**Abstract**—Ras-related GTPase (Ral) is converted to the GTP-bound form by Ral GDP dissociation stimulator (Ral-GDS), a putative effector protein of Ras. Although a number of studies indicate that Ras induces cardiac hypertrophy, the functional role of Ral-GDS/Ral signaling pathway is as yet unknown in cardiac myocytes. We investigated the role of the Ral-GDS/Ral pathway in cardiac hypertrophy. Transfection of Ral-GDS and constitutively active mutant of Ral (RalG23V) in cultured rat neonatal myocytes stimulated promoter activity of c-fos (5.4-fold and 2.6-fold, P<0.01), α-skeletal actin (2.7-fold and 2.1-fold, P<0.01), and β-myosin heavy chain–luciferase (2.8-fold and 2.3-fold, P<0.01). Ral-GDS–induced or RalG23V–induced promoter activation was increased synergistically with activated Ras (RasG12V). Dominant-negative mutant of Ral (RalS28N) partially inhibited RasG12V induced promoter activation.

Cardiac myocytes transfected with RalG23V showed increased cell size compared with nontransfected or vector-transfected cells (2.1-fold, P<0.01). Cardiotrophin-1 (CT-1) upregulated Ral-GDS mRNA expression and induced Ral activation. CT-1–induced Ral-GDS mRNA expression was inhibited by overexpression of the dominant-negative mutant of STAT3. Moreover, Ral activity was elevated in hypertrophied hearts (2.1-fold, P<0.01) by mechanical stress in association with increased CT-1 expression and signal transducer and activator of transcription 3 (STAT3) phosphorylation in the rat aortic banding model. Ral-GDS/Ral pathway is involved in a wide range of gene expressions and is activated by hypertrophic stimuli in vitro and in vivo. SAT3 may play a key role in Ral-GDS expression and Ral activation. Our data provide evidence that the Ral-GDS/Ral signaling pathway is a link to the process of cardiac hypertrophy. (Hypertension. 2003;41:956-962.)

**Key Words:** myocytes ■ hypertrophy ■ signal transduction ■ stress ■ gene expression

Cardiac hypertrophy is an adaptive response of the heart that occurs in various cardiovascular diseases; however, sustained hypertrophy is a leading predictor of future heart failure. In response to mechanical stress or neurohumoral stimuli, individual cardiomyocytes undergo hypertrophic growth. This is characterized by an increase in cell size and protein synthesis, induction of immediate early genes (eg, c-fos, c-jun, and egr-1), and reexpression of embryonic genes (eg, atrial natriuretic factor [ANF], skeletal α-actin, αSKA, β-myosin heavy chain [βMHC]).

Ras, a member of the small GTP-binding proteins, has been shown to play an important role in cardiac hypertrophy. Expression of constitutively active Ras in cultured neonatal rat ventricular myocytes or cardiac overexpression of active Ras in transgenic mice induces hypertrophy. The most thoroughly characterized effector molecules of Ras are Raf, phosphatidylinositol 3-kinase (PI3 kinase), and Ras-specific guanine nucleotide exchange factors (Ral-GEFs). Raf, a serine/threonine kinase, regulates the activity of mitogen activated/extracellular signal-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK). That pathway is well known to mediate cardiac hypertrophy. PI3-kinase has been also reported to induce hypertrophy through its several effector molecules, including Akt, p70-S6 kinase, and GSK-3β.

Although a number of studies have revealed that Raf or PI3-kinase mediates cardiac hypertrophy, the role of Ral-GEFs signaling pathway is still unclear in the heart. Ral-GEFs, including Ral-GDS, guanine nucleotide exchange factor-like factor (Rlf), RGL1, and RGL2, are directly activated by Ras and form the active GTP-bound state of Ral. Ral is one of the closest relatives of Ras. Recent reports demonstrate the crucial role of Ras in cellular transformation, differentiation, vesicle transport, and receptor endocytosis. Activated Ras binds to the Ras-binding domain of Ral-GEFs and localizes it to the membrane, where its target...
molecule Ral is located. However, indirect evidence has suggested that Ras binding may not be sufficient to activate Ral-GEFs under all conditions. Recently, a novel pathway and function of Ral-GEFs have been shown. One is that Ral-GDS expression is induced by the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3)-mediated pathway with LIF or IL-6 stimulation and leads to Ral activation in mouse myeloid cells. Another is that RIF induces ANF promoter activation in rat ventricular myocytes, providing the possibility that Ral-GEFs may mediate myocardial hypertrophy.

In the current study, we examined the role of Ral-GDS and its downstream molecule, Ral, in cardiac hypertrophy. Our findings suggest that Ral-GDS/Ral pathways are involved in the process of cardiac hypertrophy.

Methods

Materials and Cell Culture
Neonatal rat ventricular myocytes were prepared from hearts of 1- to 2-day-old Sprague-Dawley rats (Japan SLC Co Ltd) as described. Cells were distributed at a density of 18 × 10^4 cells per 60-mm dish for reporter gene experiments, Northern hybridization and Ral activation assay, or 2 × 10^5 cells per well for immunohistochemistry in DMEM/F-12 supplemented with 5% CS for 24 hours. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine. For cell stimulation, recombinant human cardiomyosin-I (PeproThch EC Ltd) was used. FITC-277, a specific farnesylase inhibitor, was from CalBiochem. Other materials and chemicals were obtained from commercial sources.

Plasmids and Transient Transfection
The c-fos luciferase reporter gene (nucleotides −404 to +41) was constructed as described. αSKα (nucleotides −394 to +24) and βMHC reporter genes (nucleotides −667 to +38) were generous gifts from Prof Michael D. Schneider (Center for Cardiovascular Development, Baylor College of Medicine, Dallas, Tex). pCGN/RalG23V (constitutively active form in which Gly23 was changed to Val), pCGN/RalG2S (dominant-negative form in which Ser28 was changed to Asn), and pCGN/Ral-GDS were constructed as described. Ral mutant and Ral-GDS were tagged with HA-epitope at their N termini. Cells were transfected with the indicated cDNAs by the calcium-phosphatase coprecipitation method. The following amounts of cDNAs were used: luciferase reporter plasmids (4 μg each); mutant Ral, Ral-GDS (8 μg each); β-galactosidase plasmid (4 μg each); and variable amounts of pCGN vector to adjust total DNA. Cells were harvested in the lysate for 48 hours after transfection. Luciferase activity was normalized to both β-galactosidase activity and protein content.

Immunofluorescence
Cells were transfected with pCGN vector or pCGN/RalG23V and 48 hours after transfection were fixed in 4% formaldehyde and permeabilized in 0.2% Triton X-100. Cells then were incubated with polyclonal goat antiserum against cardiac troponin I-C (1:100 in 1% BSA; Santa Cruz Biotechnology, Inc). Texas Red conjugated anti-goat IgG (red fluorescence, EY Labs) was used as the secondary antibody. For staining of HA, cells were incubated with mouse anti-HA antibody (1:500 in 1% BSA; Santa Cruz Biotechnology, Inc). FITC-conjugated anti-mouse IgG (green fluorescence) was used as the secondary antibody. The areas stained by troponin I-C, which is exclusively expressed in cardiac myocytes, were quantified as myocardial cell surface areas by an NIH image analyzer. The double-stained cells were recognized as the transfected myocytes. Transfection efficiency was ~1% to 2%. Two independent researchers measured the cell surface areas for nontransfected cells (n=100), pCGN vector-transfected cells (n=30), and pCGN/RalG23V transfected cells (n=30). Interobserver difference was <10%. The F-actin was detected with the use of FITC-conjugated phalloidin. All microscopic investigations were performed by a confocal laser scanning microscope.

Adenoviruses and Gene Transfer
Adenoviral vectors encoding a dominant-negative mutant of STAT3 (the substitution of Thr705 to Phe) or bacterial β-galactosidase (LacZ) was constructed as described. One day after plating, cardiac myocytes were infected with adenovirus at a multiplicity of infection of 25:1 and incubated for 2 hours. Cardiac myocytes were cultured with the serum-depleted medium for an additional 2 days. The efficiency of the expression examined by the LacZ gene expression in cultured cardiac myocytes is constantly >90% by this method.

Northern Blot Analysis
After 48 hours in the serum-depleted medium, cells were stimulated with various hypertrophic reagents. Total RNA (15 μg) was separated by 1% formaldehyde-agarose gel electrophoresis, blotted to a filter, and hybridized with 32P-labeled Ral-GDS probe. Loading conditions were determined by ethidium bromide staining of 28S ribosomal RNA. Blots were analyzed by laser densitometry.

Immunoblotting Analysis
Whole-cell extracts were prepared as described. Cells were harvested in the lysis buffer containing 50 mmol/L Tris, 5 mmol/L MgCl₂, 150 mmol/L NaCl, 1% Nonidet, 1 μg/mL leupeptin, and 1 mmol/L PMSF. An equal amount of cell lysate was electrophoresed on 12% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and hybridized with antibodies to either RalA, STAT3 (Santa Cruz Biotechnology, Inc), phosphospecific STAT3 (Cell Signaling), or human cardiotrophin-1 (PeproThch EC Ltd).

Ral Activation Assay
The activity of Ral was measured as described. Cultured cells or rat hearts were lysed and incubated with GST-RIP1 protein bound to agarose beads for 1 hour at 4°C. To produce GST-RIP1, DNA fragment corresponding to amino acids 397 to 518 of RIP1 was subcloned to pGEX-2T (Amersham Pharmacia Biotech), respectively. The beads were subjected to SDS–gel electrophoresis followed by hybridization with RalA antibody.

Animal Model
Aortic banding was performed as described. Twelve Sprague-Dawley rats (8 weeks old; weight, 260 to 280g; all male) were subjected to either an abrupt pressure overload by aortic banding (6 rats) or to sham operation (6 rats). Rats were anesthetized with isoflurane, and the aortas were constricted with 21-gauge wires, which were removed to generate defined constructions thereafter. One week after surgery, the hearts were removed. Left ventricles were frozen in liquid nitrogen.

Statistical Procedure
The results are shown as mean±SEM. Data were analyzed by 1-way ANOVA, followed by a post hoc comparison among group means. An unpaired Student t test was used when 2 groups were compared. Values of P<0.05 were considered to be statistically significant.

Results

Effect of Ral-GDS and Active Ral on c-fos, α-SKα, and β-MHC Promoter Activity in Cardiac Myocytes
First, we investigated the effect of Ral-GDS and Ral on early gene expression in cardiac myocytes. Transfection of Ral-GDS induced c-fos–luciferase expression in cardiac myocytes compared with control vector-transfected cells (5.4-fold,
RalG23V, an active Ral, also stimulated c-fos-luciferase expression (2.6-fold, \(P<0.01\)) (Figure 1A). RalS28N, an inactive Ral, did not (1.0-fold). Next, we examined the embryonic gene expressions in cardiac myocytes, using αSkA- and βMHC-luciferase. Ral-GDS induced αSkA- and βMHC-luciferase expressions in cardiac myocytes (2.7-fold and 2.8-fold, \(P<0.01\)) (Figures 1B and 1C, respectively). RalG23V also stimulated αSkA- and βMHC-luciferase (2.1-fold and 2.3-fold, \(P<0.01\), respectively).

### Ral-GDS and Active Ral Increase c-fos, αSkA, and βMHC Promoter Activity Synergistically With Active Ras

Next, we examined how Ral-GDS and Ral influence gene expressions under Ras activation. RasG12V, an active Ras, induced c-fos promoter activation (17.9-fold). Cotransfection of Ral-GDS and RasG12V synergistically elevated c-fos-luciferase activity (36.2-fold, \(P<0.01\)), and RalG23V with RasG12V also stimulated c-fos expression (31.2-fold, \(P<0.01\)) (Figure 1D). RalN28S partially inhibited RasG12V-induced c-fos–luciferase activity (9.7-fold, \(P<0.05\)); however, it was not a complete inhibition (Figure 1D). Similar results were shown for the embryonic genes. Cotransfection of Ral-GDS and RasG12V stimulated the αSkA-luciferase (5.0-fold) and βMHC-luciferase activity (8.2-fold). RalG23V also induced αSkA-luciferase activity (5.5-fold) and βMHC-luciferase activity (7.3-fold) synergistically with RasG12V (Figures 1E and 1F). The Ral-GDS/Ral pathway is involved in Ras-dependent gene expression in cardiac myocytes.

### Transfection of Constitutively Active Ral-Induces Cardiac Hypertrophy

We then examined whether active Ral can induce cardiac hypertrophy. Cells were transfected with pCGN vector or...
pCGN/RalG23V. The areas stained by troponin I-C (red fluorescence) were quantified as myocardial cell surface areas. Cells double-stained by both troponin I-C and HA (orange fluorescence) were recognized as transfected myocytes. The cardiac myocytes transfected with RalG23V displayed the increased cell surface area compared with non-transfected cells or vector-transfected cells (2.1-fold, \( P<0.01 \)) (Figures 2A and 2B). We also assessed sarcomeric actin reorganization, another important phenotypic change associated with myocyte hypertrophy. RalG23V-transfected myocytes induced an increase in the assembly and organization of sarcomeric actin compared with vector-transfected cells (Figure 2C).

CT-1 Induces RalGDS mRNA Expression and Activated Ral in Cardiac Myocytes

We then investigated which hypertrophic factors induce Ral-GDS mRNA expression or Ral activation in cardiac myocytes. We examined Ral-GDS mRNA expression with various stimuli. Cardiomyocytes were treated with physiological concentrations of phenylephrine (PE) \((1 \times 10^{-6} \approx 1 \times 10^{-4} \text{ mol/L})\), angiotensin II (Ang II) \((1 \times 10^{-10} \approx 1 \times 10^{-8} \text{ mol/L})\), or CT-1 \((1 \times 10^{-11} \approx 1 \times 10^{-9} \text{ mol/L})\). Ral-GDS mRNA was clearly induced by CT-1 stimulation in a concentration-dependent manner (Figures 3A and 3B). However, PE or Ang II, G-protein coupled receptor (GPCR) agonists, did not upregulate Ral-GDS mRNA expression (Figure 3A). Ral-GDS mRNA induction was elevated 1 hour after stimulation with CT-1, and the expression level reached peak at 2 to 3 hours (Figure 3C). CT-1, one of interleukin-6 family cytokines, has been reported to induce myocardial hypertrophy through the gp130/JAK/STAT pathway. To investigate whether Ral-GDS mRNA induction is dependent on STAT3 activation, we performed adenovirus-mediated gene transfer of LacZ or dominant-negative mutant STAT3. Overexpression of dominant-negative mutant of STAT3 reduced Ral-GDS mRNA induction by CT-1 compared with LacZ-infected cells (Figure 3D). To examine whether Ras activity is required for Ral-GDS induction by CT-1, we used FTI-277, a specific farnesylation inhibitor. Pretreatment with 1 \( \mu \text{mol/L} \) FTI-277 did not suppress the induction of Ral-GDS (Figure 3E), although this concentration of FTI-277 has been widely used for inhibition of Ras activation.18

Next, we investigated whether the enhanced expression of Ral-GDS induced Ral activation. As shown in Figure 3F, RalA was activated at 3 hours after stimulation by CT-1, corresponding to Ral-GDS induction.

Ral Activity Is Elevated in Hypertrophied Hearts Induced by Pressure Overload

We also determined whether activation of Ral occurred in the cardiac hypertrophic process in vivo by using the rat aortic banding model. Left ventricles were isolated from sham-operated or aortic banded rats. Activation assay demonstrated that the level of RalA activity in the heart from the banding group was 2.1-fold higher than sham-operated rats 1 week after surgery \(( P<0.01 \)) (Figures 4A and 4B). Immunoblots with RalA antibody showed no difference in the expression level of RalA. Since one mechanism underlying the Ral activity change in cardiac hypertrophy is Ral-GDS activation.
activation might be CT-1 expression and STAT3 phosphorylation. Western blot analysis was performed. Figure 4C shows the increases in CT-1 expression and STAT3 phosphorylation level in the hypertrophied hearts. These results suggest that Ral activation is associated with the cardiac hypertrophic process in vivo and that it might be dependent on STAT3 phosphorylation.

Discussion
Small GTP-binding proteins regulate various cell functions, including cell growth, differentiation, gene expression, vesicle transport, and cell morphological changes. Ras, RhoA, and Rac have been clarified to play a pivotal role in cardiac hypertrophy. Ral, another small GTP-binding protein, was originally identified on the basis of sequence similarity to Ras, displaying 55% amino acid sequence homology. Activated Ral can contribute to the specific biological effects induced by Ras, depending on the cell types. In this study, we investigated whether Ral and Ral-GDS are involved in cardiac hypertrophy and obtained several results as follows. (1) Ral-GDS and constitutively active Ral induced promoter activation of early and embryonic genes in neonatal rat ventricular myocytes synergistically with RasG12V. Dominant-negative Ral partially inhibited RasG12V-induced promoter activation. (2) Transfection of constitutively active Ral resulted in increased cell size of myocytes in association with sarcomeric actin reorganization. (3) Stimulation with CT-1 upregulated Ral-GDS expression and induced Ral activation. (4) Ral was activated in the heart by mechanical stress in the rat aortic banding model. This is the first report showing that Ral-GDS and Ral link to cardiac hypertrophic process.
involved in active Ras-mediated gene expressions, although some other mediators such as Raf and PI-3 kinase also might be involved. Since activated Ras is localized to the membrane, there must be some downstream molecules that mediate gene expressions. Several studies have demonstrated that the Ras-GDS/Ral pathway is involved in gene expressions, such as c-fos and NF-κB, or phosphorylation of transcriptional factors such as c-jun and AFX. Although the signals mediating molecules or pathways are still not elucidated, Post et al. reported that SRE was the necessary element for the ANF promoter activation induced by Ral. Our luciferase reporter genes also contain SRE; therefore, there is the possibility that this region of the promoter is crucial for the Ras-GDS/Ral signaling pathway. Further studies are necessary for understanding the mechanisms of gene expression by the Ras-GDS/Ral signaling pathway.

In this study, transfection of constitutively active Ras induced an increase in myocardial cell size and sarcomeric actin reorganization. Ral links to changes in cellular morphology. The dominant-negative mutant of Ral blocks developmental shape changes in Drosophila. Otherwise, activated Ral binds to filamin and induces filopodial cytoskeleton in fibroblasts; therefore, Ral-filamin interaction may influence regulation of actin cytoskeleton and induce cellular hypertrophy. Although we could show the increased cell surface area by RalG23V-transfected cells, we could not determine other phenotypic changes associated with hypertrophy, such as protein synthesis. However, RalG23V-induced sarcomeric actin reorganization and promoter activation of early and embryonic genes suggest the involvement of Ras-GDS/Ral activation in the process of cardiac hypertrophy. Besides Ral, a variety of small G proteins such as Ras and RhoA have been shown to be involved in cardiac hypertrophy. In the current study, active Ras elevated promoter activities more greatly compared with active Ral, but the relative importance of Ral among small G proteins on overall hypertrophic process was not defined, and its elucidation waits for further studies.

We then examined whether Ras-GDS mRNA is induced by various hypertrophic stimuli and found that CT-1 upregulated Ras-GDS mRNA expression and induced Ral activation. However, GPCR agonists such as PE and Ang II did not induce Ras-GDS mRNA expression. CT-1 is a potent hypertrophic factor identified as a member of the IL-6-type cytokine family, which activates the JAK/STAT3 pathway. A recent report demonstrated that STAT3 regulates Ral activation through induction of Ras-GDS expression by LIF or IL-6 stimulation in mouse myeloid cells. In our study, overexpression of dominant-negative mutant STAT3 but not FTI-277 reduced Ras-GDS mRNA induction on CT-1 stimulation. Since Ral activation induced by CT-1 was a late response, 3 hours after stimulation, the expression level of Ras-GDS might be one of the regulating mechanisms of Ral activity. We revealed that Ras-GDS induction was dependent on STAT3 activation and subsequently contributed to Ral activation in cardiac myocytes. For CT-1-induced activation of the Ras-GDS/Ral pathway, STAT3 rather than Ras might be important. We speculate that Ras activation is not always necessary for the Ras-GDS/Ral pathway. Indeed, several reports have suggested that Ras binding may not be sufficient to activate Ras-GEFs and that Ras proteins can be activated by
GEFs that do not contain Ras-binding domains. Although we could show that the Ral-GDS/Ral pathway is involved in a wide range of gene expression and is associated with hypertrophic changes, the role of Ras or STAT3 on the Ral-GDS/Ral pathway needs to be investigated further.

We demonstrated that Ral was activated in hearts by mechanical stress in the rat aortic banding model, although the total expression level of Ral was not changed. These lines of evidence imply that active Ral is involved in the process of cardiac hypertrophy. It has been reported that CT-1 induction is elevated in the hypertrophied heart in several animal models. In our model, CT-1 induction was also elevated, and phosphorylated STAT3 was increased in the hypertrophied hearts compared with the sham-operated hearts. Thus, increased CT-1 level might induce phosphorylation of STAT3 and result in Ral activation. However, there is a possibility that growth factors other than CT-1 or other signaling pathways participate in Ral activation in vivo. Further studies are needed to clarify the details.

Perspectives

In our study, we showed that the RalGDS/Ral pathway is involved in a wide range of gene expression and is associated with hypertrophic changes in the heart. To further clarify the role of the Ral-GDS/Ral pathway in the signal mechanisms of cardiac hypertrophy, we are currently working on experiments by constructing adenoviral vector encoding RalS28N. The relative significance of Ral in myocardial hypertrophy will be clarified by infection of the adenovirus into cardiac myocytes in vitro or into the hearts in vivo under various hypertrophic stimuli.

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References

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