Inactivation of Rho/ROCK Signaling Is Crucial for the Nuclear Accumulation of FKHR and Myoblast Fusion*

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Myoblast fusion is a critical process for the terminal differentiation of skeletal muscle. To elucidate the intracellular mechanisms regulating myoblast fusion, we studied the roles of signaling through the small GTPase Rho and its effector, the Rho-associated kinase ROCK, in myoblast fusion of mouse C2C12 cells. We found that Rho activity, which was high in proliferating myoblasts, decreased during myogenesis. Expression of a constitutively active form of Rho blocked myoblast fusion, but not the earlier steps of differentiation. Consistently, ROCK activity was also decreased in differentiating C2C12 cells, and an active ROCK mutant prevented their fusion. Furthermore, inactivation of ROCK by the specific inhibitor Y-27632 enhanced myoblast fusion, even in cells expressing the active Rho mutant. Thus, the down-regulation of Rho/ROCK signaling is required for myoblast fusion. We also found that Rho/ROCK signaling was required for retaining FKHR, a transcription factor implicated in myoblast fusion, in the cytoplasm and that inactivation of ROCK was essential for the nuclear accumulation of FKHR that took place just before the onset of myoblast fusion. Moreover, ROCK directly phosphorylated FKHR in vitro. We conclude that the inactivation of Rho/ROCK signaling is a prerequisite for FKHR nuclear translocation and myoblast fusion in C2C12 cells, providing evidence for a novel regulatory role of Rho/ROCK signaling in myogenic differentiation.

In the development of skeletal muscle, mononuclear myoblasts are differentiated from multipotent mesodermal precursor cells in an ordered multistep process requiring the sequential activation of myogenic transcription factors followed by myoblast fusion. In the myoblast, the myogenic basic helix-loop-helix factor MyoD, the earliest marker of muscle differentiation, initiates the myogenic program by activating the expression of various muscle-specific genes. In a later stage of myogenenesis, several reports showed Rho to be a negative regulator (20, 21). For example, high RhoA activity maintains cell polarity, and transcriptional activity (16). Rho also plays a critical role in skeletal muscle differentiation. Rho-dependent activation of serum response factor is required for myoD or a-actin gene expression, thereby promoting myogenin expression and subsequent differentiation in myoblast cell lines (17–19). Although Rho had been thought to be a positive regulator of myogenin, several reports showed Rho to be a negative regulator (20, 21). For example, high RhoA activity maintains the undifferentiated mesenchymal cell morphology and prevents smooth muscle differentiation (20). Furthermore, in rat L6 myoblasts transfected with an active RhoA mutant, no multinucleated myotubes are detected even under differentiation conditions (21). Thus, these reports imply the possibility that Rho contributes negatively to morphological differentiation; however, there is no direct evidence showing that Rho regulates myoblast fusion.

To elucidate the intracellular mechanisms regulating myoblast fusion, we focused on the function of Rho in myoblast fusion. Among a number of its effectors enabling various functions of Rho (22), we studied Rho-associated kinase ROCK/Rho kinase/ROK, which has been identified as a direct effector of...
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RhoA (23–25). The Ser/Thr kinase ROCK has been implicated in the regulation of assembly of the actin cytoskeleton and cell contractility by phosphorylating various substrates (26). Interestingly, a recent study showed that Y-27632, a ROCK inhibitor, induces the precocious expression of cardiac α-actin in chick embryos, implying that ROCK negatively affects myogenic differentiation (27). However, very little information regarding the function of ROCK in myogenesis has been obtained.

In this study, we investigated the involvement of Rho/ROCK signaling in myoblast fusion in mouse C2C12 cells. We demonstrated that both Rho and ROCK activities were decreased during myogenesis. Constitutive activation of Rho or ROCK caused a defect in myoblast fusion but did not abrogate expression of MyoD and myogenin. This phenotype was coincident with cytoplasmic retention of the transcription factor FKHR.

Furthermore, inhibition of ROCK highly enhanced myoblast fusion in association with nuclear accumulation of FKHR, which was a direct substrate of ROCK. Altogether, our results demonstrated that both Rho and ROCK activities were decreased signaling in myoblast fusion in mouse C2C12 cells. We demonstrated that both Rho and ROCK activities were decreased during myogenesis.

Preparation of Cell Lysates—C2C12 cells were washed with PBS and lysed with lysis buffer (150 mM NaCl, 1 mM 2-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 1 mM Na3VO4). Cell debris was then pelleted at 14,000 × g for 15 min at 4 °C, and the supernatant was collected as the whole cell lysate. To obtain cytosolic extracts, C2C12 cells were lysed with buffer containing 0.5% Nonidet P-40, 100 mM Hepes (pH 8.0), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4 and centrifuged at 14,000 × g for 1 min at 4 °C. The supernatant was collected for the cytosolic extract. The pellet was lysed with buffer containing 20 mM Hepes (pH 8.0), 25% glycerol, 0.4 mM NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4 and centrifuged at 14,000 × g for 15 min at 4 °C. The supernatant was collected as the nuclear extract.

GTP-bound RhoA Pull-down Assay—Cells were washed once with ice-cold Trius-buffered saline and lysed in pull-down buffer (25 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 2% glycerol, 1% Nonidet P-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). The samples were clarified at 14,000 × g for 5 min, and the supernatants were saved as the cell lysates. After the protein concentration had been determined, 200 µg of protein from the cell lysate was incubated with glutathione-Sepharose 4B (Amersham Biosciences) as a negative control or with 20 µg of GST-rohotekin Rho-binding domain-agarose (Upstate Biotechnology, Inc., Chicago, IL) for 45 min at 4 °C. Beads were washed three times with buffer B, and GTP-bound RhoA was detected by immunoblotting with anti-RhoA antibody.

Immunoprecipitation and Kinase Assay for ROCK—ROCK was immunoprecipitated from whole cell lysates by incubating equal amounts of precleared lysates proteins (100 µg) with anti-Rock antibody (5 µg/tube) or normal goat IgG (5 µg/tube) as a negative control at 4 °C with constant shaking, and then the immunoprecipitates were washed with buffer A (0.5 M NaCl, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1% mercaptoethanol) and immediately used for the kinase assay. The beads were incubated for 10 min at 30 °C with 0.6 mg/ml histone H1 (Roche Applied Science) or 2 mg/ml GST-FKHR (Upstate Biotechnology, Inc.) in the presence of [γ-32P]ATP (200 µCi/mM) and 10 µM ATP in kinase buffer. Reactions were stopped by the addition of SDS sample buffer and boiling for 5 min. Histone H1 or GST-FKHR was separated by SDS-PAGE, and 32P incorporation was measured using a Fuji BAS2000 imaging analyzer and quantified by Cerenkov counting.

RESULTS

Rho Activity Is Decreased during Myogenesis—Although a number of studies have evaluated the functions of Rho in myogenesis, the change in Rho activity during differentiation has not been clearly shown. To investigate Rho activity during myogenesis in vitro, we measured RhoA activity in mouse C2C12 myoblasts by employing a pull-down assay that captures only the active GTP-bound form of RhoA. The level of GTP-bound RhoA was high under proliferative conditions (0 h in Fig. 1A, first panel; and B); however, it decreased rapidly within 2 h after the shift to differentiation medium (2 h in Fig. 1A, first panel; and B), presumably because of serum withdrawal from the culture medium. Rho activity was maintained at a moderate level for at least 2 days and declined slightly, reaching a background level by day 4 of differentiation. These observations indicated that Rho activity decreased during myogenic differentiation occurred in a biphasic manner. In the first phase, at the beginning of differentiation, Rho activity was prominently decreased. Thereafter in the second phase, from days 2 to 4 of differentiation, residual Rho activity was completely lost. It is notable that the RhoA protein level was slightly decreased from days 2 to 4 of differentiation, with this decrease being concomitant with the second inactivation of Rho (Fig. 1A, third panel).

Excessive Rho Activity Suppresses Myoblast Fusion and MHC Expression—Because Rho activity decreased during myo-
genesis, we hypothesized that down-regulation of Rho might be important for the progression of myogenesis. To examine whether such down-regulation is required for myogenic differentiation, we established active RhoA mutant (RhoAV14) stable transfectants of C2C12 cells, which exhibited high Rho activity even under the low serum conditions (Fig. 2A). Little or no multinucleated myotubes were detected in RhoAV14-C2C12 transfectants after 6 days in differentiation medium (Fig. 2B, panels b and d), whereas a number of MHC-expressing multinucleated myotubes were formed in the control C2C12 cultures (panels a and c). No multinucleated myotubes were observed even after the RhoAV14-C2C12 cells had been allowed to grow in differentiation medium for up to 1 month (data not shown), indicating that multinucleated myotube formation was completely blocked, not simply delayed. In cultures of RhoAV14 transfectants, although MHC (late marker)- and myogenin (intermediate marker)-expressing cells were detected, none of these cells underwent cell fusion (Fig. 2B, panel d). To evaluate which stage of differentiation was blocked by RhoAV14, we performed immunoblot analysis for detection of differentiation markers. As shown in Fig. 2C, expression of MHC was remarkably suppressed in RhoAV14 transfectants, whereas no noticeable effects on the expression of MyoD (early marker) or myogenin were observed. From our detailed observation of C2C12 cell differentiation in vitro, we found that MHC expression started in a certain population of mononuclear myoblasts and rapidly increased concomitant with the initiation of myoblast fusion. Taking this observation into consideration, the defect in MHC expression in RhoAV14 transfectants may be related to the suppression of myoblast fusion, which is induced by RhoA activity.

**FIG. 1.** RhoA activity during muscle cell differentiation. A, C2C12 cells were incubated in differentiation medium for 2 h or for 2, 4, or 6 days. Cell lysates were prepared at the indicated periods and subjected to a pull-down assay for the GTP-bound form of RhoA (first panel) or immunoblotted with anti-RhoA antibody (third panel). RBD (Rho-binding domain) indicates a loading control of the GTP-bound RhoA pull-down assay (second panel). Amido Black staining is indicated as a protein normalization control of total cell lysates (lower panel). B, control. For quantification, the image intensity of GTP-bound RhoA pull-down bands was measured using NIH Image software (Version 1.63). The data were summarized from three independent sets of experiments.

**FIG. 2.** Active Rho mutant inhibits multinucleated myotube formation and MHC expression. A, C2C12 cells were stably transfected with a control vector or the constitutively active RhoA mutant Myc-RhoAV14 (RhoAV14-C2C12) and cultured in growth medium (20% fetal bovine serum (FBS)) or differentiation medium (1% fetal bovine serum) for 16 h. The levels of the GTP-bound form of Rho were measured (upper panel). RBD (Rho-binding domain) indicates a loading control of the GTP-bound RhoA pull-down assay (lower panel). B, control (panels a and c) and RhoAV14-C2C12 (panels b and d) cells were cultured in differentiation medium for 6 days. The cells were then fixed and stained for MHC (green) and myogenin (red). Cells were co-stained with Hoechst 33342 (blue) to identify nuclei. Panels a and b show phase-contrast images, and panels c and d show fluorescence microscopic images. Note that myogenin and MHC were expressed in mononuclear cells in RhoAV14-C2C12 cultures (arrows in panel d). Magnifications are ×40 (panels a and b) and ×200 (panels c and d). C, cell lysates were prepared from control or Myc-RhoAV14-expressing C2C12 (RhoAV14-C2C12) cells at day 2 (MyoD) or day 6 (MHC and myogenin) of differentiation and immunoblotted with anti-MHC, -myogenin, -anti-MyoD, or anti-Myc epitope tag (9E10, for Myc-RhoAV14) antibody. Amido Black staining is indicated as a protein normalization control.
ROCK Activity Is Down-regulated during Myogenesis—To address how RhoA regulates myogenic differentiation through its effector, we examined changes in ROCK activity during myogenesis. For this purpose, endogenous ROCK was immunoprecipitated from C2C12 cell extracts that had been prepared at different times after the shift to differentiation medium, and the immunoprecipitates were assayed for kinase activity using histone H1 as a substrate. The immunoprecipitates were assayed for kinase activity using histone H1 as a substrate, whose phosphorylation was detected by autoradiography (upper panel). Levels of ROCK-I were confirmed by immunoblotting with anti-ROCK-I antibody (lower panel). cont, control. B, shown is the quantification of ROCK activity. 

ROCK Activity during Myogenesis—To investigate whether ROCK is involved in myogenesis during the early and intermediate stages of differentiation, we tested the effect of Y-27632, a pharmacological inhibitor of ROCK/Rho kinase/ROK, whereby ROCK was inactivated in the early stage of differentiation just after the change to differentiation medium. C2C12 myoblasts were cultured in differentiation medium for 4 days in the presence or absence of Y-27632, and then the morphological change in the cells and MHC expression were monitored. As shown in Fig. 4A (panels b and d), syncytia that included a number of nuclei were dramatically increased in the presence of Y-27632. The high degree of myoblast fusion in Y-27632-treated cultures was also confirmed by determining the fusion index, which was enhanced by -4-fold in the presence of Y-27632 at day 6 of differentiation (Fig. 4B). To further examine which event of the myogenic differentiation program was promoted by Y-27632, we performed immunoblot analysis of MyoD, myogenin, and MHC at different time points of differentiation (Fig. 4C). In Y-27632-treated C2C12 cells, the expression pattern of MyoD was not significantly different from that in control cells (Fig. 4C, first panel). However, at day 6, the expression of myogenin in the treated cells was decreased (Fig. 4C, compare lanes 4 and 8), probably because these cells were fully differentiated. Furthermore, the expression pattern of MHC was also altered in the Y-27632-treated cells, i.e., MHC expression was induced strongly compared with that in control cells at day 2 (Fig. 4C, compare lanes 2 and 6), and its expression level was remarkably increased from days 2 to 4. This early onset of MHC expression indicates that myoblast fusion was induced precociously. Since the effect of Y-27632 in this assay might have been insufficient for complete ROCK inactivation during the early stage of differentiation, we added the inhibitor 4 days before differentiation. We obtained the same results: multinucleated myotube formation and MHC expression were enhanced in the presence of Y-27632 without the MyoD and myogenin expression patterns being affected (data not shown). We also demonstrated that Y-27632 did not play a negative role in either apoptosis or cellular proliferation (data not shown). Taken together, these results indicate that precocious inactivation of ROCK by Y-27632 greatly facilitates myoblast fusion, resulting in excessive formation of multinucleated myotubes. Accordingly, it seems likely that ROCK contributes negatively to myoblast fusion but does not affect the expression of MyoD and myogenin. Since ROCK activity decreased gradually during normal differentiation in C2C12 cells, we suspect that ROCK prevents myoblast fusion from the early to intermediate stages of differentiation.

Overexpression of Active ROCK Mutant Inhibits Myoblast Fusion—Taking into account our findings that ROCK activity was decreased during differentiation and that precocious inactivation of ROCK accelerated myoblast fusion, we hypothesized that ROCK has an inhibitory effect on myoblast fusion. To test this hypothesis, we asked whether the constitutively active ROCK mutant ROCK-I-C, also known as ROCK-D3 (28), would prevent myoblast fusion. ROCK-I-C, which lacks the C terminus of ROCK-I, one of two isoforms of ROCK, is constitutively active because its Rho-binding domain, PH domain, and cysteine-rich domain are removed (28). Since activation of ROCK-I (p160ROCK) is sufficient for formation of membrane blebs, which is one of the aspects of apoptotic morphology (29), it was hard to establish stable transfectants of C2C12 cells expressing ROCK-I-C. Therefore, C2C12 cells were transiently transfected with ROCK-I-C or GFP as a control; placed into differentiation medium; and then analyzed for expression of MyoD, myogenin, and MHC. We first asked whether gene expression of these differentiation markers in mononuclear myoblasts could be influenced by ROCK-I-C. As shown in Fig. 5A, after 2 days of differentiation, all of the cells transfected with GFP or...
FIG. 4. Effect of ROCK inhibitor on C2C12 cell differentiation. A, C2C12 cells were cultured in differentiation medium for 4 days in the presence (panels b and d) or absence (panels a and c) of 10 μM Y-27632, a specific inhibitor of ROCK. The cells were then fixed and stained for MHC (green; panels c and d). Cells were co-stained with Hoechst 33342 (blue; panels c and d) to identify nuclei. Panels a and b show phase-contrast images, and panels c and d show fluorescence microscopic images to detect MHC. Note that inhibition of ROCK by Y-27632 greatly facilitated formation of MHC-expressing multinucleated myotubes. Magnifications are ×40 (panels a and b) and ×200 (panels c and d). B, C2C12 cells were cultured in differentiation medium in the presence (A) or absence (C) of 10 μM Y-27632 and then fixed and stained for MHC at the indicated periods. The percentage of nuclei in multinucleated MHC-positive myotubes was calculated as a fusion index. The error bars represent the S.E. from 10 independent fields. C, cell lysates were prepared from control and Y-27632-treated C2C12 cells at the indicated periods (0, 2, 4, and 6 days after differentiation) and immunoblotted with anti-MyoD (first panel), anti-myogenin (second panel), or anti-MHC (third panel) antibody. Amidio Black staining is indicated as a protein normalization control (fourth panel). Note the acceleration of MHC expression in Y-27632-treated C2C12 cells, in which it was readily detected after only 2 days of differentiation.

ROCK-IΔC still remained mononuclear, and ~80–90% of either group expressed MyoD. At day 6, myogenin was expressed in some of the GFP- or ROCK-IΔC-transfected mononuclear myoblasts, some of which also expressed MHC because of the preceding differentiation; ~30% of either transfectant expressed myogenin and/or MHC in a mononuclear state (Fig. 5B). Thus, gene expression of these differentiation markers (MyoD, myogenin, and MHC) in mononuclear myoblasts was not influenced by the constitutive activation of ROCK. Next, we examined the effect of ROCK-IΔC on the formation of multinucleated myotubes. With respect to GFP-transfected myoblasts, some of them expressing myogenin and MHC underwent cell fusion and formed multinucleated myotubes after 6 days of differentiation, when ~25% of the cells presented as a myogenin- and MHC-expressing multinucleated myotube (Fig. 5B, lower panel, gray box). However, almost no such myotubes were observed in ROCK-IΔC-transfected cultures at all (Fig. 5B, lower panel, gray box) despite normal myogenin and MHC expression in mononuclear myoblasts as described above. Because almost no transfected cells had contact with each other (transfection efficiency was ~20% in each transfectant), the ratio of multinucleated cells may not be underestimated compared with the actual fusion events. These results strongly indicate that the high ROCK activity effectively abrogated myoblast fusion but did not inhibit MyoD, myogenin, or MHC expression in the mononuclear myoblasts.

Rho-induced Inhibition of Myoblast Fusion Is Dependent on ROCK—Since Rho and ROCK exhibited similar behavior in myogenesis, it is conceivable that Rho/ROCK signaling contributes negatively to myoblast fusion. To confirm that ROCK acts downstream of Rho, we examined whether a ROCK inhibitor (Y-27632) would be able to restore myoblast fusion in RhoAV14-C2C12 transfectants (Fig. 6A). RhoAV14 transfect-
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FIG. 6. Inhibition of ROCK induces myoblast fusion in RhoAV14-C2C12 transfectants. A, C2C12 cells were stably transfected with a control vector or RhoAV14 and cultured in differentiation medium for 4 days in the presence or absence of 20 μM Y-27632. The cells were fixed and stained for MHC (green) and myogenin (red). Cells were co-stained with Hoechst 33342 (blue) to identify nuclei. Magnifications are ×200. B, control cells (white bar) and RhoAV14 transfectants were cultured in differentiation medium for 4 days in the absence or presence of 10 (light gray bar), 20 (dark gray bar), or 40 (black bar) μM Y-27632. Cells were fixed and stained for MHC, and the percentage of nuclei in multinucleated MHC-positive myotubes was calculated as the fusion index. The error bars represent the S.E. from 10 independent fields.

Since FKHR was found to accumulate in the nucleus in proliferating myoblasts in a dose-dependent manner (Fig. 7, A, panels e and f; and B). FKHR accumulated in the nucleus in ~90% of the cells at doses of 40 and 80 μM, which are 8- and 16-fold, respectively, above the IC50 of Y-27632 for ROCK (Fig. 7B). For comparison, we treated C2C12 cells with wortmannin, an inhibitor of PI3K and a wider spectrum Ser/Thr kinase that has been reported to cause nuclear accumulation of FKHR in proliferating myoblasts at high doses (12). In the presence of wortmannin at 0.1 μM, which is 20-fold above its IC50 for PI3K, ~10% of the cells exhibited FKHR nuclear accumulation, although FKHR had accumulated in the nucleus in ~40% of the cells in the presence of 1 or 10 μM wortmannin (Fig. 7B). Immunoblot analysis of cytoplasmic and nuclear lysates also revealed that the FKHR protein in the nuclear fraction was increased in parallel with an increase in the Y-27632 concentration, whereas FKHR in the cytoplasmic fraction was decreased (Fig. 7C, upper panel). Thus, FKHR nuclear accumulation is negatively regulated by ROCK in proliferating myoblasts.

Next, we asked whether the down-regulation of ROCK is required for FKHR nuclear accumulation during differentiation. For this purpose, we used constitutively active RhoAV14-C2C12 transfectants, which are defective in myoblast fusion because of their high ROCK activity (Fig. 6). Three days after differentiation, in ~90% of the control cells, FKHR had accumulated in the nucleus (Fig. 8, A, panels a and b; B; and C, upper panel, lane 8), whereas in almost all of the RhoAV14-C2C12 transfectants, FKHR localized in the cytoplasm regardless of the differentiation conditions (Fig. 8, A, panels a and d; B, and C, upper panel, lane 10). Furthermore, treatment of RhoAV14-C2C12 transfectants with Y-27632 induced significant nuclear accumulation of FKHR (Fig. 8, A, panels e and f; B, and C, upper panel, lane 12). It is noteworthy that FKHR in the cytoplasmic fraction shifted to a lower mobility form, possibly due to phosphorylation by Akt (13), at day 3 of differentiation in both control and RhoAV14-C2C12 cells (Fig. 8C, upper panel, lanes 2, 4, and 6). From these observations, we conclude that Rho/ROCK signaling is essential for FKHR cytoplasmic retention in proliferating myoblasts and that down-regulation of ROCK is a prerequisite for FKHR nuclear accumulation during differentiation.
We finally examined whether the Ser/Thr kinase ROCK directly regulates FKHR. Endogenous ROCK was immunoprecipitated from cell lysates of proliferating C2C12 cells, and the kinase activity of immunoprecipitates was assayed using recombinant GST-tagged human FKHR as a substrate. FKHR was directly phosphorylated by ROCK in vitro (Fig. 9, lane 2), and this phosphorylation was inhibited by Y-27632 in a dose-dependent manner (lanes 3–5). This result strongly supports that direct phosphorylation of FKHR by ROCK regulates the cytoplasmic retention of FKHR.

**Fig. 7.** FKHR cytoplasmic localization is sustained by ROCK in proliferating myoblasts. A, C2C12 cells were cultured in growth medium in the absence (panels a and b) or presence (panels c and d) of 40 μM Y-27632 for 16 h or in differentiation medium for 3 days (panels e and f). The cells were fixed and stained for FKHR (panels a, c, and e) and with Hoechst 33342 for detection of nuclei (panels b, d, and f). Magnifications are ×400. B, the histogram presents the percentages of cells with FKHR localized in their nuclei. C2C12 cells were cultured in growth medium in the absence (white bar) or presence (black bars) of the indicated doses of Y-27632 (ROCK IC50 = 5 μM) or wortmannin (gray bars) for 16 h. Cells were fixed and stained for FKHR and with Hoechst 33342, and those cells with FKHR localized in their nuclei were counted. The error bars represent the S.E. from 10 independent fields. *, p < 0.001. C, cell lysates prepared from C2C12 cells treated with the indicated doses of Y-27632 were separated into cytoplasmic (left) and nuclear (right) fractions and then immunoblotted with anti-FKHR antibody (upper panel). Immunoblots with anti-RhoA (middle panel) and anti-MyoD (lower panel) antibodies indicate controls for a cytoplasmic protein and a nuclear protein, respectively.

**Fig. 8.** Down-regulation of ROCK is required for nuclear accumulation of FKHR during myogenesis. A, C2C12 cells were stably transfected with a control vector (panels a and b) or RhoAV14 (panels c–f) and cultured in differentiation medium for 3 days in the presence (panels e and f) or absence (panels a–d) of 20 μM Y-27632. The cells were fixed and stained for FKHR (panels a, c, and e) and with Hoechst 33342 to identify nuclei (panels b, d, and f). Magnifications are ×400. B, the histogram presents the percentages of cells with FKHR localized in their nuclei. C2C12 cells (control) and RhoAV14-C2C12 transfectants (RhoAV14) were cultured in differentiation medium for 3 days in the presence or absence of 20 μM Y-27632. Cells were fixed and stained for FKHR and with Hoechst 33342, and those with FKHR localized in their nuclei were counted. The error bars represent the S.E. from 10 independent fields. *, **, and *** p < 0.001. C, cell lysates prepared from control C2C12 cells and RhoAV14-C2C12 transfectants in the presence or absence of 20 μM Y-27632 at the indicated periods (days 0 and 3 of differentiation) were separated into cytoplasmic (left) and nuclear (right) fractions and then immunoblotted with anti-FKHR antibody (upper panel). Immunoblots with anti-RhoA (middle panel) and anti-MyoD (lower panel) antibodies indicate controls for a cytoplasmic protein and a nuclear protein, respectively.
In this study, we have shown, for the first time, that Rho and ROCK activities are decreased during myogenic differentiation and that down-regulation of Rho/ROCK signaling is necessary for myoblast fusion and subsequent terminal differentiation. In addition, we have revealed that ROCK activity is required for the cytoplasmic localization of FKHR, which is a direct substrate of ROCK, in both proliferating and differentiating C2C12 cells.

Originally, in myogenesis, Rho was reported to be a positive regulator required for myoD expression (17). Indeed, when Rho is inactivated, expression of MyoD and the following myogenesis are prevented (17–19). Nevertheless, our results show that constitutively active RhoA completely abrogated myoblast fusion. Several intracellular molecules have been suggested to contribute negatively to myogenesis (21, 30–32). For example, Rac and Cdc42, other members of the Rho family of GTPases, are known to interfere with myogenesis presumably through JNK activation and suppression of myogenin expression (21). However, this negative effect of Rac/Cdc42 is substantially different from that of Rho because Rho neither activates JNK nor inhibits myogenin expression (21). Moreover, Ras, another small GTPase, is also known to prevent myogenesis by inhibiting the expression of the myoD gene (30–32). However, MyoD expression in C2C12 cells transfected with the active Rho mutant was indistinguishable from that in control C2C12 cells. Thus, the inhibitory mechanism triggered by Rho is also distinct from that elicited by Ras. Taken together, the results indicate that Rho contributes to a novel intracellular signaling pathway that specifically prevents myoblast fusion without affecting expression of myoD and myogenin genes or JNK activity, which is clearly different from the case of some other small GTPases.

In our in vitro differentiation system, Rho activity decreased in a biphasic manner (Fig. 1). The first acute decrease may be largely caused by inactivation of the guanine nucleotide exchange factor, the activation of which is dependent on growth factors such as lysophosphatic acid and thrombin in sera (33, 34). The second decrease leading to complete inactivation of Rho from days 2 to 4 of differentiation is likely to depend on Rho proteolysis. To initiate myoblast fusion, Rho activity must decline to a level lower than that seen under the high serum conditions because myoblast fusion was blocked if that activity was sustained. This fact indicates that at least the first inactivation of Rho, which is caused by serum starvation, is essential for the onset of myoblast fusion. Although it is known that serum starvation induces myogenic differentiation in vitro by inhibiting cellular proliferation and up-regulating the transcriptional activity of MyoD (35), our findings propose a novel interpretation of serum starvation in myogenic differentiation, i.e. to initiate myoblast fusion via inactivation of Rho and ROCK. We do not know at present whether the second inactivation is required for initiation of myoblast fusion. However, considering that inactivation of ROCK, which regulates myoblast fusion strictly, as discussed below, is more gradual than that of Rho, the second inactivation of Rho from days 2 to 4 may not be important for the initiation of myoblast fusion at day 3 of differentiation. Although the first inactivation of Rho is essential for myoblast fusion, MyoD expression, another critical event regulated by Rho, is highly influenced by the second inactivation, but not the first, because MyoD expression remained at a high level even after the first inactivation of Rho, but it disappeared in parallel with the second inactivation, when Rho was inactivated completely. These findings strongly suggest that the moderate activity after the first inactivation of Rho is sufficient for MyoD expression during the early and intermediate stages of differentiation.

ROCK is a critical effector of Rho in the pathway negatively regulating myoblast fusion. Examination of the detailed changes in Rho and ROCK activities during differentiation revealed that ROCK, despite being a direct effector of Rho, showed a slower inactivation compared with Rho, presumably because ROCK is autophosphorylated to maintain its activity independently of Rho GTPase for a time (24, 36, 37). Importantly, myoblast fusion started at day 3 of differentiation, although the first acute inactivation of Rho occurred within 2 h after differentiation, and this time lag is presumably explained by the slower inactivation of ROCK. Taking into account that the ROCK inhibitor Y-27632 recovered myoblast fusion in a dose-dependent manner in the presence of active Rho protein (Fig. 6), it seems very likely that myoblast fusion is highly dependent on ROCK rather than Rho activity.

Then, how does Rho/ROCK signaling prevent myoblast fusion? This study also provides the first evidence that Rho/ROCK signaling sustains FKHR cytoplasmic localization. Our detailed analysis revealed that nuclear accumulation of FKHR occurred gradually from days 1 to 3 of differentiation. This slower accumulation strongly correlated with the time course of inactivation of ROCK, rather than Rho, which exhibited biphasic inactivation. Consistently, a ROCK inhibitor promoted FKHR nuclear accumulation in a dose-dependent manner even at high Rho activity. Based on the pertinent data taken together, we emphasize that the amount of FKHR accumulated in the nucleus is likely to be strictly regulated by the level of ROCK activity. Since a previous report proposed that an unknown Ser/Thr kinase prevents FKHR nuclear accumulation in primary myoblasts (12), it is plausible that ROCK, a Ser/Thr kinase, phosphorylates FKHR to regulate its localization. In fact, FKHR was directly phosphorylated by ROCK in vitro (Fig. 9). These results strongly suggest that ROCK is a candidate for the intracellular molecule that promotes FKHR cytoplasmic localization in vitro and in vivo, thereby suppressing the activity of FKHR, which up-regulates gene expression involved in remodeling of the plasma membrane or extracellular matrix. It should be noted, however, that the nuclear localization of

Fig. 9. ROCK directly phosphorylates FKHR in vitro. Whole cell lysates prepared from proliferating C2C12 cells were incubated with control IgG (lane 1) or anti-ROCK-I antibody (lanes 2–5). The immunoprecipitates (IP) were assayed for in vitro phosphorylation reaction using recombinant GST-FKHR as a substrate in the absence (lane 2) or presence (lanes 3–5) of Y-27632 (10, 20 and 40 μM, respectively), whose phosphorylation was detected by autoradiography (upper panel). Amounts of ROCK-I and GST-FKHR were confirmed by immunoblotting with anti-ROCK-I (middle panel) and anti-FKHR (lower panel) antibodies, respectively.

DISCUSSION

2 T. Nishiyama, I. Kii, and A. Kudo, unpublished data.
FKHR is not sufficient to trigger myoblast fusion since the treatment of C2C12 cells with Y-27632 caused FKHR nuclear accumulation but failed to induce myoblast fusion significantly under proliferative conditions. Accordingly, additional molecules that are possibly induced under differentiation conditions might be required to trigger myoblast fusion.

Although it has been shown in several cell lines that FKHR intracellular localization is regulated by the insulin-like growth factor/PI3K/Akt pathway (13–15), this pathway does not regulate FKHR localization in primary myoblasts (12). This notion was confirmed in this study, as we have shown that wortmannin had little effect on FKHR localization in proliferating C2C12 cells. Consistently, it has been reported that Akt-mediated phosphorylation (13), was observed as an upper band (Fig. 8c, lanes 2, 4, and 6). This implies that Akt activation occurs independently of Rho activity during differentiation and that FKHR proteins accumulated in the nucleus under differentiation conditions may be directed to be re-exported to the cytoplasm in an Akt-dependent manner. Considering that FKHR shuttles between the cytoplasm and nucleus in proliferating myoblasts (12), in which a high level of ROCK activity is maintained, ROCK is thought to promote nuclear export of FKHR, but not prevent nuclear import. Although the mechanism by which ROCK promotes nuclear export of FKHR in myoblasts is unknown at present, we speculate that FKHR might be directly phosphorylated by ROCK in the nucleus since ROCK is partially localized in the nucleus. Further experiments are necessary to elucidate the machinery for ROCK regulation of FKHR nuclear export.

In summary, the positive role of Rho signaling, i.e. activation of the expression of myoD and other myogenic genes, has been emphasized so far in the pathway of myogenic differentiation. However, in this study, we have demonstrated a negative role played by Rho signaling, which, by activating ROCK, prevents FKHR nuclear accumulation and myoblast fusion. In our current model, Rho signaling activates serum response factor to induce myogenic genes at the early and intermediate stages of differentiation. At the same time, Rho signaling also prevents myoblast fusion by activating ROCK, which enhances nuclear export of FKHR, a transcription factor essential for myoblast fusion, via direct phosphorylation. At the late stage of differentiation, when the myoblasts are ready for terminal differentiation by the accumulation of the products of myogenic genes, the Rho/ROCK signaling is inactivated to trigger myoblast fusion.

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