

The β_2 -adrenergic receptor/ β arrestin complex recruits the clathrin adaptor AP-2 during endocytosis

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ABSTRACT β arrestins mediate the desensitization of the β_2 -adrenergic receptor (β_2 AR) and many other G protein-coupled receptors (GPCRs). Additionally, β arrestins initiate the endocytosis of these receptors via clathrin coated-pits and interact directly with clathrin. Consequently, it has been proposed that β arrestins serve as clathrin adaptors for the GPCR family by linking these receptors to clathrin lattices. AP-2, the heterotetrameric clathrin adaptor protein, has been demonstrated to mediate the internalization of many types of plasma membrane proteins other than GPCRs. AP-2 interacts with the clathrin heavy chain and cytoplasmic domains of receptors such as those for epidermal growth factor and transferrin. In the present study we demonstrate the formation of an agonist-induced multimeric complex containing a GPCR, β arrestin 2, and the β_2 -adaptin subunit of AP-2. β_2 -Adaptin binds β arrestin 2 in a yeast two-hybrid assay and coimmunoprecipitates with β arrestins and β_2 AR in an agonist-dependent manner in HEK-293 cells. Moreover, β_2 -adaptin translocates from the cytosol to the plasma membrane in response to the β_2 AR agonist isoproterenol and colocalizes with β_2 AR in clathrin-coated pits. Finally, expression of β arrestin 2 minigene constructs containing the β_2 -adaptin interacting region inhibits β_2 AR endocytosis. These findings point to a role for AP-2 in GPCR endocytosis, and they suggest that AP-2 functions as a clathrin adaptor for the endocytosis of diverse classes of membrane receptors.

The agonist-mediated internalization of G protein-coupled receptors (GPCRs) represents a mechanism by which desensitized receptors are recycled to the plasma membrane as competent resensitized receptors (1–3). Many GPCRs internalize through the clathrin-coated vesicle endocytic pathway (4–7). β arrestins, which interact with the phosphorylated form of agonist-activated GPCRs to desensitize their signaling, have been demonstrated to initiate clathrin-mediated endocytosis (8, 9). The β_2 -adrenergic receptor (β_2 AR), which belongs to the GPCR family, has been postulated to endocytose via clathrin-coated pits through a direct interaction between β arrestin and clathrin (10). Consequently, it has been proposed that β arrestins serve as clathrin adaptors for GPCR endocytosis (10–12).

Clathrin-coated vesicle-mediated endocytosis of integral membrane proteins such as receptor tyrosine kinases (RTKs) involves interaction of the receptor with the clathrin adaptor complex AP-2 (13), an intrinsic component of the endocytic machinery (14). The heterotetrameric AP-2 protein complex is involved in the formation of clathrin-coated pits and functions as an adaptor by linking receptors directly to the clathrin lattice (11, 14, 15). The α subunit of AP-2 binds clathrin and has been implicated in interacting with dynamin (16, 17), a GTPase involved in the budding of clathrin-coated vesicles.

This subunit also interacts with Eps15, a protein that both binds and acts as a substrate for the epidermal growth factor receptor tyrosine kinase (18). The β_2 chain of AP-2 interacts with clathrin (19) and is able to promote clathrin lattice assembly (15). The μ_2 chain recognizes the tyrosine-based internalization signals within the cytosolic domains of some receptors (20), whereas the function of the σ_2 chain remains unclear.

Even though β arrestins bind clathrin *in vitro* with high affinity (21) they are not constitutively associated with clathrin-coated vesicles (22), nor do they promote clathrin-coat assembly like the AP-2 adaptor complex (15, 23). β arrestins are cytosolic proteins that translocate to activated GPCRs prior to initiating receptor internalization and resensitization (22). The ability of β arrestins to serve as clathrin adaptors would suggest that at least two distinct mechanisms might have arisen to direct receptor internalization through clathrin-coated pits. In this paper, we report that an agonist-mediated association occurs between the β_2 -adaptin subunit of AP-2 and β arrestin during clathrin-mediated GPCR endocytosis. This association suggests that AP-2 acts as a clathrin adaptor for GPCR endocytosis, thus indicating that different classes of receptors endocytose through common clathrin-interacting intermediates.

METHODS

Cell Transfection and β_2 AR Sequestration. The β arrestin 2 clathrin-binding-deficient mutant in pcDNA 3.1 (+) zeo (β arr2_{AAEA}) and β arrestin 2 C-terminal minigene constructs with (β arr2 310–410) or without (β arr2 310–410_{AAEA}) the clathrin-binding site were generated by PCR, and the integrity of each construct was verified by dideoxynucleotide sequencing. The hemagglutinin epitope (HA)-tagged β_2 AR construct is described elsewhere (24). HEK-293 or COS-7 cells were transiently transfected with cDNA by using a modified calcium-phosphate method (5), and β arrestin expression was assessed by Western blotting using a rabbit polyclonal antibody (25). For determination of sequestration, transfected cells expressing HA-tagged β_2 AR alone or coexpressing HA-tagged β_2 AR with β arr2, β arr2_{AAEA}, β arr2 310–410, or β arr2 310–410_{AAEA} were exposed to 10 μ M isoproterenol for 30 min at 37°C followed by washing in ice-cold phosphate-buffered saline (PBS). Cells were then incubated on ice with a mouse monoclonal anti-HA (1:500, Boehringer Mannheim) for 40–60 min followed by incubation with FITC-conjugated goat anti-mouse IgG antibody (1:250, Sigma). Sequestration was

Abbreviations: GPCR, G protein-coupled receptor; β_2 AR, β_2 -adrenergic receptor; RTK, receptor tyrosine kinase; β arr1, β arrestin 1; β arr2, β arrestin 2; HA, hemagglutinin epitope; μ OR, μ opioid receptor; GFP, enhanced green fluorescent protein.

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assessed by flow cytometry (24). Statistical significance was determined by a paired two-tailed *t* test.

Yeast Two-Hybrid Assays. A GAL4 BD- β -arrestin 2 fusion protein was constructed by excising the full-length β -arrestin 2 cDNA from a pCMV5 vector with the enzymes *Nco*I and *Sal*I and was cloned into pAS2-1 (CLONTECH) by using the same restriction sites. The GAL4 AD- α -, β 2-, μ 2-, and σ 2 constructs were kindly provided by M. S. Robinson (Univ. of Cambridge). The fusion GAL4 AD-clathrin N-terminal domain was generated by excising a 2.4-kbp fragment of the rat clathrin heavy chain cDNA (kindly provided by T. Kirchhausen, Harvard Medical School) with the enzymes *Nco*I and *Sal*I, and cloned into pACT2 digested with *Nco*I and *Xho*I. GAL4 BD fragments of β -arrestin 2 gene with *Bam*HI and *Sal*I restriction sites were generated by PCR and cloned into pAS2-1 by using the same sites. Fusion genes were transformed into the yeast Y187 or PJ69-4A strain by lithium acetate transformation as described in the instructions for the Matchmaker two-hybrid kit (CLONTECH). Transformants were allowed to grow at 30°C for 2–4 days and were assayed for β -galactosidase activity. Yeast colonies were transferred directly onto sterile filter paper for the colony-lift filter assay or grown overnight in selective medium for the liquid culture assays. Filters were assayed for β -galactosidase activity in Z-buffer containing 5-bromo-4-chloro-3-indolyl D-galactoside. β -Galactosidase activities of yeast transformants in liquid culture were assayed with a chemiluminescent β -galactosidase assay kit (CLONTECH). Constructs positive for β -galactosidase activity were also tested for their adenine prototrophy in yeast strain PJ69-4A.

Immunoprecipitation and Immunodetection of AP-2. PCR was used to introduce a Flag sequence (DYKDDDDK) at the C-terminus of β -arrestin. The functional activity of the Flag-tagged β -arrestin 2 was measured by assessing the ability of the Flag-tagged β -arrestin 2 to promote β 2AR sequestration in COS-7 cells (5, 8, 22). This construct was found to be as potent as the wild-type β -arrestin 2 for rescuing β 2AR sequestration. HEK-293 cells expressing HA-tagged β 2AR, Flag-tagged β -arrestin 1 and 2, or HA-tagged μ opioid receptor (μ OR) were serum-starved overnight in minimal essential medium (MEM). Cells were treated at 37°C for different periods of time with 10 μ M isoproterenol or 0.5 μ M etorphine. Medium was removed, and cells were solubilized in digitonin buffer [20 mM triethanolamine-HCl, pH 8.0/1% (wt/vol) digitonin/20% (vol/vol) glycerol/300 mM NaCl/1 mM EDTA containing 0.1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 1 μ g/ml pepstatin A]. Lysates were cleared by centrifugation at 100,000 \times g for 15 min at 4°C, and 15 μ g of mouse monoclonal anti-HA or M2 anti-Flag (Sigma) was added to each supernatant. Aliquots were taken for determinations of the total amount of adaptin in each sample. The supernatants were incubated for 1 h at 4°C with a 50% slurry mixture of protein A/G Sepharose beads (Pharmacia Biotech). The beads were recovered by centrifugation and washed three times with digitonin buffer, and bound proteins were solubilized in SDS-sample buffer [8% SDS/25 mM Tris-HCl, pH 6.5/10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol/0.003% bromophenol blue]. Equal amounts of proteins were loaded into wells, resolved by electrophoresis on 10% acrylamide gels, transferred onto nitrocellulose membranes, and subjected to Western blotting analysis using a mouse monoclonal anti- β 2-adaptin antibody (1:1000, Transduction Laboratories).

Immunofluorescence Microscopy. A construct of enhanced green fluorescent protein (in this paper abbreviated GFP) fused to the N terminus of β 2-adaptin was made by excising β 2-adaptin cDNA from pGAD24 with the restriction enzymes *Sma*I and *Bgl*II and ligating the resulting fragment into the *Sma*I and *Bam*HI sites of pEGFP-C1 (CLONTECH). This generated a 21-aa spacer (SGLRSRAQASNSAVDVG-

TAGPG) between the GFP C terminus and the initial methionine of β 2-adaptin (GFP/ β 2-adaptin). HEK-293 cells transfected with HA-tagged β 2AR and GFP/ β 2-adaptin were plated onto ethanol-sterilized glass coverslips (2.5×10^5 cells per well) in a 6-well plate in complete MEM at least 24 h before observation. The cells were incubated in MEM containing 20 mM Hepes with or without 10 μ M isoproterenol for 5 min at 37°C, washed, and immediately fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Receptors were labeled in PBS containing 1% bovine serum albumin (BSA) and a mouse monoclonal anti-HA conjugated with rhodamine (1:500; Boehringer Mannheim). Clathrin was labeled by using a permeabilization solution of PBS containing 0.1% Triton X-100 and 1% BSA at pH 7.4. Cells were incubated with a crude extract of mouse anti-clathrin X22 (1:70; American Type Culture Collection) for 1 h at room temperature, washed three times, and incubated with goat anti-mouse IgG conjugated with Texas red (1:200; Molecular Probes), washed, and mounted on glass slides. GFP/ β 2-adaptin fluorescence was observed at 488 nm with a fluorescein filter set. Texas red and rhodamine were observed with a rhodamine filter set as previously described (26).

RESULTS AND DISCUSSION

β -arrestins have been shown *in vitro* to bind clathrin (10), and the residues LIEF/L found in the β -arrestin 1 and 2 C termini have been shown to mediate this interaction (21). Alanine substitution of three of these residues profoundly reduces β -arrestin binding to clathrin without affecting the ability of β -arrestin to bind phosphorylated receptor (21). However, the role of the β -arrestin/clathrin interaction in cells is less well appreciated. To test whether the β -arrestin/clathrin interaction is necessary and sufficient for receptor endocytosis, we examined β 2AR endocytosis in COS-7 cells in the presence of a

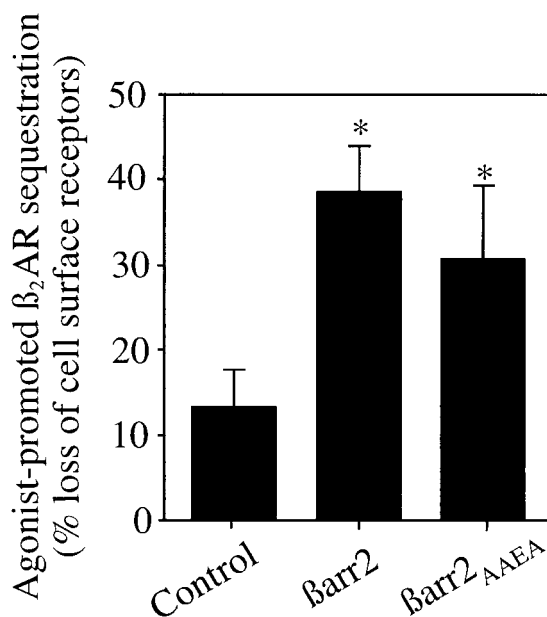


FIG. 1. Effect of β -arrestins on β 2AR sequestration in COS-7 cells. The agonist-mediated sequestration of β 2AR was measured in the presence of endogenous β -arrestin only (Control), overexpressed β -arrestin 2 (Barr2), or overexpressed β -arrestin 2_{AAEA} (Barr2_{AAEA}). Equal amounts of Barr2 and Barr2_{AAEA} were expressed (data not shown). Sequestration is defined as the fraction of total cell surface receptors that are not accessible to antibodies after exposure to agonist, and is expressed as percent loss of cell surface receptors. Results demonstrate that Barr2_{AAEA} and wild-type Barr2 expression similarly increase sequestration above control levels (*, $P < 0.01$). The data represent the mean \pm SD of three or four independent experiments.

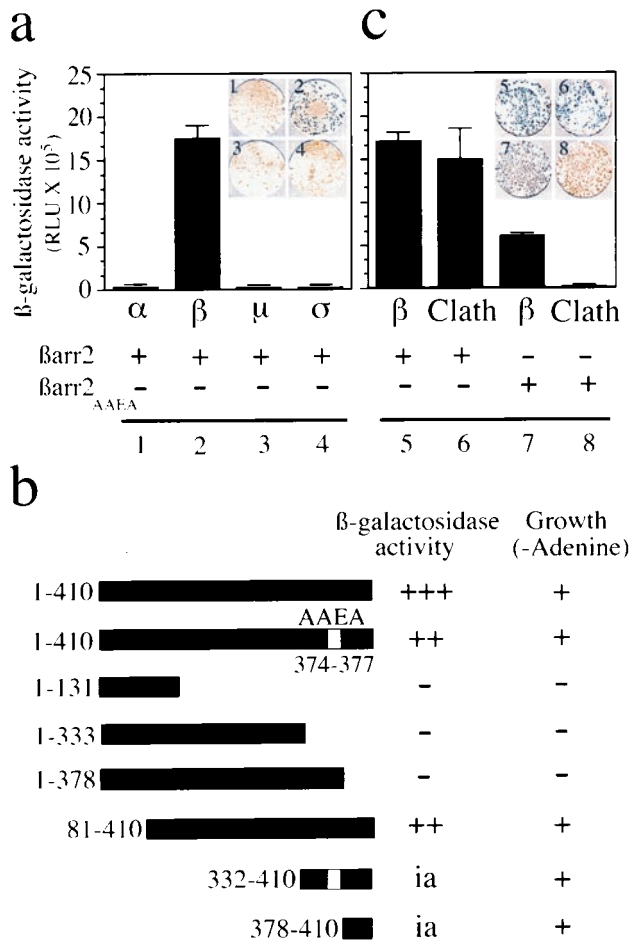


FIG. 2. Interaction between β -barrestin 2 and individual subunits of AP-2 or clathrin as assessed by β -galactosidase activity in yeast. Results of colony-lift filter assays, colony growth assays on adenine-deficient medium, and liquid culture β -galactosidase assays are shown. (a) The binding interactions between β -barrestin 2 (β arr2) and each of the four subunits of AP-2 were analyzed in yeast cells transformed with plasmids encoding GAL4 BD- β arr2 and one of the various GAL4 AD-adaptin subunits (α , β 2, μ 2, and σ 2). (b) Results of the expression of the *lacZ* reporter gene and adenine growth in yeast cells transformed with GAL4 BD- β arr2, - β arr2_{AAEA}, or different fragments of β arr2 with the GAL4 AD- β 2-adaptin subunits. Only transformants expressing interacting proteins were able to grow in the absence of adenine, eliminating the contribution of transformants with intrinsic activity (ia). Positive results for β -galactosidase activity or growth on adenine-deficient plates are indicated by (+), and no interaction is indicated by (-). (c) The interaction of β arr2 or β arr2_{AAEA} with GAL4 AD-clathrin (Clath) or GAL4 AD- β 2-adaptin (β) was examined. A more intense blue coloration on the filter indicates a stronger binding interaction between the two proteins. Results, expressed in relative light units (RLU), are the mean \pm SD of triplicate determinations. All results are representative of four to six independent experiments.

β -barrestin 2 clathrin-binding mutant (β -barrestin 2_{AAEA}: L374 \rightarrow A, I375 \rightarrow A, F377 \rightarrow A). These cells express lower levels of endogenous β -barrestins than many other cells (25), enabling an assessment of the role of β -barrestin on β 2AR endocytosis. The overexpression of β -barrestin 2_{AAEA} enhanced β 2AR internalization to the same extent as wild-type β -barrestin 2, and to a level normally observed for β 2AR sequestration in HEK-293 cells (Fig. 1) (5, 25). These results are inconsistent with the expected reduction in sequestration that should occur if the LIEF-mediated clathrin binding to β -barrestin were the only required interaction for receptor endocytosis.

The failure of the β -barrestin 2 clathrin-binding mutant to inhibit β 2AR sequestration suggests that other proteins may be

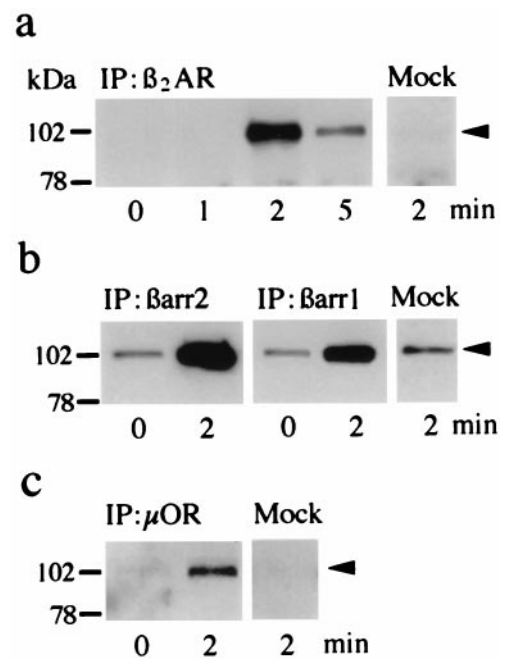


FIG. 3. β 2-Adaptin immunoprecipitation in HEK-293 cells. Cells were transfected with receptor and/or β -barrestin 1 (β arr1) or β -barrestin 2 (β arr2) cDNA and treated as described in the text. Transfected cells were exposed to agonist for the indicated times, and the cell lysates were immunoprecipitated. (a-c) Amounts of β 2-adaptin (arrowheads) that were immunoprecipitated with HA-tagged β 2AR and a monoclonal antibody against the HA epitope (a), Flag-tagged β arr1 or β arr2 and a monoclonal antibody against the Flag epitope (b), or HA-tagged μ OR and the anti-HA antibody (c). Immunoprecipitation results with agonist-treated mock-transfected cells are shown to the right of each panel for comparison. Results are representative of three to eight experiments.

required to initiate β 2AR endocytosis. One potential candidate is the AP-2 adaptor complex because of its involvement in clathrin-mediated endocytosis of other classes of membrane proteins. Therefore, we initially examined whether AP-2 interacts with β -barrestin by using a yeast two-hybrid assay, because it provides a sensitive method for revealing protein-protein interactions that may otherwise be difficult to detect biochemically. Different subunits of AP-2 were coexpressed with β -barrestin 2 in yeast, and β -galactosidase activity resulting from their interaction was assessed qualitatively by a colony-lift filter assay and quantitatively with a chemiluminescent liquid assay (Fig. 2). The results revealed that β -barrestin 2 selectively binds to the β 2-adaptin subunit (Fig. 2a). The interaction between β -barrestin 2 and β 2-adaptin resulted in a 70-fold increase in β -galactosidase activity, whereas the other three subunits of adaptin produced no significant increases over basal activity. β -barrestin 1 interacted less well with β 2-adaptin as revealed by a 4- to 5-fold increase in β -galactosidase activity (data not shown).

We next identified the region of β -barrestin involved in the binding of β 2-adaptin by using GAL4 BD fusion proteins of different domains of β -barrestin 2 (Fig. 2b). We found that the minimal β 2-adaptin binding region for transactivation of yeast reporter genes resides in residues 378-410 of the C-terminal domain of β -barrestin 2. Interestingly, these residues are immediately downstream of the clathrin-binding site. This location suggests that the LIEF clathrin-binding motif is not necessary for the interaction with β 2-adaptin. Using the same yeast-based assay, we could demonstrate that β -barrestin 2 binding sites for clathrin and β 2-adaptin are different (Fig. 2c). Whereas β arr2_{AAEA} produced a 25-fold increase in β -galactosidase activity when assayed with β 2-adaptin, no interaction

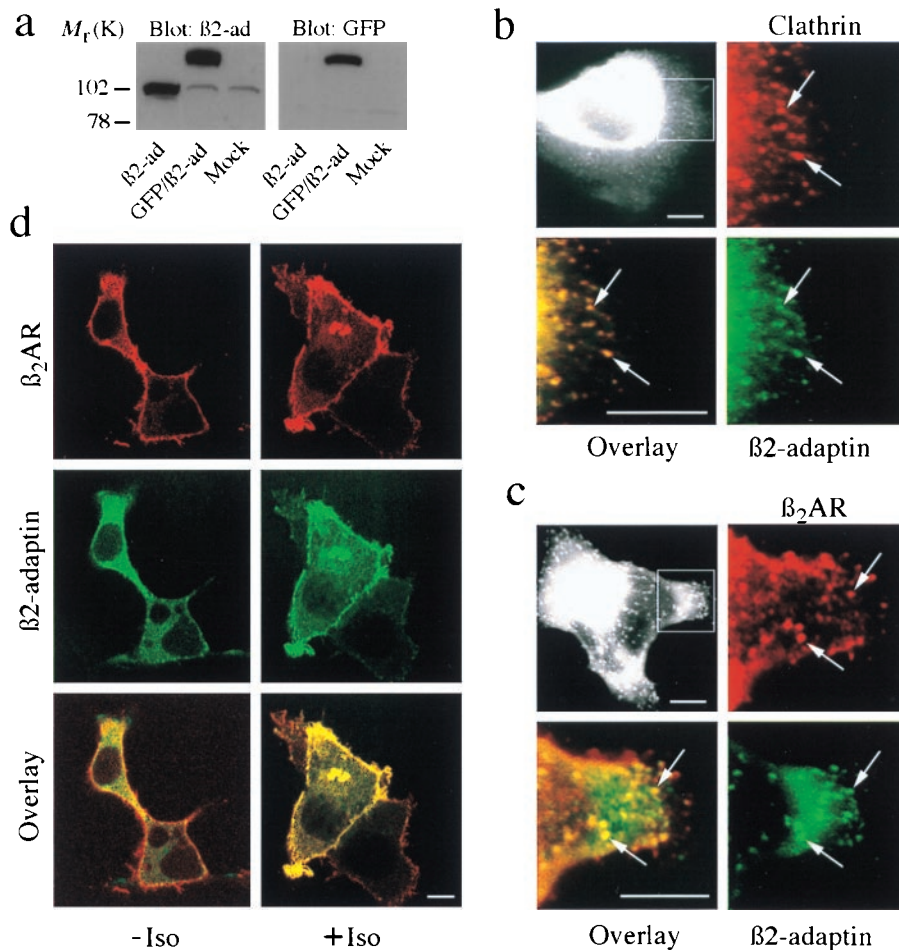


FIG. 4. Expression and distribution of GFP/ β_2 -adaptin in HEK-293 cells. (a) Immunoblots of extracts from HEK-293 cells overexpressing β_2 -adaptin, GFP/ β_2 -adaptin, or endogenous β_2 -adaptin (mock). Proteins were detected with an antibody to β_2 -adaptin (Left) or GFP (Right). (b) Epifluorescence microscopy of HEK-293 cells shows transfected GFP/ β_2 -adaptin colocalization with clathrin-coated pits. The cell area outlined in Upper Left has been enlarged in the other three panels. Upper Right shows clathrin immunostaining (red), Lower Right shows GFP/ β_2 -adaptin fluorescence (green), and Lower Left shows the overlap (yellow or arrows) obtained from the superimposed clathrin and GFP/ β_2 -adaptin images. (c) Demonstration of colocalization of GFP/ β_2 -adaptin (green, Lower Right) to plasma membrane-bound β_2 AR (red, Upper Right) after isoproterenol treatment. The cell area outlined in Upper Left has been enlarged in the other three panels. The arrows indicate punctate areas of the plasma membrane where β_2 AR and β_2 -adaptin colocalize after isoproterenol treatment. These punctate areas correspond to clathrin-coated pits (5). (d) Confocal images demonstrating the isoproterenol-induced translocation of GFP/ β_2 -adaptin to the plasma membrane. (Top) Plasma membrane distribution of β_2 AR (red) with (Right) or without (Left) isoproterenol. (Middle) Distribution of GFP/ β_2 -adaptin (green) with or without agonist stimulation. Note the significant increase in plasma membrane localization of GFP/ β_2 -adaptin in the presence of agonist at the expense of the cytosolic signal. In the absence of agonist (Bottom Left), the β_2 AR signal (red) appears at the plasma membrane, whereas that for the GFP/ β_2 -adaptin (green) is mostly cytosolic. In the presence of agonist (Bottom Right) the fluorescent signals become predominantly coincident at the plasma membrane (yellow). Epifluorescence microscopy was done as previously described (26). Confocal images were obtained on a Zeiss LSM-410 laser scanning confocal microscope. (All scale bars = 10 μ m.)

was detected with clathrin. β arrestin 2 interacted equally well with both clathrin and β_2 -adaptin (Fig. 2c). Taken together, these data confirm that removal of the LIEF motif eliminates the interaction of β arrestin2 with clathrin. The interaction of β arrestin 2 with clathrin appears to be confined to a single region of β arrestin 2, unlike what has been suggested recently (27). Moreover, the ability of β arrestin 2 to interact with the β_2 -adaptin component of the endocytic machinery even in the absence of a clathrin interaction provides a potential basis to explain how the β arrestin 2_{AAEA} mutant could retain its ability to enhance β_2 AR sequestration.

We next examined whether a functional complex consisting of β arrestin 2, β_2 AR, and β_2 -adaptin could be detected in HEK-293 cells by using immunoprecipitation of either epitope-tagged β_2 AR or β arrestins. To maximize the number of membrane-bound receptor complexes, experiments were performed in the presence of overexpressed dynamin-K44A, a dominant-negative mutant that inhibits clathrin-mediated endocytosis (28). Under these conditions the dissociation of

clathrin-coated vesicles from the plasma membrane is blocked and agonist-activated β_2 ARs accumulate in coated pits (5). Immunoprecipitation of β_2 AR from isoproterenol-treated HEK-293 cells revealed a robust time-dependent increase in the association between immunoreactive β_2 -adaptin and the receptor, which peaked at 2 min and decreased after 5 min (Fig. 3a). The transient nature of this interaction was also observed in the absence of dynamin-K44A, but the intensity of the signal was weaker (data not shown). There were no significant differences in β_2 -adaptin immunoprecipitation signals between untransfected cells (mock) and unstimulated cells expressing either β_2 AR or β arrestin 2 (Fig. 3). β_2 -Adaptin could also be recovered by immunoprecipitation of Flag-tagged β arrestin 1 and 2 (Fig. 3b) or the endogenous β arrestins (data not shown), further supporting the idea that β_2 -adaptin interacts with β arrestins in cells. Interestingly, as opposed to the yeast two-hybrid data, β arrestin 1 and 2 interacted comparably with β_2 -adaptin in HEK-293 cells. The ability of β_2 -adaptin to interact with a receptor/ β arrestin 2 complex is

not restricted to the β_2 AR. Indeed, β_2 -adaplin is also recovered in immunoprecipitates of agonist-activated μ OR, which like the β_2 AR, internalizes via clathrin-coated pits (Fig. 3c) (6).

These biochemical data suggest that β_2 -adaplin plays a dynamic role in the early stages of GPCR endocytosis. We investigated this possibility by using a fusion protein between GFP and β_2 -adaplin (GFP/ β_2 -adaplin). HEK-293 cells were transfected with β_2 -adaplin or GFP/ β_2 -adaplin, and the expression of each protein was verified by immunoblotting cell extracts with monoclonal antibodies against β_2 -adaplin or GFP (Fig. 4a). Since the AP-2 complex is a major structural component of clathrin-coated pits (11, 14, 15), we expected that a biologically active GFP/ β_2 -adaplin would colocalize with clathrin. Overexpressed GFP/ β_2 -adaplin did colocalize with clathrin in punctate regions of the plasma membrane but could also be found diffusely distributed in the cytoplasm as shown by epifluorescence microscopy (Fig. 4b). As expected, GFP/ β_2 -adaplin colocalized with activated β_2 ARs in the early stages of endocytosis because β_2 ARs internalize via clathrin-coated pits (5). When cells were treated with isoproterenol, an agonist-mediated colocalization of GFP/ β_2 -adaplin with β_2 AR became apparent as revealed by the enhanced size and intensity of punctate regions of the plasma membrane (Fig. 4b vs. 4c). Taken together, these results suggest that a redistribution of the receptor and/or the β_2 -adaplin might take place. To investigate this potential agonist-mediated recruitment of GFP/ β_2 -adaplin by activated β_2 AR, confocal microscopy was employed (Fig. 4d). In the presence of isoproterenol, the amount of plasma membrane GFP/ β_2 -adaplin was found to increase at the expense of the cytosolic fraction, colocalizing with plasma membrane β_2 AR (Fig. 4d) within the same time frame that β_2 -adaplin immunoprecipitated with β_2 AR. This increase is reflected by the enhancement of the β_2 -adaplin fluorescence at the plasma membrane (Fig. 4d Middle) and the increased coincidence of the GFP/ β_2 -adaplin and β_2 AR signals in presence of isoproterenol (Fig. 4d Bottom). These results indicate that a dynamic agonist-mediated association occurs between β_2 -adaplin and the β_2 AR/ β arrestin complex.

β arrestin C-terminal minigene constructs have been shown to inhibit β_2 AR internalization (27, 29). To investigate whether the interaction of AP-2 with β arrestin 2 was necessary for β_2 AR endocytosis, we overexpressed minigene constructs of β arrestin 2 containing the β_2 -adaplin interacting region with (β arr2 310–410) or without (β arr2 310–410_{AAEA}) the clathrin-binding site. Both constructs were found to inhibit the agonist-induced β_2 AR sequestration up to 30% (Fig. 5). The moderate level of inhibition observed with these constructs is likely attributable to their low efficiency of expression ($\approx 20\%$) compared with the expression of a similar construct of wild-type β arrestin 2 (data not shown). These results are consistent with our previous observations (Fig. 1) and suggest that the β arrestin minigenes function by competing for β_2 -adaplin binding to the endogenous β arrestins.

We have shown that β arrestins specifically interact with β_2 -adaplin, that β_2 -adaplin translocates to the plasma membrane in an agonist-dependent manner, and that a stable membrane association occurs between AP-2 and the receptor/ β arrestin 2 complex during β_2 AR (or μ OR) endocytosis. In addition, overexpression of a β arrestin C-terminal minigene construct containing the β_2 -adaplin-binding region but lacking the LIEF clathrin-binding site is able to inhibit β_2 AR endocytosis, whereas the full-length β arrestin clathrin-binding mutant retains its ability to induce sequestration. These results suggest that AP-2 acts as a clathrin adaptor for GPCRs. Our findings support the postulated role of AP-2 as a common adaptor for clathrin/receptor binding and clathrin coat assembly, and they suggest that a role of β arrestin 2 in this process is to serve as a docking protein between GPCRs and AP-2. This novel role for β arrestin 2 may be analogous to the

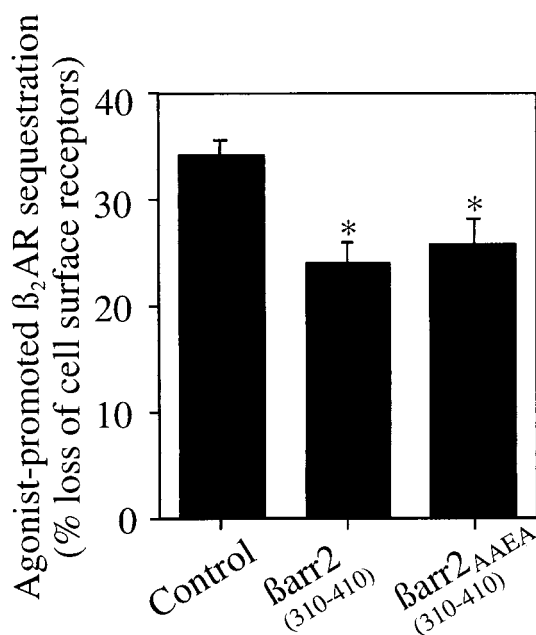


Fig. 5. Effect of minigene β arrestin on β_2 AR sequestration in HEK-293 cells. The agonist-mediated sequestration of β_2 AR was measured in the presence of endogenous β arrestin only (Control) and β arrestin 2 C-terminal minigene with (β arr2 310–410) or without (β arr2 310–410_{AAEA}) the clathrin-binding site. Similar amounts of β arr2 310–410 and β arr2 310–410_{AAEA} were expressed (data not shown). Results demonstrate that β arr2 310–410 and β arr2 310–410_{AAEA} expression similarly decrease sequestration below control levels (*, $P < 0.01$). The data represent the mean \pm SD of four independent experiments.

proposed function of Eps15 and/or epsin as AP-2 docking proteins for RTK endocytosis (18, 30–32). Our results thus challenge the paradigm that β arrestins assume the role of AP-2 in the GPCR endocytic process (11, 12).

The processing of plasma membrane proteins via clathrin-coated pits depends on the coordinated actions of several endocytic accessory proteins, the strength of their interactions, and their phosphorylation status (11, 15, 33, 34). Certain protein interactions may be required in the early stages of endocytosis, as appears to be the case for those between β arrestins and AP-2. Other associations such as those between β arrestins and clathrin may regulate later events in the process of endocytosis. Thus it might not be surprising that abrogation of the β arrestin 2/clathrin interaction does not prevent receptor internalization. For RTKs, multiple interactions of the receptor and associated proteins with components of the endocytic machinery have been demonstrated (13, 18, 30, 31). Similarly, additional interactions between GPCRs and AP-2 and/or other endocytic components may also occur and contribute to the endocytic process.

Results from these and other studies suggest that interactions between GPCRs and β arrestin 1 or 2 might differ. In contrast to β arrestin 2, β arrestin 1 was found to interact only weakly with β_2 -adaplin in the *in vitro* yeast two-hybrid assay. However, in HEK-293 cells β arrestin 1 appears just as effective as β arrestin 2 in forming a complex with the β_2 -adaplin. Interestingly, these differences may be related to the observation that the endocytic function of β arrestin 1 seems to be regulated by its phosphorylation (35). It is tempting to speculate that, like other endocytic accessory proteins (34), β arrestin 1 might be regulated by phosphorylation to interact with AP-2. Moreover, in different cells with several GPCRs, β arrestin 1 is generally less efficient than β arrestin 2 in translocating to activated receptor and promoting sequestration (unpublished data). These observations raise the possibility

that these two proteins, although highly homologous, may not have totally redundant endocytic functions.

On the basis of our results, we propose a model in which an activated membrane GPCR is first desensitized by the recruitment of β arrestin from the cytosol. This complex then serves as a membrane anchor for the docking of β 2-adaptin, and subsequently, for nucleation or association of other subunits of AP-2 and clathrin. Different subunits of AP-2 apparently have the ability to bind selectively to distinct classes of receptors or docking proteins (11, 13, 15, 36). This ultimately may explain why multiple types of receptors can simultaneously internalize via clathrin-coated pits.

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