**β-Arrestin-mediated ADP-ribosylation Factor 6 Activation and β2-Adrenergic Receptor Endocytosis**

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β-Arrestins are multifunctional adaptor proteins known to regulate internalization of agonist-stimulated G protein-coupled receptors by linking them to endocytic proteins such as clathrin and AP-2. Here we describe a previously unappreciated mechanism by which β-arrestin orchestrates the process of receptor endocytosis through the activation of ADP-ribosylation factor 6 (ARF6), a small GTP-binding protein. Involvement of ARF6 in the endocytic process is demonstrated by the ability of GTP-binding defective and GTP hydrolysis-deficient mutants to inhibit internalization of the β2-adrenergic receptor. The importance of regulation of ARF6 function is shown by the ability of the ARF GTPase-activating protein GIT1 to inhibit and of the ARF nucleotide exchange factor, ARNO, to enhance receptor endocytosis. Endogenous β-arrestin is found in complex with ARNO. Upon agonist stimulation of the receptor, β-arrestin also interacts with the GDP-ligated form of ARF6, thereby facilitating ARNO-promoted GTP loading and activation of the G protein. Thus, the agonist-driven formation of a complex including β-arrestin, ARNO, and ARF6 provides a molecular mechanism that explains how the agonist-stimulated receptor recruits a small G protein necessary for the endocytic process and controls its activation.

Agonist-triggered G protein-coupled receptor (GPCR) endocytosis is a highly regulated process involved in signaling and desensitization of numerous receptors. Agonist-dependent GPCR phosphorylation by G protein-coupled receptor kinases (GRKs) promotes binding of β-arrestins to the receptor, which in turn induces desensitization by preventing further receptor-mediated G protein activation. β-Arrestins have been shown to regulate GPCR internalization via their direct interaction with clathrin and the β subunit of the clathrin adaptor protein complex AP-2 (2–4). However, the exact molecular mechanisms regulating the protein-protein interactions involved in clathrin-coated pit formation remain unknown.

We have shown recently that a novel GTPase-activating protein for the ARF-ribosylation factor (ARF) family of small GTP-binding proteins, GIT1, can regulate internalization of the β2-adrenergic receptor (β2AR), as well as other signaling receptors, suggesting a critical role for ARF function in this process (5, 6).

The ARF proteins constitute a group of six ubiquitously expressed small GTP-binding proteins. These GTPases are essential components of the molecular machinery that regulates membrane trafficking along endocytic and biosynthetic pathways (7, 8). ARF1, the best characterized subtype, is localized to the Golgi complex and is a regulator of vesicle coat recruitment for both COP1- and clathrin-coated vesicles (9–11). ARF6 is uniquely associated with the plasma membrane (12, 13).

Activation of ARF proteins is facilitated by guanine nucleotide exchange factors that promote dissociation of bound GDP from the inactive ARF protein followed by binding of GTP to the nucleotide-free ARF. Inactivation of ARF proteins is regulated by GTPase-activating proteins (GAPs). ARF proteins have been shown to regulate various plasma membrane trafficking events such as the constitutive recycling of the transferrin receptor (12), calcium-stimulated exocytosis in chromaffin cells (14), Fcγ receptor-mediated phagocytosis in macrophages (15), endocytosis at the apical surface of epithelial Madin-Darby canine kidney cells (16), membrane recycling (17, 18), and the endothelin-mediated translocation of the GLUT4 glucose transporter (19, 20). In addition, ARF6 activation is involved in the remodeling of the actin cytoskeleton (15, 21, 22). In this study, we examine the involvement of ARF proteins in the internalization process of the β2AR and begin to elucidate the molecular mechanisms by which the function of these small GTP-binding proteins is regulated following agonist-stimulation.

**MATERIALS AND METHODS**

**Plasmids**—pBK(Δ)-β2AR, pBK(Δ)-GIT1, pGEX4T2-β2-arrestin 1 (319–418), pCDNA3-His-β2-arrestin 1, pCDNA3-Flag-β2-arrestin 1, and pCDNA3-Flag-β2-arrestin 2, were previously described (3, 5, 23, 24). pToto3 2J-hARF6 and pToto3 2J-ARF6Q67L clones were obtained from Dr. P. Stahl (Washington University, St. Louis, MO). pBKΔ-ARF6 and pBKΔ-ARF6Q67L were generated by cloning the full-length ARF6 or ARF6Q67L from pToto3 2J-hARF6 into the XbaI site of a modified pBK-CMV vector (Stratagene). pBKΔ-ARF6T27N was generated by cloning the full-length ARF6T27N from pEFGP-N1-ARF6T27N into XhoI and SfiI sites of pBKΔ. Human ARNO (3G variant) (25) was amplified from a human brain library using specific primers and subcloned into pBKΔ and pGEX4T2. All sequences were confirmed using an automated ABI DNA sequencer (Howard Hughes Medical Institute Nucleic Acid Facility, Duke University Medical Center).

**Preparation of Recombinant Proteins**—Recombinant hARF6 or myr-hARF6 were prepared as described previously (26, 27). DNA constructs 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.

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for Flag-β-arrestin 1 and 2 were prepared in pVL1393, and recombinant baculoviruses were obtained using the BD BaculoGold transfection kit (BD PharMingen). FLAG-tagged β-arrestins were purified from SF9 cells 96 h post-infection. Briefly, SF9 cells were harvested in lysis buffer (10 mM HEPES, 10% Nonidet P-40, 4 mM NaCl, 50% glycerol, 0.4 mM EDTA, protease inhibitors), cell debris were pelleted, and the supernatant was incubated with M2 affinity beads for 3–4 h at 4 °C. The beads were subsequently spun and washed three times with the lysis buffer. Purified proteins were eluted with 100 mM glycine (pH 3.5) into vials containing 1 M Tris (pH 8).

Cell Culture and Transfection—HEK 293 cells were maintained and transiently transfected as described previously (5).

Screening Assay—Receptor sequestration was determined by flow cytometry as described previously (6, 28) and defined as the percent of cell surface receptor no longer accessible to extracellular antibodies. Recycling experiments were done similarly except that the agonist was removed after 30 min of stimulation by washing the cells three times with warm media. The cells were placed back at 37 °C, and the percent of initial receptors present at the cell surface was assessed 15, 30, and 60 min after agonist removal.

Immunoprecipitation—Immunoprecipitations were done as described previously (5). Briefly, serum-starved HEK 293 cells were stimulated with isoproterenol for indicated times. Dithiobis(succinimidyl propionate) (DSP; Pierce) (25 mM) in phosphate-buffered saline was removed for direct immunoblotting of the lysates. Affinity gel FLAG beads (15 μl) (Sigma) were added to each tube, and the samples were rotated for 6 h at 4 °C. The proteins were eluted into 40 μl of SDS-polyacrylamide gel electrophoresis sample buffer containing 5% β-mercaptoethanol by heating to 95 °C for 10 min. Proteins were resolved on 14% gels and detected by immunoblot analysis.

GST Fusion Proteins and Pull-down Experiments—GEX2T plasmids bearing ARNO or β-arrestin 1 carboxyl-terminal (319–418) DNAs were prepared, and fusion proteins were purified as described previously (4). GST fusion proteins on glutathione-Sepharose 4B were incubated in 200 μl of binding buffer B (20 mM Tris, 2 mM dithiothreitol, 25 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 2.5 mM MgCl2, 1 mM ATP, pH 7.4) containing protease inhibitors for 4 h at 4 °C with purified 6xHis-β-arrestin 1 or recombinant myristoylated ARF6 protein in the absence or presence of either GDP/βS or GTP/βS. The beads were spun and washed three times with binding buffer followed by two washes with binding buffer without any detergent. Beads were resuspended in 2× SDS sample buffer, incubated at 95 °C for 10 min, resolved by electrophoresis on a 14% gel, transferred onto nitrocellulose, and analyzed by Western blot or stained with Coomassie Blue for GST-protein detection.

Co-Immunoprecipitation of Endogenous Proteins from Whole Brain Extract—Co-immunoprecipitations from whole brain extract were performed as described previously (29). β-Arrestins were immunoprecipitated from the supernatant using an antibody specific for β-arrestins (AICT) covalently cross-linked to Reactigel beads (Pierce). Immunoprecipitated proteins were detected by immunoblot analysis using a goat anti-ARNO amino-terminal antibody (Santa Cruz Biotechnology) and the β-arrestin polyclonal antibody.

GTPyS-binding Assay—[35S]GTPγS leading assays were done as described previously (30). Briefly, [35S]GTPγS binding to purified recombinant ARF6 (myristoylated and nonmyristoylated forms) was assayed in a total volume of 50 μl using a rapid filtration procedure. ARF6 (0.36 μg), [35S]GTPγS (4 μM: 1 × 106 cpm) without or with GST-ARNO (1 μg) and β-arrestin 1 (5 μg) were incubated for the indicated times at 37 °C. After the reaction was stopped by the addition of 2 ml of wash buffer and rapid filtration onto nitrocellulose, the amount of protein-bound [35S]GTPγS was quantified. Data are reported as mean ± S.E. of values from duplicate assays and expressed as the amount of GTPγS specifically bound to ARF6.

Data Analysis—The mean and standard error of the mean are expressed for values obtained from the number of separate experiments indicated performed in duplicate. Statistical analysis was performed by a two-way analysis-of-variance followed by an unpaired Student’s t test or Tukey’s statistical test.

RESULTS AND DISCUSSION

To directly investigate the role of ARF proteins in endocytosis of GPCRs, we first expressed wild type and mutant ARF proteins together with the β-AR and examined receptor internalization following isoproterenol stimulation. Expression of wild type ARF6 did not significantly affect β2AR endocytosis. Expression of ARF6Q67L and ARF6T27N, ARNO, and GIT1 on internalization of the β2AR. a, HEK 293 cells were transiently co-transfected with Flag-β-AR together with empty vector, ARF6, ARF6Q67L, or ARF6T27N. Agonist-induced internalization of epitope-tagged β2AR was measured before and after treatment with isoproterenol (10 μM) for the indicated times. b, cells were transiently co-transfected with Flag-β-AR together with empty vector, ARF6, or ARF6Q67L. Agonist-induced internalization of epitope-tagged β2AR was measured before and after 30 min of isoproterenol treatment (10 μM). Agonist was removed by washes and cell surface receptor number was quantified after 15, 30, and 60 min. Results were expressed as the percent of cell surface immunofluorescence compared with nonstimulated cells. The data represent the mean of 4–6 independent experiments done in duplicate (*, p < 0.001). c, cells transiently expressing Flag-β2AR together with either ARNO or GIT1 were stimulated with isoproterenol (10 μM) for the indicated times. Results were expressed as the percent loss of cell surface immunofluorescence compared with nonstimulated cells. The data represent the mean of 4–8 independent experiments done in duplicate (*, p < 0.05; **, p < 0.001).

proteins together with the β-AR and examined receptor internalization following isoproterenol stimulation. Expression of wild type ARF6 did not significantly affect β2AR sequestration from the cell surface at any time point, whereas overexpression of a mutant defective in GTP hydrolysis (ARF6Q67L) or in GTP binding (ARF6T27N) showed marked inhibitory effects (Fig. 1a). In contrast, expression of ARF1 or ARF1 mutants, ARF1T31N or ARF1Q71L, had no effect on β2AR endocytosis (data not shown). Presumably, this is because activated ARF1 is mainly found on intracellular membranes, whereas activated ARF6 is mainly localized at the plasma membrane.
Interestingly, expression of ARF6 mutants prevents constitutive recycling of the transferrin receptor, another process involving cell surface vesicle trafficking (12). Although expression of wild type ARF6 had no effect on the kinetics of recycling of the β2AR, expression of the ARF6Q67L mutant totally prevented reappearance of internalized receptors at the cell surface, even after 60 min of recovery when 95% of internalized receptors are normally recycled (Fig. 1b). These data suggest that ARF proteins, namely ARF6, regulate internalization as well as recycling of the β2AR, two processes requiring plasma membrane vesicle trafficking.

Like other small GTP-binding proteins, activation of ARF by GTP loading is regulated by nucleotide exchange factors, whereas inactivation by GTP hydrolysis is facilitated by GAPs. We have previously shown that expression of the ARF GTPase-activating protein GIT1 markedly inhibits internalization of the β2AR (6). Therefore, we hypothesized that expression of an appropriate ARF guanine nucleotide exchange factor would facilitate ARF6 activation, thereby leading to increased receptor internalization. In HEK 293 cells, we transiently expressed ARNO, an exchange factor for both ARF1 and ARF6 that is localized to the plasma membrane (26, 31), and examined the internalization profile of the β2AR. Increased cellular levels of ARNO led to increased agonist-stimulated internalization of receptors, whereas expression of GIT1 was inhibitory (Fig. 1c). These results suggest that expression of ARNO facilitates receptor internalization by promoting ARF6 activation. In contrast, GIT1 expression may reduce receptor internalization by triggering immediate inactivation of ARF6. Our data on the contribution of ARF regulatory proteins support the role of ARF6 in regulating agonist-promoted receptor endocytosis.

To elucidate the molecular mechanisms by which ARF6 regulates receptor internalization, we set out to identify proteins interacting with ARF6 and its regulatory proteins. The β-arrestins have been shown to play an important role in receptor internalization through their direct interaction with clathrin and its adaptor protein, AP-2 (2, 3). We hypothesized that β-arrestin, a protein recruited to GRK-phosphorylated receptors, might also play a role in the regulation of ARF6 function. Therefore, we examined whether ARF6 could interact with β-arrestin 1 and β-arrestin 2. Cells expressing HA-β2AR, Flag-β2AR, or Flag-β-arrestin 2 and ARF6 were left untreated or were stimulated with isoproterenol for 2, 5, and 10 min. Subsequently, Flag-β-arrestin proteins were immunoprecipitated, and associated ARF6 was detected by immunoblotting. Interestingly, the binding of ARF6 to β-arrestin 1 or β-arrestin 2 increased following receptor stimulation and could be detected readily after 2 min of agonist stimulation (Fig. 2a). The interaction was found to be maximal after 5 and 10 min of receptor activation. von Zastrow and Kobilka (32) have reported that β2AR targeting to clathrin-coated pits occurs after 2 min of agonist activation. Similarly, β-arrestin 2 can be found in complex with the β subunit of AP-2 following 2 min of β2AR stimulation (3). Therefore, our data suggest that ARF6 activation following receptor stimulation might regulate early events of the endocytic process. Using similar experimental conditions, we were unable to co-immunoprecipitate ARF6 with the β2AR (data not shown) suggesting that β-arrestin is required to bridge this receptor to ARF-dependent signaling pathways. Mitchell et al. (33) have reported that in 1321N1 cells, ARF proteins can be found in complex with several but not all Ca2+-mobilizing G protein-coupled receptors, activation of which lead to phospholipase D stimulation. Whether these interactions are direct or mediated via an adaptor protein has not yet been examined.

To confirm that the interactions between β-arrestin and ARF6 were direct, we used purified recombinant Flag–β-arrestin 1 and 2 together with ARF6 and analyzed their ability to interact in vitro. As illustrated in Fig. 2b, ARF6 can be co-immunoprecipitated with both β-arrestin 1 and β-arrestin 2. Mutagenesis studies of β-arrestin proteins have revealed that the binding sites for both clathrin and AP-2 are present in the carboxyl-terminal portion of the protein (2–4). To determine whether the interaction between β-arrestin and ARF6 is also mediated via the carboxyl-terminal part of β-arrestin, we used a GST fusion protein of the last 100 amino acids of β-arrestin 1 (GST–β-arrestin 1-(319–418)) and examined the interaction with ARF6. Pull-down assays revealed that a binding site for ARF6 is present within this region of β-arrestin 1 (Fig. 2c). Interestingly, the interaction between GST–β-arrestin 1-(318–419) and ARF6 was found to be regulated by the nature of the nucleotide bound to the small G protein, which is purified mainly in its GDP-bound state. The addition of GTPγS did not alter the interaction, whereas addition of GDP did completely abolish the binding of ARF6 to β-arrestin 1 (Fig. 2c). Taken together, these results suggest that β-arrestin might act as a scaffold protein bringing together the ARF protein in complex with receptors. Furthermore, this interaction is regulated by both agonist activation of the receptor and the nature of the nucleotide bound to ARF6. Once bound to GTP, activated ARF6 dissociates from β-arrestin proteins and is available to promote
were left untreated or stimulated with isoproterenol (iso/H9262) receptor (34). In addition, ARNO and ARF6 could also be found in the endocytic process of the luteinizing hormone/choriogonadotropin receptor (LH25). These results demonstrate that ARNO can promote release of a pool of GTP-S-bound ARF6 (Fig. 3b). Further, the amount of GTP-S bound to ARF6 in the presence of ARNO was significantly increased in a time-dependent fashion, consistent with its role as an ARF nucleotide exchange factor (Fig. 4). Using an amount of ARNO that leads to submaximal GTP loading, further addition of β-arrestin 1 led to a marked potentiation of the rate of activation of ARF6 stimulated by ARNO without affecting maximal loading of GTP-S. Therefore, the effect of β-arrestin 1 was most significant at early time points (5 and 10 min). In the absence of ARNO, β-arrestin 1 did not have any effect on the amount of GTP-S bound to ARF6. These data suggest that by interacting with both ARF6 and ARNO, β-arrestin 1 facilitates GTP loading of the ARF protein, thereby leading to a potentiation of the activation of this small GTP-binding protein.

Recent reports have demonstrated that ARF proteins are involved in several intracellular trafficking processes. Here, we show that ARF6 regulates internalization of the β2AR, a prototypical G protein-coupled receptor. Expression of the GTP hydrolysis-deficient mutant (ARF6Q67L) or the GTP binding-defective mutant (ARF6T27N) results in the inhibition of the internalization process. Interestingly, expression of ARF6Q67L or ARF6T27N inhibits Fcγ receptor-mediated phagocytosis in the endocytic process.

Next, we examined whether ARNO could also interact with β-arrestin proteins. Cells overexpressing HA-β2AR, Flag-ARNO, and His-β-arrestin 1 or His-β-arrestin 2 were left untreated or stimulated with isoproterenol (iso, 10 μM) for 5 min. Flag-ARNO was immunoprecipitated (IP), and associated β-arrestin 1 or β-arrestin 2 was detected by immunoblotting (IB) using a His probe antibody (Santa Cruz). Amounts of immunoprecipitated ARNO were detected using a goat anti-ARNO amino-terminal antibody (Santa Cruz). MOCK cells co-expressed the HA-β2AR, β-arrestin 1, and β-arrestin 2. Levels of β-arrestin 1 and 2 expression in the cell lysates were verified using the β-arrestin 1 A1CT antibody. Amounts of immunoprecipitated ARNO were detected using a goat anti-ARNO fusion proteins were incubated with purified Flag-β-arrestin 1 and 2 (2.5 μg). GST or GST-ARNO were captured on glutathione-Sepharose, and the amounts of β-arrestin proteins interacting were detected by immunoblotting. Data shown are representative of at least three independent experiments. c, β-arrestin was immunoprecipitated from bovine brain lysates with an antibody specific to β-arrestin (A1CT) and resolved by SDS-polyacrylamide gel electrophoresis. The presence of ARNO and β-arrestin in the immunoprecipitates was detected using antibodies to ARNO (goat anti-ARNO NT) and β-arrestin (A1CT). Immunoblot analysis of ARNO and β-arrestin in total cell lysates is also shown. Data are representative of three independent experiments. Similar results were obtained in mouse brain lysates.

To verify that these molecular interactions occur with endogenous proteins, β-arrestins were immunoprecipitated from bovine brain extracts with a β-arrestin-specific antibody covalently cross-linked to beads, and associated proteins were detected by immunoblotting. ARNO was found to be present in the β-arrestin immunoprecipitates but not in the pre-immune serum immunoprecipitates (Fig. 3c). However, under several experimental conditions, we were unable to detect ARF6 in the endogenous β-arrestin immunoprecipitates (data not shown). This is likely due to the agonist dependence of this interaction, as illustrated above. These findings demonstrate a specific interaction between ARNO and β-arrestin at endogenous levels of proteins and confirm the results obtained from cellular co-immunoprecipitations and in vitro experiments.

It is well established that the binding of ARNO to ARF6 serves to catalyze the exchange of GDP for GTP to activate the ARF protein. We next wanted to determine whether β-arrestin simply acts as an adaptor protein between activated receptors and the ARF6 complex or, rather, plays a more direct role in the regulation of ARF6 activation process. To investigate the effect of β-arrestin on ARNO-mediated GTP-S-binding to ARF6, we used recombinant β-arrestin 1, ARF6, and ARNO in an in vitro GTP-S loading assay. The rate of GTP-S binding to ARF6 was enhanced by increasing concentrations of ARNO (data not shown). Further, the amount of GTP-S bound to ARF6 in the presence of ARNO was significantly increased in a time-dependent fashion, consistent with its role as an ARF nucleotide exchange factor (Fig. 4). Using an amount of ARNO that leads to submaximal GTP loading, further addition of β-arrestin 1 led to a marked potentiation of the rate of activation of ARF6 stimulated by ARNO without affecting maximal loading of GTP-S. Therefore, the effect of β-arrestin 1 was most significant at early time points (5 and 10 min). In the absence of ARNO, β-arrestin 1 did not have any effect on the amount of GTP-S bound to ARF6. These data suggest that by interacting with both ARF6 and ARNO, β-arrestin 1 facilitates GTP loading of the ARF protein, thereby leading to a potentiation of the activation of this small GTP-binding protein.

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macrophages while stimulating endocytosis at the apical surface of Madin-Darby canine kidney cells (15, 16). Several reports have demonstrated that ARF6Q67L accumulates at the plasma membrane where it induces invaginations, whereas ARF6T27N is distributed to an internal tubulovesicular compartment (17, 18, 35). Although both ARF6 mutants affect internalization, they probably do so by different mechanisms (15, 16). Similarly, we have shown that expression of GIT1, an ARF GAP, also inhibits internalization of the ββAR. However, expression of ARNO, an ARF guanine nucleotide exchange factor, is stimulatory. By overexpressing GIT1, we might promote rapid and unregulated inactivation of ARF6, thereby leading to inhibition of vesicle formation. In contrast, expression of ARNO would result in further ARF6 activation and therefore promote vesicle formation. At endogenous levels of proteins, both ARNO and GIT probably contribute to the formation of endocytic vesicles by regulating, in a coordinated fashion, the turnover of nucleotide on ARF proteins.

According to our findings, β-arr Type 2 appears to serve as an agonist-controlled scaffold bringing together the exchange factor, ARNO, and the GDP-bound form of ARF6, thereby promoting ARF6 activation in proximity to the receptor. The agonist dependence of the ARF6 interaction with β-arr Type 2 brings this entire process under the control of the receptor. Once bound to GTP, active ARF6 proteins dissociate from β-arr Type 2 and are now available to promote the endocytic process. The biochemical details involved in this process remain to be determined. However, the closely related ARF1 protein is known to drive vesicle budding from donor membranes by promoting coat protein assembly through the recruitment of AP-1 and coatomers (COPI, COPII, and clathrin) (36, 37). Furthermore, ARF6 has been shown to activate phospholipase D, thereby increasing the levels of phosphatidic acid, and to activate phosphatidylinositol 4- and 5-kinase activity to increase phosphatidylinositol 1,4,5-trisphosphate levels (8, 38). Interestingly, the closely related ARF1 protein is known to drive vesicle budding from donor membranes by promoting coat protein assembly through the recruitment of AP-1 and coatomers (COPI, COPII, and clathrin) (36, 37). Furthermore, ARF6 has been shown to activate phospholipase D, thereby increasing the levels of phosphatidic acid, and to activate phosphatidylinositol 4- and 5-kinase activity to increase phosphatidylinositol 1,4,5-trisphosphate levels (8, 38). Interestingly, however, the closely related ARF1 protein is known to drive vesicle budding from donor membranes by promoting coat protein assembly through the recruitment of AP-1 and coatomers (COPI, COPII, and clathrin) (36, 37). Furthermore, ARF6 has been shown to activate phospholipase D, thereby increasing the levels of phosphatidic acid, and to activate phosphatidylinositol 4- and 5-kinase activity to increase phosphatidylinositol 1,4,5-trisphosphate levels (8, 38). Interestingly,
