Inhibition of Rho family GTPases by Rho GDP dissociation inhibitor disrupts cardiac morphogenesis and inhibits cardiomyocyte proliferation

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SUMMARY

Studies of Rho GTPases in Drosophila and Xenopus suggest that Rho family proteins may play an important role in embryogenesis. A reverse genetic approach was employed to explore the role of Rho GTPases in murine cardiac development. Cardiac-specific inhibition of Rho family protein activities was achieved by expressing Rho GDIα, a specific GDP dissociation inhibitor for Rho family proteins, using the α-myosin heavy chain promoter, active at embryonic day (E)8.0 during morphogenesis of the linear heart tube. RhoA, Rac1 and Cdc42 activities were significantly inhibited, as shown by decreased membrane translocation of these proteins in the transgenic hearts. Transgenic F1 mice for each of two independent lines expressing the highest levels of the transgene, died around E10.5. Homozygotes of the middle copy-number lines, in which Rho GDIα expression was increased four-fold over normal levels, were also embryonic lethal. Cardiac morphogenesis in these embryos was disrupted, with incomplete looping, lack of chamber demarcation, hypocellularity and lack of trabeculation. Cell proliferation was inhibited in the transgenic hearts, as shown by immunostaining with anti-phosphohistone H3, a marker of mitosis. In addition, ventricular hypoplasia was associated with up-regulation of p21, an inhibitor of cyclin-dependent kinases, and with down-regulation of cyclin A, while cell survival was not affected. These results reveal new biological functions for Rho family proteins as essential determinants of cell proliferation signals at looping and chamber maturation stages in mammalian cardiac development.

Key words: Rho GTPases, Rho GDI, Cardiac morphogenesis, Cardiomyocyte proliferation, Mouse

INTRODUCTION

The heart is the first organ to form during embryogenesis. Cardiac organogenesis is characterized by the precise temporal and region-specific regulation of cell proliferation, migration, death, and differentiation (reviewed by Sucov, 1998; Fishman and Chien, 1997). In mouse embryos, cardiac mesoderm involutes during gastrulation and becomes specified when it reaches its position bilaterally in the anterior lateral plate mesoderm by 7.0 days post coitus (E7.0). As neurulation proceeds, the bilateral precardiac cells then migrate toward the midline and fuse to form the definitive heart tube by E8.0. Subsequent events of looping, septation, chamber maturation, and alignment with the vascular system give rise to the mature multichambered heart by E14.0 (Kaufman, 1992). Although heart formation has been well described morphologically and a molecular basis for cardiac specification is now emerging, the underlying molecular mechanisms for cell proliferation and cell movements in cardiac morphogenesis are not as well understood.

Rho GTPase family proteins, which include RhoA, Rac1 and Cdc42, may have an important role in early mouse heart morphogenesis as they control a wide variety of cellular processes such as cell morphology, motility, proliferation, differentiation and apoptosis (reviewed by Hall, 1994; Van Aelst and D’Souza-Schorey, 1997). Functional studies in Drosophila and Xenopus suggest that Rho GTPases control a variety of developmental processes associated with cell shape changes, cell adhesion and cell migration (Murphy and Montell, 1996; Barrett et al., 1997; Magie et al., 1999; Strutt et al., 1997; Wunnenberg-Stapleton et al., 1999). However, their potential roles in mammalian development, including cardiac morphogenesis, are unknown. A recent study has shown that cardiac-specific overexpression of RhoA results in sinus and atrioventricular nodal dysfunction and contractile failure in adult transgenic mice (Sah et al., 1999). In addition, cardiac-specific expression of constitutively active Rac1 has been shown to lead to either a lethal neonatal dilated cardiomyopathy or a resolving transient cardiac hypertrophy in juveniles (Sussman et al., 2000). However, roles for RhoA and Rac1 in embryonic heart development were not addressed.

Rho GDP dissociation inhibitors (Rho GDIs), endogenous
inhibitors of Rho GTPases, play an important role in regulating the biological activities of Rho GTPases (reviewed by Sasaki and Takai, 1998). Rho GTPases possess intrinsic GTPase activity and cycle between the inactive, cytoplasmic, GDP-bound and the active, membrane-associated, GTP-bound state. Rho GDIs possess at least two biochemical functions (Ueda et al., 1990; Isomura et al., 1991). First, they preferentially interact with the inactive, GDP-bound form of Rho family proteins, and prevent them from being converted to the active, GTP-bound form that is translocated to the membrane. Second, after the active GTP-bound form is converted to the inactive GDP-bound form at the membrane, Rho GDI forms a complex with it and translocates it to the cytosol. The Rho GDI family comprises at least three isoforms: Rho GDIα, β and γ. Rho GDIα is ubiquitously expressed (including heart) and binds to all of the Rho family proteins thus far examined, including RhoA, RhoB, Rac1, Rac2, and Cdc42 (Fukumoto et al., 1990; Leonard et al., 1992). Rho GDIβ is expressed exclusively in hematopoietic tissues (Lelias et al., 1993; Scherer et al., 1993) and Rho GDIγ is preferentially expressed in brain (Zalcman et al., 1996; Adra et al., 1997). The modes of activation and action of the Rho GTPases are quite different from those of Ras GTPases, since Rho GTPases are predominantly cytosolic and associated with Rho GDIs while Ras GTPases are constitutively located on the plasma membrane, and a Ras GDI has not yet been identified.

We employed a reverse genetic approach to explore the role of Rho family GTPases in murine cardiac development. Specific inhibition of Rho GTPases in cardiomyocytes was achieved by expressing Rho GDIα using the cardiac-specific α-myosin heavy chain (αMHC) promoter, which is activated during early cardiogenesis (Subramaniam, 1991). This approach was expected to disrupt signaling by all Rho GTPases concomitantly during heart tube looping, septation and chamber maturation. We observed that targeted inhibition of Rho GTPase signaling resulted in disruption of cardiac morphogenesis and reduced cardiomyocyte proliferation, thus elucidating a critical role for Rho family proteins in heart morphogenesis.

MATERIALS AND METHODS

Generation of transgenic mice

Myc-tagged bovine Rho GDIα cDNA (0.7 kb), kindly provided by Dr Yoshimi Takai (Ueda et al., 1990), was inserted between the 5.5-kb murine αMHC promoter kindly provided by Dr Jeffrey Robbins (Gullick et al., 1991) and 0.7-kb polyadenylation signal of human G-Csf. The Rho GDIα transgene was excised from the vector, purified and injected into the pronucleus of FVB/N zygotes. The resulting founder mice were screened for the presence of the transgene in tail DNA by PCR using an αMHC promoter-specific primer (5’-ACCTACGGACACCTTAC-3’) and a Rho GDIα-specific primer (5’-GGTAGCTAGTTGCCTGC-3’). Endogenous β-casein was also amplified as a control (Chang et al., 1998). The PCR results were then confirmed by Southern blot using a 1.4-kb fragment probe containing Rho GDIα cDNA and the polyadenylation signal of human G-CSF. The copy number of the transgene in each line was determined by quantitative nucleic acid blot analysis. Serial dilutions of mouse genomic DNA were made and applied to a nitrocellulose filter that was hybridized with the transgene probe. The signals on the filter were quantitated by PhosphorImager analysis (Molecular Dynamics) and compared with a set of calibration values obtained by application of serial dilutions of the transgenic vector. For PCR screening of mouse embryos, DNA was extracted from the yolk sac, and the transgene and the endogenous β-casein were amplified as described above.

Whole mount in situ hybridization of mouse embryos

Whole mount in situ hybridization of mouse embryos was carried out as described previously (Yamada et al., 1999). The antisense probe for the transgene was generated from the polyadenylation sequences of the transgene (human G-CSF cDNA). No signal was observed with a sense probe derived from the same DNA fragment (data not shown). The cardiac α-actin antisense probe was generated from a cDNA fragment containing the 3’UTR (Wei et al., 1998). Following color development, 10-μm sections were cut from paraffin-embedded whole mounts.

RT-PCR analysis

To compare endogenous gene expression in transgenic hearts versus nontransgenic hearts, RNA samples were prepared from a pool of ten hearts from transgenic or nontransgenic E9.5 embryos of the H2 founder line using TRIZOL (Gibco-BRL, Gaithersburg, Maryland). Two RNA samples were analyzed for each group (transgenic versus nontransgenic). First strand cDNA synthesis was carried out with the SuperScrip Preamplification System (Gibco-BRL) in a volume of 50 μl using 2.5 μg of RNA and 125 ng of oligo(dT)12-18. Three reverse transcription products from each RNA sample were pooled. PCR reaction was then carried out with 2 μl of first strand cDNA and one set of specific primers. For each primer set, two or three cycle-numbers were tested to be certain that PCR product accumulates within a linear range. GAPDH (Gapd; 16 to 19 cycles) was amplified as a control marker with primers as described (Chang et al., 1998). Other genes were amplified, each with a specific primer set: p21 (19 to 22 cycles), αMHC (16 to 19 cycles) and MLC2v (16 to 19 cycles) (Chang et al., 1998); Raldh2 (Aldh1a2; 28 to 30 cycles) (Ulven et al., 2000); atrial natriuretic factor (Anf/Nppa) (16 to 19 cycles) (Xu et al., 1999); Mef2c (22 to 25 cycles) (Martin et al., 1993); Gata4 (22 to 25 cycles) (Xu et al., 1999); cardiac α-actin (19 to 22 cycles), forward 5’TGAGATGTCCTCCTCTCTTA-3’, reverse 5’-GTACCAA-TGACTGATGAG-3’; dHAND (Hand2; 19 to 22 cycles), forward 5’TACGATGAGCCTGCTCTCTA-3’, reverse 5’-TCAAGGAGGCC-AGACGTGCTG-3’; eHAND (Hand1; 23 to 26 cycles), forward 5’CCGGCGAGAGAAGATTAAA-3’, reverse 5’TCAAATGACATGACGACTGC-3’; Nkx2.5 (19 to 22 cycles), forward 5’TGGCGTCGGGGACTTGAAC-3’, reverse 5’-AGGTTGATGGCATTGGAAAC-3’; Srff (22 to 25 cycles), forward 5’-CCGGCGACGACGCAACCT-3’, reverse 5’-CCCCTCTCCTTGGCTGGAGT-3’; cyclin A (Ccn2; 22 to 22 cycles), forward 5’TACGGCTTGGTCTGTGGACC-3’, reverse 5’-CAGGGTGCCACCAATAAG-3’.

Proliferation and apoptosis assays

E9.5 embryos were separated from their yolk sacs, which were used for determining their genotype as described above. Embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned (7 μm). The sections containing cardiac structures were selected for immunohistochemical analysis. Proliferation was assayed by examining histone H3 phosphorylation as a marker of mitosis, using a rabbit polyclonal antibody raised against the Ser10 phosphopeptide of histone H3 (Upstate Biotechnology, Lake Placid, NY) (Wei, Y. et al., 1998). Sections were deparaffinized, rehydrated, and incubated with 0.05% trypsin for 15 minutes at 37°C. Sections were then exposed to anti-phosphohistone H3 antibody (5 μg/ml), followed by incubation with fluorescein-conjugated goat anti-rabbit IgG (Molecular Probes) at 1:500 dilution. After several washes, sections were counterstained with DAPI (Molecular Probes) and mounted with Vectashield (Vector Laboratories). The identical overlapping images were used to quantify the number of...
phosphohistone H3-positive cells as a percentage of total number of cells (nuclei) within the subependymal cell layer. The data are expressed as mean ± s.e.m. Student’s t-test was used for data comparison, using a significance level of P<0.05. Apoptosis was assayed using the ApopTag kit according to the manufacturer’s instructions (Intergen Company). Sections were also counterstained with DAPI (Molecular Probes) and mounted with Vectashield.

**Immunoblot analysis of Rho GDIα and subcellular fractionation and immunoblot analysis of Rho family proteins**

Protein samples were prepared from a pool of 10 hearts from E9.5-12.5 embryos or from a single heart of adult transgenic or nontransgenic mice. Hearts were quickly removed, washed, minced into small pieces then frozen in liquid nitrogen and stored at −70°C. The tissue fragments were thawed and disrupted with a Polytron homogenizer at 4°C in lysis buffer (Wei et al., 1998). The debris was pelleted with a 400 g centrifugation for 10 minutes. The supernatant (homogenate) was used for western blot analysis of Myc-Rho GDIα (transgene), endogenous Rho GDIα or Rho GTPases. Proteins (50 μg) were electrophoresed on a 12% SDS-polyacrylamide gel, transferred to Immobilon membranes (Millipore), and probed with a mouse anti-Myc antibody (Oncogene Research Product), a rabbit anti-Rho GDI antibody (Santa Cruz Biotechnology), a mouse anti-RhoA antibody (Santa Cruz Biotechnology), a mouse anti-Rac1 antibody (Upstate Biotechnology) or a rabbit anti-Cdc42 antibody (Santa Cruz Biotechnology). After incubating with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody, blots were visualized by the enhanced chemiluminescence system (Amersham).

To separate cytosolic and membrane fractions, the tissue fragments were thawed and disrupted with a Polytron homogenizer at 4°C in lysis buffer without Triton X-100. The debris was pelleted with a 400 g centrifugation for 10 minutes, and the supernatant was further centrifuged at 100,000 g for 30 minutes at 4°C. The supernatant was saved as the cytosolic fraction. The pellet was suspended in lysis buffer with 0.1% Triton X-100 and saved as the membrane fraction. Proteins (50 μg) from each fraction were then analyzed by western blotting as described above.

**RESULTS**

**Increased cardiac expression of Rho GDIα resulted in embryonic lethality**

The Rho GDIα transgene under the control of the cardiac-specific αMHC promoter was used to generate six transgenic founder lines, with copy numbers ranging from 1 to 20 genomic equivalents (Fig. 1). From the two high-copy number founders, H1 and H2 (18 and 20 copies respectively), no transgenic pups were born, suggesting that these founder mice were mosaics and the F1 transgenic heterozygotes were embryonic lethal. Indeed, transgenic embryos were detected at E8.5, E9.5, E10.5 and E11.5. At E8.5, all F1 transgenic embryos were morphologically indistinguishable from nontransgenic littermates. In contrast, all E9.5 transgenic embryos were markedly growth-retarded with severe cardiac defects as compared with nontransgenic littermates (Fig. 2A). Some of the E10.5 and all of the E11.5 transgenic embryos were dead and partially resorbed (Fig. 2B,C). The onset of detectable developmental defects at E9.5 is in agreement with the expression of endogenous αMHC at E8.0.

All lower-copy number founders, L1, L2, M1 and M2 (1, 3, 6, 7 copies respectively), were able to generate F1 heterozygotes, which had no early embryonic phenotypic defects (Fig. 3A). Interestingly, we were unable to obtain homozygous pups from two middle-copy number lines (M1 and M2), suggesting that these homozygotes were embryonic lethal. Indeed, all homozygous embryos of the M2 line at E10.5 (n=5) were markedly growth-retarded with severe cardiac defects as observed in the F1 heterozygotes of two high-copy number founders (Fig. 3). However, the onset of the developmental defects in the homozygotes of the M2 line was detected at E10.5, delayed by 1 day compared with F1 heterozygotes of two high-copy number founders, most likely due to the difference in the expression level of the transgene in these defective hearts. In contrast, the homozygous mice from two low-copy number lines (L1 and L2) had no embryonic phenotypic defects. These observations indicated a close correlation between the embryonic-lethal phenotype of the transgenic mice and the copy number of the transgene.

**Increased cardiac expression of Rho GDIα inhibited Rho GTPase activity**

Expression of the transgene in F1 heterozygotes of two high-copy number founders (H1 and H2) at E9.5 was examined by whole-mount in situ hybridization (Fig. 4A). The transgene transcripts were only present in the heart of the transgenic embryos. The level of the transgene in the embryonic and adult hearts of the heterozygotes of M2 line was determined by western blot (Fig. 4B). The ratio of transgene expression to endogenous Rho GDIα was about 1.5:1 in the embryonic hearts (E12.5) compared with 6:1 in the adult hearts of these heterozygotes, consistent with the previously reported increase of αMHC promoter activity in adult hearts than in fetal hearts (Subramaniam et al., 1991). Based on the transgene expression level in the heterozygotes of the M2 line, the ratio of the transgene expression versus endogenous Rho GDIα in the embryonic heart of homozygotes was estimated to be about 3 to 1, quite sufficient to cause early embryonic lethality. In
addition, the ratio of transgene expression to endogenous Rho GDIα was about 1:1, 3:1 and 5:1 in the adult hearts of the heterozygotes of L1, L2, and M1 respectively (Fig. 4C). The endogenous level of Rho GDIα was similar in the transgenic hearts and in nontransgenic hearts, indicating that increased expression of Rho GDIα had no effect on the expression of endogenous gene.

Rho GDIα inhibits the activity of Rho family proteins, including RhoA, Rac1 and Cdc42. These Rho GTPases and Rho GDIα are expressed in the early developing heart (Fig. 5A). To obtain evidence that the activity of Rho family proteins was inhibited in the hearts of Rho GDI transgenic mice, we examined the subcellular localization of RhoA, Rac1 and Cdc42, because the inactive GDP- and active GTP-bound forms of Rho family proteins have preferentially cytosolic and membrane subcellular localizations, respectively. Given the limited amount of protein in the defective hearts of transgenic embryos (F1 heterozygotes of H1 and H2 lines, homozygotes of M2 line), which were markedly smaller than the nontransgenic hearts, subcellular distribution of Rho family proteins was examined in the adult hearts of the heterozygotes of M2 line. For all three Rho family proteins, their content in the membrane fraction was significantly decreased in the transgenic hearts versus nontransgenic littermates (Fig. 5B), indicating that their activity was inhibited in the transgenic hearts by increased Rho GDIα expression. In contrast, the content of these Rho family proteins in the membrane or cytosolic fraction was similar in the skeletal muscle samples from transgenic mice versus nontransgenic mice. Interestingly, the content of RhoA, Rac1 and Cdc42 in the cytosolic fraction, which predominates the membrane associated form, was markedly increased in the transgenic hearts compared to the nontransgenic littermates (Fig. 5B), suggesting a negative feedback mechanism for gene regulation of Rho family proteins.

**Increased cardiac expression of Rho GDIα disrupted cardiac looping and ventricular maturation**

At E9.5, all F1 transgenic embryos from H1 and H2 founders showed severe defects in cardiac morphogenesis (Fig. 1A). In normal mouse embryos, the bilateral heart primordia migrate to the ventral midline and fuse with each other to form a single heart tube by E8.0. By E9.5, the atrial portion of the heart shifts dorsally and to the left, septations between the common atrium and primitive ventricle and between the primitive ventricle (the future left ventricle) and the bulbus cordis (the future right ventricle) become prominent, principally due to formation of the endocardial cushion. Early trabeculation in the ventricular chambers and thickening of the ventricular chamber wall become apparent at this stage. All of these developmental processes including looping, septation and ventricular maturation were severely affected in the transgenic embryos (Fig. 6). The transgenic hearts remained linear, and were dilated and surrounded by a distended pericardium (Fig. 1A; Fig. 6B,D,F). The bulboventricular groove, which demarcates
the axis of the future interventricular septum, was absent (Fig. 6D,F). Histological analysis showed very poor trabeculation, absence of endocardial cushion and a thin ventricular chamber wall (Fig. 6H,J). Thus, embryonic heart failure with cardiac dilation, pericardial effusion, hypocellularity and poor trabeculation was most likely the primary cause of embryonic lethality at this stage. However, histological analysis did not detect any defect of ventricular maturation in the M2 line heterozygous embryos at E10.5 (Fig. 6K,L), consistent with their apparent normal phenotype at this stage (Fig. 3A).

Increased cardiac expression of Rho GDIα did not enhance apoptosis, but reduced proliferation of cardiomyocytes

In order to determine the cause of arrest of heart development in the F1 heterozygotes from H1 and H2 founders, we first examined whether enhanced apoptosis of cardiomyocytes occurred in these embryos. Tissue sections from three nontransgenic and three transgenic littermates at E9.5 were stained with TUNEL reagent. No enhancement of apoptosis in the transgenic hearts was detectable, as both nontransgenic and transgenic hearts had no more than one or two positive cells per section (data not shown).

To determine if the reduced cell number was due to altered proliferation of the myocytes, tissue sections from three nontransgenic and three transgenic littermates at E9.5 were stained for phosphorylated histone H3 on Ser10, an established

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Fig. 3. Homozygotes of the middle-copy number line M2 were embryonic lethal. (A) Lateral view of E10.5 nontransgenic, heterozygous and homozygous embryos. All heterozygous embryos were phenotypically indistinguishable from the nontransgenic littermates. All E10.5 homozygous embryos were markedly growth-retarded with severe cardiac defect. (B) Genotypic and phenotypic analysis of embryos from heterozygous matings, as described in the legend of Fig. 2.

Fig. 4. Cardiac-specific expression of Rho GDIt transgene. (A) Whole-mount in situ hybridization of nontransgenic (NTG) and transgenic F1 embryos at E9.5 from the H2 founder (TG) with an antisense probe generated from the polyadenylation sequences of the transgene (human G-CSF cDNA). No signal was observed with a sense probe (data not shown). (B) Expression of the transgene was markedly increased in the adult mouse heart. Western blot analysis was performed with cardiac proteins extracted from nontransgenic and heterozygotes of the M2 founder line at E12.5 or 4 weeks after birth. The blot was probed with an anti-Rho GDIt antibody recognizing both endogenous Rho GDIt and the transgene. (C) The expression level of the transgene was proportional to the copy number. Western blot analysis was performed with cardiac proteins extracted from adult nontransgenic and transgenic heterozygotes of L1, L2, M1 and M2 founder lines using an anti-Rho GDIt polyclonal antibody. An anti-Myc antibody recognizes only the transgene (data not shown).
marker for chromosome condensation during mitotic prophase in animal cells (Wei, Y. et al., 1998). In normal embryos at E9.5, the myocardium is subdivided into a peripheral compact zone and an inner trabecular zone. The rate of cell division decreases from the periphery to the inner zone. In transgenic embryos, the inner trabecular zone was absent. The transgenic heart sections displayed significant reduction in the number of phosphorylated histone H3 cells versus nontransgenic heart sections (Fig. 7A,B) and the vast majority of the phosphorylated histone H3 cells were located immediately beneath the epicardium (the subepicardial cell layer) in both nontransgenic and transgenic embryos (Fig. 7C). In tissue sections from nontransgenic embryos, 4.7±1.2% of subepicardial cell nuclei were stained positive, while 1.9±0.5% subepicardial cell nuclei were positive in the transgenic embryos (Fig. 7E). In addition, histone H3 phosphorylation in other tissues of transgenic embryos was markedly more abundant than in the heart (Fig. 7B). These observations indicated that decreased cell proliferation caused hypocellularity in transgenic hearts.

**Increased cardiac expression of Rho GDIα induced up-regulation of p21 and down-regulation of cyclin A**

To explore the mechanisms by which cell proliferation was affected in the transgenic hearts, semi-quantitative RT-PCR analysis of cell cycle regulators was performed using RNA extracted from E9.5 transgenic and nontransgenic hearts of the H2 founder (Fig. 8). RhoA has been reported to repress the expression of p21 (Olson et al., 1998; Adnane et al., 1998), the prototype of CDK inhibitors involved in G1 phase arrest, and
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Inhibit CDKs 4, 6 and 2. In the transgenic hearts, p21 expression was up-regulated (about 2.1 fold), consistent with inhibition of RhoA by Rho GDIα. Cyclin A is an essential activator of CDKs 1 and 2, controlling the normal progression through the S phase and G2/M transition. Both RhoA and Cdc42 have been shown to induce cyclin A expression in fibroblasts or tumor cells (Udagawa and McIntyre, 1996; Philips et al., 2000). In the transgenic hearts, expression of cyclin A was down regulated (about 41%), in agreement with inhibition of RhoA and Cdc42 by Rho GDIα. These results together indicated that ventricular hypoplasia in the transgenic hearts was due to a defect in cell proliferation which was associated with up-regulation of the CDK inhibitor p21 and down-regulation of cyclin A.

Effect of increased cardiac expression of Rho GDIα on the expression of cardiogenic factors

The developmental defects of Rho GDIα transgenic embryos were similar to those of mouse embryos with null mutations in Nkx2.5 (Lyon et al., 1995), MEF2C (Lin et al., 1997), dHAND (Srivastava et al., 1997), eHAND (Riley et al., 1998) or Radlh2 (Niederreither et al., 2001). By semi-quantitative RT-PCR analysis using RNA extracted from E9.5 transgenic and nontransgenic hearts of the H2 founder, we observed that the expression level of Nkx2.5 and MEF2C was not reduced in Rho GDIα transgenic hearts, while the expression level of Raldh2, dHAND, and eHAND was reduced by 29%, 24% or 11%, respectively. Lowered expression level of each of these factors alone does not appear to be able to induce developmental defects observed in Rho GDIα transgenic embryos as the heterozygous knockout embryos of each of these factors were phenotypically normal. We also examined expression of several other cardiac contractile or regulatory genes that were implicated in heart development. Expression of these genes was either not obviously affected (ANF and cardiac α-actin) or slightly down regulated (SRF, Gata4 and MLC2v). Increased expression of Rho GDIα may modulate the expression of a subset of cardiogenic factors such as Raldh2, dHAND, GATA4 and MLC2v, by either direct and/or indirect mechanisms through its effect on cardiomyocyte proliferation.
DISCUSSION

Herein, we demonstrated that the Rho family proteins are required for cardiac looping and chamber maturation in a mammalian system. Cardiac-specific inhibition of Rho family proteins by increased expression of Rho GDIα resulted in cardiac morphogenic defects detected at E9.5, including defective looping, pericardial effusion, dilation, poor trabeculation, impaired chamber demarcation, absence of endocardial cushion and hypocellulararity. Our study also provided the first evidence for a role of Rho family proteins in control of the cardiac cell cycle. Cell proliferation in the ventricular wall of Rho GDIα transgenic heart was significantly inhibited, with up-regulation of p21, a CDK inhibitor, and down-regulation of cyclin A.

A role for Rho family proteins in cell cycle control has been extensively studied using in vitro culture systems, where Rho proteins were required for G1 cell cycle progression (reviewed by Van Aelst and D’Souza-Schorey, 1997). It is still unclear whether the effect of Rho proteins on cell proliferation is due to their effects on the actin cytoskeleton or to more direct effects on gene transcription. During normal cardiac development, progressive withdrawal of cardiac myocytes from the cell cycle is associated with up-regulation of p21 and down-regulation of cyclin A (Parker et al., 1995; Yoshizumi et al., 1995) (reviewed by MacLellan and Schneider, 2000). Our study indicated that Rho family proteins were required for cardiac cell proliferation and were involved in repressing p21 expression and in inducing cyclin A expression during looping and ventricular maturation. Interestingly, up-regulation of p21 was also observed in the transgenic hearts expressing a constitutively active mutation of ALK5, a type I TGFβ receptor, and both looping morphogenesis and chamber maturation were disrupted in activated ALK5 transgenic hearts at E9.5 (Chang et al., 1998). Thus, cell cycle regulators appear to be the common targets of Rho and TGFβ signaling pathways in cardiac morphogenesis. A hypoplastic ventricular chamber was also a characteristic defect of mouse null mutants for RXRα (Suvoc et al., 1994; Kastner et al., 1994), N-myc (Moens et al., 1993), WT-1 (Kreidberg et al., 1993), TEF-1 (Chen et al., 1994), gp130 (Yoshida et al., 1996) or bARK1 (Jaber et al., 1996) (lethal at E13-16). However, looping morphogenesis was not disrupted in these mutant mice, suggesting that Rho family proteins regulate cardiac morphogenesis via an independent regulatory pathway.

In addition to inhibition of cell proliferation, increased expression of Rho GDIα most likely interrupted other morphogenetic processes involved in heart tube looping. It is believed that looping of the embryonic heart is not a consequence of growth per se, but rather an intrinsic morphogenic property of the heart tube, partly linked to the left-right asymmetric expression of signaling molecules and extracellular matrix proteins (reviewed by Kathiriya and Srivastava, 2000). Rho family proteins may be involved in cell shape changes and cell adhesion by regulating interactions between cardiomyocytes and asymmetrically-expressed extracellular matrix proteins during heart looping. The heart tube looping defect of Rho GDIα transgenic embryos was also similar to those of mouse embryos with null mutations for Nkx2.5 (Lyon et al., 1995), MEF2C (Lin et al., 1997), dHAND (Srivastava et al., 1997), eHAND (Riley et al., 1998) or Raldh2 (Niederreither et al., 2001). We observed that the expression level of these cardiogenic factors was not dramatically affected in Rho GDIα transgenic hearts (up to 29%), and the heterozygous knockout embryos of each of these factors were previously shown to be phenotypically normal. Therefore, the interruption of looping in Rho GDIα transgenic embryos cannot be ascribed merely to the level of the expression of these factors. However, although the reduced expression of each of the factors was not sufficient to induce developmental arrest, the combined reduction in their expression levels may contribute, in part, to the morphogenic defects observed in Rho GDIα transgenic embryos.

In addition to the changes in cell cycle regulators, expression of Raldh2, MLC2V and dHAND was down-regulated by more than 20% in Rho GDIα transgenic hearts. It remains unclear whether Rho family proteins directly regulate transcription of these genes, or whether such down-regulation is secondary to the morphogenic defects of the hearts. However, RhoA regulates SRF-dependent gene activity in cultured fibroblasts (Hill et al., 1995), skeletal myoblasts (Wei et al., 1998), smooth muscle cells (Mack et al., 2001) and terminally differentiated cardiomyocytes (Wei et al., 2001a). We have also observed that inhibition of Rho kinases, downstream effectors of RhoA, before the onset of cardiomyocyte differentiation caused precocious activation of the cardiac α-actin gene in the bilateral cardiogenic regions of cultured chick embryos (Wei et al., 2001b). However, expression of these cardiac genes was affected only minimally in the Rho GDIα transgenic hearts, in which inhibition of Rho signaling occurred after the onset of cardiomyocyte differentiation. It is thus possible that RhoA-dependent signaling to SRF contributes to the regulation of cardiac genes in a stage-dependent manner: possibly Rho signaling may inhibit cardiomyocyte differentiation in precardiac cells during proliferation and migration, but they subsequently enhance cardiac gene expression in terminally differentiated cardiomyocytes which have an organized sarcomeric structure.

Our study also suggested that Rho GDIα expression must be under tight control during cardiac development as increasing its expression to four times normal levels caused severe defective cardiac morphogenesis (M2 homozygotes). However, a basal level of RhoGDIα is not required for early heart development as mice lacking Rho GDIα were initially viable and normal in appearance, but showed progressive impairment of kidneys and reproductive organs in adult mice (Togawa et al., 1999). Interestingly, although the heterozygotes of middle copy lines (M1, M2) had no detectable early embryonic phenotype, they developed an adult cardiac phenotype such as cardiac hypertrophy associated with abnormal cardiac functions (L. W., unpublished observations), most likely due to increased transgene expression in the adult hearts compared with embryonic hearts (6-fold versus 1.5-fold relative to the endogenous level).

It is also important to note that Rho GDIα expression level was unchanged during cardiac development and increased expression of Rho GDIα had no effect on the expression of the endogenous gene. In contrast, increased expression of Rho GDIα markedly up-regulated expression of RhoA, Rac1 and Cdc42, most likely due to inhibition of Rho GTPase activities, which in turn induced their gene expression through a negative feedback regulatory mechanism. These observations suggested...
that the regulation of the ratio of Rho family proteins to their endogenous inhibitor is an important mechanism controlling the activities of Rho family proteins and this ratio is regulated through modulating the expression level of Rho family proteins while the level of Rho GDIX remains stable. Further studies will be required to determine the involvement of each protein of the Rho family and the roles of downstream effectors of Rho family proteins in cardiac morphogenesis, and how Rho family members regulate their own gene expression.

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