestin on internalization of wt, stop368 and S363A mutant catfish GnRH-Rs. COS-7 cells transiently cotransfected with wt (A), stop368 (B), or S363A (C) mutant catfish GnRH-R cDNA together with either  $\beta$ -arrestin cDNA (●) or pcDNA3 vector (○) were incubated with 1 nM  $^{125}$ I-labeled cGnRH-II for the indicated time. Surface-bound radioactivity was then determined as the radioactivity that could be removed by acid wash. Internalized radioactivity was determined after solubilization of the cells as described under *Experimental Procedures*. A curve has been fitted to the data points as described by Koenig and Edwardson (1997). The results shown are the mean  $\pm$  S.E. of triplicate observations from a single representative experiment.



ence of agonist. This suggests that loss of the phospho-acceptor site at Ser363 reduces the extent of agonist-mediated internalization in agreement with reports demonstrating that mutation of serine and threonine residues in the carboxyl-terminal domains of other GPCRs results in diminished agonist-induced internalization (Benya et al., 1993; Naik et al., 1997; Pohl et al., 1997).

It has been demonstrated previously that the catfish GnRH-R is internalized at twice the rate of the tailless rat GnRH-R (Heding et al., 1998); we demonstrate in the current study that truncation of the catfish GnRH-R results in a reduction in agonist-induced internalization. This is consistent with the observation that complete truncation of the carboxyl-terminal tail of another nonmammalian (chicken) GnRH-R reduces its rate of agonist-induced internalization levels equivalent to the relatively low rate of the human GnRH-R (Pawson et al., 1998). This crucial role of the carboxyl-terminal tail within GPCRs in general is also highlighted by the observation that progressively larger truncations of the mammalian thyrotropin-releasing hormone receptor or gastrin-releasing peptide receptor increasingly impair internalization (Benya et al., 1993; Nussenzveig et al., 1993; Yu and Hinkle, 1998). We suggest that this may be associated, at least in part, with the loss of a phospho-acceptor site (or sites), which in the catfish GnRH-R is Ser363. However, our results also demonstrated that the stop368 receptor exhibited markedly impaired internalization despite containing Ser363 and undergoing agonist-dependent phosphorylation. These data suggest that multiple domains are involved in regulating internalization, as has been demonstrated for the mammalian GnRH-R, which also lacks a carboxyl-terminal tail and potential regulatory phospho-acceptor sites (Arora et al., 1995, 1997). In the case of the catfish GnRH-R, both phosphorylation and the last 12 amino acids of the carboxyl-terminal tail may, therefore, be important for tail conformation to increase the accessibility of molecular entities involved in receptor internalization. The adaptor protein  $\beta$ -arrestin might well be such an entity, and it is known that  $\beta$ -arrestin is abundantly expressed in HEK 293 cells (Menard et al., 1997). We demonstrate that  $\beta$ -arrestin plays a role in catfish GnRH-R internalization, and loss of the phospho-acceptor site Ser363, as well as truncation of the last 12 amino acids of the carboxyl-terminal tail, indeed largely impair the effect of  $\beta$ -arrestin on receptor internalization. The fact that stop368, stop356, and S363A mutant receptors showed a similar decrease in internalization strengthens our idea that stop368 and S363A mutant receptors might not have additive effects but contribute to the same process of  $\beta$ -arrestin binding. Our data agree with the current model for agonist-induced GPCR internalization, which suggests that agonist stimulation is followed by receptor phosphorylation and either the generation or stabilization of a conformation that increases the affinity for  $\beta$ -arrestin. The binding of  $\beta$ -arrestin seems not only to mediate receptor desensitization, but also to target GPCRs for clathrin-coated, vesicle-mediated endocytosis (Ferguson et al., 1996, 1998; Lefkowitz, 1998). It is unlikely that the effects of truncation, phosphorylation, and interaction with  $\beta$ -arrestin on the rate of internalization are mediated in part by a change in the fraction of GPCRs, because it has been reported that uncoupled thyrotropin-releasing hormone receptors, for

example, exhibit the same internalization as coupled receptors (Petrone et al., 1997; Yu and Hinkle, 1999).

In summary, this study demonstrates that the carboxyl-terminal tail of a nonmammalian GnRH-R is important for cell surface expression and plays a central role in determining agonist binding. In addition, we have identified a major site for agonist-induced phosphorylation within the carboxyl-terminal tail of the catfish GnRH-R and identify this phosphorylation as an important signal for agonist-induced internalization. However, agonist-mediated regulation of internalization of this GPCR is not only dependent upon receptor phosphorylation. Other domains within the carboxyl-terminal tail, at least of the catfish GnRH-R, are crucial in regulating the interaction of the receptor with accessory proteins such as  $\beta$ -arrestin. These regions directly affect processes such as agonist-induced internalization but may well impinge on other aspects of receptor function.

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**Send reprint requests to:** Dr. Jan Bogerd, Department of Experimental Zoology, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands. E-mail: j.bogerd@bio.uu.nl.

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