β1-Integrin and PI 3-kinase regulate RhoA-dependent activation of skeletal α-actin promoter in myoblasts

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β1-Integrin and PI 3-kinase regulate RhoA-dependent activation of skeletal α-actin promoter in myoblasts. Am J Physiol Heart Circ Physiol 278: H1736–H1743, 2000.—RhoA GTPase, a regulator of actin cytoskeleton, is also involved in regulating c-fos gene expression through its effect on serum response factor (SRF) transcriptional activity. We have also shown that RhoA plays a critical role in myogenesis and regulates expression of SRF-dependent muscle genes, including skeletal α-actin. In the present study, we examined whether the RhoA signaling pathway cross-talks with other myogenic signaling pathways to regulate skeletal α-actin promoter activity in myoblasts. We found that extracellular matrix proteins and the β1-integrin stimulated RhoA-dependent activation of the α-actin promoter. The muscle-specific isoform β1D selectively activated the α-actin promoter in concert with RhoA but inhibited the c-fos promoter. In addition, focal adhesion kinase (FAK) and phosphatidylinositol (PI) 3-kinase were required for full activation of the α-actin promoter by RhoA. Expression of a dominant negative mutant of FAK, application of wortmannin to cultured myoblasts, or expression of a dominant negative mutant of PI 3-kinase inhibited α-actin promoter activity induced by RhoA. These results suggest that RhoA, β1-integrin, FAK, and PI 3-kinase serve together as an important signaling network in regulating muscle gene expression.

RhoA signaling; β1D-integrin; focal adhesion kinase

THE SMALL GTPASE RhoA is involved in many actin-based cellular processes including cell adhesion, cytokinesis, stress fiber formation, and smooth muscle contractility (reviewed in Refs. 14 and 37). RhoA also modulates transcriptional activity of serum response factor (SRF), a homodimeric MADS-box-containing protein (17). SRF has been shown to be required for expression of the skeletal and cardiac α-actin genes and smooth muscle α- and γ-actin genes, which are among the earliest markers for mesoderm-derived skeletal, cardiac, and smooth muscle differentiation (4, 8, 28). We have previously shown that RhoA activates skeletal α-actin transcription in C2C12 myoblasts through SRF and that stable expression of a dominant negative mutant of RhoA reduces terminal differentiation, in part, through its effect on SRF activity (40). A recent report has also shown that inhibition of Rho family proteins by the GDP dissociation inhibitor RhoGDI suppresses myogenesis in C2C12 myoblasts (34).

One way to investigate the mechanisms by which RhoA is involved in myogenesis is to determine whether RhoA interacts with signal pathways previously shown to be important in muscle differentiation. RhoA has been shown to function both upstream and downstream of integrins in the context of focal adhesion formation and stress fiber assembly (reviewed in Ref. 5). All integrins are heterodimeric transmembrane receptors consisting of an α-subunit associated with a β-subunit and mediating association between extracellular matrix (ECM) and cytoskeleton elements. Experimental evidence for the importance of integrin interaction with ECM proteins during muscle differentiation was elucidated in fibronectin null mice, which are defective in forming somites (10). Myotube formation and myogenic differentiation were also blocked in myoblast cultures treated with antibodies to integrins (25). In addition, mice lacking focal adhesion kinase (FAK), an important mediator of integrin signaling, displayed a general defect in mesoderm formation, a phenotype similar to that observed in fibronectin-deficient mice (18). Moreover, levels of expression of several β1-integrins such as α4β1, α5β1, and α6β1 and their isoforms such as α2β1, α7β1, and α8β1 are developmentally regulated in skeletal muscle and heart (reviewed in Ref. 13), suggesting that integrin switches may be important in myogenesis and heart development. In contrast to the common β1-integrin isoform that is widely expressed and prominent in prefusion myoblasts, the β1D-integrin is restricted to skeletal muscle and heart and becomes the major isoform in these tissues (3, 38), suggesting that it may have an important function in these tissues.

Another potential signaling pathway involving RhoA during muscle differentiation is signaling through phosphatidylinositol (PI) 3-kinase (PI3K). Recent studies have shown that PI 3-kinase plays an important role in muscle differentiation and is required for insulin-like growth factor (IGF)-induced muscle differentiation (19–21). In addition, integrin-mediated signaling pathways also activate PI 3-kinase that is translocated to the cytoskeleton, which involves specific interactions of p85 subunit with actin filament and FAK (12). Moreover, PI 3-kinase functions downstream of RhoA in platelet and Swiss 3T3 cells (22, 42).

We examined whether β1-integrin-, FAK-, and PI 3-kinase-mediated myogenic signaling pathways cross talk with the RhoA signaling pathway to regulate SRF-dependent muscle gene expression in myoblasts.
dependent muscle gene expression such as skeletal α-actin promoter activity. In the present study, we found that ECM proteins and the β1-integrin affect RhoA-dependent activation of the skeletal α-actin in myoblasts. Interestingly, the muscle-specific β1-integrin potentiates RhoA-dependent activation of the skeletal α-actin promoter and inhibits RhoA-dependent activation of the c-fos promoter, suggesting that RhoA regulates these two SRF-dependent promoter activities through different signaling pathways. Moreover, FAK and PI 3-kinase are required for RhoA-dependent activation of the α-actin promoter. A dominant negative mutant of FAK (FAK-related non-kinase [FRNK]) inhibits its activation of the α-actin promoter by RhoA. Both wortmannin and a dominant negative mutant of PI 3-kinase (Δp85) inhibit α-actin promoter activity induced by RhoA. In addition, RhoA increases PI 3-kinase activity associated with exogenous p85 subunit. These results suggest that RhoA, β1-integrin, FAK, and PI 3-kinase serve together as an important signaling network in regulating muscle-specific gene expression.

MATERIALS AND METHODS

Plasmid constructs. The reporter plasmid SK-luc contains the avian skeletal α-actin promoter from −398 to +25 bp linked to the firefly luciferase reporter gene (24). The reporter plasmid c-fos-SRE-luc contains the c-fos serum response element (SRE) region (−318 to −291 bp) (24). The expression plasmids pCGN-RhoA and pCGN-V14-RhoA were constructed as previously described (40). The CDNA constructs for full-length avian FAK and the truncated COOH-terminal FAK (FRNK) were cloned into the pCMV-Myc vector and were kindly provided by Drs. Susan LaFlamme and Kenneth Yamada (23). The full-length CDNA constructs for human β1α- and β1β-integrins were kindly provided by Dr. Masato K Asagusa (15).

Tissue culture, plasmid DNA transfection, and reporter gene assays. C2C12 mouse myoblasts (27) were maintained in DMEM (GIBCO-BRL) with 10% fetal bovine serum (FBS). Cells were plated at a density of 3 × 104 cells in 60-mm tissue culture plates and were transfected after 24 h. Cells were transfected with −1 µg of total plasmid DNA containing the indicated reporter plasmid (0.1 µg of SK-luc or c-fos-SRE-luc) with expression plasmids and balanced with parental expression vector. Transfections were performed using lipofectamine (GIBCO-BRL) according to the manufacturer’s instructions. Cells were placed in DMEM with 10% FBS and harvested 48 h posttransfection. Luciferase activity and protein content were measured as previously described (24). Luciferase activity was normalized to the total protein, and data were expressed as luciferase activity normalized to baseline reporter gene. All experiments were performed in duplicate and repeated three to five times. For experiments evaluating effects of ECM proteins on skeletal α-actin promoter activity, plates were coated overnight with rat tail collagen, fibronectin (20 µg/ml), and poly-L-lysine (10 µg/ml) and then blocked with 2% BSA for 2 h before plating. For experiments evaluating PI 3-kinase activity, cells were placed in medium with or without wortmannin (1 µM) and harvested 48 h posttransfection. The medium was replaced every 8 h because of the instability of wortmannin.

Protein expression analysis. Expression levels of cDNA expression plasmids were examined by Western blot analysis. Myoblasts on 60-mm plates were transiently transfected with 2 µg of each expression plasmid using lipofectamine. After 48 h, the cells were solubilized with lysis buffer (20 mM Tris, pH 7.5, 1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 50 µg/ml antipain) for 30 min with shaking at 4°C. Cell lysates were then cleared by centrifugation. In the detection of Myc-tagged FAK and FRNK, whole cell protein extracts (50 µg) were run on an 8% SDS-PAGE gel, transferred to Immobilon membrane (Millipore), and probed with anti-Myc monoclonal antibody (OncoImaging analysis (Molecular Dynamics). Statistics. Data are expressed as means ± SE, relative to values for parallel cultures of control vector-transfected cells. Student’s t-test was used for data comparison, with a significance level of P < 0.05.

RESULTS

ECM proteins regulate activation of the skeletal α-actin promoter by RhoA. We have shown that V14-RhoA, a constitutively active RhoA mutant, activates skeletal α-actin promoter up to seven times the basal level, whereas N19-RhoA, a dominant negative mutant, represses the basal level (control vector-transfected cells) up to 40% in a dose-dependent manner in myoblasts (40). We have shown that activation of the skeletal α-actin promoter by RhoA depends on an intact SRE and a functional SRF (40). To investigate the possible interactions between integrin and RhoA signaling pathways during muscle differentiation, we first examined the role of integrin-independent (poly-L-lysine) and -dependent (collagen and fibronectin) cell adhesion on skeletal α-actin promoter activity in concert with RhoA. As shown in Fig. 1, activation of the skeletal α-actin promoter by V14-RhoA was significantly increased in myoblasts attached to collagen (P = 0.046) or fibronectin (P = 0.007) but was decreased in cells attached to...
Thus results of this experiment indicate that muscle differentiation (10, 25). We examined whether grins that have been shown to play a critical role in with RhoA-dependent activation of the skeletal

Our results show that IL2R-integrin and the expression vector served as controls. Disruption of plasmic domain of interleukin-2 receptor and the cytoplasmic domain of IL2R-α5) containing the cytoplasmic domain of α5-integrin and the expression vector served as controls. Our results show that IL2R-β1 significantly reduced the activity of the promoter in V14-Rhoa-transfected
cells (P = 0.027) with no effect on the basal activity of the promoter (P = 0.382). The noninteractive IL2R-α5 control had no significant effect on the activity of the promoter in both V14-Rhoa-transfected (P = 0.338) and control vector-transfected cells (P = 0.171) (Fig. 2B). Thus results of this experiment indicate that disruption of β1-integrin-mediated signaling interferes with RhoA-dependent activation of the skeletal α-actin promoter in myoblasts.

Muscle-specific β1D-isofrom potentiates activation of the skeletal α-actin promoter by RhoA. The β1A- and β1D-integrin isofroms are very similar and differ by only 13 amino acids in their cytoplasmic tail (3, 38). We examined whether these two integrin isofroms have different regulatory roles in skeletal α-actin promoter activity. Expression of either of these integrins alone in myoblasts did not result in increased activity of the skeletal α-actin promoter. Coexpression of β1D-integrin, but not β1A-integrin, with V14-Rhoa significantly increased RhoA-dependent activation of the SK-luc reporter plasmid (P = 0.034) (Fig. 3A), indicating a selective role in mediating RhoA signaling in regulating skeletal α-actin gene expression. It is worth noting that exogenous expression of β1A-integrin had no effect on the activation of the skeletal α-actin promoter by RhoA, whereas a dominant negative β1A-isofrom inhibited this activation. These results suggest that the endogenous level of β1A-integrin is not a limiting factor for activation of the α-actin promoter by RhoA in myoblasts.

We have previously observed that Rhoa activates both skeletal α-actin and c-fos-SRE promoters in myoblasts (40). Interestingly, in contrast to its stimulatory effect on skeletal α-actin promoter activation by RhoA, β1D-integrin inhibited activation of c-fos-SRE by RhoA (P = 0.004) (Fig. 3B), indicating that Rhoa regulates these two SRF-dependent promoter activities through different means.

FAK is required for skeletal α-actin promoter activity. We next examined whether FAK, an important downstream mediator of integrin signaling, would also be a mediator of RhoA signaling in skeletal α-actin promoter activation. A truncated mutant of FAK, FRNK (Fig. 4A), which contains the COOH-terminal domain of FAK without the kinase domain, was previously shown (31) to act as a negative inhibitor. Expression of FRNK alone reduced basal level of the skeletal α-actin promoter activity (P = 0.025). It also reduced activation of the α-actin promoter by V14-Rhoa (P = 0.029) and

![Fig. 2. RhoA-dependent activation of skeletal α-actin promoter is reduced by alteration of β1-integrin signaling. A: schematic diagram of β1- and α5-integrin chimeras. IL2R, interleukin-2 receptor. B: C2C12 myoblasts were transfected with 0.1 µg of SK-luc together with 0.75 µg of control vector plasmid or 0.25 µg of V14-Rhoa, 0.5 µg of IL2Rβ1, or 0.5 µg of IL2Rα5 alone or in combination as indicated. Data are presented as described in Fig. 1 legend. *Significantly different from control vector-transfected cells (P < 0.05).](http://ajpheart.physiology.org/ by 10.220.33.6 on April 14, 2017)
coactivation by V14-RhoA and β1D-integrin (P < 0.026) (Fig. 4B), indicating that FAK may be required for efficient activation of the α-actin promoter. However, expression of exogenous FAK did not significantly stimulate the α-actin promoter in V14-RhoA-transfected (P = 0.427) and control vector-transfected cells (P = 0.469), perhaps indicating that the level of endogenous FAK might not be a limiting factor for the activation of the skeletal α-actin promoter under these conditions. Expression of exogenous FAK and FRNK was confirmed by Western analysis (Fig. 4C).

PI 3-kinase is required for skeletal α-actin promoter activity. PI 3-kinase is another potential mediator of RhoA signaling in myogenesis. Two approaches were taken to determine whether PI 3-kinase is required for RhoA-mediated activation of the skeletal α-actin promoter. First, wortmannin was added to muscle cultures to inhibit PI 3-kinase activity (36). Second, PI 3-kinase activity was inhibited by expression of cotransfected plasmid expressing a dominant negative mutant of the p85 subunit (Δp85) that lacks the binding site for the p110 catalytic subunit (15).

As shown in Fig. 5, 1 µM wortmannin significantly inhibited RhoA-mediated activation of the skeletal α-actin promoter. Another chemical inhibitor of PI 3-kinase, LY-294002, also was found to inhibit activation of the actin promoter by RhoA (data not shown). Because wortmannin may not be PI 3-kinase specific, we then evaluated the effect of Δp85, a dominant negative PI 3-kinase mutant, on skeletal α-actin promoter activation (Fig. 6A). The expression plasmid SRα-Δp85 was transfected into C2C12 myoblasts, and its effects were compared with the empty vector SRα and the expression plasmid SRα-p85 encoding the wild-type p85 (Fig. 6B). Expression of Δp85 reduced the basal activity of skeletal α-actin promoter (P = 0.028) and the activity stimulated by V14-RhoA (P = 0.021),
whereas the wild-type p85 had no significant effect on the promoter activity under these conditions ($P < 0.125$ or 0.345, respectively). Expression of these recombinant proteins was confirmed by Western blot analysis (Fig. 6C). These observations are consistent with the results obtained with wortmannin, indicating that PI 3-kinase may mediate RhoA-dependent activation of the skeletal $\alpha$-actin promoter.

Finally, to investigate the mechanism by which PI 3-kinase modulates RhoA activation of the skeletal $\alpha$-actin promoter, we examined whether V14-RhoA increased PI 3-kinase activity in myoblasts. HA epitope-tagged p85 or $\Delta p85$ was expressed with or without V14-RhoA in myoblasts. These exogenous proteins were then immunoprecipitated with anti-HA antibody, which does not affect catalytic activity of immunoprecipitated PI 3-kinase, and assayed for lipid kinase activity, which was normalized to the p85 content in the immunocomplex determined by Western blot analysis (Fig. 7A). The activity of PI 3-kinase activity associated with the wild-type p85 was significantly increased in the presence of V14-RhoA ($P = 0.015$, $n = 3$), whereas that associated with $\Delta p85$ was not affected (Fig. 7B). One possible mechanism by which RhoA increases PI 3-kinase activity is to increase its association with FAK. However, by Western blot analysis, we found only a minor portion of the endogenous p85 subunit of PI 3-kinase associated with FAK (<10%), and this interaction was not increased by V14-RhoA. Similarly, PI 3-kinase activity coimmunoprecipitated with FAK was not increased by V14-RhoA. These observations suggest that PI 3-kinase might not be immediately downstream of FAK in regulating RhoA-dependent activation of the skeletal $\alpha$-actin promoter.

**DISCUSSION**

Integrin-mediated signaling regulates RhoA-dependent activation of the skeletal $\alpha$-actin promoter. We examined whether RhoA interacts with signaling pathways involved in muscle differentiation. The integrin-mediated signaling pathway was of particular interest because considerable amounts of evidence support a critical role for integrin-mediated signals in muscle differentiation, and functional interactions between RhoA and integrin signaling pathways have been extensively investigated in focal adhesion formation and stress fiber assembly. We showed several lines of evidence for functional interactions between RhoA and integrin-mediated signals in regulating muscle gene expression: 1) extracellular matrix proteins such as collagen and fibronectin enhanced RhoA-dependent activation of the skeletal $\alpha$-actin promoter; 2) a dominant negative mutant of $\beta_1$-integrin reduced activation of the $\alpha$-actin by RhoA; and 3) the muscle-specific $\beta_1$-integrin $\beta_1$D-isoform was found to enhance RhoA-dependent activation of the skeletal $\alpha$-actin promoter.
widely expressed, the C2C12 myoblasts represses myoblast replication, suggesting that RhoA may act through at least two different pathways to regulate skeletal α-actin and c-fos promoters. FAK is required for efficient activation of the skeletal α-actin promoter. Recent reports have shown that PI 3-kinase plays a vital role in myogenesis (19–21). Functional interactions between RhoA and PI 3-kinase have been observed between RhoA and PI 3-kinase in platelet and Swiss 3T3 cells, where RhoA is required for PI 3-kinase activation in response to extracellular stimuli (22, 42). Our results demonstrate that PI 3-kinase may be a mediator of the RhoA signaling pathway in regulating muscle-specific gene expression. Wortmannin, a known inhibitor of PI 3-kinase, and a dominant negative mutant of the p85 subunit of PI 3-kinase both inhibited RhoA-dependent activation of the skeletal α-actin promoter. PI 3-kinase activity associated with cotransfected p85 was also increased by V14-RhoA in myoblasts.

The mechanism by which PI 3-kinase interferes with RhoA signaling remains to be determined. It has been suggested that PI 3-kinase is involved in the integrin-mediated signal pathway, because PI 3-kinase is activated following cell attachment to ECM proteins and is then translocated to the cytoskeleton, which involves specific interactions of p85 subunit with actin filament and FAK (12). However, we observed that only a minor portion of the endogenous p85 subunit of PI 3-kinase was associated with FAK (16). However, a role for FAK in muscle differentiation has not been reported. The present study shows that FAK modulates RhoA-dependent activation of the skeletal α-actin promoter, suggesting a functional relationship between RhoA and FAK in regulating muscle-specific gene expressions in myoblasts. In primary cultured chicken myoblasts, we observed that FRNK reduced myosin heavy chain expression in mature myotubes (unpublished results), also supporting a role for FAK in myodifferentiation.

PI 3-kinases are required for efficient activation of the skeletal α-actin promoter while repressing c-fos promoter activity. In contrast to the common β1A-isoform, which is widely expressed, the β1D-isoform is restricted to skeletal muscle and heart and is the major isoform in these tissues, suggesting that it may have an important function in these tissues. Expression of β1D-isoform in C2C12 myoblasts represses myoblast replication, suggesting that β1D-isoform may play a role in myoblast withdrawal from the cell cycle (2). However, mice lacking the β1D-isoform (having only β1A-isoform) form normal mature muscle without obvious histological or ultrastructural abnormality (1). Our observation that β1D-isoform increased RhoA-dependent activation of the skeletal α-actin promoter, whereas β1A-isoform was ineffectual, indicates selective functional interaction between RhoA and this muscle-specific isoform in regulating muscle-specific gene expression. Our finding that β1D-isoform inhibits activation of c-fos-SRE by RhoA indicates that RhoA may act through at least two different pathways to regulate skeletal α-actin and c-fos promoters.
(30). It has been shown that IGF-dependent signal pathways through association with insulin receptor substrate 1 activate PI 3-kinase (20). Whether IGF-dependent activation of PI 3-kinase is involved in regulating the RhoA-dependent signal pathway in muscle cells is under investigation.

RhoA regulates SRF-dependent gene expressions through different signaling pathways in myoblasts and fibroblasts. SRF mediates RhoA signaling to the skeletal α-actin promoter through the proximal SRE site of the promoter (40). Since the discovery of regulation of c-fos promoter activation by RhoA (17), and PI 3-kinase was reported to function upstream RhoA to activate c-fos promoter (39) in fibroblasts. In C2C12 myoblasts, treatment with the Rho kinase inhibitor Y27632 (35) had no effect on myoblast differentiation and on activation of the skeletal kinase inhibitor Y27632 (35) had no effect on myoblast differentiation and on activation of the skeletal α-actin promoter by RhoA (unpublished results). An NF-κB inhibitor, IκBα-S32A/S36A, had no effect on the activation of the skeletal α-actin by RhoA (unpublished results). We are currently evaluating whether there is a role for LIM kinases in RhoA signaling to skeletal α-actin in myoblasts. These observations suggest that RhoA signaling to c-fos promoter in fibroblasts may be different from RhoA signaling to skeletal α-actin promoter in myoblasts. We have previously shown that activation of cardiac α-actin promoter by SRF is regulated by the formation of a functional complex of SRF with other muscle-specific cofactors such as Nkx2.5 (6) and GATA4 (unpublished results). SRF also associates with myogenic factors such as myogenin and MyoD (11). These observations may explain the discrepancies observed for nonmuscle c-fos versus myogenic SRE-dependent activities and suggest that RhoA activation of SRF-dependent muscle gene expression may involve recruitment of muscle-specific accessory factors that are susceptible to other myogenic signals.

In conclusion, regulation of muscle differentiation by RhoA is a complex process under the control of several regulatory pathways. These signaling pathways involve integrin interactions with the ECM proteins, FAK-mediated signal transduction, and generation of phospholipids. RhoA, a signaling molecule implicated in a diverse set of cellular functions, regulates muscle gene expression through cross talk with these signal pathways. Because SRF activation by RhoA appears to be regulated by different signaling pathways in fibroblasts and myoblasts, we propose that activation of SRF-dependent muscle gene expression by RhoA depends on the presence and activity of muscle-specific cofactors. Further studies are necessary to establish the physiological significance of the RhoA signaling network in muscle development, regeneration, and hypertrophy.

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