N-terminal Tyrosine Modulation of the Endocytic Adaptor Function of the β-Arrestins*

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The highly homologous β-arrestin1 and -2 adaptor proteins play important roles in the function of G protein-coupled receptors. Either β-arrestin variant can function as a molecular chaperone for clathrin-mediated receptor internalization. This role depends primarily upon two distinct, contiguous C-terminal β-arrestin motifs recognizing clathrin and the β-adaptin subunit of AP2. However, a molecular basis is lacking to explain the different endocytic efficacies of the two β-arrestin isoforms and the observation that β-arrestin N-terminal substitution mutants can act as dominant negative inhibitors of receptor endocytosis. Despite the near identity of the β-arrestins throughout their N termini, sequence variability is present at a small number of residues and includes tyrosine to phenylalanine substitutions. Here we show that corresponding N-terminal (Y/F)VTL sequences in β-arrestin1 and -2 differentially regulate μ-adaptin binding. Our results indicate that the β-arrestin1 Tyr-54 lessens the interaction with μ-adaptin and moreover is a Src phosphorylation site. A gain of endocytic function is obtained with the β-arrestin1 Y54F substitution, which improves both the β-arrestin1 interaction with μ-adaptin and the ability to enhance β2-adrenergic receptor internalization. These data indicate that β-arrestin2 utilizes μ-adaptin as an endocytic partner, and that the inability of β-arrestin1 to sustain a similar degree of interaction with μ-adaptin may result from coordination of Tyr-54 by neighboring residues or its modification by Src kinase. Additionally, these naturally occurring variations in β-arrestins may also differentially regulate the composition of the signaling complexes organized on the receptor.

The classical view of G protein-coupled receptor (GPCR) signaling describes events that occur via the activation of intracellular G proteins. This type of signaling rapidly wanes as

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5 The abbreviations used are: GPCR, G protein-coupled receptor; β2AR, β2-adrenergic receptor; MEM, minimum essential medium; EGFR, epidermal growth factor receptor; [125I]-CYP, [1125]-iodocyanopindolol; GFP, green fluorescent protein; GST, glutathione S-transferase; HT, histidine-tagged; GRK, G protein-coupled receptor kinase; RFP, red fluorescent protein; JNK, c-Jun NH2-terminal kinase.
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dendocytosis to the levels mediated by β-arrestin2. This phenylalanine substitution also results in the loss of a Src phosphorylation site. These findings provide a molecular basis for the preferential internalization of the β2AR via β-arrestin2 and uncover new regulatory functions originating from a single substitution of the β-arrestin N terminus.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody against AP-50/μ2 (μ-adaptin) was from BD Biosciences. Anti-hemagglutinin antibody was obtained from Roche Pharmaceuticals, X22 anti-clathrin antibody was purified from a hybridoma cell line (9, 25). Texas Red transferrin was purchased from Molecular Probes. Cell culture reagents were obtained from Life Sciences Technologies and the yeast two-hybrid vectors and reagents from Clontech.

Plasmid Constructs—cDNA vectors for the β2AR/β-arrestin2 full-length and N-terminal fusion proteins used for binding, adenylly cyclase, and sequestration measurements were constructed in the following manner using the vector containing β2AR/GFP (26). A 5′ primer containing a 9-base overhang, a Sall restriction site, and 18–21 bases of β-arrestin2, and a 3′ primer with a 9-base overhang, a BgIII restriction site followed by a stop codon, and 18–21 bases of β-arrestin2 were used to generate cDNA inserts by polymerase chain reaction (PCR) employing rat β-arrestin2 as a template. The fragments were digested using Sall and BgIII and ligated into the β2AR/GFP vector cut with Sall and BamH1. GFP variants of the corresponding fusion proteins were similarly constructed by eliminating the stop codon from the 3′ primer. Fusion proteins of β-arrestin and GFP containing point mutations in the N terminus were constructed using a similar strategy and cloned into the β-arrestin/GFP vector (8). The cDNA for glutathione S-transferase (GST) fusion protein was also constructed by PCR. A fragment derived from the N-terminal domain (1–80) of the human β-arrestin2 was cloned into EcoRI and XhoI of pGEX-4T-1 (Amersham Biosciences AB). Sequences were confirmed using an automated ABI DNA sequencer. Point mutations were generated in β-arrestin1(Y54F) and β-arrestin2(F55Y) using the QuikChange site-directed mutagenesis system (Stratagene).

Receptor Binding Assays—Agonist and antagonist binding assays using [3H]CYP were performed as described (27).

Adenyl Cyclase Assays—Measurement of whole cell cAMP was performed using Dowex and Alumina column chromatography as described (28).

Sequestration Assays—Measurement of receptor sequestration by flow cytometry was performed as described (27). Measurement of sequestration by [3H]CYP-12177 was performed as follows. HEK-293 cells plated at a density of 3 × 10⁵/100-mm dish were transfected with receptor cDNA augmented with empty vector cDNA to a total of 5 μg using a calcium phosphate protocol (28). The cells were trypsinized 5–6 h later and plated at a density of 750,000 cells/well in a 6-well plate and incubated in minimal essential medium (MEM) containing 10% fetal bovine serum. After 48 h, the cells were washed in phosphate-buffered saline and the media replaced with warm MEM or MEM containing 500 nM isoproterenol. After 30 min at 37° C in a 5% CO₂ incubator the cells were washed three times in ice-cold phosphate-buffered saline, covered with 1 ml of ice-cold phosphate-buffered saline, and suspended by trituration at a density of 1.25 × 10⁶/ml. 100 μl volumes of cells were incubated in 15 ml of polypropylene tubes (Fisher) with 50 μl of 30 nM [3H]CYP-12177 (Amersham Biosciences) with or without 1 μM propranolol for 90 min at 4 °C. Cells were washed and collected on a Brandel harvester with Whatman GF/C filters for β analysis.

GST Fusion Protein Purification—GST pull-down experiments using GST-β-arrestin1, GST-β-arrestin(Y54F), GST-β-arrestin2, GST-β-arrestin2-(N1–80) and GST-β-arrestin2(N1–372) were performed as described previously (29).

Yeast Two-hybrid Screening—Fusion genes expressing either β-arrestin2 or the N-terminal 80 amino acids of β-arrestin2 were transformed into Tyr-187 yeast strains or AH-109 as described previously (9). The ability of the expressed proteins to interact in yeast was assessed by a growth assay using histidine- or adenine-deficient media as opposed to a primary β-galactosidase activity screen as described previously (9). Sequences inserted into the yeast vectors were verified by dideoxy sequencing.

Peptide Phosphorylation—Peptides were synthesized (Sigma Genosys) and diluted to a working concentration of ~0.5–1 mM in sterile water. Reactions were performed according to the manufacturer’s protocol provided with the purified, active c-Src (Upstate Biotechnology). Briefly, 20 units of active c-Src was added to a reaction mixture containing substrate peptide (100 μm/assay), [α-32P]ATP (1 mCi/100 μl; 3000 Ci/mmol; PerkinElmer Life Sciences), 1 × Src reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM MgCl₂, 2 mM EGTA, 0.25 mM sodium orthovanadate, 2 mM dithiothreitol). After 10 min at 30 °C, 40% trichloroacetic acid was added to the mixture and incubated at room temperature for 5 min. The reaction was transferred to the center of a 2 × 2 cm² P81 Whatman paper square. The squares were washed five times for 5 min each wash in 0.75% phosphoric acid. The squares were then washed once in acetone, transferred to a scintillation vial with scintillation mixture, and incorporated counts were measured in a scintillation counter. Background reactions (no enzyme) for each peptide were performed. This recorded value (no enzyme added) was then subtracted from the respective reactions. The normalized (substrate concentration), background-subtracted values were plotted using GraphPad Prism. The positive control was a peptide optimized for Src substrate specificity (Upstate Biotechnology).

β-Arrestin1 Purification and Phosphorylation—β-Arrestin1-HT (histidine tagged) and β-arrestin1(Y54F)-HT were solubled into the pET-21b vector (Novagen) and expressed in DH5α cells (37 °C) with a 16 h isopropyl 1-thio-β-D-galactopyranoside induction. β-arrestins were partially purified from bacterial lysates using Probond nickel-charged resin (Invitrogen) according to the manufacturer’s protocol. Phosphorylation of β-arrestin1 proteins (done with increasing amounts of purified protein) was performed with the same reaction conditions as with the peptide reactions (described above) for 10 min at 30 °C. SDS sample buffer (Invitrogen) was added to the reac-
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RESULTS

β-Arrestin1 is identical to β-arrestin2 at −80% of their N-terminal residues. Of the 32 nonidentical residues, five occur at tyrosines, shown in bold in Fig. 1A. Four of these tyrosines occur in β-arrestin1 and are conservatively substituted in β-arrestin2 by three phenylalanines and nonconservatively by a histidine. Of particular note is the first variation corresponding to Y54/F55 (β-arrestin1/2) because mutation of Y54/D54 (β-arrestin1/2) generates internalization-deficient dominant negative β-arrestins (30). The flanking sequences (Y54/F55) VTL (β-arrestin1/2) form the only putative contiguous endocytic motifs present in the N termini of β-arrestin1 or 2 that could potentially interact with AP2. In β-arrestin1 the YXXL sequence mirrors a classical cargo AP2, μ-adaptin sorting signal, whereas in β-arrestin2 the FXXL sequence mirrors the μ-subunit sorting signal observed in membrane-associated GP180/carboxypeptidase D (13). In addition, the residues Tyr/Phe of the respective sequences in β-arrestin1/2 are conserved across species (Fig. 1B).

To determine whether β-arrestin2 could directly bind μ-adaptin we employed a yeast two-hybrid assay. cDNAs for full-length β-arrestin2, truncated β-arrestin2(N1–55), truncated β-arrestin2(N1–80), and for the substitution mutant β-arrestin2(F55A, L58A) were cloned into yeast two-hybrid bait vectors. cDNA for the full-length μ-adaptin subunit of AP2 was cloned into a yeast prey vector. We then assessed the binding interaction corresponding to a distinct bait vector and the respective sequences in membrane-associated GP180/carboxypeptidase D (13). In the absence of Tyr/Phe of the respective sequences in β-arrestin1/2 were conserved across species (Fig. 1B).

Anopheles gambiae arrestin VFOQLL
Bluebottle fly arrestin VFOQLI
Drosophila kurzt arrestin VFOQLI
Xenopus laeviscone arrestin VFOQVL
Rainbow trout arrestin VFVLLT

Bos taurus β-arrestin 1 VVVTLT
Canis familiaris β-arrestin 1 VVVTLT
Homo sapiens β-arrestin 1 VYVTLT
Mus musculus β-arrestin 1 VYVTLT
Rattus norvegicus β-arrestin 1 VYVTLT

Bos taurus β-arrestin 2 VVVTLT
Canis familiaris β-arrestin 2 VVVTLT
Homo sapiens β-arrestin 2 VVVTLT
Mus musculus β-arrestin 2 VVVTLT
Rattus norvegicus β-arrestin 2 VVVTLT

FIGURE 1. Sequence alignment of β-arrestin1 and -2. A, alignment of the first 180 residues of Rattus norvegicus β-arrestin1 and -2 is shown. Identical residues are shown in the center and conserved classes of residues are shown as plus marks. Nonconserved tyrosines are in bold. B, alignment of the N-terminal (Y/F)XXL residues in the arrestins of various species.

γ

\[ \text{Anopheles gambiae arrestin} \quad \text{VFOQLL} \]
\[ \text{Bluebottle fly arrestin} \quad \text{VFOQLI} \]
\[ \text{Drosophila kurzt arrestin} \quad \text{VFOQLI} \]
\[ \text{Xenopus laeviscone arrestin} \quad \text{VFOQVL} \]
\[ \text{Rainbow trout arrestin} \quad \text{VFVLLT} \]

\[ \text{Bos taurus β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Canis familiaris β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Homo sapiens β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Mus musculus β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Rattus norvegicus β-arrestin 1} \quad \text{VVVTLT} \]

\[ \text{Bos taurus β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Canis familiaris β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Homo sapiens β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Mus musculus β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Rattus norvegicus β-arrestin 2} \quad \text{VVVTLT} \]

\[ \gamma \]

\[ \text{Anopheles gambiae arrestin} \quad \text{VFOQLL} \]
\[ \text{Bluebottle fly arrestin} \quad \text{VFOQLI} \]
\[ \text{Drosophila kurzt arrestin} \quad \text{VFOQLI} \]
\[ \text{Xenopus laeviscone arrestin} \quad \text{VFOQVL} \]
\[ \text{Rainbow trout arrestin} \quad \text{VFVLLT} \]

\[ \text{Bos taurus β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Canis familiaris β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Homo sapiens β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Mus musculus β-arrestin 1} \quad \text{VVVTLT} \]
\[ \text{Rattus norvegicus β-arrestin 1} \quad \text{VVVTLT} \]

\[ \text{Bos taurus β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Canis familiaris β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Homo sapiens β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Mus musculus β-arrestin 2} \quad \text{VVVTLT} \]
\[ \text{Rattus norvegicus β-arrestin 2} \quad \text{VVVTLT} \]
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requires modifying the C terminus of β-arrestin2 that contains clathrin and μ-adaptin binding motifs, because isolation of μ-adaptin might occur from its association with a clathrin-β-adaptin complex. In the C-terminal β-arrestin2 truncation mutant, β-arrestin2(N1–372) the clathrin and μ-adaptin binding motifs are absent. Columns containing C-terminal GST fusion proteins of either full-length β-arrestin2 or β-arrestin2(N1–372) were exposed to a mouse brain lysate. Anti μ-adaptin antibody immunoblotting of the retained protein eluate from either the β-arrestin2 or β-arrestin2(N1–372) column demonstrated a 50-kDa band characteristic of μ-adaptin. The relative amount of each GST-fused β-arrestin protein present on a column is shown by the Coomassie staining (Fig. 2B, lower panel). This result suggests that a site responsible for μ-adaptin binding exists in β-arrestin2 that is independent of the C-terminal β-adaptin and clathrin binding motifs. Surprisingly, β-arrestin2(N1–372) bound 3-fold more μ-adaptin than full-length β-arrestin2. Han et al. (31) have hypothesized that C-terminal β-arrestin truncation mutants assume pre-activated conformations. This suggests that a μ-adaptin binding motif of β-arrestin2 may become fully exposed only upon β-arrestin-receptor binding, in a manner analogous to that postulated for the β-arrestin-μ-adaptin binding motif (31).

We next passed a mouse brain lysate through a column containing a C-terminal GST fusion protein of β-arrestin2(N1–80) to determine whether the β-arrestin2 N terminus could be responsible for the previous observations. The immunoblot corresponding to GST-β-arrestin2(N1–80), but not GST alone, also yielded a 50-kDa band when probed with anti-μ-adaptin antibody (Fig. 2C). The immunostaining of the total brain lysate, which contains μ-adaptin as part of the AP2 complex, is shown as positive control.

If residues 55–58 of β-arrestin2 are implicated in μ-adaptin binding and act as a receptor sorting motif, then fusion of the N-terminal part of β-arrestin2 to the β2AR could affect the cellular localization of the receptor. To test this hypothesis we constructed a β2AR fused to the first 78 residues of β-arrestin2, β2AR/β-arrestin2(N1–78) and characterized its response to agonist.

We first assessed some pharmacological properties of β2AR/β-arrestin2(N1–78) compared with the wild type β2AR. The two receptors have equal affinities for the antagonist iodocyanopindolol (([125]I)CYP, Table 1) and the agonist isoproterenol (as measured with membrane preparations of receptors by displacement of [125]I-CYP). They also express similarly in terms of the proportion of receptors in the G protein-bound high affinity state (Table 1), and despite a shift in the EC50 for cAMP production (Table 1), the β2AR/β-arrestin2(N1–78) chimera-acc-

**TABLE 1**

Pharmacological profile and basal sequestration of β2AR chimeras in HEK-293 cells

| Receptor | [125]ICYP | CGP-12177 |
|----------|----------|----------|
| β2AR     | Kᵢ, high | Kᵢ, low |
| EC50     | Basal sequestration |
| **[125]ICYP** |
| 37 ± 04  | 0.4 ± 0.2 | 29 ± 3 | 118 ± 28 | 2 | 0.12 ± 0.04 | 7 | 25 ± 4 | 4 |
| 560 ± 45 | ND*      |         |         |         |         |         |         |         |
| **β2AR/β-arrestin2(N1–78)** |
| 52 ± 17  | 2.1 ± 0.9 | 23 ± 4 | 301 ± 89 | 2 | 1.2 ± 0.3 | 4 | 31 ± 8 | 4 |

*Not determined.
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FIGURE 3. Sequestration of a fusion protein composed of the β2AR and the N terminus of Rat β-arrestin2. A, HEK-293 cells were transiently transfected with β2AR chimeras consisting of various segments of the β-arrestin2 N terminus fused to the receptor C-tail. Receptor sequestration after exposure to 500 nM isoproterenol for 30 min at 37 °C was then measured by [3H]CGP-12177 binding. Results are mean ± S.E. and representative of three or more independent experiments. B, HEK-293 cells transfected with cDNA for β2AR/GFP were evaluated for the ability of the receptor to localize with transferrin in cytoplasmic endosomes. In the absence of agonist, the control cell (top left panel) shows the plasma membrane distribution of β2AR/GFP (green). A representative cell in MEM containing 20 μg/ml Texas Red transferrin was treated for 30 min at 37 °C in 5% CO2 with 10 μM isoproterenol and was imaged by confocal microscopy for GFP and Texas Red fluorescence. This resulted in the redistribution of β2AR/GFP to cytoplasmic vesicles (top right panel). The internalized transferrin distribution imaged after the isoproterenol treatment is shown in the lower left panel. The codistribution of transferrin inside vesicles containing and outlined by β2AR/GFP can be observed in the overlay (bottom right panel). Bar represents 10 μm. C, the basal distribution β2AR(N1–78)/GFP in HEK-293 cells is shown in the top left panel. HEK-293 cells were transfected with both β2AR/RFP (lower left panel) and β2AR(N1–78)/GFP (top right panel) and treated with isoproterenol for 30 min. The β2AR/RFP co-localizes with the β2AR(N1–78)/GFP in cytoplasmic vesicles visualized as overlap of their fluorescence (bottom right panel; yellow). These results show a typical overlay as observed in four different sets of experiments. Bar represents 10 μm.

...tivated adenyl cyclase with similar efficacy to the β2AR (not shown).

Upon agonist treatment β2AR/β-arrestin2(N1–78) sequesters to a greater extent than β2AR as measured by flow cytometry (data not shown). This suggests that the β-arrestin2(N1–78) peptide may contain a sorting motif that underlies this enhanced ability to sequester. To identify the amino acids responsible, sequestration of β2AR/β-arrestin2 fusion proteins constructed using different peptide segments of the β-arrestin2 N terminus was assessed by the binding of the hydrophilic ligand [3H]CGP-12177 (Fig. 3A). The ability of the peptide fusions to augment sequestration disappeared with truncation at Phe-55, suggesting that amino acids 50–60 may contain a canonical sorting signal.

If β-arrestin2(N1–78) does contain an internalization signal normally employed by β-arrestin2 for endocytosis of GPCRs, then β2AR/β-arrestin2(N1–78) should traffic to an endosomal vesicular compartment containing wild type β2AR. In Fig. 3B isoproterenol-activated wild type β2AR-GFP is observed to redistribute from the plasma membrane to endosomes that contain a fluorescent transferrin marker (32). In the absence of agonist a GFP fusion protein of β2AR/β-arrestin2(N1–78) is also predominantly plasma membrane bound (Fig. 3C) like the β2AR (26). When expressed in the same cell, β2AR-RFP (Fig. 3C, lower left panel) and β2AR/β-arrestin2(N1–78)/GFP (Fig. 3C, upper right panel) redistribute to a common intracellular vesicular compartment in response to agonist (Fig. 3C, lower right panel). The type of fluorescent protein tag (RFP versus GFP) attached to the β2AR does not influence its endosomal localization (data not shown). These data establish that the β2AR and β2AR/β-arrestin2(N1–78) share a similar pattern of cellular distribution before and after agonist stimulation. Altogether, our data are consistent with a hypothesis that the N terminus of β-arrestin2 functions as an FVTL μ-adaptin binding motif (YVTL in β-arrestin1) that could participate in the regulation of β2AR internalization.

An interesting difference between the two putative μ-adaptin binding motifs is the potential for regulation of YVTL in β-arrestin1 by tyrosine phosphorylation. However, there are four tyrosines present in the N-terminal part of β-arrestin1 that are not conserved in β-arrestin2 (Fig. 4A). Using an in vitro assay, we investigated whether β-arrestin1 is a Src substrate because β2AR stimulation leads to a rapid association of the activated tyrosine kinase Src to the receptor in a β-arrestin1-dependent fashion (19).

To assess the phosphorylation of the N-terminal tyrosines in β-arrestin1 that are not conserved in β-arrestin2, we synthesized the corresponding peptides (Fig. 4, A and B). The site prediction program NetPhos (Exasy Tools) had scored two of the β-arrestin1 N-terminal tyrosines as phosphorylation sites (Fig. 4B). Even though it did not score highly as a phosphorylation candidate, β-arrestin1 peptide 2 was the only substrate that underwent significant in vitro Src phosphorylation (Fig. 4B). Interestingly, peptide 2 contains the canonical Y54XX\phi sorting signal and corresponds to the F55XX\phi sequence that we described above as a potential μ-adaptin binding motif in β-arrestin2.

Examination of the crystal structure of inactive β-arrestin1 indicates that Tyr-54 is only partially exposed. Therefore we sought to establish whether this residue could be phosphorylated by Src in full-length β-arrestin1. Co-incubation in vitro of purified Src and β-arrestin1 revealed that Src was able to phosphorylate full-length β-arrestin1 (Fig. 4C), and the single point mutation Y54F was sufficient to prevent it (Fig. 4D).

To confirm that similar Src phosphorylation occur in cells, we measured the tyrosine phosphorylation of over-expressed β-arrestin1 before and after exposing the β2AR to agonist. The specificity of the phosphotyrosine antibody used in this assay was assessed by immunoprecipitating FLAG-tagged β-arrestin1 and treating the immunoprecipitates with YOP tyrosine phosphatase (Fig. 5A). Tyrosine phosphatase treatment almost completely eliminated the anti-phosphotyrosine PY99 signal confirming the specificity. We next observed in cells that β-arrestin1 as well as β-arrestin1(Y54F) are both constitutively tyrosine phosphorylated (Fig. 5B). However, isoproterenol exposure leads to a significant increase in β-arrestin1 tyrosine phosphorylation that is not observed in β-arrestin1(Y54F) (Fig. 5, B and C).
These data suggest that β-arrestin1 is tyrosine phosphorylated on residue 54 in response to β2AR stimulation. Agonist-induced β2AR sequestration is greatly reduced in β-arrestin2 knock-out cells and unaffected in β-arrestin1 knock-out cells (33), and these observations are in agreement with the idea that β-arrestin2 is the predominant regulator of GPCR internalization. Therefore we examined whether the Y54F mutation in β-arrestin1, which recreates the FVTL motif, would provide a gain of function for β2AR internalization. HEK-293 cells expressing the β2AR were transiently transfected with either β-arrestin1, β-arrestin1(Y54F) or β-arrestin2, and agonist-induced sequestration of the β2AR was measured. Over-expression of β-arrestin1 did not change the extent of β2AR internalization (Fig. 5D). By contrast, over-expression of β-arrestin1(Y54F) or β-arrestin2 increased β2AR sequestration by 18 and 28%, respectively (Fig. 5D).

Using GST pull-down, we evaluated the interaction of β-arrestin1, β-arrestin1(Y54F), and β-arrestin2 with μ-adaptin to determine whether the gain of function of β-arrestin1(Y54F) in promoting sequestration correlated with an increase in its affinity toward μ-adaptin. The three GST β-arrestin fusion proteins interact equally well with clathrin, and the β-arrestin1 GST fusion interacts much less well with μ-adaptin than the β-arrestin2 fusion protein (Fig. 5E). Remarkably, β-arrestin1(Y54F) is able to pull-down μ-adaptin like β-arrestin2 (Fig. 5E). No clathrin or μ-adaptin was retained by the GST alone (Fig. 5E, left lane control). These data strongly support the hypothesis that the FVTL sequence in β-arrestin2 mediates an interaction with the μ-adaptin subunit of the AP2 complex during receptor endocytosis. Moreover it suggests that the tyrosine at position 54 of β-arrestin1 reduces the extent of this interaction.

**DISCUSSION**

Sequence differences between the two β-arrestin isoforms are highly conserved across species, suggesting that differences in their activities may arise even from seemingly conserved amino acid substitutions. To date, few differences between the regulatory behaviors of the β-arrestins have been demonstrated, but cell-based studies have indicated a preference of many GPCRs for β-arrestin2 during clathrin-mediated internalization (24). Our results indicate that the natural Tyr to Phe variation between position 54 in β-arrestin1 and position 55 in β-arrestin2...
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An increasing number of signaling proteins like mitogen-activated protein kinase (MAPK), JNK, and NF-\( \kappa \)B, have been inhibitor PP2 does not perturb the recruitment rate of \( \beta \)-arrestin1 to the \( \beta \)2AR (data not shown). Therefore, a Src-dependent Tyr-54 \( \beta \)-arrestin1 phosphorylation could potentially serve either as a negative regulator for stabilizing AP2/\( \beta \)-arrestin1/receptor scaffolds or possibly represents a means for recruiting SH2-directed signaling proteins modulating the nature of signaling scaffold organized by \( \beta \)-arrestins. Such a prospect is of specific interest for receptors like the mGluR1a, which can recruit, depending on the nature of the ligand, either of the \( \beta \)-arrestins or selectively only \( \beta \)-arrestin1 (34, 35).

Examination of the crystal structure of the inactive \( \beta \)-arrestin1 indicates that a direct \( \mu \)-adaptin interaction via the sequence (Y/F)XXL alone would require a major conformational change in \( \beta \)-arrestin to unmask the leucine upon receptor binding. Evidence suggests that receptor-activated \( \beta \)-arrestin can undergo substantial conformational rearrangement with receptor activation (36). However, we observed an interaction between \( \beta \)-arrestin and \( \mu \)-adaptin in the absence of receptor activation, suggesting a less substantial molecular rearrangement, but one also consistent with predicted \( \beta \)-arrestin behavior (36). Identifying the aggregate of binding determinants on the \( \beta \)-arrestin surface that engage \( \mu \)-adaptin...

tin2 generates differences in \( \beta \)-arrestin binding to \( \mu \)-adaptin and \( \beta \)-arrestin-promoted \( \beta \)2AR internalization. Interestingly, despite \( \beta \)-arrestin1 Y54LTV defining a canonical YXX\( \Phi \) \( \mu \)-adaptin binding motif, \( \beta \)-arrestin1 has less affinity for \( \mu \)-adaptin than \( \beta \)-arrestin2. However, the \( \beta \)-arrestin1(Y54F) mutant binds \( \mu \)-adaptin as well as \( \beta \)-arrestin2 and is a gain of function mutation for \( \beta \)2AR sequestration. According to available structural information, in the absence of tyrosine 54 phosphorylation, this same tyrosine is presumably engaged via a hydrogen bond with Arg-52 (31), constraining its availability for interaction with \( \mu \)-adaptin. Crystal structure of the \( \beta \)-arrestin indicates that the Tyr/Phexidentifying this putative motif is not fully exposed, however the in vitro and in cellulo phosphorylation of Tyr-54 by Src demonstrate the accessibility of this residue.

Src-mediated \( \beta \)-arrestin1 phosphorylation appears within a minute of receptor activation, and therefore most likely precedes the recruitment of activated \( \beta \)-arrestin-receptor complexes to coated pits, where Src is known to phosphorylate at a minimum both clathrin and dynamin (20–22). Notably, the Src pre- and post-receptor activation will most probably require co-crystallization of the participating components including adaptins, \( \beta \)-arrestins, and receptors.

The \( \beta \)-arrestin2 interaction with both \( \mu \)- and \( \beta \)-adaptin raises the question of the chronology for these interactions. The proposed \( \beta \)-arrestin2 C-tail conformational change upon receptor binding likely occurs before the tail is able to interact with \( \beta \)-adaptin (31) through arginine residues 394/396 (18). Indeed, arginine 394 forms part of the \( \beta \)-arrestin2 polar core and is thus unavailable for binding \( \beta \)-adaptin unless a conformational rearrangement occurs in this region secondary to receptor interaction (31). Our data show that inactive \( \beta \)-arrestin2 can interact with the \( \mu \)-subunit. Therefore inactive \( \beta \)-arrestin2 might first bind AP2 through \( \mu \)-adaptin. Next, a stronger interaction with AP2 would follow because of the additional \( \beta \)-adaptin binding that occurs after receptor-mediated conformational changes of \( \beta \)-arrestin2.

FIGURE 5. Tyrosine phosphorylation of \( \beta \)-arrestin 1 in cells and the effects of mutation of Tyr-54 to Phe on sequestration and \( \mu \)-adaptin-\( \beta \)-arrestin 1 interactions. A, \( \beta \)-arrestin1-FLAG was expressed in HEK-293 cells, immunoprecipitated, either not treated or treated with and 10–20 units of tyrosine phosphatase for 3 min at room temperature and then resolved by SDS-PAGE and Western analysis. The Western blot displayed is representative of two separate experiments. B, HEK-293/\( \beta \)2AR cells transiently expressing \( \beta \)-arrestin1-FLAG were stimulated with isoproterenol for 1 min and then lysed. \( \beta \)-arrestin1-FLAG was immunoprecipitated and then resolved by SDS-PAGE and Western analysis. C, quantification of tyrosine phosphorylation of \( \beta \)-arrestin1 wild type and mutant \( \beta \)-arrestin1(Y54F). Data are representative of three separate experiments and presented as means ± S.E. * indicates a significant difference from WT, \( p < 0.01 \). D, sequestration of \( \beta \)2AR receptor in HEK-293 cells with or without overexpression of \( \beta \)-arrestin1, \( \beta \)-arrestin1(Y54F), or \( \beta \)-arrestin2 was performed by [\( ^{3} \)H]CGP-12177 binding. Results are representative of three independent experiments and presented as means ± S.E. * indicates a significant difference from WT, \( p < 0.05 \). E, mouse brain homogenates were purified over a column containing either GST alone, GST-\( \beta \)-adaptin, GST-\( \beta \)-arrestin1(Y54F), and GST-\( \beta \)-arrestin2. The GST-conjugated proteins were removed from the columns using reduced glutathione, and equal total protein aliquots of eluate were resolved by SDS-PAGE. The loading of GST fusion proteins was confirmed by Coomassie staining (bottom panel). The three GST-\( \beta \)-arrestins were equally well able to interact with clathrin (top panel). GST-\( \beta \)-arrestin1 had a significantly reduced ability to retain \( \mu \)-adaptin compared with GST-\( \beta \)-arrestin1(Y54F) and GST-\( \beta \)-arrestin2 (middle panel). No immunoreactivity for clathrin or \( \mu \)-adaptin was detected in the control lane containing GST alone (left-most column). Immunoblots revealed characteristic 50 and 180 kDa bands corresponding to the \( \mu \)-adaptin subunit of AP2 and the clathrin heavy chain (see the lysate fraction in the right-most column). Data are representative of two independent experiments.
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associated with scaffolds formed by receptors and β-arrestins. The demonstration that β-arrestin-dependent signaling requires clathrin-dependent localization of receptor-β-arrestin complexes suggests sequence differences between β-arrestin1 and 2 that modulate the membrane localization of activated receptors could affect this novel, nonclassical means of GPCR signal transduction. Consequently, elucidating the principles underlying the dynamics of receptor-β-arrestin scaffold formation will help explain the G-protein-independent cellular response to a large variety of agonists ranging from ions to hormones.

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