Casein Kinase II Sites in the Intracellular C-terminal Domain of the Thyrotropin-releasing Hormone Receptor and Chimeric Gonadotropin-releasing Hormone Receptors Contribute to β-Arrestin-dependent Internalization*

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We have previously shown that the mammalian gonadotropin-releasing hormone receptor (GnRHR), a unique G-protein-coupled receptor (GPCR) lacking an intracellular carboxyl tail (C-tail), does not follow a β-arrestin-dependent internalization pathway. However, internalization of a chimeric GnRHR with the thyrotropin-releasing hormone receptor (TRHR) C-tail does utilize β-arrestin. Here, we have investigated the sites within the intracellular C-tail domain that are important for conferring β-arrestin-dependent internalization. In contrast to the chimeric GnRHR with a TRHR C-tail, a chimeric GnRHR with the catfish GnRHR C-tail is not β-arrestin-dependent. Sequence comparisons between these chimeric receptors show three consensus phosphorylation sites for casein kinase II (CKII) in the TRHR C-tail but none in the catfish GnRHR C-tail. We thus investigated a role for CKII sites in determining GPCR internalization via β-arrestin. Sequential introduction of three CKII sites into the chimera with the catfish C-tail (H354D, A366E, G371D) resulted in a change in the pattern of receptor phosphorylation and β-arrestin-dependence, which only occurred when all three sites were introduced. Conversely, mutation of the putative CKII sites (T365A, T371A, S383A) in the C-tail of a β-arrestin-sensitive GPCR, the TRHR, resulted in decreased receptor phosphorylation and a loss of β-arrestin-dependence. Mutation of all three CKII sites was necessary before a loss of β-arrestin-dependence was observed. Visualization of β-arrestin/GFP redistribution confirmed a loss or gain of β-arrestin sensitivity for receptor mutants. Internalization of receptors without C-tail CKII sites was promoted by a phosphorylation-independent β-arrestin mutant (R169E), suggesting that these receptors do not contain the necessary phosphorylation sites required for β-arrestin-dependent internalization. Apigenin, a specific CKII inhibitor, blocked the increase in receptor internalization by β-arrestin, thus providing further support for the involvement of CKII. This study presents evidence of a novel role for C-tail CKII consensus sites in targeting these GPCRs to the β-arrestin-dependent pathway.

Although the receptors for the hypothalamic releasing factors, gonadotropin-releasing hormone (GnRH)1 and thyrotropin-releasing hormone (TRH), belong to the rhodopsin family of G-protein-coupled receptors (GPCRs), the mammalian GnRH receptor is distinct from other members of the GPCR family, including nonmammalian GnRH receptors, in that it lacks the functionally important C-terminal domain. This structural difference raises questions about the role of the functional domains of these receptors in desensitization, internalization, and down-regulation. Chimeric GnRH and TRH receptor constructs have provided us with a useful tool for dissecting molecular interactions involved in determining the phosphorylation status of these receptors and the resultant effects on receptor function and trafficking. We have previously shown that the absence of a C-terminal tail (C-tail) is responsible for the slow internalization kinetics of the mammalian GnRH receptor and its inability to undergo acute desensitization (1, 2). The GnRHR is not phosphorylated upon activation and is β-arrestin-independent in comparison with the TRHR that does undergo phosphorylation and is internalized in a β-arrestin-dependent manner (1, 3). The addition of the C-tail from the TRHR to the carboxyl-terminal end of the GnRHR results in a chimeric receptor that is phosphorylated (3) and undergoes β-arrestin-dependent internalization (4).

The current model for regulating agonist-activated GPCRs involves recruitment of arrestins, which cause rapid desensitization by uncoupling the receptor from its cognate G-protein to attenuate signaling (5) and to target the receptor into clathrin-coated vesicles for its internalization into endosomes (6). Receptor phosphorylation is a prerequisite step in β-arrestin-mediated internalization (7).

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1 The abbreviations used are: GnRHR, gonadotropin-releasing hormone receptor; GPCR, G-protein-coupled receptor; CKII, casein kinase II; TRH, thyrotropin-releasing hormone; TRHR, TRH receptor; GRK, G-protein coupled receptor kinase; PKC, protein kinase C; C-tail, carboxyl-terminal tail; HEK, human embryonic kidney; cf, catfish; cfGnRHR, catfish GnRHR; HA, hemagglutinin; IP, inositol phosphate; GFP, green fluorescent protein; WT, wild type.
binding (7), although a few GPCRs can internalize via β-arrestin in a phosphorylation-independent manner (8, 9). G-protein receptor kinases (GRKs) have been identified as the kinases involved in GPCR phosphorylation, although consensus GRK phosphorylation sites on GPCRs have not yet been clearly defined. In some cases, GPCR internalization via the β-arrestin-dependent pathway is not regulated by the GRKs (10); however, the kinases involved remain uncertain. Other kinases implicated in GPCR internalization are the second messenger kinases, protein kinase C (PKC) and protein kinase A. These kinases have been shown to be involved in internalization of somatostatin receptor type 2A (11), secretin receptor (12), gastrin-releasing peptide receptor (13), and the β-arrestin-2 dependence of the parathyroid hormone receptor 1 (14). Phosphorylation by casein kinase 1α is involved in the regulation of M1 and M3 muscarinic receptors (15–17) and is also involved in the endocytosis of the yeast GPCR Ste3p (18).

A large number of GPCRs contain multiple consensus sites for casein kinase II (CKII). CKII is a highly conserved serine/threonine kinase expressed ubiquitously in all eukaryotic organisms (19). This tetrameric kinase is composed of two α and two β subunits, and it phosphorylates and interacts with a myriad of proteins involved in diverse cellular functions such as signal transduction, growth, and proliferation. CKII also plays a role in the internalization of single transmembrane receptors such as CD5 (20) and the transferrin receptor (21). To date, the only evidence for a role of this kinase in GPCR function has been in the regulation of the “frizzled” receptor Wnt signaling pathway (22).

We observed that chimeric GnRH receptors with extended C-tails containing CKII consensus sites gained β-arrestin-dependence, whereas GnRH receptors with C-tails lacking these sites remained β-arrestin-insensitive. This prompted us to investigate the role of CKII sites in GPCR regulation. This study provides evidence for a novel role of C-terminally located CKII sites in determining the sensitivity of a GPCR to internalize via the β-arrestin-dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—[32P]tyr-N,N'-EtGnRH, staurosporine, and apigenin were supplied by Sigma. GnRH agonist (leuprolide) was obtained from Abbott Australasia, and TRH was obtained from Peninsula Laboratories Europe Ltd. (Merseyside, United Kingdom). “H-Labeled [Me-His2]TRH was supplied by PerkinElmer Life Sciences.

**Cell Culture**—HEK 293 and COS-1 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, glutamine (0.3 mg/ml), and penicillin-streptomycin (100 units/ml) (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO2 in air. Prior to transfection, cells were plated in either 100- or 60-mm dishes at 60–80% confluency and transfected with 10 or 5 μg of total cDNA, respectively, using Superfect (Qiagen). Cells were maintained 48–72 h post-transfection.

**Expression Constructs and Mutagenesis**—The rat GnRH/rat TRHR C-tail chimera (GnRHC1TRHR tail), rat GnRH/catfish GnRHC1 C-tail chimera (GnRHC1cf tail), and the GFP/β-arrestin-1 fusion protein have been described previously (3, 4). Introduction and elimination of CK II sites was performed using the QuickChange site-directed mutagenesis kit (Stratagene, Sydney, Australia) according to the manufacturer’s instructions. β-Arrestin-1 cDNA was a gift from Prof. L. J. Benovic (Jefferson Medical College, Philadelphia, PA). The phosphorylation-independent β-arrestin-1 mutant (R119E) was kindly provided by Prof. V. V. Gurevich (Sun Health Research Institute, Sun City, AZ). To examine receptor phosphorylation, an epitope tag sequence was incorporated into the receptor to allow immunoprecipitation. HA-tagged TRHR and GnRH/cf tail have been previously validated and used for phosphorylation studies (3). Introduction of CKII sites was carried out in the HA-tagged GnRH/cf tail. To HA-tag the TRHR –3CKII, a restriction site 3’ of the coding region of HA TRHR, located in the multiple coding region of pcDNA3 (ApoE), and an internal restriction site 3’ of the HA tag TRHR (EcoRV) were chosen in order to subclone the TRHR –3CKII digested with the equivalent restriction sites. Sequences of all cDNA clones were verified using Dye Deoxy sequencing and an ABI 373 sequencer (PE Applied Biosystems).

**Iodination of GnRH Agonist**—Iodinated [32P]tyr-N,N'-EtGnRH was prepared using the lactoperoxidase method and purified by chromatography on a Sephadex G-25 column in 0.1 M acetic acid, 0.1% formic acid, 0.4 M NaCl, and 1 M sucrose. The specific activity was 47 Ci/mmol and was calculated as previously described (23).

**Total Inositoh Phosphate Assays**—Total IPs were separated and analyzed as described previously (24). Briefly, 24 h after transfection, cells were plated into 24-well plates with 0.5 ml of isotope-free Dulbecco’s modified Eagle’s medium containing 1% dialyzed fetal calf serum and 5% CO2 at 37 °C for 24 h with [3H]inositol (2 μCi/ml, Amersham Pharmacia Biotech). 48 h posttransfection, membranes were washed twice with buffer A (1 mg/ml fatty acid-free bovine serum albumin, 140 mM NaCl, 20 mM Hepes, 4 mM KCl, 8 mM Tris-glucose, 1 mM MgCl2, 1 mM CaCl2) followed by incubation for 10 min with buffer A containing 10 μM LiCl with or without the addition of either TRH or GnRH agonist (1011 to 1014 M) at 37 °C for 60 min. The assay buffer was removed, and cells were incubated at 4 °C for 30 min with 10 μM formic acid and subsequently transferred to tubes containing Dowex (AG 108) anion exchange resin (Bio-Rad). Total IPs were then eluted, and the amount of radioactivity was counted. All treatments were performed in triplicate in at least three separate experiments.

**Receptor Phosphorylation, Immunoprecipitation, and Immunoblotting**—Methods for the phosphorylation and immunoprecipitation of epitope-tagged receptors were described previously (25). In brief, COS-1 cells were transiently transfected with epitope-tagged receptors or vector in 12-well plates. Cells were serum-starved for 16 h, loaded with [32P]Pi, (200 μCi/ml), and stimulated for 20 min at 37 °C with either 100 nM GnRH agonist for the GnRH receptors or 100 nM TRH for the TRHR receptors. After stimulation, the plates were placed on ice and washed twice with 1 ml/well Hank’s buffered salt solution (4 °C), and the cells were solubilized with 300 μl of a lysis buffer containing phosphatase inhibitors (25). Cell lysates were centrifuged (14,000 × g, 15 min) and preclarified by adding bovine serum albumin and protein A-agarose for 1 h at 4 °C. The epitope-tagged receptors were immunoprecipitated from the preclarified lysates by adding 2 μg of affinity-purified 12CA5 monoclonal antibody and 20 μl of protein A-agarose. Following overnight agitation at 4 °C, the immunoprecipitates were washed five times, resuspended in 55 μl of a urea-based SDS sample buffer, heated at 60 °C for 15 min, and resolved by 10% SDS-polyacrylamide gel electrophoresis. Gels were Western blotted to polyvinylidene difluoride membrane, and phosphorylated bands were detected and quantified by PhosphorImaging. To compare the level of receptor expression for the various constructs, the membranes were subsequently probed with a rat monoclonal anti-HA high affinity antibody (3F10; Roche Molecular Biochemicals) and an anti-rat IgG-horseradish peroxidase complex (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by enhanced chemiluminescence.

**Receptor Internalization Assays**—Receptor internalization assays were done as previously described (1). Briefly, cells in 24-well plates were agonist treated with labeled agonist for time intervals from 5 min to 2 h at 37 °C. Surface-bound radioactivity was removed by washing with acid solution (50 mM acetic acid, 150 mM NaCl, pH 2.8). Internalized radioactivity was determined after solubilizing cells in 0.2 M NaOH, 1% SDS. Non-specific binding for each time point was determined under the same conditions in the presence of 1 μM unlabeled agonist. After subtraction of non-specific radioactivity, internalized radioactivity was expressed as a percentage of the total binding. All time point measurements were performed in duplicate in at least three separate experiments.

**Visualization of GFP/β-Arrestin**—Transfected HEK 293 cells were plated onto poly-l-lysine-coated eight-well chamber slides. Treatments were carried out 48 h post-transfection, and cells were fixed in 4% paraformaldehyde, mounted in FluoroGuard (Bio-Rad), and sealed with coverslips. Cells were examined under an oil immersion objective (× 60) using a Bio-Rad confocal laser microscope with a filter selective for fluorescein isothiocyanate. Optical sections (1.0 μm) were taken, and representative sections corresponding to the middle of the cells are presented.

**Statistical Analysis**—Statistical significance was determined using Student’s t test. Differences in means with significance at p < 0.05 were considered to be statistically significant.

**RESULTS**

**β-Arrestin-independent Internalization of the GnRH/cf Tail Chimera**—We have previously shown that internalization of the rat GnRH is not affected by overexpression of β-arres-
extended this study to investigate the effect of tin (p showed an increased EC50 value for the GnRHR/cf tail three added CKII sites had slightly higher basal signaling than...tion was similar for each receptor, the GnRHR/cf tail with...Tail Chimera Results in a chimera that displayed...dependent pathway. suggested that other factor(s), in addition to the presence of a...C-tail to the carboxyl terminus of the GnRHR (GnRHR/TRHR...RHR/cf tail chimera in the presence of...Agonist-dependent total IP accumulation was measured for the...receptor and pcDNA3 (white bars) or with receptor and β-arrestin (black bars). Internalization assays were carried out in duplicate samples following agonist treatment (60 min). The data represent values of at least three independent experiments. ***, p < 0.01 as compared with receptor transfected with pcDNA3.

In COS cells or by the high endogenous levels of β-arrestin when expressed in HEK 293 cells (1). The addition of the TRHR C-tail to the carboxyl terminus of the GnRHR (GnRHR/TRHR tail) created a chimera that displayed β-arrestin-dependent internalization (4). Treatment of transfected COS-1 cells with GnRH agonist for 60 min caused a significant increase in the levels of GnRHR/TRHR internalized in the presence of β-arrestin (p < 0.01) (Fig. 1), confirming previous results (4). We extended this study to investigate the effect of β-arrestin on the internalization of the GnRHR with another GPCR C-tail, the nonmammalian cfGnRHR C-tail. Interestingly, we found no increase in the levels of receptor internalized with the GnRHR/cf tail chimera in the presence of β-arrestin (Fig. 1). This suggested that other factor(s), in addition to the presence of a C-tail, were involved in targeting the GnRHR to the β-arrestin-dependent pathway.

The Addition of a Series of CKII Sites into the GnRHR/cf Tail Chimera Results in β-Arrestin-dependent Internalization—Given that the TRHR and cfGnRHR tails differed in their ability to confer β-arrestin-dependence to the GnRHR, we compared the consensus phosphorylation sites present in the C-tails of these receptors. Both the cfGnRHR and TRHR C-tails contain consensus phosphorylation sites for PKC; however, the wild type (WT) cfGnRHR C-tail contains no consensus CKII sites (Fig. 2A), while the TRHR C-tail contains three CKII sites (Fig. 2B). We employed site-directed mutagenesis to sequentially introduce one, two, and three CKII sites, utilizing existing serines, into the C-tail of the GnRHR/cf tail chimera at amino acid positions A366E (+1CKII), G371D (+2CKII), and H354D (+3CKII). These amino acids were chosen in order to create CKII consensus sites with minimal change to the C-tail sequence. Each mutated construct bound 125I-labeled GnRH agonist comparable with that of wild type (data not shown).

Agonist-dependent total IP accumulation was measured for the WT and +3CKII construct under a range of ligand concentrations (10^-5 to 10^-11 M) (Fig. 3). Although the maximal stimulation was similar for each receptor, the GnRHR/cf tail with three added CKII sites had slightly higher basal signaling than the GnRHR/cf tail receptor. The IP dose-response curves showed an increased EC50 value for the GnRHR/cf tail +3CKII compared with the WT receptor (0.667 ± 0.234 nM for WT and 4.55 ± 0.082 nM for +3CKII).

The effect of sequential addition of CKII sites into the cytoplasmic C-tail of the chimeric GnRHR/cf tail was evaluated in the absence or presence of β-arrestin (Fig. 4). COS-1 cells were transfected with either receptor and pcDNA3 vector or with receptor and β-arrestin, and the levels of internalized receptor were measured following agonist treatment (5–120 min). In the absence of β-arrestin, all mutated receptors internalized at the same rate as WT receptor (Fig. 4). Co-expression of β-arrestin had no effect on the internalization rate of WT GnRHR/cfGnRHR, +1CKII, or +2CKII chimeras. However, three CKII sites within the GnRHR/cfGnRHR C-tail (+3CKII) resulted in a significant increase in the internalization rate in the presence of β-arrestin at all time points (Fig. 4D). To determine whether the third CKII site alone was responsible for the β-arrestin-dependent increase of the +3CKII construct, a single mutation at residue 354 (H354D) was introduced into the GnRHR-R/cf tail, and the effect of β-arrestin on the internalization of GnRHR-R/cf tail H354D was assessed. Co-expression of β-arrestin with H354D receptor did not enhance GnRH-promoted internalization (Fig. 4E). Thus, we hypothesize that at least three CKII consensus sites are required for internalization of the GnRHR/cf tail via the β-arrestin-dependent pathway.

Mutation of CKII Sites in the TRHR Results in Loss of β-Arrestin-dependent Internalization—To test our hypothesis that the presence of CKII consensus sites plays a role in β-arrestin-dependent internalization of the TRHR, the three CKII sites in the TRHR C-tail were sequentially mutated by substitution of the threonine or serine in the CKII consensus site to alanine (T365A, T371A, S383A) (Fig. 2B). Each mutant TRHR construct displayed levels of ligand binding similar to the WT receptor (data not shown). Measurement of intracellular signaling under a range of agonist concentrations (10^-5 to 10^-11 M) revealed that the magnitude of agonist-induced signaling at maximal concentrations was similar between the WT TRHR and TRHR -3CKII, although removal of all three CKII sites resulted in significantly lower levels of basal signaling and thus decreased levels of IP production at lower TRH doses (Fig. 3). However, the EC50 values for each receptor were similar (7.95 ± 0.189 nM for WT and 8.83 ± 0.055 nM for -3CKII). The effect of β-arrestin on internalization of the TRHR and the mutated TRHRs lacking either one (TRHR -1CKII), two (TRHR -2CKII), or three (TRHR -3CKII) CKII sites was determined in COS-1 cells (Fig. 4). In the absence of β-arrestin, all mutated receptors internalized at the same rate as WT TRHR (Fig. 5). In the presence of β-arrestin, TRHR -1CKII and TRHR -2CKII behaved similarly to WT TRHR. Mutation of all three CKII sites from the TRHR C-tail was necessary before a total loss in β-arrestin-dependent internalization was observed (Fig. 5D). To determine if this effect was due to loss of the third CKII site alone, a TRHR with only the third mutation (S383A) was examined for its effect on β-arrestin-dependent internalization. The S383A receptor displayed a β-arrestin-dependent promotion in internalization to similar levels as exhibited by WT TRHR (Fig. 5E). These results suggest that at least one CKII consensus site in the TRHR C-tail is required to confer β-arrestin-dependent internalization.

Visualization of the Effect of Receptor C-tail CKII Sites on β-Arrestin/GFP Trafficking—The effect of C-tail CKII sites on β-arrestin sensitivity of the receptor constructs was confirmed by visualization of the cellular distribution of GFP-tagged β-arrestin using confocal microscopy. HEK 293 cells co-expressing GFP/β-arrestin with either WT or mutated receptors were analyzed for agonist-dependent redistribution of β-arrestin from the cytoplasm to the plasma membrane. The distribution of β-arrestin/GFP was mainly cytoplasmic in untreated cells expressing all receptors studied (Fig. 6, A, C, E, and G). After agonist treatment, cells expressing the GnRHR/cf tail chimera (Fig. 6B), +1CKII, or +2CKII mutants (results not shown) showed no change in β-arrestin distribution. However, in cells expressing the GnRHR/cf tail +3CKII, there was a redistribution of GFP-β-arrestin to the plasma membrane within 3 min of
agonist stimulation, thus confirming the β-arrestin dependence of this receptor (Fig. 6D). Similar experiments were carried out with the WT and mutated forms of rat GnRHR with an added cfGnRHR C-tail (GnRHR/cf tail) (A) and TRHR (B) are shown. Sequential CKII sites have been either added (GnRHR/cf tail) or removed (TRHR) by alanine substitution, and numbers refer to the relevant amino acid positions in the wild type receptor sequences. The CKII motifs ((S/T)X(D/E)) are shaded, the PKC motifs ((S/T)X(R/K)) are underlined, and mutated residues are in boldface type.

![Fig. 2. Structure of the C-terminal domains of the cfGnRHR and the TRHR, with sequential addition or removal of CKII consensus sites, respectively.](image)

![Fig. 3. Agonist mediated total IP production of wild type and mutated GnRHR/cf tail and TRHRs.](image)

![Fig. 4. Influence of β-arrestin on internalization of GnRHR/cf tail chimeras with sequentially added C-tail CKII consensus sites.](image)
ceptors can still use receptor (27) was used to determine whether the mutant protein from discriminating the phosphorylation state of the mutation of the third CKII site alone (see Fig. 2). Assays were carried to 2 h) following agonist treatment.

internalization assays were carried out at different time points (5 min compared with WT GnRHR/cf tail (Fig. 7).

suggested that the receptor kinases required for β-arrestin sensitivity of these receptors are not rate-limiting in COS-1 cells. Results obtained with R169E β-arrestin demonstrate that receptors with no CKII sites in the C-tail are able to undergo phosphorylation-independent β-arrestin internalization. This suggests that the mutations do not affect β-arrestin-dependent internalization in a nonspecific manner, but rather the phosphorylation status of the receptor affects their insensitivity.

Consequences of the C-tail CKII Sites on Receptor Phosphorylation—The capacity of β-arrestin R169E to promote the
internalization of receptors lacking C-tail CKII sites suggests that specific receptor phosphorylation sites, required for WT arrestin binding, were absent from these receptors. Therefore, we examined basal and agonist-stimulated phosphorylation of the β-arrestin-independent receptors, containing no C-tail CKII sites (GnRHR/cf tail and TRHR −3CKII), and compared this to their β-arrestin-dependent counterparts (GnRHR/cf tail +3CKII and TRHR). The HA-tagged GnRHR/cf tail and TRHR have been previously validated and used in phosphorylation studies (3). The mutagenesis was carried out in the HA GnRHR/cf tail; thus, the TRHR −3CKII was HA-tagged for this study as described under “Experimental Procedures.” Epitope tagging of this receptor did not affect receptor function or its β-arrestin-independence (data not shown). COS-1 cells transfected with HA-tagged versions of these receptors were pretreated with receptor and pcDNA3 (white bars), receptor and WT β-arrestin (black bars), or receptor and R169E β-arrestin (lined bars). Top, WT GnRHR/cf tail or GnRHR/cf tail +3CKII; bottom, WT TRHR or TRHR −3CKII. Internalization assays were carried out in duplicate samples following agonist treatment (60 min). The data represent values of four independent experiments. *, p < 0.05; **, p < 0.01 as compared with WT receptor.

The phosphorylation status and expression of WT TRHR and TRHR −3CKII were also examined and are shown in Fig. 9B. The WT and mutant TRHR are strongly phosphorylated upon the addition of agonist (Fig. 9B and Ref. 3) and run as two broad bands in the range 55–130 kDa that probably represent the mature monomer (band centered at about 60 kDa) and dimer (band centered at about 120 kDa). Phosphorylation of the CKII consensus sites in the C-tail of WT TRHR occurs upon agonist stimulation as removal of these sites resulted in an approximate 20% reduction of phosphorylated receptor (Fig. 9B, compare lane 6 with lane 4). The remaining agonist-dependent phosphorylation of the TRHR −3CKII most likely occurs on other serines/threonines present in the receptor tail, mediated by additional kinases involved in TRHR regulation.

A Specific CKII Inhibitor, Apigenin, Inhibits β-Arrestin-dependent Internalization—To support our hypothesis that CKII is involved in the β-arrestin dependence of the GnRHR/cf tail +3CKII and the WT TRHR, we used the flavonoid compound apigenin, which is known to be a specific inhibitor of CKII enzymatic activity (28). COS-1 cells were transfected with receptor in the presence or absence of β-arrestin. Internalization assays were carried out on cells pretreated with different kinase inhibitors (PKC inhibitor (staurosporine) and CKII inhibitor (apigenin)) at their effective doses (14, 22). Internalization in the absence of β-arrestin of either the GnRHR/cf tail +3CKII sites or the TRHR was similar to control values following pretreatment with staurosporine or apigenin (Fig. 10). In the
presence of β-arrestin, staurosporine had no effect on the β-arrestin-dependent increase in the internalization of either receptor when compared with control (Fig. 10), while apigenin resulted in a complete inhibition of the β-arrestin-dependent internalization of both receptors. This selective inhibition supports a role for CKII, and not PKC, in determining the sensitivity of the WT TRHR and the GnRHR/cf tail +3CKII to internalize via the β-arrestin-dependent pathway.

**DISCUSSION**

The role of β-arrestin in GPCR regulation has been extensively studied (29–31); however, the functional receptor domains that determine β-arrestin sensitivity are not fully understood. The objective of the present study was to investigate determinants associated with β-arrestin sensitivity in the C-terminal domain of GPCRs. By using GnRHR chimeric constructs, we have previously presented evidence of a causal relationship between β-arrestin sensitivity and the presence of a functional C-tail (4). Chimeric GnRHRs were generated by adding a C-tail from another GPCR to the normally β-arrestin-insensitive GnRHR and provided us with a useful tool for the study of the functionally important receptor C-tail domains. In addition, the TRHR, a GPCR known to be β-arrestin-sensitive (1, 32, 33) was examined for determinants that confer β-arrestin dependence. We present the unique finding that CKII consensus sites in the GPCR C-terminal domain can regulate β-arrestin sensitivity and subsequent receptor sequestration.

The C-tails of two β-arrestin-sensitive GPCRs, the TRHR and cGnRHR, were used to construct the chimeric GnRHRs. Intriguingly, only the chimeric GnRHR with the TRHR C-tail exhibited β-arrestin-dependent internalization. This finding suggests that the presence of a C-tail alone is not the only requirement and that additional information within this domain is needed to confer β-arrestin sensitivity. The C-tail has varying importance in the internalization of different GPCRs. Truncation of the M2 muscarinic receptor C-tail does not affect its internalization, since the main site for β-arrestin interaction is within the third intracellular loop (34, 35), whereas truncation of the TRHR C-tail abolishes internalization (33). This suggests that the information necessary for internalization is contained within the TRHR C-tail, and our finding that the addition of this region to the GnRHR results in β-arrestin independence supports this notion. Sequence comparisons between the TRHR C-tail and the cGnRHR C-tail revealed that both contained consensus sites for PKC, but there were no consensus sites for CKII in the cGnRHR C-tail. This led us to hypothesize that the presence of C-tail CKII sites can determine β-arrestin dependence. Therefore, mutant GnRHR/cf tail constructs introducing single, double, and triple CKII sites into the C-tail were tested for β-arrestin sensitivity. Introduction of CKII sites into the GnRHR/cf tail chimera resulted in β-arrestin independence; however, this was only observed when three CKII sites were present. The addition of three C-tail CKII sites did not affect levels of ligand binding or the magnitude of agonist-dependent intracellular signaling. However, for the GnRHR/cf tail +3CKII, a small but significant increase in basal signaling was evident, and the IP dose profile was slightly different with a small increase in the EC50 value compared with the receptor with no added CKII sites. All three CKII sites were needed for β-arrestin-dependent internalization, since a mutant containing the third CKII site alone (H354D) was still β-arrestin-independent. Multiple, as opposed to single, phosphorylation sites are also required for the regulation of other GPCRs (36–38); also, in a model of arrestin binding, at least three receptor phospholipase sites were
found to be required (39). Our findings were supported by confocal microscopy of cells expressing GFP/β-arrestin, in that only cells expressing the chimera with 3CKII sites displayed a redistribution of GFP/β-arrestin. The time frame of the β-arrestin translocation for the GnRH/cf tail +3CKII was similar to that observed for the GnHR/TRHR chimera (4). In addition, the higher levels of internalization for the GnRH/cf tail +3CKII compared with wild type in HEK 293 cells were similar to levels previously published for the β-arrestin-sensitive GnRHR/TRHR chimera (4).

The finding that the GnRH/cf tail chimera was β-arrestin-independent was surprising, considering that the cGnRH is β-arrestin-dependent (4, 40) and C-tail truncations of this receptor affect internalization promoted by β-arrestin (40). In addition, the GnRH/cf tail chimera undergoes agonist-dependent phosphorylation most likely at the C-tail serine 363, which is the main site of phosphorylation for the cGnRH but is more important in receptor signaling than internalization (3, 40). The finding that the GnRH/cf tail chimera is β-arrestin-insensitive suggests that this chimera does not contain the phosphorylation sites sufficient for β-arrestin-dependent internalization; this is supported also by the phosphorylation-independent internalization exhibited with β-arrestin R169E. The suggestion that multiple domains of the cGnRH are involved in β-arrestin-dependent internalization (40) may provide an explanation for this observation. Agonist-dependent phosphorylation was not increased in the GnRH/cf tail +3CKII, although additional phosphorylated bands corresponding to the molecular weight for this receptor in both untreated and treated samples were evident. A possible explanation is that the addition of consensus sites for CKII results in constitutively phosphorylated receptor and that the addition of agonist activates the receptor, which can then promote β-arrestin binding. This fulfills the two requirements of β-arrestin binding: (i) agonist receptor phosphorylation and (ii) agonist activation. The confocal studies with β-arrestin/GFP and the GnRH/cf tail +3CKII displayed no translocation prior to agonist stimulation, thus excluding the possibility that constitutive phosphorylation would result in constitutive binding of β-arrestin. In addition, there is the possibility that the mutations that introduced the CKII sites in to the cf tail also may affect receptor conformation, which, along with phosphorylation, may enhance accessibility of proteins involved in receptor endocytosis (40).

The hypothesis that CKII sites are required for β-arrestin dependence of the GnRH/cf tail chimera was further supported by the analysis of the role of these sites in a β-arrestin-dependent GPCR, the TRHR. Sequential mutation of three CKII sites in the TRHR C-tail did not alter ligand binding or affect the magnitude of agonist-dependent signaling. However, there was a significant decrease in basal and agonist-induced signaling at lower concentrations of TRH. It is unclear why these changes occur for both the TRHR or GnRH constructs, but they may reflect changes in receptor conformation in mutated receptors, which possibly alter G-protein coupling in the inactive or active state. It seems that the receptors with C-tail CKII sites (WT TRHR, GnRH/cf tail +3CKII) have higher basal signaling than those without C-tail CKII sites (TRHR −3CKII, WT GnRH/cf tail). This is an interesting observation, which is under further investigation.

Sequential mutation of CKII sites in the TRHR C-tail resulted in a receptor that was insensitive to β-arrestin; however, this only occurred when all three sites were removed. Mutation of the third CKII site alone (S383A) did not alter TRHR internalization compared with WT, suggesting that at least one CKII site in the TRHR C-tail is sufficient to promote β-arrestin-dependent internalization. Furthermore, agonist-dependent phosphorylation of the TRHR −3CKII was reduced compared with WT TRHR, suggesting that these sites are indeed phosphorylated, presumably by CKII, and may contribute to β-arrestin binding and subsequent receptor internalization. However, there was still some degree of receptor phosphorylation of the TRHR −3CKII, probably due to the many other serines/threonines in the TRHR tail. For other GPCRs (e.g., the PAR1 receptor), all serines/threonines must be mutated to alanine before phosphorylation is ablated, yet only a portion of these sites influenced receptor function (i.e., mutation of certain serines/threonines that affected receptor regulation still resulted in full receptor phosphorylation (37)).

The loss of β-arrestin dependence by the removal of CKII consensus sites is supported by two previous studies that showed that progressive truncations of the TRHR C-tail affected receptor internalization in HEK 293 cells (41, 42). It would appear from these studies that receptors with C-tail truncations that include one or more CKII sites behave like wild type receptor, and only C-tail truncations with all three CKII sites removed decrease receptor internalization. We obtained comparable results in HEK 293 cells where only the internalization of TRHR −3CKII was affected. However, this does raise the question of the significance of having more than one CKII site in the TRHR C-tail, particularly since all three CKII sites are conserved between different mammalian species. It could be argued that the introduction of mutations into these receptors has resulted in a nonspecific effect on β-arrestin sensitivity and that CKII sites may not be involved in the recruitment of β-arrestin. A mutant form of β-arrestin (R169E) that prevents the protein from discriminating the phosphorylation state of the receptor (27) was used to determine whether receptors could still use β-arrestin following modification of the CKII consensus sites. The receptors used in this study all showed an increase in internalization in the presence of β-arrestin (R169E). For WT TRHR and GnRH/cf tail +3CKII, there was no difference between wild type and R169E β-arrestin in promoting internalization of both receptors, suggesting that GRK phosphorylation is not a limiting factor (COS cells express low levels of GRKs) (26), supporting the involvement of other kinases. The fact that the two receptor constructs that were β-arrestin-insensitive (WT GnRH/cf tail and TRHR −3CKII) were capable of undergoing phosphorylation-independent internalization suggests that the C-tails of these receptors lack phosphorylation sites required for β-arrestin dependence. In addition, the decrease in total receptor phosphorylation of the TRHR −3CKII supports this hypothesis.

From the site-directed mutagenesis studies, we propose that the kinase involved in targeting the TRHR and GnRH/cf tail +3CKII to the β-arrestin pathway is CKII. This hypothesis is supported by the use of a specific CKII inhibitor, apigenin (28). Apigenin pretreatment of cells expressing the WT TRHR and the GnRH/cf tail chimera with three CKII sites completely inhibited the promotion in internalization by β-arrestin. The PKC inhibitor, staurosporine, had no such effect, suggesting that PKC is not involved in the β-arrestin sensitivity of these receptors. The presence of PKC consensus sites in the C-tails of both β-arrestin-sensitive and -insensitive receptors used in this study supports this observation.

Numerous GPCRs have been shown to require phosphorylation by a member of the GRK family for recruitment of β-arrestin (30, 43, 44). Although many receptors are phosphorylated by GRKs, this may not necessarily be involved in receptor internalization (45). In addition, phosphorylation of some GPCRs is not attributed to GRKs (8, 9), which raises the question of the regulatory mechanisms involved. It is possible that CKII or other unidentified kinases may be required in-
stead of, or in concert with, the GRKs, thus extending the repertoire of proteins available for the regulation of the GPCR superfamily. While the functions ascribed to CKII in the literature are fairly extensive, there have been various reports that provide evidence of a role for CKII in receptor endocytosis via clathrin-coated pits (21, 46–51). To date, the only role for CKII in GPCR regulation has been shown for the frizzled receptor, where CKII phosphorylates the downstream signaling molecule disheveled (22). To our knowledge, the present study is the first report of CKII involved in internalization of a GPCR via the β-arrestin-dependent pathway. However, in a study on M3 muscarinic receptor phosphorylation by casein kinase 1α, the authors report that this receptor was also a substrate for CKII, rather than the GRKs that are involved in regulating GPCR function.

To conclude, we present evidence that in order to confer β-arrestin-dependent internalization to the GnRHR, a C-tail containing consensus phosphorylation sites for CKII is required. We also propose that CKII sites have an important role in targeting the TRHR to the β-arrestin-dependent pathway, thus extending the functions ascribed to CKII in the literature.

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