The non-visual arrestins, arrestin-2 and arrestin-3, play a critical role in regulating the signaling and trafficking of many G protein-coupled receptors (GPCRs). Molecular insight into the role of arrestins in GPCR trafficking has suggested that arrestin interaction with clathrin, β2-adaptin (the β-subunit of the adaptor protein AP2), and phosphoinositides contributes to this process. In the present study, we have attempted to better define the molecular basis and functional role of arrestin-2 interaction with clathrin and β2-adaptin. Site-directed mutagenesis revealed that the C-terminal region of arrestin-2 mediated β2-adaptin and clathrin interaction with Phe-391 and Arg-395 having an essential role in β2-adaptin binding and LIELD (residues 376–380) having an essential role in clathrin binding. Interestingly, arrestin-2-R169E, an activated form of arrestin that binds to GPCRs in a phosphorylation-independent manner, has significantly enhanced binding to β2-adaptin and clathrin. This suggests that receptor-induced conformational changes in the C-terminal tail of arrestin-2 will likely play a major role in mediating arrestin interaction with clathrin-coated pits. In an effort to clarify the role of these interactions in GPCR trafficking we generated arrestin mutants that were completely and selectively defective in either clathrin (arrestin-2-ΔLIELD) or β2-adaptin (arrestin-2-F391A) interaction. Analysis of these mutants in COS-1 cells revealed that arrestin/clathrin interaction was essential for agonist-promoted internalization of the β2-adrenergic receptor, while arrestin/β2-adaptin interaction appeared less critical. Arrestin-2 mutants defective in both clathrin and β2-adaptin binding functioned as effective dominant negatives in HEK293 cells and significantly attenuated β2-adrenergic receptor internalization. These mutants should prove useful in better defining the role of arrestins in mediating receptor trafficking.

Many transmembrane signaling systems consist of specific G protein-coupled receptors (GPCRs)\(^1\) that transduce the binding of a diverse array of extracellular stimuli into intracellular signaling events \((1)\). GPCRs modulate the activity of numerous effector molecules including adenyl cyclases, phosphoinositide 3-kinase, non-receptor tyrosine kinases, small G proteins, phosphodiesterases, phospholipases, and ion channels. To ensure that extracellular stimuli are translated into intracellular signals of appropriate magnitude and specificity, these signaling cascades are tightly regulated. GPCRs are subject to three principle modes of regulation: desensitization, in which a receptor becomes refractory to continued stimuli; endocytosis, whereby receptors are removed from the cell surface; and down-regulation, where total cellular receptor levels are decreased \((2, 3)\).

GPCR desensitization is primarily mediated by second messenger-dependent kinases, such as protein kinase A and protein kinase C, and by G protein-coupled receptor kinases. G protein-coupled receptor kinases specifically phosphorylate activated GPCRs, initiating recruitment of arrestins. Arrestins are divided into two major classes, visual and non-visual, on the basis of localization. The non-visual arrestins, arrestin-2 and arrestin-3 (also termed β-arrestin-1 and -2), are broadly distributed and have been implicated in regulating multiple processes including GPCR desensitization \((4, 5)\), trafficking \((6, 7)\), and signaling via non-receptor tyrosine kinases \((8, 9)\) and mitogen-activated protein kinases \((10–12)\). Multiple interactions contribute to arrestin-mediated trafficking of GPCRs including a C-terminal insert region that interacts with the N-terminal domain of the clathrin heavy chain \((13, 14)\), a C-terminal region that interacts with the β-subunit of the heterotetrameric adaptor protein 2 (AP2) complex (β2-adaptin) \((15, 16)\), and a basic region that binds phosphoinositides \((17)\). While each of these interactions appears to contribute to the ability of non-visual arrestins to mediate GPCR trafficking, the mechanistic basis of this process remains unclear.

Here we have attempted to better define the interactions of arrestin-2 with clathrin and β2-adaptin. We were able to generate arrestin mutants that were selectively and completely defective in either clathrin or β2-adaptin interaction. These mutants were then used to elucidate the role of these interactions in β2-adrenergic receptor (β2AR) trafficking. Our studies suggested that arrestin interaction with clathrin, but not β2-adaptin, is critical for β2AR internalization. Moreover, we found that arrestin-2 mutants defective in both clathrin and β2-adaptin binding functioned as effective dominant negative mutants. These mutants should prove useful in better defining the role of arrestins in receptor trafficking.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents were generously provided by Drs. James Keen (glutathione S-transferase (GST)-clathrin-TD), Harvey McMahon (wild type and mutant GST-β2-adaptin and GST-α2-adaptin), Juan Boni-
facino (GST-β2-adaptin), and Larry Donoso (mouse monoclonal antibody F4C1).

Analysis of Arrestin Interaction with β-Adaptin and Clathrin—A GST-β2-adaptin appendage (residues 700–937) fusion protein, several various GST-β2-adaptin mutants, and a GST-clathrin terminal domain (residues 909–1003) were expressed and purified on glutathione-agarose as described previously (13, 18). Arrestin-2 and additional β2-adaptin mutations were generated by PCR and confirmed by DNA sequencing. Wild type and mutant arrestins were expressed in COS-1 cells by transient transfection as described previously (19). Arrestin extracts were prepared by lysing the cells by freeze/thaw and Polytetrafluoroethylene disruption in 20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml leupeptin, 0.2 mg/ml benzamidine. The extracts were centrifuged (50,000 x g, 20 min), and the supernatants were aliquoted, frozen, and stored at −80 °C until needed (extracts contained −2 μg of arrestin/ml).

Arrestin-containing lysates (~20 ng of arrestin) were incubated with 5 μl of glutathione-agarose beads (containing ~50 pmol of bound GST or GST fusion protein) in binding buffer (20 mM Hepes, pH 7.2, 120 mM potassium acetate, 0.1 mM dithiothreitol, 0.1% Triton X-100) for 1 h at 4 °C (in a total volume of 100 μl). The beads were then pelleted (1000 rpm, 5 min at 4 °C) and washed two to three times with 0.5 ml of ice-cold binding buffer, and bound arrestin was eluted by boiling the beads in SDS sample buffer for 10 min. The samples were electrophoresed on a 10% SDS-polycrylamide gel and transferred to nitrocellulose, and arrestin was detected by immunoblotting using monoclonal antibodies (F4C1) or rabbit polyclonal (178) anti-arrestin antibodies, horseradish peroxidase-labeled goat anti-mouse or anti-rabbit secondary antibodies, and chemiluminescence.

To prepare brain extracts, fresh bovine brain was stripped of connective tissue and minced in ~1 ml of homogenization buffer (40 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml apronitin, 0.02 mg/ml leupeptin, 0.2 mg/ml benzamidine, and 0.02 mg/ml pepstatin) per mg of tissue using a Brinkman Polytron (20,000 rpm, 30 s). The homogenate was centrifuged at 30,000 x g for 30 min, and the resulting pellet was resuspended in homogenization buffer containing 1% Triton X-100, homogenized again (20,000 rpm, 30 s), and centrifuged at 30,000 x g for 30 min. The supernatant was then centrifuged at 50,000 x g for 30 min, and the final supernatant (~20 μg of protein/ml) was aliquoted and stored at −80 °C until use.

For binding of bovine brain proteins to GST-β2-adaptin, 20 μl of brain extract (~400 μg of total protein) were incubated with 50 pmol of various GST-β2-adaptin mutants proteins bound to glutathione-agarose beads. The beads were washed, and bound proteins were eluted, electrophoresed, and transferred to a nitrocellulose membrane. Binding of AP180 and Eps15 to GST-β2-adaptin was visualized by probing the membrane with either anti-AP180 or anti-Eps15 monoclonal antibodies (Transduction Laboratories).

Cell Culture and Transient Transfection—COS-1 and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Transient transfection was done using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s recommendations.

Analysis of GPCR Interaction with Arrestins by Co-immunoprecipitation—COS-1 cells transiently transfected with pcDNA3-FLAG-β2AR and various pcDNA3-arrestin-2 constructs were stimulated with 10 μM (-)-isoproterenol for 2 min at 37 °C and then lysed in 1 ml of co-immunoprecipitation buffer (colP buffer) containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and protease inhibitors. Insoluble materials were removed by centrifugation at 50,000 x g for 30 min, and the supernatants were washed with 50 μl of 1% Triton X-100, 10% glycerol, 1% Triton X-100, and 100 mM phenylmethylsulfonyl fluoride in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Transient transfection was done using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s recommendations.

Results and Discussion

Previous studies have suggested an important role for non-visual arrestin interaction with clathrin and β2-adaptin in GPCR trafficking (7, 13, 15, 16). Arrestin interaction with clathrin is thought to occur primarily via a LôXôE motif where ϕ is a bulky aliphatic residue) found within a C-terminal insert unique to non-visual arrestins (residues 376–380 in arrestin-2) (13). Mutation of the aliphatic or acidic residues within this motif results in significant reduction in arrestin binding to clathrin (13). Non-visual arrestin interaction with β2-adaptin was first characterized by yeast two-hybrid and co-immunoprecipitation analysis (15). Additional studies revealed that Arg-394 and Arg-396 in arrestin-3 are critical for β2-adaptin binding (16), while we recently found that Phe-391 and Arg-395 (analogous to Arg-396 in arrestin-3) are essential for arrestin-2 binding to β2-adaptin (21).

In an attempt to more completely define the binding interface of arrestin and β2-adaptin we constructed several additional C-terminal mutant arrestin-2 proteins including K397E, M399K, K400E, D401R, D402R, and K403E (Fig. 1a). The mutant arrestins were then expressed in COS-1 cells and tested for binding to a GST fusion protein containing the β2-adaptin appendage domain (residues 700–937) (Fig. 1b, upper panel). The appendage domain has been implicated in interactions with multiple proteins including clathrin, AP180, Eps15, epin, and non-visual arrestins (18, 21, 22). Subsequent densitometric analysis of the blots revealed that mutation of Lys-397, Lys-400, or Lys-403 resulted in a 65–90% loss in β2-adaptin binding (Fig. 1c, left panel). The various mutants were also tested for their binding to a GST fusion protein containing residues 1–579 of the clathrin terminal domain (Fig. 1b, middle panel). Quantification of the blots suggested that mutation of Lys-397, Lys-400, or Lys-403 resulted in an ~40% loss in clathrin binding (Fig. 1c, right panel). Overall these results suggest that Lys-397, Lys-400, and Lys-403 may play a role in β2-adaptin and, to a lesser extent, clathrin binding.

It is somewhat surprising that several C-terminal residues (Lys-397, Lys-400, Lys-403, and Arg-395; Ref. 21) contribute to interaction with both β2-adaptin and clathrin. While one possible explanation is that charge inversion of these residues results in partial misfolding of the C-terminal region of arrestin, this seems unlikely since individual mutations elicited a more profound inhibitory effect on β2-adaptin binding compared with clathrin. Since binding surfaces on components of clathrin-mediated endocytic pathways are often shared by multiple interacting proteins (18, 31), we favor the explanation that these particular residues directly contribute to β2-adaptin and clathrin binding. The partial overlap of the binding surfaces for β2-adaptin and clathrin on arrestin-2 may contribute to spatial and temporal regulation of GPCR targeting to clathrin-coated pits.

We next attempted to further address the specificity of arrestin interaction with APs. Four AP complexes have been identified, each of which consists of two large (γ, α, δ, ε-adaptin and β1-γ-adaptin), one medium (μ1-γ-adaptin), and one small (φ1-γ-adaptin) subunit (23). AP2 is localized in cell sur-
face clathrin-coated pits and mediates endocytosis from the plasma membrane, while AP1, AP3, and AP4 are associated with intracellular membranes and mediate sorting at the trans-Golgi network and/or endosomes (23). Since arrestins interact with the appendage domain of β2-adaptin and arrestin interaction with other adaptins could potentially contribute to receptor trafficking, we compared the ability of arrestin-2 to bind to GST fusion proteins containing the appendage domains of α-adaptin, β2-adaptin, and β3-adaptin. These studies revealed that arrestin-2 is highly specific for binding to β2-adaptin and suggested that the primary function of this interaction will involve GPCR endocytosis (Fig. 2a).

In an effort to elucidate the molecular basis for β2-adaptin binding to arrestin-2, we systematically mutated 10 surface residues of arrestin-2 characterized in this study are in bold type, while previously reported β2-adaptin binding residues of arrestin-3 (16) are underlined. GST-pull down assays were carried out using 50 pmol of GST-β2-adaptin (upper panel) or GST-clathrin (middle panel) and 10 μl of COS-1 cell lysate expressing wild type or mutant arrestin-2 as described under “Experimental Procedures.” Bound arrestins were eluted, electrophoresed, transferred to nitrocellulose, and probed with an anti-arrestin monoclonal antibody. The lower panel shows the relative expression level of the arrestin mutants. c, arrestin binding was quantified using a densitometer, and the amount of mutant protein bound to GST-β2-adaptin (left panel) or GST-clathrin (right panel) was compared with wild type arrestin-2 (normalized to 100%). The bars represent the mean ± S.E. from six independent experiments. Statistical analysis was performed using an unpaired t test (*, p < 0.01 versus wild type (WT); **, p < 0.001 versus wild type).
residues in β2-adaptin, several of which have been implicated in β2-adaptin binding to clathrin, AP180, Eps15, and epsin (18). The various β2-adaptin mutants were expressed as GST fusions and then tested for binding to purified arrestin-2. These studies suggested that arrestin-2 interacts with multiple residues on the β2-adaptin surface including Arg-834, Trp-841, Glu-849, Tyr-888, and Glu-902 (Fig. 2b). These results confirm and extend a recent study that suggested a role for Glu-849, Tyr-888, and Glu-902 in arrestin-3 binding to β2-adaptin (22) and also confirm many of the features of our proposed model of β2-adaptin interaction with arrestin-2 (21). To verify that the various adaptins were functional, we also analyzed the binding of AP180 and Eps15 to the GST-β2-adaptin mutants. These studies largely recapitulated previous findings (18) and revealed that Lys-917 and Tyr-888 contributed to AP180 binding, while Trp-841 and Tyr-888 were involved in Eps15 binding (Fig. 2c).

Overall our results suggest that Phe-391 and Arg-395 in arrestin-2 play an essential role in interacting with β2-adaptin since mutation of either of these residues completely disrupts binding (21). Indeed, our modeling predicted that Phe-391 interacts with Trp-841 and Tyr-888 in β2-adaptin, while Arg-395 interacts with Glu-849 and Glu-902. However, our results suggest that Leu-396, Lys-397, Lys-400, and Lys-403 also contribute to β2-adaptin binding (Ref. 21 and Fig. 1). While the binding analysis of the β2-adaptin mutants fully supports this model (Ref. 22 and Fig. 2b), structural analysis will be required to completely define the binding interface of these proteins.
Structural insight has revealed that arrestin-2 contains two major domains made up of β-sheets that are joined by a polar core of buried salt bridges (21, 24). The region of arrestin-2 that interacts with β2-adaptin includes one residue in the last β-sheet (Phe-391) as well as several residues that are C-terminal to this region (Arg-395, Leu-396, Lys-397, Lys-400, and Lys-403). The C-terminal region in arrestin has been suggested to undergo significant conformational change upon receptor binding (25, 26) and potentially loses intramolecular contacts between β-sheets in the N-terminal and C-terminal regions of the protein (27). In addition, the conformational change induced by receptor binding is also thought to reflect disruption of the polar core of arrestin potentially mediated by interactions between basic residues within the polar core and phosphoserines from the receptor (28, 29). In particular, Arg-169 in arrestin-2 appears to play a key role in stabilizing the polar core.
Arrestin-2 Interaction with Clathrin and AP2

FIG. 4. Binding of wild type and ΔLIELD arrestin-2 mutants to GST-β2-adaptin and clathrin. GST-pull down assays were carried out using 50 pmol of GST-β2-adaptin (upper panel) or GST-clathrin (middle panel) and 10 μl of COS-1 cell lysate containing wild type or ΔLIELD mutant arrestin-2 as described under “Experimental Procedures.” Bound arrestins were detected using an anti-arrestin monoclonal antibody. The lower panel shows the relative expression level of the arrestin mutants. WT, wild type.

Arrestins bound to GST-β2-adaptin much better than wild type arrestin-2 (Fig. 3a). Dose-response analysis suggested that arrestin-2-R169E bound to β2-adaptin with much higher affinity than wild type arrestin-2 (Fig. 3b, left panel). Interestingly, arrestin-2-R169E also bound with higher affinity to GST-clathrin compared with wild type arrestin-2 (Fig. 3, a and b). Since the R169E mutant most likely better represents the conformation of arrestin-2 that would bind to β2-adaptin and clathrin in cells, we also assessed whether the various arrestin mutants that were defective in β2-adaptin and/or clathrin binding remained so in the background of the R169E mutation. Thus, we compared the ability of F391A, R395E, R395E, L396A, K397E, K400E, and K403E mutants of arrestin-2-R169E to bind to GST-β2-adaptin (Fig. 3c, upper panel) and GST-clathrin (Fig. 3c, lower panel). These studies revealed that the Phe-391 mutation retained its selective and complete disruption in β2-adaptin binding, while the Arg-395 mutation remained completely disrupted in β2-adaptin binding and partially disrupted in clathrin binding. Interestingly, the reduced binding of the L396A, K397E, K400E, and K403E mutants to β2-adaptin and clathrin previously observed (Ref. 21 and Fig. 1) appeared to be largely attenuated by the R169E mutation. Overall these results suggest that the conformational change induced by GPCR binding will likely play a major role in mediating β2-adaptin and clathrin binding and that the F391A mutant should prove useful in selectively dissecting the functional role of arrestin/β2-adaptin interaction.

We next focused on further characterizing the binding of arrestin-2 to clathrin. Previous work has implicated a C-terminal LΔXeE motif in clathrin binding and demonstrated that mutation of the three aliphatic residues within this motif results in an ~85% reduction in clathrin binding (13). Indeed, such a motif appears to play an important role in the ability of numerous proteins to bind clathrin (31). In an effort to generate an arrestin-2 mutant that was completely defective in clathrin binding we deleted the five-amino acid clathrin binding motif (LIELD) in arrestin-2. This mutant, arrestin-2-ΔLIELD, was expressed and initially tested for its ability to bind GST-clathrin and GST-β2-adaptin. As expected, arrestin-2-ΔLIELD was completely defective in clathrin binding (Fig. 4, middle panel), while it was largely unchanged in β2-adaptin binding (Fig. 4, top panel). These studies confirmed that the LΔXeE motif plays an essential role in arrestin-2 binding to clathrin.

A number of additional mutations were tested in the background of arrestin-2-ΔLIELD including F391A, R395E, and F391A/R395E. As expected, each of these mutants was completely defective in clathrin and β2-adaptin binding (Fig. 4). Since arrestin-2 binding to clathrin appeared to be conformationally sensitive (Fig. 3), we also tested whether incorporation of the R169E mutation in arrestin-2-ΔLIELD affected clathrin binding. Arrestin-2-R169E-ΔLIELD remained completely defective in clathrin binding and unaltered in β2-adaptin binding (not shown). This suggests that the conformational change in arrestin-2 induced by the Arg-169 mutation most likely mediates clathrin binding via the LΔXeE motif. Nevertheless, to further address whether the C-terminal domain is involved in mediating clathrin binding we analyzed binding of C-termi-

nally truncated arrestin-2 and arrestin-2-ΔLIELD to clathrin. Arrestin-2-(1–393) was completely defective in β2-adaptin binding, while it appeared to have modestly increased binding to clathrin (Fig. 4). As expected, arrestin-2-ΔLIELD-(1–395) did not bind to clathrin or β2-adaptin. Although we cannot rule out the possibility of secondary clathrin binding sites on arrestin-2, our data suggest that the LIELD motif plays an essential role in clathrin binding and that arrestin-2-ΔLIELD is an effective mutant for assessing the functional role of clathrin binding.

For the various arrestin mutants to be useful in dissecting the role of clathrin and β2-adaptin binding in cells, we also
needed to analyze whether these mutants were altered in receptor binding. The various arrestin constructs were co-expressed with a FLAG-tagged β2AR in COS-1, the cells were incubated with or without 10 μM isoproterenol for 15 min at 37 °C, and β2AR internalization was measured by enzyme-linked immunosorbent assay as described under "Experimental Procedures." The bars represent the mean ± S.E. from three to six independent experiments performed in triplicate. Statistical analysis was performed using an unpaired t test (*, p < 0.05 versus wild type (WT)).

Fig. 6. Internalization of β2AR in COS-1 cells overexpressing wild type or mutant arrestin-2. FLAG-β2AR and either wild type or mutant arrestin-2 were co-expressed in COS-1 cells. At 48 h post-transfection, cells were incubated with or without 10 μM isoproterenol for 15 min at 37 °C, and β2AR internalization was measured by enzyme-linked immunosorbent assay as described under "Experimental Procedures." The bars represent the mean ± S.E. from three to six independent experiments performed in triplicate. Statistical analysis was performed using an unpaired t test (*, p < 0.05 versus wild type).
should prove effective in further dissecting the role of clathrin and β2-adaptin interaction in arrestin function.

Previous studies using an arrestin-3 construct containing mutations of the three aliphatic residues within the LDXE motif suggested that binding of non-visual arrestins to clathrin contributes to GPCR internalization (13). A similar line of investigation identified a role for arrestin-3 interaction with β2-adaptin in GPCR internalization and further concluded that the β2-adaptin binding, and not clathrin, is necessary for the initial targeting of receptor to clathrin-coated pits (16). One concern with these previous studies is that the arrestin mutants used to dissect the role of clathrin and β2-adaptin binding in GPCR internalization were potentially flawed. For example, the LIFA mutant (containing the three aliphatic residues in the LDXE motif in arrestin-3 mutated to alamines) is only ~85% reduced in clathrin binding (13), while the R396E mutant (which corresponds to R395E in arrestin-2) used by Laporte et al. (16) is completely defective in β2-adaptin binding but is likely also reduced in clathrin binding (21). Thus, these are not ideal mutants to draw conclusions about the respective involvement of clathrin and β2-adaptin binding in arrestin function.

Since our studies demonstrate that the F391A (Ref. 21 and Fig. 3c) and ΔLIELD (Fig. 4) mutants are selectively defective in β2-adaptin and clathrin binding, respectively, we used these mutants to further dissect the role of these interactions in GPCR internalization. When co-expressed in COS-1 cells, wild type arrestin-2 promoted an ~2-fold increase in agonist-induced internalization of the β2AR (Fig. 6). In striking contrast, the ΔLIELD mutant was completely defective in promoting β2AR internalization demonstrating the important role of arrestin/clathrin interaction in this process. By comparison, arrestin-2-F391A promoted an ~2-fold increase in β2AR internalization suggesting that arrestin-2 interaction with β2-adaptin is not essential in this process. Interestingly, arrestin-2-R395E, which was completely defective in β2-adaptin binding and attenuated in clathrin binding, was also ineffective at promoting β2AR internalization (Fig. 6). This finding largely recapitulates the results of Laporte et al. who used the analogous mutation in arrestin-3 (R396E) to conclude that the binding of a receptor-arrestin complex to AP2 is necessary for the initial targeting of receptor to clathrin-coated pits (16). Analysis of additional mutants defective in either β2-adaptin binding (residues 1–393) or β2-adaptin and clathrin binding (ΔLIELD/F391A, ΔLIELD/R395E, and 1–393/ΔLIELD) confirmed that arrestin-2 binding to clathrin plays the primary role in agonist-promoted internalization of the β2AR (Fig. 6).

We next analyzed the ability of the various arrestin-2 mutants to regulate β2AR internalization in HEK293 cells. HEK293 cells have higher endogenous levels of arrestin and have proven useful for testing whether arrestin mutants can function as dominant negatives to inhibit GPCR internalization (6, 32, 33). β2AR internalization in HEK293 cells was ~25% following a 30-min treatment with agonist and was increased to ~35% by wild type arrestin-2 (Fig. 7). In contrast, arrestin-2 mutants that were completely defective in β2-adaptin binding (F391A, R395E, and residues 1–393) did not promote any additional internalization. This suggests that arrestin binding to β2-adaptin contributes to β2AR internalization in HEK293 cells, although loss of such interaction did not produce an effective dominant negative mutant. Selective loss of clathrin binding (ΔLIELD) produced a modestly effective dominant negative, although in these studies it appeared as effective as a C-terminal arrestin-2 construct (residues 319–418) that is widely used to study the role of arrestins in receptor trafficking (33). Interestingly, mutants that were defective in both clathrin and β2-adaptin binding functioned as potent dominant negative mutants with ΔLIELD/F391A and ΔLIELD/F391A/R395E being as effective at inhibiting β2AR internalization as dominant negative dynamin I. The ΔLIELD/F391A/R169E mutant was also an effective dominant negative and should be particularly useful in future studies since it overcomes the requirement for GPCR phosphorylation for arrestin binding.

We conclude that arrestin-2 binding to clathrin is essential for promoting GPCR endocytosis, while binding to β2-adaptin appears less important. These findings appear to contradict Laporte et al. (16) who concluded that binding of a receptor-arrestin complex to AP2 was critical for targeting the receptor to coated pits. How do we explain these differences in the two studies? We favor the explanation that the arrestin mutants used by Laporte et al. were either incomplete in disrupting clathrin interaction (13) or were not completely selective in disrupting β2-adaptin binding (21). This would result in an apparent diminished role of clathrin binding and exaggerated role of β2-adaptin binding. However, we cannot exclude the possibility that mechanistic differences between arrestin-2 (used in the present study) and arrestin-3 (used by Laporte et al.) may have contributed to the different findings. In addition, non-visual arrestins also interact with several additional proteins that have been implicated in GPCR endocytosis including ADP-ribosylation factor-6, ADP-ribosylation factor nucleotide binding site opener, and N-ethylmaleimide-sensitive factor (34, 35). Since the arrestin residues that mediate binding to these proteins have not been identified, we also cannot exclude the possibility that our arrestin mutants may be altered in their interaction with one or more of these proteins, although this seems unlikely.

Overall, our studies are compatible with non-visual arrestin binding to activated phosphorylated GPCRs mediating a conformational change in arrestin that promotes binding to clathrin and β2-adaptin. Although our studies do not address whether receptor-bound arrestins directly recruit clathrin or AP2 to induce de novo coated pit formation, we favor a mechanism whereby the receptor-arrestin complex moves laterally within the membrane and binds to clathrin and AP2 in a pre-existing coated pit. This mechanism is consistent with our earlier studies that demonstrated that arrestins do not promote clathrin assembly (7) as well as more recent studies that demonstrate that a receptor-arrestin-3 complex does not promote new coated pit formation (36). Our results suggest that arrestin-2 binding to clathrin is essential for mediating GPCR trafficking, while the interaction with β2-adaptin appears less critical. Nevertheless, arrestin interaction with β2-adaptin may participate in the cooperative formation of an arrestin-AP2-clathrin complex and thereby contribute to the dynamics of receptor/arrestin association with clathrin-coated pits.

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