Activation of gp130 Transduces Hypertrophic Signal Through Interaction of Scaffolding/Docking Protein Gab1 With Tyrosine Phosphatase SHP2 in Cardiomyocytes

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Abstract—Grb2-associated binder-1 (Gab1) is a scaffolding/docking protein and contains a Pleckstrin homology domain and potential binding sites for Src homology (SH) 2 and SH3 domains. Gab1 is tyrosine phosphorylated and associates with protein tyrosine phosphatase SHP2 and p85 phosphatidylinositol 3-kinase on stimulation with various cytokines and growth factors, including interleukin-6. We previously demonstrated that interleukin-6-related cytokine, leukemia inhibitory factor (LIF), induced cardiac hypertrophy through gp130. In this study, we report the role of Gab1 in gp130-mediated cardiac hypertrophy. Stimulation with LIF induced tyrosine phosphorylation of Gab1, and phosphorylated Gab1 interacted with SHP2 and p85 in cultured cardiomyocytes. We constructed three kinds of adenovirus vectors, those carrying wild-type Gab1 (AdGab1WT), mutated Gab1 lacking SHP2 binding site (AdGab1F627/659), and β-galactosidase (Adβ-gal). Compared with cardiomyocytes infected with Adβ-gal, longitudinal elongation of cardiomyocytes induced by LIF was enhanced in cardiomyocytes infected with AdGab1WT but inhibited in cardiomyocytes infected with AdGab1F627/659. Upregulation of BNP mRNA expression by LIF was evoked in cardiomyocytes infected with Adβ-gal and AdGab1WT but not in cardiomyocytes infected with AdGab1F627/659. In contrast, Gab1 repressed skeletal α-actin mRNA expression through interaction with SHP2. Furthermore, activation of extracellular signal–regulated kinase 5 (ERK5) was enhanced in cardiomyocytes infected with AdGab1WT compared with cardiomyocytes infected with Adβ-gal but repressed in cardiomyocytes infected with AdGab1F627/659. Coinfection of AdGab1WT with adenovirus vector carrying dominant-negative ERK5 abrogated longitudinal elongation of cardiomyocytes induced by LIF. Taken together, these findings indicate that Gab1-SHP2 interaction plays a crucial role in gp130-dependent longitudinal elongation of cardiomyocytes through activation of ERK5. (Circ Res. 2003;93:221-229.)

Key Words: hypertrophy • Gab1 • SHP2 • gp130 • ERK5

Grb2-associated binder-1 (Gab1) is a member of the Gab family of scaffolding/docking proteins (Gab1, Gab2, and Gab3).1,2 Gab1 contains a Pleckstrin homology (PH) domain in the amino-terminal region, as well as tyrosine-based motifs and proline-rich sequences, which are potential binding sites for various Src homology (SH) 2 domains and SH3 domains, respectively.3-6 Among these, the major binding partner of Gab1 in cells stimulated with growth factors and cytokines is SHP2, a ubiquitously expressed protein tyrosine phosphatase with two SH2 domains.8 Two tyrosine residues located in the most C-terminal ends of the Gab family proteins have been reported to fall within consensus binding motifs (YXXV/I/L) for SHP2 on tyrosine phosphorylation.9-12 The functional significance of Gab1-SHP2 interaction has been extensively studied using mutants of Gab1 unable to bind SHP2 in vitro and in vivo. The Gab1 mutant unable to bind SHP2 is defective in delivering signals for Met-dependent morphogenesis and for epidermal growth factor (EGF)-dependent epidermal proliferation and also...
blocks extracellular signal-regulated kinase 1/2 (ERK1/2) activation by EGF and lysophosphatidic acid. These findings underscore the importance of Gab1-SHP2 interaction and strongly suggest that the primary role of Gab1 might be to recruit SHP2.

To reveal the functional role of Gab1 in vivo, we and others generated mice lacking Gab1 by gene targeting. Gab1-deficient mice died in utero and displayed developmental defects in the heart, placenta, liver, and skin. Gab1 was highly expressed in embryonic heart from E10.5 to E13.5. The ventricular wall was extremely thin in all of the Gab1−/− embryos that survived past E13.5. These findings indicate that Gab1 is necessary for development of the heart.

Leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1) are interleukin-6 (IL-6)-related cytokines and bind to a heterodimer of gp130 and LIF receptor β. LIF and CT-1 are potent inducers of cardiomyocyte hypertrophy and also serve as myocyte survival factors in vitro and in vivo. The hypertrophic response in cardiomyocytes induced by LIF and CT-1 is distinct from the hypertrophic response observed after GPCR stimulation. Adrenergic agonists, endothelin-1 (ET-1), and angiotensin II (Ang II) binding to GPCR induce a rather uniform increase in cardiomyocyte size, resulting from the addition of myofibrils in parallel. In contrast, LIF and CT-1 induce a predominant increase in cell length with the addition of new sarcomeric units in series. Interestingly, recent reports have shown that the mitogen-activated protein kinase (MAPK) kinase 5 (MEK5)-MAPK extracellular signal-regulated kinase 5 (ERK5) pathway plays a critical role in gp130-mediated eccentric cardiac hypertrophy in vitro and in vivo.

In this study, we found that Gab1 was tyrosine phosphorylated and associated with SHP2 after stimulation with LIF in cardiomyocytes. It was also revealed that Gab1 plays a critical role in elongation of cardiomyocytes induced by LIF through interaction with SHP2, using adenovirus vectors expressing wild-type Gab1 and mutated Gab1, which could not bind SHP2. In addition, we found that the interaction of Gab1 with SHP2 is involved not only in the regulation of brain natriuretic polypeptide (BNP) and skeletal α-actin (SKA) gene expression but also in the activation of ERK5 after stimulation with LIF in cardiomyocytes. Furthermore, dual infection of adenovirus vectors carrying wild-type Gab1 and dominant-negative ERK5 abrogated elongation of cardiomyocytes induced by LIF, suggesting that ERK5 may be an essential component of gp130-dependent cardiomyocyte hypertrophy through Gab1-SHP2 interaction.

**Materials and Methods**

**Construction of Recombinant Adenovirus**

According to a previous study, the most C-terminal 2 tyrosine residues (Tyr-627 and Tyr-659) of Gab1 are required for binding to SHP2. Substitution of these tyrosine residues by phenylalanine renders the molecule incapable of binding to SHP2. The wild-type and mutated human Gab1 cDNAs are designated as Gab1WT and Gab1F627/659, respectively. A dominant-negative form of murine ERK5 (ERK5ΔEF) was created by mutating dual phosphorylation site (Thr-219 and Tyr-221 with alanine and phenylalanine, respectively). The adenovirus vectors expressing Gab1WT (AdGab1WT), Gab1F627/659 (AdGab1F627/659), and ERK5ΔEF (AdERK5ΔEF) were generated according to a protocol described elsewhere.

**Cell Culture and Protocol for Adenovirus Infection**

Primary cultures of neonatal rat cardiomyocytes were prepared from ventricles of 1- to 3-day-old Wistar rats (Kiwa Jikken Dobutsu) as described previously. At 16 hours after plating, cardiomyocytes were infected with adenovirus diluted in Medium-199 with 2% FBS at a multiplicity of infection (moi) of 20 and incubated for 8 hours. In the dual infection of adenovirus vectors, cardiomyocytes were infected with each virus at an moi of 10. After removal of viral suspension, cardiomyocytes were serum starved for 16 hours and stimulated with reagents. Infection efficiency, determined by lacZ gene expression in cultured cardiomyocytes, is consistently >90% with this method. Adenovirus vector expressing β-galactosidase (Adβ-gal) was used as a control.

**Immunoprecipitation and Immunoblotting**

The methods of immunoprecipitation were essentially as described previously. After stimulation, cells were immediately lysed in lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% Nonidet P-40, 500 μmol/L sodium vanadate, 1 mmol/L dithiothreitol, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride). The cleared lysates were incubated with 2 μL of anti-Gab1 serum or 4 μL of anti-SHP2 antibody and 20 μL of protein A-Sepharose for 12 hours at 4°C. Collected immune complexes were eluted with 20 μL of 2×Laemmli’s SDS loading buffer, separated on a 4% to 20% gradient polyacrylamide gel (Dai-ichi Kagaku), electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and processed for immunoblotting analysis essentially as described previously. The ECL system was used for detection.

**Northern Blot Analysis**

Northern blot analysis was performed as previously described. Total cellular RNA (8 μg) was loaded in each lane and size fractionated by 1% formaldehyde–agarose gel electrophoresis. The probes for BNP, SKA, and GAPDH were kindly donated by Dr K.R. Chien (University of California, San Diego, Calif).

**Immunofluorescence**

For immunofluorescence, cardiomyocytes were grown on glass coverslips coated with gelatin. Cells were incubated in the presence or absence of LIF 1×10−9 U/mL for 24 hours. Cells were fixed with 2% formaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with monoclonal anti-sarcomeric α-actinin antibody, followed by incubation with fluorescein-conjugated goat anti-mouse secondary antibody. Cardiomyocytes stained against sarcomeric α-actin were viewed by fluorescence microscopy. Cell size was estimated by measuring the area over which individual sarcomeric α-actin–positive cells had attached (planimetry), and cell length and cell width were determined as described previously.

**Statistics**

Statistical analysis was performed with Student’s t test. Values of P<0.05 were considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

**Gab1 and SHP2 Are Tyrosine Phosphorylated by LIF, and Phosphorylated Gab1 Interacts With SHP2 and p85 in Cardiomyocytes**

To examine which ligand induces tyrosine phosphorylation of Gab1 in cardiomyocytes, cells were incubated with LIF, norepinephrine (NE), ET-1, and Ang II for 5 minutes. Among
these, LIF exclusively induced tyrosine phosphorylation of Gab1 (Figure 1A). Furthermore, SHP2, the major binding partner of Gab1, was also tyrosine phosphorylated only by LIF (Figure 1B). Therefore, we focused on the gp130-dependent signaling pathway through Gab1 in cardiomyocytes.

Gab1 was tyrosine phosphorylated by LIF in a time-dependent and dose-dependent manner (Figures 2A and 2B). SHP2 was also tyrosine phosphorylated by LIF in a time-dependent and dose-dependent manner (Figures 2C and 2D). To elucidate the association of Gab1 with other SH2-containing molecules, cell lysates from cardiomyocytes treated with LIF were immunoprecipitated with anti-Gab1 serum and subjected to immunoblotting with anti-SHP2 and anti-p85 antibodies. SHP2 and p85 were coprecipitated with Gab1 in response to LIF (Figure 2E). Gab1 was also coprecipitated with SHP2 by the immunoprecipitation with anti-SHP2 antibody (Figure 2F). These results demonstrated that LIF induced tyrosine phosphorylation of Gab1, leading to association of Gab1 with SHP2 and p85 in cardiomyocytes.

Tyrosine Phosphorylation of Gab1 and Association of Gab1 With SHP2 in AdGab1 WT or AdGab1 F627/659-Treated Cardiomyocytes

We constructed recombinant adenovirus vectors expressing Gab1 WT and Gab1 F627/659. Figure 3A shows a schematic representation of these adenovirus vectors, which are named AdGab1 WT and AdGab1 F627/659. We examined tyrosine phosphorylation of Gab1 in Adgp-gal–treated, AdGab1 WT–treated, or AdGab1 F627/659–treated cardiomyocytes. As shown in Figure 3B, tyrosine phosphorylation of Gab1 and the amount of coprecipitated SHP2 with Gab1 were increased in AdGab1 WT–treated cardiomyocytes after LIF stimulation, compared with Adgp-gal–treated cardiomyocytes. In AdGab1 F627/659–treated cardiomyocytes, tyrosine phosphorylation of Gab1 was observed in the same manner as in AdGab1 WT–treated cardiomyocytes, but SHP2 was not coprecipitated with Gab1. As shown in Figure 3C, Gab1 was not coprecipitated with SHP2 in AdGab1 F627/659–treated cardiomyocytes. These results indicate that Gab1 WT and Gab1 F627/659, which were overexpressed...
through adenovirus-mediated gene transfer, could function effectively in cardiomyocytes.

**Elongation of Cardiomyocytes Induced by LIF Is Enhanced in AdGab1 WT-Treated Cardiomyocytes but Suppressed in AdGab1 F627/659-Treated Cardiomyocytes**

To elucidate the biological roles of Gab1 in gp130-signaling pathway in cardiomyocytes, we examined the morphological effects of Gab1<sup>WT</sup> and Gab1<sup>F627/659</sup> on cardiomyocyte hypertrophy in response to LIF. As shown in Figures 4A and 4D, LIF induced longitudinal elongation in Adβ-gal–treated cardiomyocytes. In AdGab1<sup>WT</sup>-treated cardiomyocytes, elongation of cardiomyocytes induced by LIF was enhanced compared with Adβ-gal–treated cardiomyocytes (Figures 4B and 4E). On the contrary, in cardiomyocytes expressing Gab1<sup>F627/659</sup>, this morphological change was significantly inhibited (Figures 4C and 4F).

We quantified the cell surface area of cardiomyocytes infected with these adenovirus vectors. As shown in Figure 4G, LIF increased cell surface area by 70% in Adβ-gal–treated or AdGab1<sup>WT</sup>-treated cardiomyocytes. However, in AdGab1<sup>F627/659</sup>-treated cardiomyocytes, the increase in cell surface area induced by LIF was almost abolished. To characterize the hypertrophic phenotype of these cardiomyocytes, we measured cell length and cell width according to a previously reported method. As shown in Figure 4H, cell length was significantly increased in AdGab1<sup>WT</sup>-treated cardiomyocytes, compared with that in Adβ-gal–treated cardiomyocytes. In contrast, increase in cell length was significantly suppressed in AdGab1<sup>F627/659</sup>-treated cardiomyocytes. Cell width was significantly decreased in response to LIF in AdGab1<sup>WT</sup>-treated cardiomyocytes but not altered in Adβ-gal–treated or AdGab1<sup>F627/659</sup>-treated cardiomyocytes (Figure 4I). These findings indicate that elongation of cardiomyocytes in response to LIF is enhanced by overexpression of Gab1<sup>WT</sup> but suppressed by that of Gab1<sup>F627/659</sup>, suggesting that the interaction of Gab1 with SHP2 plays a crucial role in longitudinal elongation of cardiomyocytes in response to LIF.

**Gab1 Regulates LIF-Induced Embryonic Gene Expression Through Interaction With SHP2**

Reactivation of embryonic phenotype genes, such as atrial natriuretic factor (ANF), BNP, and SKA, is known to be associated with hypertrophic responses in cardiomyocytes. We examined how the interaction of Gab1 with SHP2 contributes to the induction of BNP and SKA mRNA after LIF stimulation. BNP mRNA was upregulated by LIF in AdGab1<sup>WT</sup>-treated cardiomyocytes to the same extent as in Adβ-gal–treated cardiomyocytes. In contrast, induction of BNP mRNA was suppressed significantly in AdGab1<sup>F627/659</sup>-treated cardiomyocytes (Figures 5A and 5B). SKA mRNA expression was slightly increased in response to LIF in Adβ-gal–treated cardiomyocytes but was almost completely suppressed in AdGab1<sup>WT</sup>-treated cardiomyocytes both in basal level and after LIF stimulation. In AdGab1<sup>F627/659</sup>-treated cardiomyocytes, the expression of SKA mRNA was restored to the same extent as in Adβ-gal–treated cardiomyocytes (Figures 5A and 5C). These results indicate that the interaction of Gab1 with SHP2 is involved in the regulation of embryonic gene expression after stimulation with LIF.
Interaction of Gab1 With SHP2 Plays a Crucial Role in Activation of ERK5 by LIF in Cardiomyocytes

To elucidate a potential mechanism by which Gab1-SHP2 interaction plays a role in gp130-mediated longitudinal elongation of cardiomyocytes, we examined the effects of Gab1WT and Gab1 F627/659 on LIF-induced activation of MAP kinases (ERK5 and ERK1/2), AKT, and signal transducer and activator of transcription 3 (STAT3), which are known to mediate biological functions through gp130.21,28,31–33 These signaling molecules were rapidly activated by LIF in Adβ-gal-treated cardiomyocytes. Activation of ERK5 by LIF was augmented in AdGab1 WT-treated cardiomyocytes compared with Adβ-gal–treated cardiomyocytes. On the other hand, activation of ERK5 was reduced in AdGab1 F627/659–treated cardiomyocytes (Figure 6B). ERK1/2 was activated to the same extent in AdGab1 WT–treated cardiomyocytes as in Adβ-gal–treated cardiomyocytes. On the contrary, activation of ERK1/2 was reduced in AdGab1 F627/659–treated cardiomyocytes compared with Adβ-gal–treated cardiomyocytes (Figure 6C). Activation of AKT was enhanced in AdGab1 WT–treated or AdGab1 F627/659–treated cardiomyocytes compared with Adβ-gal–treated cardiomyocytes (Figure 6D). Activation of STAT3 was not altered in cardiomyocytes infected with Adβ-gal, AdGab1 WT, or AdGab1 F627/659. These results indicate that Gab1 plays a critical role in activation of ERK5 and ERK1/2 by LIF through interaction with SHP2 in cardiomyocytes. Based on a previous report,28 the present finding suggests that the interaction of Gab1 with SHP2 might be involved in LIF-induced elongation of cardiomyocytes through activation of ERK5.

Overexpression of the Dominant-Negative Form of ERK5 Abrogates the Effect of Gab1 WT on Longitudinal Elongation of Cardiomyocytes Induced by LIF

To determine whether ERK5 might participate in the LIF-activated signaling pathway that mediates longitudinal elonga-
tion of cardiomyocytes induced by LIF, we constructed recombinant adenovirus vector expressing dominant-negative form of ERK5 (AdERK5ΔEF). As shown in Figure 7A, overexpression of ERK5ΔEF almost abrogated LIF-induced longitudinal elongation of cardiomyocytes.

To test whether overexpression of ERK5ΔEF abrogates LIF-induced longitudinal elongation of cardiomyocytes over-expressing Gab1WT, cardiomyocytes were dual infected with AdGab1WT and Adβ-gal or with AdGab1WT and AdERK5ΔEF. LIF induced elongation of cardiomyocytes infected with AdGab1WT and Adβ-gal. On the contrary, this morphological change was significantly inhibited in cardiomyocytes infected with AdGab1WT and AdERK5ΔEF (Figure 7B). We quantified the cell surface area, cell length, and cell width of these cardiomyocytes. As shown in Figures 7C and 7D, LIF increased cell surface area by 60% and cell length by 84% in cardiomyocytes infected with AdGab1WT and Adβ-gal. However, in cardiomyocytes infected with AdGab1WT and AdERK5ΔEF, the increases in cell surface area and cell length induced by LIF were almost abrogated. Cell width was significantly decreased in response to LIF in cardiomyocytes infected with AdGab1WT and Adβ-gal but not in cardiomyocytes infected with AdGab1WT and AdERK5ΔEF (Figure 7E). The cell length to width ratio was significantly increased by LIF in cardiomyocytes infected with AdGab1WT and Adβ-gal but not in cardiomyocytes infected with AdGab1WT and AdERK5ΔEF (Figure 7F). Therefore, it seems that ERK5 might be an essential component of LIF-activated signaling.

Figure 5. Gab1 regulates embryonic gene expression through interaction with SHP2 in cardiomyocytes. A, Cardiomyocytes were infected with Adβ-gal, AdGab1WT, or AdGab1F627/659 at an moi of 20 for 8 hours and serum deprived. At 24 hours after infection, cells were treated without or with 1×10^7 U/mL LIF for an additional 24 hours. Total RNA was isolated and subjected to Northern blot analysis (8 μg/lane) using BNP and SKA cDNA probes. Equal loading and transfer conditions were confirmed by GAPDH hybridization. B and C, Relative intensity of the bands for BNP or SKA was assessed as the ratio to the intensity of GAPDH. The results were expressed as relative intensity over Adβ-gal–treated cells without LIF stimulation. Values are shown as mean±SD. *P<0.05 vs LIF(−); †P<0.05 vs Adβ-gal (n=3).

Figure 6. Gab1 is involved in activation of ERK5 by LIF through interaction with SHP2 in cardiomyocytes. A, Cardiomyocytes were infected at an moi of 20 with Adβ-gal, AdGab1WT, or AdGab1F627/659 and serum deprived. At 24 hours after infection, cells were stimulated with 3×10^2 U/mL LIF for indicated periods of time. Total cell extracts were prepared and blotted with anti-phospho ERK5 (p-ERK5), anti-ERK5, anti-phospho ERK1/2 (p-ERK1/2), anti-ERK1/2, anti-phospho AKT (pAKT), anti-AKT, anti-phospho STAT3 (p-STAT3), and anti-STAT3 antibodies. Gab1 expression was confirmed with anti-Gab1 antibody. All data shown are one representative result from 3 independent experiments that had a similar result. B through D, Phosphorylation of ERK5, ERK1/2, and AKT was normalized to total ERK5, ERK1/2, and AKT using FluorChem-8000 (Alpha-Innotech).
pathway, leading to elongated morphology of cardiomyocytes through Gab1-SHP2 interaction.

**Discussion**

The present study is the first to reveal the role of Gab1 in gp130-mediated hypertrophic signaling in cardiomyocytes in vitro. Gab1 is tyrosine phosphorylated and interacts with SHP2 and p85 after stimulation with LIF. Overexpression of Gab1 WT enhances elongation of cardiomyocytes induced by LIF. Consistent with potential involvement of Gab1 in gp130-signaling pathway, overexpression of Gab1F627/659, which could not associate with SHP2, blocks morphological change and induction of BNP mRNA in response to LIF in cardiomyocytes. Gab1 is also involved in regulation of SKA gene expression through interaction with SHP2. Moreover, Gab1 regulates LIF-induced activation of ERK5 through interaction with SHP2, leading to gp130-dependent elongation of cardiomyocytes.

Among several hypertrophic factors, we found that LIF and Ang II, did not induce tyrosine phosphorylation of Gab1 and SHP2 in cardiomyocytes. LIF and CT-1 induce cardiomyocyte hypertrophy, which is distinct from the hypertrophic phenotype observed after stimulation of GPCR agonists, both on a morphological and a molecular level. GPCR agonists induce a relatively uniform increase in myocyte size and the addition of new myofibrils in parallel. In contrast, LIF and CT-1 induce a predominant increase in cell length with the addition of new sarcomeric units in series but no concomitant increase in cell width. In the present study, we showed that Gab1 enhanced elongation of cardiomyocytes induced by LIF and that overexpression of Gab1F627/659 inhibited increase in cell size and cell length of cardiomyocytes after LIF stimulation. On the other hand, our data showed that overexpression of both Gab1 WT and Gab1F627/659 did not affect the morphological change after stimulation with GPCR agonist ET-1 in cardiomyocytes (data not shown). These findings suggest that the interaction of Gab1 with SHP2 specifically contributes to longitudinal elongation of cardiomyocytes induced by stimulation of gp130. However, the LIF-induced increase of cell length was not completely abol-
ished in AdGab1<sup>F627/659</sup>-treated cardiomyocytes (Figure 4H). This increase may be related to augmented activation of AKT in AdGab1<sup>F627/659</sup>-treated cardiomyocytes, as shown in Figures 6A and 6D.

To additionally investigate the molecular mechanisms of Gab1-mediated longitudinal elongation of cardiomyocytes, we examined the downstream signaling pathway of gp130. Interestingly, activation of ERK5 by LIF was enhanced by overexpression of Gab1<sup>WT</sup> but suppressed by that of Gab1<sup>F627/659</sup>. Furthermore, in cardiomyocytes dual infected with AdGab1<sup>WT</sup> and AdERK5<sup>AEF</sup>, the increases in cell surface area and cell length were almost abrogated. These data indicate that Gab1-SHP2 interaction plays a critical role in gp130-dependent elongation of cardiomyocytes through activation of ERK5. Although it has been reported that the interaction of Gab1 with SHP2 regulates activation of ERK1/2,9–12,14 the present study is the first demonstration that the interaction of Gab1 with SHP2 also regulates activation of ERK5. Consistent with our results, Nicol et al.<sup>28</sup> recently reported that activation of ERK5 is necessary and sufficient for elongation of cardiomyocytes induced by LIF, providing the causality between Gab1-mediated ERK5 activation and elongation of cardiomyocytes. Nicol et al.<sup>28</sup> also demonstrated that the gp130-MEK5-ERK5 pathway has a specific role in inhibition of parallel assembly of sarcomeres using adenovirus vectors expressing constitutive active and dominant-negative MEK5.<sup>28</sup> Our data showing that cell width was decreased after LIF stimulation in AdGab1<sub>WT</sub>-treated cardiomyocytes suggest that Gab1 might enhance gp130-MEK5-ERK5 signaling pathway to inhibit parallel assembly of sarcomeres.

On the other hand, ERK5 was also shown to be activated by GPCR agonist phenylephrine in cardiomyocytes.<sup>28</sup> Based on these findings, we could hypothesize that tyrosine phosphorylation of Gab1 and subsequent complex formation of Gab1 and SHP2 are primarily responsible for the specification of gp130-mediated cardiac hypertrophy (Figure 8). However, additional investigation is needed to elucidate the functional role of SHP2 in cardiac hypertrophy.

In addition to morphological change, stimulation of cardiomyocytes with LIF and CT-1 induced ANF and BNP mRNA expression but not SKA mRNA expression.<sup>20,34</sup> In contrast, GPCR agonists induced ANF, BNP, and SKA mRNA expression in a coordinate fashion.<sup>25–27,35</sup> Although overexpression of Gab1<sup>WT</sup> or Gab1<sup>F627/659</sup> did not alter the upregulation of BNP mRNA after stimulation with GPCR agonist ET-1 in cardiomyocytes (data not shown), our results showed that Gab1-SHP2 interaction mediated LIF-induced BNP and SKA mRNA in different directions. Accordingly, these findings suggest that Gab1-SHP2 interaction contributes to the unique pattern of embryonic gene expression in gp130-mediated cardiac hypertrophy. However, additional investigation is needed to reveal the molecular mechanism underlying Gab1-mediated gene regulation in gp130-mediated cardiac hypertrophy.

Finally, it is very important to investigate the concerned pathological conditions in which gp130-Gab1 pathway is involved in human clinical case or animal models. To our knowledge, one previous study has demonstrated the distinct gene expression pattern in the hearts with pressure overload (PO) and volume overload (VO) in rat model.<sup>36</sup> In this report, mRNA levels were quantified in the left ventricular myocardium from rats with cardiac hypertrophy attributable to PO or VO caused by suprarenal aortic constriction or an abdominal aortic-caval fistula, respectively. Although PO and VO caused comparable increases in LV weight and prepro-ANF mRNA, PO but not VO increased mRNA levels of SKA. This pattern of gene expression induced by VO in vivo is reminiscent of that observed in cultured cardiomyocytes after LIF stimulation. Additionally, recent reports have shown that the signaling pathway through gp130-dependent pathway is profoundly altered in patients with end-stage heart failure attributable to dilated and ischemic cardiomyopathy.<sup>37</sup> Although little is known regarding involvement of Gab1 in human clinical case or animal models, these findings and our findings suggest that Gab1 may play a role in the left ventricular remodeling in volume-overloaded hearts, providing novel insights into a therapeutic strategy for heart failure by manipulating Gab1-SHP2 interaction.

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References


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(Short Title; Gab1 in gp130-dependent cardiac hypertrophy)

Online Supplemental information

Materials

Murine leukemia inhibitory factor was purchased from Chemicon International Inc. Norepinephrine was supplied by Sankyo. Endothelin-1 was from Peptide Institute Inc. Medium-199 was from GibcoBRL. Angiotensin II and monoclonal anti-sarcomeric α-actinin (clone EA-53) antibody were obtained from Sigma. Anti-phosphotyrosine monoclonal antibody (PY99), anti-signal transducer and activator of transcription 3 (STAT3), anti-ERK1, anti-ERK2, and anti-SHP2 antibodies were from Santa Cruz Biotechnology. Anti-p85 antibody and anti-Gab1 antibody for immunoblotting and immunocytochemistry were from Upstate Biotechnology. Anti-Gab1 serum for immunoprecipitation was described previously. Anti-phospho STAT3, anti-phospho ERK1/2, anti-phospho ERK5, anti-phospho AKT, and anti-AKT antibodies were purchased from Cell Signaling Technology. Anti-ERK5 antibody was from Calbiochem.
Fluorescein-conjugated goat anti-mouse antibody and Cy3-conjugated goat anti-rabbit antibody were from Jackson Immunoresearch Laboratories. Protein A-sepharose and the ECL system were from Amersham Bioscience. All other chemicals were reagents of molecular biology grade and were obtained from standard commercial sources.

Reference

Online Figure Legends

**Online Figure 1.** LIF induces surface translocation of Gab1 in cardiomyocytes.

Cardiomyocytes were infected with AdGab1<sup>WT</sup> at an m.o.i. of 20 for 8 hours and serum-deprived. At 24 hours post-infection, cells were treated without or with 1 × 10<sup>3</sup> U/ml LIF for 5 minutes. Cells were fixed and dual-immunostained with anti-Gab1 antibody (Online Figure 1A, 1C) and anti-sarcomeric α-actinin antibody (Online Figure 1B, 1D). Gab1 and sarcomeric α-actinin were detected by Cy3-conjugated secondary goat anti-rabbit antibody and fluorescein-conjugated secondary goat anti-mouse antibody, respectively. Fluorescence microscopy revealed diffuse cytoplasmic distribution of Gab1 in vehicle-treated cells (Online Figure 1A) and translocation of Gab1 to the cell periphery in LIF-treated cells (Online Figure 1C). Representative data are shown. Experiments were repeated three times with similar results. In addition, LIF induced surface translocation of Gab1 in cardiomyocytes overexpressing Gab1<sup>F627/659</sup> (data not shown).

**Online Figure 2.** Sarcomeric organaization of cardiomyocytes infected with Adβ-gal, AdGab1<sup>WT</sup>, or AdGab1<sup>F627/659</sup>.

Cardiomyocytes were infected with Adβ-gal, AdGab1<sup>WT</sup>, or AdGab1<sup>F627/659</sup> at an m.o.i. of 20
for 8 hours and serum-deprived. At 24 hours post-infection, cells were treated with $1 \times 10^3$ U/ml LIF for additional 24 hours. Cells were dual-labeled with monoclonal anti-sarcomeric α-actinin antibody (Online Figure 2A, 2B, 2C) and rhodamine phalloidine (Online Figure 2D, 2E, 2F), to allow a simultaneous assessment of Z-band and thin filament (F-actin) assembly. Images were obtained by fluorescence microscopy. In cardiomyocytes infected with Adβ-gal and AdGab1<sup>WT</sup>, LIF induced high degree of sarcomeric organization in which myofibrils were oriented along the longitudinal cell axis and extended into the cytoplasmic projections. On the other hand, LIF could not induce longitudinal elongation of cardiomyocytes infected with AdGab1<sup>F627/659</sup>, in which myofibrils were not oriented along the longitudinal cell axis.
Online Figure 1.

LIF(-) Gab1

LIF(+) sarcomeric α-actinin

A

B

C

D
Online Figure 2.

A B C

D E F

sarcomeric

α-actinin

phalloidine

Adβ-gal AdGab1WT AdGab1F627/659