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*

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The molecular mechanism underlying the transport of G protein-coupled receptors from the endoplasmic reticulum (ER) to the cell surface is poorly understood. This issue was addressed by determining the role of Rab1, a Ras-related small GTPase that coordinates vesicular protein transport in the early secretory pathway, in the subcellular distribution and function of the angiotensin II type 1A receptor (AT1R), β2-adrenergic receptor (AR), and α2αR-AR in HEK293T cells. Inhibition of endogenous Rab1 function by transient expression of dominant-negative Rab1 mutants or Rab1 small interfering RNA (siRNA) induced a marked perinuclear accumulation and a significant reduction in cell-surface expression of AT1R and β2-AR. The accumulated receptors were colocalized with calreacin (an ER marker) and GM130 (a Golgi marker), consistent with Rab1 function in regulating protein transport from the ER to the Golgi. In contrast, dominant-negative Rab1 mutants and siRNA had no effect on the subcellular distribution of α2αR-AR. Similarly, expression of dominant-negative Rab1 mutants and siRNA depletion of Rab1 significantly attenuated AT1R-mediated inositol phosphate accumulation and ERK1/2 activation and β2-AR-mediated ERK1/2 activation, but not α2αR-AR-stimulated ERK1/2 activation. These data indicate that Rab1 GTPase selectively regulates intracellular trafficking and signaling of G protein-coupled receptors and suggest a novel, as yet undefined pathway for movement of G protein-coupled receptors from the ER to the cell surface.

Rab proteins are Ras-like small GTPases that regulate vesicular protein transport in endocytosis and exocytosis (1, 2). Since the first Rab gene (YPT1) was reported by Gullwitz et al. (3) in 1983 in Saccharomyces cerevisiae, 11 Rab family members in yeast and 63 Rab GTPases in mammalian cells have been identified. Although most of the Rab GTPases identified are ubiquitously and highly conserved in their structure and function, each Rab GTPase has a distinct intracellular localization and regulates discrete protein transport steps in secretory and endocytic pathways (1, 2, 4).

Rab1 is one of the most extensively studied and best understood Rab GTPases involved in the regulation of vesicular protein transport between intracellular organelles (5–16). Rab1 has two isoforms, Rab1a and Rab1b, with 92% identity and undistinguishable function demonstrated so far. Rab1 is localized in the endoplasmic reticulum (ER) and Golgi apparatus and regulates antegrade transport specifically from the ER to the Golgi and between the Golgi compartments of proteins, including vesicular stomatitis virus glycoprotein (5–12), the low density lipoprotein receptor (13), β-amyloid precursor protein (14), and the cystic fibrosis transmembrane conductance regulator (CFTR) (15). Interestingly, Rab1 regulates CFTR transport to the cell surface in a cell type-specific manner. CFTR transport to the cell surface is independent of Rab1 in baby hamster kidney and Chinese hamster ovary cells, but is dependent on Rab1 in HeLa and HEK293T cells (15). In addition, a dominant-negative Rab1 mutant (N124I) blocks the processing of rhodopsin from the immature 40-kDa form to the mature 35-kDa form in Drosophila (16). However, whether or not Rab1 could regulate cellular trafficking and activation of the superfamily of cell-surface receptors coupled to heterotrimeric G proteins in mammalian cells remains unknown.

Intracellular trafficking of G protein-coupled receptors is a highly regulated and dynamic process. G protein-coupled receptors are synthesized and modified in the ER, transported to the Golgi apparatus for additional post-translational modifications (e.g. glycosylation), and then further transported to the plasma membrane. The receptors at the plasma membrane may undergo internalization upon stimulation with agonists. The internalized receptors are then transported to the lysosomes for degradation or recycled back to the plasma membrane. Because most studies on receptor trafficking have focused on the events involved in receptor internalization, recycling, and degradation (17, 18), transport processes of G protein-coupled receptors from the ER through the Golgi to the cell surface itself and regulation of receptor signaling by these processes are poorly understood.

As an initial approach to these issues, we determined the role of Rab1 GTPase in intracellular trafficking and signal transduction of the angiotensin II type 1A receptor (AT1R), β2-adrenergic receptor (AR), and α2αR-AR. Our data indicate that Rab1 GTPase selectively regulates the intracellular traf-
ficking and signaling of G protein-coupled receptors and suggest a novel, as yet undefined pathway for movement of G protein-coupled receptors from the ER to the cell surface.

EXPERIMENTAL PROCEDURES

Materials—Rat AT1R in vector pCMDS, human β2-AR in vector pBC, and rat α2C-AR in vector pDNA3 were kindly provided by Dr. Kenneth E. Bernstein (Department of Pathology, Emory University, Atlanta, GA), Dr. John D. Hildebrandt (Department of Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC), and Dr. Stephen M. Lanier (Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center). Isoproterenol, UK14304, and anti-FLAG monoclonal antibody M2 were obtained from Sigma-Genzyme. Polyclonal antibody recognizing Rab1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GM130 antibodies were from Transduction Laboratories (San Diego, CA). High affinity fluorescein-conjugated anti-HA antibody 3F10 was from Roche Applied Science (Mannheim, Germany). Anti-ERK antibodies detecting total ERK2 expression were kindly provided by Dr. Andrew D. Catling (Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center). Issoproterenol, UK14304, and anti-FLAG monoclonal antibody M2 were obtained from Sigma. Fluorescently labeled secondary antibodies (Alexa Fluor 594-labeled anti-mouse and anti-rabbit), tetramethylrhodamine-conjugated cadaverin (A28000, ConA), and 4,6-diamidino-2-phenylindole were obtained from Molecular Probes, Inc. (Eugene, OR). Human angiotensin II (Ang II) was purchased from Calbiochem. Periplasmic [35S]sulfate was purchased from PerkinElmer Life Sciences. Dowex AG 1-8X was from Bio-Rad. All other materials were obtained as described elsewhere (19–22).

Plasmid Constructions—For generation of AT1R tagged with green fluorescent protein at its carboxyl terminus (AT1R-GFP), full-length AT1R in pCMDS was amplified by PCR (forward primer, 5'-GATCAGAGTGTCTGCTGATACTTACACACATTGATGGTCGACACCTACCTCAGAACTACACAG-3', in which the AT1R stop codon was mutated, and AT1R in-frame with GFP, restricted with HindIII/Sall, and ligated into the pEGFP-N1 vector (Invitrogen). A similar strategy was employed to construct β2-AR-GFP (forward primer, 5'-GATCAGAGTGTCTGCTGATACTTACACACATTGATGGTCGACACCTACCTCAGAACTACACAG-3'; and reverse primer, 5'-GATCAGAGTGTCTGCTGATACTTACACACATTGATGGTCGACACCTACCTCAGAACTACACAG-3'). The amino termini of AT1R (HA-AT1R) and β2-AR (HA-β2-AR) were also tagged with the HA epitope using primers coding YPYDVPDYA and containing appropriate restriction sites. The GFP and HA epitopes were labeled G protein-coupled receptors, including AT1R and β2-AR, resulting in receptors with similar characteristics to the wild-type receptors (23–26). cDNA encoding Rab1a was cloned from a mouse cardiac cDNA library (27) and C. The reactions were terminated with 1 ml of ice-cold 10% trichloroacetic acid (TCA) and incubated in 2 ml of Hanks' balanced salt solution containing 0.89 mM Mg2+ and 4 ml) to remove free [3H]inositol. The cells were washed three times with 2 ml of ice-cold Hanks’ balanced salt solution containing 0.8% FBS. The cells were collected, suspended in PBS containing 1% fetal calf serum at a density of 0.45–106 cells/ml, and incubated with high affinity fluorescein-conjugated anti-HA antibody 3F10 at a final concentration of 2 μg/ml for 30 min at 4 °C. After washing with PBS, the cells were resuspended, and the fluorescence was analyzed as described above. Because the staining with the 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 fluorescence measurement reflected the amount of AT1R expressed at the cell surface.

Immunofluorescence Microscopy—HEK293T cells were grown on coverslips, fixed with paraformaldehyde and 4% sucrose in PBS for 15 min, 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 primary antibody overnight. After washing with PBS (3 × 5 min), the cells were incubated with Alexa Fluor 546-labeled secondary antibody (1:2000 dilution) for 1 h at room temperature. The coverslips were mounted, and fluorescence was detected with a Leica DMRA2 epifluorescence microscope. Images were deconvolved using SlideBook software and the nearest-neighbors deconvolution algorithm (Intelligent Imaging Innovations, Denver, CO) as described (29).

Double-stranded Small Interfering RNA (siRNA)-mediated Interference—A double-stranded siRNA with a 19-nucleotide duplex and 2-nucleotide overhang (5'-UUCUCCGAACGUGUCACGUTdTdT-3' and antisense, 5'-dTdTTCUAGGUGGAAUGUUCUG-3') targetting the sequence at positions 136–156 relative to the start codon (5'-AAATCGCGACATCATGCGT-3') of human Rab1 (GenBank®/EBI Data Bank accession number NM_030981) and a control non-silencing siRNA duplex (sense, 5'-UCUCCGAACGUGUCACGUdTdT-3' and antisense, 5'-dTdTTCUAGGUGGAAUGUUCUG-3') targetting sequence at positions 136–156 relative to the start codon (5'-AAATCGCGACATCATGCGT-3') of human Rab1 (GenBank®/EBI Data Bank accession number NM_030981) and a control non-silencing siRNA duplex (sense, 5'-UCUCCGAACGUGUCACGUdTdT-3' and antisense, 5'-dTdTTCUAGGUGGAAUGUUCUG-3') targetting sequence at positions 136–156 relative to the start codon (5'-AAATCGCGACATCATGCGT-3') of human Rab1 (GenBank®/EBI Data Bank accession number NM_030981). cDNA encoding Rab1a was cloned from a mouse cardiac cDNA library (27) and cDNA encoding Rab1 was cloned from a mouse cardiac cDNA library (27). Rab1a mutants S25N and N124I were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). These dominant-negative Rab mutants were characterized by indirect immunofluorescence microscopy (28) and transient transfection of HEK293T cells (29). These constructs were then characterized using LopacTamine 2000 reagent (Invitrogen). For measurement of total and cell-surface receptor expression and ERK1/2 activation, HEK293T cells were cultured in serum-free Opti-MEM medium for 24 h, followed by transfection with siRNA. For measurement of total and cell-surface receptor expression and ERK1/2 activation, HEK293T cells were cultured in serum-free Opti-MEM medium for 24 h, followed by transfection with siRNA. For measurement of total and cell-surface receptor expression and ERK1/2 activation, HEK293T cells were cultured in serum-free Opti-MEM medium for 24 h, followed by transfection with siRNA. For measurement of total and cell-surface receptor expression and ERK1/2 activation, HEK293T cells were cultured in serum-free Opti-MEM medium for 24 h, followed by transfection with siRNA.
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1 M ammonium formate and counted by liquid scintillation spectrometry in 18 ml of Ecoscint A scintillation solution (National Diagnostics, Inc., Atlanta, GA).

Measurement of ERK1/2 Activation—HEK293T cells were cultured on 6-well dishes and transfected as described above. At 36–48 h after transient transfection, HEK293T cells were starved for at least 2 h and then stimulated with individual agonists as indicated in the figure legends. Stimulation was terminated by addition of 600 μl of 1× SDS gel loading buffer. For measurement of ERK1/2 activation by isoprenal and UK14304, 300 μl of SDS gel loading buffer was used to terminate reactions. After solubilizing the cells, 20 μl of total cell lysates was separated by 12% SDS-PAGE, ERK1/2 activation was determined by Western blotting to measure the levels of phosphorylation of ERK1/2 with phospho-specific ERK1/2 antibodies. The membranes were stripped and reprobed with anti-ERK2 antibodies to determine the total amount of ERK2 and to confirm equal loading of proteins.

Immunoblotting—Western blotting was carried out as described previously (19, 20). HEK293T cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The signal was detected using ECL Plus (PerkinElmer Life Sciences) and a Fuji Film luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program (Version 3.4).

Statistical Analysis—Differences were evaluated by one-way analysis of variance, with p < 0.05 considered statistically significant. The EC_{50} values of ERK1/2 activation in response to stimulation with receptor agonists were analyzed using PRISM software (GraphPAD, San Diego, CA). Data are expressed as the means ± S.E.

RESULTS

Regulation of AT1R Expression at the Cell Surface by Rab1 GTPase—To determine whether or not Rab1 regulates AT1R trafficking in the secretory pathway, we first determined the effect of transient expression of wild-type Rab1 (Rab1WT) and its dominant-negative mutants on AT1R expression at the cell surface and total AT1R expression. Rab1WT and its dominant-negative mutants S25N (a GDP-bound form) and N124I (a guanine nucleotide binding-deficient form) tagged with the FLAG epitope at their amino termini were transiently expressed using pcDNA3.1 vector, FLAG-Rab1WT, FLAG-Rab1S25N, or FLAG-Rab1-N124I in HEK293T cells. Expression of Rab1WT and its mutants did not alter total AT1R-GFP expression as determined by measuring GFP fluorescence by flow cytometry (Fig. 1A). In contrast, coexpression of Rab1S25N and Rab1N124I with HA-AT1R significantly attenuated AT1R expression at the cell surface by 66 and 45%, respectively, compared with transfection with HA-AT1R and the empty pcDNA3.1 vector (Fig. 1B) as quantified by flow cytometry following staining with anti-HA antibodies in unpermeabilized HEK293T cells.

We then determined the effect of expressing Rab1WT and Rab1S25N on the subcellular localization of AT1R by fluorescence microscopy analysis. As anticipated, AT1R tagged with GFP or HA was localized mainly at the cell surface in the absence of exogenous Rab1, as indicated by extensive co-localization with ConA, a plasma membrane marker (Figs. 2 and 3A). Consistent with flow cytometry data, expression of Rab1S25N markedly reduced AT1R localization at the cell surface (Fig. 2, A and B) and co-localization with ConA (Fig. 3B). Rab1S25N induced AT1R accumulation in the perinuclear regions of the transfected cells (Fig. 2), and the accumulated AT1R was co-localized with calreulin (an ER marker) and GM130 (a Golgi marker), but not with Rab4 (an endosome marker) (Fig. 3B). These data indicate that Rab1 GTPase is involved in AT1R trafficking from the ER through the Golgi to the cell surface, consistent with Rab1 function in regulating protein transport selectively from the ER to the Golgi. In contrast to the dominant-negative Rab1 mutants, expression of Rab1WT had no obvious effect on the subcellular localization of AT1R (Fig. 2), suggesting that endogenous Rab1 is not a rate-limiting factor for AT1R transport to the cell surface.

Selective Regulation of the Subcellular Distribution of AT1R, β_{2}-AR, and α_{2b}-AR by Dominant-negative Mutant Rab1S25N—The preceding data indicated that expression of Rab1S25N inhibited AT1R expression at the cell surface. To determine whether Rab1 GTPase is capable of regulating the trafficking of other G protein-coupled receptors, we determined the effect of dominant-negative mutant Rab1S25N on the subcellular distribution of β_{2}-AR and α_{2b}-AR using the same strategy followed for AT1R. Rab1S25N expression markedly reduced β_{2}-AR localization at the cell surface, with a retention of β_{2}-AR in the perinuclear regions of the transfected cells (Fig. 4A). Surprisingly, in contrast to AT1R and β_{2}-AR, expression of Rab1S25N did not affect the subcellular distribution of α_{2b}-AR compared with transfection with α_{2b}-AR alone (Fig. 4A). These data suggest that inhibition of Rab1 function may differentially regulate the intracellular trafficking of AT1R, β_{2}-AR, and α_{2b}-AR.

To confirm that Rab1 is capable of differentially regulating the intracellular trafficking of these receptors, α_{2b}-AR-GFP

Fig. 1. Effect of Rab1 on AT1R expression at the cell surface and total AT1R expression. A, Western blot analysis of transfected Rab1 expression. HEK293T cells were transfected with the pcDNA3.1 vector (Control), FLAG-Rab1WT, FLAG-Rab1S25N, or FLAG-Rab1-N124I using LipofectAMINE 2000 as described under “Experimental Procedures.” Cell homogenates (40 μg) were separated by 12% SDS-PAGE, and transfected Rab1 expression was detected by anti-FLAG antibody M2. B, effect of Rab1 on total AT1R expression and AT1R expression at the cell surface. HEK293T cells were transfected with AT1R-GFP together with the pcDNA3.1 vector, Rab1WT, Rab1S25N, or Rab1N124I, and total AT1R expression was determined by measuring GFP fluorescence using a flow cytometer. The mean values of fluorescence obtained from the untransfected cells and from cells transfected with AT1R-GFP and pcDNA3.1, Rab1WT, Rab1S25N, or Rab1N124I were 37 ± 3, 445 ± 10, 392 ± 13, 401 ± 19, and 443 ± 19, respectively. To measure AT1R expression at the cell surface, HA-AT1R was expressed together with pcDNA3.1 and Rab1 in HEK293T cells. AT1R expression at the cell surface was quantitated by flow cytometry following incubation with anti-HA antibodies as described under “Experimental Procedures.” The mean values of fluorescence obtained from the untransfected cells and from cells transfected with HA-AT1R and pcDNA3.1, Rab1WT, Rab1S25N, or Rab1N124I were 108 ± 7, 680 ± 9, 626 ± 34, 231 ± 29, and 375 ± 12, respectively. The data shown are percent of the mean value obtained from the cells transfected with AT1R and pcDNA3.1 and are presented as the means ± S.E. of three experiments. *p < 0.05 versus cells transfected HA-AT1R and pcDNA3.1.
and Rab1S25N were expressed together with HA-AT1R or HA-β2-AR in HEK293T cells. The effect of Rab1S25N on the subcellular distribution of AT1R and α2B-AR or β2-AR and α2B-AR was determined in the same transfected cells by fluorescence microscopy analysis following staining with anti-HA antibodies. As shown in Fig. 4B, expression of Rab1S25N markedly blocked the transport of AT1R and β2-AR to the cell surface without affecting the subcellular distribution of α2B-AR. These data indicate that manipulation of Rab1 GTPase function selectively modifies the trafficking of AT1R, β2-AR, and α2B-AR and that transport to the cell surface of α2B-AR is independent of Rab1 GTPase.

Differential Regulation of the Subcellular Distribution of AT1R, β2-AR, and α2B-AR by siRNA-mediated Depletion of Rab1—To further define the role of Rab1 in regulating G protein-coupled receptor trafficking, we determined the effect of siRNA depletion of Rab1 on the subcellular distribution of AT1R, β2-AR, and α2B-AR. Introduction of Rab1 siRNA into HeLa (data not shown) or HEK293T (Fig. 5A) cells markedly reduced Rab1 expression levels compared with untransfected control cells or cells transfected with control siRNA. Rab1 siRNA transfection had no effect on expression of the closely related small GTPase Rab4 (Fig. 5A), demonstrating that Rab1 siRNA selectively reduces Rab1 expression.

Similar to transient expression of dominant-negative Rab1 mutants, depletion of endogenous Rab1 by siRNA transfection resulted in a marked accumulation of AT1R and β2-AR in the perinuclear regions and inhibited transport of both receptors to the cell surface (Fig. 5B). Inhibition was specifically induced by siRNA-mediated depletion of Rab1 because the control siRNA had no effect on the subcellular distribution of AT1R and β2-AR compared with the cells without siRNA transfection. In contrast, depletion of endogenous Rab1 had no effect on the subcellular distribution of α2B-AR (Fig. 5B). These data are consistent with selective regulation of the intracellular trafficking of AT1R, β2-AR, and α2B-AR by inhibiting Rab1 function through expressing the dominant-negative Rab1 mutants and further indicate that the movement of AT1R, β2-AR, and α2B-AR from the ER to the cell surface is mediated through distinct pathways.

Selective Regulation of the Signaling of AT1R, β2-AR, and α2B-AR by Rab1 GTPase—To determine whether or not Rab1 is capable of regulating AT1R signaling through modifying receptor trafficking, we first determined the effect of transient expression of Rab1WT and its dominant-negative mutants on AT1R-mediated IP production and ERK1/2 activation. Coexpression of dominant-negative Rab1 mutants with AT1R mod-

**Fig. 2. Effect of Rab1 on the subcellular distribution of AT1R.** HEK293T cells cultured on coverslips were transfected with AT1R-GFP (A) or HA-AT1R (B) together with pcDNA3.1 (Control), Rab1WT, or Rab1S25N as described under “Experimental Procedures.” A, the subcellular distribution of AT1R-GFP was revealed by detecting GFP fluorescence. B, the subcellular distribution of HA-AT1R was revealed by fluorescence microscopy following immunostaining with rhodamine-conjugated anti-HA antibodies as described under “Experimental Procedures.” The data are representative images of five independent experiments. Blue, DNA staining by 4,6-diamidino-2-phenylindole (nuclear); green, AT1R-GFP; red, HA-AT1R. Scale bars = 10 μm.

**Fig. 3. Co-localization of AT1R with intracellular organelle marker proteins.** A, HEK293T cells were transfected with AT1R-GFP and stained with tetramethylrhodamine-conjugated ConA (a plasma membrane marker). B, HEK293T cells were transfected with AT1R-GFP and Rab1S25N and stained with ConA and antibodies against calreculin (an ER marker; 1:50 dilution), GM130 (a Golgi marker; 1:100 dilution), and Rab4 (an endosome marker; 1:100 dilution) as described under “Experimental Procedures.” The subcellular distribution and co-localization of marker proteins of AT1R were revealed by fluorescence microscopy. The data are representative images of three independent experiments. Green, AT1R-GFP; red, marker proteins; yellow, co-localization of AT1R and organelle markers. Scale bars = 10 μm.
estly (but significantly) inhibited IP production (Fig. 6A) and ERK1/2 activation (data not shown) in response to stimulation with Ang II. In contrast to dominant-negative Rab1 mutants, expression of Rab1WT did not affect AT1R-stimulated IP production (Fig. 6A) and ERK1/2 activation (data not shown). IP production in response to Ang II stimulation in HEK293T cells transfected with AT1R plus the empty pcDNA3.1 vector, Rab1WT, Rab1S25N, or Rab1N124I was increased by 5.0 ± 0.2-, 4.8 ± 0.1 (p < 0.05)-, 3.3 ± 0.3 (p < 0.05)-, and 3.2 ± 0.3 (p < 0.05)-fold, respectively. Similar to transient expression of dominant-negative Rab1 mutants, depletion of Rab1 expression by siRNA transfection significantly inhibited AT1R-mediated IP production (Fig. 6B) and ERK1/2 activation (Fig. 6C).

These data indicate that Rab1 is required for AT1R signaling. To determine whether Rab1 can differentially influence signaling responses of AT1R, β2-AR, and α2b-AR, we compared the effect of Rab1S25N on ERK1/2 activation in response to stimulation with Ang II, isoproterenol, and UK14304. The EC50 values of ERK1/2 activation in response to Ang II and isoproterenol stimulation were not different between Rab1S25N-transfected and control cells. However, the maximal activation of ERK1/2 was reduced in Rab1S25N-transfected cells compared with control cells (Fig. 7, A and B).
contrast, expression of Rab1S25N had no effect on the EC_{50} and maximal activation of ERK1/2 in response to the α_{1B}-AR agonist UK14304 in cells transfected with α_{1B}-AR (Fig. 7C). These data are consistent with the selective effects of Rab1 on the subcellular distribution of AT1R, β_{2}-AR, and α_{1B}-AR.

**DISCUSSION**

Rab1 GTPase coordinates protein transport selectively from the ER to and through the Golgi apparatus (5–16). However, whether or not Rab1 regulates the intracellular trafficking of G protein-coupled receptors in mammalian cells remains unknown. As an initial approach to understand this issue, we first determined the role of Rab1 GTPase in the subcellular distribution of AT1R and demonstrated that Rab1 GTPase is required for AT1R transport from the ER through the Golgi to the cell surface. First, expression of dominant-negative Rab1 mutants S25N and N124I significantly decreased AT1R density at the cell surface as quantified by flow cytometry. Second, expression of dominant-negative Rab1 mutants induced a marked reduction in cell-surface expression of AT1R tagged with GFP as determined by direct immunofluorescence detection. Third, siRNA depletion of Rab1 produced an effect similar to that of dominant-negative Rab1 mutants on the subcellular distribution of AT1R. Finally, AT1R was unable to co-localize with ConA, a plasma membrane marker, in the cells transfected with Rab1S25N. The reduction of AT1R at the cell surface induced by inhibiting Rab1 function was accompanied by an accumulation of AT1R in the perinuclear regions of the transfected cells. The accumulated receptor was further co-localized with calsesin (an ER marker) and GM130 (a Golgi marker), but not with Rab1 (an endosome marker), suggesting that Rab1 is involved in the AT1R transport between the ER and Golgi. This is consistent with known function of Rab1 in the ER-to-Golgi transport of proteins. Taken together, these data strongly indicate that Rab1 GTPase is an essential factor required for AT1R transport in the early secretory pathway.

The most important finding in this study is that Rab1 GTPase can selectively modify the intracellular trafficking of 

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**Fig. 6.** Effect of manipulating Rab1 function on AT1R-stimulated IP accumulation and ERK1/2 activation. A, effect of transient expression of Rab1WT and its dominant-negative mutants on AT1R-mediated IP accumulation. HEK293T cells were transfected with AT1R-GFP and the pcDNA3.1 vector, Rab1WT, Rab1S25N, or Rab1N124I using LipofectAMINE 2000 as described under ‘Experimental Procedures.’ The transfected cells were split into 60-mm culture dishes, incubated with [3H]-[H]glucosinol, and stimulated with Ang II at 1 μM. IP production was measured as described under ‘Experimental Procedures.’ The basal levels of IP production in transfected cells were set at 100% of control and expressed as fold over control. B, effect of transfection with Rab1 WT and its dominant-negative mutants on AT1R-mediated ERK1/2 activation by Rab1 siRNA targeting. HEK293T cells cultured for an additional 24 h. The cells were then stimulated with Rab1 siRNA. At 8–12 h after transfection, the cells were split and cultured for an additional 24 h. The cells were then stimulated with increasing concentrations of Ang II (1–1000 nM) for 2 min. Ang II (1μM) and 1000 nM) for 2 min at 37°C, and 514 ± 354 cpm, respectively. The data are shown as the fold increase over the respective basal levels of IP production in response to Ang II stimulation and represent the means ± S.E. of three experiments. *, p < 0.05 versus cells transfected with AT1R-GFP alone. B, inhibition of AT1R-stimulated IP production by Rab1 siRNA. HEK293T cells grown on 100-mm dishes were transfected with 4 μg of AT1R-GFP and 36 μl of 20 nM control siRNA or Rab1 siRNA. IP accumulation in response to Ang II stimulation at a final concentration of 1 μM was measured. The basal levels of IP production in cells transfected with AT1R-GFP plus control siRNA or Rab1 siRNA were 2380 ± 165 and 2489 ± 153 cpm, respectively. The data are presented as the means ± S.E. of three experiments. *, p < 0.05 versus cells transfected with control siRNA. C, inhibition of AT1R-mediated ERK1/2 activation by Rab1 siRNA targeting. HEK293T cells cultured on 6-well dishes were transfected with AT1R-GFP and control siRNA or Rab1 siRNA. At 8–12 h after transfection, the cells were split and cultured for an additional 24 h. The cells were then stimulated with increasing concentrations of Ang II (1–1000 nM) for 2 min at 37°C. ERK1/2 activation was determined by Western blot analysis using phospho-specific ERK1/2 antibodies (ERK1/2-P). Left, representative blots of ERK1/2 activation (upper panel), total ERK2 expression (middle panel), and Rab1 expression (lower panel); right, quantitative data expressed as percent of ERK1/2 activation obtained in cells transfected with individual receptors and stimulated with 1 μM Ang II (A), 10 μM isoproterenol (B), or 1 μM UK14304 (C) and presented as means ± S.E. of three experiments. The EC_{50} values of ERK1/2 activation are 5 ± 1 nM Ang II, 6 ± 3 nM Ang II, 11 ± 2 nM isoproterenol, 13 ± 2 nM isoproterenol, 30 ± 4 nM UK14304, and 33 ± 11 nM UK14304 in cells transfected with AT1R-GFP, AT1R-GFP plus Rab1S25N, β_{2}-AR-GFP, β_{2}-AR-GFP plus Rab1S25N, α_{1B}-AR-GFP, and α_{1B}-AR-GFP plus Rab1S25N, respectively. There is no significant difference between the EC_{50} values in cells transfected with receptor alone and in cells co-transfected with receptor plus Rab1S25N.

**Fig. 7.** Effect of Rab1S25N on ERK1/2 activation by AT1R, β_{2}-AR, and α_{1B}-AR. HEK293T cells transfected with AT1R-GFP (A), β_{2}-AR-GFP (B), or α_{1B}-AR-GFP (C) together with pcDNA3.1 (Control; ■) or Rab1S25N (▲) were stimulated with increasing concentrations of Ang II (1–1000 nM) for 2 min (A), isoproterenol (ISO; 0.01–10 μM) for 5 min (B), or UK14304 (0.01–10 μM) for 5 min (C). ERK1/2 activation was determined as described in the legend of Fig. 6. Left panels, representative blots of ERK1/2 activation (upper panel), total ERK2 expression (middle panel), and Rab1S25N expression detected by Western blotting using anti-FLAG antibodies (lower panel); right panels, quantitative data expressed as percent of ERK1/2 activation obtained in cells transfected with individual receptors and stimulated with 1 μM Ang II (A), 10 μM isoproterenol (B), or 1 μM UK14304 (C) and presented as means ± S.E. of three experiments. The EC_{50} values of ERK1/2 activation are 5 ± 1 nM Ang II, 6 ± 3 nM Ang II, 11 ± 2 nM isoproterenol, 13 ± 2 nM isoproterenol, 30 ± 4 nM UK14304, and 33 ± 11 nM UK14304 in cells transfected with AT1R-GFP, AT1R-GFP plus Rab1S25N, β_{2}-AR-GFP, β_{2}-AR-GFP plus Rab1S25N, α_{1B}-AR-GFP, and α_{1B}-AR-GFP plus Rab1S25N, respectively. There is no significant difference between the EC_{50} values in cells transfected with receptor alone and in cells co-transfected with receptor plus Rab1S25N.
AT1R, β2-AR, and α2B-AR. Inhibiting Rab1 function by expressing dominant-negative Rab1 mutants and Rab1 siRNA produced a similar effect on the subcellular distribution of AT1R and β2-AR, i.e. a marked reduction in receptor expression at the cell surface and an accumulation of the receptors in the perinuclear regions. In contrast to AT1R and β2-AR, manipulation of Rab1 function had no effect on the subcellular distribution of α2B-AR. These data demonstrate that Rab1 GTPase differentially modifies the intracellular transport of different G protein-coupled receptors and indicate that the receptor proteins with common structural features that track to the cell surface and couple to heterotrimeric G proteins use distinct transport pathways (i.e. Rab1-dependent and Rab1-independent) for their movement from the ER to the cell surface.

There are several possibilities regarding selective regulation of the transport of AT1R, β2-AR, and α2B-AR by Rab1 GTPase. First, transport of AT1R, β2-AR, and α2B-AR from the ER may be mediated through distinct ER-derived vesicles, which are differentially regulated by Rab1 GTPase and transported to different destinations. Consistent with this possibility, different proteins or isoforms have been reported to segregate into distinct vesicles (32). Second, the post-translational modification of these three receptors may be one of the determinants for the selection of transport pathways used by individual receptors. Of particular note, Rab1-regulated AT1R and β2-AR have putative N-linked glycosylation sites at positions 4, 176, and 188 and positions 6 and 15, respectively. In contrast, α2B-AR, which is not regulated by Rab1, does not contain glycosylation signals (33–36). Glycosylation of these receptors occurs during their transport through the Golgi apparatus, resulting in the formation of mature receptors competent for subsequent transport to the cell surface. Consistent with this hypothesis, glycosylation of AT1R and β2-AR is required for their delivery to the plasma membrane, and mutations at these glycosylation sites impair their transport to the cell surface (33–36). Because α2B-AR is not glycosylated, it may not be necessary to transport α2B-AR from the ER to the Golgi, which is a Rab1-sensitive conventional transport pathway. Third, transport of AT1R, β2-AR, and α2B-AR from the ER may be coordinated by different Rab GTPases or other G proteins. Multiple Rab GTPases, including Rab1, Rab2, and Rab6, participate in protein transport between the ER and Golgi apparatus (37, 38). In addition to Rab proteins, ADP-ribosylation factor GTPases and heterotrimeric G proteins also participate in the process of protein transport from the ER to the Golgi (39, 40). Furthermore, Rab1 selective regulation of AT1R, β2-AR, and α2B-AR transport may be related to their coupling to different heterotrimeric G proteins. Nevertheless, our data provide the first evidence that transport of different G protein-coupled receptors to the cell surface is mediated through distinct molecular mechanisms, similar to their endocytic trafficking involving different regulatory pathways.

α2B-AR transport from the ER to the cell surface is apparently mediated through a Rab1-independent non-conventional trafficking pathway in HEK293T cells. However, characteristics of the pathway are unknown. It is possible that α2B-AR is transported from the ER to the plasma membrane through direct association and fusion of the ER to the plasma membrane. Such a transport pathway is used for ER-mediated phagocytosis (41). It is also possible that α2B-AR is transported from the ER to the late Golgi and/or endosomal compartments that bypass the early Golgi compartments and subsequently to the cell surface. This pathway has been proposed for CFTR transport from the ER to the cell surface and is independent of Rab1, ADP-ribosylation factor-1, and Syn5, components required for ER-to-Golgi protein transport in the conventional secretory pathway (15).

AT1R, β2-AR, and α2B-AR mainly couple to Gp, Go, and Gi, respectively, and regulate different signaling pathways. Inhibition of Rab1 function through expression of dominant-negative Rab1 mutants or siRNA depletion of Rab1 attenuated the signaling of AT1R-mediated IP production and ERK1/2 activation and β2-AR-mediated ERK1/2 activation, but not of α2B-AR-mediated ERK1/2 activation, paralleling the effects of Rab1 on the transport of these receptors to the cell surface. Therefore, we interpret that the attenuation of AT1R- and β2-AR-mediated signaling resulting from inhibition of Rab1 function was due at least in part to the inability of the transfected cells to transport the receptors from the ER to the Golgi apparatus and subsequently to the cell surface. However, we cannot exclude that altering Rab1 function may also modulate the intracellular trafficking of other signaling molecules involved in receptor signaling systems, which may also contribute to disruption of the normal signaling of the receptors. Nevertheless, our data demonstrate that Rab1-mediated receptor transport plays an important role in the regulation of the selectivity/specificity of signaling responses of different G protein-coupled receptors.

Because most studies on the trafficking of G protein-coupled receptors have focused on the events involved in receptor internalization, recycling, and degradation (17, 18), the transport process of these receptors from the ER through the Golgi to the cell surface itself is poorly understood. Multiple Rab GTPases are involved in the endocytic trafficking of G protein-coupled receptors. Among these Rab GTPases, Rab5 coordinates the endocytic trafficking of β2-AR, AT1R, dopamine D2 receptor, endothelin, μ-opioid, m5 muscarinic acetylcholine, and neurokinin-1 receptors (42–44). Interestingly, Rab5-regulated endocytic trafficking of different G protein-coupled receptors is mediated through distinct mechanisms (44). In contrast to Rab5, Rab4 and Rab11 are involved in the recycling of the internalized receptors back to the plasma membrane for resensitization (45), and Rab7 may be involved in the transport of internalized receptors from early endosomes to lysosomes for degradation (46). For receptor exocytic transport, exit of G protein-coupled receptors from the ER requires specific chaperones or escort proteins (47–49). In this study, we have demonstrated that Rab1 is required for the transport of AT1R and β2-AR from the ER to the Golgi. Other Rab GTPases may be involved in the regulation of receptor transport from the Golgi to the plasma membrane and from the Golgi to the ER. Given the complexity of Rab GTPase regulation of vesicular protein transport, multiple Rab GTPases may coordinate transport of G protein-coupled receptors between the ER and plasma membrane.

Normal protein transport is required for maintaining cell homeostasis. Indeed, defective protein transport is associated with the pathogenesis of a variety of human diseases (50, 51). Of particular note, we have recently generated transgenic mice with cardiac-specific overexpression of Rab1 GTPase (27). Increased Rab1 expression in the myocardium results in cardiac hypertrophy that progresses to heart failure, suggesting that dysregulated Rab GTPases may play a role in the development of cardiac hypertrophy and failure. The transport from the ER to the cell surface of G protein-coupled receptors through distinct pathways is of functional significance in the regulation of the selectivity/specificity of signal processing of the receptors in the cell. Further elucidation of the molecular mechanisms of trafficking of individual receptors may provide a foundation for development of therapeutic strategies that influence specific components of the transport pathway.

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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
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