Published in final edited form as:

Methods Enzymol. 2008 ; 438: 217–226. doi:10.1016/S0076-6879(07)38015-4.

ANALYSIS OF RAB1 FUNCTION IN CARDIOMYOCYTE GROWTH

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Abstract

Protein transport between intracellular organelles is coordinated by Rab GTPases. As an initial approach to defining the function of Rab GTPases in cardiomyocytes, our laboratory focused on Rab1, which regulates protein transport specifically from the endoplasmic reticulum (ER) to the Golgi apparatus. Our studies have demonstrated that adenovirus-driven expression of Rab1 promotes cell growth of primary cultures of neonatal cardiomyocytes *in vitro* and that transgenic expression of Rab1 in the myocardium induces cardiac hypertrophy in mouse hearts *in vivo*. These data provide strong evidence implicating that ER-to-Golgi protein transport functions as a regulatory site for control of cardiomyocyte growth. Here we describe a sets of methods used in our laboratory to characterize the function of Rab1 GTPase in modulating cardiac myocyte growth.

1. INTRODUCTION

Cardiac hypertrophy is the compensatory increase in myocardial mass in response to mechanical injury or persistent increased hemodynamic loading. In cardiac hypertrophy, cardiac myocytes exhibit hypertrophic growth, which involves increases in protein synthesis and subsequent transport of newly synthesized proteins to their functional destinations. It has been well documented that intracellular protein trafficking is governed by Rab GTPases, the largest branch of the Ras-related small GTPases super-family (Balch, 1990; Martinez and Goud, 1998). However, in contrast to the small GTPases Ras, Rac, and Rho, which function as components of many cellular signal transduction pathways and have been well investigated in cardiac hypertrophy and failure (Clerk and Sugden, 2000; Hunter *et al.*, 1995; Sussman *et al.*, 2000; Sah *et al.*, 1999), the function of Rab proteins in cardiac myocytes remain largely unknown.

As an initial approach to determine the possible role of Rab GTPases in cardiomyocytes, we investigated the function of Rab1 in myocyte growth both in primary cultures of neonatal rat ventricular myocytes *in vitro* and in the mouse heart *in vivo* (Wu *et al.*, 2001; Filipeanu *et al.*, 2004, 2006a; Duvernay *et al.*, 2005). Rab1 has two isoforms, Rab1a and Rab1b, with greater than 90% identity in their amino acid sequences. Rab1 is specifically localized in the ER and the Golgi apparatus and regulates export of newly synthesized protein from the endoplasmic reticulum (ER) and subsequent anterograde transport from the ER to the Golgi (Allan *et al.*, 2000; Dugan *et al.*, 1995; Moyer *et al.*, 2001; Nuoffer *et al.*, 1994; Plutner *et al.*, 1991; Tisdale *et al.*, 1992; Wu *et al.*, 2003; Yoo *et al.*, 2002). Our studies have demonstrated that adenovirus-mediated expression of Rab1 in rat ventricular cardiomyocytes induces hypertrophic growth with an increase in the total protein synthesis, cell sizes, and sarcomeric organization at the cellular level (Filipeanu *et al.*, 2004). Consistent with the increased myocyte growth *in vitro*, transgenic mice overexpressing Rab1a specifically in the myocardium develops cardiac hypertrophy with progression to heart failure (Wu *et al.*, 2001). These data strongly suggest that protein transport between the ER and the Golgi may function as a regulatory site for control of cardiomyocyte growth. Here we describe the methods utilized in our laboratory to generate the adenoviruses expressing Rab1 and to investigate the role of Rab1 in protein synthesis, cell

size, and sarcomeric organization in neonatal rat ventricular myocytes. Finally, we will briefly discuss cardiac phenotypes observed in transgenic mice overexpressing Rab1.

2. GENERATION OF ADENOVIRUSES EXPRESSING RAB1 GTPASE

To manipulate the function of endogenous Rab1 GTPase in primary cultures of cardiomyocytes, it is necessary to achieve efficient expression of Rab1, which will allow biochemical and morphological analysis of the entire cell population. It is difficult to achieve efficient expression of proteins in primary cultures of neonatal cardiomyocytes when using commonly used transfection reagents. Viral vectors including adenovirus, retrovirus, and lentivirus have been used to successfully deliver proteins of interest into primary cultures of cardiomyocytes. In our studies, we generated the recombinant adenovirus to drive expression of Rab1a in cardiomyocytes. Rab1a (AF226873) is cloned from a mouse cardiac cDNA library (Wu *et al.*, 2001). For easy detection of Rab1a protein expression and subcellular localization, Rab1a is tagged with the FLAG epitope at its N-terminus by polymerase chain reaction (PCR) using a primer GACTACAAGGACGACGATGACAAG coding a peptide DYKDDDDK (Wu *et al.*, 2003). The FLAG epitope has been widely used to label proteins, including small GTPases without altering their characteristics. FLAG-Rab1a is inserted into the pShuttle-CMV vector at the Sal I/Hind III restriction sites and transformed into the bacteria INVαF'. The recombinant plasmid is amplified and digested with Sal I and Hind III to confirm the presence of the insert. The purified plasmid is digested with Pme I and the linearized plasmid is co-transformed into *Escherichia coli* strain BJ5183 together with the viral DNA plasmid pAdEasy-1. The recombinant adenoviral constructs are screened by restriction endonuclease digestion, digested with Pac I and transfected into 911 cells to produce viral particles. Subsequent processing and purification of adenovirus are performed as described by He *et al.* (1999).

2.1. Adenoviral Rab1 infection of primary cultures of neonatal rat ventricular myocytes

In our laboratory we have used the neonatal cardiac myocytes isolation kit from Worthington (<http://www.worthington-biochem.com/NCIS/cat.html>) to isolate neonatal cardiac myocytes. Briefly, 1- to 2-day-old Sprague Dawley rat pups are anesthetized and killed by decapitation. The beating hearts are quickly removed from 8 to 12 pups and placed into cold Ca²⁺/Mg²⁺-free Hanks balanced salt solution (HBSS). The conjunctive tissue and atria are removed under a dissecting microscope and the remaining ventricular tissue is chopped into small pieces (1 to 2 mm³) and washed repeatedly with fresh HBSS solution. The ventricular tissue is then digested overnight at 4° with 5 ml of HBSS containing trypsin at a concentration of 0.5 mg/ml. The digested ventricle is incubated with 5 ml of Leibovitz L-15 media containing 0.2 mg/ml collagenase at 37° for 30 to 45 min in a 360-degree rotator. The cardiac myocytes are gently released by repeated trituration (20 to 30 times) with a 10-ml pipette. The cell mixture is filtered through a cell strainer (diameter 70 µm) to remove the nondigested tissue. The cells are collected by centrifugation at 100 g for 5 min and resuspended in Media 199 supplemented with 15% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The fibroblasts are removed by preplating for 1 h at 37°. The remaining nonattached cells are ventricular myocytes and used for the subsequent experiments. In our hands, with this procedure, the purity of cardiac myocytes is greater than 95% as demonstrated by -actinin antibodies staining.

The isolated myocytes are plated at different densities for different experiments. The myocytes are cultured in DMEM containing 10% FBS and antibiotics for 24 h and then infected with control and Rab1 adeno-viruses for 6 h. The myocytes are divided into three groups each in triplicate: no infection, infection with control parent adenovirus, and infection with Rab1 adenovirus. The infected myocytes are cultured in DMEM without FBS and antibiotics for 2 days before experiments. To optimize the infection condition, in the preliminary studies,

isolated cardiac myocytes are infected with increasing concentrations of adenoviruses from 5 to 100 multiplicity of infection (MOI) for different periods of time (1 to 4 days).

3. ANALYSIS OF ADENOVIRUS-DRIVEN RAB1 EXPRESSION IN CARDIOMYOCYTES

To determine if Rab1 is successfully delivered into primary cultures of ventricular myocytes with adenoviruses, cardiomyocytes are cultured on 35-mm dishes at a density of 5×10^5 cells per dish and infected with the adenoviruses as above. The Rab1-infected myocytes are collected and solubilized with 300 μ l of 1X SDS-gel loading buffer. Thirty μ l of whole cardiomyocyte lysate is separated by 12% SDS-PAGE and both anti-FLAG antibody M2 (Sigma) and Rab1a (Santa Cruz Biotechnology) antibodies are used to evaluate FLAG-Rab1a expression in myocytes by Western blot analysis. Rab1a antibodies detect both endogenous Rab1a and overexpressed FLAG-Rab1a, whereas FLAG antibodies detect only overexpressed FLAG-Rab1a. Therefore, Western blots using Rab1a antibodies will determine the relative amount of endogenous and infected Rab1a. Using this protocol, Rab1 and its mutant Rab1N124I were successfully delivered into neonatal rat ventricular myocytes (Filipeanu *et al.*, 2004), as well as cardiac myocytes isolated from adult rats (Zhou, F., and Wu, G., unpublished data), and the maximal FLAG-Rab1a expression was achieved after infection at 20 MOI for 2 days (Fig. 15.1A).

3.1. Assessment of infection efficiency by Rab1 adenoviruses in cardiomyocytes

To determine the efficiency of adenoviral infection, cardiomyocytes are grown on coverslips in six-well dishes and infected as described above. Infected myocytes are 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 are then incubated with primary anti-FLAG antibody at 1:50 dilution for 1 h. After washing with PBS (3×5 min), the cells are incubated with Alexa Fluor 594-labeled secondary antibody at 1:2000 dilution for 1 h at room temperature. The coverslips are mounted, and fluorescence detected with a fluorescence microscope. In general, greater than 95% of the cardiomyocytes are infected (Filipeanu *et al.*, 2004). Importantly, consistent with the localization of endogenous Rab1 and its function in regulating protein transport between the ER and the Golgi, exogenously expressed FLAG-Rab1a is localized to the perinuclear region of the infected myocytes, presumably in the ER and the Golgi (Filipeanu *et al.*, 2004).

3.2. Measurement of cardiomyocyte viability after Rab1 infection

We have used the calcein AM retention method to measure myocyte viability after Rab1 infection (Yakovlev *et al.*, 2000). Calcein is a polyanionic fluorescein derivative whose acetoxy-methyl-ester form is plasma membrane permeable. Once inside the cell, the ester is hydrolyzed by intracellular esterases to release the fluorescent calcein molecules. The free calcein is trapped intracellularly only in the cells with intact plasma membrane and thus will indicate exclusively the viable cells. Briefly, the isolated ventricular myocytes are cultured on 24-well plates at a density of 1×10^5 cells per well in DMEM containing 10% FBS and antibiotics for 24 h. After 6 h of adenoviral infection and 40 h of culture, the myocytes are washed twice with PBS containing 145 mM NaCl, 5 mM KCl, 0.5 mM MgSO_4 , 0.5 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES (pH 7.4), and incubated with 5 μ M per well of calcein AM (Molecular Probes) at 37° for 1 h. The cells are washed twice to remove the extracellular calcein AM and allowed for dye hydrolysis for 30 min in the dark. The fluorescence is then monitored with a CytoFluor 4000 fluorimeter using 480 nm as excitation wavelength and 520 nm as emission wavelength.

4. EFFECT OF RAB1 INFECTION ON THE TOTAL PROTEIN SYNTHESIS IN NEONATAL CARDIOMYOCYTES

We used the [3H]-leucine incorporation into the newly synthesized proteins to measure the effect of Rab1 on the total protein synthesis in cardiac myocytes (Filipeanu *et al.*, 2004). The isolated cardiac myocytes are cultured on 12-well dishes at a density of 2×10^5 cells per well, and infected with control and Rab1 adenoviruses at 20 MOI as above. After infection for 6 h and culture in DMEM without FBS for 2 days, the myocytes are incubated with 0.5 ml of DMEM containing 1 μ Ci [3H]-leucine for 12 h. The myocytes are washed twice with 1 ml of ice-cold 5% trichloroacetic acid (TCA) solution and then incubated with 1 ml of TCA solution in ice for 1 h. This step is critical to remove the cytosolic [3H]-leucine, which are not incorporated into the newly synthesized proteins. After removing TCA solution, the myocytes are digested with 1 ml of 1 M NaOH for 4 to 6 h at 37°. The solution is collected into scintillation tubes and neutralized with 1 ml of 1 M HCl. The radioactivity is counted with 5 ml of Ecoscint A scintillation solution in a β -scintillation counter. Our data have demonstrated that adenoviral expression of Rab1 markedly augments total protein synthesis by 59% as measured by [3H]-leucine incorporation in neonatal rat ventricular myocytes (Filipeanu *et al.*, 2004) (see Fig. 15.1B).

5. EFFECT OF RAB1 INFECTION ON THE CELL SIZE AND SARCOMERIC ORGANIZATION OF CARDIOMYOCYTES

The effect of adenoviral expression of Rab1 on the sizes of cardiac myocytes is determined by measuring cell surface area (Filipeanu *et al.*, 2004). Isolated myocytes are grown on glass coverslips in six-well dishes at low density (1×10^4 cells/well) and made quiescent in DMEM without FBS for 48 h and infected with control and Rab1 adenoviruses. The myocytes are fixed in 4% paraformaldehyde and 4% sucrose, and stained with Alexa Fluor-594 conjugated phalloidin to visualize F-actin. Cell images from 20 randomly chosen fields of 100 cardiac myocytes are visualized ($40 \times$ objective) on each coverslip and their size is quantified using National Institutes of Health (NIH) image software. Only myocytes that are completely in the field are considered. Our data have demonstrated that adenoviral expression of Rab1 significantly enlarge the cell surface of neonatal cardiomyocytes (see Fig. 15.1C). Furthermore, the number of actin filaments as revealed by phalloidin staining is also largely increased by Rab1 infection in neonatal cardiomyocytes (Filipeanu *et al.*, 2004) (see Fig. 15.1D). Together with increased protein synthesis obtained in Rab1-infected myocytes, these results indicate that adenovirus-mediated Rab1 expression induces cellular hypertrophy in neonatal rat ventricular myocytes. These data suggest that augmentation of Rab1-mediated ER-to-Golgi transport plays an important role in regulating cardiomyocyte growth.

6. CARDIAC PHENOTYPES OF TRANSGENIC MICE OVEREXPRESSING RAB1A IN THE MYOCARDIUM

To determine the role of Rab1 GTPase in cardiac myocyte growth and function *in vivo*, we generated transgenic mice with cardiac specific expression of Rab1a (Wu *et al.*, 2001). A 1431-bp DNA fragment encompassing 28 bp of 5'-untranslated region, the mouse Rab1a coding region, and 785 bp of 3'-untranslated region is inserted into Sal I and Hind III restriction sites of the mouse α -myosin heavy-chain (MHC) promoter, which will drive Rab1 expression specifically in the myocardium (Subramaniam *et al.*, 1991; Wu *et al.*, 2000). Consistent with Rab1 function in promoting cardiomyocyte growth at the cellular level, Rab1 overexpression in the myocardium results in cardiac hypertrophy with an increase in whole heart and ventricular mass, an enlargement of myocyte size and a upregulation of fetal genes including atrial natriuretic peptide (ANF), β -myosin heavy chain (MHC), and α -skeletal actin (Fig. 15.2).

Rab1 transgenic mice develop heart failure as cardiac fractional shortening measured by echocardiography and myocyte contractility are markedly impaired in Rab1 transgenic mice (Wu *et al.*, 2001).

The most striking cardiac phenotype observed in transgenic mice over-expressing Rab1a, as compared with other transgenic mouse models of cardiac hypertrophy and failure (Filipeanu *et al.*, 2006b; Koch *et al.*, 2000), is the marked enlargement of the ER and the Golgi stacks and increased secretory atrial natriuretic peptide granules in ventricular myocytes as revealed by ultrastructural analysis (Wu *et al.*, 2001). These data are consistent with the functional role of Rab1 GTPase in modulating overall newly synthesized protein transport between the ER and the Golgi. These results indicate that altered Rab1 function in cardiomyocytes may contribute to cardiac pathology.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health grants R01-GM076167 and P20-R018766.

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Figure 15.1. Effect of adenovirus-mediated expression of Rab1a on hypertrophic growth in neonatal cardiomyocytes

(A) Adenovirus-mediated expression of FLAG-Rab1a in neonatal rat ventricular myocytes. Neonatal myocytes were infected with empty adenoviral vector (control) or recombinant FLAG-Rab1a adenovirus for 1, 2, 3, and 4 days at 20 MOI. FLAG-Rab1a expression was detected by immunoblotting with anti-FLAG antibody M2 following SDS-PAGE. (B) The effect of adenovirus-driven FLAG-Rab1 expression on total protein synthesis. Cardiomyocytes were infected with control or Rab1 adenoviruses (20 MOI), and incubated with 1 μ Ci [3H]-leucine for 12 h. The data are shown as the *x*-fold increase over the control and represent the means (plus or minus standard error) of five separate experiments, each performed in duplicate.

(C) The effect of Rab1a on the cell surface area. Cardiomyocytes were stained with phalloidin for F-actin after a 2-day infection. 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. $*p < 0.05$ versus cardiomyocytes infected with control adenovirus. (D) The effect of Rab1 on sarcomeric organization. The myocytes were stained with phalloidin and fluorescence visualized by a confocal microscope. (Filipeanu, C.M., Zhou, F., Claycomb, W. C., and Wu, G. (2004). Regulation of the cell surface expression and function of angiotensin II type 1 receptor by Rab1-mediated endoplasmic reticulum-to-Golgi transport in cardiac myocytes. *J. Biol. Chem.* 279, 41077–41084.)

Figure 15.2.

Overexpression of Rab1a specifically in the myocardium induces cardiac hypertrophy with increased ventricular weight (A) and cardiac gene expression (B). The data are obtained from six 6-week-old nontransgenic (NTG) and transgenic mice expressing Rab1a at a medium level. ANF, atrial natriuretic peptide; MHC, myosin heavy chain; SERCA, sarcoplasmic reticulum ATPase; PLB, phospholamban. * $p < 0.05$ versus NTG sibling control. (Figures adapted from 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. 2nd. (2001). Increased myocardial Rab GTPase expression: A consequence and cause of cardiomyopathy. *Circ.Res.* 89, 1130–1137.)