Characterization of Dominant Negative Arrestins That Inhibit 
\(\beta_2\)-Adrenergic Receptor Internalization by Distinct Mechanisms* 

(Received for publication, August 25, 1998, and in revised form, October 6, 1998)

Michael J. Orsini and Jeffrey L. Benovic‡

From the Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Arrestins have been shown to act as adaptor proteins that mediate the interaction of G protein-coupled receptors with the endocytic machinery. In this study, the role of arrestin-3 in receptor internalization was investigated by constructing different arrestin-3 minigenes that could potentially act as dominant negative inhibitors of arrestin function. Expression of arrestin-3 proteins containing amino acids 1–320 or 201–409 resulted in the inhibition of \(\beta_2\)-adrenergic receptor internalization in HEK-293 cells by approximately 40%. Both of these arrestins were diffusely localized within the cytoplasm of transfected cells, were unable to mediate redistribution of receptors to clathrin-coated pits, and did not localize to coated pits in either the presence or absence of receptor and agonist. Arrestin-3(1–320), but not arrestin-3(201–409), bound to light-activated phosphorylated rhodopsin with an affinity comparable with that of wild-type arrestin-3. In contrast, expression of arrestin-3 proteins composed of only the clathrin binding domain, arrestin-3(284–409), and arrestin-3(290–409) resulted in the constitutive localization of these arrestins to coated pits. Arrestin-3(284–409) and arrestin-3(290–409) acted as dominant negative inhibitors of wild-type arrestin function, inhibiting receptor internalization by 70 and 30%, respectively. Carboxyl-terminal deletions of arrestin-3 retained the ability to promote internalization until residues amino-terminal to amino acid 350 were deleted, suggesting that residues in this region also compose part of the clathrin binding domain in addition to the major binding site between residues 371–379. These studies characterize at least two distinct mechanisms, competition for either receptor or clathrin binding, by which dominant negative arrestins inhibit receptor internalization and further define residues within arrestin-3 that constitute the clathrin binding domain.

The processes of desensitization, internalization, down-regulation, and resensitization of G protein-coupled receptors (GPCRs) have been shown to be promoted in part by arrestins (reviewed in Refs. 1–3). Arrestins promote these processes by interacting with the agonist-occupied form of GPCRs. Recruitment of arrestins to receptors is promoted by phosphorylation of the receptor by G protein-coupled receptor kinases (4–6), although binding of arrestins to receptor in the absence of receptor phosphorylation has also been observed (7, 8). The binding of arrestin appears to sterically inhibit interaction of the receptor with G-proteins, thus attenuating receptor-mediated signaling (9–11). Arrestins mediate the internalization of receptors from the cell surface by interacting with clathrin, the major component of clathrin-coated pits, thus facilitating receptor endocytosis through the clathrin-coated vesicle pathway (12–14). Arrestins have recently been shown to be involved in both receptor down-regulation (15), the overall decrease in receptor number after prolonged agonist exposure, as well as resensitization (16), the dephosphorylation of the receptor and its recycling back to the cell surface after agonist removal. Recently, a role for arrestin-mediated internalization of GPCRs in the activation of the p42/p44 mitogen-activated protein kinase pathway has been proposed (17).

Extensive study of visual arrestin, which is involved in quenching phototransduction via its specific interaction with metarhodopsin II, has served as a basis for the model of nonvisual arrestin function. The four mammalian arrestins, visual arrestin (arrestin-1), \(\beta\)-arrestin (arrestin-2), \(\beta\)-arrestin-2 (arrestin-3), and cone arrestin (arrestin-4), share approximately 45% identity and 70% similarity to one another (18). Initial binding of arrestin-1 to phosphorylated metarhodopsin II occurs through the activation- and phosphorylation-recognition domains. These domains are located within the amino-terminal half of arrestins and include a conserved arginine residue (Arg-175 in arrestin-1) within the phosphorylation recognition domain (6, 19). This residue, upon interaction with receptor, is thought to act as a molecular switch that promotes a conformational change in arrestin. This conformational change is proposed to disrupt the intramolecular interaction of the basic amino terminus and acidic carboxyl terminus (20, 21), which allows binding of a hydrophobic portion of arrestin distal to Arg-175 (5, 20, 22). The recently reported crystal structure of arrestin-1 (23) has substantially confirmed the structural and functional organization of arrestin derived from biochemical and mutagenesis studies. In contrast to arrestin-1, the nonvisual arrestins, arrestin-2 and arrestin-3, contain a region within the carboxyl terminus that mediates binding to clathrin and subsequent internalization of receptors through the clathrin-coated vesicle pathway (14).

The ability to specifically interrupt arrestin-mediated inter-
nalization of GPCRs is a potentially powerful means by which to probe both receptor trafficking and the mechanism of action of arrestins. Dominant negative mutants of arrestin-2 have been described that appear to function by competing with endogenous arrestins for binding to clathrin. A Val-to-Asp mutation (V53D) in arrestin-2 has been shown to exhibit increased affinity for clathrin and decreased affinity for receptor (7, 24). Also, overexpression of a peptide comprising the arrestin-2 clathrin binding domain has been shown to inhibit internalization of both β2-adrenergic receptors (β2AR) and lutropin/choriogonadotropin receptors in a dominant negative manner (24, 25). A dominant negative mutant of dynamin that lacks GTP binding (K44A) (26) has also proven useful in assessing the role of endocytosis in various receptor-mediated events but may not be specific for the clathrin-coated vesicle pathway (27).

In this study, we have further analyzed the role of arrestin-3 in receptor trafficking by designing a panel of arrestin-3 minigenes. We assessed the effect of these constructs on β2AR internalization in both COS-1 and HEK-293 cells. These studies resulted in the characterization of several novel dominant negative arrestin-3 proteins that inhibit arrestin function at either the level of receptor or clathrin. Moreover, we identify additional residues that comprise the clathrin binding domain. The availability of arrestin-3 mutants that can interfere with arrestin function at separate steps in the endocytic pathway should prove useful in elucidating the role of arrestins in the trafficking of GPCRs mediated by different endocytic pathways and their role in GPCR-mediated signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs—**pcDNA3-Flag-β2AR, wild-type arrestin-3 and dynamin K44A plasmids were previously described (14, 15, 24, 28). The arrestin-3 minigenes 1–194, 1–320, 1–340, 1–350, 1–360, 1–370, and 1–383 were constructed as follows. A 5’ top-strand primer was designed to include a GC “clamp,” a HindIII site and a consensus Kozak sequence (CCATGG) followed by 4–5 codons encoding the amino terminus of arrestin-3. The 3’ bottom strand primer was composed of a GC clamp, an XbaI site and a stop codon (TGA) followed by the codons for the last 4–5 amino acids of the construct. These primers were used in a PCR reaction with wild-type pcDNA3-arrestin-3 template using standard PCR conditions (94 °C for 1 min, 52 °C for 30 s, 72 °C for 1 min for 30 cycles, followed by a 10-min extension at 72 °C). The resulting fragments were digested with HindIII and XbaI and ligated to pcDNA3 cut with the same enzymes. For the carboxyl-terminal arrestin-3 constructs 201–409, 204–409, 209–409, 297–409, 309–409, and 320–409, the same design was used with the exception that the 5’ top-strand primer encodes for the sequence after the initial methionine, and the 3’ bottom strand primer contained either a BglII or BamHI site. The resulting PCR products were digested with HindIII and either BglII or BamHI and ligated into pcDNA3 cut with HindIII and BamHI. All constructs were sequenced by diodeoxy dye termination.

**Cell Culture and Transient Transfection—**COS-1 and HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 95% air, 5% CO2. Before transfection, cells were grown to 70–90% confluency. Cells were transfected for 5–7 h in either 60- or 100-mm dishes (Falcon) and 10- or 30-well plates that had been coated with 0.1 mg/ml poly-L-lysine (Sigma) and allowed to adhere overnight at 4 °C in Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin. Cells were then treated with 10 μM isoproterenol for 10 min, washed, fixed with 3.7% formaldehyde, permeabilized with 0.05% Triton X-100 for 10 min, and incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200 dilution). To visualize arrestins, cells were fixed, permeabilized, and assayed as described above for arrestin internalization. The primary antibodies used to detect arrestins were either F4C1 (1:500 dilution) or the arrestin-3-specific polyclonal rabbit antibody (1:200 dilution). Clathrin-coated vesicles were visualized using a mouse monoclonal antibody (AP-6) raised against the clathrin adaptor protein, AP-2 (American Type Culture Collection). Arrestin-3 proteins were visualized with either goat anti-rabbit or anti-mouse rhodamine- or FITC-conjugated secondary antibodies diluted 1:200; AP-2 was visualized with a goat anti-mouse FITC-conjugated antibody diluted 1:100 (Mo- lycor, Inc.). Coverslips were mounted using Slow-Fade mounting medium (Molecular Probes) and examined by microscopy on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 40× objective. Cells expressing the lowest levels of transfected proteins, but clearly above those of nonexpressing cells, were chosen for view. Images were collected using QED camera software and processed with Adobe Photoshop v. 3.0.

**Binding of Arrestins to Rhodopsin—**Arrestin-3 constructs were transcribed and translated in vitro using the TnT-coupled reticulocyte lysate system (Promega) according to the manufacturer’s protocol. Briefly, arrestin-3 minigenes cloned into pcDNA3 were added to a reaction mix containing TnT buffer, 1 μl of T7 RNA polymerase, 1 μm amino acid mixture (–Leu), 1 μl RNAasin, 25 μl of rabbit reticulocyte lysate, and 5 μl of [3H]leucine (179 Ci/mmol) in a total volume of 50 μl. The reactions were incubated for 90 min at 30 °C, and incorporated radioactivity was calculated by counting 1 μl of total reaction and spotting 1 μl onto Whatman 3MM paper followed by successive incubation for 10 min in cold 10% (w/v) trichloroacetic acid, 10 min in boiling 5% (w/v) trichloroacetic acid, and 2 min in 100% ethanol. Precipitated material was solubilized in 5% SDS, 1 M sodium hydroxide, and the radioactivity was determined by counting 1 μl of the sample in scintillation fluid.

Urea-treated rod outer segment membranes were prepared, phosphorylated with rhodopsin kinase to a stoichiometry of 1 mol/mol and regenerated with 11-cis-retinal as described (20). To assess receptor binding, 2 nM translated arrestins were incubated for 5 min at 37 °C under constant illumination with 200 nM phosphorylated rhodopsin in a total volume of 50 μl in 50 mM Tris-HCl, pH 7.5, 0.5 mM magnesium chloride, 100 mM potassium acetate, and 1.5 mM dithiothreitol. Samples were quenched on ice, and receptor-bound arrestins were separated by Sepharose-2B column chromatography as described previously (22).

The ELISA assay was performed essentially as described by Daunt et al. (29). Briefly, cells were split into 24-well dishes as described above and were treated with 0.3 mM ascorbate with or without 10 μM isoproterenol, then fixed for 5 min with 3.7% formaldehyde. The primary anti-Flag antibody M1 (Sigma) and the secondary antibody, goat anti-mouse alkaline phosphatase (Bio-Rad), were used at a 1:1,000 dilution. Visualization of antibody binding was performed using an alkaline phosphatase substrate kit (Bio-Rad). Plates were read at 405 nm in a microplate reader driven by Microplate Manager software (Bio-Rad). To assess arrestin expression, cells were pelleted, resuspended in SDS sample buffer, and separated on 10% SDS-polyacrylamide gels, then transferred to nitrocellulose. To detect amino-terminal arrestin constructs, blots were probed with F4C1 (1:1,000 dilution), a mouse monoclonal antibody directed against a conserved epitope in the amino terminus of all arrestins, DGVVLD (30). To detect expression of carboxyl-terminal constructs, blots were probed with a rabbit polyclonal antibody directed against a glutathione S-transferase fusion protein containing residues 350–409 of arrestin-3 (1:2,000 dilution). Protein was visualized using the appropriate species-specific horseradish peroxidase-conjugated secondary antibodies raised in goat (Bio-Rad) diluted 1:2,000 followed by development by enhanced chemiluminescence (ECL (Amersham Pharmacia Biotech) or Supersignal (Pierce)).

**Immunofluorescence Microscopy—**COS-1 cells in 60-mm dishes were transfected as described above with 3 μg Flag-β2AR and 1 μg of the indicated arrestin-3 construct or 1 μg of the indicated arrestin-3 construct alone. After transfection, cells were allowed to adhere to glass coverslips grown in 24-well dishes. To visualize cell surface receptors, cells were incubated with M1 antibody diluted 1:500 for 1 h at 4 °C in Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin. Cells were then treated with 10 μM isoproterenol for 10 min, washed, fixed with 3.7% formaldehyde, permeabilized with 0.05% Triton X-100 for 10 min, and incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200 dilution). To visualize arrestins, cells were fixed, permeabilized, and assayed as described above for arrestin internalization. The primary antibodies used to detect arrestins were either F4C1 (1:500 dilution) or the arrestin-3-specific polyclonal rabbit antibody (1:200 dilution). Clathrin-coated vesicles were visualized using a mouse monoclonal antibody (AP-6) raised against the clathrin adaptor protein, AP-2 (American Type Culture Collection). Arrestin-3 proteins were visualized with either goat anti-rabbit or anti-mouse rhodamine- or FITC-conjugated secondary antibodies diluted 1:200; AP-2 was visualized with a goat anti-mouse FITC-conjugated antibody diluted 1:100 (Molecular Probes). Coverslips were mounted using Slow-Fade mounting medium (Molecular Probes) and examined by microscopy on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 40× objective. Cells expressing the lowest levels of transfected proteins, but clearly above those of nonexpressing cells, were chosen for view. Images were collected using QED camera software and processed with Adobe Photoshop v. 3.0.
RESULTS AND DISCUSSION

Expression and Functional Analysis of Arrestin-3 Mini- genes—Extensive mutagenesis of arrestin-1, coupled with the recent solution of its crystal structure, has revealed several distinct domains involved in arrestin function (20, 22, 23). However, much less is known about the functional organization of the nonvisual arrestins, arrestin-2 and arrestin-3. We have previously mapped the clathrin binding domain of arrestin-3 (14) and have shown that expression of the corresponding domain of arrestin-3 dominantly inhibited wild-type arrestin function (24). We therefore reasoned that expression of the arrestin-3 clathrin binding domain, which has been shown to have a higher affinity for clathrin (12), may act as a more potent inhibitor of arrestin function. Similarly, we wanted to determine whether expression of the receptor binding domain of arrestin-3 would also inhibit receptor internalization. To test these ideas, we constructed a panel of arrestin-3 minigenes containing either putative receptor or clathrin binding domains. These constructs, shown schematically in Fig. 1, were designed partially on the basis of homology of arrestin-3 domains to those in arrestin-1 (19).

Initially, we tested the ability of each of the constructs to be expressed in COS-1 cells to determine which were most suitable for further study. As shown in Fig. 2A, all of the arrestin-3 amino-terminal minigenes were detectably expressed in COS-1 cells. Compared with wild-type arrestin-3, arrestin-3(1–184) was less efficiently expressed, and arrestin-3(1–340) consisted of 3–4 discrete bands, most likely as a result of proteolytic degradation. The carboxyl-terminal minigenes were also efficiently expressed, except for arrestin-3(320–409), which was detected only after overexposure of the immunoblot (data not shown). Arrestin-3(297–409) migrated anomalously on SDS-polyacrylamide gel electrophoresis, appearing to migrate more slowly than its larger counterparts, arrestin-3(284–409) and (290–409). For these reasons, the 1–184, 1–340, 297–409, and 320–409 arrestin-3 constructs were not studied in detail.

We next evaluated the function of each arrestin-3 protein by assessing its ability to promote the internalization of the β2AR in COS-1 cells. It has previously been shown that internalization of the β2AR in COS cells is inefficient but can be promoted to significant levels by overexpression of nonvisual arrestins (15, 24, 31, 32). Two different assays were used to assess receptor internalization, an ELISA to measure the surface levels of the Flag-tagged β2AR and a ligand binding assay to measure the binding of the hydrophilic ligand [3H]CGP-12177, which should bind exclusively to cell surface receptors. The results of the ELISA assay are depicted in Fig. 3A. Of the amino-terminal minigenes, which contained progressive deletions of the carboxyl terminus, arrestin-3(1–320) and arrestin-3(1–340) were unable to promote β2AR internalization above basal levels. In contrast, the 1–350, 1–360, and 1–370 arrestin-3 proteins each promoted β2AR internalization to an extent comparable with that of wild-type arrestin-3. Because mutation or deletion of the major clathrin binding domain between residues 371–379 of arrestin-3 did not completely abolish clathrin binding or promotion of internalization (12, 14), this data suggests that additional clathrin binding determinants exist that lie between amino acids 340–370. Arrestin-3(1–393) lacks the acidic carboxyl terminus, which has been proposed to mediate an intramolecular interaction with the basic amino terminus to maintain arrestin-3 in an inactive conformation (21). Thus, perturbation of this interaction in arrestin-3 might result in a constitutively active arrestin. Indeed, arrestin-3(1–393) appears to more effectively promote high affinity agonist
binding to the β₂AR and M2 muscarinic receptor compared with wild-type arrestin-3 (33). As shown in Fig. 3A, arrestin-3(1–393) promoted receptor internalization as efficiently as wild-type arrestin-3, but receptor internalization remained dependent upon the presence of agonist. In the absence of agonist, the number of surface receptors present in cells cotransfected with arrestin-3(1–393) was similar to that present in cells cotransfected with wild-type arrestin-3 (data not shown).

Progressive deletion of the amino terminus of arrestin-3, in which both receptor binding and phosphorylation recognition domains were deleted, resulted in the proteins arrestin-3(201–409), arrestin-3(284–409), arrestin-3(290–409), arrestin-3(297–409), and arrestin-3(309–409). These arrestins were unable to promote internalization of the β₂AR (data not shown). These results show that the ability of arrestins to internalize receptors can be accurately measured in two different assays using a range of receptor expression levels.

Localization of Arrestin-3 Minigenes and Visualization of β₂AR Internalization in Cells—We then evaluated the ability of selected functional and nonfunctional arrestin-3 proteins to mediate the redistribution of the β₂AR in cells. To do this, we cotransfected COS-1 cells with Flag-tagged β₂AR and the different arrestin-3 constructs, then performed immunofluorescence analysis by incubating cells with the Flag antibody before agonist exposure. In this way, only the trafficking of receptors initially present on the cell surface would be detected. After agonist exposure, cells were permeabilized, and the Flag epitope was visualized with fluorescein-labeled secondary antibodies (1:200 dilution). WT, wild type.

Fig. 3. Analysis of the ability of arrestin-3 (Arr-3) minigenes to promote β₂AR internalization in COS-1 cells. A, COS-1 cells were cotransfected with 3 μg of pcDNA3-Flag-β₂AR and 1 μg of either empty vector or the indicated arrestin-3 constructs. 24 h after transfection, cells were treated with 10 μM (-) isoproterenol for 30 min and assayed for internalization of the β₂AR by ELISA. Values represent the mean ± S.E. of 3–5 independent experiments performed in triplicate. B, COS-1 cells were cotransfected with 5 μg of pcDNA3-Flag-β₂AR and 5 μg of the indicated arrestin-3 constructs. 24 h after transfection, cells were treated with isoproterenol, and internalization was assayed by [3H]CGP-12177 binding. The average β₂AR expression was between 3 and 3.5 pmol/mg. Values represent the mean ± S.D. of 2–3 independent experiments performed in triplicate.

Fig. 4. Immunofluorescence analysis of β₂AR trafficking. COS-1 cells were transfected with 3 μg of pcDNA3-Flag-β₂AR and 1 μg of the indicated arrestin-3 (Arr-3) constructs for 5 h then trypsinized and replated on glass coverslips. 24 h post-transfection, surface receptors were labeled by incubation of cells with M1 Flag antibody (1:500 dilution) for 1 h at 4 °C. Cells were washed with phosphate-buffered saline, exposed to 10 μM (-) isoproterenol for 10 min, fixed, and permeabilized. Localization of receptors was revealed by incubating cells with FITC-conjugated goat anti-mouse secondary antibodies (1:200 dilution). WT, wild type.
with the redistribution of $\beta_2$AR to clathrin-coated pits and endosomes.

In COS-1 cells overexpressing the $\beta_2$AR receptor and arrestin-2, arrestin-2 is only found associated with clathrin-coated pits after agonist exposure (12, 24). In contrast, overexpression of the carboxyl-terminal clathrin binding domain of arrestin-2, arrestin-2(319–418), was previously shown to display a constitutively punctate appearance and colocalized with clathrin in the absence of receptor and agonist (24). To determine the subcellular localization of the arrestin-3 minigenes in the absence of receptor and agonist (24). To determine the subcellular localization of the arrestin-3 minigenes in the absence of the indicated arrestin-3 construct then trypsinized and replated on glass coverslips. 24 h post-transfection, coverslips were processed for immunofluorescence analysis as described under “Experimental Procedures.” AP-2 was detected with a mouse monoclonal antibody (AP-6; 1:200 dilution). The carboxyl-terminal arrestin-3 proteins were detected with a polyclonal rabbit antiseraum raised against residues 350–409 of arrestin-3 (1:250 dilution), and amino-terminal proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins were visualized with goat anti-mouse or anti-rabbit FITC- or rhodamine-conjugated secondary antibodies. WT, wild type.

The $\beta_2$AR receptor and arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins arrestin-3 (1:200 dilution). The carboxyl-terminal arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins were visualized with goat anti-mouse or anti-rabbit FITC- or rhodamine-conjugated secondary antibodies. WT, wild type.

The $\beta_2$AR receptor and arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins arrestin-3 (1:200 dilution). The carboxyl-terminal arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins were visualized with goat anti-mouse or anti-rabbit FITC- or rhodamine-conjugated secondary antibodies. WT, wild type.

The $\beta_2$AR receptor and arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins arrestin-3 (1:200 dilution). The carboxyl-terminal arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins were visualized with goat anti-mouse or anti-rabbit FITC- or rhodamine-conjugated secondary antibodies. WT, wild type.

The $\beta_2$AR receptor and arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins arrestin-3 (1:200 dilution). The carboxyl-terminal arrestin-3 proteins were detected with an arrestin monoclonal antibody, F4C1 (1:250 dilution). Arrestins were visualized with goat anti-mouse or anti-rabbit FITC- or rhodamine-conjugated secondary antibodies. WT, wild type.
Mechanism of Action of Dominant Negative Arrestins

Internalization of the β2-AR in HEK-293 cells. A, HEK-293 cells were cotransfected with 1 μg of pcDNA3-Flag-β2AR and 4 μg of either empty vector or the indicated arrestin-3 construct. 24 h after transfection, cells were exposed to 10 μM (-) isoproterenol for 30 min and assayed for internalization of the β2-AR by ligand binding as described in “Experimental Procedures.” Values shown represent the mean ± S.E. of 4–8 independent experiments performed in triplicate. Expression of β2AR averaged between 0.9–1.5 pmol/mg. B, analysis of arrestin-3 expression in HEK-293 cells. Cell lysates (15 μg of total protein) were subjected to immunoblot analysis using either the mouse monoclonal antibody F4C1 or a rabbit polyclonal arrestin-3 antibody. Arrestins were visualized by ECL using the appropriate species-specific horse-radish peroxidase-conjugated secondary antibody. The results shown are representative of four independent transfections.

Proteins did not bind to rhodopsin. This result clearly indicates that arrestin-3(1–320) is likely to inhibit receptor internalization by competing for receptor binding sites and strongly suggests that arrestin-3(201–409) does not function in this manner.

In summary, we have expressed and characterized a panel of arrestin-3 minigenes to more precisely elucidate the role of arrestin-3 in receptor trafficking. We show that expression of an arrestin-3 protein composed of the major receptor activation and phosphorylation recognition domains can dominantly inhibit wild-type arrestin function by competing for receptor binding sites and strongly suggests that arrestin-3(201–409) does not function in this manner.

In vitro

FIG. 7. Analysis of receptor binding properties of arrestin-3 (Arr-3) minigenes. A, the indicated arrestin-3 constructs were transcribed and translated as described under “Experimental Procedures.” 1 μl of a 50-μl coupled transcription and translation reaction was separated on a 10% SDS-polyacrylamide gel. The gel was stained, destained, incubated with 2,5-diphenyloxazole, dried, and exposed to standard autoradiography film for 33 h at –70°C. B, 2 μl of in vitro-translated, [3H]leucine-labeled arrestin-3 proteins were incubated with 200 nM phosphorylated light-activated rhodopsin as described under “Experimental Procedures.” Bound arrestins were separated by Sephrose-2B chromatography and quantitated by scintillation counting. The results are expressed as fmol bound after subtraction of binding in the absence of receptor and represent the average ± S.E. of 4–8 independent experiments.

clathrin-coated pits despite the presence of the clathrin binding domain. One possibility is that the presence of the hydrophobic domain between amino acids 201 and 284 prevents the constitutive association of this protein with membrane-localized clathrin-coated pits. Alternately, a fraction of this protein may be associated with coated pits in amounts that are too low to be detected.

Recently, it has been reported that arrestins are recruited by agonist stimulation of at least 15 different GPCRs (34). Therefore, dominant negative arrestins are likely to be useful in dissecting the role of arrestins in the trafficking and signaling of a large number of GPCRs. The use of dominant negative arrestin-2 proteins in studies examining the role of arrestins in GPCR trafficking and signaling has been reported (7, 16, 17, 24, 25). Moreover, we have also observed that the dominant negative arrestin-3 constructs described in this study also inhibit the redistribution of both the α2-adrenergic and thromboxane A2 receptors.3 These observations further highlight the role of arrestins in regulating the trafficking of many different GPCRs. However, at present little is known about whether arrestin-2 or arrestin-3 demonstrates specificity for a given receptor. The availability of dominant negative arrestins that target the clathrin binding domain, as well as dominant negative arrestins that interfere with receptor binding, may help to resolve this issue.

Internalization of receptor tyrosine kinases such as the epidermal growth factor receptor have also been shown to proceed

3 J. DeGraff and J-L. Parent, unpublished observation.
via a clathrin-coated vesicle pathway (35–37). It is at present not known whether arrestins participate in the internalization of receptor tyrosine kinases by this pathway. The trafficking of several GPCRs including the β2AR (38), bradykinin B2 (39, 40), and M2 muscarinic receptors (41) as well as the receptor tyrosine kinases epidermal growth factor (42, 43) and platelet-derived growth factor (44) have also been shown to be mediated by noncoated vesicles known as caveolae. It is possible that arrestins may also play a role in receptor endocytosis and modulation of receptor signaling mediated by caveolae. Because mutant dynamin alleles have also been shown to inhibit the pinching off of caveolae (27), studies in which dynamin K44A alone is used to examine receptor trafficking are not likely to differentiate between clathrin and caveola-mediated endocytosis. Therefore, the use of dominant negative arrestins that inhibit receptor internalization either at the level of receptor or clathrin may prove to be a more valuable tool in elucidating the role of arrestins in receptor trafficking and signaling mediated by different endocytic pathways.

Acknowledgments—We thank the members of the Sidney Kimmel Nucleic Acid Facility for oligonucleotide synthesis and sequencing, Jessica DeGraff for assistance with minigene construction, and members of the Benovic laboratory for helpful discussions.

REFERENCES

1. Krupnick, J. G., and Benovic, J. L. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 289–319
2. Carman, C. V., and Benovic, J. L. (1998) Curr. Opin. Neurobiol. 8, 335–344
3. Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 18677–18680
4. Ferguson, S. S. G., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A., and Benovic, J. L. (1998) J. Biol. Chem. 273, 24782–24789
5. Gurevich, V. V., Richardson, R. M., Kim, C. M., Hosey, M. M., and Benovic, J. L. (1999) J. Biol. Chem. 274, 16879–16882
6. Gurevich, V. V., and Benovic, J. L. (1999) J. Biol. Chem. 274, 6010–6016
7. Ferguson, S. S. G., Downey, W. E. I., Colapietro, A.-M., Barak, L. S., Menard, L., and Benovic, J. L. (1996) Science 271, 363–365
8. Wu, G., Krupnick, J. G., Benovic, J. L., and Lanier, S. M. (1997) J. Biol. Chem. 272, 17836–17842
9. Atromadoul, H., Ariza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 17882–17890
10. Krupnick, J. G., Gurevich, V. V., and Benovic, J. L. (1997) J. Biol. Chem. 272, 18125–18131
11. Lohse, M. J., Anderxinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J.-P., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 8558–8564
12. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
13. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) J. Biol. Chem. 272, 15017–15022
14. Krupnick, J. G., Goodman, O. B., Jr., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 15011–15016
15. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) J. Biol. Chem. 273, 6976–6981
16. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. G. (1997) J. Biol. Chem. 272, 27055–27014
17. Daaka, Y., Luttrell, L. M., Ahn, S. J., Della Rocca, G. J., Ferguson, S. S. G., Caron, M. G., and Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 685–688
18. Sterne-Marr, R., and Benovic, J. L. (1995) Vitam. Horm. 51, 193–234
19. Gurevich, V. V., and Benovic, J. L. (1995) Mol. Pharmacol. 48, 161–169
20. Gurevich, V. V., and Benovic, J. L. (1995) J. Biol. Chem. 269, 11628–11638
21. Gurevich, V. V., Chen, C.-Y., Kim, C. M., and Benovic, J. L. (1994) J. Biol. Chem. 269, 8721–8727
22. Gurevich, V. V., Djon, S., Onorato, J. J., Ptatsienski, J., Kim, C. M., Sterne- Marr, R., Hosey, M. M., and Benovic, J. L. (1995) J. Biol. Chem. 270, 720–731
23. Lazzaro, M. de P. M., Bertrand, J. E., Nakamura, K., Liu, X., Krupnick, J. G., Benovic, J. L., and Ascoli, M. (1998) J. Biol. Chem. 273, 18316–18324
24. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
25. Henley, J. E., Krueger, E. W. A., Oswald, B. J., and McNiven, M. A. (1998) J. Cell Biol. 141, 85–99
26. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 32507–32512
27. Lazari, M. de P. M., Bertrand, J. E., Nakamura, K., Liu, X., Krupnick, J. G., Benovic, J. L., and Ascoli, M. (1998) J. Biol. Chem. 273, 18316–18324
28. Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A. D., and Benedetti, E. L. (1989) Eur. J. Cell Biol. 50, 340–352
29. de Woerd, W. F. C., and Leeu-Lundberg, L. M. F. (1997) J. Biol. Chem. 272, 1758–1766
30. Haasemann, M., Cartaud, J., Muller-Esterl, W., and Dunia, I. (1998) J. Cell Sci. 111, 917–928
31. Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997) J. Biol. Chem. 272, 17744–17748
32. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 30429–30434
33. Engelma, J. A., Chu, C., Lin, A., Jo, H., Ikezu, T., Okamoto, T., Kohtz, D. S., and Lisanti, M. P. (1998) FEBS Lett. 428, 205–211
34. Liu, P., Ying, Y., Ko, Y. G., and Anderson, R. G. (1996) J. Biol. Chem. 271, 10299–10303
Characterization of Dominant Negative Arrestins That Inhibit β2-Adrenergic Receptor Internalization by Distinct Mechanisms
Michael J. Orsini and Jeffrey L. Benovic

J. Biol. Chem. 1998, 273:34616-34622.
doi: 10.1074/jbc.273.51.34616

Access the most updated version of this article at http://www.jbc.org/content/273/51/34616

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 36 of which can be accessed free at http://www.jbc.org/content/273/51/34616.full.html#ref-list-1