β-Arrestin/AP-2 Interaction in G Protein-coupled Receptor Internalization

IDENTIFICATION OF A β-ARRESTIN BINDING SITE IN β2-ADAPTIN*

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β-Arrestins, proteins involved in the turn-off of G protein-coupled receptor (GPCR) activation, bind to the β2-adaptin subunit of the clathrin adaptor AP-2. The interaction of β2-adaptin with β-arrestin involves critical arginine residues in the C-terminal domain of β-arrestin and plays an important role in initiating clathrin-mediated endocytosis of the β2-adrenergic receptor (β2AR) (Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) J. Biol. Chem. 275, 23120–23126). However, the β-arrestin-binding site in β2-adaptin has not been identified, and little is known about the role of β-arrestin/AP-2 interaction in the endocytosis of other GPCRs. Using in vitro binding assays, we have identified two glutamate residues (Glu-849 and Glu-902) in β2-adaptin that are important in β-arrestin binding. These residues are located in the platform subdomain of the C terminus of β2-adaptin, where accessory/adapter endocytic proteins for other classes of receptors interact, distinct from the main site where clathrin interacts. The functional significance of the β-arrestin/AP-2/clathrin complex in the endocytosis of GPCRs such as the β2AR and vasopressin type II receptor was evaluated using mutant constructs of the β2-adaptin C terminus containing either the clathrin and the β-arrestin binding domains or the β-arrestin-binding domain alone. When expressed in human embryonic kidney 293 cells, both constructs acted as dominant negatives inhibiting the agonist-induced internalization of the β2AR and the vasopressin type II receptor. In addition, although the β2-adaptin construct containing both the clathrin and β-arrestin binding domains was able to block the endocytosis of transferrin receptors, a β2-adaptin construct capable of associating with β-arrestin but lacking its high affinity clathrin interaction did not interfere with transferrin receptor endocytosis. These results suggest that the interaction of β-arrestin with β2-adaptin represents a selective endocytic trigger for several members of the GPCR family.

β-Arrestins (β-arrestin-1 and β-arrestin-2) are cytosolic proteins involved in the homologous desensitization of many G-protein coupled receptors (GPCR)1 (1, 2). Agonist stimulation of GPCRs triggers the activation and the recruitment of specific GPCR protein kinases leading to the phosphorylation of cytosolic residues in the receptor to promote the subsequent binding of β-arrestin. For example, the interaction of β-arrestin with the phosphorylated β2-adrenergic receptor (β2AR) prevents further coupling to its cognate G protein (i.e. Gs), thus terminating the second messenger signaling events. β-Arrestins, initially appreciated exclusively for their ability to desensitize agonist-activated GPCRs, are now believed to play a much more intricate role in other cellular events such as endocytosis, trafficking, and intracellular signaling of the GPCRs (2–4). For instance, the initial observation that β-arrestin and mutants of β-arrestin could modulate the internalization of β2AR provided evidence for a role of β-arrestins in this process (5). Moreover, the findings that β-arrestins were able to associate with components of the endocytic machinery, such as clathrin and the clathrin adaptor protein AP-2, have provided an attractive mechanism to explain how β-arrestins could engage GPCRs in the internalization pathway (6, 7). Although β-arrestins can associate with a great number of agonist-stimulated GPCRs at the plasma membrane to trigger their internalization, the fate of the receptor/β-arrestin complexes differs greatly among receptors. For example, β-arrestin dissociates from β2AR at or near the plasma membrane following the internalization of the receptor, whereas β-arrestin has been shown to traffic into endosomes with other GPCRs like the vasopressin type II receptor (V2R) and the angiotensin II type 1 receptor (AT1R) (8, 9). The intracellular trafficking of β-arrestins with V2R and AT1R correlates with the slow recycling to the plasma membrane and resensitization of these receptors (8, 10). However, for some GPCRs the intracellular trafficking of β-arrestins with the receptors seem to activate specific signaling pathways. For the AT1R and the protease-activating receptor 2, the formation of endosomal receptor/β-arrestin complexes has been shown to serve as a scaffold for the recruitment and the activation of components of the mitogen-activated protein kinase pathways (11–13).

Endocytosis via clathrin-coated vesicles (CCVs) is one of the most common routes utilized by mammalian cells to internalize GPCRs (2–4). For instance, the initial observation that β-arrestin and mutants of β-arrestin could modulate the internalization of β2AR provided evidence for a role of β-arrestins in this process (5). Moreover, the findings that β-arrestins were able to associate with components of the endocytic machinery, such as clathrin and the clathrin adaptor protein AP-2, have provided an attractive mechanism to explain how β-arrestins could engage GPCRs in the internalization pathway (6, 7). Although β-arrestins can associate with a great number of agonist-stimulated GPCRs at the plasma membrane to trigger their internalization, the fate of the receptor/β-arrestin complexes differs greatly among receptors. For example, β-arrestin dissociates from β2AR at or near the plasma membrane following the internalization of the receptor, whereas β-arrestin has been shown to traffic into endosomes with other GPCRs like the vasopressin type II receptor (V2R) and the angiotensin II type 1 receptor (AT1R) (8, 9). The intracellular trafficking of β-arrestins with V2R and AT1R correlates with the slow recycling to the plasma membrane and resensitization of these receptors (8, 10). However, for some GPCRs the intracellular trafficking of β-arrestins with the receptors seem to activate specific signaling pathways. For the AT1R and the protease-activating receptor 2, the formation of endosomal receptor/β-arrestin complexes has been shown to serve as a scaffold for the recruitment and the activation of components of the mitogen-activated protein kinase pathways (11–13).

1 The abbreviations used are: GPCR, G protein-coupled receptor; AP-2, clathrin adaptor protein; AT1R, angiotensin II type 1 receptor; β2AR, β2-adrenergic receptor; CCV, clathrin-coated vesicle; Gs, glutathione S-transferase; V2R, vasopressin type II receptor; HA, hemagglutinin; TR, transferrin receptor; AD, activation domain; AVP, arginine vasopressin; PBS, phosphate-buffered saline; HEK, human embryonic kidney; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein.

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diverse classes of cargo such as extracellular molecules and membrane receptors. CCVs at the plasma membrane, also referred to as clathrin-coated pits, are composed of two main structural proteins: clathrin and the clathrin adaptor protein AP-2 (14, 15). AP-2 is a heterotetrameric complex that serves the dual role of assembling clathrin into organized cage structures and acting as an adaptor to link cargo to clathrin lattices. This is achieved through the recognition of distinct signal motifs in the cytosolic domain of membrane proteins by different subunits of AP-2. For example, the μ2-subunit of AP-2 recognizes tyrosine-based internalization signals within the cytosolic domains of receptors (16–18), whereas the β2-subunit of AP-2 (β2-adaptin) interacts with clathrin and helps to promote clathrin lattice assembly (19). The α-subunits of AP-2 bind dynamin (20), a GTPase that promotes budding of clathrin-coated vesicles (21), and recruit other endocytic accessory proteins necessary for the formation and processing of CCVs (22).

We have recently shown that the interaction of β-arrestin with β2-adaptin is necessary for the targeting of the β2AR to CCVs and have identified critical residues in β-arrestin that mediate this interaction (23). However, little information is available for the corresponding β-arrestin-binding site in β2-adaptin. Moreover, although β-arrestin/AP-2 complexes have been shown to play a role in the internalization of the β2AR, the functional significance of these complexes in clathrin-mediated endocytosis of other GPCRs remains undetermined. In this study we sought to identify critical residues within the β2-subunit of AP-2 involved in β-arrestin binding, and have investigated the functional importance of this interaction in the internalization process of different classes of membrane receptors.

EXPERIMENTAL PROCEDURES

Materials—Isoproterenol was purchased from Research Biochemical Inc., and arginine vasopressin (AVP) was obtained from Sigma. The anti-HA 12CA5 mouse monoclonal antibody was purchased from Roche Molecular Biochemicals, anti-epsin antibody was from Santa Cruz, and anti-HA 12CA5 mouse monoclonal antibody was purchased from Roche Molecular Biochemicals. Isopropyl β-D-thiogalactopyranoside was from Novagen.

Plasmid Constructs—Recombinant DNA procedures were carried out following standard protocols. Glutathione S-transferase (GST) and GAL4-AD fusion proteins were constructed by polymerase chain reaction (PCR). Fragments derived from the N-terminal domain (1–585) and the C-terminal domain (589–937, 664–937, 784–937, and 784–840) of the human β2-adaptin were cloned into BamHI and XhoI of pGEX-5X-2 (Amersham Biosciences AB). The GST-β-arrestin-1 C-terminal construct (331–418) has been described elsewhere (22). β2-Adaptin-(664–937)-Y888A, -E849A, and -E902A were constructed by PCR by generating two fragments: an N-terminal fragment containing a ClaI restriction site and the residues to be substituted, and a C-terminal fragment starting at the residue following the mutation and ending with the stop codon of β2-adaptin followed by a BamHI restriction site. The PCR fragments were cut with ClaI and BamHI, and blunt end-ligated into pGEX-5X-2-β2-adaptin-(664–937) cut with ClaI/BamHI. Silent mutations were introduced into the N-terminal fragment of β2-adaptin-(664–937)-E902A (BamHI), and in the C-terminal fragments of β2-adaptin-(664–937)-E849A (MluI) and 664–937-Y888A (HindIII) for screening purposes. PCR fragments derived from the C-terminal domain of β2-adaptin (592–937, 664–937, and 825–937) were cloned into BamHI and XhoI of pACT-2 (Invitrogen). The GALA-2-β-arrestin-2 and the GALA-AD-β2-adaptin fusion proteins have been described elsewhere (7).

Yeast Two-hybrid Assays—Fusion genes expressing β-arrestin-2 or β2-adaptin wild type and mutants were co-transformed into PJA69-4A yeast strains using the lithium acetate method (CLONTECH). Protein-protein interactions were assayed for their adenine (Ado) auxotrophy by growth on synthetic complete (SC) plates lacking adenine (SC−Ade) or by measuring β-galactosidase activity levels in cells grown on SC−Ade plates.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Eagle’s minimal essential medium with Earle’s salt supplemented with 10% (v/v) heat-inactivated fetal bovine serum and gentamicin (100 μg/ml). Transient transfections were performed using calcium phosphate coprecipitation method as described previously (24). Twenty-four hours after transfection, cells were split into appropriate plates and experiments were performed the following day. For internalization experiments using the β2AR or the V2R, cells were seeded at a density of 5.0–7.5 × 104 cells/well in 6-well plates and 2.5 × 105 cells/well in 12-well plates, respectively.

Receptor Sequestration—Adenylate receptor sequestration was assessed by flow cytometry as described previously (8). Transfected HEK 293 cells were incubated with or without isoproterenol (10 μM) for 20 min. Sequestration of receptors was defined as the fraction of cell surface receptors that was removed from the surface after exposure to agonist. Internalization of the V2R was assessed using a previously described assay with minor modifications (8). In brief, cells expressing the V2R were incubated at 37°C in Eagle’s minimal essential medium with Earle’s salt containing 0.2% (w/v) of bovine serum albumin in presence of 1 nM [3H]AVP for 20 min. Internalization of receptors was stopped on ice by rapidly washing the cells with ice-cold phosphate-buffered saline (PBS). Cells were washed three times with either ice-cold PBS to remove the unbound agonist or ice-cold acid wash buffer (50 mM Hepes acid, pH 2.5, 1 mM NaCl) to remove unbound AVP and the cell surface receptor-bound agonist. Sequestration of receptors was defined as the percentage of radioligand that was acid-resistant after incubating cells for 20 min at 37°C. Internalization of transferrin was assessed using confocal microscopy to measure the uptake of FITC-conjugated transferrin in HEK 293 cells. Cells were incubated with FITC-transferrin for 15 min at 37°C and then fixed in PBS containing 4% paraformaldehyde. For the detection of β2-adaptin mutants, fixed cells were stained in PBS containing 2% bovine serum albumin (w/v) and 0.2% Triton X-100 (v/v) using a primary HA antibody followed by secondary Texas red labeling. For quantification of transferrin uptake, 100 cells showing comparable expression of β2-adaptin minigene constructs (as estimated by the intensity of the fluorescence) were analyzed. Inhibition of transferrin uptake was defined as maximal when transfected cells showed a FITC signal of less than 25% of that of adjacent cells lacking the expression of the β2-adaptin constructs (i.e. when transfected cells showed a reduction of transferrin fluorescence signal of more than 75%). The level of transferrin and β2-adaptin fluorescence (in intensity per pixel) was measured in different areas of the cytoplasm using the LSM 510 microscope software as described previously (25).

GST Fusion Protein Purification and Pull-down Experiments—β2-adaptin and β-arrestin-1 C-terminal constructs in pGEX-5X-2 were transformed in Escherichia coli BL21-gold (DE3) cells, and GST fusion proteins were prepared as previously described (23). HEK 293 cells expressing the Flag-tagged β-arrestins or HA-tagged β2-adaptin constructs were solubilized in TGH buffer (50 mM HEPEs, pH 7.4, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM EDTA) containing protease inhibitors. Cells were solubilized for 1 h at 4°C, centrifuged at 10,000 × g for 30 min, and the supernatant was recovered for GST fusion protein binding assays. For experiments involving mouse whole brain extract, a similar procedure was followed for protein solubilization, with the exception that whole brain was homogenized in TE buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% (v/v) Triton X-100) containing protein inhibitors. Binding assays were performed using 10 μg of GST fusion proteins on glutathione-Sepharose beads that were incubated for 1 h at 4°C with solubilized proteins. Beads were recovered by centrifugation and washed three times with cold TGH or TE buffer, and the protein complexes were separated by SDS-PAGE. Proteins were transferred to nitrocellulose, analyzed by Ponceau S staining to detect the integrity of the GST fusion proteins, and subjected to immunoblotting.

RESULTS

The β2-subunit of the clathrin adaptor protein AP-2 (i.e. β2-adaptin) interacts with two arginine residues (Arg-394 and Arg-399) in the C-terminal domain of β-arrestin to mediate the targeting of β2AR to CCVs (23). To identify the sites in β2-adaptin involved in β-arrestin binding, the entire coding sequence of β2-adaptin was divided into two halves: an N-terminal half (β2-adaptin 1–588) and a C-terminal half (β2-adaptin 592–937). GST fusion proteins of both regions were generated. Fusion proteins were incubated with cytosolic ex
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β-Arrestin interacts with the C terminus of β₂-adaptin. HEK 293 cells expressing either Flag-tagged β-arrestin-1 (bar1) or Flag-tagged β-arrestin-2 (bar2) were solubilized as described under “Experimental Procedures,” and the solubilized proteins were incubated with GST-β₂-adaptin fusion proteins of the N terminus (GST-β₂Ad 1–588), the C terminus (GST-β₂Ad 592–937), or GST alone. Affinity-purified proteins were resolved on SDS-PAGE, transferred onto nitrocellulose membrane, and Ponceau S-stained to reveal GST proteins (lower panel: GST, ~31 kDa; GST-β₂-adaptin-(1–588), ~80 kDa; GST-β₂-adaptin-(592–937), ~70 kDa). β-Arrestins were detected by Western blot using a Flag antibody (top panels). Results show that both β-arrestin-1 and β-arrestin-2 interact with the C terminus of β₂-adaptin (592–937). Input represents 2.5% of the total amount of starting material used in the assay. Results are representative of at least three independent experiments.

![Figure 1](http://www.jbc.org/)

**Fig. 1. β-Arrestin interacts with the C terminus of β₂-adaptin.** HEK 293 cells expressing either Flag-tagged β-arrestin-1 (bar1) or Flag-tagged β-arrestin-2 (bar2) were solubilized as described under “Experimental Procedures,” and the solubilized proteins were incubated with GST-β₂-adaptin fusion proteins of the N terminus (GST-β₂Ad 1–588), the C terminus (GST-β₂Ad 592–937), or GST alone. Affinity-purified proteins were resolved on SDS-PAGE, transferred onto nitrocellulose membrane, and Ponceau S-stained to reveal GST proteins (lower panel: GST, ~31 kDa; GST-β₂-adaptin-(1–588), ~80 kDa; GST-β₂-adaptin-(592–937), ~70 kDa). β-Arrestins were detected by Western blot using a Flag antibody (top panels). Results show that both β-arrestin-1 and β-arrestin-2 interact with the C terminus of β₂-adaptin (592–937). Input represents 2.5% of the total amount of starting material used in the assay. Results are representative of at least three independent experiments.

The crystal structure of the ear domain of β₂-adaptin has recently been solved (28). Analysis of the structure reveals the presence of two regions: an N-terminal subdomain (residues 705–825) and a C-terminal or “platform” subdomain (residues 826–937) (Fig. 3). Our results indicate that the β₂-adaptin binding site is located within residues 825–937 of the β₂-adaptin C terminus. The crystal structure of the ear domain of β₂-adaptin has recently been solved (28). Analysis of the structure reveals the presence of two regions: an N-terminal subdomain (residues 705–825) and a C-terminal or “platform” subdomain (residues 826–937) (Fig. 3). Our results indicate that the β₂-adaptin binding site is located within residues 825–937 of the β₂-adaptin C terminus.
394 and Arg-396) (23). We hypothesized that the positively charged guanidino group of the arginine residues in β-arrestin might form an ionic bond with the carboxyl group of acidic residues in β2-adaptin (i.e. aspartate or glutamate). Initial attempts at defining the β-arrestin binding site by replacing pairs or triplets of consecutive acidic residues in the β2-adaptin C-terminal domain yielded only limited information on the residues involved. Indeed, most of the β2-adaptin mutants by themselves were trans-activating in the yeast two-hybrid system, with the exception of the β2-adaptin-(825–937) (E828A, D829A, E833A) and β2-adaptin-(664–937) (E922A, D932A, D932K) constructs, which did not show any trans-activation when expressed by themselves and were both found to still interact with β-arrestin-2 (data not shown).

Alternative strategies to map the β-arrestin-binding site in β2-adaptin were considered. We took advantage of the solved structure of β2-adaptin C terminus to identify neighboring acidic residues in the platform subdomain that might participate in this interaction (28). This region of β2-adaptin contains 13 acidic residues, and analysis of their relative position in the β2-adaptin platform reveals the presence of a candidate pair: residue Glu-849, located in the first β-sheet strand between the α1 and α2 helices; and residue Glu-902, located in the fourth β-sheet strand (Fig. 3). Although these residues are distant with respect to the primary sequence, structurally and molecularly the side chains of Glu-849 and Glu-902 residues are in close proximity to one another (≈6–10 Å). These two charged residues are located within a hydrophobic pocket in the center of the β2-adaptin platform subdomain (28). The location of electrostatically charged residues such as Glu-849 and Glu-902 in this polar patch of β2-adaptin is reminiscent of other protein–protein binding domains (29), and might provide both the specificity and strength required for β-arrestin interaction. We first tested whether individually substituting residues Glu-849 and Glu-902 for alanine would affect the association of β2-adaptin with β-arrestin. GAL4 fusion proteins of the full-length β2-adaptin or E849A and E902A mutants were co-transformed in yeast with β-arrestin-2, and the interaction between the β2-adaptin proteins and β-arrestin was assessed (Fig. 4). Results show that, although full-length β2-adaptin interacts with β-arrestin, replacement of glutamic acid residues 849 and 902 by alanine in β2-adaptin impaired the association of both mutants with β-arrestin. To confirm that residues Glu-849 and Glu-902 were involved in the binding of β-arrestin, HA-tagged β2-adaptin-(664–937) constructs containing wild type or mutant amino acids at positions 849 and 902 were generated and expressed in HEK 293 cells. Cytosolic extracts from transfected cells were incubated with GST alone, or with a GST fusion protein of the C terminus of β-arrestin-1 (GST-βarr-CT), which contains both the β2-adaptin and the clathrin binding domain (23). The presence of wild type or mutant β2-adaptin proteins and clathrin in the affinity-purified complex was revealed by immunoblotting with either anti-HA or anti-clathrin heavy chain antibodies. Results show that both wild type β2-adaptin-(664–937) and clathrin associated with GST-βarr-CT, but did not associate with GST alone (Fig. 5). When GST-βarr-CT was incubated with cytosol from cells expressing the E849A or E902A β2-adaptin-(664–937) mutants, β-arrestin binding was eliminated. However, under the same conditions, clathrin binding was unaffected.

The polar residue tyrosine 888 (Tyr-888) in the surface-exposed hydrophobic patch of the β2-adaptin platform shares a hydrogen bond with the residue Glu-902 and is therefore another good candidate to regulate the formation of a β2-adaptin/β-arrestin complex (Fig. 3). Substitution of residue Tyr-888 for alanine in β2-adaptin-(664–937) (Y888A) resulted in a loss in the ability of the GST-βarr-CT to complex with β2-adaptin (Fig. 5). These results suggest that the site for β-arrestin binding is contained within the platform subdomain of β2-adaptin, and might involve residues Glu-849, Glu-902, and Tyr-888. However, the failure of β2-adaptin mutants to bind β-arrestin might simply reflect a change in the β2-adaptin subdomain structure rather than the removal of critical determinants involved in the association of the two proteins. To rule out this possibility, we examined the ability of β2-adaptin-(664–937)-Y888A, -E849A, and -E902A mutants to bind other endocytic proteins. We hypothesized that if such a structural alteration occurred in the ear domain of β2-adaptin, then the mutants may be affected in their ability to bind other high affinity and/or low affinity β2-adaptin-interacting-proteins, such as epsin and clathrin (28). GST fusion proteins of wild type and the mutant β2-adaptin (Y888A, E849A, or E902A) were incubated with cytosolic extracts from mouse brain. Associated epsin and clathrin in the affinity-purified complex were revealed by immunoblotting (Fig. 6). The results indicate that the β2-adaptin-(664–937) wild type, -E849A, and -E902A proteins were equally effective at binding epsin and clathrin. However, we did not detect any association of epsin or clathrin with GST-β2-adaptin-(664–937)-Y888A or GST alone. These results indicate that the replacement of Glu-849 and Glu-902 by alanine residues does not significantly alter the tertiary structure of β2-adaptin C-terminal subdomain, and suggest that β-arrestin directly interacts with these residues. Replacement of the tyrosine residue may result in a more drastic effect on the folding of the molecule because it prevents the interaction of β2-adaptin with other endocytic proteins, or Tyr-888 may play a direct role in epsin and clathrin binding. Taken together, our results strongly suggest that Glu-849 and Glu-902 in the C terminus of β2-adaptin participate in β-arrestin binding.

The interaction of β-arrestin with both AP-2 and clathrin has been shown to play an important role in the regulation of β2AR endocytosis (7, 23). We next assessed whether preventing the recruitment of AP-2 to agonist-activated β2AR/β-arrestin complexes would interfere with the initial steps of receptor endocytosis. We used fragments of β2-adaptin containing different binding domains (i.e. clathrin and/or β-arrestin) as minigene constructs. We reasoned that these mutants would act as selective dominant negative by preventing the recruitment of clathrin and/or β2-adaptin to the β-arrestin/receptor complex without affecting the biology of endogenous AP-2 proteins. HEK 293 cells were transfected with a green fluorescence-tagged β2AR (β2AR-GFP) and HA-tagged β2-adaptin-(664–937) mutant, stimulated with isoproterenol for 5 min or left unstimulated, and receptor internalization was visualized by con-
focal microscopy (Fig. 7). In the absence of agonist, β2AR-GFP was uniformly distributed at the plasma membrane, whereas β2-adaptin mutants were found both in the cytosol and at the plasma membrane (Fig. 7, left panels). Upon isoproterenol stimulation of cells expressing β2AR-GFP alone, the membrane-delimited fluorescence almost totally disappeared in favor of the emergence of numerous puncta inside the cell (Fig. 7). However, in cells expressing both β2AR-GFP and the β2-adaptin-(664–937) mutant and stimulated with agonist, the receptors did not appear to internalize. This is shown by the lack of significant decrease in receptor fluorescence from the plasma membrane (compare the lower cell expressing both β2AR-GFP and β2-adaptin-(664–937) with the upper cell expressing the receptor alone). Similar results were also obtained when the β2-adaptin-(592–937) mutant was expressed, although this construct appeared to affect significantly the expression level of β2AR (data not shown).

The ability of β2-adaptin C-terminal constructs to interact with proteins involved in the endocytic process such as clathrin suggests that the expression of β2-adaptin-(592–937) or β2-adaptin-(664–937) might affect the internalization of several different classes of receptors. For instance, the transferrin receptor (TfR), which internalizes via clathrin-coated vesicles, is believed to be linked to the clathrin cages through the interac-
The detection of $\beta_2$-arrestin and clathrin and epsin. Cytosol extracts from whole mouse brain were incubated with GST fusion proteins of $\beta_2$-arrestin-(664–937) wild type, mutant constructs ($\beta_2$-arrestin-(664–937)-Y888A, -E849A, and -E902A), or GST alone. Protein complexes were resolved on SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted for the detection of either clathrin heavy chain (upper panel) or epsin (middle panel). Results show that the interaction of GST-$\beta_2$-arrestin-(664–937)-E849A and -E902A with epsin and clathrin is comparable with that of the wild type construct. However, replacement of Tyr-888 residue by an alanine residue greatly impaired the ability of the GST-$\beta_2$-arrestin-(664–937) to complex with both endocytic proteins. The bottom panel shows the amount of GST and GST fusion protein used in the assay. Results are representative of at least three independent experiments.

**FIG. 6.** $\beta_2$-Adaptin mutants impaired in $\beta_2$-arrestin binding interaction with clathrin and epsin. Cytosol extracts from whole mouse brain were incubated with GST fusion proteins of $\beta_2$-adapatin-(664–937) Y888A, -E849A, and -E902A, or GST alone. Protein complexes were resolved on SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted for the detection of either clathrin heavy chain (upper panel) or epsin (middle panel). Results show that the interaction of GST-$\beta_2$-adapatin-(664–937) Y888A and -E902A with epsin and clathrin is comparable with that of the wild type construct. However, replacement of Tyr-888 residue by an alanine residue greatly impaired the ability of the GST-$\beta_2$-adapatin-(664–937) to complex with both endocytic proteins. The bottom panel shows the amount of GST and GST fusion protein used in the assay. Results are representative of at least three independent experiments.

**FIG. 7.** Overexpression $\beta_2$-adapatin C-terminal subdomain inhibits the agonist-mediated internalization of $\beta_2$AR. HEK 293 cells were transfected with the green fluorescent-tagged protein of the $\beta_2$AR ($\beta_2$AR-GFP) and HA-tagged $\beta_2$-adapatin-(664–937) minigene construct. Cells were then either left unstimulated (-Iso) or stimulated with 10 µM isoproterenol (+Iso) for 5 min at 37 °C, fixed, and stained for the detection of $\beta_2$-adapatin-(664–937) expression using anti-HA and Texas Red-conjugated antibodies (right panels). Internalization of $\beta_2$AR was visualized on a Zeiss LSM-510 confocal microscope using sequential line excitation filters at 488 and 568 nm, and emission filter sets at 505 to 550 nm for GFP detection and 585 nm for Texas Red detection. Results show that there is little internalization of the $\beta_2$AR in a cell expressing the $\beta_2$-adapatin dominant negative construct, as the majority of receptor fluorescence remains at the plasma membrane after isoproterenol stimulation (lower left panel; compare the bottom cell expressing $\beta_2$-adapatin-(664–937) with the upper cell indicated with arrows that lacks the minigene construct). Results are representative of three independent experiments.

**FIG. 8.** Effect of $\beta_2$-adapatin C-terminal minigene constructs on transferrin uptake. A, HEK 293 cells were transfected with HA-tagged $\beta_2$-adapatin-(592–937) or -(664–937) minigene constructs and incubated with FITC-labeled transferrin (TF-FITC) as described under “Experimental Procedures.” Cells were fixed, and the expression of minigenes was revealed by immunofluorescence using rabbit anti-HA and anti-rabbit Texas Red-conjugated antibodies. Transferrin uptake and minigene expressions were visualizing by confocal microscopy. The contours of cells expressing $\beta_2$-adapatin minigenes are highlighted in the FITC channel (right panels, cyan). Results are representative of three independent experiments. B, quantification was done by qualitatively assessing the level of FITC fluorescence in 100 cells expressing the $\beta_2$-adapatin minigene construct and comparing it with the fluorescence of neighboring cells lacking the dominant negative construct.

(32), but has never been shown to require $\beta$-arrestin. Therefore, expression of $\beta_2$-adapatin-(592–937) or $\beta_2$-adapatin-(664–937) mutants, which have comparable $\beta$-arrestin binding capability but different affinities for clathrin (see Fig. 2), should result in different effects on TfR internalization. As expected, overexpression of the $\beta_2$-adapatin-(664–937) minigene only affected the internalization of FITC-labeled transferrin in a minority of cells (Fig. 8). In marked contrast, more than 65% of cells expressing the $\beta_2$-adapatin-(592–937) mutant, which contains a high affinity clathrin-binding site, showed no transferrin uptake (Fig. 8). These results are consistent with the involvement of $\beta_2$-adapatin in clathrin assembly and TfR internalization (28), but suggest that $\beta$-arrestin/$\beta_2$-adapatin interaction does not influence TfR internalization.

Finally, we looked at the ability of $\beta_2$-adapatin-(592–937) and -(664–937) mutants to block the endocytosis of other GPCRs. We chose the V2R, another receptor that internalizes in a $\beta$-arrestin- and clathrin-dependent manner (8), and compared its internalization pattern to that of the $\beta_2$AR. Cells expressing either receptor with or without the $\beta_2$-adapatin mutants were
stimulated with their respective agonist for 20 min, and the level of receptor internalization was assessed (Fig. 9). The inhibitory effect of the β2-adaptin mutants was also compared with another dominant negative construct of the clathrin-mediated pathway, K44A dynamin. When expressed with the β2AR, K44A dynamin inhibited 60% of receptor internalization compared with cells expressing β2AR alone (Fig. 9A). The β2-adaptin-(592–937) and -(664–937) minigene constructs, K44A dynamin, and pcDNA 3.1 vector (Empty) were expressed in HEK 293 cells with agonist-mediated internalization of receptors assessed as described under “Experimental Procedures” and represents the loss of receptor fluorescence for the β2AR after 20 min of isoproterenol stimulation or the fraction of [3H]AVP bound to V2R after the same incubation period that was resistant to acid washes. Results are the mean ± S.E. of three to four or three to five experiments for β2AR and V2R, respectively. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Fig. 9. Effect of different endocytic dominant negative constructs on GPCR endocytosis.** β2-Adaptin-(592–937) and β2-adaptin-(664–937) minigene constructs, K44A dynamin, and pcDNA 3.1 vector (Empty) were expressed in HEK 293 cells with β2AR (A) or V2R (B). Agonist-mediated internalization of receptors was expressed in HEK 293 cells with AT1R (data not shown). When the internalization of AT1R was assessed in the presence of the β2-adaptin-(592–937) mutant, a significant reduction in the endocytosis of the receptors was observed at early time points of agonist stimulation (i.e. a reduction of 50% in receptor internalization was already detected after 2 min of agonist stimulation compared with cells expressing wild type receptors alone; data not shown). These data indicate that the association of AP-2, via its ear domain, to receptor/β-arrestin complexes is important for clathrin-mediated endocytosis of GPCRs.

**DISCUSSION**

In the present study, we have identified critical residues in the β2-adaptin C-terminal domain responsible for the binding to β-arrestins. Expressing an AP-2 β-subunit mutant containing the β-arrestin-binding domain acts as a dominant negative of GPCR endocytosis without affecting the internalization of other classes of receptors such as the transferrin receptor. These results extend our previous findings that β-arrestins are endocytic scaffold proteins and indicate that the association of β-arrestin with AP-2 plays an important role in the clathrin-mediated internalization of other members of the GPCR family. We find that residues Glu-849 and Glu-902 in the platform C-terminal subdomain of β2-adaptin are involved in the interaction of AP-2 with β-arrestin. Mutational analysis of both proteins indicates that residues Glu-849 and Glu-902 in β2-adaptin are potentially linked through ionic interactions with residues Arg-394 and Arg-396 of β-arrestin (23). These findings, however, do not exclude the possibility that other residues and/or contact points may be required to stabilize and regulate the association between the two proteins. The platform subdomain of β2-adaptin has been shown to contain binding sites for other endocytic adaptor/accessory proteins such as AP180, epsin, and eps15 (28). However, residues in β2-adaptin implicated in these interactions seem to differ from those involved in the association of β-arrestin. For example, Owen et al. (28) have reported that substituting residue Glu-902 in β2-adaptin for an arginine (E902R) had no effect on the binding of AP180, epsin, and eps15. Consistent with these data, we find that a similar mutation in β2-adaptin (E902A) retains its binding capability for epsin, but does not associate with β-arrestin. Other residues such as Tyr-888 located in the platform subdomain of β2-adaptin seem to be involved in many, if not most of these interactions. We showed, however, that replacement of residue Tyr-888 with alanine (Y888A) not only impaired β2-adaptin binding to β-arrestin but also abrogated its association with clathrin and epsin. Owen et al. (28) also reported that the replacement of residue Tyr-888 by a valine (Y888V) compromised the binding of β2-arrestin to clathrin, AP180, epsin, and, to a lesser extent, eps15. This would suggest that Tyr-888 in β2-adaptin is a critically conserved residue that acts as a regulatory point of contact for different endocytic proteins. Alternatively, the loss of multiple interactions between different endocytic proteins and the β2-adaptin tyrosine mutant may reflect a structural change in the platform subdomain.

Clathrin-mediated endocytosis of membrane proteins requires the coordinate regulation, both spatially and temporally, of multiple protein-protein interactions. For example, the AP-2 complex recruits different accessory proteins and links receptors to CCVs through direct interactions or via accessory/adaptor proteins associated with the AP-2 complex (18, 22). Some of these interactions may affect the stability of the receptor in the CCV. The β2AR and V2R have been shown to internalize in a β-arrestin-dependent fashion (5, 8, 33), and β-arrestin may serve this function by linking the receptors to CCVs by simultaneously binding the β-subunit of AP-2 as well as clathrin. Therefore, by preventing the association of both clathrin and AP-2 with β-arrestin, this may have additive inhibitory effects on the endocytosis of the receptors compared with conditions where only each individual interaction is blocked. Unexpectedly, the β2-adaptin-(664–937) mutant, which binds poorly to clathrin but retains its β-arrestin-binding capability, was able to
block the endocytosis of both receptors as efficiently as the β2-adaptin-(592–937) mutant, which contains a high affinity binding site for β-arrestin and clathrin. An explanation for this effect could come from the fact that both β2-adaptin constructs are blocking the binding of β-arrestin to the receptors, thus preventing the targeting of the receptors to CCVs. However, when β2-adaptin mutants were expressed with a green fluorescent protein-tagged β-arrestin (GFP-β-arrestin-2) and the β2AR, GFP-β-arrestin-2 was recruited to the plasma membrane following agonist stimulation (data not shown). Alternatively, the association of plasma membrane following agonist stimulation (data not shown). Alternatively, the association of

In conclusion, we have mapped a β-arrestin binding domain in β2-adaptin. This interaction involves two glutamic acid residues (Glu-849 and Glu-902) in the platform subdomain of the ear of β2-adaptin, where other endocytic accessory/adaptor proteins have been shown to interact. Our data provide additional evidence for the role of β-arrestins as endocytic scaffold proteins and indicate that the interaction of β2-adaptin with β-arrestin is important in recruiting GPCRs for clathrin-coated vesicle-mediated endocytosis.

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β-Arrestin/AP-2 Interaction in G Protein-coupled Receptor Internalization: IDENTIFICATION OF A β-ARRESTIN BINDING SITE IN β2-ADAPTIN
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