Identification of a Short Linear Sequence Present in the C-terminal Tail of the Rat Follitropin Receptor That Modulates Arrestin-3 Binding in a Phosphorylation-independent Fashion*

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The rat follitropin receptor (rFSHR) is an unusual G protein-coupled receptor in that agonist-induced activation leads to the phosphorylation of the first and third intracellular loops instead of the C-terminal tail. To determine regions of G protein-coupled receptors that affect internalization independently of phosphorylation we examined the effects of truncations of the C-terminal tail of the rFSHR on agonist-induced internalization. Our studies show that progressive truncations of a region flanked by residues 642 and 651 enhance the internalization of human follicle-stimulating hormone (hFSH). Further characterization of a mutant truncated at residue 649 (designated rFSHR-t649) and another mutant in which the 642–651 region was deleted in the context of the full-length rFSHR, designated rFSHR(A642–651), showed that both of them internalized hFSH at rates that were 2–3 times faster than rFSHR-wild type (wt). Like rFSHR-wt, however, the internalization of hFSH mediated by rFSHR-t649 and rFSHR(A642–651) can be inhibited with dominant-negative mutants of the non-visual arrestins or dynamin. Alanine-scanning mutagenesis of the 642–651 region suggests that the effects on internalization are not mediated by a single residue, however. In an attempt to understand the molecular basis of the enhanced internalization of hFSH mediated by these mutants we used an assay that can be readily used to assess the association of the rFSHR with the arrestin-3 in co-transfected cells. Using this assay we were able to show that, when compared with rFSHR-wt, rFSHR(A642–651) displays an ~4-fold enhancement in binding affinity for arrestin-3 and an ~1.7-fold reduction in maximal arrestin-3 binding capacity. We conclude that a short linear sequence present in the C-terminal tail of the rFSHR (642-SATIH-FARKK651) that is not phosphorylated limits internalization by lowering the affinity of the rFSHR for the endogenous non-visual arrestins.

A large number of studies performed during the past several years have established that the binary complex formed by GPCRs and the non-visual arrestins (arrestin-2 and arrestin-3, also known as β-arrestin-1 and β-arrestin-2, respectively) serves as a common molecular intermediate in the agonist-induced G protein uncoupling and internalization of these receptors and in the generation/propagation of G protein-independent signals. The formation of this complex is in turn facilitated by agonist-induced activation and/or by the G protein-coupled receptor kinase (GRK)-catalyzed phosphorylation of the GPCRs (reviewed in Refs. 1 and 2).

The follitropin receptor (FSHR) is a large GPCR (~700 amino acids) that binds a large and complex protein ligand (FSH, M, ~30,000) with nanomolar affinity (3–5). The binding of hFSH to the rat FSHR (rFSHR) promotes the rapid internalization of the agonist-receptor complex (6–8). Agonist-induced internalization of the rFSHR proceeds via a dynamin-dependent pathway, and it involves the GRK-catalyzed phosphorylation of the rFSHR in the first and third intracellular loops and the interaction of the activated/phosphorylated rFSHR with the non-visual arrestins (6–10). The internalization of the rFSHR seems to be more dependent on receptor activation and non-visual arrestin binding than on receptor phosphorylation, however. Thus, co-transfection of cells with the clathrin-binding domains of arrestin-2 or -3 or with a dominant-negative mutant of dynamin induces a substantial inhibition of internalization, whereas mutation of the rFSHR phosphorylation sites or dominant-negative inhibitors of GRKs is generally less effective in inhibiting internalization (6–8). Additional studies conducted with two signaling-impaired mutants of the rFSHR that retain the phosphorylation sites (designated rFSHR-D389N and rFSHR-Y530F) also underscored the importance of receptor activation on non-visual arrestin-mediated internalization (7). These two mutants display impairments in agonist-induced activation and phosphorylation and internalize the bound agonist at a slow rate. Co-transfection with GRK2 partially rescues the phosphorylation of both mutants but rescues only the internalization of rFSHR-D389N, while co-transfection with arrestin-3 rescues the internalization of both mutants (7).

With this information in mind, we initiated a new series of experiments designed to define the structural features of the rFSHR that are important for internalization and for the binding of the non-visual arrestins. Because we are particularly interested in defining features that affect these processes without affecting phosphorylation, we concentrated on the C-terminal tail of the rFSHR because this region of the rFSHR is not phosphorylated upon agonist-induced activation (6, 7, 10).

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1 The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; FSH, follicle-stimulating hormone; FSHR, follitropin receptor; rFSHR, rat FSHR; GFP, green fluorescent protein; hFSH, human FSH; wt, wild type.
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MATERIALS AND METHODS

Plasmids and Cells—A full-length cDNA encoding the rFSHR in pcDNAI/Neo has been described (11). Truncations of the C-terminal tail of the rFSHR were constructed using PCR strategies to introduce a stop codon in the position immediately following the new desired C terminus. The deletion of the 642–651 region from the rFSHR and the simultaneous mutation of these 10 residues to alanines were also accomplished using PCR strategies. The mutation of individual residues in the 642–651 region was accomplished using the Stratagene QuikChange site-directed mutagenesis kit. The rFSHR-wt, rFSHR-K44A, and rFSHR-K44A-wt, and the point and cluster mutations of the 642–651 region were epitope-tagged by introducing the Myc epitope between the predicted C terminus of the signal peptide and the predicted N terminus of the mature receptor as described earlier for the rat and human lutropin receptor (15). Briefly, cells were co-transfected with the pCMV-rFSHR-wt or pCMV-rFSHR-K44A, FLAG-arrestin-3, and dynamin-K44A as indicated in the figure legends. The cells were then incubated with or without a saturating concentration of hFSH (50 nM) at 37 °C as indicated in the figure legends, and then the receptor-arrestin-3 complex was stabilized by cross-linking with dithiobis(succinimidyl propionate), as described elsewhere (16). The methods used to use the cross-linked cells, immunoprecipitate the complexes, and immunoblot the immunoprecipitates have also been described (16).

The immunoprecipitates were resolved on polyacrylamide gels, and electrophoretic blots were prepared as described elsewhere (25). Blots of the immunoprecipitates were incubated with anti-FLAG (M2) or anti-Myc monoclonal antibodies covalently coupled to horseradish peroxidase (final dilution of 1:500 or 1:1000, respectively) for 1 h at room temperature using the blocking and incubation conditions described elsewhere (25). The amount of arrestin-3 expressed was similarly determined using small aliquots of the whole cell lysate containing equivalent amounts of protein. Proteins were directly visualized and quantitated using a combination of the Super Signal West Femto Maximum Sensitivity system of detection (Pierce) and a Kodak digital imaging system. This image-capturing system is set up to alert us when image saturation occurs and to prevent us from measuring the intensity of such images.

Apparent binding constants for the FLAG-arrestin-3/rFSHR interaction were measured using cells co-transfected with increasing amounts of the FLAG-arrestin-3 expression vector as shown in Fig. 6. The amount of FLAG-arrestin-3 present in the immunoprecipitates was corrected for the amount of receptor present in the immunoprecipitates, and the binding data were plotted against the amount of FLAG-arrestin-3 plasmid used for transfection. The binding data were then directly fitted to a hyperbola using the non-linear regression analysis included in the Prism Software package (the regression coefficients for the non-linear regressions were at least 0.98).

Confocal Microscopy—Cells were plated in 8-chamber coverslip culture vessels coated with polylysine (BioCoat from BD PharMingen). They were co-transfected (in a total volume of 400 μl) with 400 ng of Myc-tagged rFSHR, 4 ng of arrestin-3-GFP, and 100 ng of dynamin-K44A using the methods described above. Two days after transfection the cells were incubated with or without hFSH for 20 min at 37 °C as described above for the arrestin-3 binding assays. The preparation of the cells was done as recently described (16). The rFSHR was visualized with the 9E10 monoclonal antibody followed by a secondary antibody labeled with rhodamine, and the arrestin-3-GFP was visualized with a BioRad confocal microscope at the Central Microscopy Facility of The University of Iowa.

Hormones and Supplies—Human kidney 293T cells are a derivative of 293 cells that express the SV40T antigen (26) and were provided to us by Dr. Marlene Hosey (Northwestern University, Chicago, IL). Purified hFSH (AFP-5720D, prepared from human pituitaries) was kindly donated by Dr. George Bousfield (Wichita State University). The 9E10 monoclonal antibody was prepared by the Hybridoma Facility of the Cancer Center of The University of Iowa. The secondary antibody used for detection (pH 7.4) was kindly provided by Dr. George Bousfield (Wichita State University). The 9E10 monoclonal antibody was prepared by the Hybridoma Facility of the Cancer Center of The University of Iowa. The secondary antibody used for detection (pH 7.4) was kindly provided by Dr. George Bousfield (Wichita State University). The 9E10 monoclonal antibody was prepared by the Hybridoma Facility of the Cancer Center of The University of Iowa. The secondary antibody used for detection (pH 7.4) was kindly provided by Dr. George Bousfield (Wichita State University). The 9E10 monoclonal antibody was prepared by the Hybridoma Facility of the Cancer Center of The University of Iowa. The secondary antibody used for detection (pH 7.4) was kindly provided by Dr. George Bousfield (Wichita State University). The 9E10 monoclonal antibody was prepared by the Hybridoma Facility of the Cancer Center of The University of Iowa. The secondary antibody used for detection (pH 7.4) was kindly provided by Dr. George Bousfield (Wichita State University).
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RESULTS

Fig. 1A shows the effect of 23 progressive truncations of the C-terminal tail of the rFSHR on the internalization of hFSH. Each truncated construct was transiently transfected in 293T cells, and internalization was measured during a 9-min incubation at 37 °C with a concentration of 125I-hFSH equivalent to the $K_d$. The results are expressed as internalization index, which is defined as the ratio of internalized/surface-bound ligand (24). This index can be readily used to approximate the rate of internalization because a plot of the internalization index versus time is linear for at least 20 min and the slope of this plot gives the rate constant of internalization (see “Materials and Methods”).

The results of these experiments (Fig. 1A) show that truncations of the C-terminal tail up to residue 652 have no effect on internalization and further truncations to residue 642 enhance the rate of internalization of 125I-hFSH, whereas even more severe truncations (starting at residue 640) inhibit internalization. The effect of truncations that enhance internalization was confirmed by deletion of the appropriate residues (i.e. the 642–665 region) from the full-length rFSHR. As shown in Fig. 1B, a mutant lacking this region, designated rFSHR(Δ642–651), also displayed and enhanced the rate of internalization of hFSH. Alanine scanning mutagenesis showed that mutation of the individual residues present in the 642–651 region does not enhance internalization (Fig. 2). In fact, the only effect on internalization detected with the alanine scanning mutagenesis was a slight decrease in internalization induced by mutation of Ser$^{642}$ (Fig. 2). Because the only charged residues in the 642–651 region are four basic residues (His$^{645}$, His$^{646}$, Arg$^{647}$, and Lys$^{651}$), we also examined the importance of the charge of this region by analyzing an additional mutant in which these four basic residues were simultaneously mutated to alanine. The 9-min internalization index of this cluster mutant (0.45 ± 0.02, n = 3) was not statistically different from that of the rFSHR-wt (0.40 ± 0.02, n = 3).

The molecular basis of the enhanced internalization was next examined using one of the shortest truncations that enhances internalization (i.e. rFSHR-649 and rFSHR(Δ642–651)). Table I shows that the expression and the ligand binding affinity of rFSHR-649 and rFSHR(Δ642–651) are comparable with those of rFSHR-wt. Because the rFSHR can stimulate cAMP and inositol phosphate accumulation (9, 10), we measured the signaling properties of rFSHR-649 and rFSHR(Δ642–651) using the activation of these two pathways as end points. Cells expressing rFSHR-649 or rFSHR(Δ642–651) display basal levels of cAMP comparable with those of cells expressing rFSHR-wt, but their maximal cAMP response to hFSH is reduced by 50 and 20%, respectively (Table I). The EC$^{50}$ for the hFSH-induced cAMP accumulation was also increased 1.6-fold in cells expressing rFSHR(Δ642–651) but was not changed in cells expressing rFSHR-649 (Table I). The basal levels of inositol phosphates were similar in cells expressing rFSHR-wt, rFSHR-649, or rFSHR(Δ642–651), but cells expressing either of these two mutants display a 60–70% reduction in the maximal hFSH-induced inositol phosphate response (Fig. 3). Together these results clearly show that rFSHR activation is impaired by the two mutations examined. These changes are unlikely to be responsible for the increased internalization of hFSH mediated by either of these two mutations, however, because impairments in rFSHR activation have been previously shown to inhibit the internalization of hFSH rather than to enhance it (7). It is also of interest to note that rFSHR-635, a more extensive truncation that impairs internalization (cf. Fig. 1A), has no effect on hFSH binding affinity or receptor expres-
Internalization of the Follitropin Receptor

TABLE I

| Receptor | Kd (nM) | Maximal binding (pmol/10⁶ cells) |
|----------|---------|---------------------------------|
| rFSHR-wt | 1.8 ± 0.2 | 340,000 ± 30,000 |
| rFSHR(t649) | 1.6 ± 0.1 | 333,000 ± 54,000 |
| rFSHR(Δ642–651) | 2.0 ± 0.3 | 390,000 ± 30,000 |

TABLE II

| Receptor | pcDNA3.1 | Arrestin-3(284–409) | Dynamin-K44A |
|----------|----------|---------------------|-------------|
| rFSHR-wt | 15 ± 2 | 98 ± 10* | 236 ± 17* |
| rFSHR(t649) | 3 ± 1 | 45 ± 6* | 121 ± 10* |
| rFSHR(Δ642–651) | 7 ± 1 | 47 ± 5* | 180 ± 14* |

FIG. 3. Human FSH-induced stimulation of inositol phosphate accumulation in 293 cells transiently transfected with rFSHR-wt or mutants thereof. 293T cells were transiently transfected with rFSHR-wt, rFSHR-t649, or rFSHR(Δ642–651) (all at 1 μg/35-mm well) as indicated. The accumulation of [3H]inositol phosphates was then measured in cells incubated with the indicated concentrations of hFSH as described under “Materials and Methods,” and it is expressed as -fold over basal. The basal levels of [3H]inositol phosphates were ~6000 cpm/10⁶ cells and were similar in the cells transfected with the different constructs. Each point shows the average ± S.E. of three independent transfections. The responses of cells expressing rFSHR-t649 or rFSHR(Δ642–651) were statistically different (p < 0.05) from those of cells expressing rFSHR-wt at all concentrations of hFSH tested.

This hypothesis used a recently developed co-transfection/cross-linking/co-immunoprecipitation approach to examine the interaction of the rFSHR-wt and rFSHR(Δ642–651) with arrestin-3 in vivo. In this approach 293 cells are co-transfected with an epitope-tagged GPCR together with an epitope-tagged non-visual arrestin and dynamin-K44A, and the agonist-dependent formation of the GPCR/non-visual arrestin complex is quantitated by detecting the presence of the non-visual arrestin in immunoprecipitates of the GPCR prepared from cross-linked cells. This method has been recently used by us (16) and others (32) to examine the interaction of three GPCRs, the lutropin, β2-adrenergic, and the angiotensin type 2 receptors with arrestin-2 or arrestin-3. Instead of using rFSHR-t649 and rFSHR(Δ642–651) in the arrestin binding assays we chose to use only the deletion mutant. This was done because it is possible that some of the residues deleted could directly participate in the cross-linking step needed to detect arrestin binding (see above and “Materials and Methods”). If this is the case then the deletion of 10 residues (as it occurred in rFSHR(Δ642–651)) is less likely to have a direct effect on cross-linking than the deletion of 26 residues caused by the truncation of the rFSHR at residue 649.

In cells co-transfected with the Myc-rFSHR (wt or Δ642–651), FLAG-arrestin-3, and dynamin-K44A, there is an agonist and time-dependent increase in the formation of the arrestin-3-rFSHR complex that reaches a steady state within a few minutes of addition of hFSH (Figs. 4 and 5). At the end of a 20-min incubation, hFSH stimulation increased arrestin-3 binding to either receptor construct ~5-fold. It is important to stress that co-transfection of the cells with dynamin-K44A prevents internalization (cf. Table II) thus ensuring that changes in the formation of the rFSHR-arrestin-3 complexes are a reflection of events that occur at the cell surface rather than a reflection of differences in the rates of internalization of the rFSHR-wt and rFSHR(Δ642–651). That the association of arrestin-3 with the rFSHR occurs at the cell surface was independently documented using confocal microscopy (Fig. 6). These results are presented in Fig. 6 and show that arrestin-3-GFP is mostly cytosolic, whereas rFSHR-wt and rFSHR(Δ642–651) are localized inside the cell and at the cell.
membrane. Although we did not attempt to ascertain the exact location of the intracellular rFSHR, this is likely to represent a previously described precursor of the mature rFSHR that is located in the endoplasmic reticulum (33). The relative distribution of the intracellular and cell surface forms of rFSHR-wt and rFSHRΔ642–651 appears to be similar, however, and this finding is consistent with the observation that cells expressing either of these two constructs have a comparable density of cell surface receptors (Table I). Most importantly the expression of either wt or Δ642–651 region increases the arrestin-3 binding affinity ~4-fold and decreases the arrestin-3 binding capacity of the rFSHR ~1.7-fold.3

DISCUSSION

The studies presented herein identified a region of the C-terminal tail of the rFSHR, Δ642SATHNFHARKΔ651, that impairs internalization and inhibits the binding of arrestin-3 to the rFSHR. Thus, the deletion of this region in the context of the full-length rFSHR enhances the rate of internalization of hFSH ~2-fold, enhances the binding affinity of arrestin-3 for the rFSHR ~4-fold, and decreases the arrestin-3 binding capacity of the rFSHR ~1.7-fold. The finding that the mutation of any individual residue present in this region fails to affect internalization suggests that this effect is directly or indirectly caused by a combination of at least two residues.

We conclude from these experiments that the enhanced affinity of rFSHRΔ642–651 for arrestin-3 is responsible for the enhanced rate of internalization of hFSH mediated by this mutant. At low levels of endogenous non-visual arrestins, the enhanced affinity of rFSHRΔ642–651 for the non-visual arrestins would result in an increase in non-visual arrestin binding (cf. Figs. 7 and 8) and thus an increase in internalization (cf. Table II). Because the non-visual arrestins also participate in receptor desensitization (2), the enhanced association of rFSHRΔ642–651 with the endogenous non-visual arrestins could also be responsible for the reduction in the signaling properties of this mutant (cf. Fig. 3 and Table I). The magnitude of the changes in signaling properties of rFSHRΔ642–651 and rFSHRΔ649 is in fact similar to the magnitude of changes in signaling of other GPCRs induced by a number of manipulations that either prevent or enhance their ability to

Note that the data presented in Figs. 7 and 8 show that differences in the amount of arrestin-3 bound to rFSHR-wt and rFSHRΔ642–651 would be minimally detectable at the concentration of arrestin-3 used in the time courses displayed in Figs. 4 and 5.

FIG. 4. Time course of the association of the rFSHR-wt and rFSHRΔ642–651 with arrestin-3 in intact cells. 293T cells (plated in 100-mm dishes) were transiently co-transfected with 7.5 μg of Myc-rFSHR-wt + 0.5 μg of FLAG-arrestin-3 + 2.5 μg of dynamin-K44A or with 7.5 μg of Myc-rFSHRΔ642–651 + 0.5 μg of FLAG-arrestin-3 + 2.5 μg of dynamin-K44A. The transfected cells were washed and cross-linked immediately (labeled as 0 min with hFSH) or incubated at 37 °C with 50 nM hFSH for the times indicated prior to cross-linking. The cross-linked cells were washed and lysed with detergents as described under “Materials and Methods.” Aliquots of the different lysates (~18 μl for the top panel and ~500 μl for the middle and bottom panels) containing equivalent amounts of protein were used for measuring the amount of FLAG-arrestin-3 present in the cell lysates (top panels) or the amounts of FLAG-arrestin-3 (middle panel) or Myc-rFSHR (bottom panel) immunoprecipitated with the 9E10 monoclonal antibody. The presence of FLAG-arrestin-3 and Myc-rFSHR was detected using anti-FLAG monoclonal antibody (52) and anti-Myc monoclonal antibody (9E10) covalently modified with horseradish peroxidase, respectively, the Super Signal West Femto Maximum Sensitivity system of detection from Pierce, and a Kodak digital imaging system (see “Materials and Methods” for details). The results of a representative experiment showing only the relevant areas of each blot are shown. IP, immunoprecipitate.

FIG. 5. Quantitation of the time course of the association of the rFSHR-wt and rFSHRΔ642–651 with arrestin-3 in intact cells. The time course of association of arrestin-3 with the rFSHR-wt or rFSHRΔ642–651 was measured as described in Fig. 5. The y axis shows the amount of arrestin-3 bound to either receptor corrected for the amount of receptor present in the immunoprecipitates. Each point is the mean of two independent transfections. The error bars extend to the individual values obtained in each transfection.

are at the cell surface; see Figs. 4–6), and the complexes were cross-linked, immunoprecipitated, and analyzed as described above. The representative experiment illustrated in Fig. 7 shows that the amount of the rFSHR-arrestin-3 complex formed in the hFSH-stimulated cells is dependent on the amount of arrestin-3 expressed and that the formation of the rFSHR-arrestin-3 complex is higher with rFSHRΔ642–651 than with rFSHR-wt. When plotted as a hyperbolic binding isotherm (Fig. 8) these data can be used to calculate the relative affinities and binding capacities of rFSHR-wt and rFSHRΔ642–651 for arrestin-3. The results of this type of analysis (Table III) show that deletion of the 642–651 region increases the arrestin-3 binding affinity ~4-fold and decreases the arrestin-3 binding capacity of the rFSHR ~1.7-fold.
The presence of FLAG-arrestin-3 and Myc-rFSHR was determined in intact cells transfected with increasing amounts of Myc-rFSHR-wt or Myc-rFSHR(Δ642–651). 293T cells (plated in 100-mm dishes) were transiently co-transfected (in a total volume of 400 μl) with 400 ng of Myc-rFSHR-wt or Myc-rFSHR(Δ642–651), 100 ng of dynamin-K44A, and 4 ng of arrestin-3-GFP. The transfected cells were washed and incubated with (50 nM) or without hFSH at 37 °C for 20 min as indicated. The cells were fixed, and the receptors (in red) were visualized using an anti-Myc monoclonal antibody (9E10) and a rhodamine-conjugated anti-mouse antibody. Arrestin-3-GFP is shown in green, and co-localized components are shown in yellow. The cells were observed and analyzed using a BioRad confocal microscope at the Central Microscopy Facility of The University of Iowa.

**Fig. 6.** Co-localization of arrestin-3-GFP and rFSHR-wt or rFSHR(Δ642–651). 293T cells (plated in 8-chamber cover slip culture vessels) were co-transfected with a constant amount (7.5 μg) of Myc-rFSHR-wt or Myc-rFSHR(Δ642–651), a constant amount of dynamin-K44A (2.5 μg), and increasing amounts of FLAG-arrestin-3 as indicated. The transfected cells were washed and incubated with (50 nM) or without hFSH at 37 °C for 20 min as indicated. The corrected binding data were plotted as a simple binding isotherm and described under “Materials and Methods.” The amount of immunoprecipitated arrestin-3 was corrected for the amount of rFSHR-wt or rFSHR(Δ642–651) present in the immunoprecipitates. The corrected binding data were plotted as a simple binding isotherm against the amount of arrestin-3 used to transfect the cells. Each point represents the mean ± S.E. of three independent transfections. The lines shown through these points were calculated non-linear regression analysis of the data points using the Prism Software package.

**Fig. 7.** Association of the rFSHR-wt or rFSHR(Δ642–651) with arrestin-3 in intact cells transfected with increasing amounts of arrestin-3. 293T cells (plated in 100-mm dishes) were transiently co-transfected with a constant amount (7.5 μg) of a given receptor plasmid (Myc-rFSHR-wt or Myc-rFSHR(Δ642–651)), a constant amount of dynamin-K44A (2.5 μg), and increasing amounts of FLAG-arrestin-3 as indicated. The transfected cells were washed, incubated for 20 min at 37 °C with 50 nM hFSH, and cross-linked. The cross-linked cells were washed and lysed with detergents as described under “Materials and Methods.” Aliquots of the different lysates (~18 μl for the top panel and ~500 μl for the middle and bottom panels) containing equivalent amounts of protein were used for measuring the amount of FLAG-arrestin-3 present in the cell lysates (top panel), FLAG-arrestin-3 immunoprecipitated with the 9E10 monoclonal antibody (middle panel), and Myc-rFSHR immunoprecipitated with the 9E10 monoclonal antibody (bottom panel) as described under “Materials and Methods.” The presence of FLAG-arrestin-3 and Myc-rFSHR was detected using an anti-FLAG monoclonal antibody (M2) or anti-Myc monoclonal antibody (9E10) covalently modified with horseradish peroxidase, the Super Signal West Femto Maximum Sensitivity system of detection from Pierce, and a Kodak digital imaging system (see “Materials and Methods” for details). Only the relevant portions of the blots of a representative experiment are shown. IP, immunoprecipitate.

**Table III**

| Receptor | Apparent Kd μg arrestin-3/100-mm dish | Maximal binding arbitrary units |
|----------|--------------------------------------|--------------------------------|
| rFSHR-wt | 5.0 ± 1.1                            | 9.3 ± 0.7                      |
| rFSHR(Δ642–651) | 1.3 ± 0.3* | 5.6 ± 0.4*                      |

The binding of arrestin-3 to the rFSHR-wt or rFSHR(Δ642–651) was measured in intact cells as shown in Fig. 7 and quantitated using non-linear regression analysis of the binding data as shown in Fig. 8 and described under “Materials and Methods.” Each value shown represents the average ± S.E. obtained in three independent experiments. * indicate statistically significant differences (p ≤ 0.05) when compared to rFSHR-wt.

In contrast, the decreased binding capacity of rFSHR(Δ642–651) for arrestin-3 is less likely to have a functional effect because this would not result in a reduction in the formation of the endogenous non-visual arrestins (30, 34–36).
the receptor-non-visual arrestin complex at the low levels of endogenous arrestin-2/3 present in 293 cells. Thus, the functional effects of the reduction in maximal binding capacity would be detectable only at high concentrations of the non-visual arrestins (cf. Fig. 8) that are achieved by overexpression. Nevertheless, the dual effect of this region on arrestin-3 binding affinity and capacity is rather unique and could be brought about by a number of mechanisms. For example, some of the residues present in the 642–651 region could directly inhibit arrestin-3 binding (thus accounting for the effect on affinity), whereas others could form part of an arrestin-3 binding site by interacting with other receptor regions. Such a possibility is in fact supported by the results of the alanine-scanning mutagenesis, which failed to identify any single residue of the 642–651 region as being responsible for the internalization effect. Alternatively, the 642–651 region may have overlapping binding sites for arrestin-3 and for another rFSHR binding protein. It is possible that some of the residues present in the 642–651 region become cross-linked to arrestin-3, and thus their removal would result in a reduction in the maximal amount of bound arrestin-3 that is detectable in this assay.

Assessments of the in vitro interaction of the arrestins with the β2-adrenergic receptor, the m2-muscarinic receptor, and rhodopsin have defined at least two primary sites in these GPCRs that participate in arrestin binding, a phosphorylation recognition site and an activation recognition site (37–39). The phosphorylation recognition site is thought to be composed of the GPCR residues that become phosphorylated by G protein-coupled receptor kinases in response to agonist stimulation. Because most GPCRs are phosphorylated at Ser/Thr residues located in their C-terminal tail it is likely that the phosphorylation recognition site of most GPCRs is present in their C-terminal tails. In fact, the importance of Ser/Thr residues in the C-terminal tail of several GPCRs in the formation of stable GPCR-arrestin complexes has been recently documented (32, 40–44). The location of the activation recognition site has not been well defined in any GPCR, but its existence is supported by the finding that the non-visual arrestins can bind in vitro to GPCR fragments (notably the third intracellular loop) that do not become phosphorylated upon agonist activation (40, 41, 45, 46). Likewise, synthetic peptides derived from the second and third intracellular loops of rhodopsin can compete for the binding of visual arrestin to light-activated rhodopsin in vitro (47). Although the 642–651/NFHPR2651 sequence identified here as being involved in non-visual arrestin binding is located in the C-terminal tail of the rFSHR, this sequence is not part of a phosphorylation recognition site because the agonist-induced phosphorylation of the rFSHR occurs in Ser/Thr residues located in the first and third intracellular loops rather than the C-terminal tail (6, 7, 10).

The inhibitory effect of the 642–651 region of the rFSHR on arrestin-3 binding affinity is interesting in view of the involvement of the C-terminal tail of other GPCRs in arrestin binding (44, 48). The distal portion (residues 344–372) of the C-terminal tail of the δ opioid receptor, a region that may comprise the phosphorylation recognition domain of this GPCR (44, 49), appears to exert an inhibitory effect on non-visual arrestin association when its phosphorylation sites are mutated. Because the agonist-induced non-visual arrestin binding and internalization of the δ opioid receptor are facilitated by the phosphorylation of at least two Ser/Thr residues present in this region (49) it was hypothesized that the phosphorylation of these residues relieves an intrinsic inhibitory effect of this region of the δ opioid receptor on arrestin binding (44). Although a comparison of the 642–651 region of the rFSHR with the 344–372 region of the δ opioid receptor failed to reveal any amino acid sequence homology, both regions are highly basic (their pI values are estimated to be 11.14 for the rFSHR peptide and 12.30 for the δ opioid receptor peptide). Moreover residues 318–330 of the C-terminal tail of the platelet-activating factor receptor, a peptide with a pI of ~4 has been shown to participate in the binding of arrestin-2 to this receptor (48). The charge of these regions could be considered as an important component of their inhibitory or stimulatory effect on non-visual arrestin binding because the interaction of arrestins with GPCRs is known to be facilitated by the presence of acidic residues either in the GPCRs or in the arrestins. As already mentioned above the introduction of acidic charges that occurs during GPCR phosphorylation is known to promote arrestin binding (37–39), and a single Arg to Glu mutation in the arrestin molecule can readily induce binding of arrestins to GPCRs in a phosphorylation-independent fashion (38, 39). Because the individual or simultaneous mutation of the four basic residues present in the 642–651 region of the rFSHR failed to affect internalization (see “Results” and Fig. 2) we can readily conclude that the charge of this peptide is not responsible for the observed effects, however.

In summary our results show that the 642–651 region of the C-terminal tail of the rFSHR has important effects on arrestin-3 binding that are independent of receptor phosphorylation. The possible existence of discrete GPCR sequences that inhibit arrestin binding should be considered in revising current models for the interaction between these two important signaling components.

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Identification of a Short Linear Sequence Present in the C-terminal Tail of the Rat Follitropin Receptor That Modulates Arrestin-3 Binding in a Phosphorylation-independent Fashion

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