Rac1 Inhibits Myogenic Differentiation by Preventing the Complete Withdrawal of Myoblasts from the Cell Cycle*

Received for publication, April 10, 2001, and in revised form, July 19, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M103195200

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The small GTPase protein Rac1 is involved in a wide range of biological processes, yet its role in cell differentiation is mostly unknown. Here we show that Rac1 activity is high in proliferating myoblasts and decreases during the differentiation process. To analyze the involvement of Rac1 in muscle differentiation, different forms of the protein were expressed in muscle cells. A constitutively activated form of Rac1 (Rac1Q61L) inhibited the activity of MyoD in promoting muscle differentiation, whereas a dominant negative form of Rac1 (Rac1T17N) induced the activity of MyoD in promoting muscle differentiation. Expression of Rac1T17N imposed myogenic differentiation on myoblasts growing under mitogenic conditions. In inquiring whether Rac1 affected the withdrawal of myoblasts from the cell cycle, we analyzed the expression of cyclin D1 and p21WAF1 and the phosphorylation state of the retinoblastoma protein. According to these markers and bromodeoxyuridine incorporation, C2 myoblasts expressing Rac1T17N exited the cell cycle earlier than control C2 cells. Myoblasts expressing Rac1Q61L did not permanently withdraw from the cell cycle. An indication of the possible involvement of the mitogen-activated protein kinase (MAPK) pathway in Rac1-mediated myoblast proliferation was obtained by the use of MAPK kinase inhibitors U0126 and PD098059. These inhibitors arrested C2-Rac1Q61L cell cycling. Taken together, our results show that Rac1 activation interferes with myoblast exit from the cell cycle via or in concert with the MAPK pathway.

The Rho family of small GTP-binding proteins, which includes Rho, Rac, and CDC42, is involved in a wide range of biological processes, including cell motility, cell adhesion, cell morphology, cytokinesis, and cell proliferation (for reviews, see Refs. 1 and 2).

In mammalian cells, Rac functions by generating the actin-rich lamellipodial protrusions and membrane ruffling that are thought to be a major part of the driving force for cell movement (3). Independent of this effect of cytoskeletal rearrangements, Rac1 and CDC42 induce the activation of mitogen-activated protein kinase cascades. Some reports suggest that Rac1 and CDC42 activate the c-Jun N-terminal kinase (JNK) and the p38 MAPK (4–7), whereas other studies indicate that Rac1 cross-talks with the ERK MAPK pathway. Rac and CDC42 can synergize with Raf1 or MEK1 to promote ERK activation (8). It turns out that p21-activated kinase (PAK), which is a CDC42 and Rac target, positively regulates Raf1 and MEK1 activities through phosphorylation of specific serine residues (9, 10). These functions of Rac and CDC42 in cytoskeletal reorganization and the induction of MAPK signaling pathways may explain their role in the regulation of a variety of cellular processes in development and morphogenesis (11).

The differentiation of skeletal muscle cells involves two major stages: (a) withdrawal of myoblasts from the cell cycle, and (b) subsequent expression of myotube-specific genes. Proliferating myoblasts express two myogenic transcription factors from the basic helix-loop-helix family, MyoD and Myf5, before the onset of muscle differentiation (12–14). Once activated, MyoD and Myf5 induce the withdrawal of myoblasts from the cell cycle and the expression of another myogenic basic helix-loop-helix factor, myogenin, as well as transcription factors from the MEF2 family. Together, myogenin and MEF2 family members cooperate in the activation of many muscle structural genes (15, 16).

Although the molecular mechanisms controlling myogenesis at the transcriptional level are well characterized, the signaling molecules that mediate the transduction of extracellular cues and affect the muscle regulatory factors are only partially uncovered. The role of Rho GTPases, particularly that of Rac proteins in skeletal muscle differentiation, is controversial. Ramocki et al. (17) suggested that the Rho family did not affect the ability of MyoD to convert 10T1/2 fibroblasts to myoblasts. A study by Takano et al. (18) indicated that the different members of the Rho family played a positive role in the differentiation of C2 myoblasts. In their study, the expression of dominant negative forms of Rho family members suppressed transcription of muscle-specific genes, whereas constitutively activated forms induced their transcription (18). A more recent work by Gallo et al. (19) that studied avian myoblasts suggested that constitutively active Rac1 and Cdc42 caused inhibition of myogenin expression and blocked myogenic differentiation. This inhibition was mainly a consequence of posttranscriptional block to the expression of muscle-specific proteins. The study also indicated that cells expressing constitutively active Rac1 failed to assemble ordered sarcomeres,

* This work was supported by a grant from the Israel Science Foundation (to E. B.) and by funds from the Rappaport Foundation for Medical Research and the Foundation for the Promotion of Research in the Technion, Israel Institute of Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PAK, p21 activated kinase; pRb, retinoblastoma protein; MHC, myosin heavy chain; BrdUrd, bromodeoxyuridine; ER, estrogen receptor; GM, growth medium; DM, differentiation medium; CAT, chloramphenicol acetyltransferase; MLC2, myosin light chain 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4',6-diamino-2-phenylindole.
whereas the expression of dominant negative Rac1 accelerated sarcomere maturation.

In view of the disputed and diverse roles of Rho members in myogenesis, we investigated the involvement of Rac1 in muscle cell differentiation. The activity of Rac1 was high in proliferating myoblasts and decreased during their differentiation to myotubes. We generated stable myoblast cell lines expressing the different forms of Rac1 and found that constitutively activated Rac1 prevented withdrawal from the cell cycle and muscle differentiation, whereas dominant negative Rac1 induced growth arrest and differentiation. The activity of Rac1 induced the expression of cyclin D1 and repressed the induction of the cyclin-dependent kinase inhibitor p21. Consequently, pRb remained hyperphosphorylated, and myoblasts did not withdraw properly from the cell cycle. Inhibition of the MAPK pathway rescued the withdrawal of activated Rac1-expressing myoblasts from the cell cycle. This study therefore indicates that the activity of Rac1 has to be blocked in order for myoblasts to exit the cell cycle and differentiate into mature myotubes.

**EXPERIMENTAL PROCEDURES**

**Materials**—U0126 and SB203580 were supplied by Calbiochem. PD098059 was from Sigma. ERK1/2, phospho-specific ERK1/2, p38 MAPK, and phospho-specific p38 MAPK antibodies were supplied by New England Biolabs. An antibody to cyclin D1 protein (SC-717) was purchased from Santa Cruz Biotechnology. A monoclonal antibody to CDK2 (143D10) was from Cell Signaling Technology. A monoclonal antibody to cyclin-dependent kinase inhibitor p21. Consequently, pRb remained hyperphosphorylated, and myoblasts did not withdraw properly from the cell cycle. Inhibition of the MAPK pathway rescued the withdrawal of activated Rac1-expressing myoblasts from the cell cycle. This study therefore indicates that the activity of Rac1 has to be blocked in order for myoblasts to exit the cell cycle and differentiate into mature myotubes.

**RESULTS**

**Rac1 Activity Is Reduced during Muscle Differentiation**—To study the activity of endogenous Rac1 during differentiation of C2 myoblasts, GTP-bound Rac1 was specifically precipitated using glutathione S-transferase fused to the G-protein-binding domain of PAK1 (30) and then detected with a Rac1-specific antibody (Fig. 1A). The amount of GTP-bound Rac1 was high in proliferating myoblasts and was significantly reduced after myoblasts were grown in DM (Fig. 1A). This pattern of activity suggested that Rac1 was necessary for myoblast proliferation, but not for the differentiation to myotubes.

**Generation of C2 Myoblast Cell Lines Expressing Different Forms of Rac1**—To study how the activated GTPase Rac1 affects the differentiation of myoblasts, we generated several C2 cell lines expressing wild type (WT-Rac1), constitutively active (RacQ61L and Act-Rac1), and dominant negative (Rac1T17N and DN-Rac1) forms of Rac1 and a control C2 cell line containing integrated copies of the empty expression vector (Neo). Cells were drug-selected, and surviving colonies were pooled.
together. Myoblasts transfected with Rac1Q61L appeared flat and enlarged, whereas those transfected with Rac1T17N showed reduced cellular dimensions (data not shown). The amount of GTP-bound Rac1 in C2-Rac1Q61L cells (Act-Rac1 cells) was high and remained high after 24 h in DM, whereas the amount of GTP-bound Rac1 in C2-Rac1T17N cells (DN-Rac1 cells) was almost undetectable (Fig. 1B).

Constitutively Active Rac1 Inhibits Myogenic Differentiation—Next we analyzed how the expression of different forms of Rac1 affected the differentiation process. Myoblasts were induced to differentiate in DM, and the expression of several genes was analyzed by Northern blotting (Fig. 1D). The differentiation state of cells can be represented by the expression levels of MLC2. The expression of MLC2 was induced in all cell lines as a result of their growth in DM; however, we observed differences in its levels of expression. MLC2 expression was induced to similar levels in the C2 control cell line (Neo) and in myoblasts expressing the wild type form of Rac1 (WT-Rac1) (Fig. 1D, compare lanes 1–4 and 13–16). The expression of MLC2 in cells expressing Rac1Q61L (Act-Rac1) was initially induced, but it was reduced to very low levels after 72 h in DM (Fig. 1D, lanes 9–12). Expression of MLC2 in cells expressing Rac1T17N (DN-Rac1) was induced to levels higher than those seen in all other cell lines (Fig. 1D, lanes 5–8). Transcripts of the transfected Rac1 were of a different size than the endogenous transcript and were identified in cell lines transfected with plasmids encoding for the different forms of Rac1, but not in the control cell line (Fig. 1D, bottom panel).

We conclude that expression of dominant negative Rac1 stimulates the expression of MLC2, whereas constitutively active Rac1 inhibits the expression of the myogenic marker MLC2.

To further substantiate these results and find out whether the above-mentioned effect of Rac1 proteins is quantitative, single cell colonies were isolated from each cell line. These colonies were expanded, and levels of transfected Rac1 were analyzed. We chose to analyze two colonies expressing different levels of either dominant negative or activated Rac1. The expression of MLC2 was compared with that in a clone of myo-
blasts that did not express significant levels of the exogenous gene. Induction of MLC2 was proportional to the transcript levels of dominant negative Rac1 (Fig. 2, DN-Rac#1 and DN-Rac#2). In cells expressing the dominant negative form of Rac1, transcripts of MLC2 were accumulated earlier and to higher levels than those in control cells. In contrast, induction of MLC2 decreased proportionally to the increasing levels of constitutively activated Rac1 (Fig. 2, Act-Rac#1 and Act-Rac#2). High expression levels of activated Rac1 completely abolished any accumulation of MLC2 (Fig. 2, bottom panel). Therefore, these results suggest a quantitative effect of Rac1 mutants on the expression of MLC2.

Rac1 Affects the Morphology and Size of Myotubes—Next we analyzed whether the expression of Rac1 mutants affected the morphology of multinucleated myotubes. We hypothesized that Rac1 could affect the morphology of myotubes via its function on the organization of actin filaments and stress fibers that control cell motility and cell-cell interactions (2). Under a light microscope, we could see that the cell line expressing Rac1Q61L did not develop typical myotubes (data not shown). Immunostaining of the different Rac1-expressing cell lines at 48 h in DM allowed a closer inspection of myotube morphology. We found that cells expressing activated Rac1 formed very few myotubes, which were, on average, much larger in size and contained significantly more nuclei/cell than did the control C2 cells (Neo) (Fig. 3). These myotubes also differed in their shape, which was wide rather than elongated. In contrast, the cells expressing dominant negative Rac1 developed multiple elongated myotubes that were significantly smaller in size and contained fewer nuclei/cell compared with the control C2 cells (Fig. 3). The differences in cell size and fusion rates between cell lines do not appear to be due to differences in the density of cells, as can be seen by the nuclear staining of the same microscopic field (Fig. 3, right panels). In addition, we were able to observe differences in the intensity of myosin staining between the different cell lines. Intensive myosin staining was observed in dominant negative Rac1-expressing cells (DN-Rac1), whereas the staining of myotubes expressing activated Rac1 (Act-Rac1) was the weakest (Fig. 3).

The Transcriptional Activity of MyoD Is Modulated by Rac1—The effects of Rac1 on muscle-specific transcription observed in Fig. 1D prompted us to analyze whether the activity of the myogenic transcription factor MyoD was also affected. To that end, 10T1/2 fibroblasts were transiently transfected with expression vectors of MyoD and the different forms of Rac1. By immunostaining of these cells, we found that transfected MyoD induced the expression of endogenous MHC in about 50% of the transfected cells (Fig. 4A, lane 1). Under the same conditions, cells that were cotransfected with the activated allele of Rac1 exhibited a significantly lower proportion of MHC staining in the cytoplasm of MyoD-transfected cells (Fig. 4A, lane 2), whereas cells cotransfected with the dominant negative Rac1 showed higher MHC staining (Fig. 4A, lane 3). These results suggest that Rac1 affected MyoD activity in inducing muscle differentiation. The results also indicate that the different forms of Rac1 affected muscle differentiation in a similar way, regardless of whether they were transiently or stably expressed following a selective pressure. To further analyze the transcriptional activity of MyoD, the same expression vectors were cotransfected with two MyoD-responsive reporter genes. One reporter contained the enhancer element of the muscle creatine kinase gene (peAT-CAT), and the other was a minimal promoter that contained several MyoD binding sites (4R-tk-CAT). Transfection of the activated form of Rac1 inhibited MyoD-mediated transcription of both reporter genes (Fig. 4B, lanes 2 and 6). Wild-type Rac1 inhibited the transcriptional activity of MyoD to a lesser extent (Fig. 4B, lanes 4 and 8). Transfection of the dominant negative form of Rac1 did not significantly affect MyoD-mediated transcription (Fig. 4B, lanes 3 and 7). A different approach for analyzing the regulation of MyoD by Rac1
used a fibroblast cell line that stably expressed a conditional MyoD protein (25). The protein is a MyoD-estrogen receptor chimera whose activity is induced by the addition of (E)-estradiol to the medium. Induction of myogenesis in this cell line follows a very precise timetable because it depends on the activation of the MyoD-ER protein. We infected dominant negative Rac1 to these cells and selected for cells that stably expressed the transcript of exogenous Rac1 (Fig. 5A, lanes 3 and 4). When the MyoD protein was activated under conditions that promoted differentiation (DM), the expression of MLC2 was higher in the cell line expressing dominant negative Rac1 (pBABE-D.N.-Rac) as compared with the control cell line (pBABE-puro). The differences between these cell lines were much more significant when the MyoD-ER protein was activated during the growth of cells in serum-rich medium (GM) that usually prevented the activity of the MyoD protein. The activation of MyoD-ER protein induced the transcription of p21, myogenin, and MLC2 in the cell line expressing dominant negative Rac1 (Fig. 5B, lanes 4–6), but it did not affect the transcription of these genes in the control cell line (Fig. 5B, lanes 1–3). The activation of p21 and myogenin, two direct target genes of MyoD, suggests that inactivation of Rac1 in growth-promoting conditions (GM) allows transcriptional activity of the MyoD protein that is otherwise repressed.

The differentiation of myoblasts is always preceded by their withdrawal from the cell cycle. The cooperation of dominant negative Rac1 with MyoD in the induction of differentiation under medium conditions that promote cell proliferation suggested that together they induced the withdrawal from the cell cycle that allowed the subsequent activation of muscle-specific genes.

Activation State of Rac1 Affects the Expression and Activity of Regulators of the Cell Cycle—Our subsequent experiments were aimed at studying whether the effect of Rac1 on myoblast differentiation was a consequence of its possible involvement in
myoblast proliferation. Previous studies have demonstrated that early events obligatory for the differentiation process are the induction of the cyclin-dependent kinase inhibitor p21WAF1 and the subsequent dephosphorylation and activation of the pRb (31). First, we analyzed the expression of p21WAF1 in myoblasts expressing Rac1 mutants that were induced to differentiate in DM (Fig. 6A). Transcripts of p21WAF1 were accumulated during the differentiation of control cells (Fig. 6A, lanes 4–6). They accumulated more significantly in the cells expressing dominant negative Rac1 and grown under the same conditions (Fig. 6B, lanes 7–9). Unlike the previous experiment, this experiment was done under conditions that prevented cell-cell interactions and contact inhibition. The total level and the ratio of the hyperphosphorylated:underphosphorylated forms of pRb remained unchanged in myoblasts expressing the activated form of Rac1 during their growth in DM (Fig. 6B, bottom panel, lanes 1–3). In the control cell line (Neo), both hyperphosphorylated and underphosphorylated forms of pRb accumulated during growth in DM (Fig. 6B, bottom panel, lanes 4–6). Unlike the two other cell lines, cells expressing dominant negative Rac1 exhibited a significant induction of underphosphorylated pRb and almost undetectable amounts of the hyperphosphorylated form, indicating that pRb was fully activated in this cell line (Fig. 6B, bottom panel, lanes 7–9).

Previous studies indicated that the levels of cyclin D1 protein declined during the differentiation of myoblasts, and its forced constitutive expression induced myoblast proliferation and inhibited differentiation (33, 34). Our analysis of cyclin D1 confirmed that the protein level declined during differentiation of C2 cells (Fig. 6C, lanes 4–6). Similarly, cyclin D1 protein declined to almost undetectable levels in myoblasts expressing dominant negative Rac1 after 24 h of growth in DM (lanes 7–9). Interestingly, the level of cyclin D1 protein was substantially higher in myoblasts expressing activated Rac1, and although it declined during myoblast growth in differentiation medium, it was still significantly higher than that in C2 cells growing under the same conditions (Fig. 6C, lanes 1–3).

These changes in p21WAF1, cyclin D1, and pRb indicate that Rac1 may affect the exit of myoblasts from the cell cycle.

**Rac1 Activity Affects Proliferation of Myoblasts**—The distinct differences in the levels of p21WAF1 and cyclin D1 and in the phosphorylation states of pRb between the Rac1-expressing cell lines suggested that when triggered to differentiate, these cells may differ in their exit from the cell cycle. To analyze the withdrawal from the cell cycle independently of cell-cell contact inhibition, cells were plated at 20–30% confluence in DM. Cells in the S phase were identified by BrdUrd incorporation after their growth in DM for different periods of time (Fig. 7). A total of 40–60% of the cells were in S phase after 24 h of growth in DM in all three cell lines (Fig. 7). However, an obvious difference was found between cells that were grown in DM for 48 h: whereas cells expressing the dominant negative Rac1 were resting, those expressing the activated allele and the control cells continued to proliferate (Fig. 7). Nevertheless, the number of control cells and cells expressing activated Rac1 in the S phase was reduced between 24 and 48 h in DM (see the histogram in Fig. 7). This experiment suggests that inactivation of Rac1 facilitates the withdrawal of myoblasts from the cell cycle.

**MAPK Is Involved in the Mitogenic Effect of Rac1 on Muscle Cells**—Previous studies have linked the ERK MAPK pathway to the proliferation of myoblasts and suggested that activation of this pathway in myoblasts interfered with their withdrawal from the cell cycle (35–38). To test the possibility that MAPK is involved with Rac1 in the proliferation of myoblasts, we analyzed how the inhibition of MAPK affected the withdrawal of
myoblasts expressing constitutively active Rac1 (Rac1Q61L) from the cell cycle. Cells at 50% confluence were induced to differentiate in DM without or with the presence of the MAPK kinase-specific inhibitors U0126 and PD098059. Three parameters were analyzed: (a) the level and phosphorylation state of pRb, (b) the expression of cyclin D1, and (c) the percentage of cells found in the S phase. The hyperphosphorylated and underphosphorylated forms of pRb were observed in Rac1Q61L-expressing cells at the time of DM addition (Fig. 8A, lane 1). The underphosphorylated form was accumulated, whereas the hyperphosphorylated form remained unchanged after 10 and 24 h in DM (Fig. 8A, lanes 2 and 3). Yet the hyperphosphorylated form disappeared when cells were grown for 24 h in DM in the presence of U0126 or PD098059 (Fig. 8A, lanes 7 and 9). The protein level of cyclin D1 was high and mildly reduced after 10 and 24 h in DM (Fig. 8A, lanes 1–3). Treatment of cells with U0126 significantly reduced the levels of cyclin D1 protein, and treatment with PD098059 partially reduced the levels of cyclin D1 protein (lanes 6–9). The differences in the effects of these inhibitors on cyclin D1 may be attributed to their relative potencies. Whereas U0126 completely abolished phosphorylated ERKs, PD098059 only reduced the levels of phosphorylated ERKs (compare Fig. 8A, lanes 6 and 7 to lanes 8 and 9).

The two MEK inhibitors were specific to the ERK MAPK pathway because they did not affect the amounts of phosphorylated p38 MAPK, a closely related member of the MAPK family (Fig. 8A, lanes 4 and 5). Thus, the p38 pathway was not involved with Rac1 in myoblast proliferation.

The effect of MAPK kinase inhibitors U0126 and PD098059 on the phosphorylation of pRb and the expression of cyclin D1 in C2 cells expressing activated Rac1 suggested that inhibition of the MAPK pathway induced the exit of myoblasts from the cell cycle. To further test this possibility, the relative amount of cells in S phase was analyzed by BrdUrd staining of cells that were grown for 24 h in the absence or presence of U0126 or PD0980580 (Fig. 8B). Treatment with U0126 or PD980580 caused a dramatic decrease in the number of cells in the S phase relative to untreated cells or cells grown in the presence of SB203580 under the same confluence and medium conditions (Fig. 8B). Interestingly, treatment of myoblasts expressing the dominant negative form of Rac1 with U0126 did not affect the rate of their withdrawal from the cell cycle (data not shown). We therefore conclude that inhibition of the ERK MAPK pathway, but not of the p38 MAPK pathway, in cells expressing an active Rac1 protein induces growth arrest. Consequently, the ERK MAPK pathway may be downstream to Rac1 or may cooperate with it in driving the proliferation of C2 myoblasts.

**DISCUSSION**

Differentiation of skeletal muscle cells involves two major stages: (a) the withdrawal of myoblasts from the cell cycle, and (b) the subsequent expression of myotube-specific genes. Many extracellular growth factors and intracellular signaling pathways are involved in the growth control of muscle cells and...
their differentiation. In this report, we show that the small GTP-binding protein Rac1 plays a role in the proliferation of myoblasts and suggest that it has to be inactivated before myoblasts withdraw from the cell cycle.

Activation of Rac1 Inhibits Muscle Differentiation, Whereas Inactivation of Rac1 Promotes Muscle Differentiation—The study of C2 cell lines expressing either a constitutively active Rac1 (Rac1Q61L) or a dominant negative protein (Rac1T17N) proved that Rac1 protein was involved in muscle differentiation. Activation of Rac1 largely inhibited the expression of MHC and reduced the number of multinucleated myotubes. Inactivation of Rac1 increased the relative expression of MHC and increased the number of myotubes. Two recent reports support the hypothesis that activation of Rac1 in myoblasts prevents their subsequent differentiation after serum withdrawal (19, 39). Although similar, these two works emphasized different aspects of differentiation and in some respects differ from our results. Gallo et al. (19) transiently expressed a constitutively active and dominant negative Rac1 in primary avian myoblasts. In agreement with our study, they found that active Rac1 disrupted myogenic differentiation, whereas dominant negative Rac1 accelerated the process. On the basis of their results, they claim that activated Rac1 did not affect the levels of muscle-specific gene transcripts but inhibited the accumulation of muscle-specific proteins (19). Their conclusion was that active Rac1 affected a posttranscriptional event. Contrarily, expression of active Rac1 in our study reduced the steady-state levels of MHC transcripts in a dose-dependent manner (see Figs. 1 and 2). The difference may be due to the different cellular systems used in the two studies. Gallo et al. (19) also analyzed how Rac1 affected the structure and maturation of sarcomeres during the formation of myotubes. They found that the expression of active Rac1 inhibited the organization of skeletal proteins into ordered sarcomeres, whereas dominant negative Rac1 accelerated maturation of sarcomeres. In their study, myotubes expressing active Rac1 were also more flattened and larger in size than the control myotubes. Interestingly, in the present work, the few myotubes formed by the fusion of cells expressing active Rac1 were significantly larger than the myotubes of the control cells, and the structure of these myotubes was atypical (Fig. 3). Cells expressing dominant negative Rac1 formed smaller myotubes than did control cells, but these myotubes had an elongated structure (Fig. 3).

Therefore, in our study as well, Rac1 affected the formation and structure of myotubes, an effect that could be mediated by the assembly of sarcomeres. The third work, by Meriane et al. (39), did not agree with the findings of the other two studies in two major aspects: (a) in the work of Meriane et al. (39), a dominant negative form of Rac1 (Rac1T17N) inhibited myotube formation, and (b) Meriane et al. claim that inhibition of myogenesis by active Rac1 is mediated by the JNK pathway. The first point is difficult to reconcile, given the differences in cell types and experimental approaches used. The second point is based on studies with mutants of Rac1 that differentially activate downstream effectors (40). Meriane et al. (39) found that the Y40C mutant of active Rac1, which induces membrane ruffling but loses most of its potential to activate JNK, failed to inhibit myogenin expression. In contrast, Gallo et al. (19) and our observations suggest that the same Y40C mutant of active Rac1 inhibited myogenesis to the same extent as the control active protein (Rac1Q61L). Moreover, a second mutant of Rac1, F37A, which induces JNK but is less efficient in the induction of membrane ruffling, did not efficiently inhibit differentiation (19). Therefore, these studies suggested that Rac1 could inhibit myogenin independently of the activation of the JNK pathway.

Rac1 Affects the Transcriptional Activity of MyoD—In the present work, we show that mutants of Rac1 affect the expression of the early differentiation markers myogenin and p21WAF1 (Fig. 5). The transcription of these two genes is directly up-regulated by the MyoD protein (25, 41). The coexpression of MyoD with different mutants of Rac1 in 10T1/2 fibroblasts indicated that Rac1 affected the activity of MyoD (Fig. 4). The activated form of Rac1 inhibited MyoD-mediated differentiation, whereas the dominant negative protein induced this activity of MyoD relative to control cells (Fig. 4A). In addition, the activated form of Rac1 inhibited the transcriptional activity of MyoD in a reporter gene assay (Fig. 4B). Another experiment

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supporting the same conclusion used a fibroblast cell line expressing an inducible MyoD (Fig. 5). Activation of MyoD in this fibroblast cell line induces the expression of MyoD target genes such as myogenin and p21WAF1. The activity of MyoD was repressed when cells were grown in serum-rich medium; however, the expression of a dominant negative form of Rac1 in the same cells rescued the activity of MyoD, as reflected by the induction of myogenin and p21WAF1 expression. Therefore, abrogation of Rac1 activity releases MyoD from the inhibitory effect of serum.

Rac1 Controls Proliferation of Myoblasts—The activity of MyoD is regulated by the mitotic state of muscle cells. In dividing myoblasts grown in serum-rich medium, MyoD protein is transcriptionally inactive, and it is activated only after serum is removed and myoblasts withdraw from the cell cycle (reviewed in Ref. 13). The effects of Rac1 on the transcriptional activity of MyoD suggested to us that Rac1 could be involved in the regulation of myoblast proliferation. Indeed, recent studies have demonstrated the involvement of Rac1 in the cell cycle. The expression of constitutively activated forms of Rac and Raf was sufficient to stimulate G1-S transition, indicating that these signals act synergistically to regulate cell cycle progression (42). Activated Rac1 induces the expression of cyclin D1, and dominant negative Rac1 partially interferes with Ras-induced cyclin D1 expression (43, 44). Therefore, it appears that the collaborative action of the Rac1 protein and the ERK cascade causes sufficient levels of cyclin D1 to stimulate G1-S transition. Another function of the Rho family in stimulating cell proliferation involves the interference with the induction of the cyclin-dependent kinase inhibitor p21WAF1 by Ras (45). Under certain circumstances, overexpression of activated Ras or Raf can lead to cell cycle arrest due to the induction of p21WAF1 (46, 47). However, activated Ras did not induce p21WAF1 and consequently stimulated cell cycle progression of cells expressing activated Rho (45). Therefore, it appears that the Rho family contributes to cell cycle progression by two distinct mechanisms, induction of cyclin D1 and inhibition of p21WAF1 expression.

To study the possible involvement of Rac1 in myoblast cell growth, several indicators of cell cycle progression were analyzed in the myoblast cell lines expressing the different Rac1 proteins (Figs. 6 and 7). As expected, expression of p21WAF1 was induced during the differentiation of control C2 cells. Weaker and transient induction of p21WAF1 was observed in cells expressing activated Rac1. A profoundly stronger induction of p21WAF1 occurred in cells expressing dominant negative Rac1 (Fig. 6A). The level of the retinoblastoma protein is induced, and it becomes dephosphorylated during the withdrawal of myoblasts from the cell cycle (31). Control cells and those expressing the dominant negative Rac1 underwent these changes. However, part of pRb remained hyperphosphorylated in cells expressing activated Rac1 (Fig. 6B). The amount of cyclin D1 protein declined as myoblasts withdrew from the cell cycle (Ref. 33; Fig. 6). The cyclin D1 level was higher in myoblasts expressing active Rac1 and marginally declined relatively to other cell lines after their growth in DM. Finally, to analyze the actual withdrawal from the cell cycle, cells found during the S phase were detected using a BrdUrd incorporation assay. To avoid any effect of contact inhibition on cell growth, the experiment was done with a subconfluent population of myoblasts. Cells expressing the dominant negative form of Rac1 were the only ones to completely withdraw from the cell cycle after 48 h in DM (Fig. 7). Together, these results indicate that in myoblasts, Rac1 affects the cyclin D1/cyclin-dependent kinase pathway; activation of Rac1 induces the levels of cyclin D1 and inhibits the induction of p21WAF1, resulting in a hyper-phosphorylated pRb that fails to function in the withdrawal of myoblasts from the cell cycle.

Interestingly, the stable expression of dominant negative Rac1 did not prevent myoblast proliferation in GM. Based on the results presented in Fig. 1B that show that Rac1 activity was blocked in these cells, it can be concluded that its activity is not essential for myoblast growth. The existence of redundant pathways controlling myoblast proliferation that can replace Rac1 may explain this observation. The activity of Rac1 may be rate-limiting during the exit of myoblasts from the cell cycle. In such a case, its functional inactivation by expression of the dominant negative form may accelerate exit from the cell cycle.

Does Rac1 Cross-talk with RhoA during Myoblast Proliferation?—Rac1 is just one GTPase that belongs to a big Rho family involved in the regulation of multiple cellular processes. There is a very complex cross-talk between these GTPases that is specific to each biological process. The cross-talk between two members, Rac1 and RhoA, was analyzed in several cellular systems, and it was found that in some cases they exert synergistic functions, whereas in others, they exert antagonistic functions (48–51). It was nicely demonstrated by others that RhoA induces muscle differentiation, whereas Rac1, investigated in the present work, inhibits muscle differentiation. To begin to understand the possible cross-talk between Rac1 and RhoA, we blocked RhoA activity in the myoblast cell line expressing activated Rac1. The addition of the specific RhoA inhibitor, C3 transferase, to these cells growing in DM did not reverse the incomplete cell cycle withdrawal mediated by activated Rac1. Thus, it is unlikely that Rac1 and RhoA cooperate in this activity, and it is more likely that they antagonize each other’s function. Additional studies are needed to understand the possible cross-talk between these and other members of the Rho family.

Rac1 Cooperates with the ERK Pathway in Myoblast Proliferation—As indicated above, Rac1 and the ERK MAPK pathway cooperate in the passage through G1 and entry into the S phase (42). Moreover, it was suggested that the two pathways cross-talk. Rac1 was shown to activate ERK through PAK-mediated phosphorylation of MEK and Raf (8, 9). ERK activity contributes to the proliferation of myoblasts, and this activity has to decline as myoblasts exit the cell cycle (35–38). The cooperation between Rac1 and ERK in the proliferation of myoblasts was studied in the present work. The myoblasts expressing constitutively active Rac1 were treated with specific inhibitors of the ERK pathway (Fig. 8). Inhibition of ERK in these cells induced the dephosphorylation of pRb, reduced the expression of cyclin D1, and increased the withdrawal of myoblasts from the cell cycle. Inhibition of p38 MAPK, representing another MAPK pathway, did not affect the withdrawal from the cell cycle, suggesting that Rac1 specifically cooperated with ERK and not with p38 MAPK. Future studies should reveal the exact mechanism of cross-talk between these pathways in differentiating myoblasts.

In conclusion, it is becoming evident that the Rho family plays complex roles in the differentiation of muscle cells. RhoA, a member of the Rho family, induces muscle transcription and differentiation through the activation of serum response factor (52, 53). Using several mechanisms, CDC42H and Rac inhibit differentiation (Refs. 19 and 39 and the present work). These functions of the Rho family may regulate the migration of undifferentiated myoblasts to their destination and their subsequent differentiation at the correct sites during embryo development.

3 H. Heller and E. Bengal, unpublished results.
Acknowledgments—We thank Uri Nudel and David Yaffe for the C2 cells. We thank Muriel Zohar and Silvio Gutkind for Rac1 expression vectors. We thank Stephen Tappert for the generous gifts of reagents, including the 10T1/2 MyoD-ER cells. We thank Bianca Raikhl-Eisenkraft and Abbie Rosner for critical reading of the manuscript.

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J. Biol. Chem. 2001, 276:37307-37316.
doi: 10.1074/jbc.M103195200 originally published online August 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103195200

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