Fine Regulation of RhoA and Rock Is Required for Skeletal Muscle Differentiation*

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Loriana Castellani†‡, Erica Salvati†‡, Stefano Alemà†‡, and Germana Falcone‡

From the †Istituto di Biologia Cellulare, Consiglio Nazionale delle Ricerche, 00016 Monterotondo Scalo (RM), Italy and ‡Dipartimento di Scienze Motorie e della Salute, Università di Cassino, 03043 Cassino (FR), Italy

The RhoA GTPase controls a variety of cell functions such as cell motility, cell growth, and gene expression. Previous studies suggested that RhoA mediates signaling inputs that promote skeletal myogenic differentiation. We show here that levels and activity of RhoA protein are down-regulated in both primary avian myoblasts and mouse satellite cells undergoing differentiation, suggesting that a fine regulation of this GTPase is required. In addition, ectopic expression of activated RhoA in primary quail myocytes, but not in mouse myocytes, inhibits accumulation of muscle-specific proteins and cell fusion. By disrupting RhoA signaling with specific inhibitors, we have shown that this GTPase, although required for cell identity in proliferating myoblasts, is not essential for commitment to terminal differentiation and muscle gene expression. Ectopic expression of an activated form of its downstream effector, Rock, impairs differentiation of both avian and mouse myoblasts. Conversely, Rock inhibition with specific inhibitors and small interfering RNA-mediated gene silencing leads to accelerated progression in the lineage and enhanced cell fusion, underscoring a negative regulatory function of Rock in myogenesis. Finally, we have reported that Rock acts independently from RhoA in preventing myoblast exit from the cell cycle and commitment to differentiation and may receive signaling inputs from Raf-1 kinase.

Proliferating myoblasts in vitro are characterized by the expression of the myogenic regulatory factors MyoD and/or Myf5, specific markers of skeletal myogenesis commitment (1). When subjected to differentiation cues, such as serum removal, myoblasts withdraw from the cell cycle and initiate the myogenic differentiation program through the accumulation of the transcription factor myogenin (1) and of tissue-specific contractile proteins. Full maturation is attained through fusion of postmitotic myocytes into multinucleated myotubes and the assembly of myofibrils. Myogenesis can therefore be seen as a two-state process consisting of commitment and progression, both requiring an active interplay between positive and negative regulatory signals.

Members of the Rho family of small GTPases (RhoA, Rac1, and Cdc42), initially identified as regulators of the actin cytoskeleton, are involved in modulating multiple signal transduction pathways affecting processes such as cell migration, cell cycle progression, and gene expression (2, 3). In myogenic cells, Rho GTPases have been shown to play a critical role in skeletal muscle differentiation. Whereas it is generally agreed that Rac1 and Cdc42 act as negative regulators of the onset of myogenesis (4–6), the role of RhoA and of its downstream effector, Rock (7), is not fully established, although extensively investigated. Forced expression of RhoA has been shown to bring about the myogenic potential of mouse embryo fibroblasts (8) to accelerate myoblast differentiation (5, 9) and to promote α-actin gene expression through serum response factor activation (10–12). Conversely, Rock activity diminishes myogenin and myosin accumulation in primary avian myoblasts induced to differentiate (4) and inhibits myoblast fusion and myosin accumulation in the murine myogenic cell line C2C12 through Rock (13, 14). Only recently it has been reported that Rock activity and localization can be modulated by Raf-1 during migration of mouse keratinocytes and fibroblasts (15), suggesting that Rock may receive inputs from other signaling molecules.

The accumulated evidence describes apparently contrasting roles of RhoA in skeletal myogenesis, possibly because of the heterogeneity of the experimental strategy adopted and the variety of the myogenic cell contexts utilized. In this report, we set out to evaluate the contribution of RhoA and its downstream effector, Rock, in temporally distinct phases of skeletal muscle differentiation (proliferating myoblasts versus postmitotic myocytes) and in two different cell contexts, primary avian myoblasts and established murine cell lines, to define common functions and possible cell-specific differences. Ectopic expression of constitutively active RhoA diminished the accumulation of muscle-specific proteins in avian myocytes but not in murine myocytes, whereas expression of activated Rock impaired differentiation in both avian and mouse cells. Specific alteration of endogenous RhoA and Rock in both cell types, by different means, resulted in distinct phenotypes, whereas inhibition of endogenous RhoA did not significantly perturb myogenic differentiation, inhibition of Rock improved differentiation of myoblasts, and fusion of myocytes. Together our findings indicate that RhoA and Rock may act independently in myoblasts, as they play distinct roles in maintaining myoblast identity and controlling commitment to terminal differentiation. Finally, we show that, in addition to RhoA, Rock may receive signaling inputs by Raf-1 kinase.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Highly purified Triton X-100 and Nonidet P-40 were from Roche Applied Science. C3 transferase was a gift of A. Hall (University College, London, UK). Y-27632 was provided by A. Yoshimura (Yoshitomi Pharmaceutical Industries). Monoclonal antibody (mAb)§ to RhoA (26C4) was purchased from Santa Cruz Biotech-
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tology. mAbs to α-sarcomeric actin (5C5), β-actin (AC-15), vinculin (VIN-11–5), and TRITC-conjugated phalloidin were from Sigma. mAbs to troponin T (TnT) and to BrdUrd (BU-1) were from Amersham Biosciences. mAbs to ROCK-I (clone 46), ROCK-II (clone 21), Ras (clone 18), and Rac (clone 102) were from BD Transduction Laboratories. mAb to green fluorescent protein (GFP) was from Clontech. mAb to MyoD (5.8 A) was from Dako. mAb to avian vinculin (VIN3–24) was obtained from the Developmental Studies Hybridoma Bank. mAb to Myc tag (9E10) was provided by G. Evan (University of California, San Francisco, CA), to skeletal α-actin (9A2B8) and to myosin (MF-20) by D. Fischman (Cornell University, New York, NY), and to murine myogenin (F5D) by G. Cosset (Vita-salute San Raffaele University, Milan, Italy). Rabbit serum to chicken myosin was provided by B. Paterson (National Institutes of Health, Bethesda, MD). A polyclonal antibody to chicken skeletal muscle myosin was developed in our laboratory using purified chicken muscle myosin as the immunogen (4). Rabbit polyclonal antibodies to total and phosphorylated ERKs were from Cell Signaling Technologies. Fluorescein isothiocyanate- and TRITC-conjugated goat anti-rabbit and anti-mouse antibodies were from Jackson ImmunoResearch. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad.

Cell Cultures and Viral Infection—Primary cultures of quail myoblasts (QMb) were prepared as described previously (16) and maintained proliferating in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% tryptose phosphate broth, and 3% quail embryo extract (growth medium (GM)) at 37 °C. Polyclonal populations of quail myoblasts transformed by a temperature-sensitive mutant of the Rous sarcoma virus (QMb-LA29) were established as described previously (16). Differentiation was induced by plating the cells on collagen-coated dishes in GM and the following day by substituting GM with F-14 medium supplemented with 2% fetal calf serum (differentiation medium (DM)). C2C12 myoblasts were maintained proliferating at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum (GM), as described (17). Differentiation was induced by incubating the cultures in Dulbecco’s modified Eagle’s medium with 2% horse serum (DM). For endogenous RhoA quantitation, C2C12 were induced to differentiate in DM also containing 25 μM 1-β-d-arabinofuranosylcytosine to inhibit cell proliferation.

To induce myogenic differentiation without cellular fusion, QMb-LA29 and C2C12 were cultivated in a DM supplemented with 1.85 and 1.4 mM EGTA, respectively (low calcium DM). Note that Western blot analysis of extracts from both QMb and C2C12 myoblasts induced to differentiate in low calcium DM showed that EGTA did not affect accumulation of muscle-specific proteins. This finding differs from a previous report showing that inhibition of N-cadherin-dependent adhesion impairs muscle-specific gene expression (18).

High-titer stocks of adenoviruses expressing GFP were a gift of M. Crescenzi (Istituto Superiore di Sanita, Rome, Italy), and adenoviruses co-expressing Myc-tagged RhoAV14 and GFP were provided by G. P. Dotto (University of Lausanne, Lausanne, Switzerland) (19). After infection, QMb-LA29 were kept in GM for 24 h to allow virus expression before induction of differentiation in a modified DM containing 10% fetal calf serum. This medium was chosen because high serum was found to improve expression of the transgene. The percentage of infection was measured by counting GFP-expressing cells kept proliferating at 35 °C and found to be close to 90%.

High-titer stocks of retroviruses carrying the activated human Raf-1 C terminus half, fused to human estrogen receptor (pBP3ΔRaf-1DD:ER) (20), were used to infect C2C12 myoblasts. After puromycin (1.5 μg/ml) selection, cells were analyzed for ΔRaf-1:ER protein expression using an anti-ER antibody (Santa Cruz Biotechnology). ΔRaf-1:ER expressing myoblasts were cultivated and induced to differentiate as parental C2C12 cells. Induction of the protein was obtained by the addition of 1 μM β-estradiol to either GM or DM.

Expression Vectors and Reporter Constructs—The expression vectors used were the following: pEGFP (Promega Italia), pEGFP-Rac1V12 (Rho-Rac1V12 fusion protein), and pEGFP-RhoAV14 (Rho-RhoAV14) (21), RK5-RhoAN19 (Myc-tagged RhoAN19, provided by A. Hall), pCAG-Rock1Δ3 (RockΔ3) (22), pCAGGS-Rock1-KD/IA (DN-Rock) (22), and pSG5-HRasV12 (RasV12, provided by C. J. Marshall, Institute of Cancer Research, London, UK). Plasmid pGEX3X-C21 (Rhoetkin) for bacterial expression of recombinant fusion protein GST-C21 was provided by J. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands), and plasmid pGEX2TK-RhoAV14 for recombinant GST-RhoAV14 was from A. Hall.

Transient Transfections—Proliferating myoblasts were transfected with the Lipofectamine reagent (Invitrogen) in serum-free Opti-MEM (Invitrogen). Alternatively, quail myoblasts were transfected in GM using the N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid/calcium phosphate precipitation technique (23). After transfection, the cells were kept in GM for 24 h to allow transgene expression before being further processed.

Small interfering RNA (siRNA) sequences against Rock-I and -II were from Dharmacon (siGenome duplexes D-046504-04 and D-040429-02, respectively), and a nonspecific duplex was used as the control. C2C12 cells were transfected with 100 nM individual oligonucleotide using serum-free Opti-MEM and Lipofectamine. After 5 h, the medium was replaced with GM and maintained for a minimum of 24 h. The extent of siRNA-mediated inhibition of Rock-I and -II protein accumulation was evaluated by Western blot analysis with specific antibodies, and only the experiments in which at least 50% inhibition of both proteins was detected were further processed.

For transient transgene expression, 1–2 μg of plasmid DNA was transfected per 35-mm dish. In co-transfection experiments, 0.2 μg of RhoAV14 DNA was transfected along with 2 μg of DN-Rock DNA. Empty vector was used to normalize for DNA content when necessary. 24 h after transfection, QMb-LA29 were shifted to 41 °C in low calcium DM for 2 days. C2C12 myoblasts were maintained in GM or induced to differentiate in DM for two days at low density to minimize the extent of fusion. All of the cultures were then fixed and processed for immunofluorescence for the transfected proteins and for the endogenous muscle-specific proteins. Levels of expression of the transfected proteins were monitored by Western blot in parallel cultures.

Bromodeoxyuridine (BrdUrd) Labeling and Immunofluorescence Analysis—for BrdUrd incorporation studies, QMb-LA29 transfected at 35 °C were shifted to 41 °C in DM and labeled with 20 μM BrdUrd for various time periods following shift up, as required. 48 h after siRNA transfection or 24 h after the addition of 10 μM Y27632 inhibitor, C2C12 were labeled with 20 μM BrdUrd for 3 h, fixed, and then processed for immunofluorescence.

For immunofluorescence analysis, cultures were routinely fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 in phosphate-buffered saline, and processed as previously described (17, 24). The samples were examined with an Olympus microscope. Images were recorded on a charge-coupled device camera and processed using software by Delta Sistemi (Rome, Italy) and Adobe Photoshop.

Whole-cell Extracts and Western Blot Analysis—Cells were lysed in radioimmunoprecipitation assay buffer supplemented with a mixture of protease inhibitors as previously described (4). Western blots were carried out using horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies and revealed with a chemiluminescence detec-
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RESULTS
RhoA is Down-regulated upon Skeletal Muscle Terminal Differentiation—To investigate the role of RhoA during differentiation of skeletal muscle cells, we first set out to determine the levels of expression and activity of endogenous RhoA protein in both primary cells and established cell lines of avian and mammalian origin. Specifically, we compared primary embryonal QMb and QMb expressing a temperature-sensitive mutant of v-Src tyrosine kinase to C2C12 murine myoblasts, because QMb are highly prone to differentiation, whereas mammalian myoblasts have been widely used to study RhoA function during in vitro myogenesis (11–13). The additional advantage of QMb-LA29 is that they can be kept proliferating both in GM and DM at the permissive temperature for the v-Src kinase (35 °C) and can synchronously differentiate into multinucleate myotubes exhibiting a high degree of structural maturation when shifted to the restrictive temperature (41 °C) (4, 24).

Western blot analysis of cellular extracts derived from QMb and C2C12 kept proliferating in GM or differentiating for various days in DM showed a progressive reduction of RhoA protein as differentiation proceeded (Fig. 1, A and B). To establish whether the diminished accumulation of RhoA was due to the reduction of growth factors in DM or to myogenic differentiation, QMb-LA29 were also analyzed in DM at 35 °C. As shown in Fig. 1C, a pronounced reduction of RhoA protein paralleling the maturation process of myotubes at 41 °C was observed.

The degree of muscle differentiation was evaluated in the three cell types by monitoring the accumulation of sarcomeric α-actin and the reduction of the β-actin isoform (Fig. 1, A–C).

To investigate whether the reduction of the RhoA protein was due to down-regulation of the transcript, mRNAs were prepared from QMb-LA29 (Fig. 1C), kept proliferating at 35 °C or induced to differentiate at 41 °C for up to three days, and analyzed by Northern blot. As shown in Fig. 1C, accumulation of the RhoA transcript was diminished in myotubes as compared with proliferating myoblasts, paralleling the reduction of the protein.

To measure RhoA activity in myocytes, given the reduction of RhoA protein during differentiation, cellular extracts were normalized for total RhoA content before being subjected to a pull-down assay using as bait Rhotekin, a RhoA effector protein that binds only to active RhoA-GTP (26). RhoA-GTP content was equally diminished in myotubes as compared with proliferating QMb and QMb-LA29 kept in GM and DM at 35 °C (Fig. 1D). RhoA-GTP was equally reduced in QMb differentiated at 37 or 41 °C and in QMb-LA29 kept in GM and DM at 35 °C.
induced to differentiate at high density in GM at 41 °C (not shown). The amount of RhoA-GTP was slightly augmented in extracts of differentiated C2C12 myotubes normalized for total RhoA content, as compared with that of proliferating myoblasts (Fig. 1D). Two days later, the cells were processed for immunofluorescence for the transfected protein and endogenous myogenin. The percentage of transfected cells expressing myogenin, referred to as control GFP taken as 100%, is shown in the histogram. At least 300 transfected cells were examined for each condition in every experiment. The levels of the transfected proteins, compared with endogenous RhoA, were analyzed by Western blot analysis (Fig. 2A). Scoring of the cultures for co-expression of the transgene and of myogenin clearly showed that RhoAV14 effectively reduced the number of myocytes expressing myogenin only in QMb-LA29, whereas RockΔ3 exerted its inhibitory action on both C2C12 and QMb-LA29, as did also Rac1V12 and RasV12 (Fig. 2A). RhoAN19 did not alter differentiation of both avian and mammalian myocytes, suggesting that endogenous RhoA activity was not required to attain terminal differentiation in both cell types. Because RhoAV14 did not exert any apparent effect on differentiation of C2C12 myoblasts, we verified whether its expression was effective in increasing actin stress fiber formation, as reported for many other cell types (2). Indeed, C2C12 myoblasts expressing RhoAV14 exhibit...
incorporated pronounced stress fibers compared with control cells expressing GFP (Fig. 2B). In addition, to confirm that, as described in other cells, actin stress fiber induction was mediated by Rock (7, 22), proliferating C2C12 myoblasts were co-transfected with RhoAV14 and DN-Rock or transfected with RhoAV14 and treated with the Rock pharmacological inhibitor Y27632 (10 μM, 27) and stained for F-actin. Both DN-Rock and Y27632 efficiently inhibited RhoAV14-induced stress fibers (Fig. 2B) and reduced F-actin content in GFP-expressing and control cells, clearly indicating that the RhoA/Rock axis is active for the control of the actin cytoskeleton.

Because Rock is a major downstream effector of RhoA in many cellular functions (7), we sought to establish whether the inhibition of myogenin induced by RhoAV14 in QMb-LA29 were also exerted through Rock. Proliferating QMb-LA29 was co-transfected with RhoAV14 and DN-Rock and induced to differentiate for two days before being processed for immunofluorescence. As shown in Fig. 2A, DN-Rock fully reversed the inhibitory action of RhoAV14 on myogenin expression, identifying Rock as a necessary downstream effector of RhoAV14 for the inhibition of terminal differentiation of avian cells.

To investigate whether RhoAV14 inhibited myogenic differentiation of avian cells by interfering with the exit from the cell cycle, QMb-LA29 expressing RhoAV14, RhoAN19, Rac1V12, RasV12, and GFP were allowed to incorporate BrdUrd for two 10-h intervals while being induced to differentiate at 41 °C. Scoring for double positive cells by immunofluorescence indicated that the majority of control GFP-expressing cells exited the cell cycle within the first 10-h period and accumulated myogenin (Fig. 3A). RhoAV14-expressing cells, although exhibiting a reduced BrdUrd incorporation, showed a significant reduction of myogenin accumulation (Fig. 3A). Inhibition of endogenous RhoA with RhoAN19 influenced the rate of exit from the cell cycle in a similar manner to RhoAV14 but did not alter the expression of myogenin (Fig. 3A), indicating the lack of correlation between cell cycle exit kinetics and the inhibition of differentiation exerted by RhoAV14. As expected, RasV12 and, to a lesser extent, Rac1V12 maintained the cells proliferating under differentiation conditions, although inhibiting myogenesis (Fig. 3A).

The ability of RhoAV14 in negatively regulating myogenic differentiation of avian cells was further investigated in the entire cell population by infection of QMb-LA29 with adenoviruses encoding for GFP-RhoAV14 or GFP as the control. Infected myoblasts, kept proliferating at 35 °C for 24 h to allow for the expression of the transgene, were shifted to 41 °C to induce differentiation (Fig. 3B). The histogram in A (left panel) illustrates the percentage of transfected cells expressing myogenin, referred to as control GFP-positive cells after 20 h in DM at 41 °C (100%). The histogram in A (right panel) illustrates the percentage of transfected cells expressing myogenin, referred to as control GFP-positive cells after 20 h in DM at 41 °C (100%). Bars represent S.D. B, micrographs of QMb-LA29 infected with adenoviruses expressing GFP or GFP-RhoAV14 (RhoAV14) induced to differentiate for two days and stained for skeletal myosin (upper panels) and counterstained with Hoechst 33342 nuclear dye (lower panels). Scale bar, 100 μm. C, analysis of total RNAs extracted from QMb-LA29 infected with adenoviruses expressing GFP or RhoAV14, as described above, and probed for myosin heavy chain (MHC), myosin light chain (MLC), and α-actin mRNAs. Quantitation of the indicated RNAs, normalized for glyceraldehyde-3-phosphate-dehydrogenase mRNA (see “Experimental Procedures”), is expressed as the percentage of those measured in differentiated GFP-expressing myotubes to which was assigned the value of 100. Bars represent S.D.

RhoA with C3 transferase in QMb kept in GM or DM for 2 days did not change the number of differentiated cells (Fig. 4B), as also observed by ectopic expression of RhoAN19 in QMb-LA29 (Fig. 2A), but induced a noticeable inhibition of cellular fusion only in GM (Fig. 4). Note that a fraction of QMb undergoes terminal differentiation also in GM, as often observed in primary muscle cultures. Similar to QMb, C2C12 myoblasts

FIGURE 3. RhoA inhibits fusion and expression of muscle-specific genes without affecting progression of the cell cycle. A, QMb-LA29 were transfected in GM with the expression constructs indicated and after one day were shifted to low calcium DM either at 35 or 41 °C and allowed to incorporate BrdUrd for two periods of 10 h (0–10 h, 10–20 h) (left panel) or monitored for myogenin expression during the same time intervals (right panel) by immunofluorescence analysis. The histogram in A (left panel) represents the percentage of transfected cells positive for BrdUrd, having chosen as a reference (100%) the number of cells double-positive for GFP and BrdUrd at 35 °C in DM for 10 h. The histogram in A (right panel) illustrates the percentage of transfected cells expressing myogenin, referred to as control GFP-positive cells after 20 h in DM at 41 °C (100%). Bars represent S.D. B, micrographs of QMb-LA29 infected with adenoviruses expressing GFP or GFP-RhoAV14 (RhoAV14) induced to differentiate for two days and stained for skeletal myosin (upper panels) and counterstained with Hoechst 33342 nuclear dye (lower panels). Scale bar, 100 μm. C, analysis of total RNAs extracted from QMb-LA29 infected with adenoviruses expressing GFP or RhoAV14, as described above, and probed for myosin heavy chain (MHC), myosin light chain (MLC), and α-actin mRNAs. Quantitation of the indicated RNAs, normalized for glyceraldehyde-3-phosphate-dehydrogenase mRNA (see “Experimental Procedures”), is expressed as the percentage of those measured in differentiated GFP-expressing myotubes to which was assigned the value of 100. Bars represent S.D.
induced to differentiate in DM for two days in the presence of C3 transferase showed a percentage of differentiation comparable with that of control cultures, consistent with the lack of effect of RhoAN19 on the expression of myogenin (Fig. 2A). The shape of C2C12 myocytes, however, was altered by C3 transferase treatment, becoming skinny and elongated (Fig. 4A), suggesting a role of RhoA in modulating myotube morphology.

To further explore the role of RhoA in mammalian myogenic cells, C2C12 myoblasts kept growing at low density in GM (a condition that allows the sole expression of the myogenic regulatory factor MyoD (17)) were treated with C3 transferase before being processed for Western blot analysis. As shown in Fig. 5A, a marked reduction of MyoD accumulation was observed in extracts of C3 transferase-treated myoblasts, as previously reported (9, 10), suggesting that RhoA is required to maintain myoblast identity. Interestingly, MyoD accumulation, as well as that of muscle-specific markers such as myogenin, myosin, and α-actin, was not affected when C3 transferase was added upon shift to DM (Fig. 5A). Moreover, the reduced levels of MyoD in C3 transferase-treated myoblasts did not affect the ability of these cells to differentiate when shifted to DM with or without C3 transferase (Fig. 5A). Note that long term inhibition of RhoA by C3 transferase resulted in a marked reduction of RhoA accumulation (Fig. 5A), as previously observed (29, 30).

A Novel Role for Rock in Regulation of Myogenesis—Given the effectiveness of Rock (35) in inhibiting the myogenic differentiation of both mammalian and avian cells, we sought to investigate the role of endogenous Rock in the myoblast compartment and in the transition from proliferating myoblasts to postmitotic myocytes. Inhibition of Rock in QMb in GM by Y27632 (10 μM) showed a significant induction of α-actinin expression and myotube formation (Fig. 4). Upon mitogen removal, however, treatment with Y27632 showed a negligible effect on the number of differentiated myocytes and cell fusion (Fig. 4B), probably because of the fast kinetics and the high degree of differentiation of these cells. Similarly, Y27632 treatment of C2C12 myoblasts led to the promotion of differentiation. Morphologically, C2C12 myotubes formed in the presence of Y27632 were larger in size and higher in number (Fig. 4A). To establish the relative contribution of promoted muscle-specific protein accumulation and augmented cell fusion to the observed effect of Y27632, C2C12 myoblasts were induced to differentiate for two days in a low calcium DM, culturing conditions that inhibit cell fusion but do not alter the number of differentiated cells as compared with standard DM. As shown in Fig. 4B, the number of myocytes expressing TnT was comparably increased by the addition of Y27632 in both low calcium and standard DM, clearly indicating that Rock inhibition promotes differentiation in addition to cell fusion.

To define the mechanism through which Rock affected differentiation, we measured the rate of exit from the cell cycle and the expression of myogenic markers in C2C12 myoblasts, in which Rock was inhibited, by three independent means. First, C2C12 grown in GM in the presence of Y27632 (10 μM) showed a reduced BrdUrd incorporation compared with control cells, unaltered levels of MyoD accumulation, and a marked increase in myogenin expression, measured as both the number of positive cells and as protein accumulation (Table 1 and Fig. 5C). Upon induction of differentiation in DM, Y27632 induced an increase in the number of myogenin-positive cells at day 1, compared with control (Table 1). At day 2, both Y27632-treated and untreated cells showed the same number of myogenin-expressing cells, as myogenin accumulation is maximum at that time in C2C12 cells (Table 1 and Fig. 5C). The promoting effect on differentiation operated by Y27632 was confirmed by an increase in sarcomeric protein accumulation, noticeable at day 2 and continuing up to day 4 in DM (Fig. 5B). Second, the expression of DN-Rock analyzed at the single cell level in DM showed an inhibition of BrdUrd incorporation and an increase in expression of TnT (not shown). As expected, myogenin expression was not affected by DN-Rock at two days in DM (Fig. 2A). Finally, C2C12 transfected with siRNAs for either Rock-1, -II, or both showed reduced expression levels of the respective proteins. RNA interference for both proteins was more effective in accelerating the exit from the cell cycle and increasing the number of myogenin-positive cells (Table 1) than the RNA interference for Rock-I and -II separately (not shown). Similarly, the increase in accumulation of myogenin in GM and of sarcomeric proteins in DM (Fig. 5D) was most evident when levels of both Rock-I and -II were reduced by at least 50%, indicating that both kinases are required to prevent myoblast commitment to differentiation. Indeed, immunofluorescence analysis of myocytes, in which both Rock proteins were down-regulated by RNA interference, showed an increase (∼2-fold) in the number of TnT-expressing cells and fusion (Fig. 5E). Together these findings indicate that Rock is required for myoblast proliferation and
prevents commitment to differentiation, possibly through RhoA-independent signals.

Ectopic expression of activated members of the Ras-Raf-ERK signaling pathway has been shown to inhibit myogenic differentiation (31–33). In addition, it has recently been reported that, in mouse keratinocytes, Raf-1 regulates Rock-II localization and activity (15). We therefore investigated whether Rock could be modulated by Raf also in myoblasts. To this aim, C2C12 cells were infected with retroviruses carrying estrogen-inducible activated Raf-1-ER (C2C12-Raf:ER) and analyzed for Raf-1:ER expression. In the absence of inducer, C2C12-Raf:ER behaved as uninfected parental C2C12 cells, whereas in the presence of 17β-estradiol, C2C12-Raf:ER acquired a transformed morphology and failed to differentiate in DM (Fig. 6). C2C12-Raf:ER were grown in GM or induced to differentiate in DM in the presence of Y27632 or C3 transferase. As shown in Fig. 6, inhibition of Rock, but not of RhoA, induced the expression of myogenin in GM and rescued the inhibition of muscle-specific proteins induced by Raf-1:ER in DM. Interestingly, the observed increase in myogenin and myosin accumulation following Y27632 treatment occurred in the presence of high levels of phosphorylated ERKs (Fig. 6), indicating that the Raf-1-dependent activation of ERK is insensitive to Rock inhibition. When both C3 transferase and Y27632 were added to the cultures, the rescuing effect of Rock inhibition was maintained both in GM and DM (Fig. 6), indicating that Rock is on a pathway insensitive to RhoA activity. These results suggest that Rock is endowed with RhoA-independent functions in the regulation of myogenic differentiation and can receive signaling inputs from Raf-1 kinase.

DISCUSSION

Skeletal myogenic cells, when subjected to differentiation cues, withdraw from the cell cycle, synthesize muscle-specific proteins, and fuse into multinucleated myotubes. The question addressed here has been whether RhoA, a small GTPase that orchestrates the actin cytoskeleton and related functions (2) but is also involved in the promotion of cell cycle progression and gene expression (3), plays a role in the onset and/or maintenance of skeletal muscle differentiation. The experimental strategy adopted has been that of contrasting the overexpression of constitutively active forms of RhoA and Rock, a major RhoA effector (7), with the inhibition of their endogenous counterparts in primary and established myogenic cells.

RhoA Activity Is Potentially Detrimental for Myogenic Differentiation—Inhibition of RhoA in established myoblasts reduces both the accumulation of the myogenic regulatory factor MyoD and the activity of its promoter (9, 10) in accordance with the induction of MyoD

![FIGURE 5. Silencing of Rock leads to increased myogenin and sarcomeric protein accumulation. C2C12 myoblasts were maintained in GM or induced to differentiate in DM in the presence of C3 transferase (7.5 μg/ml) or Y27632 (10 μg/ml). Cellular homogenates of C2C12 kept in GM for one day or in DM for two days with or without C3 transferase (A, C3) or kept in DM from one to four days with or without Y27632 (B), normalized for protein content, were immunoblotted with antibodies specific for the proteins indicated. Note that, in A, some cultures were treated with C3 transferase both in GM for one day and in DM for two days, as indicated. C, Western blot analysis of C2C12 cultured in GM for one day in the presence of C3 transferase (C) or Y27632 (Y), as indicated. D, C2C12 myoblasts were transfected at low cell density with nonspecific control (scr), siRNA for Rock-I, Rock-II, or both (Rock-I+II) and kept in GM for 24 h. The next day they were either maintained in GM for an additional 24 h (left panel) or shifted to DM for two days (right panel); cellular homogenates, normalized for protein content, were immunoblotted with antibodies specific for the proteins indicated. E, immunofluorescence micrographs of C2C12 myoblasts transfected with nonspecific control (scrambled) or siRNA for Rock-I and Rock-II (Rock-I+II), kept in GM for one day and in DM for two days, and double-labeled with antibody to TnT (first and third panels) and with the fluorescent nuclear dye Hoechst 33258 (second and fourth panels). Scale bar, 100 μm.](#)
Table 1: Inhibition of Rock accelerates exit from cell cycle and commitment to terminal differentiation

| Condition | BrdUrd | Myogenin | Myogenin |
|-----------|--------|----------|----------|
| Control   | 50±5   | 3±4      | 4±6      |
| Y27632    | 35±6   | 11±3     | 8±7      |
| Scrambled | 32±3   | 10±2     | ND       |
| siRock    | 39±4   | 6±7      | ND       |

The down-regulation of RhoA upon terminal differentiation, the effects of RhoA inhibition in myoblasts, and the inhibitory activity of RhoAV14 in muscle-specific protein accumulation and/or fusion suggest a novel interpretation of the role of RhoA during myogenesis. Although RhoA activity in myoblasts appears important in maintaining cell identity, it is no longer required for commitment to terminal differentiation. In addition, forced RhoA expression in myocytes results in a negative regulation of myogenesis that is independent of Rac1V12 activity. In avian myocytes, the inhibitory action of RhoAV14 can be rescued by co-expression of a dominant negative mutant of Rock, indicating that active RhoA utilizes Rock for this function, whereas in murine myocytes, activated RhoA and Rock do not appear to act in cascade, because RockΔ3 (but not RhoAV14) inhibits myogenin expression through a delay in transition to the postmitotic state but rather on accumulation of muscle-specific gene products in myocytes.
sion. Induction of stress fibers by RhoAV14 in C2C12 myoblasts, however, is inhibited by DN-Rock or by Y27632 treatment, indicating that Rock lies downstream of RhoA in the regulation of the actin cytoskeleton. It appears that, although in avian myoblasts the RhoA/Rock axis is maintained for both control of differentiation and actin regulation (4), in murine myoblasts, RhoA utilizes Rock for the control of the actin cytoskeleton, whereas Rock affects differentiation independently from RhoA. Therefore, depending on the specific cell function and cellular context, RhoA and Rock may act in concert or independently.

Notably, in both avian and murine myoblasts, ectopic expression of activated Rock inhibits differentiation. Conversely, pharmacological inhibition of Rock improves differentiation and fusion, thus suggesting that Rock is endowed with specific functions in myogenic differentiation in a cell type-independent manner. In addition, silencing of Rock in C2C12 myoblasts, although not affecting MyoD levels, results in an accelerated exit from the cell cycle and in induction of myogenin expression, suggesting that Rock prevents commitment to differentiation. These findings highlight a functional difference between RhoA and Rock in myoblasts.

Rock behaves as the major RhoA effector in many biological contexts (7). We have shown here that RhoA and Rock may act independently. Therefore, it seems likely that Rock, in addition to RhoA, receives inputs by other signaling molecules. In a recent report, it has been shown that Raf-1 regulates Rock-II localization and function in keratinocyte and fibroblast migration (15). We show here that the block of differentiation induced by activated Raf-1 kinase can be partially recovered by Rock inhibition but not by the inhibition of RhoA, clearly indicating that Rock, but not RhoA, lies downstream of activated Raf-1. Interestingly, the inhibition of Rock in C2C12-RafER by Y27632 did not alter ERK phosphorylation, suggesting that ERK and Rock may act independently downstream of Raf-1. Indeed, in contrast to Rock inhibition, inhibition of the ERK upstream activator MEK by its specific inhibitor UO126 (42) did not rescue muscle-specific protein expression in C2C12-RafER, indicating that MEK may be required for other functions than the control of differentiation. Because we have shown that Rock inhibition results in the accelerated exit from the cell cycle in myoblasts, it could be hypothesized that, in normal growth conditions (i.e., in the presence of high mitogen concentration when the Ras-Raf-ERK pathway is normally activated) (43), Rock activity contributes to sustained myoblast proliferation.

CONCLUSIONS

Based on the studies presented here, we propose that, during development, high RhoA activity in mesenchymal precursor cells may help in regulating lineage choices (8) as well as skeletal myoblast identity (10, 9). However, once specific differentiation cues are set in myoblasts, RhoA is progressively and specifically down-regulated to levels that are compatible with proper muscle-specific gene expression, execution of tissue-specific morphogenetic events such as fusion into multinucleated syncitia, and maintenance of the terminally differentiated phenotype. Rock instead appears to concur in keeping myoblasts cycling and in preventing commitment to terminal differentiation. Therefore, we suggest that RhoA and Rock play distinct and independent roles in myogenic differentiation, depending on the cellular background, and that Rock can be targeted by other signaling molecules such as Raf-1.

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