ADP-Ribosylation Factors Modulate the Cell Surface Transport of G Protein-Coupled Receptors^S

Chunmin Dong, Xiaoping Zhang, Fuguo Zhou, Huijuan Dou, Matthew T. Duvernay, Ping Zhang, and Guangyu Wu

Departments of Pharmacology and Experimental Therapeutics (C.D., X.Z., F.Z., H.D., M.T.D., G.W.) and Medicine (P.Z.), Louisiana State University Health Sciences Center, New Orleans, Louisiana

Received September 10, 2009; accepted January 20, 2010

ABSTRACT

ADP-ribosylation factors (ARFs) regulate vesicular traffic through recruiting coat proteins. However, their functions in the anterograde transport of nascent G protein-coupled receptors (GPCRs) from the endoplasmic reticulum to the plasma membrane remain poorly explored. Here we show that treatment with brefeldin A, an inhibitor of guanine nucleotide exchange on ARFs, markedly attenuated the cell surface numbers of α_{2B} -adrenergic receptor (AR), β_2 -AR, angiotensin II type 1 receptor, and chemokine (CXC motif) receptor 4. Functional inhibition of individual ARF GTPases by transient expression of the GDP-bound, GTP-bound, and guanine nucleotide-deficient mutants showed that the five human ARFs differentially modulated receptor cell surface expression and that the ARF1 mutants

produced the most profound inhibitory effect. Furthermore, expression of the ARF1 GTPase-activating protein (GAP) ARFGAP1 significantly blocked receptor transport. Interestingly, the GDP- and GTP-bound ARF1 mutants arrested the receptors in distinct intracellular compartments. Consistent with the reduced receptor cell surface expression, extracellular signal-regulated kinase 1 and 2 activation by receptor agonists was significantly attenuated by the GDP-bound mutant ARF1T31N. Moreover, coimmunoprecipitation showed that α_{2B} -AR associated with ARF1 and glutathione transferase pull-down assay indicated that the α_{2B} -AR C terminus directly interacted with ARF1. These data show that ARF1 GTPase is involved in the regulation of cell surface expression of GPCRs at multiple transport steps.

ADP-ribosylation factors (ARFs) belong to the superfamily of Ras-related small GTPases and modulate vesicle-mediated transport (D'Souza-Schorey and Chavrier, 2006). Six ARF members (ARF1–6) have been identified in mammalian cells, but ARF2 is not expressed in humans. Based on their amino acid sequence homology and gene organization, ARFs are divided into three classes: class I (ARF1–3), class II (ARF4–5), and class III (ARF6). Among these ARF GTPases, ARF1 and ARF6 are the best studied and well understood members. ARF1 plays a crucial role in both anterograde and

retrograde trafficking, whereas ARF6 is mainly involved in regulation of endocytosis and actin cytoskeleton remodeling (Stearns et al., 1990; Palacios et al., 2001; Spang, 2002). Although ARF3 has not been well studied, it is generally considered that the functions of ARF1 and ARF3 are interchangeable. In contrast, the physiological roles for the class II ARFs remain poorly characterized.

Like other Ras-related GTPases, the function of ARFs is highly regulated by their recycling between active GTP-bound and inactive GDP-bound conformations (Gsandtner et al., 2005; Lee et al., 2005). Inactive GDP-bound ARFs may be recruited from cytosol onto the membrane by interacting with receptor proteins, and their association with the membrane is mediated through the N-terminal myristoylated amphipathic helix domain. On the membrane, ARFs undergo the exchange of GDP for GTP, which is catalyzed by guanine nucleotide exchange factors (GEFs). Active GTP-bound ARFs

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant GM076167].

C.D. and X.Z. contributed equally to this work.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.109.161489.

^S The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum-Golgi intermediate complex; TGN, *trans*-Golgi network; GAP, GTPase-activating protein; GPCR, G protein-coupled receptor; AR, adrenergic receptor; AT1R, angiotensin II type 1 receptor; CXCR4, chemokine (CXC motif) receptor 4; ERK1/2, extracellular signal-regulated kinase 1 and 2; HA, hemagglutinin; ISO, isoproterenol; UK-14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine; BFA, brefeldin A; Ang II, angiotensin II; SDF-1 α , stromal cell-derived factor 1 α ; CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one; RX 821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; GFP, green fluorescent protein; GST, glutathione transferase; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; M₃-MR, M₃-muscarinic receptor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ARFGAP1, ADP-ribosylation factor GTPase-activating protein 1.

subsequently interact with downstream effectors. It has been shown that the GTP-bound ARF1 recruits distinct protein complexes onto different intracellular compartments, resulting in the formation of different transport vesicles. In the early secretory pathway, activation of ARF1 results in the recruitment of a complex of cytosolic proteins, collectively known as coatomers, leading to the formation of COPI-coated vesicles, which mediate cargo transport from the Golgi to the endoplasmic reticulum (ER), from the ER-Golgi intermediate complex (ERGIC) to the Golgi, and between Golgi cisternae (Spang, 2002). In the post-Golgi transport, the activated ARF1 recruits the adaptor protein complex and Golgi-localized γ -ear-containing ARF1-binding proteins (Bonifacio, 2004) to initiate the formation of the clathrin-coated vesicles, which mediate protein transport between the *trans*-Golgi network (TGN) and the endosomal compartment. The release of the cargo-carrying vesicles from the membrane is controlled by the hydrolysis of GTP to GDP of ARFs, a process facilitated by GTPase-activating proteins (GAPs).

G protein-coupled receptors (GPCRs) represent the largest family of cell surface receptors and regulate a variety of cell functions (Pierce et al., 2002). The magnitude of receptor-elicited cellular response to a given stimulus is dictated by elaborately regulated intracellular trafficking processes that control the cell surface expression and subcellular compartment targeting of the receptors. Similar to many plasma membrane proteins, the life of GPCRs begins at the ER, where they are synthesized, folded, and assembled. Properly folded receptors are transported from the ER through the ERGIC, the Golgi apparatus, and the TGN to the plasma membrane (Dong et al., 2007). At the plasma membrane, GPCRs may undergo internalization on stimulation by their ligands. The internalized receptors in the early endosome may be sorted to the lysosome for degradation or to the recycling endosome for return to the plasma membrane. In contrast to the extensive studies on the endocytic, recycling, and degradation pathways (Goodman et al., 1996; Puthenveedu and von Zastrow, 2006), the molecular mechanism underlying the export of newly synthesized GPCRs from the ER to the cell surface remains less well understood.

As an initial approach to elucidate the mechanism of nascent GPCR targeting to the cell surface, we have determined the role of Sar1 and several Rab GTPases in regulating cell surface transport of representative GPCRs, including the angiotensin II and adrenergic receptor (AR) subfamilies (Wu et al., 2003; Filipeanu et al., 2004; Filipeanu et al., 2006; Dong and Wu, 2007; Dong et al., 2008; Zhang et al., 2009). Our studies indicate that the cell surface transport of different GPCRs may be mediated through distinct pathways and suggest a novel pathway mediating α_{2B} -AR traffic from the ER to the Golgi apparatus (Wu et al., 2003). In this study we investigated the role of ARF GTPases in the anterograde transport from the ER to the cell surface of four family A GPCRs, including α_{2B} -AR, β_2 -AR, angiotensin II type 1 receptor (AT1R), and chemokine (CXC motif) receptor 4 (CXCR4).

Materials and Methods

Materials. Antibodies against ARF1, ARF5, and ARF6 were purchased from Assay Designs, Inc. (Ann Arbor, MI). Antibodies against ARF3, GM130, and p230 and PE-conjugated monoclonal

anti-CXCR4 and IgG2a antibodies were from BD Biosciences (San Jose, CA). ERGIC-53 antibodies were from Alexis Laboratories (San Diego, CA). Antibodies against phospho-extracellular signal-regulated kinase 1 and 2 (ERK1/2) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ERK1/2 antibodies were from Cell Signaling Technology Inc. (Danvers, MA). High affinity fluorescein-conjugated anti-hemagglutinin (HA) antibody 3F10 and anti-HA mouse monoclonal antibodies 12CA5 were from Roche Diagnostics (Mannheim, Germany). Isoproterenol (ISO), UK-14,034, brefeldin A (BFA), and anti-His antibodies were from Sigma-Aldrich (St. Louis, MO). Human angiotensin II (Ang II) was purchased from EMD Chemicals (Gibbstown, NJ). Human stromal cell-derived factor 1 α (SDF-1 α) was purchased from PeproTech (Rocky Hill, NJ). Penicillin/streptomycin, L-glutamine, and trypsin/EDTA were from Invitrogen (Carlsbad, CA). [3 H]CGP-12177 (specific activity = 51 Ci/mmol), [3 H]RX 821002 (41 Ci/mmol), *l*-[N-methyl- 3 H]scopolamine methyl chloride ([3 H]NMS, 80 Ci/mmol), and (3-(125 I)-iodotyrosyl 4) Ang II (125 I-Ang II) (2000 Ci/mmol) were purchased from GE Healthcare (Piscataway, NJ). Alexa Fluor 594-labeled secondary antibodies and 4,6-diamidino-2-phenylindole were from Invitrogen. All the other materials were obtained as described elsewhere (Wu et al., 2003; Duvernay et al., 2009a).

Plasmid Constructions. α_{2B} -AR, β_2 -AR, and AT1R tagged with green fluorescent protein (GFP) at their C termini were generated as described previously (Wu et al., 2003). α_{2B} -AR, AT1R, and ARF4 tagged with three HA at their N termini were generated as described previously (Duvernay et al., 2009a). CXCR4 tagged with HA at its N terminus was obtained from Missouri S&T cDNA Resource Center (Rolla, MO). GFP and HA have been widely used to tag GPCRs without altering their trafficking and function (Wu et al., 2003; Duvernay et al., 2009a). Glutathione transferase (GST) fusion proteins encoding the C terminus of α_{2B} -AR were generated into the pGEX-4T-1 vector using a strategy as described previously (Wu et al., 1997, 1998). ARF mutants were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA). The sequence of each construct used in this study was verified by restriction mapping and nucleotide sequence analysis.

Cell Culture and Transient Transfection. Human embryonic kidney (HEK) 293 cells and ghost cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Wu et al., 2003). NG108-15 neuroblastoma-glioma cells expressing endogenous α_{2B} -AR subtype were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 100 μ M hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine. Transient transfection of the cells was carried out using Lipofectamine 2000 reagent (Invitrogen) as described previously (Wu et al., 2003). The transfection efficiency was estimated to be greater than 75% based on the GFP fluorescence. To determine the effect of BFA treatment on the cell surface expression and subcellular distribution of receptors, the cells transfected with the receptors were incubated with BFA at a concentration of 5 μ g/ml culture medium for 8 h.

Radioligand Binding. Cell surface expression of α_{2B} -AR, β_2 -AR, M_3 -muscarinic receptor (M_3 -MR), and AT1R in HEK293 cells was measured by ligand binding of intact live cells as previously described using [3 H]RX 821002, [3 H]CGP-12177, [3 H]NMS, and 125 I-Ang II (Filipeanu et al., 2004; Duvernay et al., 2009a), respectively. In brief, HEK293 cells were cultured on six-well dishes and transfected with 200 ng of the α_{2B} -AR, β_2 -AR, or M_3 -MR plasmid together with 800 ng of pcDNA3 or ARFs. After 6 h, the cells were split into 12-well plates at a density of 4×10^5 cells/well and cultured for an additional 24 h. For measurement of β_2 -AR expression at the cell surface, the cells were incubated with DMEM containing the ligand [3 H]CGP-12177 at a concentration of 20 nM for 90 min at room temperature. Nonspecific binding was determined by preincubation with alprenolol at a concentration of 20 μ M for 30 min followed by incubation with [3 H]CGP-12177 (20 nM) in the presence of alprenolol (20 μ M). For measurement of the cell surface expression of the

exogenously expressed α_{2B} -AR in HEK293 cells and endogenous α_{2B} -AR in NG108-15 cells, the cells were incubated with DMEM plus [3 H]RX 821002 at a concentration of 20 nM for 90 min at room temperature. The nonspecific binding was determined in the presence of rauwalscine (10 μ M). For measurement of M_3 -MR expression at the cell surface, the cells were incubated with the ligand [3 H]NMS at a concentration of 20 nM for 2 h at 4°C. Nonspecific binding was determined in the presence of atropine (20 μ M). The cells were washed twice with 1 ml of phosphate-buffered saline (PBS), and the cell surface-bound ligands were extracted by 1 M NaOH treatment for 2 h. The radioactivity was counted by liquid scintillation spectrometry in 3.5 ml of Ecoscint A scintillation solution (National Diagnostics, Atlanta, GA).

For measurement of AT1R expression at the cell surface, HEK293 cells were cultured on six-well plates and transfected with 0.5 μ g of AT1R together with 1.5 μ g of pcDNA3 or individual ARF1 mutants. The cells then were split into 12-well plates and starved for 24 h followed by an incubation with 400 μ l of DMEM containing [125 I]-Ang II at a concentration of 20 pM for 5 h at 4°C to avoid AT1R internalization. The nonspecific binding was determined in the presence of nonradioactive Ang II (3 μ M). After washing the cells twice with 1 ml of DMEM, the bound ligand was extracted by mild acid treatment (2 $\times$ 5 min with 0.5 ml of buffer containing 50 mM glycine and 125 mM NaCl, pH 3). The radioactivity was counted in a gamma counter. All the radioligand binding assays were performed in triplicate.

Flow Cytometric Analysis of the Cell Surface and Total Expression of Receptors. The cell surface expression of α_{2B} -AR, AT1R, and CXCR4 was measured by flow cytometry as described previously (Zhang et al., 2009). In brief, the receptors were tagged with three HA at their N termini and transiently expressed into HEK293 cells. The cells were suspended at a density of 1×10^7 cells/ml in PBS containing 1% FBS and incubated with high affinity anti-HA-fluorescein (3F10) at a final concentration of 2 μ g/ml for 30 min at 4°C. For measurement of the cell surface expression of endogenous CXCR4 in HEK293 and ghost cells, the cells were incubated with anti-CXCR4 antibodies or the control antibodies IgG2a (20 μ l of antibodies/100 μ l of cells) for 30 min at room temperature. After washing three times with 1.0 ml of PBS plus 1% FBS, the cells were resuspended and the fluorescence was analyzed on a flow cytometer (FACSCalibur; BD Biosciences). Because the staining with anti-HA antibodies was carried out in the unpermeabilized cells and only those receptors expressed at the cell surface were accessible to the anti-HA antibodies, the measured fluorescence reflected the amount of receptor expressed at the cell surface.

Immunofluorescence Microscopy. Fluorescence microscopic analysis of receptor subcellular localization was carried out as described previously (Wu et al., 2003). HEK293 cells were grown on coverslips precoated with poly-L-lysine in six-well plates and transfected with 40 ng of GFP-tagged receptors with or without cotransfection with 400 ng of the pcDNA3 vector or the ARF1 mutants. For colocalization of the receptors with the ER marker DsRed2-ER, HEK293 cells were transfected with 100 ng of GFP-tagged receptors and 100 ng of pDsRed2-ER with 400 ng of the pcDNA3 vector or ARF1 mutants. The cells were fixed with 4% paraformaldehyde/4% sucrose mixture in PBS for 15 min and stained with 4,6-diamidino-2-phenylindole for 5 min. For colocalization of the receptors with the ERGIC, Golgi, and TGN markers, HEK293 cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and blocked with 5% normal donkey serum for 1 h. The cells were then incubated with antibodies against ERGIC-53, GM130, or p230 for another 2 h. After washing with PBS (3 $\times$ 5 min), the cells were incubated with Alexa Fluor 594-labeled secondary antibody (1:2000 dilution) for 1 h at room temperature. The coverslips were mounted, and fluorescence was detected with a Leica DMRA2 epifluorescent microscope (Leica Microsystems, Inc., Bannockburn, IL). Images were deconvolved using SlideBook software and the nearest neighbor deconvolution algorithm (Intelligent Imaging Innovations, Denver, CO).

Measurement of ERK1/2 Activation. HEK293 or ghost cells were cultured on six-well plates and transfected with 1.5 μ g of the pcDNA3 vector or ARF1T31N with or without cotransfection with 0.5 μ g of the receptor plasmids. At 6 to 8 h after transfection, the cells were split and cultured for 24 to 30 h. The cells were starved for at least 2 h and then stimulated with agonists as indicated in the figure legends. The stimulation time and drug concentrations used in these experiments will produce the maximal ERK1/2 in response to each agonist (Wu et al., 2003; Duvernay et al., 2004). Stimulation was terminated by addition of 1 $\times$ SDS gel loading buffer as described previously (Wu et al., 2003). After solubilizing the cells, 20 μ l of total cell lysates was separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. ERK1/2 activation was determined by Western blotting measuring the levels of phosphorylation of ERK1/2 with phosphospecific ERK1/2 antibodies. The membranes were then stripped and reprobed with anti-ERK1/2 or anti-ARF1 antibodies to determine the total amount of ERK1/2 and ARF1, respectively. The signal was detected using Enhanced Chemiluminescence Plus (PerkinElmer Life and Analytical Sciences, Waltham, MA) and a Fujifilm (Tokyo, Japan) luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program (version 3.4; Fujifilm).

Coimmunoprecipitation. For coimmunoprecipitation of α_{2B} -AR and ARF1, HEK293 cells cultured on 100-mm dishes were transfected with 2 μ g of HA- or GFP-tagged α_{2B} -AR or the pEGFP-N1 vector together with ARF1 for 36 h. The cells were washed twice with PBS, harvested, and lysed with 500 μ l of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete Mini protease inhibitor mixture (Roche Diagnostics). After gentle rotation for 1 h, samples were centrifuged for 15 min at 14,000g, and the supernatant was incubated with 50 μ l of protein G-Sepharose for 1 h at 4°C to remove nonspecific bound proteins. Samples were then incubated with 1 μ g of anti-HA or anti-GFP antibodies overnight at 4°C with gentle rotation followed by incubation with 50 μ l of protein G-Sepharose 4B beads for 5 h. Resin was collected by centrifugation and washed three times with 500 μ l of lysis buffer without SDS. Immunoprecipitated receptors were eluted with SDS gel loading buffer, separated by 10% SDS-PAGE, and visualized by immunoblotting using anti-HA or anti-GFP antibodies (Dong and Wu, 2006). ARF1 in the immunoprecipitates was detected by using anti-ARF1 antibodies.

GST Fusion Protein Pull-Down Assay. The GST fusion proteins containing the C terminus of α_{2B} -AR were expressed in bacteria and purified using a glutathione affinity matrix as described previously (Wu et al., 1997, 1998). The fusion proteins immobilized on the glutathione resin were either used immediately or stored at 4°C for no longer than 3 days. Each batch of fusion protein used in experiments was first analyzed by Coomassie Blue staining after SDS-PAGE. GST fusion proteins tethered to the glutathione resin were incubated with cell lysate prepared from HEK293 cells transfected with GFP-tagged ARF1 in 500 μ l of binding buffer (20 mM Tris-HCl, pH 7.5, 2% Nonidet P40, and 70 mM NaCl) at 4°C overnight. To determine whether the α_{2B} -AR C terminus could directly interact with ARF1, ARF1 was tagged with the epitope His at its N terminus, and His-ARF1 was purified and eluted by using the His SpinTrap kit from GE Healthcare. One microgram of purified His-ARF1 was incubated with GST- α_{2B} -AR C-terminal fusion protein at 4°C for 2 h. The resin was washed four times with 1 ml of binding buffer, and the retained proteins were solubilized in 1 $\times$ SDS gel loading buffer and separated by SDS-PAGE. Bound ARF1-GFP and His-ARF1 were detected by immunoblotting using anti-GFP and anti-His antibodies, respectively.

Statistical Analysis. Differences were evaluated using Student's *t* test, and *P* < 0.05 was considered statistically significant. Data are expressed as the mean $\pm$ S.E.

Results

Inhibition of the Cell Surface Expression of α_{2B} -AR, β_2 -AR, AT1R, and CXCR4 by BFA Treatment. To investigate the role of ARF GTPases in the anterograde transport of GPCRs, we first determined the effect of BFA treatment on the cell surface expression of four family A GPCRs, including α_{2B} -AR, β_2 -AR, AT1R, and CXCR4. BFA treatment is the well characterized tool for studying the function of ARF GTPases. BFA is a fungal metabolite that inserts at the interface between GDP-ARF and the catalytic Sec7 domain of GEFs, thus preventing the function of GEFs in facilitating the displacement of GDP with GTP. α_{2B} -AR-GFP, β_2 -AR-GFP, AT1R-GFP, or HA-CXCR4 was transiently expressed into HEK293 cells, and their cell surface expression was measured by radioligand binding in intact live cells (GFP-tagged receptors) or flow cytometry after staining with anti-HA antibodies in unpermeabilized cells (HA-tagged receptors). BFA treatment at a concentration of 5 μ g/ml for 8 h dramatically inhibited the cell surface expression of all four receptors by approximately 80% (Fig. 1A).

We then determined the effect of the BFA treatment on the subcellular distribution of the receptors. GFP-tagged α_{2B} -AR, β_2 -AR, and AT1R HA-tagged CXCR4 were transiently expressed in HEK293 cells, and their subcellular localization at steady state was revealed by fluorescence microscopy analysis. As anticipated, each receptor robustly expressed at the cell surface in control cells. BFA treatment arrested the receptors in the perinuclear regions of the cell (Fig. 1B), and the intracellularly accumulated receptors were extensively colocalized with the ER marker DsRed2-ER (data not shown). These data suggest that ARF GTPases may play a crucial role in the anterograde transport from the ER to the cell surface of a group of GPCRs.

Five Human ARF GTPases Differentially Modulate the Cell Surface Transport of α_{2B} -AR. We next asked the question of which ARF GTPases regulate GPCR transport to the cell surface. To address this question, we defined the role of each of the five identified human ARF GTPases in the cell surface transport of α_{2B} -AR. The loss of function of each ARF GTPase was achieved by expressing its GTP-bound (Q mutated to L), GDP-bound (T mutated to N), and guanine nucleotide-deficient (N mutated to I) mutants, which have been shown to function as the dominant negative mutants. Expression of ARF GTPases was determined by immunoblotting (Fig. 2A). Transient expression of each of the three ARF1 mutants markedly attenuated the cell surface expression of α_{2B} -AR as measured by intact cell ligand binding. Expression of ARF3Q71L and ARF3N126I also strongly inhibited α_{2B} -AR transport to the cell surface. In contrast, ARF3T31N, ARF5T31N, and ARF6Q67L mutants only moderately attenuated α_{2B} -AR transport by approximately 25%, whereas all three ARF4 mutants, ARF5Q71L, ARF5T31N, ARF6T27N, and ARF6N122I did not clearly alter the cell surface expression of α_{2B} -AR (Fig. 2B). These data show that five ARF GTPases differentially modulate the cell surface targeting of nascent α_{2B} -AR.

Attenuation of ARF1 Function Inhibits the Cell Surface Expression of α_{2B} -AR, β_2 -AR, AT1R, CXCR4, and M_3 -MR. Our preceding data show that expression of the ARF1 mutants produced the most profound inhibitory effect

Fig. 1. Inhibition of the cell surface expression of α_{2B} -AR, β_2 -AR, AT1R, and CXCR4 by BFA treatment. A, the cell surface expression of the receptors in cells treated with BFA. HEK293 cells were transfected with α_{2B} -AR-GFP, β_2 -AR-GFP, AT1R-GFP, or HA-CXCR4 and treated with BFA at a concentration of 5 μ g/ml for 8 h. The cell surface expression of α_{2B} -AR, β_2 -AR, and AT1R was determined by intact cell ligand binding using [3 H]RX 821002, [3 H]CGP-12177, and [125 I]-Ang II, respectively, as described under *Materials and Methods*. The mean values of specific ligand binding were $22,737 \pm 714$, $17,718 \pm 771$, and $16,754 \pm 782$ ($n = 3$, each in triplicate) from cells transfected with α_{2B} -AR, β_2 -AR, and AT1R, respectively. The cell surface expression of HA-CXCR4 was measured by flow cytometry after staining with anti-HA antibodies in unpermeabilized cells. The data shown are percentages of the mean value obtained from cells transfected with individual receptor and treated with ethanol (control) and are presented as the mean $\pm$ S.E. of three experiments. *, $P < 0.05$ versus control. B, fluorescence microscopy analysis of the subcellular localization of the receptors. HEK293 cells cultured on coverslips were transfected with α_{2B} -AR-GFP, β_2 -AR-GFP, AT1R-GFP, or HA-CXCR4 (40 ng) and treated with BFA as above. The subcellular distribution of GFP-tagged receptors was revealed by detecting the GFP signals, whereas HA-CXCR4 was detected after staining with anti-HA antibodies as described under *Materials and Methods*. Scale bar, 10 μ m.

on α_{2B} -AR expression at the cell surface as measured by intact cell ligand binding. To eliminate the possible effect of the ARF1 mutants on the ligand binding ability of α_{2B} -AR, HA- α_{2B} -AR was expressed together with the ARF1 mutants, and its cell surface expression was measured by flow cytometry. Consistent with the intact cell ligand binding data, the cell surface expression of α_{2B} -AR was dramatically attenuated by the ARF1 mutants (Fig. 3A). In contrast, expression of the ARF1 mutants had no noticeable effects on the total α_{2B} -AR expression as quantified by measuring the GFP signal using flow cytometry (Fig. 3A).

We next determined the effect of expression of the ARF1 mutants on the cell surface expression of β_2 -AR, AT1R, and

Fig. 2. Effect of transient expression of ARF mutants on the cell surface expression of α_{2B} -AR. **A**, Western blot analysis of the expression of wild-type ARF GTPases (WT) and their mutants. HEK293 cells cultured on six-well plates were transfected with the pcDNA3 vector (Ctrl) or individual ARF GTPase. Cell homogenates were separated by SDS-PAGE. The expression of ARF1, ARF3, ARF5, and ARF6 was detected by Western blotting using ARF isoform-specific antibodies and ARF4 by anti-HA antibodies (top). β -Actin expression (bottom) is used as a loading control. **B**, effect of ARF mutants on the cell surface expression of α_{2B} -AR. HEK293 cells were transfected with α_{2B} -AR together with WT ARF GTPases or their mutants. The expression of α_{2B} -AR at the cell surface was determined by intact cell ligand binding using [3 H]RX 821002. The data shown are percentages of the mean value obtained from cells transfected with α_{2B} -AR and individual WT ARF and are presented as the mean $\pm$ S.E. of three experiments. *, $P < 0.05$ versus the cells transfected with respective WT ARF.

CXCR4. Similar to α_{2B} -AR, the cell surface expression of β_2 -AR, AT1R, and CXCR4 was markedly inhibited by each of the three ARF1 mutants by 60 to 80% (Fig. 3B). These data strongly show that ARF1 is essential for the cell surface transport of α_{2B} -AR, β_2 -AR, AT1R, and CXCR4.

It was reported that transient expression of ARF1T31N did not significantly influence the plasma membrane expression of M_3 -MR in 1321N1 human astrocytoma cells (Mitchell et al., 2003). To determine whether ARF1 could selectively modulate the transport of distinct GPCRs, we measured the effect of the three ARF1 mutants on the cell surface expression of M_3 -MR in HEK293 cells using the same system as for α_{2B} -AR, β_2 -AR, AT1R, and CXCR4. Our data show that transient expression of each of the three ARF1 mutants markedly inhibited the cell surface expression of M_3 -MR as measured by intact cell ligand binding (Fig. 3B).

Fig. 3. Effect of ARF1 mutants on the cell surface expression of α_{2B} -AR, β_2 -AR, AT1R, CXCR4, and M_3 -MR. **A**, effect of ARF1 mutants on the cell surface and total expression of α_{2B} -AR measured by flow cytometry. For the cell surface expression, HEK293 cells were transfected with HA- α_{2B} -AR together with the pcDNA3 vector (control) or ARF1 mutants. The expression of α_{2B} -AR at the cell surface was determined by flow cytometry after staining with anti-HA antibodies in unpermeabilized cells. For the total receptor expression, the cells were transfected with GFP- α_{2B} -AR and the ARF1 mutants, and the total receptor expression was determined by measuring the GFP fluorescence. **B**, effect of the ARF1 mutants on the cell surface expression of β_2 -AR, AT1R, CXCR4, and M_3 -MR. HEK293 cells were transfected with individual receptor and ARF1 mutants, and the cell surface expression of β_2 -AR, AT1R, and CXCR4 was measured as described in the legend of Fig. 1. M_3 -MR expression at the cell surface was measured by intact cell ligand binding using the ligand [3 H]NMS as described under *Materials and Methods*. The mean value of specific [3 H]NMS binding was $22,447 \pm 1342$ cpm ($n = 3$, each in triplicate) from cells transfected with M_3 -MR alone. **C**, effect of the expression of cytohesin-2 and ARFGAP1 on the cell surface expression of α_{2B} -AR. The data shown are percentages of the mean value obtained from cells transfected with the pcDNA3 vector and are presented as the mean $\pm$ S.E. of three experiments. *, $P < 0.05$ versus control.

We then determined the effect of transient expression of cytohesin-2 and ARF GTPase-activating protein 1 (ARFGAP1), which facilitates the exchange of GDP for GTP and the GTP hydrolysis of ARF1, respectively, on the cell surface transport of α_{2B} -AR. Expression of cytohesin-2 did not alter α_{2B} -AR cell surface expression, whereas expression of ARFGAP1 significantly inhibited by 22% (Fig. 3C). These data suggest that the GTP hydrolysis of ARF1 may play an important role in α_{2B} -AR transport.

ARF1T31N and ARF1Q71L Arrest the Receptors in Different Intracellular Compartments. To further characterize how ARF1 regulates GPCR anterograde transport, we visualized the subcellular distribution of the receptors in cells expressing the GDP-bound ARF1T31N or GTP-bound ARF1Q71L mutants. Consistent with the marked reduction

in the cell surface expression as measured by radioligand binding and flow cytometry, GFP-tagged α_{2B} -AR, β_2 -AR, and AT1R were unable to transport to the cell surface in cells transfected with ARF1T31N or ARF1Q71L. Interestingly, these two ARF1 mutants clearly arrested the receptors in distinct intracellular compartments (Fig. 4). Similar effects were obtained for CXCR4 (data not shown). These data suggest that inhibition of the ARF1 function by expressing its GDP- and GTP-bound mutants may selectively block receptor transport at different intracellular compartments along the biosynthetic pathway.

Subcellular colocalization of the intracellularly accumulated α_{2B} -AR with different organelle markers revealed that α_{2B} -AR was strongly colocalized with the ER marker DsRed2-ER in cells expressing ARF1T31N (Fig. 5A), whereas the receptor was partially colocalized with the ERGIC marker ERGIC-53, the Golgi marker GM130, and the TGN marker p230 in cells expressing ARF1Q71L (Fig. 5B). Similar colocalization patterns were obtained for β_2 -AR, AT1R, and CXCR4 (data not shown). These data suggest that, similar to the BFA treatment, expression of the GDP-bound ARF1T31N mutant blocks receptor transport from the ER, whereas the GTP-bound ARF1Q71L mutant inhibits receptor export at the ERGIC, the Golgi, and the TGN (Fig. 5C).

Expression of ARF1T31N Blocks ERK1/2 Activation by α_{2B} -AR, β_2 -AR, AT1R, and CXCR4. To evaluate whether the attenuated receptor cell surface expression caused by expression of the ARF1 mutants could result in a concomitant defective signaling, we measured the activation of the mitogen-activated protein kinase pathway in response to stimulation with receptor agonists in cells expressing ARF1T31N. Stimulation with the agonists UK-14,304 (Fig. 6A), ISO (Fig. 6B), Ang II (Fig. 6C), and SDF-1 α (Fig. 6D and Supplemental Fig. 1) strongly activated ERK1/2 in cells expressing α_{2B} -AR, β_2 -AR, AT1R, and CXCR4, respectively. ERK1/2 activation by each agonist was significantly compromised in cells coexpressing ARF1T31N and individual receptor compared with cells expressing receptor alone (Fig. 6, A–E). These data are consistent

Fig. 4. The subcellular distribution of α_{2B} -AR, β_2 -AR, and AT1R in cells expressing ARF1T31N and ARF1Q71L. HEK293 cells cultured on coverslips were transfected with GFP-conjugated α_{2B} -AR, β_2 -AR, or AT1R (40 ng) together with the pcDNA3 vector (control), ARF1T31N, or ARF1Q71L (400 ng). The subcellular distribution of the receptors was revealed by detecting the GFP fluorescence as described under *Materials and Methods*. The data are representative images of at least five independent experiments. Scale bar, 10 μ m.

Fig. 5. Colocalization of α_{2B} -AR with different organelle markers in cells expressing ARF1T31N and ARF1Q71L. A, colocalization of α_{2B} -AR with the ER marker DsRed2-ER in cells expressing ARF1T31N. HEK293 cells were transfected with α_{2B} -AR-GFP (40 ng) together with pDsRed2-ER (40 ng) and ARF1T31N (400 ng). The subcellular distribution and colocalization with DsRed2-ER of α_{2B} -AR were revealed by fluorescence microscopy. B, colocalization of α_{2B} -AR with the ERGIC, Golgi, and TGN markers in cells expressing ARF1Q71L. HEK293 cells were transfected with α_{2B} -AR-GFP together with ARF1Q71L (400 ng) and then stained with antibodies against the ERGIC marker ERGIC-53, the Golgi marker GM130, or the TGN marker p230 as described under *Materials and Methods*. The data are representative images of four independent experiments. Green, α_{2B} -AR-GFP; red, DsRed2-ER (A), ERGIC-53 (B, top), GM130 (B, middle), and p230 (B, bottom); blue, DNA staining by 4,6-diamidino-2-phenylindole (nucleus); yellow, colocalization of the receptor and different organelle markers. Scale bars, 10 μ m. C, a diagram summarizing the effects of BFA and the ARF1 mutants on the transport of the receptors from the ER, through the ERGIC, the Golgi, and the TGN, to the cell surface. PM, plasma membrane.

with the inhibitory effect of ARF1T31N on the cell surface expression of the receptors.

Expression of ARF1T31N Attenuates the Cell Surface Expression and Signaling of Endogenous α_{2B} -AR and CXCR4. To further explore the physiological significance of ARF1 regulation of GPCR trafficking, we determined the effect of ARF1T31N on the cell surface expression of endogenous α_{2B} -AR in NG108-15 cells and endogenous CXCR4 in HEK293 and ghost cells. Expression of ARF1T31N moderately but consistently reduced the cell surface expression of endogenous α_{2B} -AR by 25% in NG108-15 cells as measured by intact cell ligand binding, whereas it markedly reduced the cell surface transport of endogenous CXCR4 by 70 and 44% in HEK293 and ghost cells, respectively (Fig. 7A) as measured by flow cytometry after staining with anti-CXCR4 antibodies. Consistent with the reduction in the

Fig. 6. Effect of transient expression of ARF1T31N on ERK1/2 activation by α_{2B} -AR, β_2 -AR, AT1R, and CXCR4. HEK293 cells were transfected with α_{2B} -AR (A), β_2 -AR (B), AT1R (C), or CXCR4 (D) together with pcDNA3 (control) or ARF1T31N and stimulated with UK-14,304 (1 μ M for 5 min) (A), ISO (1 μ M for 5 min) (B), Ang II (1 μ M for 2 min), or SDF-1 α (50 nM for 5 min) at 37°C. ERK1/2 activation was determined by Western blot analysis using phosphospecific ERK1/2 antibodies (pERK1/2). A through D, representative blots of ERK1/2 activation (top), total ERK1/2 expression (middle), and ARF1 expression (bottom); E, quantitative data expressed as percentages of ERK1/2 activation obtained from cells transfected with individual receptors and the pcDNA3 vector and presented as the mean $\pm$ S.E. of three experiments. *, $P < 0.05$ versus control.

CXCR4 cell surface expression, ERK1/2 activation by SDF-1 α was clearly reduced in both HEK293 and ghost cells expressing ARF1T31N (Fig. 7B). These data indicate that ARF1 modulates the cell surface expression of endogenous α_{2B} -AR and CXCR4.

Interaction of α_{2B} -AR with ARF1. To elucidate the possible molecular mechanism underlying the function of ARF1 in regulating GPCR transport, we determined whether α_{2B} -AR is able to physically associate with ARF1 in vivo. α_{2B} -AR tagged with HA or GFP was transiently expressed in HEK293 cells, and the interaction between α_{2B} -AR and ARF1 was measured by coimmunoprecipitation with anti-HA or anti-GFP antibodies. A significant amount of ARF1 was found in the anti-HA and anti-GFP immunoprecipitates (Fig. 8), suggesting that α_{2B} -AR may associate with ARF1.

We then sought to identify the ARF1-binding domain in α_{2B} -AR by focusing on the C terminus as the C termini have been shown to modulate the ER-to-cell surface transport for many GPCRs, including α_{2B} -AR (Duvernay et al., 2004, 2009b). The C terminus was generated as a GST fusion protein (Fig. 9, A and B), and its interaction with ARF1 was determined in the GST fusion protein pull-down assay. The

Fig. 7. Effect of ARF1T31N on the cell surface expression and signaling of endogenous α_{2B} -AR and CXCR4. A, effect of ARF1T31N on the cell surface expression of endogenous α_{2B} -AR in NG108 cells and endogenous CXCR4 in HEK293 and ghost cells. The cells were transfected with pcDNA3 vector (control) or ARF1T31N (2 μ g) for 48 h. The cell surface expression of endogenous α_{2B} -AR and CXCR4 was measured by intact cell ligand binding using [3 H]RX 821002 and flow cytometry after staining with CXCR4 antibodies, respectively, as described under *Materials and Methods*. The data shown are percentages of the mean value obtained from cells transfected with the pcDNA3 vector and are presented as the mean $\pm$ S.E. of three experiments. *, $P < 0.05$ versus respective control. B, effect of ARF1T31N on ERK1/2 activation by endogenous CXCR4. HEK293 and ghost cells transfected with pcDNA3 or ARF1T31N were stimulated with SDF-1 α (50 nM for 5 min) at 37°C. ERK1/2 activation was determined by Western blot analysis using phospho-specific ERK1/2 antibodies. Top, ERK1/2 activation; middle, total ERK1/2 expression; and bottom, ARF1 expression. Similar results were obtained in at least three separate experiments.

Fig. 8. Association of α_{2B} -AR with ARF1. α_{2B} -AR tagged with HA at its N terminus or GFP at its C terminus was transiently expressed together with ARF1 in HEK293 cells and immunoprecipitated with anti-HA or anti-GFP antibodies. The cells transfected with the pEGFP-N1 vector were used as a negative control. ARF1, HA- α_{2B} -AR, and α_{2B} -AR-GFP in the immunoprecipitates were detected by immunoblotting using anti-ARF1 (top), anti-GFP (middle), and anti-HA (bottom) antibodies, respectively. Molecular mass is indicated at the left. Similar results were obtained in at least four separate experiments.

GST fusion protein encoding the C terminus of α_{2B} -AR, but not GST alone, was able to interact with ARF1 (Fig. 9C).

To determine whether the interaction between the α_{2B} -AR

Fig. 9. Interaction between the α_{2B} -AR C terminus and ARF1. **A**, the sequence of the α_{2B} -AR C terminus. **B**, the purified GST and GST fusion proteins encoding the C terminus of α_{2B} -AR (GST-CT) stained with Coomassie Blue. Molecular mass is indicated at the left. **C**, interaction of GST- α_{2B} -AR C terminus with ARF1 measured by GST fusion protein pull-down assay. GFP-tagged ARF1 was expressed in HEK293 cells, and total cell homogenates were incubated with the GST and GST- α_{2B} -AR C-terminal fusion proteins as described under *Materials and Methods*. Bound ARF1-GFP was detected by immunoblotting using anti-GFP antibodies. Similar results were obtained in five separate experiments. **D**, direct interaction between the α_{2B} -AR C terminus and ARF1. ARF1 was tagged with six His at its N terminus, and purified His-ARF1 was incubated with GST- α_{2B} -AR C-terminal fusion protein as described under *Materials and Methods*. Bound His-ARF1 was detected by immunoblotting using anti-His antibodies. This experiment was repeated three times with similar results.

C terminus and ARF1 is direct or indirect, ARF1 was generated as His-tagged fusion proteins, and the purified His-ARF1 was also able to interact with GST- α_{2B} -AR C terminus fusion protein (Fig. 9D). These data show that the small GTPase ARF1 directly interacts with the α_{2B} -AR C terminus.

Discussion

As a continuous effort to elucidate the molecular mechanism underlying the export trafficking of GPCRs, we determined the function of the small GTPase ARFs in regulating cell surface expression, subcellular distribution, and signaling of GPCRs. The results presented here have shown for the first time that, of the five ARFs identified in humans, ARF1 plays a crucial role in the anterograde transport of a group of the family A GPCRs. Transient expression of three different ARF1 mutants dramatically inhibited the cell surface transport of α_{2B} -AR, β_2 -AR, AT1R, and CXCR4. Consistent with the remarked reduction in the cell surface expression, the

receptors were expressed intracellularly, and receptor-mediated signaling measured as ERK1/2 activation was significantly attenuated in cells transfected with the ARF1 mutants. An important role for ARF1 in receptor export trafficking was further supported by a reduction of α_{2B} -AR cell surface expression in cells expressing ARFGAP1, which facilitates the GTP hydrolysis of ARF1 (Lee et al., 2005). These data strongly indicate that the cell surface transport of α_{2B} -AR, β_2 -AR, AT1R, and CXCR4 uses an ARF1-dependent pathway.

Interestingly, expression of the GDP-bound mutant ARF1T31N strongly arrested the receptors in the ER, whereas the GTP-bound mutant ARF1Q71L inhibited receptor transport from the ERGIC, the Golgi, and the TGN. These data are consistent with other studies showing that expression of different ARF1 mutants blocked protein transport at distinct intracellular compartments (Dascher and Balch, 1994; Ward et al., 2001). ARF1 GTPase has been shown to modulate the assembly and budding of the COPI-coated transport vesicles that are involved in anterograde transport from the ERGIC to the Golgi and retrograde transport from the Golgi to the ER (Stearns et al., 1990; Balch et al., 1992; D'Souza-Schorey and Chavrier, 2006). Expression of the GDP-bound mutant ARF1T31N would induce malfunction of the COPI-mediated retrograde transport system and disrupt the recycling of components of transport machinery required for anterograde transport from the ER. Therefore, expression of ARF1T31N results in the receptor accumulation in the ER. Similar to the expression of ARF1T31N, the treatment with BFA, which blocks the function of GEFs and arrests ARFs in the GDP-bound state, induced an ER accumulation of the receptors. On the contrary, expression of the GTP-bound ARF1Q71L mutant would disrupt the release of the COPI-coated vesicles from the ERGIC and the Golgi, leading to receptor accumulation in these compartments.

The molecular mechanism underlying the transport of GPCRs from the TGN to the plasma membrane remains poorly defined, and the vesicles that mediate the post-Golgi transport of GPCRs have not been identified. The fact that the expression of ARF1Q71L arrested receptors in the TGN suggests that, in addition to modulating receptor traffic at the ERGIC and the Golgi, ARF1 is also involved in the regulation of the post-Golgi transport of the receptors. It has been shown that ARF1 plays an important role in the formation of the TGN-derived clathrin-coated transport vesicles, which are mainly responsible for the transport of lysosomal enzymes between the TGN and the endosomal compartment (Puertollano et al., 2001; Misra et al., 2002; Bonifacio, 2004). Furthermore, a recent study has shown that ARF1 modulates the cytoplasmic protein complex termed exomer, which is important for the TGN-to-plasma membrane transport in yeast (Wang et al., 2006). In addition, ARF1 has been shown to regulate the Golgi-to-plasma membrane transport of internalized protease-activated receptor-2 (Luo et al., 2007). It is also interesting to note that ARF1 has been shown to directly interact with GPCRs (Mitchell et al., 1998), which may contribute to the mechanism of receptor transport regulation by ARF1. Our current study shows that ARF1 modulates multiple transport steps involved in the biosynthetic pathway, including export from the TGN, of a group of the family A GPCRs. We have also shown that ARF1 is able to physically associate with α_{2B} -AR, and the interaction is me-

diated through the C terminus of the receptor. The interaction between α_{2B} -AR and ARF1 appears to be direct as two purified fusion proteins containing the α_{2B} -AR C terminus and ARF1 (i.e., GST-C terminus and His-ARF1) are able to interact, forming a complex. These data imply that the function of ARF1 in coordinating GPCR transport may be mediated through its direct interaction with the intracellular domains of the receptors.

The current studies, together with previous reports, have shown that the regulation of cell surface targeting of nascent GPCRs by ARF GTPases appears complicated. ARF1 is involved in the cell surface transport of all the GPCRs, including α_{2B} -AR, β_2 -AR, AT1R, CXCR4, and M₃-MR, as examined in this study. Inhibition of M₃-MR cell surface expression by three ARF1 mutants is in contrast to a previous study showing that ARF1T31N did not alter the plasma membrane numbers of M₃-MR (Mitchell et al., 2003). The differential effects of the ARF1 mutants on M₃-MR cell surface transport could be a result of the different systems (e.g., different cell types) used in these two studies. Similar to ARF1, ARF3 also clearly modulates α_{2B} -AR cell surface expression, as expression of each of the three ARF3 mutants significantly attenuated the α_{2B} -AR numbers at the cell surface. Strong inhibition of α_{2B} -AR transport by the ARF1 and ARF3 mutants supports the notion that ARF1 and ARF3 may have overlapping functions (Volpicelli-Daley et al., 2005). It has been shown that ARF4 modulates the post-Golgi traffic of rhodopsin, which is mediated via a direct interaction between ARF4 and the C terminus of the receptor (Deretic et al., 2005). However, expression of any of the three ARF4 mutants did not influence α_{2B} -AR expression at the cell surface, whereas expression of ARF5N126I moderately inhibited. These data show that ARF5, but not ARF4, may play a role in the α_{2B} -AR biosynthesis. These data also implicate that the class II ARF GTPases, ARF4 and ARF5, may selectively modulate the cell surface transport of distinct GPCRs. Furthermore, ARF6 has been showed to modulate the cell surface expression of vasopressin V2 receptor (Madziva and Birnbaumer, 2006). Moderate inhibition of α_{2B} -AR transport by ARF6Q67L suggests that ARF6 may also regulate α_{2B} -AR cell surface transport. However, the reason for differential effects of the GTP-bound, the GDP-bound, and the guanidine nucleotide-deficient mutants of ARF3, ARF5, and ARF6 on the α_{2B} -AR transport remains unknown. Nevertheless, these studies show that different ARF GTPases may differentially modulate the transport of distinct GPCRs. As our previous studies have shown that Rab1 GTPase selectively modulates the cell surface transport of GPCRs (Wu et al., 2003; Filipeanu et al., 2006), it will be interesting to study the effect of ARF-Rab1 combination on the export trafficking of distinct GPCRs.

α_{2B} -AR, β_2 -AR, AT1R, and CXCR4 couple to different heterotrimeric G proteins and regulate distinct signaling pathways. Expression of the ARF1T31N mutant significantly attenuated ERK1/2 activation by the receptor agonists, paralleling its inhibitory effects on the cell surface transport of the receptors. Therefore, the attenuation of receptor-mediated ERK1/2 activation is caused, at least in part, by less receptor transport to the cell surface in the ARF1T31N-transfected cells. However, we cannot exclude the possibility that ARF1T31N expression may also influence the intracellular trafficking and function of other signaling molecules that are involved in regulation of receptor-mediated ERK1/2

activation. It is also possible that, in addition to its well established function in regulating vesicle-mediated transport, ARF1 may function as a signaling entity directly involved in the activation of the ERK1/2 pathway. Consistent with this possibility, ARF1 has been shown to be involved in receptor-mediated phospholipase D activation (Brown et al., 1993; Cockcroft et al., 1994; Massenburg et al., 1994).

In contrast to extensive investigation on the endocytic process of GPCRs, the studies on the export trafficking of GPCRs from the ER through the Golgi to the cell surface have just begun (Dong et al., 2007; Achour et al., 2008; Ge et al., 2009). Recent studies on the functional roles of Ras-like small GTPases, particularly the Rab subfamily, in regulating GPCR expression at the cell surface and identification of highly conserved export motifs, which dictate receptor exit at distinct intracellular organelles, have greatly advanced our understanding of the cell surface targeting of newly synthesized GPCRs and have also shown that receptor export trafficking plays a crucial role in regulating receptor function and in the development of a number of diseases (Duvernay et al., 2004, 2009a; Dong et al., 2007). To further explore the regulatory mechanisms of the ER-to-cell surface transport of GPCRs may provide an important foundation for developing new therapeutic strategies in treating diseases involving abnormal trafficking and signaling of the receptors.

Acknowledgments

We thank Drs. Stephen M. Lanier, John D. Hildebrandt, Kenneth E. Bernstein, Jürgen Zetzula, and Victor W. Hsu for sharing reagents.

References

- Achour L, Labbé-Jullié C, Scott MG, and Marullo S (2008) An escort for GPCRs: implications for regulation of receptor density at the cell surface. *Trends Pharmacol Sci* **29**:528–535.
- Balch WE, Kahn RA, and Schwaninger R (1992) ADP-ribosylation factor is required for vesicular trafficking between the endoplasmic reticulum and the cis-Golgi compartment. *J Biol Chem* **267**:13053–13061.
- Bonifacino JS (2004) The GGA proteins: adaptors on the move. *Nat Rev Mol Cell Biol* **5**:23–32.
- Brown HA, Gutowski S, Moomaw CR, Slaughter C, and Sternweis PC (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* **75**:1137–1144.
- Cockcroft S, Thomas GM, Fensome A, Geny B, Cunningham E, Gout I, Hiles I, Totty NF, Truong O, and Hsuan JJ (1994) Phospholipase D: a downstream effector of ARF in granulocytes. *Science* **263**:523–526.
- D'Souza-Schorey C and Chavrier P (2006) ARF proteins: roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol* **7**:347–358.
- Dascher C and Balch WE (1994) Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus. *J Biol Chem* **269**:1437–1448.
- Deretic D, Williams AH, Ransom N, Morel V, Hargrave PA, and Arendt A (2005) Rhodopsin C terminus, the site of mutations causing retinal disease, regulates trafficking by binding to ADP-ribosylation factor 4 (ARF4). *Proc Natl Acad Sci U S A* **102**:3301–3306.
- Dong C, Filipeanu CM, Duvernay MT, and Wu G (2007) Regulation of G protein-coupled receptor export trafficking. *Biochim Biophys Acta* **1768**:853–870.
- Dong C and Wu G (2006) Regulation of anterograde transport of alpha2-adrenergic receptors by the N termini at multiple intracellular compartments. *J Biol Chem* **281**:38543–38554.
- Dong C and Wu G (2007) Regulation of anterograde transport of adrenergic and angiotensin II receptors by Rab2 and Rab6 GTPases. *Cell Signal* **19**:2388–2399.
- Dong C, Zhou F, Fugetta EK, Filipeanu CM, and Wu G (2008) Endoplasmic reticulum export of adrenergic and angiotensin II receptors is differentially regulated by Sar1 GTPase. *Cell Signal* **20**:1035–1043.
- Duvernay MT, Dong C, Zhang X, Robitaille M, Hebert TE, and Wu G (2009a) A single conserved leucine residue on the first intracellular loop regulates ER export of G protein-coupled receptors. *Traffic* **10**:552–566.
- Duvernay MT, Dong C, Zhang X, Zhou F, Nichols CD, and Wu G (2009b) Anterograde trafficking of G protein-coupled receptors: function of the C-terminal F(X)6LL motif in export from the endoplasmic reticulum. *Mol Pharmacol* **75**:751–761.
- Duvernay MT, Zhou F, and Wu G (2004) A conserved motif for the transport of G protein-coupled receptors from the endoplasmic reticulum to the cell surface. *J Biol Chem* **279**:30741–30750.
- Filipeanu CM, Zhou F, Claycomb WC, and Wu G (2004) Regulation of the cell surface expression and function of angiotensin II type 1 receptor by Rab1-mediated endoplasmic reticulum-to-Golgi transport in cardiac myocytes. *J Biol Chem* **279**:41077–41084.

- Filipeanu CM, Zhou F, Fugetta EK, and Wu G (2006) Differential regulation of the cell-surface targeting and function of beta- and alpha1-adrenergic receptors by Rab1 GTPase in cardiac myocytes. *Mol Pharmacol* **69**:1571–1578.
- Ge X, Loh HH, and Law PY (2009) μ -Opioid receptor cell surface expression is regulated by its direct interaction with Ribophorin I. *Mol Pharmacol* **75**:1307–1316.
- Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, and Benovic JL (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383**:447–450.
- Gsandtner I, Charalambous C, Stefan E, Ogris E, Freissmuth M, and Zetzl J (2005) Heterotrimeric G protein-independent signaling of a G protein-coupled receptor. Direct binding of ARNO/cytohesin-2 to the carboxyl terminus of the A2A adenosine receptor is necessary for sustained activation of the ERK/MAP kinase pathway. *J Biol Chem* **280**:31898–31905.
- Lee SY, Yang JS, Hong W, Premont RT, and Hsu VW (2005) ARFGAP1 plays a central role in coupling COPI cargo sorting with vesicle formation. *J Cell Biol* **168**:281–290.
- Luo W, Wang Y, and Reiser G (2007) p24A, a type I transmembrane protein, controls ARF1-dependent resensitization of protease-activated receptor-2 by influence on receptor trafficking. *J Biol Chem* **282**:30246–30255.
- Madziva MT and Birnbaumer M (2006) A role for ADP-ribosylation factor 6 in the processing of G-protein-coupled receptors. *J Biol Chem* **281**:12178–12186.
- Massenburg D, Han JS, Liyanage M, Patton WA, Rhee SG, Moss J, and Vaughan M (1994) Activation of rat brain phospholipase D by ADP-ribosylation factors 1, 5, and 6: separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. *Proc Natl Acad Sci U S A* **91**:11718–11722.
- Misra S, Puertollano R, Kato Y, Bonifacino JS, and Hurley JH (2002) Structural basis for acidic-cluster-dileucine sorting-signal recognition by VHS domains. *Nature* **415**:933–937.
- Mitchell R, McCulloch D, Lutz E, Johnson M, MacKenzie C, Fennell M, Fink G, Zhou W, and Sealfon SC (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature* **392**:411–414.
- Mitchell R, Robertson DN, Holland PJ, Collins D, Lutz EM, and Johnson MS (2003) ADP-ribosylation factor-dependent phospholipase D activation by the M3 muscarinic receptor. *J Biol Chem* **278**:33818–33830.
- Palacios F, Price L, Schweitzer J, Collard JG, and D'Souza-Schorey C (2001) An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J* **20**:4973–4986.
- Pierce KL, Premont RT, and Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**:639–650.
- Puertollano R, Randazzo PA, Presley JF, Hartnell LM, and Bonifacino JS (2001) The GGAs promote ARF-dependent recruitment of clathrin to the TGN. *Cell* **105**:93–102.
- Puthenveedu MA and von Zastrow M (2006) Cargo regulates clathrin-coated pit dynamics. *Cell* **127**:113–124.
- Spang A (2002) ARF1 regulatory factors and COPI vesicle formation. *Curr Opin Cell Biol* **14**:423–427.
- Stearns T, Willingham MC, Botstein D, and Kahn RA (1990) ADP-ribosylation factor is functionally and physically associated with the Golgi complex. *Proc Natl Acad Sci U S A* **87**:1238–1242.
- Volpicelli-Daley LA, Li Y, Zhang CJ, and Kahn RA (2005) Isoform-selective effects of the depletion of ADP-ribosylation factors 1–5 on membrane traffic. *Mol Biol Cell* **16**:4495–4508.
- Wang CW, Hamamoto S, Orci L, and Schekman R (2006) Exomer: a coat complex for transport of select membrane proteins from the trans-Golgi network to the plasma membrane in yeast. *J Cell Biol* **174**:973–983.
- Ward TH, Polishchuk RS, Caplan S, Hirschberg K, and Lippincott-Schwartz J (2001) Maintenance of Golgi structure and function depends on the integrity of ER export. *J Cell Biol* **155**:557–570.
- Wu G, Benovic JL, Hildebrandt JD, and Lanier SM (1998) Receptor docking sites for G-protein betagamma subunits. Implications for signal regulation. *J Biol Chem* **273**:7197–7200.
- Wu G, Krupnick JG, Benovic JL, and Lanier SM (1997) Interaction of arrestins with intracellular domains of muscarinic and alpha2-adrenergic receptors. *J Biol Chem* **272**:17836–17842.
- Wu G, Zhao G, and He Y (2003) Distinct pathways for the trafficking of angiotensin II and adrenergic receptors from the endoplasmic reticulum to the cell surface: Rab1-independent transport of a G protein-coupled receptor. *J Biol Chem* **278**:47062–47069.
- Zhang X, Wang G, Dupré DJ, Feng Y, Robitaille M, Lazartigues E, Feng YH, Hébert TE, and Wu G (2009) Rab1 GTPase and dimerization in the cell surface expression of angiotensin II type 2 receptor. *J Pharmacol Exp Ther* **330**:109–117.

Address correspondence to: Guangyu Wu, Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112. E-mail: gwu@lsuhsc.edu