

The Rho Family G Proteins Play a Critical Role in Muscle Differentiation

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Received 19 May 1997/Returned for modification 3 August 1997/Accepted 26 November 1997

The Rho family GTP-binding proteins play a critical role in a variety of cytoskeleton-dependent cell functions. In this study, we examined the role of Rho family G proteins in muscle differentiation. Dominant negative forms of Rho family proteins and RhoGDI, a GDP dissociation inhibitor, suppressed transcription of muscle-specific genes, while mutationally activated forms of Rho family proteins strongly activated their transcription. C2C12 cells overexpressing RhoGDI (C2C12RhoGDI cells) did not differentiate into myotubes, and expression levels of myogenin, MRF4, and contractile protein genes but not MyoD and myf5 genes were markedly reduced in C2C12RhoGDI cells. The promoter activity of the myogenin gene was suppressed by dominant negative mutants of Rho family proteins and was reduced in C2C12RhoGDI cells. Expression of myocyte enhancer binding factor 2 (MEF2), which has been reported to be required for the expression of the myogenin gene, was reduced at the mRNA and protein levels in C2C12RhoGDI cells. These results suggest that the Rho family proteins play a critical role in muscle differentiation, possibly by regulating the expression of the myogenin and MEF2 genes.

Myogenic basic helix-loop-helix (bHLH) proteins are master regulatory proteins that activate the transcription of many muscle-specific genes during myogenesis (reviewed in references 60 and 89). Each of the four myogenic bHLH proteins, MyoD (17), myogenin (18, 90), myf5 (7), and MRF4 (8, 47, 67), can activate the skeletal myogenic program when introduced into a variety of cells derived from all three germ layers of the embryo. The bHLH motif mediates dimerization of myogenic factors with ubiquitous bHLH proteins such as E12/E47, and these heterodimeric complexes bind to a conserved DNA sequence known as the E box, which is present in the promoters and enhancers of most muscle-specific genes (54). Myocyte enhancer binding factor 2 (MEF2), which is a member of the MADS box family, also plays an important role in muscle differentiation (reviewed in reference 61). MEF2 activates transcription by binding to the consensus sequence, called the MEF2-binding site, which is also found in the control regions of numerous muscle-specific genes (23, 64). In embryos with loss-of-function mutations of the single *mef2* gene in *Drosophila* (*D-mef2*), somatic, cardiac, and visceral muscle cells did not differentiate (6, 38, 66). These results indicated that MEF2 is necessary for the differentiation of all types of muscle cells. MEF2 and myogenic bHLH proteins have been suggested to activate mutual expression in an autoregulatory network and maintain the expression of muscle-specific genes (5, 16, 31, 43, 56). Moreover, a recent study demonstrated that MEF2 and myogenic bHLH proteins synergistically activate expressions of muscle-specific genes via protein-protein interactions between DNA-binding domains of these heterologous classes of transcription factors (51). In addition, it has been reported that a variety of factors, such as fibroblast growth factor, transform-

ing growth factor β , and Ras, modulate muscle differentiation by regulating expression of myogenic bHLH proteins (9, 34, 36, 37).

The Rho family GTP-binding proteins (G proteins) consist of three subfamilies, Rho, Rac, and Cdc42 (25, 58, 77, 78). All members of the Rho family exhibit both GDP/GTP binding and GTPase activities; they are inactive when bound to GDP and active when bound to GTP (58). The GDP/GTP exchange reaction is regulated by guanine nucleotide exchange factors and GTPase-activating proteins. Among guanine nucleotide exchange factors, GDP dissociation stimulators catalyze the dissociation of GDP and convert Rho proteins into an active GTP-bound form, while RhoGDI, a GDP dissociation inhibitor, inhibits the dissociation of GDP (reviewed in references 58 and 78). The intrinsic GTPase activity of the GTP-bound form is stimulated by GTPase-activating proteins. The Rho family proteins have been shown to regulate a variety of cytoskeleton-dependent cell functions, such as cell morphology (11, 63, 71), formation of focal adhesions and stress fibers (63, 68, 75), cell motility (79, 80), platelet and lymphocyte aggregation (53, 85), growth factor- and phorbol ester-induced membrane ruffings (57, 69), contractile ring formation and cytokinesis (33, 39), smooth muscle contraction (29), cell cycle progression (91), neurite retraction (30), and bud formation in the yeast *Saccharomyces cerevisiae* (92). There is a regulatory cascade among the three subfamily members. Bradykinin stimulates the formation of filopodia by activating Cdc42 first, which is followed by the formation of lamellipodia induced by Rac activation, leading finally to the formation of stress fibers and focal contacts induced by Rho activation (35, 59). Recently, the Rho family of G proteins has also been shown to function in protein kinase cascades. Phosphatidylinositol 3-kinase (PI3K) has been reported to function upstream (27, 58, 69) and downstream (84, 94) of the Rho family proteins. It has been reported that Cdc42 and Rac proteins directly bind to and activate a family of highly related serine/threonine kinases referred to as p21-activated kinases (PAKs) (41, 42). They also regulate members of the mitogen-activated protein kinase

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(MAPK) family, such as c-Jun N-terminal kinase (JNK) and p38MAPK (4, 15, 46) and 70-kDa S6 kinase (13).

Recently, the Rho family of G proteins has been reported to be required for transcriptional activation of the *c-fos* serum response element (SRE) induced by extracellular signals and to activate its transcription via the MADS box-containing transcription factor serum response factor (SRF) (28). SRE in the promoter of the *c-fos* gene contains a core sequence known as the CArG box (48). The CArG box sequence is also found in the promoters of many muscle-specific genes and is essential for the expression of these genes (49, 50, 65). Although the precise mechanism by which the CArG box acts in the expression of these muscle-specific genes remains unknown, SRF has been shown to bind to the CArG box (81) and to be required for muscle differentiation (87). It has also been reported that SRF is necessary for the expression of some muscle-specific genes in concert with other myogenic factors (72) and that SRF physically interacts with myogenic bHLH proteins (24). In the present study, we examined the role of Rho family proteins in muscle differentiation. We show here that inhibition of Rho family functions suppresses transcription of muscle-specific genes irrespective of the existence of CArG box in their promoters. C2C12 cells overexpressing RhoGDI did not differentiate into myotubes, and expression levels of the myogenin, MRF4, and contractile protein genes but not the MyoD and myf5 genes were markedly reduced in the cell lines. The promoter activity of the myogenin gene was suppressed by dominant negative mutants of the Rho family and was reduced in RhoGDI-expressing cells. Expression of MEF2, which has been shown to be required for expression of the myogenin gene, was reduced at the mRNA and protein levels in those cells. These results suggest that the Rho family plays a critical role in muscle differentiation, possibly by regulating the expression of myogenin and MEF2.

MATERIALS AND METHODS

Cell culture and transfections. C2C12 mouse myoblasts were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 15% fetal bovine serum (FBS). Differentiation of C2C12 cells was induced by changing the culture medium from growth medium (GM) (DMEM with 15% FBS) to differentiation medium (DM) (DMEM with 0.1% FBS). To isolate stable transfectants, a 1.8-kb human RhoGDI cDNA (22) subcloned into the pMAM2-BSV vector (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) (32), which harbors a blasticidin S resistance gene and a dexamethasone (Dexa)-inducible mouse mammary tumor virus long terminal repeat-Rous sarcoma virus promoter, was transfected into C2C12 myoblasts by the calcium phosphate method. Permanently transfected cells were selected with 20 μ g of blasticidin S per ml, and two independent cell lines designated C2C12RhoGDI-1 and -2 were isolated. Transient transfections were performed by the calcium phosphate method. At 36 h after transfection, culture media were changed from GM to DM and cells were incubated for an additional 36 h so that they fully differentiated into myocytes. Then cells were harvested and subjected to luciferase assays. In preliminary experiments, we cotransfected simian virus 40 (SV40)-driven β -galactosidase as an internal control. The relative promoter activity was not changed with or without the correction by the β -galactosidase activity. Thus, the luciferase activity in the same amount of cell extracts was measured and presented without the correction by the β -galactosidase activity. Western blot analysis revealed that various transfected proteins were expressed at almost the same levels from 2 days after transfection through at least 1 week.

Plasmids. Expression vectors encoding RhoGDI and various mutants of RhoA, Rac1, and Cdc42 were provided by Y. Takai (22) and J. S. Gutkind (15), respectively. pMHC25, pTnT15, and pSRMyoDneo were provided by T. Endo (20). EMSV-Myo8 and MEF2C were gifts from E. Olson (18). pMSVmyf5 and pEMSVmyf6 were gifts from H. Arnold (7, 8). The myogenin promoter was from J. Schmidt (40). The reporter constructs of the skeletal α -actin promoter with various mutations were provided by M. D. Schneider (62). All plasmid DNAs were prepared by using Qiagen (Chatsworth, Calif.) plasmid DNA preparation kits.

Immunofluorescence. To assess the myogenic conversion of C2C12 myoblasts to myotubes, cells were immunostained with a monoclonal antibody (MF20) against sarcomeric myosin heavy chain (MHC) as described previously (3). Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G was used as the secondary antibody.

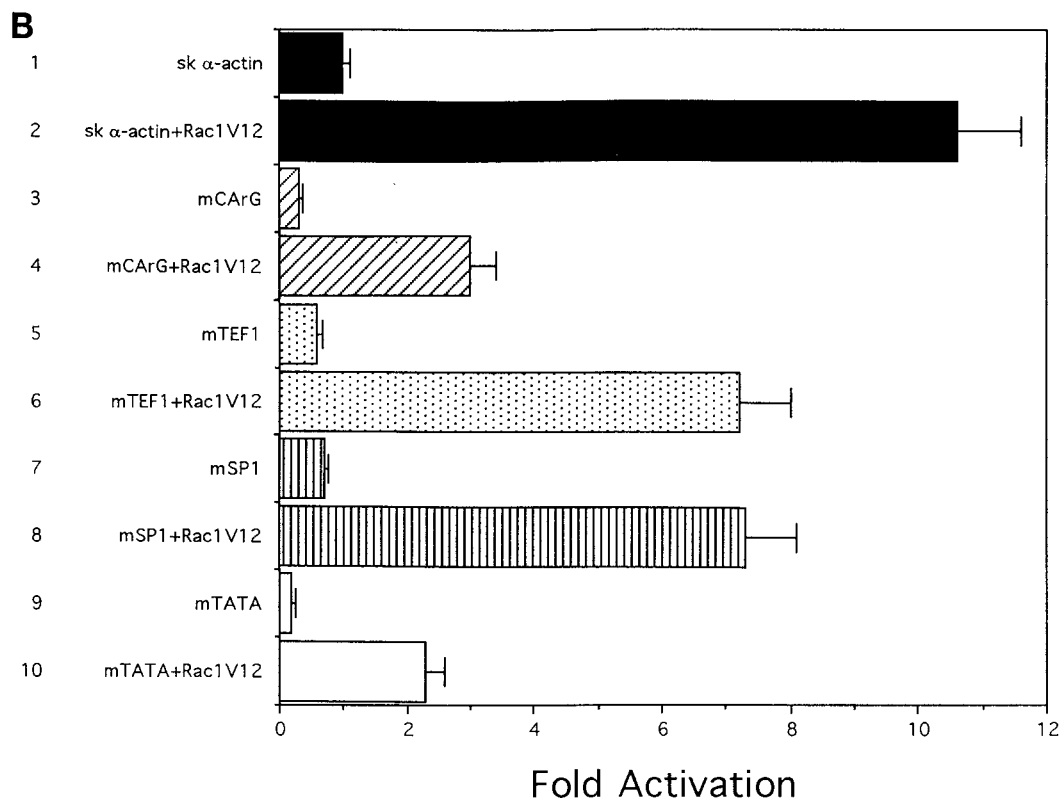
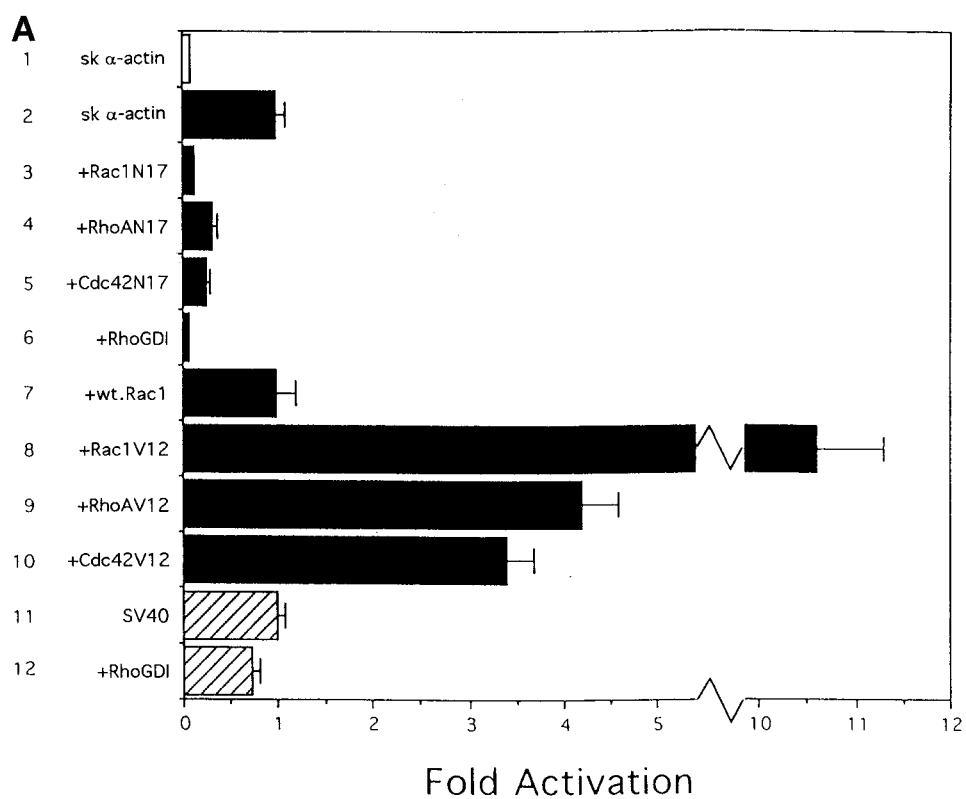
Northern blot analysis. Stable transfectants were maintained in GM, and 1 μ M Dexa was added to the culture medium 24 h before confluence. When cells were grown to 100% confluence, the culture medium was changed to DM with Dexa (1 μ M), and cells were maintained under these conditions for 3 days. Total RNA was extracted by the acid guanidine method, and 10 μ g of total RNA was loaded in each lane for Northern blot analysis. The following cDNA fragments were used as probes: the *Pst*I fragment of pMHC25 containing the rat skeletal muscle MHC cDNA, the *Pst*I fragment of pTnT15 containing the rat skeletal muscle troponin T (TnT) cDNA, the *Eco*RI fragment of pSRMyoDneo containing the MyoD cDNA (17), the *Eco*RI fragment of EMSV-Myo8 containing the murine myogenin cDNA (18), the *Eco*RI fragment of pMSVmyf5 containing human myf5 cDNA (7), and the *Eco*RI fragment of pEMSVmyf6 containing human MRF4 cDNA (8).

Gel mobility shift assay. Nuclear extracts were prepared from C2C12 cells or C2C12RhoGDI cells as described previously (23). Ten micrograms of nuclear extracts was incubated with the 32 P-labeled oligonucleotide probe corresponding to the MEF2 site of the myogenin promoter. The electrophoretic mobility shift assay was performed as described previously (23).

RESULTS

Rho family proteins are required for the transcription of muscle-specific genes. To test whether Rho family G proteins are involved in transcription of muscle-specific genes, we first examined the role of Rho family proteins in the transcription of the skeletal α -actin gene, which depends on the CArG box (14). A luciferase reporter plasmid containing the skeletal α -actin promoter (bp -394 to +24) was transiently transfected into C2C12 myoblasts together with dominant interfering mutants of Rac1 (Rac1N17), RhoA (RhoAN17), and Cdc42 (Cdc42N17) (15) or the GDP dissociation inhibitor RhoGDI (22). At 36 h after induction of C2C12 cell differentiation by changing the culture medium from GM to DM, luciferase activities were measured. The transcriptional activity of the skeletal α -actin gene was much higher in differentiated myotubes than in undifferentiated myoblasts (Fig. 1A, bars 1 and 2) and was reduced by cotransfection of either dominant interfering plasmid and RhoGDI (Fig. 1A, bars 2 to 6). RhoGDI, which inhibits the functions of all Rho family members, most strongly inhibited the transcriptional activity of the skeletal α -actin gene during muscle differentiation (Fig. 1A, bar 6), and the inhibitory activity was strongest in Rac1N17 among the three dominant interfering mutants (Fig. 1A, bar 3). Transfection of these interfering mutants of the Rho family proteins and RhoGDI did not show such a strong inhibitory effect on transcription of nonmuscle gene promoters such as the SV40-derived promoter (Fig. 1A, bars 11 and 12). We next examined the effects of wild-type and mutationally activated forms of Rho family G proteins (wild-type Rac1, Rac1V12, RhoAV12, or Cdc42V12) (35, 68, 69) on the activity of the skeletal α -actin promoter (Fig. 1A, bars 7 to 10). Although overexpression of wild-type Rac1 had no significant effects on the activity of the skeletal α -actin promoter (Fig. 1A, bar 7), all constitutively active mutants of Rho family proteins activated the promoter to various degrees (Fig. 1A, bars 8 to 10). Rac1V12 most strongly activated the transcription, by more than 10-fold (Fig. 1A, bar 8).

To examine whether the regulation of the skeletal α -actin gene by Rho proteins requires the CArG box, luciferase reporter plasmids containing various mutations in the skeletal α -actin promoter were transiently cotransfected with Rac1V12, which activated the skeletal α -actin promoter most strongly among Rho family proteins (Fig. 1B). Mutations in the CArG box (62) more strongly reduced responsiveness to Rac1V12 as compared with mutations in the TEF-1 binding site or SP1 binding site (62) (Fig. 1B, bars 1 to 8). These results suggest that the CArG box plays an important role in Rho protein-induced activation of skeletal α -actin transcription. However, since there remains some responsiveness to Rac1V12 even in



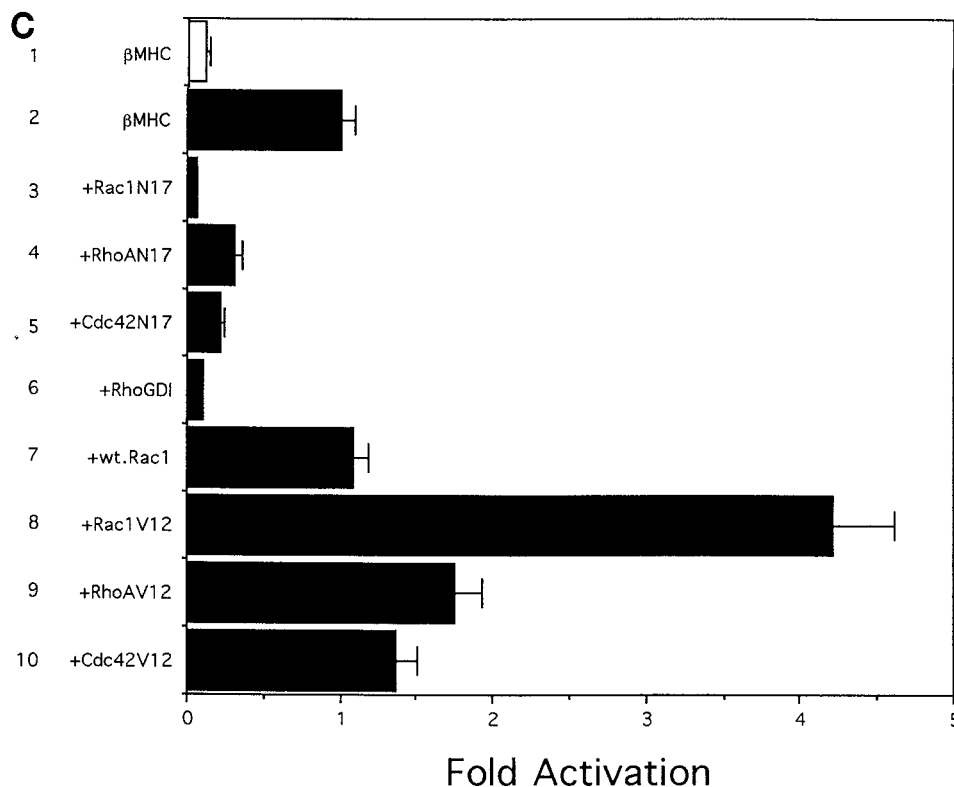


FIG. 1. Rho family proteins are involved in transcriptional regulation of muscle-specific genes. Luciferase reporter plasmids containing the wild-type (wt) skeletal α -actin promoter (A), mutants of the skeletal α -actin promoter (B), or the β MHC promoter (C) were transiently transfected into C2C12 myoblasts by the calcium phosphate method with effector plasmids encoding dominant interfering mutants (Rac1N17, RhoAN17, or Cdc42N17) or constitutively active mutants (Rac1V12, RhoAV12, or Cdc42V12) of Rac1, RhoA, or Cdc42, RhoGDI, or wild-type Rac1. Cell differentiation was induced by changing the culture medium from GM to DM at 36 h after transfection. Cells were cultured for an additional 36 h, and luciferase activities were measured by using 10 μ g of cell extracts. (A) Luciferase reporter plasmids containing the skeletal α -actin promoter (bars 1 to 10) and the SV40 promoter (bars 11 and 12) were transfected with various mutants of Rho proteins. (B) Luciferase reporter plasmids containing the wild-type skeletal α -actin promoter (bars 1 and 2) or the skeletal α -actin promoter with mutations in the CArG box (bars 3 and 4), the TEF-1 site (bars 5 and 6), the SP1 site (bars 7 and 8), or the TATA box (bars 9 and 10) were transfected with Rac1V12. (C) Luciferase reporter plasmids containing the β MHC promoter were transfected with various mutants of Rho proteins. Bars 1 in panels A and C represent the luciferase activity in undifferentiated myoblasts. Results are expressed relative to the levels of luciferase activity in cells transfected with vector alone (bars 2 and 11 in panel A, bar 1 in panel B, and bar lane 2 in panel C). The results are shown as means \pm standard errors for four independent experiments.

the CArG box mutant, there may be CArG box-dependent and -independent pathways in Rho protein-induced transactivation of the skeletal α -actin gene.

We next examined the role of Rho proteins in transcriptional regulation of the β MHC gene, whose transcription does not depend on the CArG box (83). When dominant interfering mutants of Rac1, RhoA, and Cdc42 or RhoGDI were overexpressed, luciferase activity of the rat β MHC promoter (bp -354 to +34) was also suppressed (Fig. 1C, bars 1 to 6). Among the interfering mutants, Rac1N17 showed the strongest inhibitory activity on the β MHC promoter (Fig. 1C, bar 3). Although wild-type Rac1 had no effects on the promoter activity of the β MHC gene (Fig. 1C, bar 7), constitutively active mutants of Rho proteins activated transcription of the β MHC gene to a lesser extent than that of the skeletal α -actin gene (Fig. 1C, bars 8 to 10). Rac1V12 most strongly (by ~4-fold) among the three constitutively active mutants activated the expression of the β MHC gene. These results suggest that Rho proteins may regulate the expression of muscle-specific genes during muscle differentiation, regardless of the presence of the CArG box in their promoters.

Inhibition of Rho protein functions suppresses myogenesis. To determine whether Rho family G proteins are necessary for myogenesis, we isolated two independent C2C12 cell lines

which were permanently transfected by human RhoGDI cDNA in a Dexamethasone-inducible promoter vector and designated them C2C12RhoGDI-1 and -2 cells. When the culture medium was changed from GM to DM, C2C12 myoblasts differentiated into myocytes, and many anti-MHC antibody (MF20)-positive myotubes were formed (Fig. 2A to C). The addition of 1 μ M Dexamethasone to the culture medium had no effect on muscle differentiation in parental C2C12 cells (Fig. 2D). When cultured in DM, C2C12RhoGDI-1 cells also differentiated into myotubes as did parental C2C12 cells in the absence of Dexamethasone (Fig. 2E). When expression of RhoGDI was induced by the addition of 1 μ M Dexamethasone, however, myogenesis was completely suppressed (Fig. 2F and G). There were no MF20-positive myotubes in C2C12RhoGDI-1 cells in the presence of Dexamethasone. When C2C12 myoblasts were pretreated for 24 h before differentiation with the ADP-ribosylation exoenzyme C3 from *Clostridium botulinum* (C3 exoenzyme), which selectively ribosylates and inhibits functions of Rho protein but not of Rac or Cdc42 protein, C2C12 cells formed very thin myotubes that were stained faintly with MF20 (Fig. 2H). Similar results were obtained with C2C12RhoGDI-2 cells. These results suggest that Rho family proteins play a critical role in terminal muscle differentiation.

Expression of muscle-specific genes in C2C12RhoGDI cells. To elucidate the mechanism of how Rho family proteins are

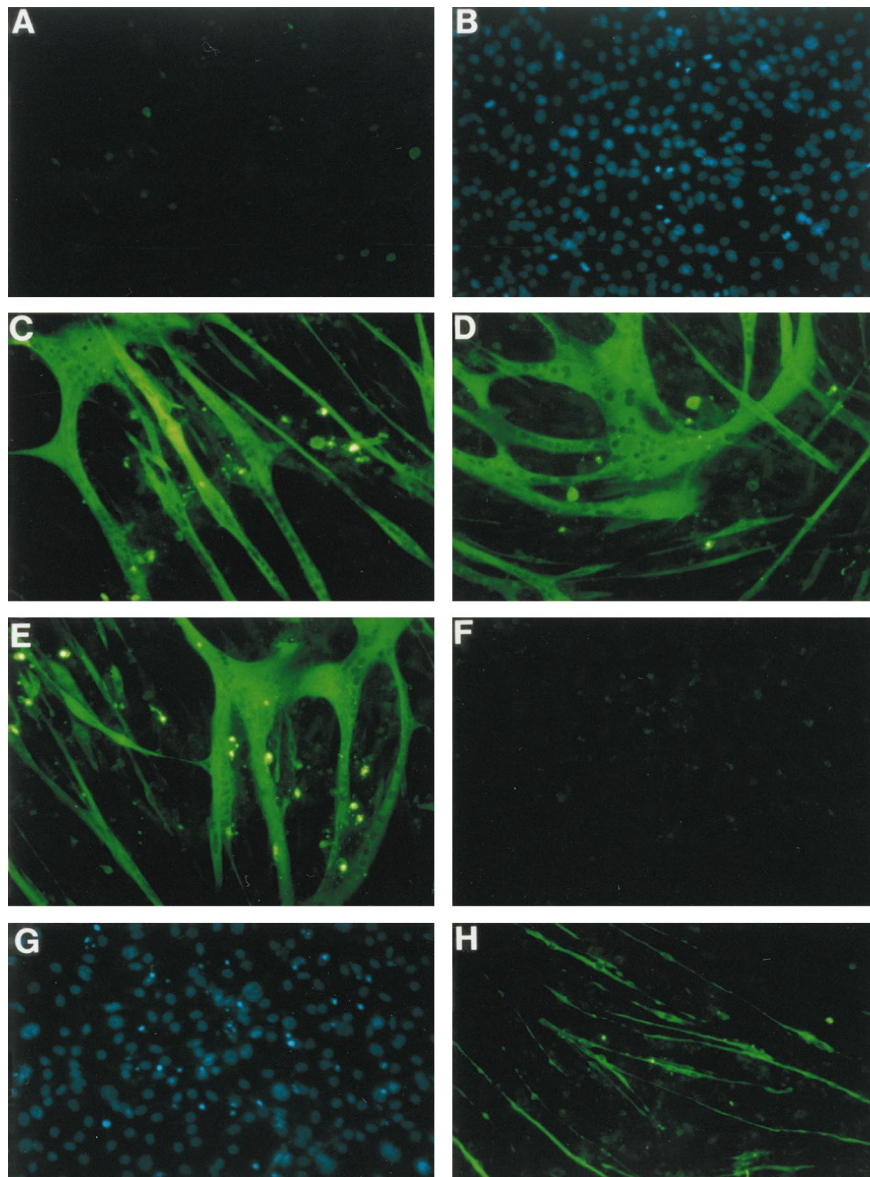


FIG. 2. Inhibition of muscle differentiation by overexpression of RhoGDI. C2C12 cells (A, B, C, D, and H) or C2C12RhoGDI-1 cells (E, F, and G), which bear the Dexamethasone-inducible RhoGDI, were cultured in GM (A and B) or in DM (C, D, E, F, G, and H). For differentiation, the culture medium was changed from GM to DM after confluency, and the cells were cultured for additional 3 days. A separate set of cultures was treated identically but was exposed to 1 μ M Dexamethasone (D and F) or to 10 μ g of *C. botulinum* ADP-ribosyltransferase C3 (H) from 24 h before the culture medium was changed. The cells were stained with anti-sarcomeric MHC antibody (MF20) (A, C, D, E, F, and H) or Hoechst dye (B and G).

involved in muscle differentiation, expression of muscle-specific genes was examined by Northern blot analysis. When C2C12 myoblasts were induced to differentiate into myotubes by serum deprivation, expression of myogenic bHLH protein genes such as the myogenin and MRF4 genes and of muscle-specific contractile protein genes such as the MHC and TnT genes was induced (Fig. 3, lanes 1 and 2). Transcripts of myf5 and MyoD were detected in both myoblasts and myotubes, as reported previously (7, 17). The addition of Dexamethasone to parental C2C12 cells did not affect the expression levels of any of these genes (Fig. 3, lanes 2 and 3). When induced to differentiate, C2C12RhoGDI-1 cells also expressed these muscle-specific

genes as abundantly as parental C2C12 cells in the absence of Dexamethasone (Fig. 3, lanes 2 and 5). When expression of RhoGDI was induced by Dexamethasone, although the mRNA levels of MyoD and myf5 were not changed, induction of the myogenin and MRF4 genes was markedly suppressed (Fig. 3, lanes 5 and 6). All contractile protein genes were also expressed at very low levels in C2C12RhoGDI-1 cells in the presence of Dexamethasone. Similar expression patterns were obtained with C2C12RhoGDI-2 cells. Since myogenin and MRF4 have been reported to play important roles in terminal muscle differentiation, we speculate that Rho family proteins are involved in muscle differentiation, possibly by regulating expression of these myogenic genes.

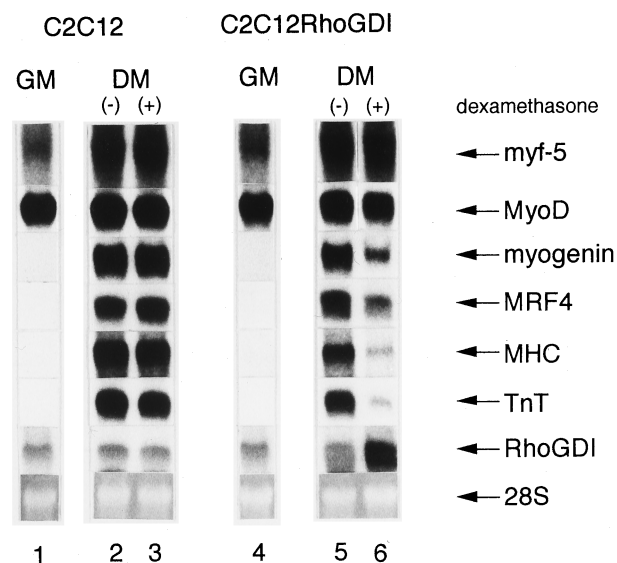


FIG. 3. RhoGDI inhibits induction of myogenin and muscle-specific genes. RNA was prepared from C2C12 cells and C2C12RhoGDI cells cultured in GM or in DM for 3 days. Ten micrograms of RNA from each sample was subjected to Northern blot analysis. Ethidium bromide staining of rRNA is presented at the bottom to show that same amount of intact RNA was loaded in each lane.

Rho family proteins are required for the transcription of the myogenin gene during myogenesis. Among myogenic bHLH genes, induction of the myogenin and MRF4 genes was inhibited during muscle differentiation by overexpressing RhoGDI. During mouse development, each myogenic bHLH factor is expressed in a precise temporal and spatial pattern to give rise to muscle. *myf5* is the first of the bHLH factors to be expressed during myogenesis; this is followed shortly thereafter by the expression of MyoD and myogenin and finally by that of MRF4 (73, 89). Myogenin has been shown to activate the MRF4 promoter during myogenesis (5, 56), and knockout experiments have clearly demonstrated that myogenin is required for differentiation from myoblasts to myocytes (26, 55). These previous observations suggest that reduced myogenin gene expression could result in low MRF4 and contractile protein mRNA levels and failure in myogenesis. We therefore examined the transcriptional activity of the myogenin gene in C2C12RhoGDI cells. A reporter gene containing the myogenin promoter (bp -222 to +40), which can confer its muscle-specific expression (40), was transfected together with the expression plasmid encoding either RhoGDI or dominant interfering or constitutively active mutants of the Rho family proteins. The activity of the myogenin promoter was upregulated during muscle differentiation (Fig. 4, bars 1 and 2) and was strongly suppressed by RhoGDI or dominant interfering mutants of Rho family proteins (Fig. 4, bars 3 to 6). The promoter activity was, conversely, upregulated by the activated mutants of Rho family proteins (Fig. 4, bars 8 to 10). In

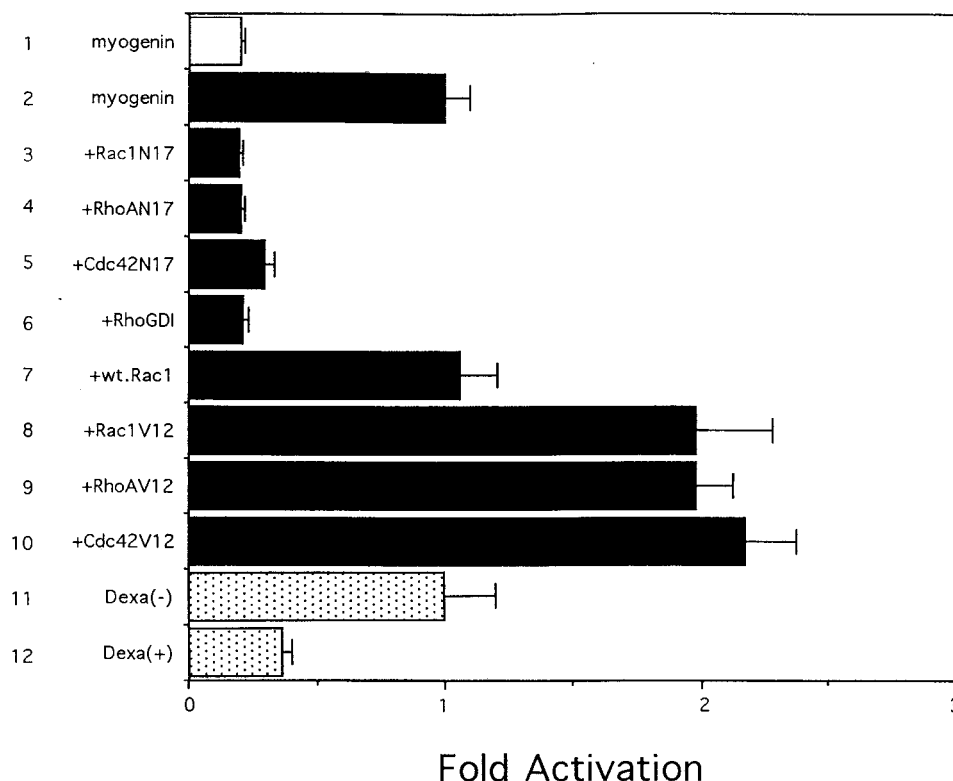


FIG. 4. Rho family proteins regulate the transcription of the myogenin gene. The luciferase reporter gene containing the myogenin promoter was transiently transfected into C2C12 cells together with effector plasmids encoding dominant interfering mutants, RhoGDI, wild-type (wt) Rac1, or constitutively active mutants of Rac1, RhoA, and Cdc42 (bars 1 to 10). The reporter plasmids were also transiently transfected into C2C12RhoGDI cells in the absence (bar 11) or presence (bar 12) of 1 μ M Dexa. The culture medium was changed from GM to DM at 36 h after transfection, and luciferase activity was determined 36 h later by using cell extracts prepared from myoblasts (bar 1) or myocytes (bars 2 to 12). The results are expressed relative to the luciferase activity in cells cotransfected with the vehicle vector (bar 2) or C2C12RhoGDI cells in the absence of Dexa (bar 11). The results are shown as means \pm standard errors for four independent experiments.

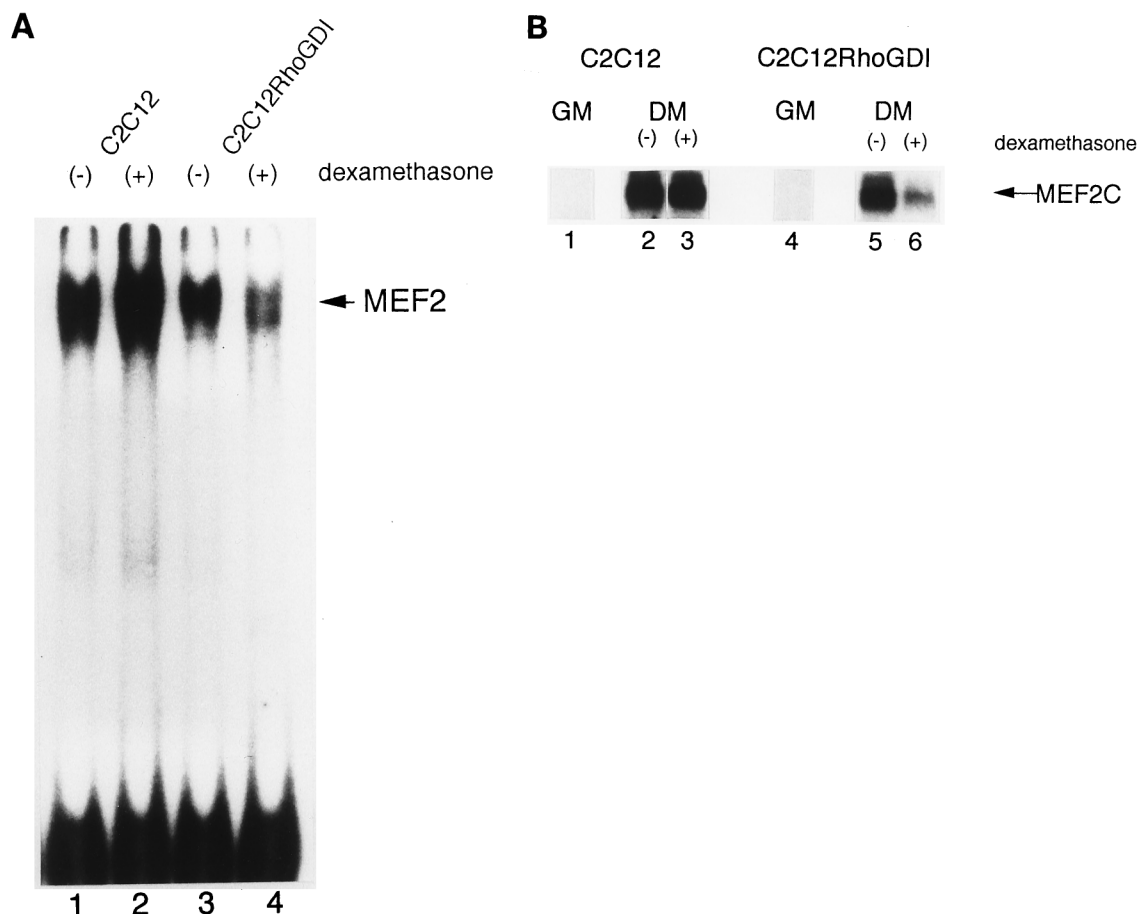


FIG. 5. Rho family proteins regulate the expression of the MEF2 protein. (A) The MEF2 DNA binding activity was determined by electrophoretic mobility shift assay as described in Materials and Methods. Nuclear extracts were prepared from C2C12 cells (lanes 1 and 2) or C2C12RhoGDI cells (lanes 3 and 4) after inducing differentiation into myotubes in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 μ M Dexa. Equal amounts (10 μ g) of nuclear extracts were incubated with radiolabeled myogenin MEF2-binding site and electrophoresed in Tris-acetate-buffered gels. (B) RNA was prepared from C2C12 cells (lanes 1 to 3) and C2C12RhoGDI cells (lanes 4 to 6) cultured in GM (lanes 1 and 4) or in DM (lanes 2, 3, 5, and 6) for 3 days. Ten micrograms of RNA from each sample was subjected to Northern blot analysis. Human MEF2C cDNA was used as a probe.

C2C12RhoGDI cells, the luciferase activity of the reporter gene increased during muscle differentiation in the absence of Dexa but was markedly suppressed when expression of RhoGDI was induced by Dexa (Fig. 4, bars 11 and 12). These results suggest that Rho family G proteins are critically involved in the transcription of the myogenin gene.

Rho family proteins are involved in transcription of the myogenin gene through MEF2. We next examined how the Rho family is involved in the transcription of the myogenin gene. It has been demonstrated that an E box and a MEF2-binding site in the myogenin promoter are required for the transcription of the myogenin gene (10, 12, 19, 40, 93). Because the E box-binding proteins myf5 and MyoD, which are thought to be involved in the regulation of myogenin gene expression during terminal muscle differentiation, were expressed in cells overexpressing RhoGDI as abundantly as in parental cells (Fig. 3), we focused on MEF2 proteins. Electrophoretic mobility shift assay revealed that the level of proteins bound to the MEF2 site of the myogenin promoter was much less in cells overexpressing RhoGDI than in C2C12 cells and C2C12 RhoGDI cells without Dexa (Fig. 5A). Northern blot analysis showed that the expression of MEF2C was induced in both parental and C2C12RhoGDI cells without Dexa by serum de-

privation (Fig. 5B, lanes 2, 3, and 5). When Dexa was added to the culture medium, expression levels of MEF2C were markedly reduced in C2C12RhoGDI cells (Fig. 5B, lane 6). These results suggest that low expression levels of the myogenin gene may be due to reduced expression of the MEF2 gene in C2C12RhoGDI cells.

DISCUSSION

The Rho family of GTP-binding proteins consists of the Rho, Rac, and Cdc42 subfamilies and has been demonstrated to regulate numerous aspects of cytoskeleton function (25, 58, 77, 78). Recently it has been reported that Rho family proteins also play a critical role in transcriptional regulation of the *c-fos* gene by modulating the transcription factor SRF (28). Since SRF also binds to the CArG box, which is a critical *cis* element in the promoters of many muscle-specific genes (49, 50, 65, 81, 87), we examined whether the Rho family plays an important role in the expression of muscle-specific genes. Since the skeletal α -actin promoter with mutations in the CArG box showed less transactivation by constitutively activated mutants of the Rho family proteins than did the wild-type promoter (Fig. 1B) and since effects of Rho proteins were more prominent in the

CARg box-containing skeletal α -actin promoter than the CARg box-less β MHC promoter (Fig. 1A and C), the CARg box may play an important role in Rho-induced gene expression. However, since Rho proteins have some effects on the skeletal α -actin promoter with mutations in the CARg box (Fig. 1B) and on β MHC genes which have no CARg box in their promoters (Fig. 1C), Rho family proteins are involved in muscle gene expression through CARg box-dependent and -independent mechanisms. Since neither RhoGDI nor dominant negative mutants of the Rho family showed such a strong inhibitory activity on the promoter of SV40 (Fig. 1A) or thymidine kinase (data not shown), the inhibitory effects of these molecules were considered specific to muscle genes, unlike dominant negative Ras mutants (1). We isolated C2C12 cell lines which were permanently transfected with RhoGDI in an inducible promoter vector. Two independent cell lines, C2C12RhoGDI-1 and -2 cells, differentiated into myotubes like wild-type parental C2C12 cells in the absence of Dexa. When RhoGDI was induced by Dexa, however, C2C12RhoGDI cells did not differentiate into myotubes. Since Dexa itself did not have any effects on differentiation of parental cells, the inhibition of myogenesis in C2C12RhoGDI cells in the presence of Dexa should be due to overexpression of RhoGDI. These results strongly suggest that Rho family G proteins play a critical role in muscle differentiation. There is a study suggesting that Rho family proteins are required for epidermal cell differentiation (76).

Although inhibition of muscle differentiation was not complete as in the case of cells overexpressing RhoGDI, C3 exoenzyme also inhibited muscle differentiation (Fig. 2H). The different effects of C3 exoenzyme and RhoGDI may come from the specificities of the molecules. C3 exoenzyme selectively inhibits the functions of Rho protein but not those of Rac or Cdc42 protein, while RhoGDI suppressed the functions of all three Rho family proteins. Although there were differences in inhibitory activity among dominant interfering mutants of the three Rho family members (Rac1N17, RhoAN17, and Cdc42N17), all of the mutants strongly suppressed the transcription of muscle-specific genes. These results suggest that each member of the Rho family proteins may be involved in the transcription of muscle-specific genes or that there may be a hierarchy among the three subfamilies of Rho proteins and muscle gene transcription may be activated by the cascade of these Rho subfamilies (35, 59). It remains to be determined how each of these Rho family proteins is involved in the regulation of muscle differentiation.

The myogenic bHLH proteins MyoD, myf5, myogenin, and MRF4 are master regulatory proteins and regulate the transcription of all muscle-specific genes during muscle differentiation (60, 89). Each bHLH protein shows auto- and cross-activation of each protein (7, 18, 47, 67, 82, 89). Although all four bHLH proteins have many common features, the temporal and spatial expression patterns of individual genes are different (73). It has been reported that both the MyoD and myf5 genes are expressed in myoblasts as well as in myotubes, while myogenin and MRF4 are not expressed in undifferentiated myoblasts and their expression was induced during terminal differentiation (7, 17, 18, 47, 67, 73, 82, 90). Evidence provided from mouse mutants carrying an inactivated myogenin gene suggests that the expression of myogenin leads to overt terminal muscle differentiation (12, 55). In cells overexpressing RhoGDI, MyoD and myf5 were expressed as abundantly as in parental C2C12 cells but expression levels of myogenin and MRF4 were very low, suggesting that inhibition of Rho family functions did not change the state of committed myoblasts but inhibited terminal differentiation. Since MRF4 functions

downstream of the other myogenic bHLH factors (5, 8, 47, 67) and since myogenin and MEF2 synergistically activated the MRF4 promoter during myogenesis (5, 56), we speculated that a lack of an increase in expression of the myogenin gene, but not the MRF4 gene, in C2C12RhoGDI cells may be a primary cause for failure of myogenesis. The transcriptional activity of the myogenin gene was low in cells overexpressing RhoGDI and was suppressed by cotransfection with dominant interfering mutants of Rho family proteins. It has been reported that transcription of the myogenin gene is regulated by bHLH proteins and preexisting MEF2 (10, 12, 19, 40, 93). Since a recent study demonstrated that MEF2 and myogenic bHLH proteins synergistically activate muscle gene expression via protein-protein interactions between the DNA-binding domains of these heterologous classes of transcription factors, it is difficult to dissect the roles of two different transcription factors in the activation of the myogenin gene. However, because MyoD and myf5, which might regulate myogenin gene expression during terminal muscle differentiation, were expressed abundantly in C2C12RhoGDI cells, we postulated that loss of the upregulation of myogenin gene expression after serum deprivation may be due to low expression of MEF2 proteins but not of bHLH proteins.

The results of Northern blot analysis and electrophoretic mobility shift assay showed that the expression of MEF2 was reduced in C2C12RhoGDI cells. Although the regulatory mechanism of MEF2 gene transcription has not been clarified as yet, overexpression of bHLH proteins was reported to induce the expression of MEF2 in fibroblasts (16, 43). Thus, there seems to be a cross-activating loop between bHLH proteins and MEF2. The molecular mechanism involved in the reduced expression of MEF2 in C2C12RhoGDI cells is unknown at present. Recently, many target proteins of the Rho family have been identified, and Rho family proteins have been shown to participate in the protein kinase cascade. Rac1 and Cdc42 activate serine/threonine kinase PAKp65 (41, 42), and the p85 subunit of PI3K is also directly associated with activated Rac and Cdc42 (84, 94). Rho binds to and activates protein kinase N (2, 88) and a novel serine/threonine kinase, Rho kinase (44). Rac1 and Cdc42 have also been reported to activate JNK and the 70-kDa S6 kinase (13, 15, 46). It has been reported that phosphorylation of the serine residue located between the MADS and MEF2 domains of the MEF2 protein enhances its DNA binding activity and transcriptional activity (52). These observations suggest that Rho family proteins may be involved in the increase of the binding activity of MEF2 to the myogenin promoter by phosphorylation. Upregulation of myogenin gene expression may in turn increase MEF2 expression. There have also been many reports suggesting that occupation of the extracellular matrix receptor, integrin, is required for terminal muscle differentiation (21, 45, 70, 74). A recent report has suggested that Rho family proteins work as mediators of integrin signaling (86). Taking these observations and our results together, it is tempting for us to speculate that integrin regulates muscle differentiation via the Rho family proteins. How Rho family proteins regulate the expression of MEF2 and myogenin and how the members of this family interact with each other during muscle differentiation remain to be clarified.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work.

We thank Y. Takai, J. S. Gutkind, T. Endo, J. Schmidt, E. N. Olson, M. D. Schneider, and H. H. Arnold for providing plasmids and S. Narumiya for providing C3 exoenzyme.

This study was supported by a grant-in-aid for scientific research and

developmental science research from the Ministry of Education, Science and Culture; a grant from Pfizer Pharmaceutical Co. Ltd.; and a grant from Tanabe Medical Frontier (all to I.K.).

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