Interaction of Scaffolding Adaptor Protein Gab1 with Tyrosine Phosphatase SHP2 Negatively Regulates IGF-1-dependent Myogenic Differentiation via the ERK1/2 Signaling Pathway

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Grb2-associated binder 1 (Gab1) coordinates various receptor tyrosine kinase signaling pathways. Although skeletal muscle differentiation is regulated by some growth factors, it remains elusive whether Gab1 coordinates myogenic signals. Here, we examined the molecular mechanism of insulin-like growth factor-1 (IGF-I)-mediated myogenic differentiation, focusing on Gab1 and its downstream signaling. Gab1 underwent tyrosine phosphorylation and subsequent complex formation with protein-tyrosine phosphatase SHP2 upon IGF-I stimulation in C2C12 myoblasts. On the other hand, Gab1 constitutively associated with phosphatidylinositol 3-kinase regulatory subunit p85. To delineate the role of Gab1 in IGF-I-dependent signaling, we examined the effect of adenovirus-mediated forced expression of wild-type Gab1 (Gab1WT), mutated Gab1 that is unable to bind SHP2 (Gab1ΔSHP2), or mutated Gab1 that is unable to bind p85 (Gab1Δp85), on the differentiation of C2C12 myoblasts. IGF-I-induced myogenic differentiation was enhanced in myoblasts overexpressing Gab1ΔSHP2, but inhibited in those overexpressing either Gab1WT or Gab1Δp85. Conversely, IGF-I-induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation was significantly repressed in myoblasts overexpressing Gab1ΔSHP2 but enhanced in those overexpressing either Gab1WT or Gab1Δp85. Furthermore, small interference RNA-mediated Gab1 knockdown enhanced myogenic differentiation. Overexpression of catalytic-inactive SHP2 modulated IGF-I-induced myogenic differentiation and ERK1/2 activation similarly to that of Gab1ΔSHP2, suggesting that Gab1-ΔSHP2 complex inhibits IGF-I-dependent myogenesis through ERK1/2. Consistently, the blockade of ERK1/2 pathway reversed the inhibitory effect of Gab1WT overexpression on myogenic differentiation, and constitutive activation of the ERK1/2 pathway suppressed the enhanced myogenic differentiation by overexpression of Gab1ΔSHP2. Collectively, these data suggest that the Gab1-ΔSHP2-ERK1/2 signaling pathway comprises an inhibitory axis for IGF-I-dependent myogenic differentiation.

Skeletal muscle differentiation is a multistep process in which multipotent mesodermal cells give rise to myoblasts that subsequently withdraw from the cell cycle and differentiate into multinucleated myotubes. Most skeletal muscle cell lines from rodents proliferate in high serum conditions containing various mitogens, and post-confluent cells spontaneously differentiate after several days in low serum conditions (1, 2). Among various growth factors, the insulin-like growth factors (IGFs), 3 included
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EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Anti-phospho-p44/42 ERK1/2 (Thr-202/Tyr-204), anti-phospho-AKT (Thr-308), anti-ERK1/2, and anti-AKT antibodies were purchased from Cell Signaling Technology. Anti-Gab1 and anti-Gab2 sera for immunoprecipitation were described previously (15, 16, 22). The antibodies against the following molecules used for immunoblotting, Gab1, Gab2, insulin receptor substrate-1 (IRS-1), and p85 PI3K were from Millipore; PY99, SHP2, MEK1, and myogenin were from Santa Cruz Biotechnology. Anti-myosin heavy chain (MHC) monoclonal antibody (MF20) was purchased from the Developmental Hybridoma Bank (Dr. D. A. Fischman, University of Iowa, Iowa City, IA). Hoechst 33342 nuclear dye was from Sigma. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from GE Health Science. U0126 was from Promega (Madison, WI). Serum and cell culture reagents were from Invitrogen. Human recombinant IGF-I was kindly provided by Astellas Pharmaceuticals.

Adenovirus Vector Construction—The generation of adenovirus vectors expressing human Gab1WT and Gab1ΔSHP2 (mutated on the two tyrosine residues responsible for binding with SHP2) were described previously (22). In this study, we constructed the adenovirus vectors expressing Gab1Δp85, which can’t bind with p85 due to the substitution of tyrosine residues 447, 472, and 589 of human Gab1, corresponding to the YXXM motifs, to phenylalanines by PCR-based mutagenesis described previously (35). Substitution of these tyrosine residues by phenylalanine renders the molecule incapable of binding with p85. We also constructed adenovirus vectors expressing wild-type SHP2 (SHP2WT) and phosphatase-inactive SHP2 (SHP2C/S) using the plasmid vectors described previously (15). For adenovirus production, the sequence encoding Gab1Δp85, SHP2WT, or SHP2C/S was subcloned into the shuttle plasmid pACCMVpLpA. Recombinant adenoviruses were then obtained according to the homologous recombination system described elsewhere (36). The adenovirus vectors expressing constitutive-active MEK1 and dominant-negative MEK1 were kindly provided by Dr. S. Kawashima (Kobe University) and described previously (37). The construction of adenovirus vector expressing human Gab2ΔSHP2, which can’t bind with SHP2, is described in the supplemental data.

Cell Culture, Stimulation, and Adenoviral Infection—C2C12 murine myoblast cells were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose, 0.58 g/liter L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin supplemented with in postnatal maintenance of cardiac function via the neuregulin-1/ErbB signaling pathway (33). In addition, liver-specific Gab1 knock-out mice displayed enhanced hepatic insulin sensitivity with reduced glycemia and improved glucose tolerance as a result of insufficient insulin-elicited activation of ERK1/2 (34). However, it remains elusive whether Gab1 has a specific role in skeletal muscle differentiation. In this study, we demonstrate for the first time that Gab1-SHP2 interaction exerts an inhibitory effect on IGF-I-induced myogenic differentiation via activation of the ERK1/2 signaling pathway.

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20% fetal bovine serum (FBS). Before stimulation, cells were serum-starved overnight. Stimulations were performed using 80 ng/ml IGF-I for 10 min, unless otherwise indicated. For adenoviral infection of C2C12 myoblasts, subconfluent cells were cultured in DMEM with 20% FBS and plated on 3.5-cm collagen type I-coated plastic dishes. To each dish, 140 μl of RNAi duplex-Lipofectamine™ RNAiMax complex diluted in OptiMEM I medium was added. After incubation for 72 h, the cells were used for the experiments.

Statistics—All data are expressed as mean ± S.D. Differences among multiple groups were compared by one-way ANOVA followed by a post hoc comparison tested with Scheffe’s method. Values of p < 0.05 were considered significant.

RESULTS

Gab1 Undergoes Tyrosine Phosphorylation and Subsequently Associates with SHP2 upon IGF-I Stimulation in C2C12 Myoblasts—We examined the effect of IGF-I on tyrosine-phosphorylation of Gab1 and its association with SH2 domain-containing signaling molecules in C2C12 myoblasts. IGF-I indeed induced tyrosine phosphorylation of Gab1 and subsequent association of Gab1 with SHP2 in C2C12 myoblasts (Fig. 1A, left panel). Furthermore, SHP2 was also tyrosine-phosphorylated and associated with Gab1 after stimulation with IGF-I (Fig. 1A, right panel). On the other hand, Gab1 constitutively associated with p85 both before and after IGF-I stimulation (Fig. 1A, left panel). In the IGF-I-dependent signaling pathway, IRS-1 has been reported to be a major binding partner of p85 and essential for IGF-I-dependent PI3K-AKT signaling in skeletal muscle cells (38). Consistently, IRS-1 underwent strong tyrosine phosphorylation after stimulation with IGF-I in C2C12 myoblasts. In clear contrast to Gab1, IRS-1 associated with p85 in a manner dependent on IGF-I stimulation (Fig. 1B). In addition, we could not detect the complex formation of IRS-1 with SHP2 either before or after stimulation with IGF-I (Fig. 1B). These results demonstrate that IGF-I induces tyrosine phosphorylation of Gab1, leading to complex formation of Gab1 with SHP2 in C2C12 myoblasts.

IGF-I-induced Tyrosine Phosphorylation of Gab1 and Association of Gab1 with SH2 Domain-containing Molecules in the C2C12 Myoblasts Infected with Adenovirus Vectors—IGFs have been reported to stimulate both proliferation and differentiation of cultured skeletal muscle cells (3, 4). The effect of IGF-I on the proliferation of myoblasts has been reported to be attributed mainly to ERK1/2 pathway (3). In the present study, we tried to reveal the role of Gab1 in IGF-I-dependent differentiation from myoblasts into myotubes. To discern the role of Gab1-SHP2 interaction from that of Gab1-p85 interaction in IGF-I-dependent differentiation, we used recombinant adenovirus vectors carrying β-gal (control), Gab1WT, or Gab1ΔSHP2 as described previously (22) and created an adenovirus vector overexpressing Gab1Δp85. We examined tyrosine phosphorylation of Gab1 upon stimulation with IGF-I in myoblasts overexpressing β-gal, Gab1WT, Gab1ΔSHP2, or Gab1Δp85. As shown in Fig. 2A, tyrosine phosphorylation of Gab1 and the amount of co-immunoprecipitated SHP2 with Gab1 were increased after stimulation with IGF-I in control myoblasts expressing β-gal. On the other hand, tyrosine phosphorylation of Gab1 in the
myoblasts overexpressing Gab1\textsuperscript{WT}, Gab1\textsuperscript{△SHP2}, or Gab1\textsuperscript{△p85} increased much more at baseline compared with those overexpressing β-gal. In these cells, tyrosine phosphorylation of Gab1 decreased after stimulation with IGF-I. The IGF-I-induced association of Gab1 with SHP2 increased in the C2C12 myoblasts overexpressing Gab1\textsuperscript{WT}, or Gab1\textsuperscript{△SHP2} compared with those overexpressing β-gal, but was almost abrogated in those overexpressing Gab1\textsuperscript{△SHP2} (Fig. 2A). The co-immunoprecipitation of Gab1 with p85 was increased in cells expressing Gab1\textsuperscript{WT} or Gab1\textsuperscript{△SHP2} compared with those expressing β-gal, but was almost abrogated in those expressing Gab1\textsuperscript{△p85} at the baseline. The association of Gab1 with p85 decreased in response to IGF-I in the cells overexpressing Gab1\textsuperscript{WT} or Gab1\textsuperscript{△SHP2} (Fig. 2A). We observed much more dissociation of p85 from Gab1 in myoblasts overexpressing Gab1\textsuperscript{WT} compared with those overexpressing Gab1\textsuperscript{△SHP2}, which might be attributed to the increased activation of SHP2. Presumably, SHP2 dephosphorylates the tyrosine residues for p85 binding site of Gab1 consistent with the previous report on EGF-dependent signaling (39). These data demonstrate that overexpression of
differentiation. The extent of myogenic differentiation was induced in myoblasts overexpressing Gab1ΔSHP2, but inhibited in myoblasts overexpressing either Gab1WT or Gab1Δp85, compared with myoblasts infected with control adenovirus vector expressing β-gal (Fig. 3A). Western blot analysis also revealed that the expression of MHC was significantly increased by the overexpression of Gab1ΔSHP2, but seemed to be repressed by the overexpression either Gab1WT or Gab1Δp85, compared with control (Fig. 3, B and C). These findings indicate that Gab1-SHP2 interaction exerts an inhibitory effect on myogenic differentiation induced by a low serum condition.

Gab1-SHP2 complex formation has been reported to result in an increase of phosphatase activity of SHP2 upon stimulation with EGF (20, 21, 24). To confirm the requirement of catalytic activity of SHP2 for inhibition of myogenesis, we created adenovirus vectors expressing wild-type SHP2 (SHP2WT) and phosphatase-inactive SHP2 (SHP2C/S). After infection with adenovirus vectors for 24 h, confluent C2C12 myoblasts were cultured in the DMEM containing 2% HS. The extent of myogenic differentiation determined by immunocytochemistry was significantly increased by the overexpression of SHP2WT, but similar increase of myogenic differentiation was not observed by overexpression of SHP2C/S compared with control (Fig. 4, A and B). Western blot analysis also showed that overexpression of SHP2WT in C2C12 cells induced significant increase of MHC expression compared with control (Fig. 4, C).

On the other hand, tyrosine phosphorylation of IRS-1 and interaction between IRS-1 and p85 were comparable among the four groups of cells after stimulation with IGF-I (Fig. 2B). Thus, these findings indicate that overexpression of Gab1ΔSHP2 or Gab1Δp85 specifically perturbs the IGF-I-dependent interaction of Gab1 with SHP2 or p85, respectively.

Myogenic Differentiation Induced by a Low Serum Condition Is Negatively Regulated by Gab1-SHP2 Complex through Activating SHP2 in C2C12 Myoblasts—C2C12 myoblasts undergo myogenic differentiation under a low-serum condition such as 2% HS (1, 2). Therefore, we examined the effects of overexpression of Gab1WT, Gab1ΔSHP2, or Gab1Δp85, on myogenic differentiation under low serum condition. After infection with adenovirus vectors for 24 h, confluent C2C12 myoblasts were cultured in the DMEM containing 2% HS. On the third day after induction of myogenic differentiation, cells were immunostained with anti-MHC antibody for evaluation of myogenic differentiation. The extent of myogenic differentiation was enhanced in myoblasts overexpressing Gab1ΔSHP2, but inhibited in myoblasts overexpressing either Gab1WT or Gab1Δp85.
expression, compared with control (Fig. 3, B and C). Taken together, these findings suggest that Gab1 exerts an inhibitory effect on myogenic differentiation of C2C12 myoblasts under low serum condition through association with SHP2 and increase of SHP2 catalytic activity.

IGF-I-induced Myogenic Differentiation Is Negatively Regulated by Gab1-SHP2 Complex through Increasing Catalytic Activity of SHP2 in C2C12 Myoblasts—The IGF family, including IGF-I and -II, induces myogenic differentiation of myoblasts after myoblasts fully proliferate and become ready for differentiation (1–3). Therefore, we examined the effect of overexpression of various Gab1 proteins on the myogenic differentiation of C2C12 myoblasts cultured in IGF-I-containing differentiation medium. After infection with adenovirus vectors for 24 h, post-confluent C2C12 myoblasts were cultured in DMEM containing 80 ng/ml IGF-I. On the third day after induction of myogenic differentiation, cells were immunostained with anti-MHC antibody and post-stained with Hoechst 33342 nuclear dye. Myogenic differentiation was enhanced in myoblasts overexpressing Gab1WT or Gab1Ap85, compared with control (Fig. 4B). The extent of myogenic differentiation was significantly increased in the myoblasts overexpressing Gab1Ap85, although it seemed to repressed in those overexpressing Gab1WT or Gab1Ap85, compared with control (Fig. 4, B and C). Furthermore, the expression of myogenin was enhanced in myoblasts overexpressing Gab1Ap85, but repressed in myoblasts overexpressing Gab1WT or Gab1Ap85, compared with control (Fig. 4B). These data coincide with the results observed in the low serum condition, suggesting that Gab1-SHP2 complex has an inhibitory role in the IGF-I-induced myogenic differentiation.

Gab2, another Gab family protein, has been reported to complement the function of Gab1 in some signaling pathways such as EGF-dependent signaling (33, 40). To confirm the specific role of Gab1 in myogenic differentiation, we examined the effect of overexpression of Gab2Ap85, the Gab2 mutant that cannot associate with SHP2 (41), on IGF-I-induced myogenic differentiation. The extent of myogenic differentiation was comparable between myoblasts overexpressing Gab2Ap85 and those expressing β-gal (supplemental Fig. S1, A and B). We also confirmed that Gab2Ap85 did not associate with SHP2 either before or after stimulation with IGF-I (supplemental Fig. S1C). These data suggested that IGF-I-induced myogenic differential-

![FIGURE 5. SHP2 phosphatase activity is required for inhibition of myogenic differentiation. A, C2C12 myoblasts overexpressing β-gal, SHP2WT, or SHP2Ap85 were cultured in differentiation medium containing IGF-I. The third day after exposure to the differentiation medium, cells were immunostained with anti-MHC antibody and post-stained with Hoechst 33342 nuclear dye. Myogenic differentiation was enhanced in myoblasts overexpressing SHP2Ap85 compared with myoblasts overexpressing β-gal and SHP2WT. Experiments were repeated three times with similar results. B, cell lysates were collected from C2C12 cells overexpressing β-gal, SHP2WT, or SHP2Ap85 at the indicated time after cultivation in the differentiation medium containing IGF-I. Cell lysates were subjected to Western blot analysis for analyzing the expression of MHC and myogenin. The membrane was reprobed with anti-SHP2 antibody for confirmation of adenoviral overexpression of SHP2. AKT was examined as a loading control. C, the relative expression level of MHC was quantified by normalizing the expression of MHC by that of AKT. Values are shown as means ± S.D. (*, p < 0.05 or **, p < 0.01 compared with control cells expressing β-gal on the same day after myogenic induction, by one-way ANOVA). a.u., arbitrary unit(s).

FIGURE 6. siRNA-mediated Gab1 knockdown enhances myogenic differentiation. A, C2C12 myoblasts were transfected with control siRNA (control) or with two independent siRNAs targeting different sequences of Gab1 (Gab1#1 and Gab1#2) for 72 h. Cell lysates were subjected to Western blot analysis. AKT was also checked as a loading control. B, C2C12 myoblasts were transfected with siRNAs as described in Fig. 6A. On the third day after transfection, the medium was changed from growth medium containing 20% FBS to differentiation medium containing 80 ng/ml IGF-I. Cells were cultured in the presence of IGF-I for 2 days. Cells were immunostained with anti-MHC antibody and post-stained with Hoechst 33342 nuclear dye. Experiments were repeated three times with similar results. C, cell lysates were collected at the indicated time after induction of myogenic differentiation and subjected to Western blot analysis. On the first and second day after myogenic induction, the expression level of MHC and myogenin were increased in cells transfected with Gab1 siRNAs (#1 or #2) compared with control. AKT was examined as a loading control. D, the relative expression level of MHC was quantified by normalizing the expression of MHC by that of AKT. Values are shown as means ± S.D. (*, p < 0.01 compared with control cells on the same day after myogenic induction, by one-way ANOVA). a.u., arbitrary unit(s).]
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To elucidate a potential mechanism how Gab1 is involved in IGF-I-mediated myogenic differentiation, the extent of myogenic differentiation was enhanced in myoblasts transfected with Gab1-targeted siRNAs compared with control (Fig. 6B). Consistently, Western blot analysis revealed a significant increase of MHC expression in myoblasts transfected with Gab1-targeted siRNAs compared with control (Fig. 6C). Therefore, we could not evaluate the myogenic differentiation in C2C12 myoblasts transfected with SHP2-targeted siRNAs (supplemental Fig. S2B). Moreover, we performed siRNAs-mediated SHP2 knockdown in C2C12 myoblasts. SHP2 protein expression was reduced by 70% in the myoblasts transfected with SHP2-targeted siRNAs compared with control (supplemental Fig. S2A). On the contrary to the results obtained by Gab1-targeted siRNAs experiments, SHP2 knockdown inhibited proliferation of C2C12 myoblasts. Therefore, we could not evaluate the myogenic differentiation in C2C12 myoblasts transfected with SHP2-targeted siRNAs (supplemental Fig. S2B).

IGF-I-induced Activation of ERK1/2 Is Regulated by Gab1-SHP2 Complex through Activation of SHP2 in C2C12 Myoblasts—To elucidate a potential mechanism how Gab1 is involved in IGF-I-mediated myogenic differentiation of C2C12 myoblasts, we examined the effects of adenovirus-mediated overexpression of Gab1 WT, Gab1 SHP2−/−, and Gab1 p85−/− on the anti-MHC antibody. The extent of myogenic differentiation was enhanced in myoblasts transfected with Gab1-targeted siRNAs compared with control (Fig. 6B). Consistently, Western blot analysis revealed a significant increase of MHC expression in myoblasts transfected with Gab1-targeted siRNAs compared with control (Fig. 6C). Furthermore, the expression of myogenin was increased in myoblasts transfected with Gab1-targeted siRNAs (Fig. 6C). These data coincide with the results obtained via overexpression experiments using adenovirus vectors and suggest that Gab1 has an inhibitory role in the IGF-I-induced myogenic differentiation.

Moreover, we performed siRNAs-mediated SHP2 knockdown in C2C12 myoblasts. SHP2 protein expression was reduced by 70% in the myoblasts transfected with SHP2-targeted siRNAs compared with control (supplemental Fig. S2A). On the contrary to the results obtained by Gab1-targeted siRNAs experiments, SHP2 knockdown inhibited proliferation of C2C12 myoblasts. Therefore, we could not evaluate the myogenic differentiation in C2C12 myoblasts transfected with SHP2-targeted siRNAs (supplemental Fig. S2B).
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IGF-I-dependent signaling pathways downstream of Gab1. IGF-I induced activation of ERK1/2 and AKT in control myoblasts expressing β-gal. IGF-I-induced activation of ERK1/2 was significantly augmented in myoblasts overexpressing Gab1WT or Gab1Δp85 compared with control myoblasts expressing β-gal. On the other hand, activation of ERK1/2 was significantly reduced in myoblasts expressing Gab1ΔSHP2 (Fig. 7, A and B). On the other hand, activation of ERK1/2 was not changed in myoblasts expressing Gab2ΔSHP2 compared with control myoblasts expressing β-gal (supplemental Fig. S1D). These data indicate that Gab1 plays a critical role in IGF-I-induced ERK1/2 activation through interaction with SHP2 in C2C12 myoblasts.

IGF-I-induced AKT activation was almost comparable in myoblasts expressing β-gal, Gab1WT, or Gab1Δp85. On the contrary, activation of AKT seemed to be enhanced in cells overexpressing Gab1Δp85, compared with the other three groups (Fig. 7B). These data indicate that Gab1 might have a competitive role for sequestering p85 in the cytoplasm from other scaffolding adaptor proteins such as IRS-1.

To confirm the requirement of SHP2 activity for activation of ERK1/2 upon IGF-I stimulation, we examined the effects of adenovirus-mediated overexpression of SHP2WT or SHP2C/S inhibitor, U0126, before inducing myoblasts into myogenic differentiation in the presence of IGF-I. U0126 enhanced IGF-I-induced up-regulation of MHC and myogenin in control myoblasts expressing β-gal (Fig. 9, A and B). Furthermore, U0126 reversed the inhibitory effect of Gab1WT overexpression on IGF-I-induced myogenic differentiation (Fig. 9, C and D). These findings suggest that Gab1 inhibits IGF-I-dependent myogenesis through activating MEK1/2-ERK1/2 pathway.

Finally, we examined the effect of adenoviral overexpression of constitutively active MEK1 (MEK1CA). Overexpression of MEK1CA repressed the enhanced myogenic differentiation observed in myoblasts overexpressing Gab1ΔSHP2 (Fig. 10, A and B). Collectively, interaction of Gab1 with SHP2 negatively regulates IGF-I-dependent myogenic differentiation in C2C12 myoblasts via activation of the MEK1/2-ERK1/2 pathway (Fig. 11).

**DISCUSSION**

In this study, we investigated the role of Gab1 in skeletal muscle differentiation. To our knowledge, this study demon-
strates for the first time that Gab1 has an inhibitory role in IGF-I-induced myogenic differentiation through activating SHP2-MEK1/2-ERK1/2 signaling pathway.

IGF-I induced complex formation of Gab1 with SHP2 in C2C12 myoblasts. It has been reported that Gab1 undergoes dramatic tyrosine-dephosphorylation upon changing growth medium to differentiation medium containing 2% HS in C2C12 cells (42). This finding indicated that Gab1 might have a key role in myogenic differentiation. Recently, it has been reported that Gab1 itself is a substrate of SHP2 (39). Furthermore, it has been reported that phosphorylation of ERK1/2 decreases while phosphorylation of AKT increases during differentiation with either insulin or 2% HS (9). Therefore, tyrosine dephosphorylation of Gab1 and subsequent dissociation of SHP2 from dephosphorylated Gab1 might be essential steps for the diminution of ERK1/2 activity during myogenic differentiation.

The Gab1-SHP2 complex formation is indispensable for IGF-I-induced activation of ERK1/2. It has been reported that Gab1-SHP2 interaction has an essential role for ERK1/2 activation downstream of EGF family/ErbB receptor signaling or hepatocyte growth factor/c-Met signaling (20–23). Furthermore, the recent study creating liver-specific Gab1 knock-out mice also demonstrated that Gab1 is required for both insulin-elicited ERK1/2 activation and subsequent inhibition of IRS-1-P3K-AKT signaling (34). Here, we demonstrated that overexpression of Gab1/SHP2, which is incapable of associating with SHP2, repressed IGF-I-dependent activation of ERK1/2 in C2C12 myoblasts. In a similar context, SHP2 itself has been reported to have crucial roles for ERK1/2 activation downstream of various growth factors, including IGF-I (24, 43–46).

We also observed that overexpression of Gab1/SHP2, which is incapable of associating with SHP2, repressed IGF-I-dependent activation of ERK1/2 in C2C12 myoblasts. In a similar context, SHP2 itself has been reported to have crucial roles for ERK1/2 activation downstream of various growth factors, including IGF-I (24, 43–46). We also observed that overexpression of phosphatase-inactive SHP2 (SHP2/−/−) inhibited IGF-I-dependent activation of ERK1/2 in C2C12 myoblasts. These findings demonstrate that Gab1-SHP2 complex formation is requisite for both activation of SHP2 itself and ERK1/2 upon IGF-1 stimulation in C2C12 myoblasts. Because the mechanism how SHP2 regulates receptor tyrosine kinase-mediated ERK1/2 activation remains controversial (18, 19), further analyses are needed for uncovering the mechanism how Gab1-SHP2 complex regulates IGF-I-induced activation of ERK1/2.

The Gab1-SHP2 complex exerts an inhibitory effect on the IGF-I-induced myogenic differentiation through activation of ERK1/2. IGFs have been reported to stimulate both proliferation and differentiation of skeletal muscle cells in culture (3, 4). IGFs activate two major cytoplasmic signaling pathways, PI3K-AKT cascade and Raf-MEK1/2-ERK1/2 MAPK cascade. The former has been reported to have positive effects on myogenesis, and the latter MAPK-signaling cascade has been reported to have detrimental effects on the myogenic differentiation
induced by insulin or IGFs (3, 9, 10). SHP2 has been also reported to play an inhibitory role for myogenic differentiation in the presence of fibroblast growth factor 2 (FGF2) in C2C12 myoblasts partly in an ERK1/2-dependent manner (44). We found that IGF-I-induced myogenic differentiation was inhibited by overexpression of either Gab1WT or Gab1Δp85, but not by that of SHP2WT. In addition, IGF-I-induced ERK1/2 activation in C2C12 myoblasts was enhanced by overexpression of either Gab1WT or Gab1Δp85, but not by that of SHP2WT. SHP2 can be specifically activated through binding directly with tyrosine-phosphorylated docking proteins such as Gab1 or FRS2α (18), suggesting that a sufficient amount of Gab1 is required to fully activate SHP2 by binding with SHP2 and that an insufficient amount of endogenous Gab1 might be the major limiting factor in overexpressed SHP2WT-mediated myogenic differentiation. Collectively, these data suggest that Gab1 is a crucial negative regulator for IGF-I-dependent myogenic differentiation through activation of the SHP2-MEK1/2-ERK1/2 signaling pathway (Fig. 10).

The migration of muscle progenitor cells into the limb anlage from somites is strongly impaired in Gab1-knock-out embryos similarly in c-Met-knock-out mice (32). Furthermore, it has been reported that the knock-in mice, which carry mutant Gab1 incapable of binding with SHP2, displayed quite similar defects in migration of muscle progenitor cells from somites into the limb anlage during embryogenesis as observed in Gab1-knock-out mice (29). In clear contrast, the knock-in mice, which carry mutant Gab1 incapable of binding either Grb2 or c-Met, did not show any defects in migration of muscle progenitor cells, suggesting the specific role of Gab1-SHP2 complex in the migration of muscle progenitor cells (29). These previous data coincide with our findings that Gab1-SHP2 interaction has an inhibitory effect on IGF-I-induced myogenic differentiation. Taken together, Gab1 might play a key role not only for inhibition of myogenesis but also for maintenance of the undifferentiated state of mesenchymal cells through activation of SHP2.

Although the PI3K-AKT signaling pathway is central to IGF-I-dependent signaling and myogenic differentiation, our data demonstrate that the diminution of ERK1/2 activity is a prerequisite for IGF-I-induced myogenesis. IGFs promote skeletal muscle differentiation through PI3K-AKT-dependent signaling pathway (3, 6, 7, 47–49). It has been reported that the activity of AKT increased during myogenic differentiation under cultivation in 2% HS or IGF-I-containing differentiation medium (9). We found that overexpression of Gab1Δp85 in C2C12 myoblasts resulted in enhanced activation of AKT upon stimulation with IGF-I, compared with those overexpressing Gab1WT or Gab1ΔSHP2. However, overexpression of Gab1Δp85 did not enhance, but repressed IGF-I-induced myogenic differentiation to a similar extent to Gab1WT. In clear contrast, overexpression of Gab1ΔSHP2 significantly enhanced IGF-I-induced myogenic differentiation compared with control. These findings indicate that myogenic differentiation requires the diminution of Gab1-SHP2-ERK1/2 signaling pathway prior to full activation of PI3K-AKT signaling pathway. It has been reported that the blockade of ERK1/2 signaling pathway enhances myogenic differentiation (3, 8–10). Inhibition of the ERK1/2 pathway has been reported to up-regulate Mirk/dyrk1B, a RhoA-dependent serine/threonine kinase that positively regulates skeletal muscle differentiation and inhibits apoptosis of myoblasts (50). Mirk/dyrk1B might be one of the candidate molecules through which ERK1/2 exerts an inhibitory effect on skeletal muscle differentiation.

In conclusion, the present study reveals that Gab1-SHP2 interaction exerts an inhibitory effect on IGF-I-dependent myogenic differentiation via activation of ERK1/2 signaling pathway.

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