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Distinct Effects of Rac1 on Differentiation of Primary Avian Myoblasts

Rita Gallo,* Marco Serafini,* Loriana Castellani,† Germana Falcone,* and Stefano Alemà*‡

*Istituto di Biologia Cellulare, Consiglio Nazionale delle Richerche, 00137 Rome, Italy; and †Dipartimento di Neuroscienze and Istituto Nazionale di Fisica della Materia, sez B, Università di Tor Vergata, 00133 Rome, Italy

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Rho family GTPases have been implicated in the regulation of the actin cytoskeleton in response to extracellular cues and in the transduction of signals from the membrane to the nucleus. Their role in development and cell differentiation, however, is little understood. Here we show that the transient expression of constitutively active Rac1 and Cdc42 in unestablished avian myoblasts is sufficient to cause inhibition of myogenin expression and block of the transition to the myocyte compartment, whereas activated RhoA affects myogenic differentiation only marginally. Activation of c-Jun N-terminal kinase (JNK) appears not to be essential for block of differentiation because, although Rac1 and Cdc42 GTPases modestly activate JNK in quail myoblasts, a Rac1 mutant defective for JNK activation can still inhibit myogenic differentiation. Stable expression of active Rac1, attained by infection with a recombinant retrovirus, is permissive for terminal differentiation, but the resulting myotubes accumulate severely reduced levels of muscle-specific proteins. This inhibition is the consequence of posttranscriptional events and suggests the presence of a novel level of regulation of myogenesis. We also show that myotubes expressing constitutively active Rac1 fail to assemble ordered sarcomeres. Conversely, a dominant-negative Rac1 variant accelerates sarcomere maturation and inhibits v-Src–induced selective disassembly of I-Z-I complexes. Collectively, our findings provide a role for Rac1 during skeletal muscle differentiation and strongly suggest that Rac1 is required downstream of v-Src in the signaling pathways responsible for the dismantling of tissue-specific supramolecular structures.

INTRODUCTION

The Rho subfamily of small GTP-binding proteins, which includes Rho, Rac, and Cdc42, has been implicated in the regulation of a range of biological processes, including cell motility, cell adhesion, cytokinesis, cell morphology, and cell growth (for reviews, see Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). A major function of Rho family members is to act as molecular switches in the control of the actin cytoskeleton and in the assembly of associated integrin complexes. In fibroblasts, RhoA is required for the formation of stress fibers, Rac1 regulates membrane ruffling, and Cdc42 is involved in filopodia formation (Ridley and Hall, 1992; Nobes and Hall, 1995). In addition, there is increasing evidence that Rho GTPases play an important role in cell proliferation (Olson *et al.*, 1995) and that they are essential components of Ras- (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995; Joneson *et al.*, 1996) and Src-induced (Minden *et al.*, 1995; Provenzano, Falcone, and Alemà, unpublished observations) cell transformation. Consistent with these observations, several groups have reported that Rac1 and Cdc42, but not RhoA, activate the c-Jun N-terminal kinase (JNK) and the p38/MAPK cascades (Bragodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995; Zhang *et al.*, 1995) independent of cytoskeletal rearrangements (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). RhoA, on the other hand, is necessary for serum-induced activation of the transcription factor SRF (Hill *et al.*, 1995; Alberts *et al.*, 1998).

Clearly, the GTPases of the Rho family are linked to multiple signaling pathways and consequently are likely to regulate a variety of cellular processes in development and morphogenesis (Van Aelst and D'Souza-Schorey, 1997). In *Drosophila*, for example, Rho is required for gastrulation (Barrett *et al.*, 1997) and Rho, Rac1, and Cdc42 are required for the generation of tissue polarity (Strutt *et al.*, 1997) and to control actin-dependent processes in wing-disk epithelium (Eaton *et al.*, 1995). Perturbation of GTPase activities of Rac1 and Cdc42 in neurons by expression of constitutively active and dominant-negative mutants results in specific defects in

‡ Corresponding author. E-mail address: alema@ibc.rm.cnr.it.

axon and dendrite outgrowth (Luo *et al.*, 1996), and Rho signaling appears to be selectively required in early development for proliferative expansion and survival of thymocytes (Henning *et al.*, 1997). Rac appears to be involved in muscle morphogenesis, because it has been reported that myoblasts fail to fuse properly when a constitutively active *Drosophila* Rac homologue is expressed in the muscle precursors of the embryo; conversely, expression of a dominant-negative form generates excessively fused muscle fibers (Luo *et al.*, 1994). Moreover, Myoblast city, a homologue of mammalian DOCK180, has been identified as a specific mediator of Rac1 activity in several morphogenetic processes in *Drosophila*, including myogenesis (Nolan *et al.*, 1998).

Although the molecular mechanisms controlling myogenesis are well characterized at the transcriptional level both in vivo and in vitro (Emerson, 1993; Sassoon, 1993; Olson and Klein, 1994), the signaling molecules that mediate the transduction of extracellular cues to the nucleus, resulting in the activation of the muscle regulatory factors (MRFs), are far from being identified (Olson, 1992; Alemà and Tatò, 1994; Ludolph and Konieczny, 1995; Maione and Amati, 1997). MRFs represent key elements in the induction of myogenic differentiation in that they both control transcription of muscle-specific genes and repress cell proliferation by interacting with cell cycle regulators (reviewed by Lassar *et al.*, 1994; Olson and Klein, 1994; Maione and Amati, 1997; Walsh and Perlman, 1997). Much insight into the control of myoblast differentiation was gathered from the study of signaling molecules that act as negative regulators of myogenesis in vitro. Serum mitogens, fibroblast growth factor 2, and transforming growth factor β1 have been shown to inhibit differentiation through interference with MRF functions (Olson, 1992; Lassar *et al.*, 1994; Ludolph and Konieczny, 1995). Expression in myogenic cells of a number of exogenous oncoproteins, including the Src tyrosine kinase, the Ras GTPase, and the transcription factors Myc, Fos, and Jun, invariably resulted in inhibition of differentiation (Alemà and Tatò, 1994; Lassar *et al.*, 1994). Several independent studies have shown that transformation by Ras and Src oncogenes prevents myogenesis in both primary quail myoblasts and mouse myogenic cell lines by inhibiting the expression (Konieczny *et al.*, 1989; Lassar *et al.*, 1989, Falcone *et al.*, 1990, 1991; Yoon and Boettiger, 1994; Russo *et al.*, 1997) and the function (Kong *et al.*, 1995; Hirayama *et al.*, 1997; Gauzzi, Ciuffini, Falcone, and Alemà, unpublished observations) of MRFs. Attempts to delineate the downstream components of Ras signal transduction pathways leading to suppression of MRF functions have led to the conclusion that neither the MAPK pathway nor the Rac/Rho pathway are involved (Ramocki *et al.*, 1997; Weyman *et al.*, 1997).

Here we set out to address the issue of the role of Rho family GTPases during myogenesis in vitro with the aim of gaining clues as to their biological functions during development and their possible role as key regulators of the assembly of tissue-specific cytoskeletal structures. This issue has been addressed before, but it remains controversial because it was concluded in one instance that Rho family members are silent when expressed in an artificial myogenic context (Ramocki *et al.*, 1997) and in another instance that the activity of all Rho family GTPases is actually required for differentiation to occur in an established murine cell line (Takano *et al.*, 1998). The data reported in the present paper support very different conclusions. Taking advantage of the use of unestablished myoblasts derived from avian embryos that, contrary to the murine models, have normal control of proliferation and differentiation and can assemble highly ordered sarcomeric structures (Castellani *et al.*, 1995, 1996), we find that, although the activity of Rac1 is not required for commitment to terminal differentiation, the unscheduled expression of constitutively active Rac1 and Cdc42, but not RhoA, disrupts the orderly progression of the myogenic program. Moreover, we highlight novel roles of Rac1 in the maintenance of the differentiated state and in the disassembly of sarcomeres induced by the deliberate activation of the v-Src tyrosine kinase in terminally differentiated myotubes.

MATERIALS AND METHODS

Materials and Antibodies

DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Other chemicals were purchased from Sigma Chemical (St. Louis, MO). Highly purified Triton X-100 was from Boehringer Mannheim (Indianapolis, IN). BODIPY Fl phallacidin was from Molecular Probes (Eugene, OR). C3 transferase was a gift from A. Hall (University College London, London, United Kingdom). mAb to β-galactosidase was purchased from Boehringer Mannheim, and mAb to vinculin (VIN3-24) (Saga *et al.*, 1985) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit polyclonal antibody to JNK (C-17) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to myc tag (mAb 9E10) was provided by Gerard Evan (Imperial Cancer Research Fund, London), antibody to skeletal α-actinin (mAb 9A2B8) was provided by Donald Fischman (Cornell University Medical College, Ithaca, NY), and antibody to hemagglutinin (HA; mAb 12CA5) was provided by Oreste Segatto (Istituto Regina Elena, Rome, Italy). Rabbit sera to chicken myogenin were kindly provided by Bruce Paterson (National Cancer Institute, Bethesda, MD). Rabbit serum to viral capsid protein p27 was provided by Michael Hayman (New York University, Stony Brook, NY). A polyclonal antibody to chicken skeletal muscle myosin was developed in our laboratory with purified chicken muscle myosin as immunogen (Castellani *et al.*, 1996). Rabbit and goat anti-mouse antibodies, and TRITC- and FITC-conjugated goat anti-rabbit and anti-mouse antibodies, were from Jackson Immunoresearch Laboratories (West Grove, PA). HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad (Richmond, CA).

Cell Cultures

Chicken embryo fibroblasts were prepared from 10-d-old SPAFAS embryos as described previously (La Rocca *et al.*, 1989) and maintained at 37°C in DMEM, supplemented with 10% FCS, 10% tryptose phosphate broth, and 1% chicken serum (referred to as growth medium [GM]). Primary cultures of quail (*Coturnix japonica*) myoblasts (QMb) were prepared as described previously (La Rocca *et al.*, 1989; Falcone *et al.*, 1991) and maintained proliferating in GM also containing 3% quail embryo extract at 37°C. Differentiation was induced by plating 10^5 cells on 35-mm collagen-coated dishes in GM and, the next day, by substituting GM with F14 medium supplemented with 2% FCS (referred to as differentiation medium [DM]). Polyclonal populations of transformed QMb were established as described previously from primary passage cultures infected at high multiplicity with high-titer viral stocks of tsLA29, a temperature-sensitive mutant of Rous sarcoma virus (RSV) (Falcone *et al.*, 1991). tsLA29-transformed QMb (QMb-LA29) were propagated at 35°C (permissive temperature) on collagen-coated dishes in GM, and myogenic differentiation was assayed at 41°C (restrictive temperature) in DM as described for QMb. Activation of temperature-

sensitive v-Src in terminally differentiated myotubes was carried out by shifting the cultures from 41 to 35°C for the appropriate lengths of time in DM. To induce myogenic differentiation without cellular fusion, both QMb and QMb-LA29 were cultivated in DM containing 1.85 mM EGTA to reduce the free calcium concentration in the medium. COS-7 cells were cultured in DMEM supplemented with 10% FCS.

A cell line expressing Rac1V12 was established by transfection of QMb-LA29 at 35°C with pEXVmyc-Rac1V12 along with pRSV-neo in a 10:1 ratio and by subsequent selection in GM containing 1 mg/ml G418. Polyclonal populations were obtained that expressed detectable levels of myc-tagged Rac1V12 protein, as assessed by immunoblotting with anti-myc mAb 9E10. Differentiation of QMb-LA29Rac1 cells was induced in DM at 41°C as described for parental cells.

Construction and Growth of RCASBP Rac1V12 Virus

To obtain replication-competent retroviruses expressing an activated allele of Rac1, cDNA encoding the myc-tagged mutant Rac1V12 (from pEXVmyc-V12Rac1) was subcloned into the helper-independent retroviral vector RCASBP(A) (Petropoulos and Hughes, 1991), kindly provided by S. Hughes (National Cancer Institute, Frederick, MD), which encodes an envelope subgroup A virus. To allow cloning into the unique *Cla*I site of the vector, the cDNA was previously inserted into an adaptor plasmid (Hughes *et al.*, 1987). Chicken embryo fibroblasts were transfected in GM with the viral plasmids RCASBP and RSCABP Rac1V12 by means of an optimized calcium phosphate transfection procedure (Chen and Okayama, 1987) and passaged twice to allow virus spread. One week later, when all of the cells expressed viral proteins, as monitored by immunostaining with antibodies reacting to p27 viral capsid proteins and anti-myc antibodies for expression of Rac1 protein, viral stocks were harvested in GM containing 1% DMSO. Primary QMb were infected at high multiplicity of infection and passaged once, and 4 d after infection they were plated on collagen-coated dishes in DM to allow differentiation.

Transient Transfections and Chloramphenicol Acetyl Transferase Assay

Transient transfections were carried out in v-Src–transformed QMb by means of the calcium phosphate procedure (Chen and Okayama, 1987) and in primary myoblasts with the Lipofectamine reagent (Life Technologies-BRL, San Giuliano Milanese, Italy), according to the manufacturer's recommendations. The following expression vectors were used: pEXVmyc-V12Rac1, pEXVmyc-V12N17Rac1, pEXVmyc-V14RhoA, pRKmyc-V14N19RhoA, pRK5myc-L61Rac1, pRK5myc-L61Y40CRac1, pRK5myc-L61F37ARac1, pRK5myc-L61Cdc42 (from A. Hall), pcDNA3-JNK-HA (from S. Gutkind, National Institutes of Health, Bethesda, MD), pcDNA-βGal (Invitrogen, Carlsbad, CA), and pcDNAI-src, obtained by cloning the SR-A v-*src* gene in pcDNAI vector and the Green Fluorescent Protein (GFP) vector pGreen-Lantern (Life Technologies-BRL). Expression vectors for MEKK1 and MEKK2 were kindly provided by S. Gutkind and M. Karin (University of California, San Diego, CA) respectively.

Cells for transient expression of chloramphenicol acetyl transferase (CAT) reporter constructs were transfected in duplicate with Lipofectamine. Reporter plasmids for muscle-specific transcription included the pMCK-CAT plasmid (Sternberg *et al.*, 1988), the 4R-*tk*CAT reporter (Weintraub *et al.*, 1990), and the myogenin reporter construct pMyo1565CAT (kindly provided by E.N. Olson, University of Texas Southwestern Medical Center, Dallas, TX). The chicken β-actin-CAT and the RSV-CAT constructs (kindly provided by Bruce Paterson) were used as controls. CAT activity was assayed in total cell extracts and normalized for protein content with an enzymatic immunoassay kit (Boehringer Mannheim).

RNA Isolation and Northern Blot Analysis

Total RNA was prepared by the Ultraspec RNA isolation system (Biotecx, Houston, TX). Ten-microgram aliquots of the obtained RNA were resolved on 0.9% agarose/2.2 M formaldehyde gels. Transfer to nitrocellulose membranes and high-stringency hybridization were carried out according to standard procedures. Probes were labeled with a random-primed DNA-labeling kit (Amersham, Arlington Heights, IL). For detection of muscle-specific and constitutive transcripts, inserts of the following plasmids were cut with the appropriate restriction enzymes and used as probes: cC127, containing a 600-base pair quail myosin light-chain cDNA (provided by C. Emerson, University of Pennsylvania, Philadelphia, PA); cC128, containing a 500-base pair quail myosin heavy-chain cDNA (provided by C. Emerson); and a plasmid containing a 1.2-kilobase avian GAPDH cDNA (obtained from C. Schneider, University of Udine, Udine, Italy).

Whole-cell Extracts and Western Blot Analysis

Cells were briefly rinsed with PBS containing 0.5 mM sodium orthovanadate and collected with 0.2 ml of SDS sample buffer (8 M urea, 0.14 M β-mercaptoethanol, 0.04 M DTT, 2% SDS, 0.075 M Tris-Cl, pH 8.0) per 35-mm plate. SDS-PAGE (2–20 μg of total proteins per well) and Western blot analysis were carried out as described previously (Castellani *et al.*, 1995; Gallo *et al.*, 1997) with HRP-conjugated goat anti-rabbit and anti-mouse antibodies, and the results were revealed with the ECL detection system (Amersham).

Assays for Activation of JNK

Primary myoblasts were plated at 3×10^5 per 60-mm dish in GM and cotransfected with 2.5 μg of the various expression vectors and 1.25 μg of pcDNA3-HA-JNK. The next day, transfected cells were transferred to F14 medium supplemented with 0.5% FCS for about 30 h, and controls for JNK activity were treated with 600 mM sorbitol for 30 min before lysis. Subconfluent COS-7 cells were cotransfected with 0.6 μg of the various expression vectors and 2 μg of pcDNA3-JNK-HA per 60-mm dish by the DEAE-dextran method, as described previously (Olson *et al.*, 1995). All transfected cells were rinsed with PBS containing 0.5 mM sodium orthovanadate before cell lysis. Both primary myoblasts and COS-7 cells were lysed with a buffer solution containing 0.3 M NaCl, 50 mM NaF, 0.1 mM orthovanadate, 5 mM EDTA, 5 mM EGTA, 40 mM sodium pyrophosphate, 25 mM HEPES, pH 7.6, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (40 μg/ml leupeptin, 40 μg/ml aprotinin, 40 μg/ml soybean trypsin inhibitor, 1 mM PMSF) (Olson *et al.*, 1995). Parallel plates were used to evaluate the amount of transfected HA-JNK by Western blots probed with mAb 12CA5. JNK was immunoprecipitated from normalized lysates with 3 μg of mAb 12CA5 and 20 μl of protein G–Sepharose and assayed for GST-c-Jun phosphorylation activity in 30 μl of kinase reaction buffer containing 25 mM $MgCl_2$, 2 mM DTT, 0.1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 μM ATP, 4 μCi of [γ-^{32}P]ATP, 25 mM HEPES, pH 7.6, and 3 μg of GST-c-Jun. After incubation at 30°C for 30 min, kinase reaction was stopped by the addition of boiling SDS sample buffer, and the reaction products were resolved on a 10% SDS-polyacrylamide gel. Analysis and quantitation of phosphorylated species was carried out by PhosphorImager analysis with the use of ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of immunoprecipitated HA-JNK was evaluated by Western blot analysis with ECL, and bands were quantitated by scanning films recorded at different exposure times.

To analyze the activation of endogenous JNK, cells were plated on a 90-mm dish in GM and the next day transferred to F14 medium supplemented with 0.5% FCS for 2 d. Controls for JNK activity were treated with UV light 30 min before lysis. Cells were then rinsed with PBS containing 0.5 mM sodium orthovanadate and extracted

for 30 min on ice with Triton lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 0.5% Triton X-100, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.5 mM DTT) containing phosphatase and protease inhibitors. After dilution to 0.15% Triton X-100 and normalization of cell extracts for protein content, endogenous JNK was precipitated with 3 μg of GST-c-Jun fusion protein coupled to glutathione-agarose beads (Mainiero *et al.*, 1998). After washing, the beads were incubated with 30 μl of kinase reaction buffer as described previously, and the samples were boiled in sample buffer and separated by 12% SDS-PAGE. Analysis and quantitation of phosphorylated species was carried out by PhosphorImager analysis.

Immunofluorescence and Confocal Analysis

Cultures were routinely fixed for 10 min with 4% paraformaldehyde (Fluka, Buchs, Switzerland) in PBS at room temperature, permeabilized with 0.25% Triton X-100 in PBS for 10 min, and incubated with primary antibodies at the appropriate dilution. To enhance the signal derived from labeling with mAb to α-actinin, a triple-sandwich technique was used (Provenzano *et al.*, 1998). After washing with PBS, cells were incubated with secondary antibodies and/or BODIPY Fl phallacidin and, after a final wash, stained with 1 μg/ml Hoechst 33258 (Calbiochem, La Jolla, CA) before being mounted in Mowiol (Calbiochem). The samples were routinely examined with a Zeiss (Thornwood, NY) microscope equipped with 40× and 50× water-immersion objectives. Confocal analysis was carried out with a Leica (Heidelberg, Germany) TCS 4D system equipped with 40x1.00–0.5 and 100x1.3–0.6 oil-immersion lenses.

RESULTS

Rho Family GTPases Impose Distinct Phenotypes on QMb

The effect of the expression of Rho family members on myogenic cells was investigated on both early-passage (p1–p3) QMb and myoblasts transformed by a temperature-sensitive mutant of RSV (QMb-LA29) (Falcone *et al.*, 1991). QMb grown in GM appeared heterogeneous when analyzed for expression of myogenin, a muscle regulatory factor required for the execution of the myogenic program (Olson and Klein, 1994). Analysis of QMb by indirect immunofluorescence with antibodies to myogenin and to myosin showed that both myogenin$^+$ (25–30%) and myosin$^+$ (15–25%) cells were present in these cultures, the latter representing terminally differentiated cells. The full myogenic potential of QMb was assayed after induction of differentiation in DM and ranged between 70 and 80%. Parallel experiments carried out with QMb-LA29 indicated that the percentage of myogenin$^+$ cells was reduced to 2–4% at the permissive temperature (35°C) for the v-Src oncoprotein in both GM and DM, whereas the vast majority of cells undergo terminal differentiation in DM only at the restrictive temperature (41°C) (Falcone *et al.*, 1991).

Primary and temperature-sensitive v-Src–transformed QMb were transiently transfected with constructs encoding constitutively activated myc-tagged forms of Rho family members (RhoAV14, Rac1V12, Rac1L61, and Cdc42L61); plasmids encoding β-galactosidase or GFP were used as controls. After transfection, cells were kept in GM for 36 h, and the organization of the actin cytoskeleton was monitored by immunofluorescence (Figures 1 and 2). Primary QMb expressing activated Rac1 (Figure 1A), compared with cells expressing β-galactosidase (Figure 1F), showed changes in cell morphology and in the organization of the actin cytoskeleton, highlighted by labeling with phallacidin. Rac1L61 transfectants appeared flat and enlarged, with a poor complement of stress fibers but pronounced lamellipodia and ruffles; occasionally, multinucleated cells were also observed. Expression of Cdc42L61 induced cytoskeletal changes (Figure 1D) resembling those observed in Rac1L61-expressing cells, and occasionally filopodia were observed. QMb expressing RhoAV14 showed reduced cellular dimensions accompanied by an apparently augmented actin polymerization (Figure 1E). The ability of activated Rac1 to modify the overall morphology of myoblasts was further investigated with the use of Rac1 mutants that have been shown to separate the ability of Rac1 to interact with different downstream effectors and to induce cytoskeletal changes in fibroblasts (Joneson *et al.*, 1996; Lamarche *et al.*, 1996; Westwick *et al.*, 1997). QMb expressing Rac1L61C40, a mutant defective in stimulating Pak-1 and JNK activity, were very similar to those expressing Rac1L61, both in cellular morphology and in the organization of polymerized actin (Figure 1B), as observed in fibroblasts (Lamarche *et al.* 1996). On the other hand, Rac1L61A37, a mutant that retains the ability to stimulate Pak-1 and JNK activity but not to affect the actin cytoskeleton, did not significantly modify the QMb cellular phenotype (Figure 1C).

At variance with primary QMb, v-Src–transformed myoblasts at 35°C showed a cytoskeleton characterized by a poor complement of actin stress fibers and aggregation of F-actin into small globular complexes that also contain vinculin and other cytoskeletal proteins typically found in adhesion plaques (Figure 2, E and J). Expression of the activated forms of Rac1 and Cdc42, monitored by labeling with antibody to the myc tag (data not shown), imposed on these cells pronounced changes in cell morphology accompanied by the appearance of thin actin fibers (Figure 2, A and B) and arrowhead-shaped focal adhesion plaques, as revealed by labeling with phallacidin and antibody to vinculin (Figure 2, F and G). Expression of RhoAV14 in QMb-LA29 induced a pronounced increase in polymerized actin and focal adhesion plaque formation (Figure 2, C and H) compared with cells expressing β-galactosidase (Figure 2, E and J), occasionally accompanied by a reduction in cellular dimensions. Expression of Rac1N17, a dominant-negative mutant of Rac1, on the other hand, had no discernible effect on either cell shape and dimensions or the organization of the actin cytoskeleton (Figure 2, D and I), making Rac1N17-expressing cells indistinguishable from neighboring cells in the same dish or from those expressing β-galactosidase (Figure 2, E and J). The levels of expression of Rac1N17, revealed by labeling with antibody to the myc tag, appeared to be comparable to those of the activated forms of the Rho family members, suggesting that inhibition of endogenous Rac1 is unable to rescue the cytoskeletal changes imposed on myoblasts by v-Src. Taken together, these findings indicate that each activated form of the Rho family GTPases is able to impose a characteristic phenotype in myoblasts independent of the starting phenotype (primary versus v-Src–transformed myoblasts). This conclusion is supported by the finding that Rac1-induced changes in QMb-LA29 are not modified by coexpression of RhoAN19 or exposure of the cultures to C3 transferase, a potent inhibitor of Rho function (Nobes and Hall, 1995),

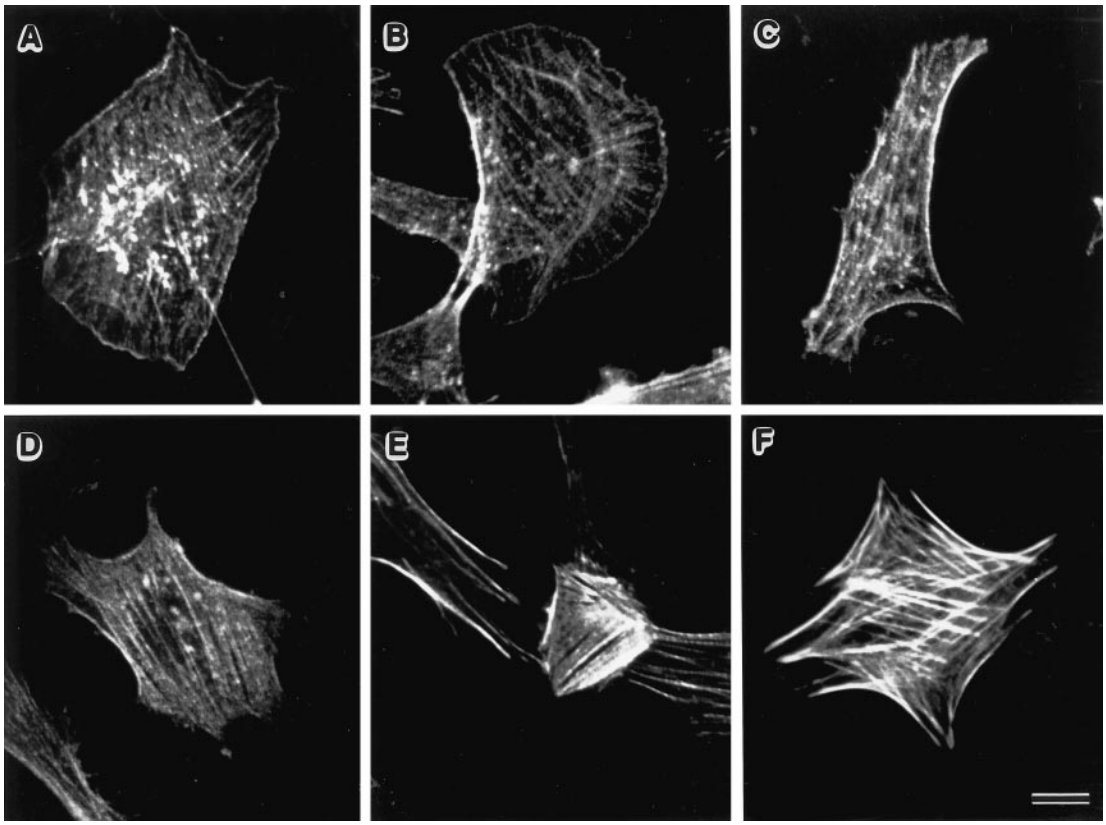

Figure 1. Rho family members induce changes in cell shape and actin cytoskeleton in primary QMb. Immunofluorescence micrographs of BODIPY phallacidin–labeled QMb in GM, transiently expressing myc epitope–tagged Rac1L61 (A), Rac1L61C40 (B), Rac1L61A37 (C), Cdc42L61 (D), or RhoAV14 (E), or β-galactosidase (F) used as control. Cells expressing the various transfected constructs were identified by immunolabeling for the myc epitope or β-galactosidase (data not shown); representative cells positive for expression of the myc epitope (A–E) and β-galactosidase (F) are shown. Bar, 10 μm.

suggesting that in these cells Rac1 operates in a Rho-independent manner (data not shown).

Constitutively Active Rac1 and Cdc42 Disrupt Myogenic Differentiation

QMb and QMb-LA29 transiently transfected with plasmids encoding either β-galactosidase/GFP or mutant alleles of the Rho family members were allowed to differentiate in a modified DM containing low levels of free calcium ions (DM containing 1.85 mM EGTA) to inhibit fusion into multinucleated myotubes (Adamo *et al.*, 1976). Inhibition of fusion was desirable to prevent the possible functional attenuation of exogenous proteins when recruited into myotubes formed by transfected and untransfected cells, thereby allowing a quantitative assessment of differentiation by single-cell analysis. Double labeling of myocytes with anti-myc mAb 9E10 and antibodies against myogenin and myosin ensured that only transfected cells were studied (Figure 3). The percentage of cells expressing myogenin or myosin was strongly inhibited in cells transfected with activated mutants of Rac1 (Rac1V12 and Rac1L61) and Cdc42 (Cdc42L61), whereas it was only marginally reduced by expression of RhoAV14 (Figure 3, A and B). Intriguingly, the small number of Rac1-expressing cells that scored as myosin positive accumulated lower levels of myosin compared with controls (data not shown). Given the distinct effects imposed by RacL61C40 and Rac1L61A37 on the actin cytoskeleton of QMb, their ability to influence myogenic differentiation was also measured. As shown in Figure 3A, Rac1L61C40 was very effective in inhibiting myogenin expression, whereas Rac1L61A37 exerted a comparatively weaker inhibition of differentiation.

To ascertain whether endogenous Rac1 function is necessary to attain myogenic terminal differentiation, growing QMb and QMb-LA29 were transfected with an expression vector encoding the myc-tagged Rac1N17 protein. Upon the shift to DM, the percentage of Rac1N17-expressing cells (Figure 3A and data not shown) undergoing differentiation remained comparable to that of controls. QMb-LA29 expressing Rac1N17 were also analyzed at the permissive temperature for the oncoprotein because it has been shown that Rac1 is required for focus formation induced by v-Src in NIH 3T3 fibroblasts (Minden *et al.*, 1995; our unpublished observations). Expression of Rac1N17 in temperature-sensitive v-Src–transformed myoblasts had no apparent effect on rescue of terminal differentiation, measured as expression of

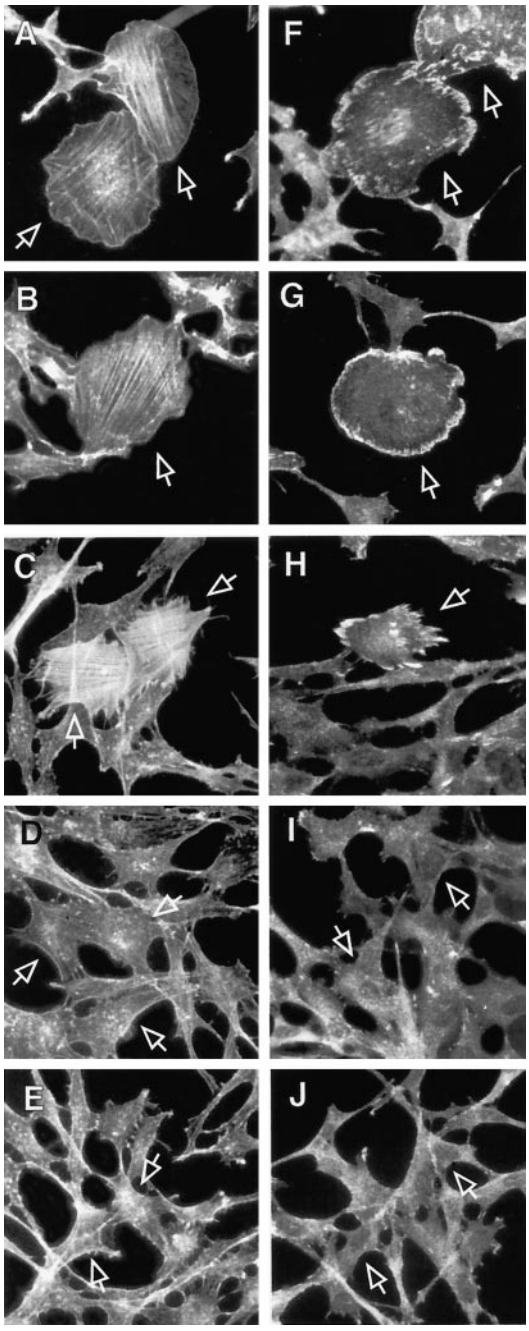

Figure 2. v-Src–transformed myoblasts expressing Rho family GTPases display distinct phenotypes. Immunofluorescence micrographs of QMb-LA29 kept in GM at 35°C after transfection with Rac1L61 (A and F), Cdc42L61 (B and G), RhoAV14 (C and H), Rac1N17 (D and I), and β-galactosidase (E and J) labeled with BODIPY phallacidin (A–E) and antibody to vinculin (F–J). Cells expressing the transfected constructs were identified by double labeling with antibody to the myc epitope or to β-galactosidase (arrows). Bar, 10 μm.

myogenin and myosin (data not shown), suggesting that the block of myogenesis exerted by v-Src may not be mediated by endogenous Rac1.

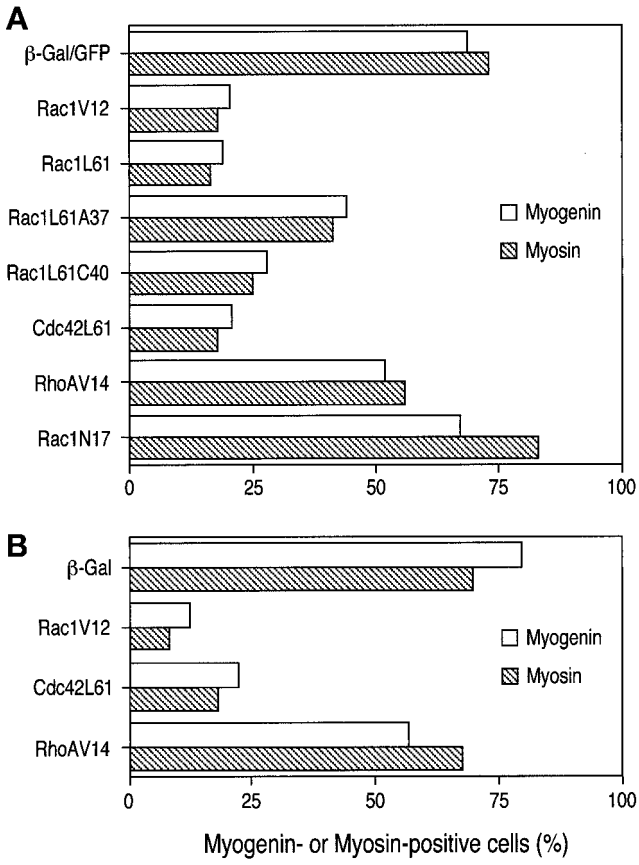

Figure 3. Transient expression of activated Rac1 and Cdc42 alleles, but not of RhoA, is sufficient to inhibit the expression of myogenin and myosin in QMb. Primary QMb (A) and QMb-LA29 (B) at 35°C were transiently transfected with expression vectors for β-galactosidase (β-Gal) and myc epitope–tagged Rac1V12, Rac1L61, Rac1L61A37, Rac1L61C40, Cdc42L61, RhoAV14, and Rac1N17. After transfection, QMb and QMb-LA29 were cultured in DM containing 1.85 mM EGTA for 2 d at 37 and 41°C, respectively. After fixation, cells were double labeled with mAbs to β-galactosidase or to the myc tag and with polyclonal antibodies to chicken myogenin, a muscle-specific regulatory factor, or to chicken myosin. Double scoring of cells for either β-Gal/myogenin and β-Gal/myosin or myc/myogenin and myc/myosin is shown as percentage of cells expressing the transfected proteins. Very similar results were obtained when β-Gal was substituted with GFP. Transfection followed by immunolocalization was performed in five independent experiments, and in all experiments at least 200 transfected cells were examined for each condition.

The JNK pathway has been shown to be activated downstream of Rac1 and Cdc42, but not of RhoA, in many mammalian cell lines, and it has been held responsible, at least in part, for the effect of these small G proteins on gene expression (reviewed by Van Aelst and D'Souza-Schorey, 1997). Therefore, experiments were carried out to measure whether JNK was activated by Rho family members and v-Src in QMb. QMb were transiently transfected with expression vectors for activated Rac1, Cdc42, and RhoA, as well as with those for Rac1L61C40, Rac1L61A37, and wild-type v-Src and with HA-tagged mammalian JNK, and after 2 d of serum

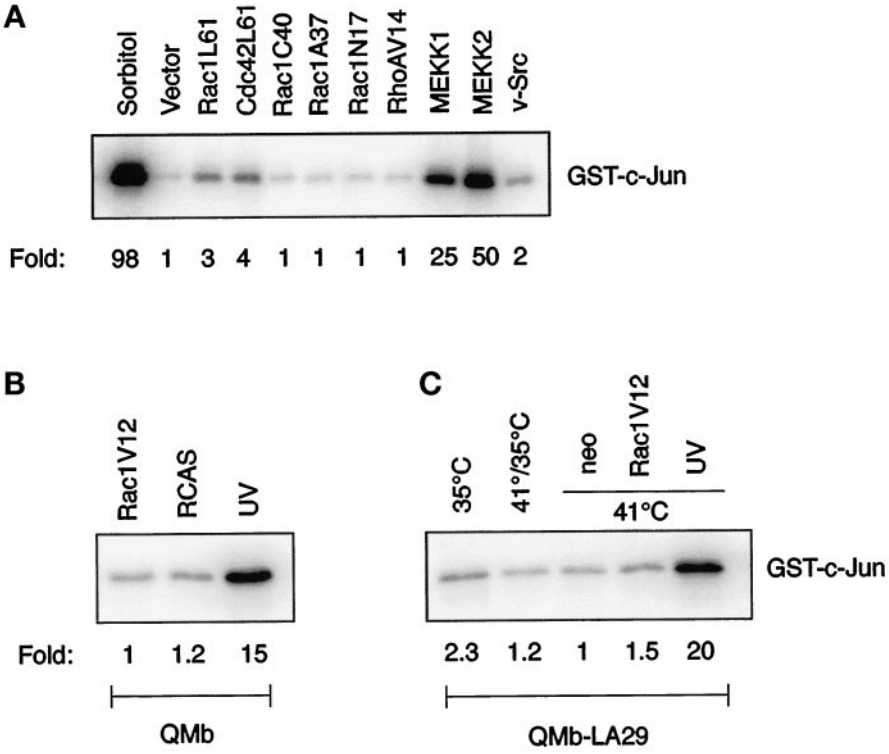

Figure 4. Rac1 and Cdc42 are weak activators of JNK in QMb. (A) QMb were cotransfected with a HA-tagged JNK expression vector along with vectors expressing activated and dominant-negative Rho family members, MEKK1, MEKK2, and v-Src as indicated. QMb cotransfected with HA-JNK and empty vector with or without treatment with sorbitol were used as controls. The activity of immunoprecipitated HA-JNK was assayed with GST-c-Jun protein as substrate. The numbers represent fold stimulation above the activity of HA-JNK (referred to as 1) normalized for HA-JNK content. (B and C) Endogenous JNK activity was measured with glutathione beads coated with GST-c-Jun fusion protein as bait in QMb infected with RCASBP (RCAS) or RCASBP-Rac1V12 (Rac1V12) at 37°C (B) and QMb-LA29 expressing the neomycin-resistance gene (neo) at the indicated temperatures or Rac1V12 at 41°C (C). RCAS-infected QMb and QMb-LA29 were treated with UV as controls for JNK activation. Fold stimulation above basal JNK activity in RCAS-infected QMb (B) and QMb-LA29 at 41°C (C), referred to as 1, is indicated.

starvation they were assayed for JNK activity with GST-c-Jun as substrate (Olson *et al.*, 1995). In this assay, the osmotic stress inducer sorbitol and potent upstream inducers of JNK such as MEKK1 and MEKK2 (Xia *et al.*, 1998) were used to assess the range of JNK activation attainable in avian cells and to confirm that mammalian JNK can indeed be activated by avian upstream kinases. As shown in Figure 4, whereas sorbitol (90-fold), MEKK1 (25-fold), and MEKK2 (50-fold) induced high JNK activity, Rac1L61 and Cdc42L61 activated cotransfected JNK only 3- to 4-fold. Moreover, Rac1L61C40 and Rac1L61A37 mutants, like RhoA and Rac1N17, had no measurable effect on JNK activity. The same constructs, with the exception of RhoA, Rac1L61C40, and Rac1L61N17, efficiently activated the kinase in COS-7 cells (data not shown), as reported previously (Coso *et al.*, 1995; Olson *et al.*, 1995). Transfected v-Src induced a weak activation of JNK activity (twofold), consistent with the measurements of Bojovic *et al.* (1996) in chicken embryo fibroblasts. Together, the modest activation of JNK exerted by Rac1 and Cdc42, and the observation that the Rac1L61C40 mutant still inhibits differentiation, raise questions about the involvement of the JNK pathway with respect to the block of differentiation by expression of these proteins.

Terminal Differentiation Is Unperturbed but Accumulation of Muscle-specific Proteins Is Inhibited in Myoblasts Constitutively Expressing Rac1V12

The experiments described above show that the activated forms of Rac1 and Cdc42 transiently expressed at high levels inhibit the expression of muscle-specific proteins, resulting in block of terminal differentiation. However, to obtain a quantitative assessment of the effect of activated Rho family members on muscle-specific protein accumulation and to study the fusion and maturation of sarcomeric structures in multinucleated myotubes, a cell population stably expressing the exogenous mutant proteins would be required. To obtain such a cell population, an RCASBP retroviral vector containing the myc-tagged Rac1V12 cDNA (RCAS-Rac1V12) and competent for replication in avian cells was constructed. High-titer retroviral stocks of RCASBP, RCASBP-Rac1V12, and the PR-A strain of RSV (RSV-PR-A), to be used as a control of inhibition of myogenic differentiation, were used to infect primary QMb. Infected myoblasts were cultured in DM for 2 or 3 d to assay for morphological differentiation and muscle-specific gene expression at both the RNA and protein levels. Although myc-tagged Rac1 expression in infected myoblasts was below detection by immunofluorescence, the protein was readily detected by immunoblotting (see Figure 6A).

RCAS-Rac1V12–infected myoblasts grown in DM fused into atypical myotubes with an efficiency comparable to that of RCASBP-infected controls (data not shown), whereas RSV-PR-A–infected cells remained mostly proliferating, as reported previously (Falcone *et al.*, 1985). Accumulation of muscle-specific transcripts for myosin heavy and light chains, analyzed by Northern blot, was not affected by Rac1V12 compared with controls but was fully inhibited by v-Src after 2 d (Figure 5, lanes 1–3) and 3 d (Figure 5, lanes 4–6) in DM. Accordingly, transcription of reporter genes under the control of muscle-specific promoters, including that of myogenin, was not inhib-

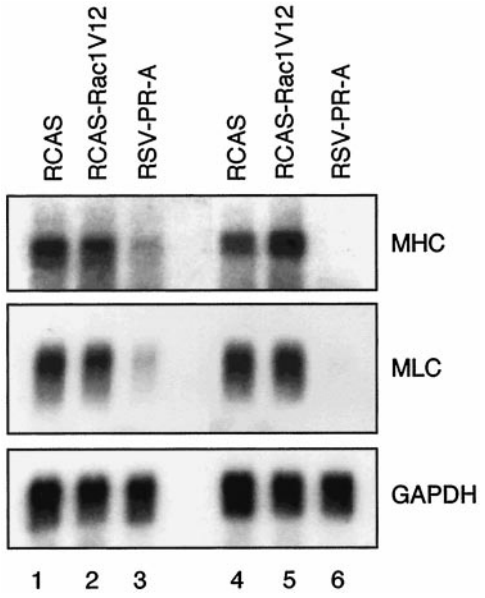

Figure 5. Accumulation of muscle-specific gene transcripts is not affected by stable expression of Rac1V12 protein. RNAs extracted from QMb infected with high-titer viral stocks of replication-competent RCASBP retroviruses (RCAS) (lanes 1 and 4), RCASBP viruses expressing Rac1V12 (RCAS-Rac1V12) (lanes 2 and 5), and RSV-PR-A strain (lanes 3 and 6). Total RNAs were harvested from cells kept in DM for 2 d (lanes 1–3) and 3 d (lanes 4–6). Filters were hybridized with probes for myosin heavy chains (MHC), myosin light chains (MLC), and GAPDH as indicated.

ited by Rac1V12 (data not shown). In contrast, accumulation of structural muscle-specific proteins, including myosin heavy chain, α-actinin, α-actin, and calsequestrin, measured by Western blotting, was severely inhibited in Rac1V12-expressing myotubes, whereas that of vinculin, a ubiquitous cytoskeletal protein, was not significantly affected (Figure 6A). As expected, the accumulation of myosin (Figure 6A) and other muscle-specific contractile proteins (data not shown) was negligible in v-Src–transformed myoblasts.

To obtain independent evidence that the stable expression of activated Rac1 is compatible with incomplete differentiation, QMb-LA29 constitutively expressing Rac1V12 (QMb-LA29-Rac1) were also established by cotransfection with the neomycin-resistance gene and selection at 35°C. As observed in RCASBP-Rac1V12–infected QMb undergoing differentiation, accumulation of muscle-specific proteins, but not of talin or β-actin, was severely inhibited in QMb-LA29 myotubes expressing Rac1V12 compared with controls (Figure 6B). Altogether, these findings indicate that the efficiency of fusion into myotubes is preserved in myogenic cells stably expressing Rac1V12 and that the inhibition of myogenic differentiation is exerted at a posttranscriptional level.

To further address the issue of the role of the JNK pathway in the Rac1-induced phenotype, endogenous JNK activity was measured in both QMb and QMb-LA29 stably expressing Rac1V12 by a sensitive assay with the use of GST-c-Jun–agarose as a bait for activated JNK (Mainiero *et al.*, 1998). These measurements, shown in Figure 4, B and C, indicate that Rac1V12 is unable to induce JNK activity. Interestingly, v-Src also appears as a weak activator of JNK when expressed transiently (Figure 4A) or stably (Figure 4C) in myoblasts, and it has no effect on endogenous JNK activity after its activation in myotubes (Figure 4C) (see Bojovic *et al.*, 1996).

Rac1 Regulates Sarcomere Assembly in Myotubes

QMb-LA29 myotubes appear highly three dimensional and are characterized by pronounced and extensive cross-striations highlighted by staining for myosin and α-actinin, major protein components of thick filaments and Z-discs (Castellani *et al.*, 1996). Stable expression of Rac1V12 in these cells altered myotube morphology without affecting cellular fusion, as shown in Figure 7. Rac1V12-expressing myotubes appeared very wide and flat with a poor complement of myofibrils, highlighted by immunostaining for α-actinin and myosin (Figure 7, C and D). To ascertain whether the lack of bona fide sarcomeric structures in ts LA29-Rac1 myotubes, typically observed in control neomycin-resistance gene myotubes (Figure 7, A and B), could be ascribed only to the reduced accumulation of the constituent proteins, myotubes in which the accumulation of sarcomeric proteins seemed comparable to that in controls were analyzed by confocal microscopy. No sarcomeric organization was observed in Rac1-expressing myotubes independently of contractile protein accumulation (Figure 7).

To investigate the role of endogenous Rac1 in attaining full maturation of sarcomeric structures, QMb-LA29 were transfected with an expression vector encoding the myc-tagged Rac1N17 protein. Upon the shift to the restrictive temperature in DM, an accelerated maturation of myotubes expressing Rac1N17 was revealed by the early appearance of cross-striations (Figure 8). Indirect immunofluorescence of 2-d-old myotubes, in fact, showed a better-defined sarcomeric banding of both myosin (Figure 8C) and α-actinin (data not shown) in Rac1N17-expressing cells, identified by labeling for the myc tag (Figure 8D), compared with myotubes negative for the myc tag in the same dish or myotubes expressing β-galactosidase (Figure 8, A and B) used as controls. Together, these data support the view that Rac1 may be involved in modulating myofibrillar structure and alignment.

To specifically define the role of endogenous Rac1 in the maintenance of sarcomeric myofibrils, fully differentiated QMb-LA29 myotubes expressing Rac1N17 or β-galactosidase, as control, were shifted from 41 to 35°C to activate the temperature-sensitive v-Src kinase. It has previously been shown that activation of v-Src in fully differentiated QMb-LA29 myotubes causes a selective reorganization of sarcomeres and cytoskeleton, characterized by the appearance of F-actin–containing bodies originating from the progressive dismantling of the I-Z-I segments (Castellani *et al.*, 1995, 1996). Immunofluorescence of Rac1N17-expressing myotubes kept at 35°C for 4 h and double labeled with phallacidin and antibodies to the myc tag showed that Rac1N17 exerted a complete block of actin body formation induced by v-Src (Figure 9). The efficacy of Rac1N17 in inhibiting sarcomeric dismantling was further verified by prolonged incubation of the cultures at 35°C for up to 12 hours and by confocal microscopy analysis. It has previously been shown, in fact, that formation of actin bodies begins in the ventral myofibrils of myotubes, not easily visualized by conventional fluorescence microscopy (Castellani *et al.*, 1995). Thus, these findings suggests that Rac1 is likely involved in sarcomere turnover and

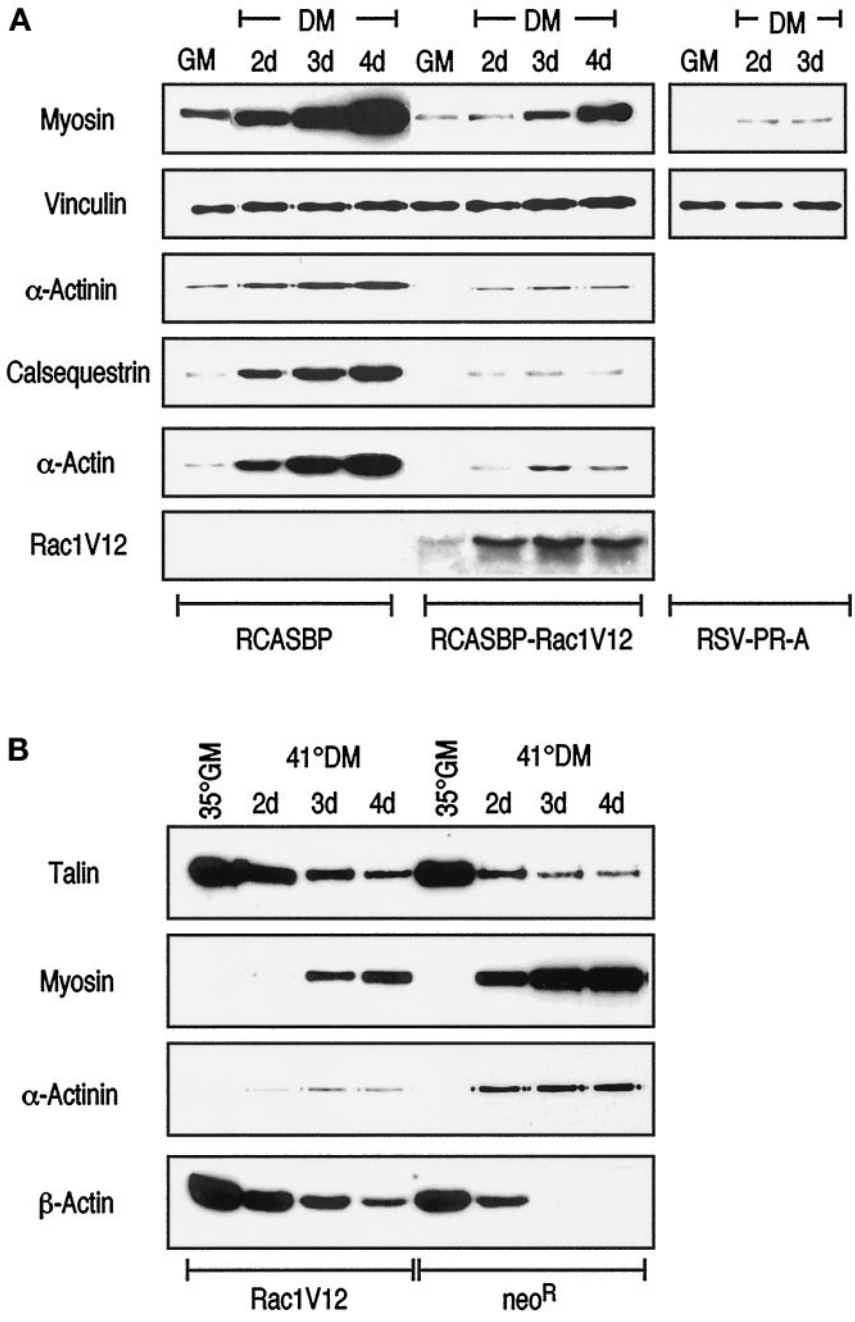

Figure 6. Rac1V12 inhibits accumulation of muscle-specific proteins in myotubes. Western blot analysis of cell extracts from QMb infected with RCASBP, RCASBP-Rac1V12, or RSV-PR-A retroviruses and cultured in DM for 2, 3, and 4 d (A) and polyclonal populations of QMb-LA29 stably transfected with expression vectors encoding a neomycin-resistance gene (neo^R) or Rac1V12 cultured in GM at the permissive temperature (35°C) or in DM at the restrictive temperature (41°C) for 2, 3, and 4 d (B). Cell extracts were then analyzed for expression of cytoskeletal and muscle-specific proteins by immunoblotting with specific antibodies, as indicated. Probing of the blots with antibodies to cytoskeletal and muscle-specific proteins shows that the accumulation of myofibrillar proteins such as myosin, α-actinin, and calsequestrin during in vitro maturation is highly reduced in myotubes expressing Rac1V12 compared with controls. Conversely, the reduction of cytoskeletal proteins such as β-actin and talin, which usually accompany myotube maturation, is partly inhibited by the presence of Rac1V12. The data shown are representative of three independent experiments.

can be placed downstream of v-Src in the signaling pathway leading to cytoskeletal remodeling of myotubes.

DISCUSSION

Overexpression of Activated Rac1 and Cdc42 Inhibits Myogenic Differentiation

Differentiation of skeletal muscle precursor cells is a multistep process involving expression of muscle regulatory factors, withdrawal from the cell cycle, induction of muscle-specific gene expression, and changes of the cytoskeleton into specialized structures. The issue addressed here is whether the small G proteins of the Rho family, namely Rac1, RhoA, and Cdc42, which have been shown to exert profound effects on both the morphology and the biology of a variety of cell types (Hall, 1998), play a role in the complex series of events leading to full myogenic differentiation.

A reassuring finding resulting from the phenotypes imposed on QMb by constitutively active Rho GTPases is that

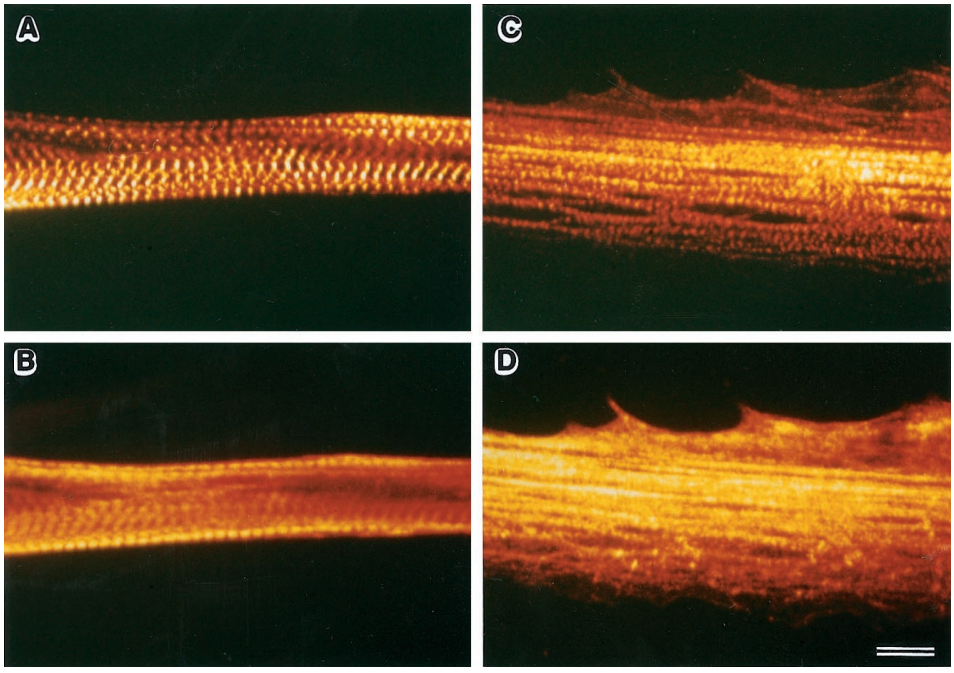

Figure 7. Rac1V12 inhibits the organization of α-actinin and myosin into sarcomeres. QMb-LA29 stably expressing neomycin resistance (A and B) or Rac1V12 (C and D) were cultured in DM at 41°C for 3 d and then processed for immunofluorescence. Confocal immunofluorescence micrographs of myotubes double labeled for α-actinin (A and C) and myosin (B and D) show that myotubes expressing Rac1V12 are flattened onto the substrate and that both α-actinin and myosin are organized in bundle-like assemblies, lacking both lateral alignment and periodic cross-striation, which are easily detected in controls. The two chromophores are depicted with the same color scale in panels A and B and panels C and D. Bar, 10 μm.

they are distinct for each GTPase and on the whole similar to those reported for mammalian fibroblasts. Surprisingly, the GTPases impose their characteristic phenotype also in QMb-LA29, which otherwise exhibit an overall morphology typical of transformed cells. Therefore, it can be assumed that, although the recipient cell types may show strikingly different starting morphologies, the signaling pathways responsible for cytoskeletal rearrangements and actin polymerization by Rho family members are largely conserved in a number of cell contexts. A close examination of the individual phenotypes reveals that RhoA imposes in both cell types an actin cytoskeleton that is similar to the one exhibited by the naive quail myoblast. A prima facie conclusion is that in QMb-LA29 endogenous RhoA activity is inhibited by v-Src to attain the morphologically transformed phenotype. Rac1 and Cdc42, instead, impose a cell morphology and an actin

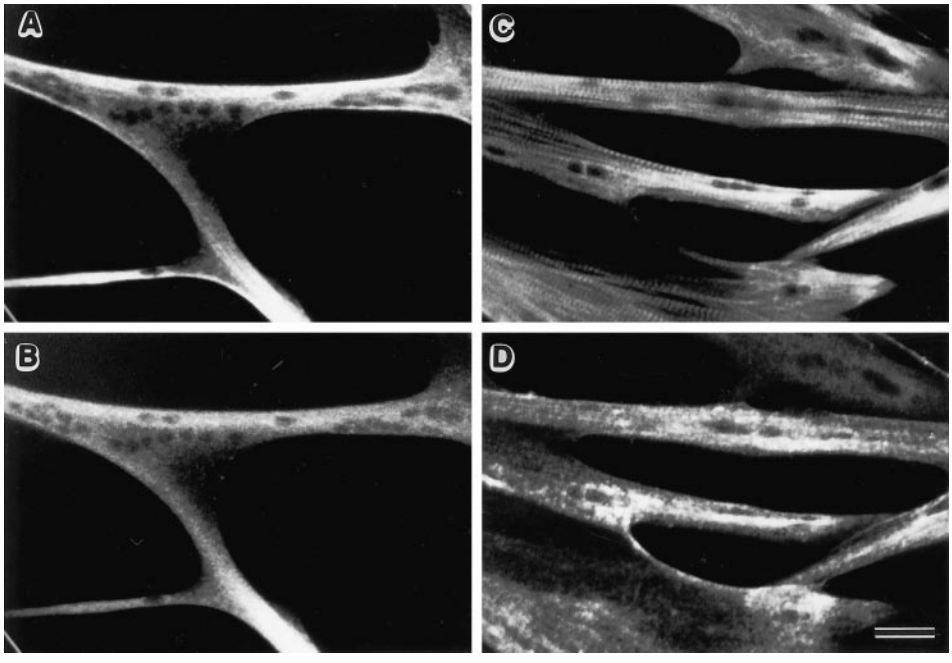

Figure 8. Expression of the dominant-negative mutant Rac1N17 accelerates maturation of sarcomeres. QMb-LA29 transiently transfected with expression vectors encoding for β-galactosidase (A and B) and the myc epitope–tagged Rac1N17 (C and D) were allowed to differentiate for 2 d at 41°C in DM and then processed for immunofluorescence. Confocal micrographs of myotubes double labeled with antibodies to myosin (A and C) and either β-galactosidase (B) or the myc epitope (D) show that the cross-striations due to the organization of the A bands are more prominent in myotubes expressing Rac1N17 than in controls expressing β-galactosidase. Bar, 25 μm.

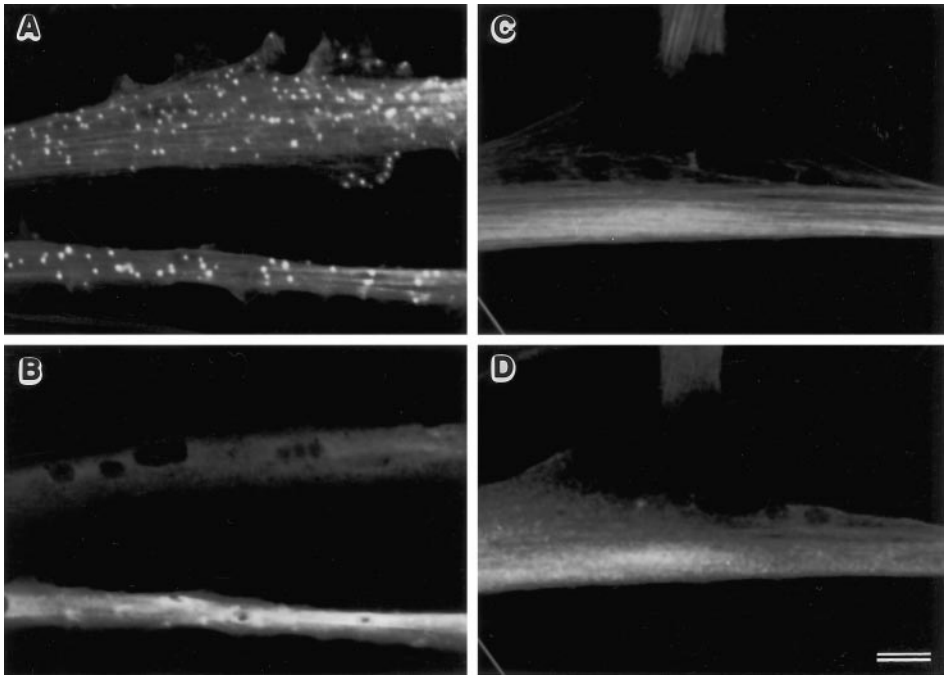

Figure 9. Expression of Rac1N17 inhibits the selective disassembly of the I-Z-I segments induced by v-Src in myotubes. QMb-LA29 transiently transfected with expression vectors encoding for β-galactosidase (A and B) and myc epitope–tagged Rac1N17 (C and D) were allowed to differentiate in DM at 41°C for 3 d and then shifted to 35°C for 4 h. Confocal micrographs of myotubes double labeled with phallacidin (A and C) and antibodies to β-galactosidase (B) or the myc epitope (D) show that Rac1N17 inhibits the v-Src–induced formation of actin bodies apparent in control cultures. Bar, 10 μm.

cytoskeleton largely different from those of the naive myoblast and of the v-Src–transformed myoblast. Thus, the function of v-Src in establishing the transformed phenotype may not require the activation of Rac1 and/or Cdc42, as also supported by the finding that expression of Rac1N17 is silent in v-Src myoblasts.

The expression of Rac1 and Cdc42 in cultures of QMb subjected to differentiation cues blocks the expression of myogenin, one of the muscle regulatory factors expressed early in development and necessary to activate muscle-specific gene transcription. On the contrary, expression of RhoA has no apparent effect on the myogenic progression of myoblasts, suggesting that this small GTP-binding protein may not participate in the signal transduction pathways leading to inhibition of skeletal muscle differentiation. Indeed, both Rac1 and Cdc42, but not RhoA, have been reported to activate the JNK and p38-MAPK signaling pathways (Coso *et al.* 1995; Minden *et al.* 1995), thereby affecting gene transcription. Previous mutational analysis studies have suggested that Rac1 triggers actin polymerization and JNK activity by bifurcating pathways that can be distinguished by specific amino acid substitutions in the effector domain (Joneson *et al.*, 1996; Lamarche *et al.*, 1996). To delineate the contribution of distinct Rac-stimulated signaling pathways to the inhibition of myogenesis, two Rac1 double mutants, Rac1A37 and Rac1C40, were also expressed in myoblasts. As reported for fibroblasts, only Rac1C40 induces lamellipodia in growing myoblasts and causes significant inhibition of myogenic differentiation. Furthermore, Rac1 and Cdc42 only modestly activate the JNK pathway in primary avian myoblasts, and neither Rac1C40 nor Rac1A37 activates JNK. Hence, it appears that activation of JNK is not essential for inhibition of differentiation by constitutively active Rac1. The attractive hypothesis remains that the altered pattern of polymerized actin imposed by Rac1 and Cdc42, at variance with that of RhoA, renders the myoblast insensitive to differentiation cues. The inhibition of QMb differentiation by Rac1, therefore, may be tentatively assigned to those Rac1 targets that are responsible for changes in cytoskeleton and cell transformation in fibroblasts (Van Aelst and D'Souza-Schorey, 1997; Westwick *et al.*, 1997). Alternatively, in avian myoblasts Rac1 may inhibit progression to muscle differentiation by means of novel and possibly tissue-specific effector pathways.

There is evidence that Rac is involved in muscle morphogenesis in *Drosophila*, although an analysis of its mode of action at the cellular and molecular level is lacking (Luo *et al.*, 1994). The role of Rac1 in vertebrate skeletal muscle development remains to be fully established. Our attempts to disrupt the function of endogenous Rac1 in QMb undergoing differentiation with Rac1N17 have resulted in the absence of a distinct phenotype. Thus, it could be concluded that endogenous Rac1 does not need to be activated during commitment; in fact, transient expression of Rac1V12 inhibits myogenesis. However, expression of Rac1V12 affects myogenic differentiation in a dose-dependent manner and at different levels, suggesting that constitutively active Rac1 protein may sequester downstream effectors that either are not normally used by endogenous Rac1 or are used, albeit to a lesser extent. The finding that the transient expression of Rac1N17 accelerates sarcomere assembly indicates that inactivation of Rac1 function may be required for this process in myotubes. This conclusion is reinforced by the observation that Rac1V12 alters sarcomere assembly (see below).

While the work described in this paper was in progress, two reports were published describing attempts to assess the role of Rho family members in myogenic cells (Ramocki *et al.*, 1997; Takano *et al.*, 1998). Although consistent with the conclusion that activated Rac1 weakly transforms established fibroblasts (Symons, 1995; Joneson *et al.*, 1996;

Westwick *et al.*, 1997), our results differ considerably from those of Ramocki *et al.*, (1997). In their study, transient transfection of murine C3H10T1/2 cells with activated members of the Rho family had no apparent effect on the ability of MyoD to convert them to myogenic cells. Two points relevant to this issue can be made. First, part of the apparent discrepancy may have arisen from our use of primary avian myoblasts rather than established cell lines, which may be endowed with constitutive signaling pathways sensitive to the action of Rac1 and Cdc42. A second, complementary argument stems from the fact that we introduced Rac1 in a variety of ways into committed myoblasts expressing physiological levels of muscle-regulatory endogenous factors, whereas the myogenic conversion experiments used by Ramocki and collaborators entailed the transient cotransfection of MyoD, resulting in high expression levels, which may overcome the effect of Rac1, as previously shown for Ras (Lassar *et al.*, 1989). The results described by Takano *et al.* (1998) that activated mutants of Rho family proteins strongly activate the transcription of muscle-specific genes in established murine C2C12 cells are in great disagreement with both our findings and those of Ramocki and collaborators. These contrasting results are hard to reconcile, given the differences in cell types and in experimental protocols used. Two sets of preliminary data, however, appear to basically confirm the data described here for QMb. First, RhoA and Rac1 transiently expressed in C2C12 cells, either in their active or their dominant-negative form, exhibit a similar phenotype to that of QMb when assayed for transcription from muscle-specific, promoter-driven reporter genes and for myogenin and myosin expression (our unpublished results). Second, treatment of QMb with CNF-1, a bacterial exotoxin that activates all endogenous Rho family GTPases (Lerm *et al.*, 1999), efficiently inhibits terminal differentiation in a reversible manner (Tatò, personal communication).

Rac1 Disrupts Myogenesis in a Dose-dependent Manner

The efficacy of Rac1 in affecting myogenic differentiation was further investigated in stable populations of myoblasts expressing the Rac1V12 protein obtained by either viral infection or selection after transfection. At variance with transiently transfected cells, these cultures express levels of exogenous Rac1 protein that are equal to or twofold higher than that of the endogenous protein (our unpublished results). Here, Rac1V12 expression yields an incomplete differentiated phenotype, characterized by an atypical morphology of the myotubes and a highly reduced accumulation of myofibrillar proteins. Intriguingly, both accumulation of myogenin and transcription of muscle-specific genes were unaffected. Such a level of posttranscriptional regulation of genes involved in myogenic differentiation is not without precedent, both during embryo development and in myogenic cells in culture. For example, myogenin transcripts are detectable in mouse somites 1 d earlier than the corresponding protein, suggesting a posttranscriptional regulation of myogenin expression during early myogenesis (Cusella-De Angelis *et al.*, 1992). Similarly, the transcripts of two muscle-specific genes, neonatal myosin heavy chain and cardiac troponin I, are present in the embryo several days earlier than the corresponding proteins (Lyons *et al.*, 1990; Ausoni *et al.*, 1991). Another example of reversible posttranscriptional control has been reported in fusion-blocked rat myogenic cells, in which a battery of muscle-specific transcripts is detected in the absence of the corresponding proteins (Endo and Nadal-Ginard, 1987).

Rac1 Regulates the Organization of Sarcomeric Structures

In QMb, besides a reduced accumulation of muscle-specific proteins, the constitutive expression of Rac1V12 also results in an altered organization of sarcomeric structures. In Swiss 3T3 fibroblasts, activation of Rac1 promotes the formation of a cortical meshwork of polymerized actin yielding lamellipodia and ruffles (Nobes and Hall, 1995; Hall, 1998). Constitutive expression of activated Rac1 in myotubes may force the organization of the cortical actin cytoskeleton into structures that are not compatible with the proper tethering of sarcomeres to the sarcolemma. This would result in negative feedback on the ability to stabilize the lateral alignment of nascent myofibrils between each other and to the submembranous cytoskeleton and on the ensuing stability of the contractile proteins forming the individual filaments. Indeed, myotubes expressing lower levels of activated Rac1 appear flat, with a poor complement of myofibrils, which only occasionally show limited areas of cross-striations. This interpretation of the Rac1 phenotype in differentiated myotubes receives support from the finding that transient expression of Rac1N17 in differentiating myotubes accelerates the appearance of cross-striations. Furthermore, the robust activation of JNK in QMb-LA29 myotubes by UV treatment does not alter sarcomeric structure (our unpublished observations), suggesting that Rac1 does not operate through the JNK signaling pathway in the remodeling of sarcomeres. Therefore, it is tempting to speculate that the reduced accumulation of sarcomeric proteins and their altered organization may be related; for instance, the inability of contractile proteins to be properly assembled could affect their stability and render them more susceptible to degradation. On more general terms, the posttranscriptional phenotype imposed by Rac1 suggests the existence of a signaling pathway generated upon cytoskeleton remodeling that controls gene expression, at least at the level of translation or protein stability. Clearly, this is an issue for future experimentation.

The striking changes induced by the deliberate activation of v-Src tyrosine kinase in myotubes (Castellani *et al.*, 1995, 1996) are fully inhibited by Rac1N17, indicating that Rac1 is required by v-Src to bring about tissue-specific cytoskeletal rearrangements, such as disassembly of the I-Z-I segments, and may be relevant to the mechanisms normally used for the turnover of sarcomeres. How can we reconcile the observation that Rac1N17 is incapable of reverting morphological transformation and block of differentiation induced by v-Src in myoblasts yet it abolishes v-Src–induced actin body formation in differentiated myotubes? One simple explanation holds that v-Src affects the onset of differentiation and the maintenance of the differentiated state by activating multiple and distinct pathways. Block of differentiation at the myoblast stage may require only activation of the Ras pathway and not of the Rac1 pathway, as shown by the ability of a dominant-negative Ras mutant to recover the differentiated phenotype of v-Src–transformed myoblasts (our unpublished observation). On the contrary, disman-

tling of tissue-specific organelles and remodeling of postmitotic myotubes is achieved through Rac1 function and does not appear to involve Ras activation, because the forced expression of RasN17 does not affect the genesis of actin bodies (our unpublished observations).

In conclusion, it is becoming increasingly clear that Rho family GTPases play critical roles during development and cell differentiation. The present study underscores their role as regulators of myogenesis and support the contention that Rac1 participates in the complex regulatory processes underlying dynamic remodeling and maintenance of the differentiated state of skeletal muscle cells.

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REFERENCES

Adamo, S., Zani, B., Siracusa, G., and Molinaro, M. (1976). Expression of differentiative traits in the absence of cell fusion during myogenesis in culture. Cell Differ. *5*, 53–67.

Alberts, A.S., Geneste, O., and Treisman, R. (1998). Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation. Cell *92*, 475–487.

Alemà, S., and Tatò, F. (1994). Oncogenes interfere with myogenesis at multiple levels. Semin. Cancer Biol. *5*, 147–156.

Ausoni, S., De Nardi, C., Moretti, P., Gorza, L., and Schiaffino, S. (1991). Developmental expression of rat cardiac troponin I mRNA. Development *112*, 1041–1051.

Barrett, K., Leptin, M., and Settleman, J. (1997). The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in *Drosophila* gastrulation. Cell *91*, 905–915.

Bojovic, B., Rodrigues, N., Dehbi, M., and Bedard, P.-A. (1996). Multiple signaling pathways control the activation of the CEF-4/9E3 cytokine gene by $pp60^{v\text{-}src}$. J. Biol. Chem. *271*, 22528–22537.

Bragodia, S., Derijard, B., Davis, R.J., and Cerione, R.A. (1995). Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem. *270*, 27995–27998.

Castellani, L., Reedy, M., Airey, J.A., Gallo, R., Ciotti, M.T., Falcone, G., and Alemà, S. (1996). Remodeling of cytoskeleton and triads following activation of v-Src tyrosine kinase in quail myotubes. J. Cell Sci. *109*, 1335–1346.

Castellani, L., Reedy, M.C., Gauzzi, M.C., Provenzano, C., Alemà, S., and Falcone, G. (1995). Maintenance of the differentiated state in skeletal muscle: activation of v-Src disrupts sarcomeres in quail myotubes. J. Cell Biol. *130*, 871–885.

Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. *7*, 2745–2752.

Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell *81*, 1137–1146.

Cusella-De Angelis, M.G., et al. (1992). MyoD, myogenin independent differentiation of primordial myoblasts in mouse somites. J. Cell Biol. *116*, 1243–1255.

Eaton, S., Auvinen, P., Luo, L., Jan, Y.N., and Simons, K. (1995). Cdc42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disk epithelium. J. Cell Biol. *131*, 151–164.

Emerson, C.P. (1993). Skeletal myogenesis: genetics and embryology to the fore. Curr. Opin. Genet. Dev. *3*, 265–274.

Endo, T., and Nadal-Ginard, B. (1987). Three types of muscle-specific gene expression in fusion-blocked rat skeletal muscle cells: translational control in EGTA-treated cells. Cell *49*, 515–526.

Falcone, G., Alemà, S., and Tatò, F. (1991). Transcription of muscle-specific genes is repressed by reactivation of $pp60^{v\text{-}src}$ in postmitotic quail myotubes. Mol. Cell. Biol. *11*, 3331–3338.

Falcone, G., Gauzzi, M.C., Tatò, F., and Alemà, S. (1990). Differential control of muscle-specific gene expression specified by *src* and *myc* oncogenes in myogenic cells. Ciba Found. Symp. *150*, 250–261.

Falcone, G., Tatò, F., and Alemà, S. (1985). Distinctive effects of the viral oncogenes *myc*, *erb*, *fps* and *src* on the differentiation of quail myogenic cells. Proc. Natl. Acad. Sci. USA *82*, 426–430.

Gallo, R., Provenzano, C., Carbone, R., Di Fiore, P.P., Castellani, L., Falcone, G., and Alemà, S. (1997). Regulation of the tyrosine kinase substrate Eps8 expression by growth factors, v-Src and terminal differentiation. Oncogene *15*, 1929–1936.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509–514.

Henning, S.W., Galandrini, R., Hall, A., and Cantrell, D.A. (1997). The GTPase Rho has a critical regulatory role in thymus development. EMBO J. *16*, 2397–2407.

Hill, C.S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activation by SRF. Cell *81*, 1159–1170.

Hirayama, E., Isobe, A., Okamoto, H., Nishioka, M., and Kim, J. (1997). Myogenin expression is necessary for commitment to differentiation and is closely related to src tyrosine kinase activity in quail myoblasts transformed with Rous sarcoma virus. Eur. J. Cell Biol. *72*, 133–141.

Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. *61*, 3004–3012.

Joneson, T., McDonough, D., Bar-Sagi, D., and Van Aelst, L. (1996). Rac regulation of actin polymerization and proliferation by a distinct pathway from JNK kinase. Science *274*, 1374–1376.

Khosravi-Far, R., Solski, P.A., Clark, G.J., Kinch, M.S., and Der, C.J. (1995). Activation of Rac1, RhoA and mitogen-activated protein kinases is required for Ras transformation. Mol. Cell. Biol. *15*, 6443–6453.

Kong, Y., Johnson, S.E., Taparowsky, E.J., and Konieczny, S.F. (1995). Ras p21Val inhibits myogenesis without altering the DNA binding or transcriptional activities of the myogenic basic helix-loop-helix factors. Mol. Cell. Biol. *15*, 5205–5213.

Konieczny, S.F., Drobes, B.L., Menke, S.L., and Taparowsky, E.J. (1989). Inhibition of myogenic differentiation by the H-Ras oncogene is associated with the down-regulation of the MyoD gene. Oncogene *4*, 473–481.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell *87*, 519–529.

La Rocca, S.A., Grossi, M., Falcone, G., Alemà, S., and Tatò, F. (1989). Interaction with normal cells suppresses the transformed phenotype of v-*myc*-transformed quail muscle cells. Cell *58*, 123–131.

Lassar, A.B., Skapek, S.X., and Novitch, B. (1994). Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. Curr. Opin. Cell Biol. *6*, 788–794.

Lassar, A.B., Thayer, M.J., Overell, R.W., and Weintraub, H. (1989). Transformation by activated *ras* or *fos* prevents myogenesis by inhibiting expression of MyoD1. Cell *58*, 659–667.

Lerm, M., Selzer, L.M., Hoffmeyer, A., Rapp, U.R., Aktories, K., and Schmidt, G. (1999). Deamidation of Cdc42 and Rac by *Escherichia coli* cytotoxic necrotizing factor 1: activation of c-Jun-terminal kinase in HeLa cells. Infect. Immun. *67*, 496–503.

Ludolph, D.C., and Konieczny, S.F. (1995). Transcription factor families: muscling in on the myogenic program. FASEB J. *9*, 1595–1604.

Luo, L., Jan, L.Y., and Jan, Y.N. (1996). Small GTPases in axon outgrowth. Perspect. Dev. Neurobiol. *4*, 199–204.

Luo, L., Liao, Y.J., Jan, L.Y., and Jan, Y.N. (1994). Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblasts fusion. Genes Dev. *8*, 1787–1802.

Lyons, G.E., Ontell, M., Cox, R., Sassoon, D., and Buckingam, M. (1990). The expression of myosin genes in developing skeletal muscle in the mouse embryo. J. Cell Biol. *111*, 1465–1476.

Mainiero, F., Gismondi, A., Soriani, A., Cippitelli, M., Palmieri, G., Jacobelli, J., Piccoli, M., Frati, L., and Santoni, A. (1998). Integrin-mediated Ras-extracellular regulated kinase (ERK) signaling regulates interferon production in human natural killer cells. J. Exp. Med. *188*, 1267–1275.

Maione, R., and Amati, P. (1997). Interdependence between muscle differentiation and cell-cycle control. Biochim. Biophys. Acta *1332*, M19–M30.

Minden, A., Lin, A., Claret, F.X., Abo, A., and Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell *81*, 1147–1157.

Nobes, C.D., and Hall, A. (1995). Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. Cell *81*, 53–62.

Nolan, K.M., Barrett, K., Lu, Y., Hu, K.-Q., Vincent, S., and Settleman, J. (1998). Myoblast city, the *Drosophila* homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes. Genes Dev. *12*, 3337–3342.

Olson, E.N. (1992). Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. *154*, 261–272.

Olson, E.N., and Klein, W.H. (1994). bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev. *8*, 1–8.

Olson, F.M., Ashworth, A., and Hall, A. (1995). An essential role for Rho, Rac and Cdc42 GTPases in cell cycle progression through G1. Science *269*, 1270–1272.

Petropoulos, C.J., and Hughes, S.H. (1991). Replication-competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. J. Virol. *65*, 3728–3737.

Provenzano, C., Gallo, R., Carbone, R., Di Fiore, P.P., Falcone, G., Castellani, L., and Alemà, S. (1998). Eps8, a tyrosine kinase substrate, is recruited to the cell cortex and dynamic F-actin upon cytoskeleton remodelling. Exp. Cell Res. *242*, 186–200.

Qiu, R.-G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995). An essential role for Rac in Ras transformation. Nature *374*, 457–459.

Ramocki, M.B., Johnson, S.E., White, M.A., Ashendel, C.L., Konieczny, S.F., and Taparowski, E.J. (1997). Signaling through mitogen-activated-protein kinase and Rac/Rho does not duplicate the effects of activated Ras on skeletal myogenesis. Mol. Cell. Biol. *17*, 3547–3555.

Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factor. Cell *70*, 389–399.

Russo, S., Tatò, F., and Grossi, M. (1997). Transcriptional down-regulation of myogenin expression is associated with v-ras-induced block of differentiation in unestablished quail muscle cells. Oncogene *9*, 63–73.

Saga, S., Hamaguchi, M., Hoshino, M., and Kojima, K. (1985). Expression of meta-vinculin associated with differentiation of chicken embryonal muscle cells. Exp. Cell Res. *156*, 45–56.

Sassoon, D.A. (1993) Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. Dev. Biol. *156*, 11–23.

Sternberg, E.A., Spizz, G., Perry, W.M., Vizard, D., Weil, T., and Olson, E.N. (1988). A ras-dependent pathway abolishes activity of a muscle-specific enhancer upstream from the muscle creatine kinase gene. Mol. Cell. Biol. *8*, 2896–2909.

Strutt, D.I., Weber, U., and Mlodzik, M. (1997). The role of RhoA in tissue polarity and Frizzled signaling. Nature *387*, 292–295.

Symons, M. (1995). The Rac and Rho pathways as a source of drug targets for ras-mediated malignancies. Curr. Opin. Biotechnol. *6*, 668–674.

Takano, H., Komuro, I, Oka, T., Shiojima, I., Hiroi, Y., Mizuno, T., and Yazaki, Y. (1998). The Rho family G proteins play a critical role in muscle differentiation. Mol. Cell. Biol. *18*, 1580–1589.

Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. Genes Dev. *11*, 2295–2322.

Walsh, K., and Perlman, H. (1997). Cell cycle exit upon myogenic differentiation. Curr. Opin. Genet. Dev. *7*, 597–602.

Weintraub, H., Davis, R., Lockshon, D., and Lassar, A. (1990). MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proc. Natl. Acad. Sci. USA *87*, 5623–5627.

Westwick, J., Lambert, Q., Clark, G., Symons, M., Van Aelst, L., Pestell, R., and Der, C. (1997). Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol. Cell. Biol. *17*, 1324–1335.

Weyman, C.M., Ramocki, M.B., Taparowsky, E.J., and Wolfman, A. (1997). Distinct signaling pathways regulate transformation and inhibition of skeletal muscle differentiation by oncogenic Ras. Oncogene *14*, 697–704.

Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. (1998). JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. Genes Dev. *12*, 3369–3381.

Yoon, H., and Boettiger, D. (1994). Expression of v-*src* alters the expression of myogenic regulatory factor genes. Oncogene *9*, 801–807.

Zhang, S., Han, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J., and Bokoch, G.M. (1995). Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J. Biol. Chem. *270*, 23934–23936.