THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 39, Issue of September 24, pp. 41077–41084, 2004
Printed in U.S.A.

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*

Received for publication, May 28, 2004, and in revised form, July 2, 2004
Published, JBC Papers in Press, July 12, 2004, DOI 10.1074/jbc.M405988200

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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),[1] 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 G_q 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 AT1Rs 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 through 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

* This work was supported by National Institutes of Health Grant 1P20RR018766 and Louisiana Board of Regents Grant LEQSF (2002-05)-RD-A-18 (to G. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; ER, endoplasmic reticulum; WT, wild type; AR, adrenergic receptor; GFP, green fluorescent protein; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; BFA, brefeldin A; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; m.o.i., multiplicity of infection.

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 antegrade 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 dileucine 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 adenovirus-mediated gene transfer of Rab1WT and its dominant-negative mutant elicit 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). Adenoviral 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, calregulin, and rhodamine-conjugated anti-hemagglutinin (HA) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phosphor-MEK1 were kindly provided by Dr. Andrew Catling (Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, LA). Anti-GM130 antibody was from BD Transduction Laboratories. High affinity fluorescein-conjugated anti-HA antibody 3F10 was from Roche Applied Science. Brefeldin A (BFA), PD123319, isoproterenol, norepinephrine, L-ascorbic acid, fibronectin, 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 Calbiochem. Penicillin-streptomycin and L-glutamine were from Invitrogen. Alexa Fluor 594- and 488-labeled anti-mouse or anti-rabbit secondary antibodies, Alexa Fluor 594-labeled phalloidin, and 4,6-diamidino-2-phenylindole were obtained from Molecular Probes (Eugene, OR). Normal donkey serum was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). [^{3}H]Leucine (specific activity = 173 Ci/mmol) and [^{3}H]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 epitopes 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 AF226873) cloned from a mouse cardiac 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 well 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 SalI/HindIII restriction sites by PCR. After linearization with PmeI, the plasmid was co-transformed into *Escherichia coli* strain BJ5183 together with pAdEasy-1, the viral DNA plasmid. The recombinant adenoviral constructs were screened by restriction endonuclease digestion. The adenoviral constructs were digested with PacI and transfected into 911 cells to produce viral particles. Subsequent processing and purification of adenovirus were performed in the Louisiana State University Health Sciences Center vector core facility by standard procedures.

Isolation, Culture, and Adenoviral Infection 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 preplated 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 cardiomyocytes. Isolated neonatal 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 adenovirus 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.

Culture and Transfection of HL-1 Cardiomyocyte—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 norepinephrine, and 2 mM L-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 dishes 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 [^{3}H]Ang II binding to intact myocytes as described (36–38) with modifications. Briefly, neonatal myocytes were plated at a density of 5×10^5 cells/well in 12-well plates and infected with adenoviral constructs as described above. Infected cells were incubated with phosphate-buffered saline (PBS) containing [^{3}H]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 [^{3}H]Ang II was extracted by mild acid treatment (2×5 min with 0.5 ml of buffer containing 50 mM glycine, pH 3, and 125 mM NaCl). The radioactivity was counted by liquid scintillation spectrometry in 6 ml of Ecoscint 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 increased linearly at concentrations between 2.5 and 15 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 4×10^6 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 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, 39). Cardiac myocytes were plated at a density of 1×10^6/well in 6-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.

[^{3}H]Leucine Incorporation—Protein synthesis rate was determined as described (40, 41). Briefly, neonatal cardiomyocytes were plated in 12-well plates at a density of 6×10^5/well in DMEM supplemented with 10% fetal bovine serum. After infection with the desired construct, the myocytes were made quiescent by incubation for 48 h in DMEM without fetal bovine serum. The cardiomyocytes were then incubated with [^{3}H]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 cardiomyocytes 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 cells were lysed with 1 ml of 1 M 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, cardiomyocytes 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 measured in three separate experiments using NIH image software. Only these myocytes that were completely in the field were measured. The effect of Rab1 on the Ang II-mediated increase in cell size was determined using the same formula as for the protein synthesis described above.

Immunoblotting—Western blotting was carried out as described previously (29, 42). For measurement of Rab1 expression, cardiomyocytes were harvested and homogenized in buffer containing 5 mM Tris-HCl, pH 7.4, 5 mM EGTA, and 5 mM EDTA supplemented with Complete Mini protease inhibitor mixture (Roche Applied Sciences). Fifty μg of total homogenate was separated by 12% SDS-PAGE, and Rab1 expression was detected by Western blotting using anti-FLAG antibodies. ERK1/2 and MEK1 activation was determined by measuring their phosphorylation with phospho-specific antibodies. The membranes were stripped and reprobed with anti-MEK1 or ERK2 antibodies to determine the total amount of kinases and to confirm equal loading of proteins. 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 using Student's t test or one-way analysis of variance, and $p < 0.05$ was considered as statistically significant. Data are expressed as the means ± S.E.

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

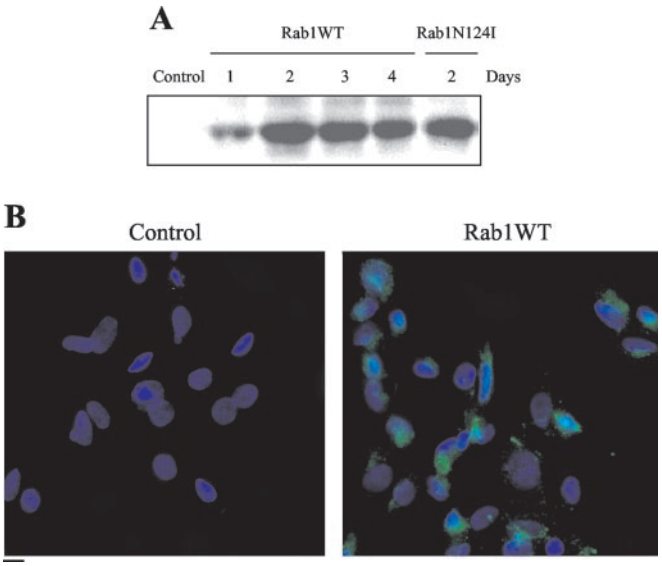

FIG. 1. **Adenovirus-mediated expression of Rab1 in neonatal rat ventricular myocytes.** *A*, Western blot analysis of expression of Rab1WT and dominant-negative mutant Rab1N124I driven by adenovirus. Neonatal myocytes were infected with empty adenoviral vector (*Control*) or recombinant FLAG-Rab1WT adenovirus for 1, 2, 3, and 4 days or with Rab1N124I for 2 days at an m.o.i. of 20. Fifty μg of whole cardiomyocyte lysate was separated by 12% SDS-PAGE, and FLAG-Rab1 expression was detected by immunoblotting with anti-FLAG antibody M2. The immunoblot is representative of results obtained in two different experiments. *B*, localization of Rab1WT and estimation of infection efficiency. Cardiomyocytes were cultured on coverslips and infected with control or FLAG-Rab1WT adenovirus as above. Localization of FLAG-Rab1WT in infected cardiomyocytes was revealed by fluorescence microscopy following immunostaining with anti-FLAG antibodies as described under "Experimental Procedures." Similar results were obtained in at least three separate experiments. *Scale bar*, 10 μm.

Rab1 on AT1R expression at the cell surface. Recombinant adenovirus encoding FLAG-tagged Rab1WT and its dominant-negative mutant Rab1N124I were generated to infect cardiomyocytes isolated from neonatal rat ventricles. Rab1 expression was determined by Western blotting using a FLAG high affinity monoclonal antibody (Fig. 1*A*). Fluorescent microscopic analyses following immunostaining with anti-FLAG antibodies revealed that greater than 95% of the cardiomyocytes were infected (Fig. 1*B*). 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 [^{3}H]Ang II ligand binding in intact neonatal cardiomyocytes. The cell surface expression of AT1R as measured at a saturating concentration of Ang II (20 nM) was significantly augmented in cardiomyocytes infected with Rab1WT adenovirus as compared with that from cardiomyocytes infected with control adenovirus. In contrast, AT1R expression at the cell surface was markedly attenuated in cardiomyocytes 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 cardiomyocytes.

Effect of Rab1 on the ER-to-Golgi Transport of AT1R in Cardiomyocytes—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-

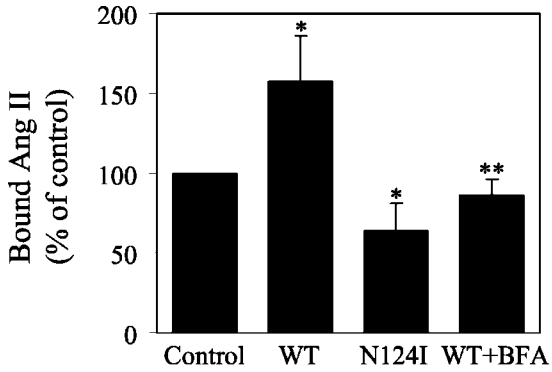

FIG. 2. **Effect of adenovirus-mediated expression of Rab1WT and Rab1N124I on AT1R expression at the cell surface in neonatal cardiomyocytes.** Cardiomyocytes were cultured and infected with control, Rab1WT, or Rab1N124I adenovirus. For BFA treatment, the Rab1WT-infected cardiomyocytes were incubated with BFA at a concentration of 5 μg/ml for 8 h. Two days after adenoviral infection, the cell surface expression of AT1R was determined by ligand [^{3}H]Ang II binding as described under "Experimental Procedures." Nonspecific binding obtained in the presence of 10 μM nonradioactive Ang II was subtracted from the values presented. The mean values of specific binding were 496 ± 112, 779 ± 145, and 316 ± 85 cpm (n = 8 each in duplicate) from the cardiomyocytes infected with control, Rab1WT, or Rab1N124I adenovirus, respectively, and 426 ± 50 cpm (n = 3) from the cardiomyocytes infected with Rab1WT adenovirus and treated with BFA. The data shown are the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus and are presented as the means ± S.E. *, $p < 0.05$ *versus* cardiomyocytes infected control adenovirus; **, $p < 0.05$ *versus* cardiomyocytes infected with Rab1WT adenovirus alone.

olite that disrupts the structures of the Golgi and blocks protein transport from the ER to the Golgi (43, 44). BFA treatment significantly blocked Rab1-mediated increase in AT1R expression at the cell surface (Fig. 2), suggesting Rab1WT-mediated AT1R increase in cell surface is due to the modification of AT1R transport from the ER to the Golgi, consistent with the well established Rab1 function.

To further characterize the functional role of Rab1 in regulating AT1R expression at the cell surface, we sought to determine the effect of transient expression of the dominant-negative mutant Rab1N124I and the constitutively active mutant Rab1Q70L on the subcellular distribution of AT1R. To this end, we choose HL-1 cardiomyocytes, an immortal cardiac muscle cell line that proliferates and retains phenotypic characteristics of cardiomyocytes (35, 45). HL-1 myocytes can be easily transfected with epitope-tagged AT1R, allowing us to directly visualize subcellular localization of the receptor. AT1R tagged with GFP or HA was co-expressed together with FLAG-tagged Rab1N124I or Rab1Q70L in HL-1 myocytes. Rab1 expression was detected by immunoblotting with anti-FLAG antibodies (Fig. 3*A*), and the subcellular distribution of AT1R was visualized by fluorescence microscopy.

As anticipated, AT1R was mainly localized at the plasma membrane (Fig. 3*B*) in the absence of exogenous Rab1, which was confirmed by co-localization with tetramethylrhodamine-conjugated concanavalin A, a plasma membrane marker (data not shown). Expression of Rab1N124I markedly reduced the cell surface expression of AT1R as compared with myocytes transfected with the receptor and empty pcDNA3.1 vector (Fig. 3*B*). The receptors were retained in the perinuclear regions of the transfected myocytes, presumably in the ER and the Golgi (29). Consistent with microscopic analysis of receptor subcellular localization, the number of HA-AT1Rs in the plasma membrane quantitated by flow cytometry following staining with anti-HA antibodies in unpermeabilized HL-1 myocytes was significantly reduced by 44% (Fig. 3*D*).

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. 3*C*). The intracellularly accumulated AT1R was extensively co-localized with GM130, a Golgi marker (Fig. 3*C*), 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 [^{3}H]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. 5*A*). 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. 5*B*).

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. 5*C*). 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

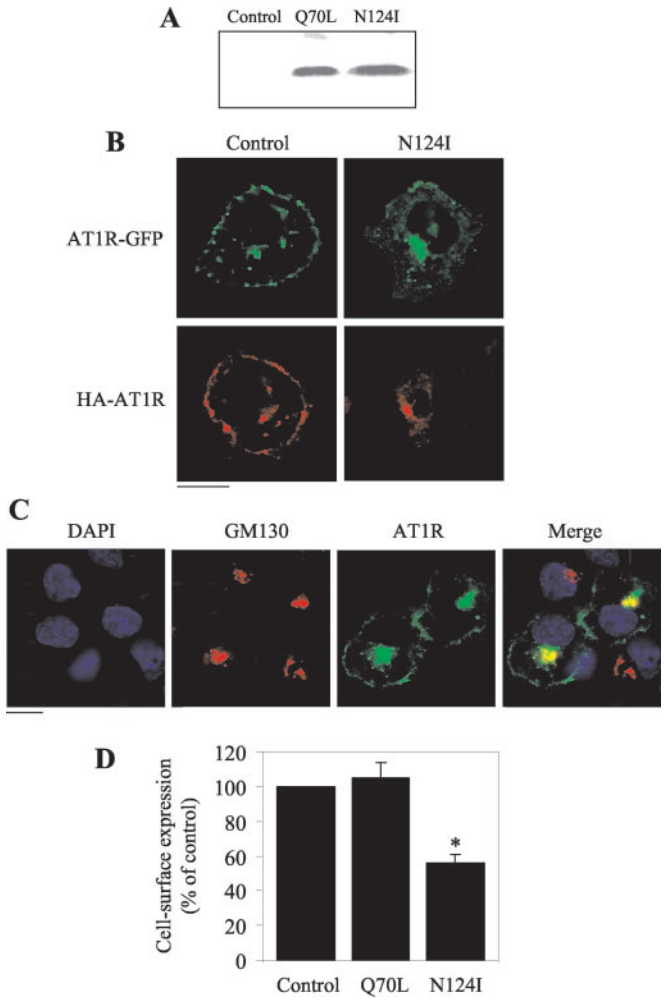

FIG. 3. **Effect of Rab1 on the subcellular distribution of AT1R in HL-1 cardiomyocytes.** *A*, Western blot analysis of transfected Rab1 expression. HL-1 myocytes were transfected with the pcDNA3.1 vector (*Control*), FLAG-Rab1Q70L, or FLAG-Rab1N124I using LipofectAMINE 2000 as described under "Experimental Procedures." Fifty μg of whole myocyte lysate was separated by 12% SDS-PAGE, and transfected Rab1 expression was detected by immunoblotting with anti-FLAG antibody M2. *B*, the effect of dominant-negative mutant Rab1N124I on the subcellular distribution of AT1R. HL-1 myocytes cultured on coverslips were transfected with AT1R-GFP (*upper panel*) or HA-AT1R (*lower panel*) plus pcDNA3.1 (*Control*) or Rab1N124I. The subcellular distribution of the receptor was revealed by detecting GFP fluorescence (*upper panel*) or by fluorescence microscopy following immunostaining with rhodamine-conjugated anti-HA antibodies (*lower panel*) as described under "Experimental Procedures." The data are representative images of three independent experiments. *C*, the effect of the constitutively active mutant Rab1Q70L on the subcellular distribution of AT1R. HL-1 myocytes cultured on coverslips were transfected with AT1R-GFP and FLAG-Rab1Q70L. The cells were stained with 4,6-diamidino-2-phenylindole (*DAPI*) and antibodies against GM130 (a Golgi marker; 1:100 dilution). The subcellular distribution and co-localization with GM130 of AT1R-GFP were revealed by fluorescence microscopy. The data are representative images of at least three independent experiments. *Blue*, DNA staining by 4,6-diamidino-2-phenylindole (nuclear); *red*, GM130 (Golgi); *green*, GFP-AT1R; *yellow*, co-localization of AT1R and GM130. *D*, the effect of Rab1Q70L and Rab1N124I on the cell surface expression of AT1R. HL-1 myocytes were transfected with HA-AT1R together with pcDNA3.1 (*Control*), Rab1Q70L, or Rab1N124I. 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 myocytes and from myocytes transfected with HA-AT1R plus pcDNA3.1, Rab1Q70L, or Rab1N124I were 92 ± 7, 520 ± 14, 541 ± 39, and 240 ± 21, respectively. The data shown are the percentage of the mean value obtained from the cells transfected with pcDNA3.1 and HA-AT1R and are presented as the means ± S.E. of three experiments. *, $p < 0.05$ *versus* cells transfected HA-AT1R and pcDNA3.1 (*Control*). *Scale bars*, 10 μm.

cardiomyocyte hypertrophy as indicated by sarcomeric reorganization and a moderate increase in protein synthesis and cell size (7–10). Protein synthesis and the cell surface area in cardiomyocytes infected with parent adenoviral vector were increased by about 25 and 35%, respectively, in response to Ang II (100 nM) after blockade of AT2R function using PD123319 (10 μM) (data not shown). Ang II effects on hypertrophic growth were dramatically increased in cardiomyocytes expressing Rab1WT (Fig. 6, *A* and *B*). After subtracting the Rab1 effect, Ang II-stimulated protein synthesis and the cell surface area were dramatically increased by 88 and 115%, respectively, as compared with cardiomyocytes infected with control adenovirus (Fig. 6, *A* and *B*).

In contrast to Rab1WT, expression of the dominant-negative mutant Rab1N124I significantly attenuated the Ang II-mediated hypertrophic response (Fig. 6). Increases in total protein synthesis and the cell surface area in response to stimulation with Ang II were markedly attenuated by 90 and 68%, respectively, in cardiomyocytes infected with Rab1N124I as compared with cardiomyocytes infected with control adenovirus (Fig. 6, *A* and *B*). Furthermore, the formation of striated sarcomeric structures in response to Ang II stimulation was also reduced by Rab1N124I (Fig. 6*C*). These data indicate that the reduction of Rab1 function prevents Ang II-mediated cardiomyocyte hypertrophic growth, consistent with a decrease in the cell surface expression and signaling of AT1R induced by the dominant-negative Rab1 mutant in cardiomyocytes.

DISCUSSION

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, β_2-adrenergic receptor (AR), and α_{2B}-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. 3*D*) 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-

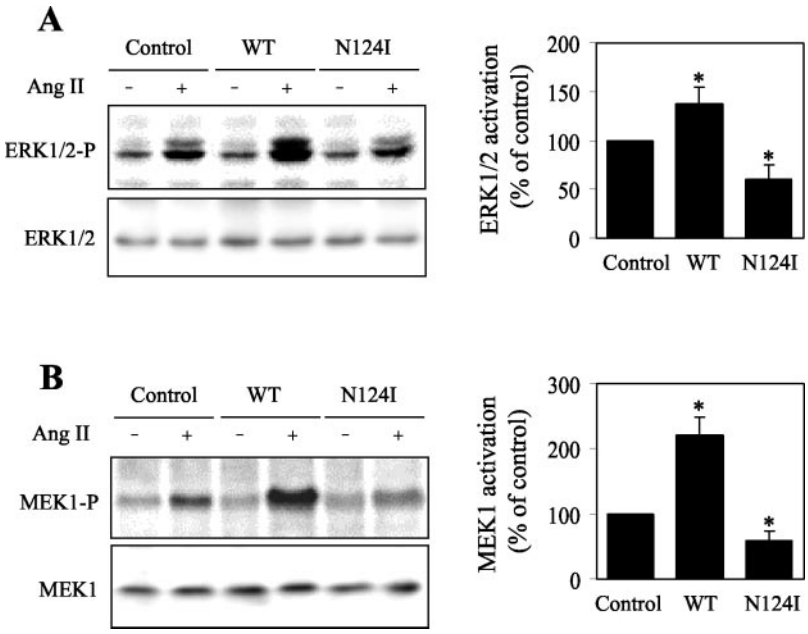

FIG. 4. **Effect of manipulating Rab1 function on AT1R-mediated ERK1/2 and MEK1 activation in neonatal cardiomyocytes.** Cardiomyocytes cultured in 6-well dishes were infected with parent (*Control*), Rab1WT, or Rab1N124I adenoviruses at an m.o.i. of 20 for 2 days. The cardiomyocytes were then stimulated 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.

lowing results. First, Rab1WT-induced enhancement in AT1R expression at the cell surface was prevented by BFA, which impairs Golgi function and blocks the ER-to-Golgi protein transport (43, 44). Second, the dominant-negative mutant Rab1N124I induced AT1R accumulation in the perinuclear regions, presumably the ER and Golgi (29), with an impairment of AT1R transport to the plasma membrane. Third, the constitutively active mutant Rab1Q70L induced AT1R accumulation in the Golgi without influencing normal cell surface expression of the receptor, suggesting that the AT1R transport from the ER to the Golgi is facilitated by the Rab1Q70L. These data also imply that AT1R transport from the ER to the Golgi and from the Golgi to the plasma membrane are two separate rate-limiting steps.

To determine whether the modification of AT1R transport from the ER to the Golgi by Rab1 influenced AT1R function in neonatal cardiomyocytes, we determined the effect of Rab1 on AT1R-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 RabN124I oppositely influenced Ang II-induced neonatal cardiomyocytes 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 responses 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 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 vivo* 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 is required for β_2-AR transport from the ER through the Golgi to the cell surface in HEK293T cells (29). Our preliminary studies have shown that Rab1 is also required for the transport of β_2-AR from the ER to the cell surface and cAMP production in response to stimulation with isoproterenol, but not forskolin, in HL-1 cardiac cells.[2] As the β-AR also plays a crucial role in regulating cardiomyocyte growth, it will be interesting to determine whether Rab1 regulates the trafficking and function of endogenous β-AR in primary cultures of cardiomyocytes. By virtue of the ability to regulate ER-to-Golgi traffic and signal propagation of G protein-coupled receptor systems in cardiac myocytes, Rab1 may play an important role in regulating cardiomyocyte and cardiac function.

Elucidation of the functional role of Rab GTPases in regulating the intracellular trafficking and signal transduction of G

[2] M. Sato and G. Wu, unpublished data.

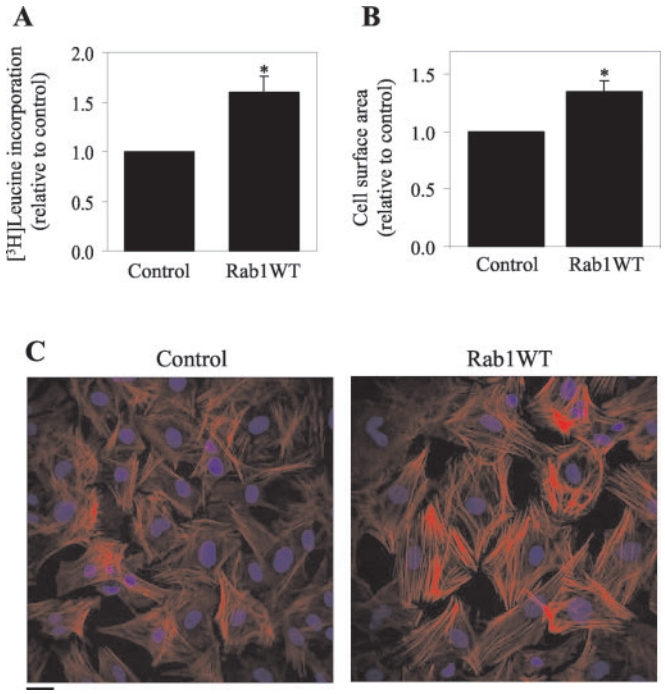

FIG. 5. **Effect of adenovirus-mediated expression of Rab1WT on hypertrophy in neonatal cardiomyocytes.** *A*, the effect of adenovirus-driven Rab1 expression on total protein synthesis. Cardiomyocytes were cultured in 12-well plates at a density of 6×10^5/well, infected with empty (*Control*) or Rab1WT adenoviruses (20 m.o.i.), and incubated with 1 μCi of [^{3}H]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 infection, 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.

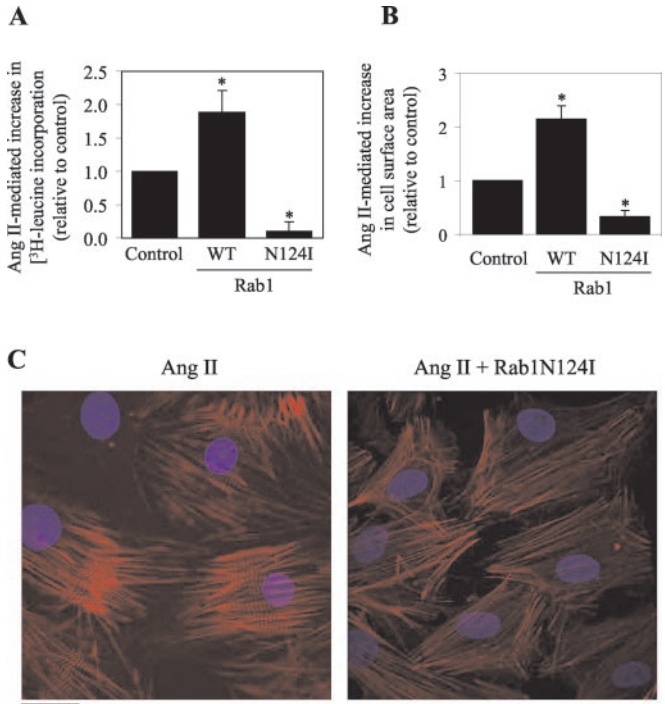

FIG. 6. **Effect of adenovirus-mediated expression of Rab1WT and Rab1N124I on Ang II-stimulated hypertrophic response in neonatal cardiomyocyte.** To reflect the effect of Rab1 on Ang II-mediated stimulation, the contribution of Rab1 itself to protein synthesis and the cell surface area was subtracted as described under "Experimental Procedures." Cardiomyocytes were cultured and then infected as described in the legend for Fig. 5. After adenoviral infection for 2 days, cardiomyocytes were stimulated with Ang II at a concentration of 100 nM for 24 h at 37 °C. *A*, the effect of Rab1 on Ang II-stimulated total protein synthesis measured by [^{3}H]leucine incorporation as described in the legend for Fig. 5. *B*, the effect of Rab1 on Ang II-mediated 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 Rab1N124N 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.

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 endocytic trafficking from the plasma membrane to the endosome of G protein-coupled receptors including β_2-AR, AT1R, dopamine D_2, endothelin, μ-opioid, m4 muscarinic acetylcholine, and neurokinin 1 receptors (38, 47–50). Rab4 and Rab11 may control the recycling of internalized β_2-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 Youe He in the early stages of this project. We also thank Marella E. Kuriakose and Emel 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.

REFERENCES

1. Griendling, K. K., Lassegue, B., and Alexander, R. W. (1996) *Annu. Rev. Pharmacol. Toxicol.* **36,** 281–306
2. Murphy, T. J., Alexander, R.W., Griendling, K. K., Runge, M. S., and Bernstein, K. E. (1991) *Nature* **351,** 233–236
3. Matsubara, H. (1998) *Circ. Res.* **83,** 1182–1191
4. Touyz, R. M., and Schiffrin, E. L. (2000) *Pharmacol. Rev.* **52,** 639–672
5. Sadoshima, J., Qiu, Z., Morgan, J. P., and Izumo, S. (1995) *Circ. Res.* **76,** 1–15
6. Filipeanu, C. M., Henning, R. H., Nelemans, S. A., and de Zeeuw, D. (2001) *J. Renin Angiotensin Aldosterone Syst.* **2,** 219–226
7. Sadoshima, J., and Izumo, S. (1993) *Circ. Res.* **73,** 413–423
8. Aoki, H., Izumo, S., and Sadoshima, J. (1998) *Circ. Res.* **82,** 666–676
9. Baker, K. M., and Aceto, J. F. (1990) *Am. J. Physiol.* **259,** H610–618
10. Wada, H., Zile, M. R., Ivester, C. T., Cooper, G. T., and McDermott, P. J. (1996) *Am. J. Physiol.* **271,** H29–H37
11. Hein, L., Stevens, M. E., Barsh, G. S., Pratt, R. E., Kobilka, B. K., and Dzau, V. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6391–6396
12. Paradis, P., Dali-Youcef, N., Paradis, F. W., Thibault, G., and Nemer, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 931–936
13. De Rosa, M. L., Cardace, P., Rossi, M., Baiano, A., and de Cristofaro, A. (2002) *J. Hum. Hypertens.* **16,** 133–140
14. Lanctot, P. M., Leclerc, P. C., Escher, E., Leduc, R., and Guillemette, G. (1999) *Biochemistry* **38,** 8621–8627
15. Mundell, S. J., and Benovic, J. L. (2000) *J. Biol. Chem.* **275,** 12900–12908
16. Hein, L., Meinel, L., Pratt, R. E., Dzau, V. J., and Kobilka, B. K. (1997) *Mol. Endocrinol.* **11,** 1266–1277

17. Hunyady, L., Catt, K. J., Clark, A. J., and Gaborik, Z. (2000) *Regul. Pept.* **91,** 29–44
18. Claing, A., Laporte, S. A., Caron, M. G., and Lefkowitz, R. J. (2002) *Prog. Neurobiol. (N. Y.)* **66,** 61–79
19. Marchese, A., Chen, C., Kim, Y. M., and Benovic, J. L. (2003) *Trends. Biochem. Sci.* **28,** 369–376
20. Takai, Y., Sasaki, T., and Matozaki, T. (2001) *Physiol. Rev.* **81,** 153–208
21. Martinez, O., and Goud, B. (1998) *Biochim. Biophys. Acta* **1404,** 101–112
22. Plutner, H., Cox, A. D., Pind, S., Khosravi-Far, R., Bourne, J. R., Schwaninger, R., Der, C. J., and Balch, W. E. (1991) *J. Cell Biol.* **115,** 31–43
23. Wilson, B. S., Nuoffer, C., Meinkoth, J. L., McCaffery, M., Feramisco, J. R., Balch, W. E., and Farquhar, M. G. (1994) *J. Cell Biol.* **125,** 557–571
24. Jin, M., Saucan, L., Farquhar, M. G., and Palade, G. E. (1996) *J. Biol. Chem.* **271,** 30105–30113
25. Lupashin, V. V., and Waters, M. G. (1997) *Science* **276,** 1255–1258
26. Allan, B. B., Moyer, B. D., and Balch, W. E. (2000) *Science* **289,** 444–448
27. Dugan, J. M., deWit, C., McConlogue, L., and Maltese, W. A. (1995) *J. Biol. Chem.* **270,** 10982–10989
28. Duvernay, M. T., Zhou, F., and Wu, G. (2004) *J. Biol. Chem.* **279,** 30741–30750
29. Wu, G., Zhao, G., and He, Y. (2003) *J. Biol. Chem.* **278,** 47062–47069
30. Wu, G., Yussman, M. G., Barrett, T. J., Hahn, H. S., Osinska, H., Hilliard, G. M., Wang X, Toyokawa, T., Yatani, A., Lynch, R. A., Robbins, J., and Dorn, G. W., II (2001) *Circ. Res.* **89,** 1130–1137
31. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2509–2514
32. Wu, G., Bogatkevich, G. S., Mukhin, Y. V., Benovic, J. L., Hildebrandt, J. D., and Lanier, S. M. (2000) *J. Biol. Chem.* **275,** 9026–9034
33. Wu, G., Benovic, J. L., Hildebrandt, J. D., and Lanier, S. M. (1998) *J. Biol. Chem.* **273,** 7197–7200
34. Wu, G., Krupnick, J. G., Benovic, J. L., and Lanier, S. M. (1997) *J. Biol. Chem.* **272,** 17836–17842
35. Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A., and Izzo, N. J., Jr. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2979–2984
36. Bouscarel, B., Wilson, P. B., Blackmore, P. F., Lynch, C. J., and Exton, J. H. (1988) *J. Biol. Chem.* **263,** 14920–14924
37. Vanderheyden, P. M., Fierens, F. L., De Backer, J. P., Fraeyman, N., and Vauquelin, G. (1999) *Br. J. Pharmacol.* **126,** 1057–1065
38. Hunyady, L., Baukal, A. J., Gaborik, Z., Olivares-Reyes, J. A., Bor, M., Szaszak, M., Lodge, R., Catt, K. J., and Balla, T. (2002) *J. Cell Biol.* **157,** 1211–1222
39. Slack-Davis, J. K., Eblen, S. T., Zecevic, M., Boerner, S. A., Tarcsafalvi, A., Diaz, H. B., Marshall, M. S., Weber, M. J., Parsons, J. T., and Catling, A. D. (2003) *J. Cell Biol.* **162,** 281–291
40. Thaik, C. M., Calderone, A., Takahashi, N., and Colucci, W. S. (1995) *J. Clin. Invest.* **96,** 1093–1099
41. van Kesteren, C. A., van Heugten, H. A., Lamers, J. M., Saxena, P. R., Schalekamp, M. A., and Danser, A. H. (1997) *J. Mol. Cell. Cardiol.* **29,** 2147–2157
42. Wu, G., Toyokawa, T., Hahn, H., and Dorn, G. W., II (2000) *J. Biol. Chem.* **275,** 29927–29930
43. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz. J. (1992) *J. Cell Biol.* **116,** 1071–1080
44. Yoo, J. S., Moyer, B. D., Bannykh, S., Yoo, H. M., Riordan, J. R., and Balch, W. E. (2002) *J. Biol. Chem.* **277,** 11401–11409
45. White, S. M., Constantin, P. E., and Claycomb, W. C. (2004) *Am. J. Physiol.* **286,** H823–H829
46. Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000) *J. Biol. Chem.* **275,** 13727–13736
47. Volpicelli, L. A., Lah, J. J., and Levey, A. I. (2001) *J. Biol. Chem.* **276,** 47590–47598
48. Iwata, K., Ito, K., Fukuzaki, A., Inaki, K., and Haga, T. (1999) *Eur. J. Biochem.* **263,** 596–602
49. Schmidlin, F., Dery, O., DeFea, K. O., Slice, L., Patierno, S., Sternini, C., Grady, E. F., and Bunnett, N. W. (2001) *J. Biol. Chem.* **276,** 25427–25437
50. Seachrist, J. L., Anborgh, P. H., and Ferguson, S. S. (2000) *J. Biol. Chem.* **275,** 27221–27228
51. Dale, L. B., Seachrist, J. L., Babwah, A. V., and Ferguson, S. S. (2004) *J. Biol. Chem.* **279,** 13110–13118