RhoA controls myoblast survival by inducing the phosphatidylinositol 3-kinase-Akt signaling pathway

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Abstract The small GTPase RhoA regulates the expression of the myogenic transcription factor, MyoD, and the transcription of muscle-specific genes. We report that RhoA also affects the survival of differentiating myoblasts. Two signaling pathways, extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3-K)-Akt, are involved in myoblast survival. Here, we show that inhibition of RhoA prevents the phosphorylation of Akt, but does not affect the phosphorylation of ERK. Constitutive expression of an active form of Akt prevents apoptosis in myoblasts treated with the Rho inhibitor C3-transferase. These results indicate that RhoA functions to prevent myoblast death by inducing the PI3-K-Akt pathway.

Keywords: Muscle differentiation; Rho GTPase; PI3-K-Akt signaling pathway; Apoptosis

1. Introduction

The Rho GTPases affect various cellular functions [1] including cytoskeleton rearrangement, cell motility, cell growth and cytokinesis [2]. This group of GTPases works as molecular switches that transmit intracellular signals from growth factors or G protein coupled receptors.

The Rho GTPases have been implicated in different aspects of the differentiation of myoblasts. Rac1 and Cdc42 members of the family induce myoblast proliferation and inhibit the expression of muscle-specific genes [3,4], while RhoA induces differentiation [5]. RhoA is directly involved in muscle-specific transcription [3,5]. The activity of RhoA is required for the expression of MyoD, a key regulator of skeletal muscle differentiation, but not of another family member, Myf5 [5]. The transcriptional regulator, serum response factor (SRF), mediates the effect of RhoA on MyoD transcription. Recently, the DNA binding site for SRF was identified at one of the regulatory regions of the MyoD gene [6]. Thus, a signaling pathway emerges that links RhoA via SRF to the expression of MyoD, and is probably the reason for the positive effect of RhoA on muscle-specific gene expression.

A recent study strengthened the concept of Rho involvement in skeletal muscle specification [7]. Cells derived from embryos lacking p190-BRhoGAP and exhibiting excessive Rho activity were defective for adipogenesis, but underwent myogenesis under the proper growth conditions.

In the present work, we investigated other aspects of Rho involvement in muscle differentiation than the already known effect on muscle transcription. Our study shows that RhoA activity which is induced during myoblast differentiation affects myotube-structure and prevents the programmed cell death (PCD) of myoblasts. Our study suggests that the known survival pathway, phosphatidylinositol 3-kinase (PI3-K)-Akt [8,9], is found downstream to RhoA.

2. Materials and methods

2.1. Materials

RhoA monoclonal antibody (SC-418), MyoD polyclonal antibody (SC-760), and Myogenin monoclonal antibody (SC-12732) were from Santa Cruz Biotechnology. A monoclonal antibody to MyoD (5.8A) was from Novocastra. A monoclonal antibody to myosin heavy chain (MHC) (MF-20) was a gift from Dr. S. Tapscott. A monoclonal antibody to α tubulin was from Sigma. Phospho-Akt polyclonal antibody (#9270), Akt polyclonal antibody (#9272), p44/42 MAP kinase (extracellular signal-regulated kinase, ERK) polyclonal antibody (#9102) and phospho-p44/42 MAP kinase (phospho-ERK) polyclonal antibody (#9102) were from Cell signaling. An antibody to active caspase 3 was from R&D systems.

2.2. Plasmids

pCEFL AU5 RhoAQ63L and pCEFL AU5 RhoAN19 and pCEFL AU5 RhoA wt were a generous gift from Dr. JS Gutkind (NIH). These pCEFL-AU5 expression vectors were described in an article by Teramoto and colleagues [10]. A *Bam*H1–*Eco*R1 fragments encoding for the ORF of RhoA N19 and a *Hin*dIII–*Eco*RI fragment encoding for the ORF of the RhoAQ63L were cloned into the pBABE-Puro retroviral vector. Myr-Akt-HA fragment [11] was cloned into the retroviral vector pLNCX v.2 [12] to generate pCL-Myr-Akt-HA-NCX. Expression vectors of HA-Akt mutants (CMV5) were described before [13]. pBABE-MyoD:ER-puro was obtained from S. Tapscott (FHCRC, Seattle). Construction of the bacterial expression vector of C3-Tat was described before [14].

2.3. Cell culture

C2 cells were a gift from Dr. David Yaffe [15]. Cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM)

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Abbreviations: ERK, extracellular signal-regulated kinase; PI3-K, phosphatidylinositol 3-kinase; GM, growth medium; DM, differentiation medium; MHC, myosin heavy chain; DN, dominant negative; Act, activated; MD:ER, MyoD-estrogen receptor; ROK, Rho kinase

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supplemented with 15% calf serum (Hyclone), penicillin, and streptomycin (Growth Medium, GM). To induce differentiation, we used DMEM supplemented with 10 μg of insulin per ml and 10 μg of transferrin per ml (Differentiation medium, DM).

2.4. Preparation of C3 transferase-Tat

A bacterially expressed chimera protein of *Clostridium botulinum* C3 fused to the transduction domain of the HIV Tat protein was produced in *Escherichia coli* and purified as previously described [14]. The C3 Tat protein was added directly to DM at a concentration of 50 μg/ml. The medium was replaced every 24 h by medium containing fresh inhibitor.

2.5. Generation of stable C2 clones

C2 cells were infected with replication-defective viruses carrying pBABE-Puro, pBABE-Puro RhoAQ63L, pBABE-Puro RhoAN19, pCL-Myr-Akt-HA-NCX and pBABE-MyoD:ER-puro vectors. Retroviruses were generated as described [12]. Infected cells were selected with Puromycin (3 μg/ml) or Neomycin (1 mg/ml).

2.6. Transfection of 293T cells, immunoprecipitations and kinase assay

Calcium phosphate method was used to transfect cells. Cells were lysed and whole cell extracts were collected. Tagged proteins were immunoprecipitated using an anti-HA antibody (Babco inc.) Kinase activity of Akt was analyzed as described before using the peptide GRPRTSSFAEG as a substrate [16].

2.7. Immunohistochemical staining

Cells were fixed and immunostained as described [17]. The primary antibodies used were anti active caspase 3 and anti MHC. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

2.8. GTP loading of RhoA

Specific isolation of RhoA-GTP was performed as described previously [18].

2.9. Western blot analysis

Cells were lysed and whole cell extracts were collected as described [17]. Equal amounts of extracted proteins (40 μg) were loaded and separated over 12.5% SDS–PAGE and transferred to nitrocellulose membranes. Immunoblotting was conducted as described [17]. Proteins were visualized using the enhanced chemiluminescence kit of Pierce Inc.

3. Results and discussion

3.1. The activity of RhoA is induced during growth of C2 cells in differentiation medium

For studying the involvement of RhoA in the differentiation of myoblasts, the endogenous activity of RhoA was determined in a C2 myoblast cell line. In its active form, Rho is bound to GTP and interacts with its effector proteins. Protein extracts from C2 cells grown for different time periods in DM were incubated with a bacterially purified chimera effector, GST-Rhotekin. Levels of activated RhoA were determined by their interaction with GST-Rhotekin and identified by Western blotting (Fig. 1). The amount of RhoA-GTP was low in dividing myoblasts and was higher in cells grown in DM for different periods of time.

3.2. RhoA affects the structure of myotubes

For understanding RhoA function during myoblast differentiation, C2 cells were infected with retroviruses encoding for a constitutively active form of RhoA (RhoA Q63L) or a dominant negative (DN) form of the protein (RhoA N19). Expression levels of exogenous Rho mutant proteins in these cells were up to three fold higher than endogenous RhoA (Fig. 2A, compare lanes 1–4 to 4–8 and 9–12).

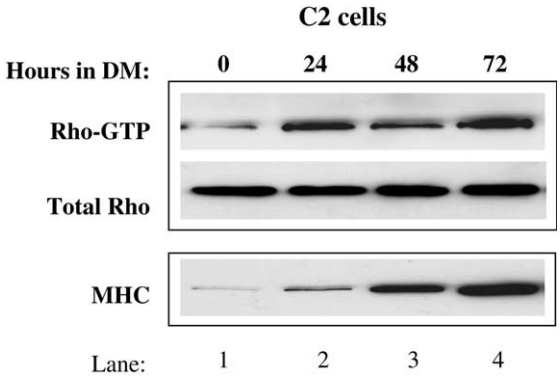

Fig. 1. The activity of endogenous RhoA increases in differentiating C2 cells. The amount of Rho-GTP (active Rho) was analyzed as described in Section 2 using extracts that were prepared from C2 cells grown in DM for different periods of time as indicated. One fifth of the protein extract used to isolate Rho-GTP was used to detect total Rho. Rho-GTP, total-RhoA and MHC were detected by Western blotting.

RhoA was demonstrated before to affect the expression of MyoD [5]. We analyzed the expression of several myogenic markers during the growth of these cell lines in DM (Fig. 2A). Indeed, MyoD protein levels were significantly reduced at all times in the DN-RhoA expressing cells relative to the control C2 cells. The same cells expressed low levels of the early marker, myogenin, and undetectable levels of the late marker, MHC. The cells expressing the active form of RhoA expressed high levels of all myogenic markers, however, the expression was delayed relative to the control cells (Fig. 2A). The effect of RhoA mutants on myogenic differentiation was analyzed also by immunostaining of myotubes with an antibody to MHC (Fig. 2B). Myotubes were detected in the control cells, but not in the cells expressing DN-RhoA. Myotubes were also detected in the cells expressing Act-RhoA, but their structure was very different from that of the control cells. These myotubes were much thicker and shorter (Fig. 2B, compare C2-puro to Act-RhoA). The average number of nuclei per myotube was similar in these two cell lines (data not shown). Together, these data suggest that besides its effect on the expression of myogenic markers, RhoA also affects the structure of myotubes.

3.3. RhoA does not affect the exit of myoblasts from the cell cycle

Since the withdrawal of myoblasts from the cell cycle is directly linked to their differentiation, we compared the withdrawal of the different cell lines from the cell cycle. By several criteria: the expression of cyclin D1, phosphorylation state of pRb, and BrdU staining of nuclei, RhoA expression did not significantly alter the withdrawal of myoblasts from the cell cycle (data not shown).

Another cell cycle regulator, the cyclin-dependent kinase inhibitor $p21^{waf1}$ normally induced during the withdrawal of myoblasts from the cell cycle, was affected by the expression of RhoA mutants (Fig. 3A) [19]. The levels of $p21^{waf1}$ in the parental cell line and in the cells expressing activated Rho were induced during differentiation, though induction of $p21^{waf1}$

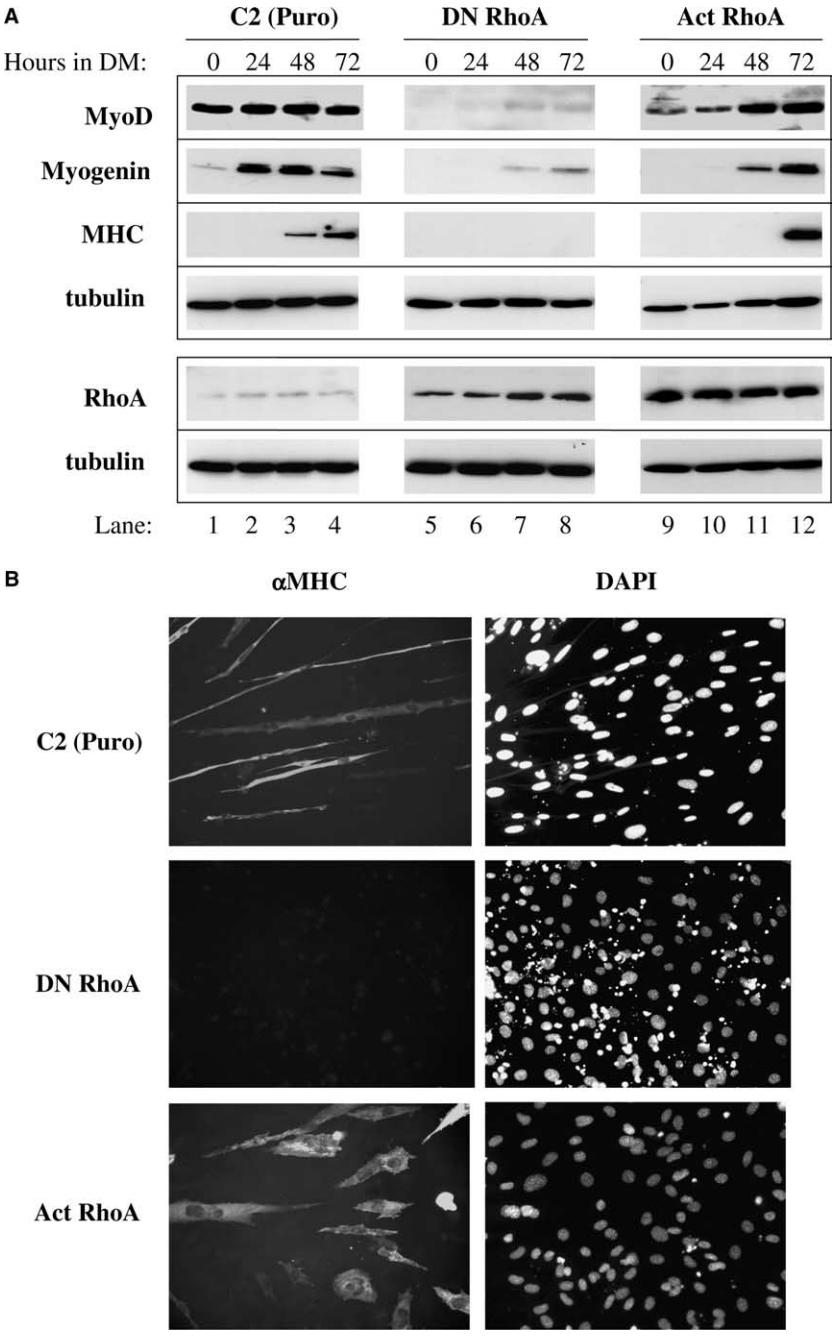

Fig. 2. RhoA affects both the expression of myogenic markers and the structure of myotubes. (A) C2 cells expressing the different forms of RhoA and a control vector (Puro) were grown in GM to 80% confluence and then in DM for the indicated time periods. Proteins were extracted and separated over SDS–PAGE and analyzed by Western blotting. The blot was reacted with different muscle-specific antibodies as indicated and with anti α tubulin antibody. The levels of RhoA were determined in a separate experiment. The amount of α tubulin served as a control for the loading of proteins in each lane. (B) C2 cells expressing the different forms of RhoA and the control cells were grown in DM for 48 h. Cells were fixed and myotubes were identified by immunostaining with MHC antibody (left panel). Nuclei of the same microscopic fields were stained with DAPI (right panel).

was delayed in the latter (Fig. 3A, compare lanes 1–4 to lanes 9–12). The levels of $p21^{waf1}$ were low and did not change during the growth of cells expressing DN RhoA in DM (lanes 5–8). The low levels of $p21^{waf1}$ in these cells could affect their survival state. It is well documented that the resistance of myoblasts to apoptosis is correlated to the expression of $p21^{waf1}$ [19]. Therefore, we further investigated the possible link between Rho and the survival of myoblasts.

3.4. Inhibition of RhoA causes apoptotic cell death of myoblasts

To find out whether the absence of Rho activity could induce cell death, we added C3 transferase to the medium of C2 myoblasts (see Section 2). The exoenzyme C3 transferase inactivates Rho proteins by ADP-ribosylation but not CDC42 and Rac [20].

Following 48 h of growth in DM, C2 cell lines and C2 cells treated with C3-transferase were immunostained with an antibody that recognized the active form of caspase 3 (Fig. 3B).

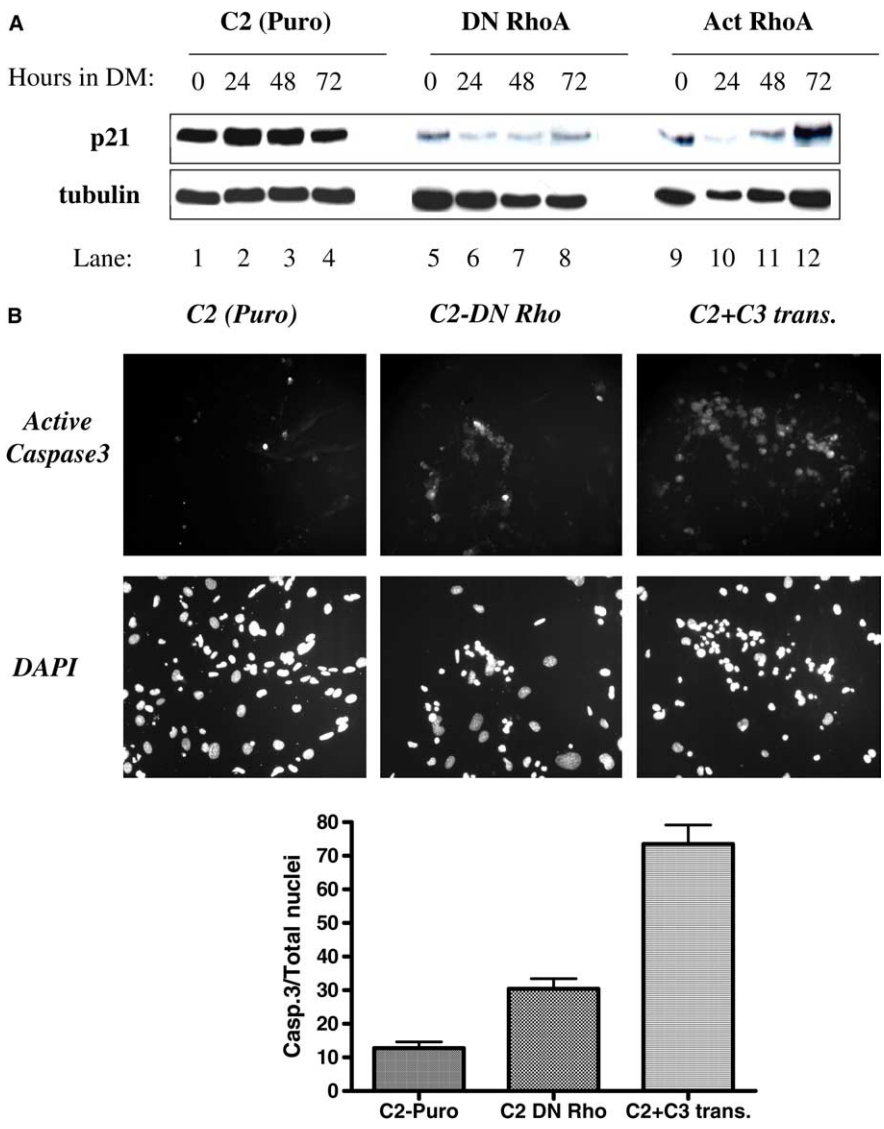

Fig. 3. Inhibition of RhoA causes apoptosis of differentiating myoblasts. (A) C2 cells expressing the different forms of RhoA were grown as described in Fig. 2. Proteins were extracted and separated over SDS–PAGE and analyzed by Western blotting. The blot was reacted with antibodies to p21 and α tubulin. (B) Confluent C2 cells expressing DN Rho and confluent parental C2 cells grown in the absence or presence of C3-transferase (50 μg/ml) were left in DM for 48 h. Upper panel: Apoptotic cells were detected by immunostaining with an antibody recognizing the active processed form of caspase 3. Nuclei of the same fields were stained with DAPI. Lower panel: Quantification of the caspase 3 experiment. For each experiment, about 600 nuclei were counted. The percentage of apoptotic cells was calculated by dividing the number of caspase 3-stained cells by the total number of nuclei. Values are means from three independent experiments. Error bars represent standard errors.

Around 15% of the parental C2 cells were positively stained for active caspase 3. More than 30% of the Rho DN expressing cells and 70% of the cells treated with C3 transferase stained positively for active caspase 3. Similar results were obtained using a TUNEL assay (data not shown). Therefore, these data suggest that inhibition of Rho activity results in a substantial percentage of cells undergoing apoptosis.

3.5. *Phosphorylation of Akt is decreased while the phosphorylation of ERK is unaffected in C2 cells treated with Rho inhibitor*

Two signaling pathways, ERK and PI3-K-Akt, are involved in the expression of $p21^{waf1}$ and promote myoblast survival [9,21]. To find out whether these pathways are found downstream to RhoA, C2 cells were grown in DM in the presence or absence of C3-transferase, and the phosphorylation status of Akt and ERK was investigated (Fig. 4A). Phosphorylation of Akt and ERK is transiently induced during the differentiation of C2 cells (Fig. 4A, lanes 1–4) [17,22]. Treatment of C2 cells with C3 transferase decreased the phosphorylation of Akt but not of ERK during differentiation (Fig. 4A, compare lanes 2–4 to 5–7). This result indicates that Akt is downstream relative to RhoA, and therefore may explain the involvement of RhoA in the expression of $p21^{waf1}$ and the survival of myoblasts [19]. To further explore this possibility, we transfected RhoA mutants into 293T cells and analyzed the kinase activity of Akt in extracts from these cells (Fig. 4B). Wild type and activated RhoA increased the kinase activity of Akt, while treatment of cells with LY294002 blocked its activity. Therefore, RhoA affects PI3-K and consequently the kinase activity of Akt. Rho proteins were previously shown to activate PI3-K and Akt proteins [23,24], yet the mechanism of activation remains

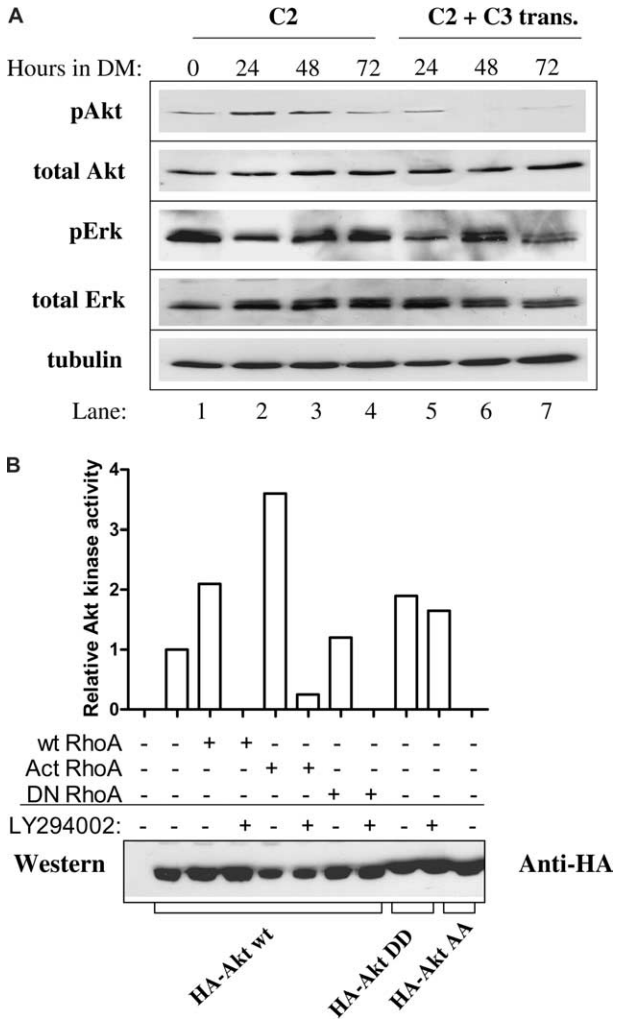

Fig. 4. Inhibition of Rho prevents the phosphorylation of Akt in differentiating myoblasts. (A) C2 cells were grown in GM and then in DM in the presence or absence of C3 transferase. At the indicated time points in DM, cells were lysed and proteins were extracted, separated over SDS–PAGE and analyzed by Western blotting. Membrane was reacted with antibodies to phosphorylated Akt (Ser 473; pAkt), total Akt, phosphorylated ERK (Thr 202/Tyr 204; pERK), total ERK and α tubulin. (B) 293T cells were transfected with expression vectors coding for different RhoA and Akt proteins. 48 h later, cells were lysed and proteins were extracted. HA-tagged Akt proteins were immunoprecipitated and their kinase activity was measured as described [16]. The data are presented as fold activation relative to wt HA-Akt. The relative amount of immunoprecipitated Akt proteins was analyzed by Western blot. HA-Akt DD-constitutively active Akt; HA-Akt AA-kinase dead Akt.

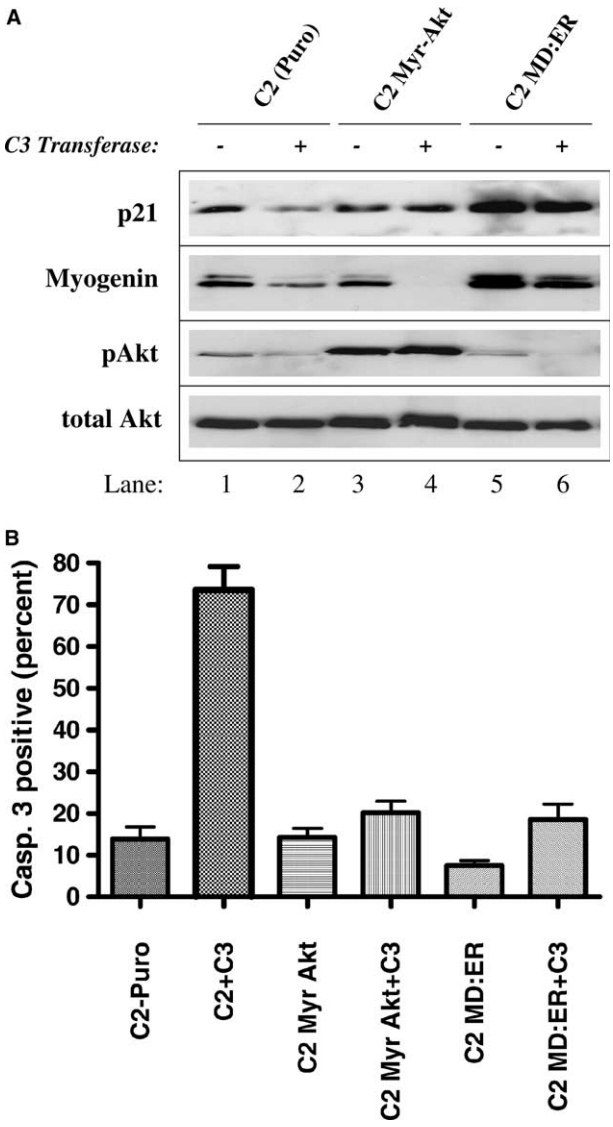

Fig. 5. Myr-Akt can restore the expression of $p21^{waf1}$ and prevents programmed-cell death in C2 cells treated with C3-transferase. C2 cells, C2 cells expressing myristoylated Akt and C2 cells expressing MyoD-ER proteins were grown in GM to 80% confluence and then for additional 48 h in DM in the presence or absence of C3-transferase. (A) Proteins were extracted and separated over SDS–PAGE and analyzed by Western blotting. The blot was reacted with antibodies to $p21^{waf1}$, myogenin, phosphorylated Akt (Ser 473; pAkt) and total Akt. (B) Cells were grown as described above and then fixed. Apoptotic cells were detected by immunostaining with an antibody recognizing the active processed form of caspase 3. For each experiment, about 600 nuclei in at least 10 microscopic fields were counted. The percentage of apoptotic cells was calculated by dividing the number of caspase 3-stained cells by the total number of nuclei. Error bars represent standard errors.

unknown. Some studies demonstrated direct interactions between subunits of PI3-K and small GTPases [25–28]. We examined this possibility, and our preliminary results indicate association of the catalytic subunit of PI3-K, p110 with RhoA in transfected cells (data not shown).

3.6. *Expression of activated-Akt rescues C2 cells from apoptosis induced by RhoA inhibition*

If Akt is indeed downstream to RhoA, one would expect that the expression of constitutively active Akt can reverse the effects of Rho inhibition in differentiating C2 cells. A C2 cell line expressing myristoylated-Akt, a constitutively active membrane-bound Akt, was isolated. The myristoylated-Akt was highly phosphorylated also when this cell line was grown in the presence of C3-transferase, suggesting that the protein was constitutively active (Fig. 5A, lanes 3, 4). In contrast to the control C2 cells, growth of this cell line in the presence of C3-transferase did not decrease the levels of $p21^{waf1}$ as compared to cells grown in its absence (Fig. 5A, compare lanes 1, 2 to 3, 4). Nevertheless, the expression of myristoylated Akt did not restore the expression of myogenin that was significantly inhibited by C3 transferase (Fig. 5A, lanes 1–4). Since the

inhibition of RhoA results in a pronounced decrease in MyoD expression (Fig. 2A) [5], we further studied the ability of forced expression of MyoD to rescue muscle differentiation. For that, an inducible MyoD protein (MD:ER; a chimera protein of MyoD and the hormone binding domain of estrogen receptor) was constitutively expressed in C2 cells. The active MD:ER protein was able to rescue the expression of both $p21^{waf1}$ and the myogenin proteins in the presence of C3 transferase. Still, the MD:ER protein did not rescue the phosphorylation of Akt that was almost undetectable in the presence of C3 transferase (Fig. 5A, lane 6). To further investigate whether activated Akt or forced expression of MyoD could rescue the substantial apoptosis occurring in myoblasts grown in the presence of C3 transferase, the different cell lines were immunostained with an antibody that recognized the active form of caspase 3 following their growth in DM (Fig. 5B). Whereas C3 transferase induced a sharp increase from about 15 to 70% in parental C2 cells positively stained for active caspase 3, it barely changed the relative amount of stained-cells expressing myristoylated Akt or MD:ER.

From this set of experiments we can conclude that: (a) Active Akt can rescue apoptosis that is induced by lack of Rho activity, but not the expression of myogenin. (b) MD:ER protein can rescue both the expression of myogenin and apoptosis independently of the phosphorylation state of Akt.

Overall, our results suggest additional functions for RhoA in myogenesis besides those known so far. One function is to promote the survival of myoblasts via the PI3-kinase-Akt pathway. This pathway prevents myoblast apoptosis, but does not affect muscle transcription (Fig. 5). A second fucntion is to affect the structure of myotubes (Fig. 1). Experiments not presented here suggest that this function is mediated by Rho kinase (ROK). However, ROK does not function as an effector of RhoA in the activation of PI3-K-Akt. A third, previously described function is the transcriptional activation of MyoD [5]. As a major regulator of MyoD, of the PI3-kinase pathway and of the structure of myotubes, RhoA proves to be a key modulator of skeletal muscle formation.

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