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
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 oncogenes, 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., 1996). and the function (Kong et al., 1995; Hirayama et al., 1997; Gauzzi, Ciuflitti, 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 (V13N-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-actin (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-day-old SPF AFS 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 10^6 cells on 35-mm collagen-coated dishes in GM and, the next day, by substituting GM with FI4 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-dependent sarcomeres induced by the deliberate activation of the v-Src tyrosine kinase in terminally differentiated myotubes.
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 Ccl 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 cotransfected with 2.5 μg of pdDNA3-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 pdDNA3-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, 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 nontransformed 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 MgCl2, 2 mM DTT, 0.1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 μM ATP, 4 μCi of [γ-32P]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 with 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 (20–20 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).
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 MgCl₂, 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-κ-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-actin, 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 100× water-immersion objectives. Confocal analysis was carried out with a Leica (Heidelberg, Germany) TCS 4D system equipped with 40×1.00–0.5 and 100×1.3–0.6 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 GM 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 oncprotein 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).
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 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 Rac1V12 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...
myogenin and myosin (data not shown), suggesting that the block of myogenesis exerted by v-Src may not be mediated by endogenous Rac1.

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 HA-tagged mammalian JNK, and after 2 d of serum

Figure 2. v-Src-transformed myoblasts expressing Rho family GT-Pases 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.

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.
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-
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.

To specifically define the role of endogenous Rac1 in the maintenance 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, 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 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) (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 alters 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 myotubes (Figure 4C) (Bojovic et al., 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 suggest that Rac1 is likely involved in sarcomere turnover and...
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 multi-step 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...
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

![Image](https://example.com/image1)

**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.

![Image](https://example.com/image2)

**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.
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 used by 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 (Tato, 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-
ting 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 underlyng dynamic remodeling and maintenance of the differentiated state of skeletal muscle cells.

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REFERENCES


