

The Interaction of β -Arrestin with the AP-2 Adaptor Is Required for the Clustering of β_2 -Adrenergic Receptor into Clathrin-coated Pits*

Received for publication, March 27, 2000
Published, JBC Papers in Press, April 17, 2000, DOI 10.1074/jbc.M002581200

Stéphane A. Laporte, Robert H. Oakley, Jason A. Holt, Larry S. Barak, and Marc G. Caron‡

From the Howard Hughes Medical Institute Laboratories and the Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

β -Arrestins are cytosolic proteins that regulate the signaling and the internalization of G protein-coupled receptors (GPCRs). Although termination of receptor coupling requires β -arrestin binding to agonist-activated receptors, GPCR endocytosis involves the coordinate interactions between receptor- β -arrestin complexes and other endocytic proteins such as adaptor protein 2 (AP-2) and clathrin. Clathrin interacts with a conserved motif in the β -arrestin C-terminal tail; however, the specific molecular determinants in β -arrestin that bind AP-2 have not been identified. Moreover, the respective contributions of the interactions of β -arrestin with AP-2 and clathrin toward the targeting of GPCRs to clathrin-coated vesicles have not been established. Here, we identify specific arginine residues (Arg³⁹⁴ and Arg³⁹⁶) in the β -arrestin 2 C terminus that mediate β -arrestin binding to AP-2 and show, *in vitro*, that these domains in β -arrestin 1 and 2 interact equally well with AP-2 independently of clathrin binding. We demonstrate in HEK 293 cells by fluorescence microscopy that β_2 -adrenergic receptor- β -arrestin complexes lacking the β -arrestin-clathrin binding motif are still targeted to clathrin-coated pits. In marked contrast, receptor- β -arrestin complexes lacking the β -arrestin/AP-2 interactions are not effectively compartmentalized in punctated areas of the plasma membrane. These results reveal that the binding of a receptor- β -arrestin complex to AP-2, not to clathrin, is necessary for the initial targeting of β_2 -adrenergic receptor to clathrin-coated pits.

β -Arrestins are cytosolic proteins that participate in the desensitization of many G proteins-coupled receptors (GPCRs)¹ by binding to agonist-activated phosphorylated receptors and uncoupling them from their cognate G proteins (1–3). β -Arrestins not only mediate the desensitization of GPCRs but also trigger their internalization through clathrin-coated vesicles.

* This work was supported in part by a fellowship award from the Heart and Stroke Foundation of Canada and a fellowship from the Medical Research Council of Canada (to S. A. L.), National Institutes of Health Grant NS 19576, an unrestricted Neuroscience Award from Bristol-Myers Squibb (to M. G. C.), and National Institutes of Health Grant HL 61365 (to L. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Inst., Box 3287, Duke University Medical Center, Durham, NC 27710. Fax: 919-681-8641; E-mail: caron002@me.duke.edu.

¹ The abbreviations used are: AP-2, adaptor protein 2; β_2 AR, β_2 -adrenergic receptor; CT, C terminus; GFP, green fluorescent protein; GST, glutathione S-transferase; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid.

The evidence for the participation of β -arrestin in GPCR endocytosis comes from observations that overexpression of β -arrestin can rescue a β_2 AR sequestration-defective mutant (4). In addition, overexpression of dominant negative forms of β -arrestin or other endocytic proteins related to the clathrin pathway, such as dynamin, inhibit β_2 AR internalization (4–6). Moreover, we and others have shown that upon activation of the β_2 AR, β -arrestins translocate to receptors and that receptor- β -arrestin complexes concentrate in punctated areas of the plasma membrane with clathrin and the adaptor protein AP-2 (7–9). Endocytosis of receptors via clathrin-coated vesicles appears to be necessary for dephosphorylation, recycling, and resensitization of many GPCRs (10–15).

Receptors entering the endocytic pathway through clathrin-coated vesicles are linked to clathrin via their interactions with specific adaptor proteins (16–18). One such adaptor is the AP-2 protein that plays a critical role in the recruitment of clathrin and the assembly of lattices that constitute the coat of the internalized membrane during vesiculation. AP-2 is a heterotetrameric complex composed of two large subunits of ~100 kDa: the α and β_2 , and two smaller subunits: the μ_2 and σ_2 subunits of 50 and 17 kDa, respectively. By interacting with the cytoplasmic tail of the epidermal growth factor or transferrin receptors, AP-2 is believed to link these receptors to the clathrin-coated vesicles and initiate their endocytosis (19, 20). The interaction of AP-2 with these receptors appears to be mediated via specific sorting signals. For instance the μ_2 has been reported to recognize tyrosine-based internalization signals such as YXX ϕ (where ϕ is a bulky hydrophobic residue) and NPXY and dileucine motifs often found in the cytoplasmic tail of receptors (21–24). The β -subunit, which provides the link between the receptor and clathrin lattices by binding to the clathrin heavy chain, also appears to recognize dileucine motifs (25). β -Arrestins, which bind to and desensitize GPCRs, have been shown to interact with clathrin through a conserved motif in their C-terminal domain (8, 26). The receptor- β -arrestin complex also interacts with the β -subunit of the AP-2 (9), thus providing a potential mechanism by which GPCRs are targeted to clathrin-coated vesicles. However, the extent to which each interaction participates in clathrin-mediated GPCR endocytosis is unknown.

Here, we identify the specific residues in β -arrestin that mediate its binding to AP-2 and demonstrate that, like clathrin, AP-2 interacts directly with β -arrestin and that these interactions occur independently. Using β -arrestin mutants deficient in either AP-2 or clathrin binding, we evaluated the respective contribution of these interactions to the GPCR endocytic process. Although each interaction can be shown to participate in the internalization of the β_2 AR, only the β -arrestin/AP-2 interaction seems to be required for the initial targeting of the receptor to clathrin-coated pits.

MATERIALS AND METHODS

Plasmids Constructs—Recombinant DNA procedures were carried out following standard protocols. The GAL4 BD- β -arrestin 2 fusion protein mutants were generated by polymerase chain reaction (PCR). Residues 381–410 of β -arrestin 2 were substituted with quintuple alanines by replacing the *EcoRI-SalI* fragments of pAS2-1- β -arrestin 2 with the PCR products. A similar strategy was employed to introduce a single alanine substitution in β -arrestin 2 at position 396 (β -arrestin 2 R396A) or position 398 (β -arrestin 2 K398A). The GAL4-AD- β_2 -adaptin construct is described elsewhere (9).

Glutathione *S*-transferase (GST) fusion proteins of arrestin C-terminal domains were constructed from PCR fragments derived from the C terminus of rat β -arrestin 1 (331–418), rat β -arrestin 2 (333–410 or 377–410), β -arrestin 2 R396A (377–410), and β -arrestin 2 K398A (377–410) and cloned into *BamHI* and *XhoI* of pGEX-5X-2. Wild type bovine visual arrestin C terminus (322–404) and the N384R mutant were cloned into *BamHI* and *EcoRI* of the same vector.

A green fluorescent protein conjugated to the N-terminal domain of β -arrestin 2 (GFP- β -arrestin 2) or β -arrestin 2 mutant deficient in clathrin-binding (GFP- β -arrestin 2 AAEA) were generated by cloning the full-length β -arrestin 2 or β -arrestin 2 AAEA from pcDNA 3.1 Zeo into the *HindIII* and *ApaI* sites of pEGFP-C3. GFP- β -arrestin 2 R396A and GFP- β -arrestin 2 K398A were generated by replacing the *PstI-SalI* fragment from pEGFP- β -arrestin 2 with the corresponding digested fragment from pAS2-1- β -arrestin 2 R396A or - β -arrestin 2 K398A.

β -Arrestin 2 C-terminal minigene constructs (285–410) were constructed from PCR fragments derived from β -arrestin 2, β -arrestin 2 AAEA, β -arrestin 2 R396A, or β -arrestin 2 AAEA/R396A and cloned into the *BamHI* and *XbaI* sites of pcDNA 3.1 Zeo. A *NcoI* site was introduced at the N terminus of the minigene constructs, creating an initiating methionine followed by a glycine before the first conserved residue of β -arrestin 2. All constructs were verified by DNA sequencing (Howard Hughes Medical Institute DNA Sequencing Facility, Duke University).

Yeast Two-hybrid Assays—Fusion genes expressing either β -arrestin 2 or β -arrestin 2 mutants and β_2 -adaptin were transformed into Y187 or PJ69-4A yeast strains as described previously (9). Protein/protein interactions were assayed in PJ69-4A for their adenine auxotrophy complementation or in Y187 for β -galactosidase activity using a chemiluminescent β -galactosidase assay kit (CLONTECH).

Purification of Clathrin and AP-2 Complexes—Clathrin and AP-2 complexes were purified from 4–6 cow brains as described by Manfredi and Bazari (27) with minor modifications. Briefly, brains were homogenized in 400 ml of isolation buffer A (100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin) and spun at 8,000 rpm for 45 min in a Sorvall GS-3 rotor. The supernatant was recovered and spun at 45,000 rpm in a Ti-45 rotor for 60 min, and the pellets were resuspended in isolation buffer A, combined, and homogenized with a glass-on-glass Dounce homogenizer. Homogenates were diluted with equal volume of isolation buffer A containing 12.5% Ficoll 400, 12.5% sucrose and spun at 19,000 rpm for 40 min in a Sorvall SS-34 rotor. Supernatants were diluted in 400 ml of isolation buffer A and spun at 45,000 rpm in a Ti-45 rotor for 60 min. The pellets were combined and homogenized in Buffer B (1.0 mM Tris-HCl, pH 7.0, 200 mM dithiothreitol) and left on ice for 30 min. Samples were spun at 90,000 rpm in a TL-100 ultracentrifuge. The supernatant was saved, and the pellets were again homogenized in Buffer B and immediately spun at 90,000 rpm. Supernatants from two ultraspins were combined and loaded on an S-300 Sepharose 26/60 Sephacryl column (Amersham Pharmacia Biotech) equilibrated with 0.5 mM Tris-HCl, pH 7.0, 0.1 mM dithiothreitol, and 0.02% sodium azide. Samples were collected at a rate of 0.25 ml/min, and fractions were analyzed by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue. Fractions containing purified clathrin or AP-2 were pooled and concentrated using a Centrprep 10K according to the manufacturer's instructions. Purified proteins were either used immediately or stored on ice for up to 2–3 weeks.

GST Fusion Protein Expression and Pull-down Experiments—Plasmid DNAs were transformed in *Escherichia coli* BL21 cells. Overnight cultures were grown in LB medium supplemented with ampicillin (100 μ g/ml), diluted to an A_{600} of 0.2 in the same medium and grown for another 1 h at 37 °C. Cultured cells were then induced with 0.1 mM isopropyl-1-thio- β -D-galactopyranoside for 2 h at room temperature. Cells were then pelleted, washed once with phosphate-buffered saline (PBS) and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml lysozyme and incubated for 15 min on ice. Cells were lysed by adding Triton X-100 (1% v/v) immediately followed by two

freeze-thaw cycles. Solubilized cells were incubated with DNase (300 units) for 15 min on ice and centrifuged at 13,000 rpm for 10 min. Glutathione-Sepharose beads were added to the supernatant and gently agitated at 4 °C for 2 h. Beads were washed three times with cold PBS containing 1% Triton X-100 followed by three washes with cold PBS without any detergent. Protein concentration was determined using a DC protein assay kit (Bio-Rad), and the integrity of the fusion proteins was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

GST fusion proteins (1–2.5 μ g) on beads were incubated in 0.5 ml of binding buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA 0.2% Triton X-100) for 2 h at 4 °C with purified clathrin (10 μ g) or AP-2 (5 μ g). The beads were spun and washed three times with binding buffer followed by three washes with binding buffer without any detergent. Beads were resuspended in SDS sample buffer (8% SDS, 25 mM Tris-HCl, pH 6.5, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromphenol blue), and proteins were resolved by electrophoresis on a 4–20% gel, transferred onto nitrocellulose, and analyzed by Western blot using mouse monoclonal anti- β_2 -adaptin or clathrin antibodies (Transduction Laboratories) or stained with Coomassie Blue for GST protein detection.

Receptor Endocytosis—HEK 293 were transiently transfected with cDNA using a modified calcium-phosphate method (28). Transfected cells expressing hemagglutinin-tagged β_2 AR alone or co-expressing hemagglutinin-tagged β_2 AR with β -arrestin 2 C-terminal minigene constructs were exposed to 10 μ M isoproterenol for 30 min at 37 °C, and sequestration was assessed by flow cytometry as described previously (29). The expression of β -arrestin 2 minigenes was assessed by Western blot using a rabbit polyclonal antibody (30). Statistical significance was determined by a paired two-tailed *t* test.

GFP- β -Arrestin 2 Translocation in Live Cells and AP-2 Colocalization in Fixed Cells—GFP- β -arrestin 2 translocation was visualized in real time on a 37 °C heated stage Zeiss laser scanning microscope (LSM-510) as described previously (15). Cells expressing β_2 AR with GFP- β -arrestin 2, β -arrestin 2 AAEA, and β -arrestin 2 R396A were stimulated with 10 μ M isoproterenol in serum-free medium buffered with 20 mM HEPES, and images were collected sequentially every 30 s for a period of 5 min using a single line excitation filter of 488 nm and emission filters at 505–550 nm. For AP-2 staining, cells were fixed in 3.7% paraformaldehyde in PBS for 30 min following a 2-min agonist stimulation. Cells were washed in PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and incubated with crude extract of the anti- α -adaptin antibody AP.6 (1:1; American Type Culture Collection) for 1 h at room temperature. Cells were washed and incubated with goat anti-mouse IgG conjugated with Texas Red (1:250; Molecular Probes) for 1 h at room temperature. Samples were visualized using single sequential line excitation filters at 488 and 568 nm and emission filter sets at 505–550 nm for GFP detection and 585 nm for Texas Red detection.

RESULTS

We previously identified a region of 32 amino acids in the β -arrestin 2 C terminus (amino acids 378–410) that binds to the β_2 subunit of the AP-2 adaptor complex (9). To identify the specific residues within this region that mediate the β -arrestin/AP-2 interaction, β -arrestin 2 mutants were assessed for their ability to interact with β_2 -adaptin in a yeast two-hybrid assay. Alanine scanning substitutions of β -arrestin 2 residues 381–410 were generated. As shown in Fig. 1*a*, successive quintuple alanine substitution of residues 391–400 in the full-length β -arrestin 2 greatly impaired the ability of β -arrestin 2 to interact with β_2 -adaptin. This region of β -arrestin 2 is highly conserved among the arrestin family with the exception of the two charged residues arginine 396 and lysine 398 and the residues leucine 395 and glycine 399 (Fig. 1*b*). Because arrestins can participate in ionic interactions (31, 32), we focused our attention on the two conserved positively charged residues. We replaced arginine 396 and lysine 398 with alanine and compared both β -arrestin 2 mutants (R396A and K398A) with the wild type β -arrestin 2 for their interaction with β_2 -adaptin (Fig. 1*c*). The interaction between β -arrestin 2 or β -arrestin 2 K398A with β_2 -adaptin resulted in 25- and 15-fold increases in β -galactosidase activity, respectively, whereas no significant increase over basal activity was detected when the β -arrestin 2 R396A mutant was expressed with β_2 -adaptin in yeast (Fig.

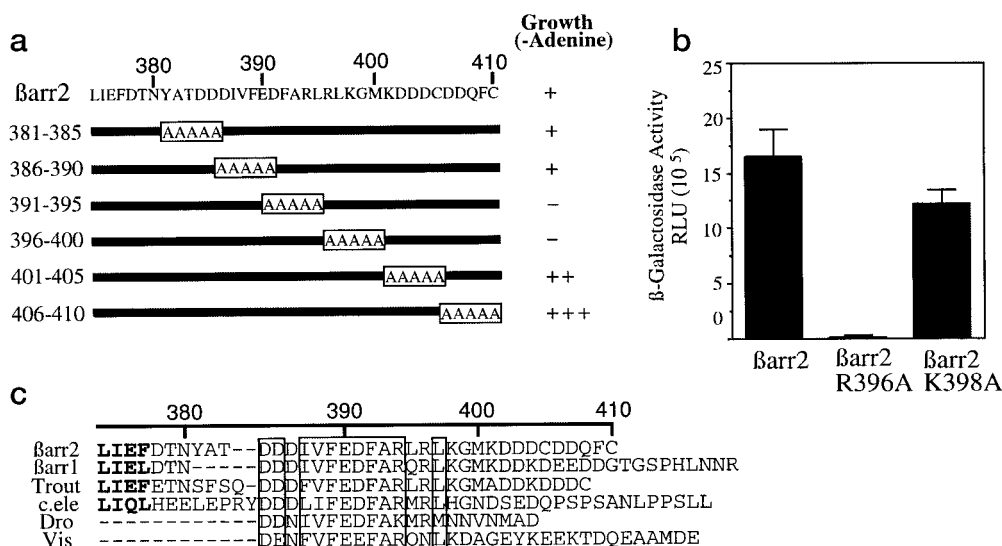


FIG. 1. Identification of residues in the C terminus of β -arrestin 2 involved in β_2 -adaptin binding. *a*, residues 391–400 in β -arrestin 2 are involved in β_2 -adaptin binding. Plasmids encoding the fusion protein GAL4 BD- β -arrestin 2 wild type or quintuple alanine mutants were co-transformed with the GAL4 AD- β_2 -adaptin fusion protein in PJ69-4A. Protein/protein interactions were assessed for adenine auxotroph complementation. Growth on adenine-deficient plates is indicated by +, and absence of growth, resulting from the lack of protein interactions, is indicated by -. *b*, sequence homology among arrestin C-terminal domains from rat β -arrestin 2 (accession number P29067), rat β -arrestin 1 (P29066), rainbow trout (P51466 or P51468), *Drosophila melanogaster* (P19107), *Caenorhabditis elegans* (P51485), and bovine visual arrestin (retinal S-antigen; P08168) C terminus. The boxed letters represent highly conserved residues. The putative clathrin binding site in β -arrestin 1, β -arrestin 2, trout arrestin, and *C. elegans* arrestin is highlighted in bold letters. Residues in β -arrestin 2 are numbered from amino acids 380 to 410. *c*, arginine 396 in β -arrestin is necessary for β_2 -adaptin binding. Protein interactions were assessed in Y187 yeast strain for β -galactosidase activity using a liquid assay as described under “Materials and Methods.” Results for the β -galactosidase activity, expressed in relative light units (RLU), are the means \pm S.D. of triplicate determinations and are representative of at least three independent experiments.

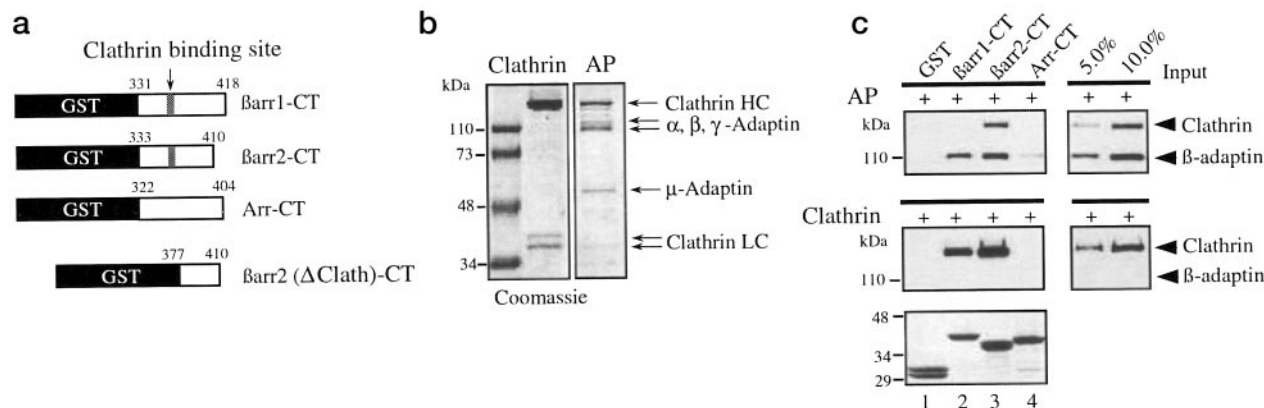


FIG. 2. Binding of arrestin C termini to purified clathrin and AP-2 adaptor proteins. *a*, GST fusion proteins were immobilized on Sepharose beads and incubated with purified clathrin or AP-2 adaptor proteins. *b*, Coomassie staining of purified clathrin and adaptor proteins (AP). Depicted are the clathrin heavy (HC) and light chains (LC), the α and β subunits of AP-2, and the γ subunit of AP-1. Note that the AP preparation also contains clathrin heavy and light chains. *c*, arrestin interactions with clathrin and AP-2. Purified adaptor (upper panels) or clathrin (middle panels) preparations were incubated with GST fusion proteins of β -arrestin 1, β -arrestin 2, or visual arrestin C termini (lower panel) and affinity purified as described under “Materials and Methods.” Affinity purified proteins were transferred onto membranes and immunodetected with both β_2 -adaptin and clathrin heavy chain (HC) antibodies or Coomassie stained to examine the expression of the GST fusion proteins. Results show that both β -arrestin 1 and 2 (lanes 2 and 3) can interact with AP-2 adaptor complex and clathrin, whereas visual arrestin only interacts marginally with AP-2 (lane 4). Input represents 5 or 10% of the total amount of starting material used in each assay (right panels). Results are representative of at least four independent experiments.

1c). Under the same conditions, β -arrestin mutants showed no differences in their interaction with clathrin when compared with wild type β -arrestin 2 (data not shown). These results reveal that arginine 396 in β -arrestin 2 is important for β_2 -adaptin binding.

We next examined whether arrestins could bind β_2 -adaptin in its heterotetrameric form (AP-2). GST fusion proteins of the C terminus (CT) of arrestins immobilized on Sepharose beads were incubated with purified clathrin and/or AP-2 adaptor proteins (Fig. 2, *a* and *b*). Recovered proteins were analyzed by SDS-polyacrylamide gel electrophoresis to detect immunoreactive clathrin heavy chain and AP-2 using specific antibodies

against the clathrin and the β_2 -adaptin (Fig. 2c). Both GST- β -arrestin 1-CT and β -arrestin 2-CT were equally effective in coprecipitating AP-2 (Fig. 2c, upper panel, lanes 2 and 3, respectively). In contrast, visual arrestin-CT, which does not contain the arginine 396 residue found in β -arrestin 2 or the clathrin binding motif, only weakly bound AP-2 (Fig. 2c, lower panel, lane 4). GST- β -arrestin 2-CT also co-precipitated clathrin (Fig. 2c, upper panel, lane 3), which is consistent with the presence of low levels of clathrin in the AP-2 preparation (Fig. 2b, right panel). Under these conditions, no immunoreactive clathrin heavy chain was detected with GST- β -arrestin 1-CT or visual arrestin-CT, in agreement with the reported lower affinity of β -ar-

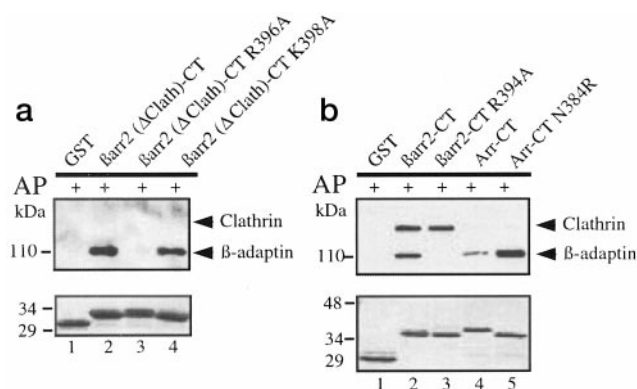


FIG. 3. The interaction of β -arrestin with AP-2 involves arginine residues in the β -arrestin 2 C terminus and is independent of clathrin binding. *a*, β -arrestin interaction with AP-2 does not require clathrin interaction. Results show that the GST- β -arrestin 2 C-terminal fusion protein lacking the clathrin binding domain (β arr2 (Δ Clath CT)-CT) was still able to affinity purify AP-2 but not clathrin. Mutation of the arginine 396 (R396A) but not the lysine 398 (K398A) impaired the ability of β -arrestin 2 to bind AP-2. Shown in the lower panel is the amount of GST fusion proteins used in each assay as revealed by Coomassie Blue staining. *b*, arginine 394 in β -arrestin 2 is involved in AP-2 binding. GST- β -arrestin 2 C-terminal fusion proteins were incubated with purified adaptor proteins (AP), affinity purified, and analyzed as described previously under "Materials and Methods." Results show that substitution of arginine 394 for an alanine residue impaired the ability of β -arrestin 2 to bind AP-2 without affecting its association with clathrin (lane 2 versus lane 3). Substitution of asparagine 384 in visual arrestin for an arginine enhanced the ability of visual arrestin to interact with AP-2. Results are representative of at least three independent experiments.

restin 1 for clathrin (8). Incubation of purified clathrin with similar amounts of GST- β -arrestin 1-CT, β -arrestin 2-CT or visual arrestin-CT resulted in the association of clathrin with β -arrestin 1 and 2 but not visual arrestin (Fig. 2*c*, middle panel, lanes 2–4, respectively). Again, β -arrestin 2 exhibited stronger interaction with clathrin than did β -arrestin 1.

To test whether β -arrestin binding to AP-2 was independent of the association of β -arrestin with clathrin, a GST- β -arrestin 2-CT fusion protein lacking the putative clathrin-binding site (Δ Clath; see Fig. 2*a*) was incubated with the adaptor protein preparation containing both AP-2 and clathrin. Results showed that GST- β -arrestin 2 (Δ Clath)-CT interacted with AP-2 even in the absence of its clathrin-binding site (Fig. 3*a*, lane 2). Substitution of the arginine 396 to alanine (R396A) in GST- β -arrestin 2 (Δ Clath)-CT abolished AP-2 binding, whereas the lysine 398 to alanine substitution (K398A) had no significant effect on AP-2 interaction (Fig. 3*a*, lanes 3 and 4, respectively). This is consistent with the yeast two-hybrid results.

Visual arrestin lacks both the putative clathrin-binding site and the conserved arginine 396 found in β -arrestin 2 (Fig. 1*b*). It is therefore not surprising to detect no interaction between visual arrestin and clathrin. However, the modest interaction detected between the visual arrestin and AP-2 suggests the participation of other residues. Indeed, alanine replacement of residues 391–395 greatly impaired the ability of β -arrestin to interact with β_2 -adapting (Fig. 1*a*). Another charged residue, arginine 394 in β -arrestin 2, is conserved throughout the arrestin family. This residue was substituted with an alanine residue (R394A), and its ability to interact with AP-2 was assessed (Fig. 3*b*). GST β -arrestin 2-CT R394A was found to bind to clathrin but failed to precipitate any detectable AP-2, whereas GST β -arrestin 2-CT interacted with both clathrin and AP-2 (Fig. 3*b*, lanes 2 and 3). These results suggest that both arginine residues 394 and 396 are involved in AP-2 binding. To further substantiate the involvement of these two residues, AP-2 binding was also investigated using visual arrestin in a

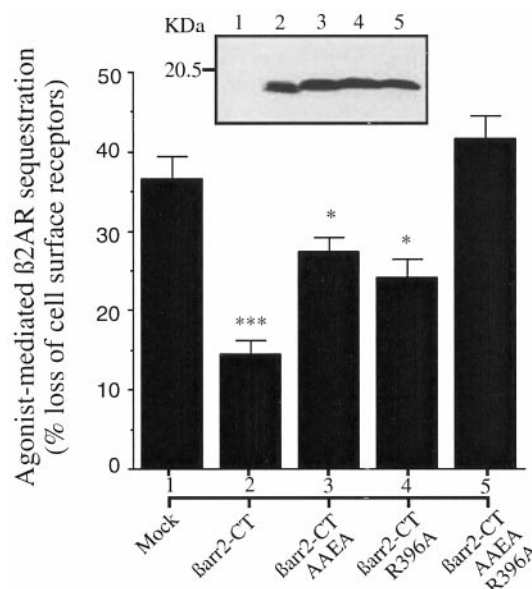


FIG. 4. Effect of β -arrestin 2 minigenes on agonist-induced β_2 AR sequestration in HEK-293 cells. The sequestration of β_2 AR was assessed in the presence of endogenous β -arrestin 2 (Mock), or overexpressed minigene constructs containing the wild type β -arrestin 2 C terminus (*Barr2-CT*) or deficient in clathrin or AP-2 binding sites (*Barr2-CT AAEA* and *Barr2-CT R396A*, respectively), or the β -arrestin 2 C terminus deficient in both clathrin and AP-2 binding sites (*Barr2-CT AAEA R396A*). Results show that a maximal inhibitory effect on β_2 AR sequestration was achieved when both sites were present in the β -arrestin 2 minigene (second bar). Removal of either the clathrin or the AP-2 binding site in β -arrestin 2 minigenes partially inhibited β_2 AR internalization compared with the minigene constructs containing both sites (bars 3 and 4 versus bar 2). The minigene construct lacking both the clathrin and AP-2 binding site had no effect on β_2 AR internalization (bar 5 versus bar 1). The level of β -arrestin 2 minigene expression was assessed by Western blot using a rabbit polyclonal antibody directed against the C-terminal domain of β -arrestin 2 (inset) (30). The data represent the means \pm S.D. of five to eight independent experiments.

gain-of-function paradigm. The asparagine 384 in visual arrestin, corresponding to position 396 in β -arrestin 2, was replaced by an arginine residue, and the mutant GST-arrestin-CT fusion protein was incubated with the AP-2 adaptor proteins (Fig. 3*b*). Although GST-arrestin-CT showed only a modest interaction with AP-2, GST-arrestin-CT N384R showed a robust association with AP-2 without any change in its interaction with clathrin as compared with the wild type arrestin-CT fusion protein (Fig. 3*b*, lanes 4 and 5). These results reveal that highly conserved arginine residues in β -arrestin 2 C terminus (arginine 394 and 396 in β -arrestin 2) are involved in AP-2 binding and suggest that the clathrin and AP-2 binding sites in β -arrestin 2 are distinct.

Our results provide biochemical evidence to support the premise that β -arrestin plays a role as an endocytic scaffold protein for both AP-2 and clathrin. To evaluate the function of these interactions in cells, we used minigene constructs containing β -arrestin 2 C-terminal domains. A similar construct containing the wild type β -arrestin 1 C-terminal domain has been shown previously to inhibit GPCR endocytosis (6, 33–35). Endocytosis of β_2 AR was assessed in HEK 293 cells expressing different C-terminal constructs of β -arrestin 2 lacking the clathrin-binding site, the AP-2-binding site, or both the clathrin- and AP-2-binding sites (Fig. 4). Results show that in cells expressing each individual construct at a similar level (Fig. 4, inset), the wild type β -arrestin C-terminal minigene had the most significant effect on the β_2 AR, inhibiting its internalization by more than 50% compared with cells expressing the receptor alone (mock). Expression of minigene constructs lack-

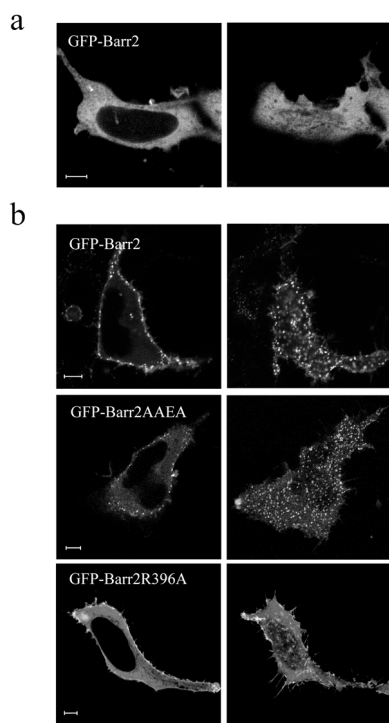


FIG. 5. Translocation of GFP- β -arrestin 2 to agonist-activated β_2 AR in HEK-293 cells. Wild type β -arrestin 2 or β -arrestin 2 deficient in clathrin or AP-2 binding were assessed for their translocation to β_2 AR using N-terminal GFP conjugates of β -arrestins. *a*, in unstimulated cells expressing both the β_2 AR and GFP- β -arrestin 2, the fluorescence is distributed uniformly throughout the cytoplasm as visualized both in Z sections of the middle (*left panel*) or bottom of the cell (*right panel*). A similar distribution of fluorescence was observed for the GFP- β -arrestin 2 clathrin-deficient or AP-2-deficient binding mutants (GFP-BarrAAEA and GFP-BarrR396A, respectively) in the absence of agonist (data not shown). *b*, in the presence of 10 μ M of isoproterenol, GFP- β -arrestin 2 translocates to β_2 AR and displayed a punctated distribution at the plasma membrane (*top panels*). Activation of β_2 AR in cells expressing GFP- β -arrestin 2 AAEA showed a similar punctated distribution at the plasma membrane, although the puncta appeared smaller in size (*middle panels*). GFP- β -arrestin 2 R396A translocated to agonist-activated receptor (*bottom panels*) but exhibited a more diffuse pattern at the plasma membrane with few puncta (*right panels*). The images represent 0.5- μ m Z sections of the middle of cells (*left panels*) or Z sections of the bottom of the cell (*right panels*). All scale bars are 5 μ m.

ing either the clathrin or the AP-2 binding sites resulted in 27 and 32% inhibitions on β_2 AR internalization, respectively. Removal of both sites abrogated the ability of the β -arrestin C-terminal minigene to inhibit β_2 AR internalization. These results suggest that β -arrestin interactions with both clathrin and AP-2 are important for β_2 AR endocytosis.

The endocytosis of GPCRs involves the interaction of multiple proteins. The impairment of β_2 AR endocytosis resulting from the inhibition of AP-2 and/or clathrin binding to β -arrestin, however, does not discriminate at which step in the endocytic pathway these interactions occur. To study the dynamic regulation of these interactions in the initial steps of β_2 AR endocytosis (*i.e.* targeting of receptor to clathrin-coated pits), we fused GFP to the N terminus of β -arrestin and assessed in real time the cellular distribution of agonist-activated β_2 AR/ β -arrestin complexes (Fig. 5). Shown are confocal images of sections running through the middle of the cell (*left panels*) or images that focus on AP-2 clusters on the plasma membrane at the bottom of the same cell (*right panels*). In the absence of agonist, GFP- β -arrestin 2 and GFP- β -arrestin 2 mutants were present in a predominantly diffuse, cytoplasmic distribution (Fig. 5*a* and data not shown). Upon agonist treatment, GFP- β -arrestin 2 translocated from the cytoplasm to the receptor at

the plasma membrane and clustered in puncta (Fig. 5*b*, *top panels*). Similarly, a GFP- β -arrestin 2 mutant deficient in clathrin binding (β -arrestin 2 AAEA) localized in punctated regions of the plasma membrane that appear smaller in size from that observed with the wild type GFP- β -arrestin 2 (Fig. 5*b*, *middle panels*). The GFP- β -arrestin 2 mutant deficient in AP-2 binding (β -arrestin 2 R396A) translocated to the receptor but failed to coalesce into puncta at the plasma membrane (Fig. 5*b*, *bottom panels*). A similar diffuse pattern of fluorescence at the plasma membrane was observed with GFP- β -arrestin 2 that lacked both the clathrin and the AP-2 binding sites (data not shown).

To test whether the β_2 AR/ β -arrestin 2 complexes were targeted to punctated regions of the plasma membrane representing clathrin-coated pits, agonist-stimulated cells expressing the β_2 AR with GFP- β -arrestin 2 or GFP- β -arrestin 2 R396A were fixed and immunostained for AP-2 (Fig. 6). Results show that GFP- β -arrestin 2 translocated to activated β_2 AR in punctated regions of the plasma membrane that coincided with AP-2 staining (Fig. 6*a*, *upper panels* and *inset*). These clusters were apparent in Z sections showing GFP- β -arrestin 2 fluorescence or AP-2 staining of cross-sections of the middle or the bottom of the cell (*upper* and *lower panels*, respectively). Similar distribution and colocalization was observed in stimulated cells expressing both the β_2 AR and GFP- β -arrestin 2 and immunostained for clathrin (data not shown). Stimulation of the β_2 AR in cells expressing the GFP- β -arrestin 2-AP-2-deficient mutant (GFP- β -arrestin R396A) showed β -arrestin translocation to the plasma membrane but in a uniformly distributed pattern with little colocalization with AP-2 (Fig. 6*b*, *upper panels* and *inset*). The presence of coated pits, comparable with cells expressing the wild type GFP- β -arrestin, were still detectable as visualized by the cross-section of the bottom of the cell stained for AP-2 (compare Fig. 6*b*, *bottom left panel* with same panel in Fig. 6*a*). However, in the same cross-section, clustering of β -arrestin 2 R396A in clathrin-coated pits was only sporadically detected (Fig. 6*b*, *bottom right panel* and *inset*). These results show that AP-2 binding to β -arrestin is required for agonist-mediated targeting of GPCR to coated pits and suggest that the interaction of clathrin with β -arrestin may not be involved in the initial event of endocytosis and must regulate downstream events of this process.

DISCUSSION

β -Arrestins play an important role in the endocytosis of many GPCRs. However, the molecular events involved between the activation of GPCRs and their concentration in clathrin-coated pits have not been determined. Here we show, using purified preparations of AP-2 and clathrin, that β -arrestin can bind independently to both proteins. The independent nature of these interactions is confirmed by the identification of two arginine residues essential for AP-2 binding in the β -arrestin C terminus downstream of the clathrin-binding site. Using β -arrestin mutants lacking either the AP-2 or the clathrin binding sites, we provide evidence that the interaction of β -arrestin with AP-2, rather than clathrin, is the necessary step for the clustering of β_2 AR into clathrin-coated pits. These results imply that the initial recruitment of AP-2 to diverse classes of membrane bound receptors may be a common step for endocytosis.

Evidence suggests that AP-2 sorts cargo (*i.e.* receptors) into coated vesicles by interacting with specific recognition sequences such as tyrosine-based or dileucine motifs (36). The β_2 -adaptin interaction with β -arrestin, which requires arginine residues, may yet represent another means by which AP-2 links cargo to coated pits. Similar interactions involving multiple arginine residues have also been described for the specific

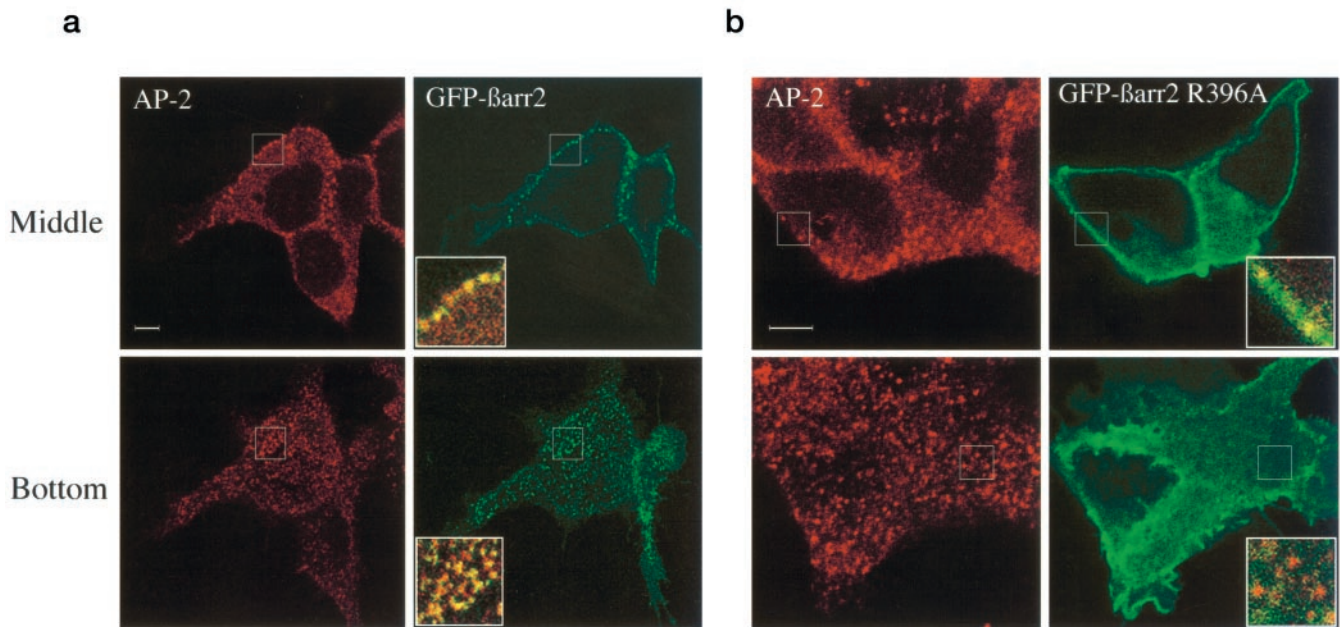
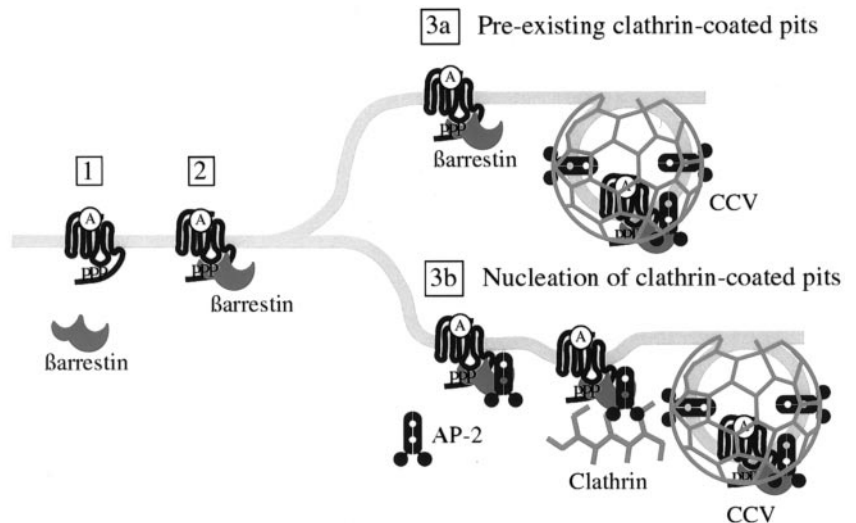


FIG. 6. **GFP- β -arrestin 2 colocalization with AP-2 in coated pits of agonist-treated HEK 293 cells expressing the β_2 AR.** Confocal images of Z sections representing regions of the middle (upper panels) or the bottom (lower panels) of the same cell expressing the β_2 AR with GFP- β -arrestin 2 wild type (a) or GFP- β -arrestin 2 deficient in AP-2 binding (R396A) and stained for AP-2 (left panels). Insets show the enlarged overlay images of β -arrestin fluorescence and AP-2 staining of the same boxed region of the cell. In presence of $10 \mu\text{M}$ of isoproterenol, GFP- β -arrestin 2 translocates to β_2 AR in punctated regions of the plasma membrane and colocalizes with AP-2. Under the same conditions, GFP- β -arrestin 2 R396A is recruited to the receptor but remains in a more diffuse pattern at the plasma membrane and is found only rarely in coated pits as seen by the low frequency of colocalization with AP-2. All scale bars are $5 \mu\text{m}$.

FIG. 7. **A model for the role of β -arrestin in GPCR targeting to clathrin-coated vesicles.** Upon activation of the β_2 AR, the receptor becomes phosphorylated in a G protein-coupled receptor kinase fashion (step 1), and β -arrestins translocate to the receptor (step 2). The receptor- β -arrestin complex may be targeted to pre-existing clathrin-coated vesicles (step 3a). Alternatively, the receptor- β -arrestin complex may recruit the AP-2 adaptor protein (step 3b), and this complex may initiate the assembly of clathrin and the formation of the clathrin-coated vesicle. The networks of contacts between β -arrestin, AP-2, and clathrin can cooperatively stabilize receptors in clathrin-coated pits. β -Arrestins can bind both to AP-2 and clathrin through their C-terminal domain, whereas AP-2 can also bind clathrin via its β -subunit. CCV, clathrin-coated vesicle.



binding of amphiphysin-2 with the SH3 domain of dynamin, two proteins involved in clathrin-mediated endocytosis (37). Visual arrestin lacks the paired arginine residues and interacts weakly with AP-2 as compared with β -arrestin 1 and 2. Reconstitution of the doublet of arginines in visual arrestin establishes the high efficacy interaction with AP-2.

A model for the interaction of β -arrestin 2 and the β -subunit of the AP-3 adaptor protein with clathrin has recently been proposed by ter Haar *et al.* (38). This model is based on the crystal structure of the N-terminal domain of clathrin and short peptides containing clathrin-binding motifs derived from the sequences of either β -arrestin 2 or the β -subunit of adaptor proteins. The structural data predict that these two peptides can interact with the same groove on the β -propeller surface of clathrin. Based on these observations, the authors proposed

that β -arrestin and AP-2 could either compete for the same binding site or more likely bind to adjacent clathrin heads in clathrin cages or cooperate in stabilizing complexes into coated pits (38). Our findings that the sites of interactions for clathrin and AP-2 reside within a 25-amino acid stretch in the C terminus of β -arrestin are interesting with respect to this model. The demonstration that *in vitro* β -arrestin can co-precipitate with both purified clathrin and AP-2 based on their individual interactions with β -arrestins might be consistent with the cooperation of these proteins in establishing networks of contacts in coated pits.

The visualization of the translocation of GFP- β -arrestin to activated receptors and their colocalization with AP-2 in coated pits provides a means to assess the relative contribution of these proteins to the endocytic process in live cells. β -Arrestin

mutants deficient in clathrin binding are still able to translocate to the β_2 AR and colocalize in pits, whereas β -arrestin mutants lacking the AP-2 binding site translocate to receptors, but the receptor- β -arrestin complexes are essentially excluded from pits. These results establish that the interaction of β -arrestin with AP-2 is a required step for the concentration of β_2 AR into coated pits and suggest that this interaction facilitate the recruitment and/or the assembly of clathrin coats. In this respect, β -arrestin may play the same role as AP-180, a monomeric clathrin adaptor protein that has been shown to have cooperative effects on clathrin assembly when interacting with AP-2 (39). Although the association of β -arrestin with clathrin may not be necessary for the initial targeting of β_2 AR to coated pits, this interaction is nonetheless of functional importance for receptor endocytosis. Expression of a dominant negative mutant of β -arrestin 2 containing both the clathrin and AP-2 binding sites is found to have additive inhibitory effect on the agonist-induced internalization of β_2 AR compared with β -arrestin mutants containing either one alone. Perhaps the interaction of β -arrestins with clathrin helps to stabilize receptor- β -arrestin-AP-2 complexes into individual coated pits. This interpretation is consistent with the cooperative model describe above and is substantiated by our observation that the intensity of puncta as visualized by the translocation of a GFP- β -arrestin 2 mutant lacking the clathrin-binding site was lower than the intensity observed with the GFP- β -arrestin 2. This may reflect a decrease in the number of receptors contained in pits or a decrease in the clustering of individual pits together. The clustering of multiple pits might be necessary for the effective endocytosis of the GPCRs. Indeed, such clusters of clathrin-coated pits have recently been observed upon agonist treatment of cells (40).

A pivotal unresolved question in the cell biology of receptor endocytosis is whether the cargo can initiate the nucleation of coated pits or whether the cargo is simply recruited to existing pits through its interaction with endocytic adaptor proteins. According to our data and as presented in the model in Fig. 7, β -arrestin translocation and its binding to agonist-activated receptors initiate the recruitment of the AP-2 adaptor protein. The receptor- β -arrestin-AP-2 complex could then initiate the assembly of clathrin lattices and the formation of cages (*step 3b*). Alternatively, the β_2 AR- β -arrestin complex could encounter the AP-2 adaptor in a pre-existing pit, and this interaction could be stabilized by the subsequent interaction of the complex with clathrin (*step 3a*). Although our data do not discriminate which pathway predominates, AP-2 interaction with β -arrestin most likely represents the required common step in both models. Whereas the interaction of β -arrestin with AP-2 is important in clustering the β_2 AR into coated pits, we cannot exclude the existence of other protein/protein interactions that may play a role in this process. Nonetheless, our results indicate that AP-2, rather than clathrin, is the proximal adaptor for β -arrestin-mediated targeting of β_2 AR into clathrin-coated pits and suggest that β -arrestin/clathrin interaction serves an ulterior role in the endocytosis of the β_2 AR.

Acknowledgments—We thank S. S. G. Ferguson and L. M. Luttrell for helpful comments on this manuscript and P. McDonald and R. T. Premont for help in purifying clathrin and AP-2.

REFERENCES

- Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) *Can. J. Physiol. Pharmacol.* **74**, 1095–1110
- Krupnick, J. G., and Benovic, J. L. (1998) *Annu. Rev. Pharmacol. Toxicol.* **38**, 289–319
- Lefkowitz, R. J. (1998) *J. Biol. Chem.* **273**, 18677–18680
- Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* **271**, 363–366
- Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J. Biol. Chem.* **271**, 18302–18305
- Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* **272**, 32507–32512
- Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) *J. Biol. Chem.* **272**, 27497–27500
- 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
- Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3712–3717
- Pippig, S., Andexinger, S., and Lohse, M. J. (1995) *Mol. Pharmacol.* **47**, 666–676
- Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 5–8
- Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. (1997) *J. Biol. Chem.* **272**, 27005–27014
- Vogler, O., Bogatkewitsch, G. S., Wriske, C., Krummnerl, P., Jakobs, K. H., and van Koppen, C. J. (1998) *J. Biol. Chem.* **273**, 12155–12160
- Ishii, I., Saito, E., Izumi, T., Ui, M., and Shimizu, T. (1998) *J. Biol. Chem.* **273**, 9878–9885
- Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *J. Biol. Chem.* **274**, 32248–32257
- Schmid, S. L. (1997) *Annu. Rev. Biochem.* **66**, 511–548
- Hirst, J., and Robinson, M. S. (1998) *Biochim. Biophys. Acta* **1404**, 173–193
- Kirchhausen, T. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 705–732
- Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996) *J. Biol. Chem.* **271**, 13377–13384
- Nesterov, A., Carter, R. E., Sorkina, T., Gill, G. N., and Sorkin, A. (1999) *EMBO J.* **18**, 2489–2499
- Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) *Science* **269**, 1872–1875
- Rapoport, I., Miyazaki, M., Boll, W., Duckworth, B., Cantley, L. C., Shoelson, S., and Kirchhausen, T. (1997) *EMBO J.* **16**, 2240–2250
- Bremnes, T., Lauvrak, V., Lindqvist, B., and Bakke, O. (1998) *J. Biol. Chem.* **273**, 8638–8645
- Hofmann, M. W., Honing, S., Rodionov, D., Dobberstein, B., von Figura, K., and Bakke, O. (1999) *J. Biol. Chem.* **274**, 36153–36158
- Rapoport, I., Chen, Y. C., Cupers, P., Shoelson, S. E., and Kirchhausen, T. (1998) *EMBO J.* **17**, 2148–2155
- Krupnick, J. G., Goodman, O. B., Jr., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* **272**, 15011–15016
- Manfredi, J. J., and Bazari, W. L. (1987) *J. Biol. Chem.* **262**, 12182–12188
- Ferguson, S. S., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A. M., and Caron, M. G. (1995) *J. Biol. Chem.* **270**, 24782–24789
- Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caron, M. G. (1994) *J. Biol. Chem.* **269**, 2790–2795
- Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 17882–17890
- Gurevich, V. V., and Benovic, J. L. (1995) *J. Biol. Chem.* **270**, 6010–6016
- Gaidarov, I., Krupnick, J. G., Falck, J. R., Benovic, J. L., and Keen, J. H. (1999) *EMBO J.* **18**, 871–881
- Vrecl, M., Anderson, L., Hanyaloglu, A., McGregor, A. M., Groarke, A. D., Milligan, G., Taylor, P. L., and Eidne, K. A. (1998) *Mol. Endocrinol.* **12**, 1818–1829
- Troispoux, C., Guillou, F., Elalouf, J. M., Firsov, D., Iacovelli, L., De Blasi, A., Combarnous, Y., and Reiter, E. (1999) *Mol. Endocrinol.* **13**, 1599–1614
- Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L., and Liu-Chen, L. Y. (1999) *J. Biol. Chem.* **274**, 12087–12094
- Kirchhausen, T., Bonifacino, J. S., and Riezman, H. (1997) *Curr. Opin. Cell Biol.* **9**, 488–495
- Owen, D. J., Wigge, P., Vallis, Y., Moore, J. D., Evans, P. R., and McMahon, H. T. (1998) *EMBO J.* **17**, 5273–5285
- ter Haar, E., Harrison, S. C., and Kirchhausen, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1096–1100
- Hao, W., Luo, Z., Zheng, L., Prasad, K., and Lafer, E. M. (1999) *J. Biol. Chem.* **274**, 22785–22794
- Gaidarov, I., Santini, F., Warren, R. A., and Keen, J. H. (1999) *Nat. Cell Biol.* **1**, 1–7

The Interaction of β -Arrestin with the AP-2 Adaptor Is Required for the Clustering of β_2 -Adrenergic Receptor into Clathrin-coated Pits
Stéphane A. Laporte, Robert H. Oakley, Jason A. Holt, Larry S. Barak and Marc G. Caron

J. Biol. Chem. 2000, 275:23120-23126.

doi: 10.1074/jbc.M002581200 originally published online April 17, 2000

Access the most updated version of this article at doi: [10.1074/jbc.M002581200](https://doi.org/10.1074/jbc.M002581200)

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 40 references, 30 of which can be accessed free at <http://www.jbc.org/content/275/30/23120.full.html#ref-list-1>