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*

Catalin M. Filipeanu‡, Fuguo Zhou‡, William C. Claycomb§, and Guangyu Wu‡†

From the ‡Departments of Pharmacology and Experimental Therapeutics and §Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112

Rab1 GTPase coordinates vesicle-mediated protein transport specifically from the endoplasmic reticulum (ER) to the Golgi apparatus. We recently demonstrated that Rab1 is involved in the export of angiotensin II (Ang II) type 1 receptor (AT1R) to the cell surface in HEK293 cells and that transgenic mice overexpressing Rab1 in the myocardium develop cardiac hypertrophy. To expand these studies, we determined in this report whether the modification of Rab1-mediated ER-to-Golgi transport can alter the cell surface expression and function of endogenous AT1R and AT1R-mediated hypertrophic growth in primary cultures of neonatal rat ventricular myocytes. Adenovirus-mediated gene transfer of wild-type Rab1 (Rab1WT) significantly increased cell surface expression of endogenous AT1R in neonatal cardiomyocytes, whereas the dominant-negative mutant Rab1N124I had the opposite effect. Brefeldin A treatment blocked the Rab1WT-induced increase in AT1R cell surface expression. Fluorescence analysis of the subcellular localization of AT1R revealed that Rab1 regulated AT1R transport specifically from the ER to the Golgi in HL-1 cardiomyocytes. Consistent with their effects on AT1R export, Rab1WT and Rab1N124I differentially modified the AT1R-mediated activation of ERK1/2 and its upstream kinase MEK1. More importantly, adenovirus-mediated expression of Rab1N124I markedly attenuated the Ang II-stimulated hypertrophic growth as measured by protein synthesis, cell size, and sarcomeric organization in neonatal cardiomyocytes. In contrast, Rab1WT expression augmented the Ang II-mediated hypertrophic response. These data strongly indicate that AT1R function in cardiomyocytes can be modulated through manipulating AT1R traffic from the ER to the Golgi and provide the first evidence implicating the ER-to-Golgi transport as a regulatory site for control of cardiomyocyte growth.

Angiotensin II (Ang II), an octapeptide hormone, plays an important role in the development of cardiac hypertrophy through the activation of cell surface Ang II receptors (1). There are two major subtypes of Ang II receptors, the Ang II type 1 receptor (AT1R) and the Ang II type 2 receptor (AT2R), and both receptor subtypes belong to the seven transmembrane-spanning receptor superfamily coupling to heterotrimeric G proteins (2, 3). In cardiomyocytes, AT1R couples to the Gαs protein to stimulate phospholipase C, leading to the formation of intracellular inositol 1,4,5-triphosphate, the release of calcium from intracellular stores, and the activation of mitogen-activated protein kinases (MAPK) (4–6). A number of studies have demonstrated that AT1R activation induces hypertrophic growth in both cultured cardiomyocytes and animal hearts. In cultured cardiomyocytes, Ang II stimulation induces many characteristics of cardiomyocyte hypertrophy including increased expression of fetal genes, enhanced protein synthesis, enlargement of overall cell size, and sarcomeric organization (7–10). In transgenic animal models, increased AT1R expression in the myocardium is sufficient to initiate hypertrophic growth (11, 12). Furthermore, AT1R antagonist treatment prevents the development of cardiac hypertrophy in experimental animal models and in hypertensive patients (13).

The intracellular trafficking of AT1R is a critical event in regulating its function. After being synthesized, folded, and assembled in the endoplasmic reticulum (ER), the AT1R is transported to the Golgi apparatus, where post-translational modifications (e.g. glycosylation) occur (14). Then, it is transported to the plasma membrane. The AT1R at the plasma membrane undergoes internalization in the continuous presence of the agonist Ang II. The internalized AT1R may then be transported to the lysosome for degradation or recycled back to the plasma membrane (15–19). Therefore, the number of AT1R at the plasma membrane is determined by the overall balance of AT1R export to the cell surface, internalization, recycling, and degradation. Although most studies on AT1R trafficking have focused on the events involved in the internalization, recycling, and degradation (15–19), the export of AT1R from the ER to the Golgi to the cell surface and regulation of receptor function by these processes remain poorly understood.

Intracellular protein trafficking between organelles is coordinated by Rab proteins, the largest branch of the Ras-like small GTPases, consisting of 11 members in yeast and 63 members in mammalian cells (20, 21). Although most of the Rab proteins identified so far are ubiquitous and highly conserved in their structure and function, each Rab protein has a distinct intracellular localization and regulates discrete protein functions.
Transport steps in secretory and endocytic pathways. Among multiple Rab GTPases involved in the regulation of vesicular protein transport between intracellular organelles, Rab1 is one of the most extensively studied and best understood Rab GTPases (22–27). Rab1 is localized in the ER and Golgi and regulates anterograde protein transport specifically from the ER to the Golgi and between the Golgi compartments.

We recently demonstrated that AT1R exit from the ER is mediated by a motif consisting of a phenylalanine and a di-leucine spaced by six residues in the membrane-proximal carboxyl terminus of the receptor (28) and that Rab1 regulates the transport of AT1R from the ER to the cell surface in HEK293T cells (29). We also demonstrated that transgenic overexpression of Rab1 in the myocardium induces cardiac hypertrophy (30), suggesting that an alteration in Rab1 function may play a role in the development of cardiomyocyte hypertrophy. The present studies were undertaken to determine whether modifying the ER-to-Golgi transport, as a consequence of manipulating Rab1 function, could alter the cell surface expression and function of endogenous AT1R in cardiomyocytes, and if so, whether Rab1-mediated alteration in AT1R function contributes to the development of cardiomyocyte hypertrophy. We demonstrated that adenosine-mediated gene transfer of Rab1WT and its dominant-negative mutant elicited opposing effects on the ER-to-Golgi transport and cell surface expression of AT1R as well as AT1R-mediated signaling and hypertrophic response in cardiomyocytes. These data indicate that AT1R function can be modulated through manipulating AT1R traffic from the ER to the Golgi in cardiomyocytes and provide the first evidence implicating the ER-to-Golgi transport as a regulatory site for control of cardiomyocyte growth.

EXPERIMENTAL PROCEDURES

Materials—Angiotensin II type 1A receptor in vector pCDM8 was kindly provided by Dr. Kenneth E. Bernstein (Department of Pathology, Emory University, Atlanta, GA). Adenoaviral AdEasy system components (31) were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MD). Antibodies against Rab1, phospho-ERK1/2, MEK1, calreculin, and rhodamine-conjugated anti-hemagglutinin (HA) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-MEK1 were kindly provided by Dr. Andrew Catling (Department of Pharmaceutical and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, LA). Anti-GM130 antibody was from BD Transduction Laboratories. High affinity fluoroescin-conjugated anti-HA antibody 3F10 was from Roche Applied Science. Brefeldin A (BFA), PD123319, isoproterenol, norepinephrine, L-ascorbic acid, vitrogen. Alexa Fluor 594- and 488-labeled anti-mouse or anti-rabbit antibodies were from Rhodamine. Angiotensin II type 1A receptor in vector pCDM8 was kindly provided by Dr. Kenneth E. Bernstein (Department of Pathology, Emory University, Atlanta, GA). Adenoaviral AdEasy system components (31) were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MD). Antibodies against Rab1, phospho-ERK1/2, MEK1, calreculin, and rhodamine-conjugated anti-hemagglutinin (HA) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-MEK1 were kindly provided by Dr. Andrew Catling (Department of Pharmaceutical and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, LA). Anti-GM130 antibody was from BD Transduction Laboratories. High affinity fluoroescin-conjugated anti-HA antibody 3F10 was from Roche Applied Science. Brefeldin A (BFA), PD123319, isoproterenol, norepinephrine, L-ascorbic acid, fluoroescin, and anti-FLAG M2 monoclonal antibody were obtained from Sigma. Claycomb medium and fetal bovine serum were purchased from JRH Biosciences (Lenexa, KS). Human Ang II was purchased from R & D Systems. Penicillin-streptomycin and 1-glutamine were from Invitrogen. Alexa Fluor 594- and 488-labeled anti-mouse or anti-rabbit secondary antibodies, Alexa Fluor 594-labeled phallolidin, and 4,6-diamidino-2-phenyldole were obtained from Molecular Probes (Eugene, OR). Normal donkey serum was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). [3H]Leucine (specific activity = 173 Ci/mmol) and [3H]Ang II (specific activity 50.5 Ci/mmol) were purchased from PerkinElmer Life Sciences. All other materials were obtained as described elsewhere (32–34).

Plasmid Constructions—AT1Rs tagged with green fluorescent protein (GFP) at its carboxyl terminus (AT1R-GFP) and with HA epitope at its amino terminus (HA-AT1R) were generated as described previously (28, 29). The GFP and HA epitope have been used to label G protein-coupled receptors, including AT1R, resulting in the receptors with similar characteristics to the wild-type receptors (28, 29). Rab1 (accession number J05576) cloned from a mouse cDNA library (30) was tagged with the FLAG epitope at its amino terminus (FLAG-Rab1) as described previously (29). Constitutively active Rab1 (Q70L, a GTP-bound and GTPase-deficient form) and dominant-negative (S25N, a GDP-bound form, and N124I, a guanine nucleotide binding-deficient form) mutants were generated using the QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). These Rab1 mutants have been characterized and extensively utilized to explore the function of endogenous Rab1 (22–27, 29). The structure of each construct used in the present study was verified by restriction mapping and nucleotide sequence analysis (Louisiana State University Health Sciences Center DNA sequence core).

Generation of Adenovirus Stocks—Wild-type or mutant Rab1 was inserted into pShuttle-CMV at the Sall/HindIII restriction sites by PCR. After linearization with PmeI, the plasmid was co-transformed into Escherichia coli strain B15183 together with pAdEasy-1, the viral DNA plasmid. The recombinant adenoaviral constructs were screened by restriction endonuclease digestion. The adenoaviral constructs were digested with PacI and transfected into 911 cells to produce viral particles by subsequent packaging of recombinant virus performed in the Louisiana State University Health Sciences Center vector core facility by standard procedures.

Isolation, Culture, and Adenoviral Injection of Neonatal Rat Ventricular Myocytes—Neonatal ventricular myocytes were isolated from the hearts of 1–2-day-old Sprague-Dawley rats through trypsin digestion (50 μg/ml, overnight at 4 °C) in calcium- and magnesium-free Hank’s balanced salt solution (pH 7.4) and collagenase digestion in Leibovitz’s L-15 media (Worthington Biochemical Corp.). The myocytes were plated on a noncoated dish to reduce contamination of fibroblast cells. Based on immunofluorescence analysis after staining with antibodies against α-actinin, greater than 95% cells isolated by this procedure are cardiac myocytes. Isolated cardiac myocytes were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics. After a 24-h culture, myocytes were switched to serum-free DMEM and infected with control parent adenovirus or adenoaviral expressing Rab1WT or its dominant-negative mutant Rab1N124I at a multiplicity of infection (m.o.i.) of 20. After infection for 6 h, the medium was changed, and the myocytes were further cultured in DMEM. The efficiency of adenoviral infection of neonatal myocytes was evaluated by immunostaining using anti-FLAG antibodies.

Cell Culture and Transfection of HL-1 Cardiomyocytes—HL-1 myocytes were plated onto fibronectin-gelatin-coated plates or coverslips and cultured in Claycomb medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-pinephrine, and 2 mM 1-glutamine as described (35). Transient transfection of the HL-1 myocytes was carried out using LipofectAMINE 2000 reagent (Invitrogen). The efficiency of transfection based on the GFP fluorescence was between 60 and 80% in HL-1 cardiomyocytes. For measurement of Rab1 expression and AT1R expression at the cell surface, HL-1 myocytes were cultured in 6-well plates and transfected with 0.8 μg of AT1R-GFP and 2.0 μg of pcDNA3.1 or FLAG-Rab1 constructs. For immunofluorescence microscopy analysis, HL-1 myocytes were cultured in 6-well dishes and transfected with 30 ng of GFP- or HA-tagged AT1R and 300 ng of Rab1 constructs.

Measurement of AT1R Expression at the Cell Surface—Cell surface AT1R expression in neonatal cardiomyocytes was measured by [3H]Ang II binding to intact myocytes as described (36–38) with modifications. Briefly, neonatal myocytes were plated at a density of 5 × 105 cells/well in 12-well plates and infected with adenoviral constructs as described above. Infected cells were incubated with phosphate-buffered saline (PBS) containing [3H]Ang II at 4 °C (to limit AT1R internalization induced by ligand Ang II during the binding) overnight with constant shaking. To exclude the contribution of AT2R to ligand binding, all solutions were supplemented with 1 μM PD123319, a specific AT2R antagonist. The nonspecific binding was determined in the presence of nonradioactive Ang II (10 μM). The cells were washed twice with 1 ml of ice-cold PBS, and the cell surface-bound [3H]Ang II was extracted by mild acid treatment (2 × 5 min with 0.5 ml of 0.7 M H2SO4). The cell surface-bound radioactivity was counted by liquid scintillation spectrometry in 6 ml of Ecosint A scintillation solution (National Diagnostics, Inc., Atlanta, GA). We first determined the Ang II dose-dependent binding curve (2.5–20 nM) in cardiomyocytes infected with control parent adenovirus. Ang II binding to cardiomyocytes decreased linearly at Ang II concentrations between 1 nM and reached maximal binding at the concentration of 15 nM. Therefore, the cell surface expression of AT1R in neonatal cardiomyocytes infected with Rab1WT adenovirus was measured at a saturating concentration of 20 nM.

Total AT1R expression at the cell surface in HL-1 myocytes was measured by flow cytometry as described previously (29). Briefly, HL-1 myocytes were transfected with HA-AT1R, collected and resuspended in PBS containing 1% fetal calf serum at a density of 4 × 106 cells/ml, and incubated with high affinity fluoroescin-conjugated anti-HA antibody 3F10 at a final concentration of 2 μg/ml for 30 min at 4 °C. After

Downloaded by [**] at 06/15/2018
washing twice with 0.5 ml of PBS and 1% fetal calf serum, the cells were resuspended, and the fluorescence was measured in a flow cytometer (BD Biosciences FACSCalibur). 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 receptor expressed at the cell surface.

**Immunofluorescence Microscopy**—Cardiomyocytes were grown on coverslips, fixed with a mixture of 4% 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 for 1 h. After washing with PBS (3 × 5 min), the cells were incubated with Alexa Fluor 594- or -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 or a Bio-Rad radiance confocal microscope (28, 29).

**Measurement of ERK1/2 and MEK1 Activation**—The activation of ERK1/2 and its upstream kinase MEK1 was measured as described previously (28, 29, 30). Cardiac myocytes were plated at a density of 1 × 10⁶/well in 12-well plates, infected with Rab1 constructs as described above, and cultured in DMEM without serum for 48 h. The cells were then preincubated with 10 μM PD123319 for 5 min and stimulated with 100 nM Ang II for 2 min. The reaction was stopped by the addition of 600 μl of 1× SDS gel loading buffer. After solubilizing the cells, 30 μl of total cell lysates was separated by 10% SDS-PAGE, and ERK1/2 and MEK1 activation was determined by immunoblotting.

**[3H]Leucine Incorporation**—Protein synthesis rate was determined as described (40, 41). Briefly, neonatal cardiac myocytes were plated in 12-well plates at a density of 6 × 10⁵/well in DMEM supplemented with 10% fetal bovine serum. After infection with the desired construct, the myocytes were washed twice with 1 ml of 5% trichloroacetic acid for 1 h in ice. The cells were then washed twice with 1 ml of 10% trichloroacetic acid for 1 h in DMEM without fetal bovine serum. The cardiomycocytes were then incubated with [3H]Leucine (1 μci) for 24 h with or without 100 nM Ang II and 10 μM PD123319. The reaction was terminated by aspirating the medium. The cardiomycocytes were washed twice with 1 ml of 5% trichloroacetic acid followed by an extraction with 1 ml of 5% trichloroacetic acid for 1 h in ice. The extracts were then lyced with 1 ml of 1× NaOH for 6 h. The lysate was transferred to scintillation vials, neutralized with 1 ml of 1 M HCl, and counted by liquid scintillation spectrometry in 5 ml of Ecoscint A scintillation solution. Since Rab1 influences protein synthesis, the effect of Rab1 on Ang II-stimulated protein synthesis was calculated using the following formula: ([Ang II and Rab1] — [Rab1])/(Ang II and control adenovirus) — [control adenovirus].

**Measurement of Cell Surface Area**—To measure the cell surface area, cardiomycocytes were stained with Alexa Fluor 594-conjugated phalloidin (1:50 dilution) for 10 min to visualize F-actin. Cell images from at least 20 randomly chosen fields (×40 objective) of 100 cardiomyocytes were obtained in at least three separate experiments. Scale bar, 10 μm.

**RESULTS**

**Effect of Adenovirus-mediated Gene Transfer of Rab1 into Neonatal Rat Ventricular Myocytes on AT1R Expression at the Cell Surface**—To determine whether Rab1 regulates the export of endogenous AT1R from the ER to the cell surface, we first determined the effect of adenovirus-mediated expression of Rab1 on AT1R expression at the cell surface. Recombinant adenovirus encoding FLAG-tagged Rab1WT and its dominant-negative mutant Rab1N124I were generated to infect cardiomycocytes isolated from neonatal rat ventricles. Rab1 expression was determined by Western blotting using a FLAG high affinity monoclonal antibody (Fig. 1A). Fluorescent microscopic analyses following immunostaining with anti-FLAG antibodies revealed that greater than 95% of the cardiomycocytes were infected (Fig. 1B). Consistent with the localization of endogenous Rab1 in other cell types, infected Rab1WT was localized to the perinuclear region of the myocytes, presumably in the ER and the Golgi (22, 29). These data indicate that Rab1 was successfully transferred into primary cultures of neonatal rat ventricular myocytes with adenoviruses.

We next evaluated the effect of adenovirus-mediated gene transfer of Rab1 on the cell surface expression of AT1R using [3H]Ang II ligand binding in intact neonatal cardiomycocytes. The cell surface expression of AT1R as measured at a saturating concentration of Ang II (20 nM) was significantly augmented in cardiomycocytes infected with Rab1WT adenovirus as compared with that from cardiomycocytes infected with control adenovirus. In contrast, AT1R expression at the cell surface was markedly attenuated in cardiomycocytes infected with the dominant-negative mutant Rab1N124I adenovirus (Fig. 2). These data indicate that manipulation of Rab1 function modifies the cell surface expression of endogenous AT1R in neonatal cardiomycocytes.

**Effect of Rab1 on the ER-to-Golgi Transport of AT1R in Cardiomycocytes**—To determine whether the effect of Rab1 on AT1R expression at the cell surface is mediated through modifying receptor transport from the ER to the Golgi, we tested whether BFA could block Rab1 effect. BFA is a fungal metab...
Expression of the active mutant Rab1Q70L did not significantly change AT1R distribution at the plasma membrane (Fig. 3, C and D) but induced a marked accumulation of AT1R in the perinuclear region (Fig. 3C). The intracellularly accumulated AT1R was extensively co-localized with GM130, a Golgi marker (Fig. 3C), suggesting that the constitutive active mutant Rab1Q70L facilitated AT1R transport to the Golgi from the ER in HL-1 myocytes. These data indicate that the activation of Rab1 GTPase is required for normal export of AT1R from the ER to the Golgi in cardiomyocytes.

Modulation of AT1R Signaling by Rab1 in Neonatal Cardiomyocytes—To determine whether Rab1 is capable of regulating AT1R signaling through modifying the ER-to-Golgi transport of AT1R, we determined the effect of Rab1 on the AT1R-mediated activation of ERK1/2 and its upstream kinase MEK1. Adenovirus-mediated Rab1WT expression significantly enhanced ERK1/2 and MEK1 activation in response to stimulation with Ang II in neonatal cardiomyocytes (Fig. 4). In contrast, the dominant-negative Rab1N124I expression markedly reduced Ang II-induced ERK1/2 and MEK1 activation. These data indicate that Rab1 modulates not only AT1R traffic but also its signal transduction.

Cellular Hypertrophy Induced by Rab1WT in Neonatal Cardiomyocytes—We previously demonstrated that transgenic overexpression of Rab1 in the myocardium induces cardiac hypertrophy (31). To determine whether cardiac hypertrophy in Rab1 transgenic mice is the consequence of hemodynamic regulation or the direct effect of increased Rab1 expression on cardiomyocytes, we evaluated the effect of increased expression of Rab1 on hypertrophy of primary cultures of neonatal rat ventricular myocytes. We first determined the effects of adenovirus-mediated gene transfer of Rab1WT on total protein synthesis and overall size of neonatal cardiomyocytes. Total protein synthesis measured by [3H]leucine incorporation was significantly increased by 59% (p < 0.05) in cardiomyocytes infected with Rab1WT at 20 m.o.i. as compared with cardiomyocytes infected with parent adenoviral vector (Fig. 5A). Consistent with the augmented protein synthesis, the cell surface area was also significantly enlarged by 35% (p < 0.05) in myocytes infected with Rab1WT (Fig. 5B).

Because cardiomyocyte hypertrophy is accompanied by sarcomeric organization, we next examined whether Rab1WT infection induces such changes. The number of actin filaments as revealed by phalloidin staining was largely increased by Rab1WT infection (Fig. 5C). However, Rab1WT expression was not enough to induce the formation of highly organized sarcomeric structures. The cell surface area, total protein synthesis, and sarcomeric organization obtained from myocytes infected with parent adenoviral vector were almost the same as those obtained from noninfected myocytes (data not shown), suggesting that the influence of Rab1 infection on these hypertrophic parameters could not be attributed to nonspecific effects of adenoviral infection. These results indicate that adenovirus-mediated Rab1WT expression induces cellular hypertrophy in neonatal rat ventricular myocytes, consistent with the cardiomyocyte hypertrophy observed in the transgenic mouse hearts overexpressing Rab1.

Effect of Rab1 on Ang II-stimulated Hypertrophic Responses in Neonatal Cardiomyocytes—The preceding data indicated that adenovirus-mediated expression of Rab1WT and Rab1N124I produced opposite effects on AT1R expression at the cell surface and on cell signaling. We then determined whether manipulation of Rab1 function through adenovirus-mediated expression of Rab1WT and Rab1N124I could differentially influence Ang II-stimulated hypertrophic growth in neonatal cardiomyocytes. Ang II stimulation induces neonatal...
The ER-to-Golgi Transport Controls AT1R Function

The molecular mechanisms underlying the export process of G protein-coupled receptors from the ER through the Golgi to the cell surface and its pathophysiological significance in regulating receptor function remain largely unknown. As an initial approach to these issues, we previously determined the role of Rab1 in the export and signal transduction of AT1R, β2-adrenergic receptor (AR), and α2-AR using co-transfection systems (29). To expand these studies, in this report, we determined whether Rab1 controls the export traffic and function of endogenous AT1R in cardiomyocytes. The endogenous Rab1 function was manipulated through adenovirus-mediated gene transfer of Rab1WT and its dominant-negative mutant Rab1N124I. We demonstrated that Rab1WT and its dominant-negative mutant have opposing effects on the ER-to-Golgi transport and cell surface expression of AT1R as well as AT1R-mediated signaling and cell growth in cardiomyocytes.

We first determined the effect of Rab1 on endogenous AT1R expression at the cell surface. Consistent with our previous data in HEK293T cells (29), inhibition of Rab1 function by expressing dominant-negative mutant Rab1N124I attenuated the cell surface expression of AT1R in neonatal cardiomyocytes, further indicating that Rab1 is an essential factor for normal export of AT1R. Interestingly, expression of Rab1WT significantly increased endogenous AT1R expression at the cell surface in neonatal cardiomyocytes, suggesting that Rab1 may function as a rate-limiting factor for the transport of endogenous AT1R to the cell surface. However, Rab1WT and its constitutively active mutant Rab1Q70L had no significant effect on the overall cell surface expression of AT1R in transfected HEK293 cells (29) and HL-1 cardiomyocytes (Fig. 3D) overexpressing AT1R. Probably, AT1R overexpression has saturated the export potential of the cell; thus, further increase in Rab1 would have no effect on the AT1R transport. These data indicate that Rab1 is involved in the regulation of AT1R expression at the cell surface.

The influence of Rab1 on AT1R expression at the cell surface is due to its effect on the export of the receptor from the ER-to-Golgi compartment. This is supported by the fol-
Cardiomyocytes cultured in 6-well dishes were infected with parent (Control), Rab1WT, or Rab1N124I adenoviruses at an m.o.i. of 20 for 2 days. The cardiomyocytes were then stimulated with Ang II at a concentration of 100 nM for 2 min at 37 °C. The activation of ERK1/2 (A) and MEK1 (B) was determined by Western blot analysis using phospho-specific ERK1/2 (ERK1/2-P) and MEK1 (MEK1-P) antibodies, respectively. Representative blots of ERK1/2 and MEK1 activation (upper panel) and total ERK2 and MEK1 expression (lower panel) are shown. Right panel, quantitative data expressed as the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus and presented as the means ± S.E. of three individual experiments. *, p < 0.05 versus cardiomyocyte infected with control adenovirus.

We have previously demonstrated that transgenic overexpression of Rab1 in the myocardium induces cardiac hypertrophy with an increase in myocyte size, ventricular mass, and expression of embryonic cardiac genes (30). Consistent with cardiac hypertrophy observed in in vitro mouse hearts, increased expression of Rab1WT induced hypertrophy in in vitro neonatal cardiomyocytes as indicated by an increase in total protein synthesis, enlargement of the size of individual myocytes, and enhanced actin organization. These data further suggest that the cardiac hypertrophy observed in transgenic mouse hearts overexpressing Rab1 is a direct consequence of Rab1-facilitated protein transport in cardiomyocytes, rather than an indirect effect.

The transport from the ER to the Golgi compartment of G protein-coupled receptors represents the first step in intracellular trafficking of the receptors and influences the cell surface expression and function of the receptors (46). In addition to AT1R, the transport of many other G protein-coupled receptors from the ER to the Golgi may also be regulated by Rab1 in cardiomyocytes. Indeed, we have demonstrated that Rab1 expression of Rab1 in the myocardium induces cardiac hypertrophy, in which mouse hearts overexpressing Rab1 is a direct consequence of Rab1-mediated MAPK activation and hypertrophic growth.

Adenoviral expression of Rab1WT significantly augmented and Rab1N124I attenuated Ang II-stimulated ERK1/2 and MEK1 activation in neonatal cardiomyocytes, mirroring the effects of Rab1 on AT1R expression at the cell surface. Therefore, we conclude that the modulation of AT1R-mediated signaling was due at least in part to the influence of Rab1 on AT1R transport from the ER to the Golgi apparatus and subsequently to the cell surface. However, we cannot exclude the possibility that altering Rab1 function may also modulate the intracellular trafficking of other molecules involved in AT1R signaling systems, which may contribute to the modulation of AT1R function.

We also determined the effect of adenovirus-mediated expression of Rab1WT and Rab1N124I on Ang II-mediated hypertrophic responses in neonatal cardiomyocytes. Cardiomyocyte hypertrophy is generally characterized by increased protein synthesis, morphological alteration in cell size, and/or enhanced sarcomeric organization. Expression of Rab1WT and Rab1N124I oppositely influenced Ang II-induced neonatal cardiomyocyte hypertrophy, in which Rab1WT promoted and Rab1N124I attenuated Ang II-mediated increase in protein synthesis, cell size, and sarcomeric organization, consistent with the effect of Rab1 on AT1R expression at the cell surface and on signaling. Of particular note, the protein synthesis in response to Ang II stimulation was markedly increased in cardiomyocytes infected with Rab1WT and almost abolished in cardiomyocytes infected with Rab1N124I as compared with that from cardiomyocytes infected with control adenovirus. These data imply that cardiomyocyte growth can be manipulated by controlling the transport of AT1R at the level of the ER and the Golgi compartment.

We have shown that Rab1 is also required for the transport of AT1R to the plasma membrane. However, we cannot exclude the possibility that alteration of AT1R function on AT1R-mediated ERK1/2 activation in neonatal cardiomyocytes, mirroring the effects of Rab1 on AT1R-mediated MAPK activation and hypertrophic growth.

Elucidation of the functional role of Rab GTPases in regulating cardiomyocyte and cardiac function.

M. Sato and G. Wu, unpublished data.
protein-coupled receptors has just begun. Our results have demonstrated that Rab1 critically regulates the ER-to-Golgi transport and function of AT1R. In addition to Rab1, several other Rab GTPases have also been implicated in the regulation of intracellular trafficking of G protein-coupled receptors at other transport processes. For example, Rab5 regulates the recycling of internalized β2-AR and AT1R, dopamine D₂, endothelin, µ-opioid, m4 muscarinic acetylcholine, and neurokinin 1 receptors (38, 47–50). Rab4 and Rab11 may control the recycling of internalized β2-AR and AT1R from the endosome to the plasma membrane (38, 50, 51). Rab7 may be involved in the targeting of G protein-coupled receptors to the lysosome for degradation (51). Therefore, defining the functional role of individual Rab GTPases in cardiomyocyte growth by modifying the transport of selective G protein-coupled receptors at distinct steps may provide a novel foundation for the development of strategies in treating cardiac disease.

Acknowledgments—We acknowledge Stephen M. Lanier and Pamela A. Lucchesi (Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center (LSUHSC), New Orleans, LA) for every helpful suggestion and encouragement of this work. We thank Andrew D. Catling (Department of Pharmacology and Experimental Therapeutics, LSUHSC, New Orleans, LA) for comments and reagents. We appreciate the initial efforts of Phansak Karam and Debabrata Das and the constructive reviews of the referee.

REFERENCES

14. Youe He in the early stages of this project. We also thank Marella E. Kurikase and Emil Songu-Mize (Cell and Molecular Core, Department of Pharmacology and Experimental Therapeutics) for assistance in isolation of neonatal cardiomyocytes, Connie Porretta (Department of Medicine) for measuring AT1R expression by flow cytometry, and Robert Kutner (Vector Core, Department of Medicine) for purification of adenoviruses.

Fig. 5. Effect of adenovirus-mediated expression of Rab1WT on hypertrophy in neonatal cardiomyocytes. A, the effect of adenovirus-driven Rab1 expression on total protein synthesis. Cardiomyocytes were cultured in 12-well plates at a density of 6 × 10⁵/well, infected with empty (Control) or Rab1WT adenoviruses (20 m.o.i.), and incubated with 1 μCi of [³H]leucine for 24 h. Total protein synthesis was measured as described under “Experimental Procedures.” The data are shown as the fold increase over the control and represent the means ± S.E. of five separate experiments each performed in duplicate. B, the effect of Rab1 on the cell surface area. Myocytes were cultured and infected with control or Rab1WT adenovirus as above. After a 2-day incubation, cardiomyocytes were stained with phalloidin for F-actin. The cell surface area was measured by using the NIH Image program. At least 100 myocytes from 20 randomly selected fields in three separate experiments were measured as described under “Experimental Procedures.” C, the effect of Rab1 on sarcomeric organization. The myocytes were stained with phalloidin, and fluorescence was detected as described under “Experimental Procedures.” The images shown are representative of three experiments. *p < 0.05 versus cardiomyocytes infected with control adenovirus. Scale bar, 10 μm.

Fig. 6. Effect of adenovirus-mediated expression of Rab1WT and Rab1N124I on Ang II-stimulated hypertrophic response in neonatal cardiomyocyte. To reflect the effect of Rab1 on Ang II-stimulated hypertrophy, the contribution of Rab1 itself to protein synthesis and the cell surface area was subtracted as described under “Experimental Procedures.” Cardiomyocytes were cultured and then infected as described in the legend for Fig. 5. A, the effect of Rab1 on Ang II-stimulated total protein synthesis measured by [³H]leucine incorporation as described in the legend for Fig. 5. B, the effect of Rab1 on Ang II-stimulated increase in the cell surface area. C, the effect of Rab1 on Ang II-stimulated sarcomeric organization revealed by staining with phalloidin for F-actin. For easy comparison, the images obtained from the neonatal cardiomyocytes infected with control and Rab1N124I adenovirus after Ang II stimulation are shown. Similar results were obtained in three experiments. *p < 0.05 versus cardiomyocytes infected with control adenovirus. Scale bar, 10 μm.