Mirk/Dyrk1B Mediates Survival during the Differentiation of C2C12 Myoblasts*

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Stephen E. Mercer, Daina Z. Ewton, Xiaobing Deng, Seunghwan Lim, Thomas R. Mazur, and Eileen Friedman‡

From the Department of Pathology, Upstate Medical University, State University of New York, Syracuse, New York 13210

The kinase Mirk/dyrk1B is essential for the differentiation of C2C12 myoblasts. Mirk reinforces the G0/G1 arrest state in which differentiation occurs by directly phosphorylating and stabilizing p27Kip1 and destabilizing cyclin D1. We now demonstrate that Mirk is anti-apoptotic in myoblasts. Knockdown of endogenous Mirk by RNA interference activated caspase 3 and decreased myoblast survival by 75%, whereas transient overexpression of Mirk increased cell survival. Mirk exerts its anti-apoptotic effects during muscle differentiation at least in part through effects on the cell cycle inhibitor and pro-survival molecule p21Cip1. Overexpression and RNA interference experiments demonstrated that Mirk phosphorylates p21 within its nuclear localization domain at Ser-153 causing a portion of the typically nuclear p21 to localize in the cytoplasm. Phosphomimetic GFP-p21-S153D was pancellular in both cycling C2C12 myoblasts and NIH3T3 cells. Endogenous Mirk in myotubes and overexpressed Mirk in NIH3T3 cells were able to cause the pancellular localization of wild-type GFP-p21 but not the nonphosphorylatable mutant GFP-p21-S153A. Translocation to the cytoplasm enables p21 to block apoptosis through inhibitory interaction with pro-apoptotic molecules. Phosphomimetic p21-S153D was more effective than wild-type p21 in blocking the activation of caspase 3. Transient expression of p21-S153D also increased myoblast viability in colony forming assays, whereas the p21-S153A mutant had no effect. This Mirk-dependent change in p21 intracellular localization is a natural part of myoblast differentiation. Endogenous p21 localized exclusively to the nuclei of proliferating myoblasts but was also found in the cytoplasm of post-mitotic multinucleated myotubes and adult human skeletal myofibers.

Although there is a large body of work describing the functions of myogenic regulatory factors in skeletal muscle commitment and differentiation, much less is known regarding the signaling pathways that control the specific molecular events involved in terminal differentiation and maintenance of skeletal muscle. We demonstrated recently (1) that a novel gene, which we cloned in 1997, the kinase Mirk,1 plays a critical role in controlling C2C12 myoblast differentiation following the commitment stage of myogenesis. Depletion of endogenous Mirk by RNA interference blocked the transcription of myogenin and the subsequent myoblast differentiation program (1). Mirk controls the activation of the myogenin transcription factor MEF2 by regulating nuclear accumulation of the MEF2 inhibitors, class II histone deacetylases (2). However, it has been reported that embryonic knockout of Mirk did not block skeletal muscle development in the early embryo (3), suggesting that Mirk may have a more significant function in muscle repair than in initial myogenesis.

Mirk (Minibrain-related kinase; also known as Dyrk1B) is a member of an evolutionarily conserved family of proteins, the Dyrk/Minibrain family of arginine-directed serine/threonine protein kinases (4–7), that play roles in controlling the switch from proliferation to differentiation in a wide variety of organisms. The roles of Dyrk/Minibrain/Mirk homologues in yeast (Yak1) and slime mold (YakA) suggest that this group of kinases helps to regulate the transition from growth to differentiation in response to environmental stresses (8, 9).

Mirk is a unique, multifunctional kinase with primary activity in G0 phase and early differentiation. Specifically, Mirk elongates the G0 phase of the cell cycle in C2C12 myoblasts, Mv1Lu epithelial cells, and NIH3T3 fibroblasts by stabilizing the CDK inhibitor p27Kip1 (10) and by destabilizing the G1 cyclin, cyclin D1 (11). Mirk has also been shown to act as a transcriptional activator (2, 12, 13) and to inhibit cell motility (14). Because of these actions of Mirk on cell cycle regulators active in G0/G1 during myoblast differentiation, we extended our study of Mirk substrates to p21Cip1. The CDK inhibitor p21 is highly expressed in differentiating muscle in vivo (15) and has been implicated as playing a central role in mediating the pre-differentiation growth arrest induced by the major muscle regulatory factor MyoD (16). It is believed that this effect of p21 during myogenesis is because of inhibition of G1 cyclin-CDK complexes and subsequent blockade of retinoblastoma protein phosphorylation (16).

Studies using mice deficient in members of the Cip/Kip CDK inhibitor family have demonstrated that in the absence of p21, mice can develop into normal adults (17). This appears to be due to redundant functions of p57, because mice deficient in both p21 and p57 demonstrate severely arrested muscle development (18). Specifically, the double mutant mice displayed...
increased proliferation and apoptosis of myoblasts and failed to form myotubes. Significantly, although p21-deficient mice appear to develop normally, p21 is essential for normal regeneration through the muscle progenitor (or satellite) cells that are responsible for repairing muscle following injury (19). It has been suggested that muscle differentiation during embryogenesis and during regeneration are fundamentally different, with p57 playing a dominant role in embryogenesis and p21 being the critical CDK inhibitor during regeneration (18).

In the current study, we demonstrate that in the C2C12 myoblast model of muscle regeneration, Mirk functions to promote cell survival during the initial stages of differentiation, similar to the known function of Mirk in mediating survival of colon carcinoma cells in serum-free conditions (20). Undifferentiated or partly differentiated cells are removed from cultures of fusing myoblasts by apoptosis, causing a loss of 20–30% of C2C12 myoblasts during the first 48 h in differentiation medium (15). Mirk diminishes the extent of myoblast apoptosis during the differentiation process, at least in part by direct modulation of p21Cip1 localization. Mirk phosphorylates p21 within its nuclear localization domain. This phosphorylation maintains a portion of p21 protein in the cytoplasm where p21 has been reported to be unable to mediate cell cycle arrest (21) and where p21 blocks caspase 3 activation (22).

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal antibodies were raised to unique sequences at the N terminus (amino acids 1–19) (20) and C terminus (amino acids 595–624) of Mirk and affinity-purified. The C-terminal antibody was raised to a slightly longer peptide than the C-terminal antibody used in all our previous studies on Mirk (20), but it was only homologous to Mirk/dyrk1B in BLAST analysis. This antibody detected all three Mirk splice variants found in normal muscle as follows: 69, 70, and 75 kDa, the 69- and 70-kDa variants found in C2C12 cells (1) and the inducible 70-kDa Mirk form stably expressed in Mv1Lu cells (14), but no other proteins at the dilutions used in the current study (Western blotting data not shown). The C-terminal antibody was labeled with Alexa Fluor 594 using the Zenon rabbit IgG labeling kit (Molecular Probes) for use in direct immunofluorescence experiments. Alexa Fluor 488 and 594 (highly cross-adsorbed) secondary antibody conjugates and Alexa Fluor 488 phalloidin were purchased from Molecular Probes. An anti–phospho-hsp70 antibody was purchased from CalBiochem. Phospho-hsp70 was phosphorylated at Thr40A, which is conserved in all hsp70 homologues. An antibody was raised to a slightly longer peptide than the C-terminal peptide and homologous to Mirk/dyrk1B in BLAST analysis. This antibody detected Mirk in C2C12 myoblasts and tended to copurify with high quantities of the bacterial hsp70 homologue, the dnaK gene product. This protein has been reported to play a role in degradation of abnormally folded proteins in E. coli (24). The following protocol allowed the majority of the heat shock protein to be removed from the bacterial lysate and the SCP expression preparation. The conditions also promoted hsp-dependent refolding of GST-Mirk (25, 26), resulting in a highly purified and active enzyme preparation. 300 ml of LB medium was seeded with 10% of an overnight culture of BL-21 carrying the desired plasmid and incubated at 37 °C for 1–2 h. Protein expression was induced with 0.1 mM isopropyl β-D-thiogalactoside for 4–6 h at 24 °C, and bacteria were collected by centrifugation and resuspended in 3 ml of ice-cold sonication buffer (140 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, 10 mM MgCl2, 5 mM ATP, 5 mM DTT, 20% glycerol, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 µg/ml lysozyme, EDTA-free complete protease inhibitor set, pH 7.3; Roche Applied Science). The ATP and MgCl2 are required for the activity of the heat shock protein. The resuspended bacteria were incubated on ice for 15 min, and the bacteria were then lysed on ice with a microtub (Misonix, setting 2.5) for 3 min with 5-s bursts separated by 5 s of rest. Lysates were clarified by centrifugation for 15 min at 15,000 × g to remove insoluble material. 13-mL portions of the cleared lysate were added to 1 ml (bed volume) of glutathione-Sepharose 4B (Amersham Biosciences) in a 15-ml conical tube, and an additional 750 µl of 100 mM ATP, 150 µl of 1 mM MgCl2, and a Complete Protease Inhibitor Mini-Tab (Roche Applied Science) was then added to each sample. The beads were then incubated at 4 °C overnight with end-on rotation. Beads were washed four times for 10 min with 10 ml of standard wash buffer (290 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, 10% glycerol, 0.1% Triton X-100, pH 7.3) to remove the majority of contaminants from the beads. The beads were then washed twice after 1 h with 10-mL portions of Mg/ATP wash buffer (290 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, 5 mM MgCl2, 2 mM ATP, 2 mM DTT, 10% glycerol, 0.1% Triton X-100, pH 7.3) to remove the majority of the heat shock protein contaminant. The beads were then washed three times for 5 min with standard wash buffer. All washes and incubations were performed at 4 °C with end-on rotation. The beads were massaged by 4 h with 10-mL portions of Mg/ATP wash buffer (290 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, 5 mM MgCl2, 2 mM ATP, 2 mM DTT, 10% glycerol, 0.1% Triton X-100, pH 7.3) to remove the majority of the heat shock protein contaminant. The beads were then washed three times for 5 min with standard wash buffer. All washes and incubations were performed at 4 °C with end-on rotation. The eluate was concentrated; the glutathione was dialyzed out, and the elution buffer was exchanged for PreScission...
cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100, pH 7.0) by three concentrations with an Amicon Ultra-4 30,000 molecular weight cut-off centrifugal filter device (Millipore). Protein concentration was measured using the Bradford protein assay, and 20 units of PreScission enzyme were added per mg of protein. Proteolytic cleavage was carried out overnight at 4 °C with end-on rotation. The cleaved protein solution was then incubated two times for 4 h at 4 °C with equal volumes (bed volume) of glutathione-Sepharose 4B in order to clear the sample of uncleaved protein, GST, and the PreScission enzyme (also a GST fusion protein). The resulting sample was concentrated, and the cleavage buffer was replaced with PBS with 10% glyceral and protease inhibitors using centrifugal filtration. The product retained high activity for up to 1 year when stored at −20 °C.

**In Vitro Kinase Assays**—2–5 μg of GST-p21 fusion proteins bound to glutathione-Sepharose beads were washed twice with kinase assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.5 mM dithiothreitol) and then incubated with 100 μg of recombinant Mirk for 20 min at 30 °C in 30 μl of kinase assay buffer containing 10 μM unlabelled ATP plus 5 μCi of [γ-32P]ATP. Reaction products were analyzed by SDS-PAGE and autoradiography.

**In Vitro Labeling and Immunoprecipitations**—2 × 10^6 C2C12 cells were plated in 60-mm dishes and transfected immediately with 12 μg of FLAG-p21 constructs and 4 μg of Hisa-Mirk using 32 μl of Lipofectamine 2000 in 1 ml of DMEM containing 10% FBS. Following 24 h of expression, cells were switched to differentiation media for 19 h, then to phosphate-free media for 1 h, and then incubated with 400 μCi of [32P]orthophosphate for 4 h in 2 ml of reduced phosphate medium (75% reagent, and 25% phosphate-free media for 1 h, then incubated with 400 μCi of [32P]orthophosphate for 4 h in 2 ml of reduced phosphate medium (75% phosphate-free DMEM, 25% DMEM). Cells were washed twice and then lysed in 0.5 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, Roche Complete Proteinase Inhibitor Mixture, and Sigma Phosphatase Inhibitor Mixture to remove insoluble material. FLAG constructs were isolated using 15 μl of Protein A-agarose (Santa Cruz Biotechnology) for 1 h at room temperature with 500 μl of Lipofectamine 2000 in DMEM containing 10% FBS. After 24 h of expression, cells were plated in 60-mm dishes and transfected immediately with 12 μg of plasmid DNA, 5 μl of Lipofectamine per well. Following 24 h of expression, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 5 min, washed twice with 5 ml of PBS, permeabilized with 0.1% Triton X-100, and mounted with Biomedia Gel/Mount. Preparations were analyzed and photographed as described below.

**Statistical Analysis of Localization**—Cell counts were obtained at 400× using a Nikon Eclipse E60i fluorescent microscope. For C2C12 cells, at least 300 cells were observed in 5–10 random fields in each of four separate preparations, and the number of cells with localization of green fluorescence in the nucleus only and localization of green fluorescence in the nucleus and cytoplasm was determined. Two separate preparations of NIH3T3 fibroblasts were examined in the same way. Combined counts were analyzed by the χ² test (Minitab) to determine the significance of differences between the WT and mutant constructs.

**Immunofluorescence of Cultured Cells**—C2C12 myoblasts were plated in Lab-Tek 2-well chamber slides (2 × 10^5 cells per well), cultured for 1 day in growth media, and then switched to differentiation media. After 24 h of expression in GM or 48 h in DM, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 5 min, washed twice for 5 min with PBS, permeabilized with 0.1% Triton X-100, and mounted with Biomedia Gel/Mount. Preparations were analyzed and photographed as described below.

**Immunofluorescence of Human Muscle Frozen Sections**—Anony- mous samples of flash-frozen human muscle tissue on slides were obtained from the Department of Pathology, Stony Brook, NY, Upstate Medical University, in accordance with institutional review procedures for clinical specimen use. Slides were thawed at room temperature for 15 min, hydrated with PBS for 15 min, and then fixed in 4% paraformaldehyde for 1 min at room temperature or ice-cold acetone for 10 min at 0 °C. Slides were rinsed three times for 5 min with PBS. The sections were then demarcated with a PAF pen (Zymed Laboratories Inc.), permeabilized for 20 min with 0.2% Triton X-100 in PBS (wash buffer; used in all subsequent steps), and blocked with 10% normal goat serum, 0.2% Triton, PBS for 30 min. Mirk was visualized with polyclonal antibody to either the N or C terminus of Mirk (both at 1:500 dilution for 1 h), whereas p21 was visualized with either the polyclonal C-19 antibody (1:250 for 1 h) or the monoclonal F-5 antibody (1:50 for 2 h). Nonspecific IgG isolated from the same species as the primary antibody diluted to an equivalent mass concentration was used as a negative control. All labeling incubations were diluted in 10% normal goat serum, 0.2% Triton, PBS for 30 min. Mirk was visualized with polyclonal antibody to either the N or C terminus of Mirk (both at 1:500 dilution for 1 h), whereas p21 was visualized with either the polyclonal C-19 antibody (1:250 for 1 h) or the monoclonal F-5 antibody (1:50 for 2 h). Nonspecific IgG isolated from the same species as the primary antibody diluted to an equivalent mass concentration was used as a negative control. All labeling incubations were diluted in 10% normal goat serum, 0.2% Triton, PBS. After three 5-min washes, cells were incubated for 30 min with 1:1000 dilution of anti-p21 mouse monoclonal antibody (F-5, Santa Cruz Biotechnology) and anti-C-terminal Mirk antibody conjugated to Alexa Fluor 594. As a control for nonspecific antibody interactions, we also performed identical experiments with the C-19 polyclonal antibody (Santa Cruz Biotechnology) and our anti-N-terminal Mirk antibody. Additional controls included use of direct immunofluorescence (using the Zenon labeling system) and substitution of the primary antibody with nonspecific mouse or rabbit IgG as appropriate. All labeling incubations were diluted in 10% normal goat serum/PBS. After three 5-min washes, cells were incubated with a 1:1000 dilution of goat anti-mouse anti- body conjugated to Alexa Fluor 594 (Molecular Probes). Slides were washed twice for 5 min. During single-labeling experiments for detection of p21, cells were incubated for 20 min with 2 units of Alexa Fluor 594 phallolidin (Molecular Probes), diluted in 3% BSA/PBS, and washed twice. When using direct labeling, cells were fixed a second time (4% paraformaldehyde for 5 min) to stabilize the Zenon label after the secondary antibody washes. Nuclei were counterstained with 1 μg/ml of 4',6-diamidino-2-phenylindole hydrochloride (DAPI). Following three 5-min washes, slides were rinsed with distilled water, blown dry, and mounted with Biomedia Gel/Mount. Images were obtained as described below.

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**Imaging**—Monochrome fluorescence images were obtained at ×400 or ×1000 (as indicated) using a Diagnostic Instruments SPOT RT
camera mounted on a Nikon Eclipse E500 fluorescent microscope. SPOT RT software version 4.0.9 was used to pseudocolor the images, adjust the RGB histogram, and merge the images. Image manipulation consisted of resetting the zero point of the RGB histogram of the green and red fluorescent channels to stretch the darker areas of the image yielding a uniform black background consistent with the image viewed through the microscope. Final figures were arranged using Adobe Photoshop version 7.0.

**BrdUrd Incorporation Assay**—C2C12 myoblasts were plated overnight in Labtek 2-well chamber slides (106 cells per well) and then transfected (2 μg of plasmid DNA, 4 μl of PLUS, and 4 μl of Lipofectamine per well) with GFP vector, GFP-p21-WT, or the phosphomimetic GFP-p21-S153D. Cells were transfected in serum-free media for 4 h, and an equal volume of 20% FBS/DMEM was then added. BrdUrd incorporation was measured using the cell proliferation assay kit (Amersham Biosciences). After 24 h of expression, cells were incubated for 1 h with BrdUrd labeling reagent diluted 1:500 in 10% FBS/DMEM. Cells were rinsed twice with PBS and fixed for 30 min at room temperature with acid alcohol (90% absolute ethanol, 5% glacial acetic acid, 5% water). Cells were then rinsed three times with PBS, blocked for 30 min in 10% normal goat serum/PBST, and then incubated for 1 h at room temperature with a DNase I solution containing a 1:100 dilution of anti-BrdUrd antibody (Amersham Biosciences). The denatured GFP protein was labeled by a 30-min incubation with anti-GFP antibody (Santa Cruz Biotechnology 8334; 1:500). BrdUrd was visualized using anti-mouse Alexa Fluor 594; GFP was visualized with anti-rabbit Alexa (Santa Cruz Biotechnology 8451; 1:500). The denatured GFP was labeled by a 30 min incubation with anti-GFP antibody (Santa Cruz Biotechnology 8334; 1:500). BrdUrd was visualized using anti-mouse Alexa Fluor 594; GFP was visualized with anti-rabbit Alexa Fluor 488. At least 300 GFP-expressing cells were observed in 8–10 random fields in each of three separate preparations, and the number of cells labeled for both GFP and BrdUrd was determined using a Green/Orange V2 filter set (Chroma Technology) that allowed simultaneous visualization of both fluorophores. An additional control was the proportion of cells incorporating BrdUrd was also assessed in a set of non-transfected slides stained with DAPI. Combined counts were analyzed by the χ2 test (Minitab) to determine the significance of differences between the GFP vector and GFP-p21 constructs.

**RESULTS**

**Mirk Promotes Myoblast Survival**—Undifferentiated or partly differentiated cells are removed from cultures of fusing myoblasts by apoptosis. Programmed cell death of 20–30% of C2C12 myoblasts is seen during the first 48 h after myoblasts are transferred from growth medium to differentiation medium (15). We had observed in earlier studies that the serine/threonine kinase Mirk afforded colon carcinoma cells increased survival capabilities, whereas mutant forms of kinase-inactive Mirk did not (20). Mirk is highly expressed in skeletal muscle cells and in myoblasts, so we speculated that one function of Mirk was to enhance the survival of differentiating myoblasts. Mirk is enriched in NIH3T3 cells in G0/G1 and down-regulated when cells are treated with mitogens (10). Likewise, Mirk was found to be enriched in C2C12 myoblasts undergoing mitosis and in early G1 (Fig. 1A). Levels of Mirk rapidly increase in C2C12 cells arresting in G0/G1 when placed in DM (1, 11). We hypothesized that if Mirk mediated cell survival, C2C12 cells expressing ectopic wild-type Mirk would survive in greater numbers during myoblast differentiation than cells expressing kinase-inactive YF-Mirk. C2C12 cells were transfected with either wild-type Mirk, YF mutant Mirk, or vector alone. The constructs were allowed to express for 18 h, and the cells were then transferred to differentiation medium for 0–48 h. Expression of Mirk or YF-Mirk was determined by immunofluorescence analysis. Each construct was expressed in roughly the same percentage of C2C12 cells, 30–34%, after culture for 18 h in growth medium. When cells were cultured in DM for 48 h, four times as many cells in the differentiating cultures were found expressing ectopic wild-type Mirk as expressing kinase-inactive YF-Mirk. These data supported the hypothesis that Mirk provided some survival advantage to differentiating myoblasts. In parallel transfections, cell lysates were analyzed for expression of ectopic Mirk and YF-Mirk normalized to β-tubulin by Western blotting (Fig. 1B, top). The amount of wild-type Mirk declined about 2-fold by 48 h, during which time cells in the cultures were undergoing apoptosis. In contrast, the amount of YF-Mirk decreased far more, about 10-fold. To determine what percentage of Mirk left after 48 h was endogenous, another set of transfections was performed. Cell lysates were analyzed for expression of ectopic YF-Mirk and for endogenous Mirk from the cultures transfected with the vector alone (Fig. 1B, bottom). These blots were exposed for a much longer time to visualize endogenous Mirk, which accounted for ½ of the Mirk detected in cultures transfected with YF-Mirk at the 48-h point. Only about 8% of the YF-Mirk was left after 48 h, whereas about 50% of wild-type Mirk was retained. Half-life measurements showed that this effect was not because of differences in protein stability (data not shown). Thus both biochemical analysis and immunofluorescence studies demonstrated that about 4–5 times as many C2C12 myoblasts survived apoptosis in cultures undergoing differentiation if they expressed ectopic wild-type Mirk than if they expressed ectopic kinase-inactive YF-Mirk.

In a recent report (1), we showed that depletion of endogenous Mirk by RNA interference in C2C12 myoblasts undergoing differentiation blocked the induction of myogenin and contractile proteins and subsequent myoblast fusion. In these experiments, we also observed a loss of those cells in which Mirk had been depleted, visualized by expression of cotransfected DsRed by fluorescence microscopy. To test directly whether Mirk played some role in myoblast survival, we depleted Mirk in C2C12 cells by RNA interference using RNAi to Mirk and a mutant RNAi as the control, and we measured cell viability by colony formation after Mirk depletion (Fig. 1, C and D). C2C12 cells were cotransfected with the neomycin resistance gene and with the pH silencer vector encoding either RNAi to Mirk, a mutant RNAi, or vector alone, and the extent of Mirk depletion was analyzed by Western blotting (Fig. 1C). The Mirk-depleted cells and controls were plated at single cell density and selected in growth medium containing G418 for 3 weeks to determine the number of cells capable of proliferation to form a colony. Colony formation was reduced 75% in the C2C12 cells experiencing Mirk knockdown at the time of plating compared with cells transfected with the mutant RNAi or the vector control (Fig. 1D). Parallel studies with NIH3T3 cells showed that the cells in colonies that did arise after Mirk knockdown expressed Mirk (data not shown). Thus these colonies presumably had arisen either from cells that had not been transfected or from cells that had lost the Mirk RNAi plasmid. These data indicate that knockdown of Mirk levels at the time C2C12 myoblasts were seeded as single cells had reduced their viability and thus their ability to give rise to colonies, and indicate that Mirk functions as a survival factor.

We next assayed whether depletion of Mirk would render cells more susceptible to apoptosis in short term experiments without chemical selection of transfected cells (Fig. 1E). We depleted Mirk in C2C12 cells by RNA interference, with a mutant RNAi and the empty vector as controls, and we used cotransfected DsRed to mark the transfectants. After 24 h to induce Mirk knockdown, we transferred the cells to differentiation medium for 1 day, and we identified the apoptotic cells within the DsRed+ population by incubation with a fluorescent conjugate of the permeable caspase inhibitor VAD-fmk. 48% of cells with Mirk knockdown were undergoing apoptosis compared with 22.9 and 20.1%, respectively, of cells treated with mutant RNAi or vector, a highly significant difference (Fig. 1E). A similar knockdown experiment was performed in a time course format by measuring the abundance of Mirk, and the apoptosis effector activated caspase 3 by Western blotting (Fig. 1F). After 1 day, Mirk levels were depleted to about 50% of control values, and caspase 3 was activated to 160% of control.
**FIG. 1. Mirk mediates myoblast survival.** A, Mirk is expressed in cycling C2C12 myoblasts. Fluorescence microscopy demonstrates that Mirk expression is greatest in C2C12 cells in mitosis and in cells in early G1. Affinity-purified anti-peptide antibody to the Mirk C terminus was conjugated to Alexa Fluor 594 and used to detect endogenous Mirk in C2C12 cells in growth medium. Nuclei were stained with DAPI, and identical fields were merged with SPOT RT software. Similar results were obtained with antibody directed to the unique N terminus of Mirk. B, top, ectopic Mirk maintains the survival of differentiating C2C12 cells. Cells were transiently transfected with wild-type Mirk or kinase-inactive mutant YF-Mirk, allowed to express for 18 h, and then transferred to differentiation medium for up to 48 h. Lysates were analyzed for expression of Mirk.
values. After 2 days, there was less Mirk knockdown and less activation of caspase 3. After 3 days, there was no Mirk knockdown and no activation of caspase 3 (Fig. 1F). Thus, depletion of Mirk protein correlated with increased abundance of activated caspase 3 in multiple experiments and led to loss of cells by apoptosis. In another experiment, we measured the effect of the caspase inhibitor benzoxycarbonyl-VDV-dmfk on differentiation-induced apoptosis, as assayed by the TUNEL reaction (Fig. 1G). The caspase inhibitor blocked apoptosis to a similar extent, 40% in control cultures, 50% in mutant RNAi-treated cultures, and 33% in cultures where Mirk knockdown occurred. These studies employing either Mirk knockdown or Mirk overexpression demonstrate that Mirk has anti-apoptotic survival functions in C2C12 myoblasts.

Mirk Phosphorylates p21 in Vivo—The CDK inhibitor p21Cip1 has been implicated in the control of apoptosis during myofiber fusion (15), possibly through stabilization by ERKs (27). Mirk phosphorylates another CDK inhibitor p27Kip1 in vivo (10), so we hypothesized that Mirk might also phosphorylate the closely related p21Cip1. A wild-type p21 construct with an N-terminal FLAG epitope tag was cotransfected into NIH3T3 fibroblasts with either wild-type or kinase-inactive Mirk and allowed to coexpress for 24 h. After 4 h of labeling with [32P]orthophosphate, FLAG-p21 was immunoprecipitated with anti-FLAG M2 antibody, and the immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography (Fig. 2). FLAG-p21 migrated at the expected molecular weight but was phosphorylated only in the cotransfections with wild-type Mirk. FLAG-p21 was not labeled when coexpressed with kinase-inactive KR-Mirk or vector (Fig. 2, upper panel). Western blotting for the FLAG epitope (Fig. 2, lower panel) demonstrated that equal amounts of FLAG-p21 were immunoprecipitated. The lower band of the doublet was FLAG-p21, and the upper band was light chain. The FLAG-p21 protein exhibited retarded mobility only when coexpressed with wild-type Mirk (Fig. 2, center lane), consistent with its phosphorylation. Similar data were obtained when Mirk was coexpressed with p21 in C2C12 myoblasts (data not shown). The many functions of p21 rely on its association with a variety of proteins, including cyclins, CDKs, PCNA, and others, which associate with p21 within specific domains. It was essential to determine the phosphorylation site of Mirk in p21 in order to be able to postulate the biological effect of this phosphorylation. For initial studies, we measured the capacity of Mirk to phosphorylate in vitro a series of GST-p21 constructs that were mutated to the non-phosphorylatable alanine residue at known phosphorylation sites: Ser-130 (the p38 MAPK site (23)), Thr-57 (the GSK3 site (28)), and Ser-146 (a protein kinase C site (29) and one of two Akt sites (30)). Mirk phosphorylated each of these constructs to a similar degree (data not shown), indicating that none of these sites was the Mirk phosphorylation site. We then compared human and mouse p21 sequences for

and β-tubulin by Western blotting. Bottom, to determine whether the amount of Mirk remaining in cells expressing ectopic YF-Mirk after 48 h was either ectopic or endogenous Mirk, cells were transiently transfected with kinase-inactive mutant YF-Mirk, or vector, allowed to express for 18 h, and then transferred to differentiation medium for up to 48 h. Lysates were analyzed for expression of Mirk and β-tubulin by Western blotting. The DNA content vector blots were exposed for the same amount of time to allow direct comparisons of duplicate experiments with similar results is shown. C, depletion of Mirk in cycling myoblasts by RNA interference reduced C2C12 cell viability in colony formation assays. Cells were plated at 5 × 103 per 60-mm dish, three dishes per point for 24 h, and then were transfected for 4 h in serum-free medium with 5 μg of pSilencer DNA encoding either RNAi to Mirk (RNAi), a mutant RNAi (Mt), or vector (Vc) together with 0.5 μg of neomycin resistance plasmid, using 10 μl of Lipofectamine and PLUS reagent. Fetal bovine serum was then added to 10% to maintain cell viability during expression. Cells were incubated for 20 h before trypsinization and then lysed for Western blotting (WB). Ct, control, cross-reacting band showing similar loading. D, same experiment as in C, but transfected cells were reseeded at the same cell density and selected in 400 μg/ml G418 for 3 weeks. Mean colony number ± S.E. is shown. E, Mirk knockdown increases apoptosis measured by fluorescence microscopy using an activated caspase 3 inhibitor. C2C12 myoblasts were plated overnight in LabTec 2-well chamber slides (2 × 105 cells per well) and then cotransfected with 0.5 μg of pDsRed and 1.5 μg of pSilencer vector (Vc, mutant si or RNAi to Mirk (4 μl of PLUS and 4 μl of Lipofectamine per well). Cells were transfected in serum-free media for 4 h, and an equal volume of 20% FBS/DMEM was then added. Following 24 h of expression, cells were incubated with differentiation media for 24 h. Activated caspase was then labeled by a 30-min incubation with 10 μl CaspACE FITC-VDV-dmfk in situ marker (Promega) diluted in DM. This is a fluorescent conjugate of the permeable caspase inhibitor VAD-fmk that was used as an in situ marker for apoptosis. Slides were washed and mounted with BioMeda GelMount. At least 200 DsRed expressing cells were observed in each of four separate preparations, and the number of cells labeled for both DsRed and the FITC-VAD-fmk marker was determined using a Green/Orange V2 filter set (Chroma) that allowed simultaneous visualization of both fluorophores. Combined counts were analyzed by the χ2 test to determine the significance of differences between the RNAi constructs. Efficiency of cotransfection was determined to be greater than 85% in parallel experiments using a combination of GFP and DsRed. The number of cells scored per assay conditions is shown. F, knockdown of endogenous Mirk by RNA interference inhibits the activation of caspase 3 in differentiating C2C12 cells. C2C12 cells (5 × 105 per 60-mm dish) were transfected with pSilencer plasmid encoding RNAi to Mirk (RNAi) or mutant RNAi (Mt) for 24 h and then switched to differentiation medium for 1–3 days, as noted. The relative abundance of Mirk/tubulin and of activated caspase 3/tubulin in lysates was determined by Western blotting on the upper and lower sections of same blot that was cut in half and then reunited for the exposure, with tubulin used as the loading control. The relative abundance of caspase 3/tubulin in cells treated with RNAi to Mirk was then normalized to their relative abundance in cells treated with mutant RNAi. Mean ± S.E. (if greater than 5%) is shown from data from three experiments. G, apoptosis induced by differentiation medium or Mirk knockdown blocked to a similar extent by a caspase inhibitor. C2C12 myoblasts were plated, transfected, and cultured as in E, except they were cotransfected with 0.5 μg of pEGFP. A parallel set of cultures was incubated with DM containing 20 μM of the cell-permeable caspase inhibitor benzoxycarbonyl-VDV-dmfk (Promega). After 24 h in DM, DNA breaks in apoptotic cells were labeled with tetramethylrhodamine-dUTP by terminal deoxynucleotidyl transferase-mediated in situ end labeling (TUNEL) using the in situ cell death detection kit (Roche Applied Science). At least 300 GFP-expressing cells were observed in each of four separate preparations, so that an average of 1250 cells were scored per point. The number of GFP-expressing cells labeled with the TUNEL marker was determined using a Green/Orange V2 filter set (Chroma) that allowed simultaneous visualization of both fluorophores. Combined counts were analyzed by the χ2 test. Efficiency of cotransfection was determined to be greater than 85% in parallel experiments using a combination of GFP and DsRed.
mutant p21-S153A constructs were phosphorylated in vitro consisting of amino acids 143–154 is shown by two-dimensional peptide mapping. The peptide containing Ser-153 and recombinant Mirk, digested with trypsin, and then subjected to two-dimensional peptide mapping following digestion of the phosphorylated p21 protein by trypsin. Three phosphopeptides were derived from wild-type p21 after in vitro phosphorylation by Mirk, but only one phosphopeptide was seen after phosphorylation of the mutant p21-S153A construct (Fig. 3A). The slower migrating peptide on chromatography (Fig. 3A, long arrow) was probably a partial digestion product. The tryptic peptide containing serine 153 consists of amino acids 143–154 and is flanked by two KRR sequences, so trypsin could cleave at multiple points within both of these regions. Alternative cleavage may also have been promoted by steric hindrance resulting from phosphorylation of Ser-153. Supporting this interpretation was the observation that the relative abundance of the two peptides varied in different experiments. However, the same general pattern was seen in three separate experiments, demonstrating that Mirk phosphorylates p21 at serine 153.

**In Vivo Phosphorylation of FLAG-p21 by Cotransfected Mirk Occurs at Ser-153 in Differentiating Myoblasts**—In vivo phosphorylation is known to be more specific than in vitro phospho-

`FIG. 3. Mirk phosphorylates p21 at Ser-153 in vitro and in differentiating myoblasts. A, Mirk phosphorylates p21 at Ser-153 in vitro as shown by peptide mapping with trypsin. Wild-type (Wt) and mutant p21-S153A constructs were phosphorylated in vitro by purified recombinant Mirk, digested with trypsin, and then subjected to two-dimensional peptide mapping. The peptide containing Ser-153 and consisting of amino acids 143–154 is shown by a short arrow in both panels. The peptide identified by the longer arrow may be a partial digestion product containing Ser-153, due to steric interference of the enzyme by the phosphate group. Similar data were seen in two additional peptide maps. B, Mirk phosphorylates p21 at Ser-153 in vitro as shown by peptide mapping with chymotrypsin. Wild-type FLAG-p21, mutant p21-S153A, and mutant p21-S130A were each cotransfected with wild-type Mirk into C2C12 cells. Constructs were allowed to express overnight, and cells were then placed in myoblast differentiation medium for 24 h, the last 4 h of which cells were labeled with [32P]orthophosphate. In one dish transfected with wild-type p21, 10 μM SB203580 was added at the same time as the label. FLAG-p21 was immunoprecipitated with anti-FLAG M2 antibody, tryptic digestes were digested with chymotrypsin and then subjected to two-dimensional peptide mapping. The p21-S153A mutant incorporated about 40% as much label as wild-type p21 when normalized to the total amount of p21 immunoprecipitated. Chymotrypsin was used because it yields a simpler map with a broader distribution of peptides. The peptide indicated by the arrow is lost in the p21-S153A mutant and is the presumed 152–159-amino-acid peptide. C, depletion of endogenous Mirk by RNA interference blocks the phosphorylation of transfected wild-type p21 but not p21-S153A mutant at the Mirk phosphorylation site. Cells were plated at 5 × 10^5 per 60-mm dish, cultured for 16 h, and then were transfected for 4 h in serum-free medium with 2.5 μg of either FLAG-p21 or FLAG-p21-S153A, and 2.5 μg of pSilencer DNA encoding either RNAi to Mirk (RNAi) or vector. As controls, FLAG-p21 was cotransfected with either wild-type Mirk (M) or kinase-inactive YF-Mirk (YF). Fetal bovine serum was then added to 10% to maintain cell viability during overnight expression, and cells were switched to Opti-MEM differentiation medium for 24 h, with 325 μCi of [32P]orthophosphate added to each dish for the last 4 h following 1 h in phosphate-free medium. Anti-FLAG epitope immunoprecipitates were separated by SDS-PAGE, and the FLAG-p21 bands were detected by autoradiography (top lanes) and then by Western blotting for FLAG. The amounts of p21 and Mirk in the lysates were determined by Western blotting (lower 2 bands). D, SB203580 inhibits myoblast differentiation as measured by the level of expression of the myogenenic regulatory factor myogenin that controls the differentiation program. C2C12 cells were transfected with either mutant FLAG-p21-S153A or wild-type FLAG-p21, and constructs were allowed to express for 24 h. Cycloheximide at 20 μg/ml was added to each culture, and FLAG-p21 and tubulin abundances were determined at the indicated times by Western blotting and normalized to the 0 time values. Mean ± S.E. is shown from two separate experiments.

The peptide identified by the longer arrow may be a partial digestion product containing Ser-153, due to steric interference of the enzyme by the phosphate group. Similar data were seen in two additional peptide maps. B, Mirk phosphorylates p21 at Ser-153 in vitro as shown by peptide mapping with chymotrypsin. Wild-type FLAG-p21, mutant p21-S153A, and mutant p21-S130A were each cotransfected with wild-type Mirk into C2C12 cells. Constructs were allowed to express overnight, and cells were then placed in myoblast differentiation medium for 24 h, the last 4 h of which cells were labeled with [32P]orthophosphate. In one dish transfected with wild-type p21, 10 μM SB203580 was added at the same time as the label. FLAG-p21 was immunoprecipitated with anti-FLAG M2 antibody, tryptic digestes were digested with chymotrypsin and then subjected to two-dimensional peptide mapping. The p21-S153A mutant incorporated about 40% as much label as wild-type p21 when normalized to the total amount of p21 immunoprecipitated. Chymotrypsin was used because it yields a simpler map with a broader distribution of peptides. The peptide indicated by the arrow is lost in the p21-S153A mutant and is the presumed 152–159-amino-acid peptide. C, depletion of endogenous Mirk by RNA interference blocks the phosphorylation of transfected wild-type p21 but not p21-S153A mutant at the Mirk phosphorylation site. Cells were plated at 5 × 10^5 per 60-mm dish, cultured for 16 h, and then were transfected for 4 h in serum-free medium with 2.5 μg of either FLAG-p21 or FLAG-p21-S153A, and 2.5 μg of pSilencer DNA encoding either RNAi to Mirk (RNAi) or vector. As controls, FLAG-p21 was cotransfected with either wild-type Mirk (M) or kinase-inactive YF-Mirk (YF). Fetal bovine serum was then added to 10% to maintain cell viability during overnight expression, and cells were switched to Opti-MEM differentiation medium for 24 h, with 325 μCi of [32P]orthophosphate added to each dish for the last 4 h following 1 h in phosphate-free medium. Anti-FLAG epitope immunoprecipitates were separated by SDS-PAGE, and the FLAG-p21 bands were detected by autoradiography (top lanes) and then by Western blotting for FLAG. The amounts of p21 and Mirk in the lysates were determined by Western blotting (lower 2 bands). D, SB203580 inhibits myoblast differentiation as measured by the level of expression of the myogenenic regulatory factor myogenin that controls the differentiation program. C2C12 cells were transfected with either mutant FLAG-p21-S153A or wild-type FLAG-p21, and constructs were allowed to express for 24 h. Cycloheximide at 20 μg/ml was added to each culture, and FLAG-p21 and tubulin abundances were determined at the indicated times by Western blotting and normalized to the 0 time values. Mean ± S.E. is shown from two separate experiments.
rlation, especially on a substrate such as p21, which exists in vivo, in several different contexts, and is only found as a free molecule immediately after synthesis (31). In fact, in our previous study, p38 MAPK phosphorylated p21 strongly at Thr-57 and weakly at Ser-130 in vitro, but in colon carcinoma cells p38 MAPK only phosphorylated p21 at Ser-130 (23). Mirk was cotransfected into proliferating myoblasts with either wild-type FLAG-p21, the Mirk site mutant FLAG-p21-S153A, or the p38 site mutant FLAG-p21-S130A. The cells were then switched to differentiation media for 24 h and labeled with $[^{32}P]$orthophosphate for the last 4 h. The immunoprecipitated p21 constructs were digested with chymotrypsin before phosphopeptide mapping. Trypsin digestion yields 11, and only 6 of these peptide fragments contain either serine or threonine residues, resulting in a much simpler pattern. Additionally, the position of the peptide containing the p38 MAPK phosphorylation site on p21 is readily identifiable in the chymotrypsin map (23). Mirk was found to phosphorylate p21 at Ser-153 in differentiating myoblasts, in accordance with Mirk’s in vitro phosphorylation of p21. Mutation of p21 to S153A at the Mirk phosphorylation site blocked phosphorylation in vivo. FLAG-p21 was phosphorylated on three phosphopeptides in vivo, whereas FLAG-p21-S153A was phosphorylated on only two phosphopeptides (Fig. 3B). The peptide containing Ser-153 is indicated by an arrow in each panel of Fig. 3B. This peptide consists of amino acids 152–159 and contains only one serine and no threonines. Mutation of this peptide at serine 153 to alanine blocked its in vivo phosphorylation. Therefore, p21 is phosphorylated at Ser-153 in differentiating myoblasts by Mirk.

Knockdown of endogenous Mirk by RNAi blocked phosphorylation of wild-type FLAG-p21, but not FLAG-p21-S153A, mutant at the Mirk phosphorylation site (Fig. 3C), confirming that Mirk phosphorylates p21 at Ser-153 in differentiating myoblasts in vivo. C2C12 cells were transfected with either wild-type or mutant p21 together with the pSilencer plasmid for Mirk RNAi or the vector alone. Parallel cultures were transfected with wild-type p21 and either wild-type Mirk or kinase-inactive YF-Mirk. Cells in DM were metabolically labeled with $[^{32}P]$orthophosphate. The p21 constructs were immunoprecipitated by their FLAG epitopes, and the extent of incorporation of the label was determined by autoradiography after SDS-PAGE. Ectopic Mirk phosphorylated FLAG-p21 in vivo, whereas the kinase-inactive YF-Mirk did not (Fig. 3C, 1st 2 lanes), and served as the positive and negative controls, respectively. Endogenous Mirk in the differentiating C2C12 cells phosphorylated wild-type p21 but not mutant p21-S153A (Fig. 3C, 3rd and 4th lanes), although similar amounts of p21 were immunoprecipitated. In the cultures in which Mirk knockdown was accomplished (Fig. 3C, last 2 lanes), neither wild-type Mirk nor the p21-S153A mutant was phosphorylated much above background levels. Therefore, endogenous Mirk phosphorylates p21 at Ser-153 in vivo in differentiating myoblasts.

Mirk and p38 MAPK interact in vivo. Mirk is activated by the p38 MAPK kinase MKK3 (12), and p38 MAPK can sequester Mirk in vivo (13). Thus, we questioned whether phosphorylation of p21 by p38 MAPK occurred in differentiating myoblasts, as it does in colon carcinoma cells (23), and if so, whether it was an essential prerequisite for phosphorylation of p21 by Mirk. In one set of cultures in the peptide mapping studies (Fig. 3B), 10 μM SB203580 was added to inhibit p38 MAPK. During myoblast differentiation, p38 MAPK is activated and in turn activates the myogenic regulatory factors MEF2A, MEF2C, and MyoD (32–34). However, the in vivo peptide mapping studies (Fig. 3B) show that p38 MAPK does not appear to phosphorylate p21 in differentiating myoblasts, at least within the time frame of these experiments. Mutation of p21 at the p38 MAPK site of Ser-130 and treatment of cells with the p38 inhibitor SB203580 did not eliminate any of the three phosphorylated peptides. In addition, the Ser-153-containing peptide was phosphorylated in the FLAG-p21-S130A mutant construct to a similar extent as in wild-type p21, demonstrating that phosphorylation of p21 at Ser-130 is not an essential prerequisite for phosphorylation by Mirk (Fig. 3B). The SB203580 preparation used in these experiments was active because exposure of C2C12 cells in DM for 1 or 2 days to this drug prevented induction of myogenin, an essential myogenic regulatory factor, whose transcription is mediated by the p38 MAPK substrate MEF2 (Fig. 3D). Treatment of differentiating C2 myoblasts with SB203580 at 5 μM for 48 h has been reported to block the kinase activity of p38 on the exogenous substrate myelin basic protein but not to block the phosphorylation of p38 (35). Other investigators as well as ourselves (data not shown) have also noted this discrepancy. However, our studies do not entirely depend on the use of the inhibitor. There was no loss in phosphorylation of the p38 site mutant, p21-S130A, during myoblast differentiation because the peptide containing this mutant sequence was as equally phosphorylated as the wild-type peptide (Fig. 3B). Therefore, we could detect no phosphorylation of p21 by p38 MAPK during this initial period of myogenic differentiation.

The phosphorylation of p21 by p38 MAPK at Ser-130 increased the stability of p21 in colon carcinoma cells (23). However, phosphorylation of p21 by Mirk did not stabilize p21 in C2C12 cells. In fact, p21-S153D was slightly less stable than wild-type p21, as demonstrated by cycloheximide arrest experiments (Fig. 3E). Therefore, neither Mirk nor p38 MAPK stabilizes p21 during the initial stages of myoblast differentiation. Furthermore, at this stage in differentiation, when Mirk acts as a survival factor for myoblasts, only Mirk, not p38 MAPK, phosphorylates p21.

**Table I**

| BrdUrd incorporation in C2C12 cells expressing GFP-p21 constructs | None | Vector | p21(WT) | p21(S153D) |
|---|---|---|---|---|
| GFP | 910 | 973 | 992 | 935 |
| GFP + BrdUrd | 272 | 292 | 23 | 18 |
| % S phase | 29.9 | 30.0 | 2.5 | 1.9 |

*P < 0.0001*
Mirk Blocks Apoptosis through Translocation of p21

A. Cycling C2C12 Myoblasts

GFP-p21  DAPI  Merge

WT

S153A

S153D

B. Cycling NIH/3T3 Fibroblasts

GFP-p21  DAPI  Merge

WT

S153A

S153D

FIG. 4. Fluorescence microscopy demonstrated that the Mirk phosphorylation site phosphomimetic mutant p21-S153D is found in both the cytoplasm and the nucleus, whereas wild-type p21 and the nonphosphorylatable p21-S153A are localized exclusively in the nucleus in the majority of cells. GFP-p21-wild-type, GFP-p21-S153A which is not phosphorylated by Mirk, and GFP-p21-S153D, the Mirk site-phosphomimetic construct, were transiently expressed in C2C12 cells (A, ×400 magnification) and in NIH3T3 cells (B, ×1000 magnification). Nuclei were stained with DAPI, and identical fields were photographed for GFP and DAPI. Images were merged with SPOT RT software. Scale bars = 50 μm.

| TABLE II |
| Localization of GFP-p21 constructs in C2C12 cells and NIH3T3 cells |
| Cycling C2C12 myoblasts |
| WT | S153A | S153D |
| Nucleus + cytoplasm | 246 | 200 | 998 |
| Nucleus only | 1022 | 1082 | 278 |
| % nucleus + cytoplasm | 19.4 | 15.6 | 78.3 |
| (p < 0.02) | (p < 0.0001) |
| NIH3T3 fibroblasts |
| WT | S153A | S153D |
| Nucleus + cytoplasm | 164 | 141 | 420 |
| Nucleus only | 453 | 488 | 209 |
| % nucleus + cytoplasm | 26.6 | 22.4 | 66.8 |
| (p < 0.0001) |

More Wild-type p21 Is Found in the Cytoplasm When Mirk Is Induced during Myoblast Differentiation—The localization studies described above (Figs. 4 and 5) were performed in cycling myoblasts. We next transfected the same three GFP-p21 constructs into C2C12 cells, and we then stimulated them to differentiate. Only the fused myoblasts were examined, and trials had demonstrated that GFP-p21 is always localized in the nucleus in this system, we used a one-tailed analysis to compare the proportion of transfected cells expressing the GFP constructs in both the cytoplasm and the nucleus to the proportion of cells expressing GFP-p21 exclusively in the nucleus. GFP-p21-WT was found in the cytoplasm in only 19.4% of myoblasts. Four times as many cells, 78.3%, expressed the Mirk-phosphomimetic GFP-p21-S153D construct in the cytoplasm. This gave a highly significant (p < 0.0001) difference in localization. In contrast, the nonphosphorylatable GFP-p21-S153A construct remained localized in the nucleus in myoblasts, with only 15.6% of the cells expressing this construct in the cytoplasm, giving a statistically significant value of p < 0.02 compared with wild-type p21. Similar results were obtained with NIH3T3 cells (Table II). Wild-type p21 was found in the cytoplasm of only 26.6% of transfected fibroblasts, whereas over twice as many transfected fibroblasts expressed the Mirk-phosphonimetic GFP-p21-S153D construct in the cytoplasm. This was also a highly significant difference in localization (p < 0.0001). The nonphosphorylatable GFP-p21-S153A construct remained localized in the nucleus in fibroblasts, with only 22.4% of the cells containing this construct in the cytoplasm, a value that was not significantly different from cells expressing wild-type p21. These data clearly demonstrate that mutation of p21 to mimic phosphorylation by Mirk caused a much larger proportion of the p21 to localize in the cytoplasm in both myoblasts and fibroblasts. Mutation of an additional serine in the nuclear localization signal (Ser-160 to S160A or S160D) did not affect the cellular distribution of GFP-p21 (data not shown).

Ectopic Mirk Translocates GFP-p21 to the Cytoplasm through Phosphorylation at Ser-153—NIH3T3 fibroblasts were transfected with ectopic wild-type Mirk or kinase-inactive YFP-Mirk and either wild-type GFP-p21 or GFP-p21-S153A. The cells expressing exogenous p21 and exogenous Mirk constructs were visualized by immunofluorescence. Cells expressing both constructs were visualized by using a Green/Orange V2 filter set, and the percentage of such cells in which p21 was pancellular (versus restricted solely to the nucleus) was twice as large as the controls (Fig. 5). χ² analysis showed this to be a highly significant difference (p < 0.0001). Expression of elevated levels of ectopic Mirk was sufficient to make the majority of wild-type p21 pancellular, while having no effect on the p21 mutant at the Mirk site (S153A). Moreover, the p21 translocation was dependent on the kinase activity of Mirk.

H11021 p 0.02) (H11021 p 0.0001) difference in localization.

h11021 p 0.0001) (H11021 p 0.0001) difference in localization.

h11021 p 0.0001) difference in localization.

h11021 p 0.0001) difference in localization.

h11021 p 0.0001) difference in localization.

h11021 p 0.0001) difference in localization.

h11021 p 0.0001) difference in localization.

h11021 p 0.0001) difference in localization.


counts were analyzed by the allowed simultaneous visualization of both fluorophores. Combined Mirk was determined using a Green/Orange V2 filter set (Chroma) that
tions, and the number of cells labeled for both GFP-p21 and transfected
were scored in 8–10 random fields in each of three separate prepara-
GFP-p21 expressing cells were observed for each transfectant. Cells
alyzed by using rabbit polyclonal antibody to the Mirk C terminus at
8334; 1:500), followed by anti-mouse Alexa Fluor 488. Mirk was visu-
serum. After 24 h of expression, cells were fixed with 4% paraformal-
Mirk. Cells were transfected in DMEM containing 10% bovine calf
YF-M
YF-M
YF-M
S153A, together with either wild-type (M) or kinase inactive (YF-M)
Mirk. Cells were transfected in DMEM containing 10% bovine calf serum. After 24 h of expression, cells were fixed with 4% paraformaldehyde, and the GFP-p21 protein was labeled by a 30-min incubation
in paraffin sections of human muscle
even after antigen retrieval with citrate, EDTA, or proteolytic digestion. This result is not unexpected given that p21 is normally found in complexes, where the epitope may not be accessible to the antibody, particularly after formalin fixation. We therefore performed immunofluorescence on frozen sections of adult human muscle. We were able to detect p21 in adult muscle using the C-19 antibody at a dilution of 1:200, followed by goat anti-rabbit Alexa Fluor 594 at 1:500 (Fig. 8). The distribution of p21 in the adult muscle fibers paralleled our observations of p21 localization in myotubes. p21 remained concentrated in the nucleus but was distributed throughout the myofibrils. We were also able to see this pattern using the F-5 anti-p21 antibody, thus demonstrating the specificity of our immunolabeling results (data not shown). Therefore, examination of human skeletal muscle tissue confirmed that endogenous p21 partitioned into the cytoplasm as a natural part of muscle differentiation.

Changes in the Localization of Endogenous p21 during Differen-
tiation of C2C12 Myoblasts—The data thus far confirmed that a significant proportion of exogenous p21 is localized in the cytoplasm of differentiating myoblasts following phosphorylation by Mirk. We next investigated the localization of endogenous p21 during differentiation of myoblasts. C2C12 myoblasts were cultured for 1 day in growth medium (GM), and then switched to differentiation medium (DM). After 24 h in GM or 48 h in DM, cells were fixed in paraformaldehyde, and endogenous p21 was then visualized with the monoclonal antibody F-5. This antibody was raised against full-length p21, so antibody binding should not be affected by phosphorylation at Ser-153 in the C terminus (Fig. 6). As an additional control, p21 was labeled in separate experiments using the polyclonal antibody C-19 (data not shown). The anti-p21 antibody was de-
was labeled in separate experiments using the polyclonal an-
tibody C-19 (data not shown). The anti-p21 antibody was de-
tected with goat anti-mouse secondary antibody conjugated to green fluorescent Alexa Fluor 488. Cells were also incubated with Alexa Fluor 594 coupled to phalloidin to detect actin and DAPI for nuclear staining. All of the fields were merged to form the composite shown in the right-most column Fig. 7.

Endogenous p21 was exclusively localized in the nucleus in proliferating myoblasts in growth medium. The signals for p21 and for DAPI completely coincided (Fig. 7, top 2 rows). When myoblasts were cultured for 2 days in differentiation medium, however, p21 was found in the cytoplasm as well as the nucleus in fused myoblasts (Fig. 7, bottom two rows). Note in particular in the Merge column of Fig. 7, the large myotubes that are stained with actin to delineate the entire cell body and with DAPI to delineate nuclei. 11 nuclei were found in the myotube in the 3rd row, whereas four nuclei were found in the myotube in the 4th row. Both of these myotubes show extensive localization of endoge-
nous p21 in their cytoplasm (Fig. 7, p21 column, rows 3 and 4). These data clearly demonstrate that localization of endogenous p21 in the cytoplasm occurs as a natural part of myoblast differentiation. Thus the increase in concentration of the exogenous FLAG-p21 within the cytoplasm of differentiating myotubes (Fig. 6) is reflected in a similar cytoplasmic enrichment of the endogenous p21.

Endogenous p21 Is Found within the Cytoplasm of Adult Human Muscle—p21 is known to play critical roles in early muscle differentiation (18) and in the maintenance of adult muscle tissue through the muscle progenitor (satellite) cell population (19). However, there do not appear to be any exten-
sive reports describing the presence or function of p21 in normal mature muscle. Therefore, we performed SAGE analysis using the NCBI/GEO data base, and we found that p21 is expressed to a higher extent in muscle biopsies from young individuals, aged 21–31 years old, than in biopsies from most older patients, aged 62–77 (GDS156), but that all skeletal muscle biopsies contained p21 mRNA. We were unable to de-
tect p21 in formalin-fixed paraffin sections of human muscle even after antigen retrieval with citrate, EDTA, or proteolytic digestion. This result is not unexpected given that p21 is normally found in complexes, where the epitope may not be accessible to the antibody, particularly after formalin fixation. We therefore performed immunofluorescence on frozen sections of adult human muscle. We were able to detect p21 in adult muscle using the C-19 antibody at a dilution of 1:200, followed by goat anti-rabbit Alexa Fluor 594 at 1:500 (Fig. 8). The distribution of p21 in the adult muscle fibers paralleled our observations of p21 localization in myotubes. p21 remained concentrated in the nucleus but was distributed throughout the myofibrils. We were also able to see this pattern using the F-5 anti-p21 antibody, thus demonstrating the specificity of our immunolabeling results (data not shown). Therefore, examination of human skeletal muscle tissue confirmed that endogenous p21 partitioned into the cytoplasm as a natural part of muscle differentiation.

Phosphomimetic p21-S153D is More Effective than Wild-type p21 in Blocking Caspase 3 Activation—Caspase 3 is the pri-
mary effector caspase in apoptosis. Caspase 3 is activated by cleavage of its precursor, procaspase 3. C2C12 myoblasts were transiently transfected with either wild-type FLAG-p21, the phosphomimetic FLAG-p21-S153D, the nonphosphorylatable FLAG-p21-S153A, or vector alone. The constructs were allowed to express overnight; the cells were then cultured in differen-
tiation medium for 16 h, and the amount of active caspase 3, released by cleavage, was determined by Western blotting. Wild-type p21 caused a small 10% inhibition in the amount of activated caspase 3, whereas the Mirk phosphomimetic p21-S153D construct blocked caspase 3 activation five times as much (Fig. 9). In contrast, little inhibition by the nonphosphoryl-
latable construct was observed. In addition, the phosphomimetic p21 construct communoprecipitated with ASK1 (data not shown), one upstream activator of this caspase cascade. ASK1 has been shown to be sequestered by wild-type p21 in other studies (22). Therefore, one mechanism by which Mirk blocks apoptosis in myoblasts is through phosphorylation of
A subpopulation of the p21 molecules is then retained in the cytoplasm where it blocks apoptosis through inhibition of the activation of caspase 3.

**Transient Expression of Phosphomimetic p21 Aids Myoblast Survival**—C2C12 cells were transiently transfected with either wild-type FLAG-p21, the phosphomimetic FLAG-p21-S153D, or the nonphosphorylatable FLAG-p21-S153A, and the length of time each construct was expressed was determined (Fig. 10A). After 24 h of transfection and expression, the cells were placed in growth medium for 0–13 days. All of the constructs expressed for only about 1 day in growth medium (Fig. 10A) and then were lost. We used this transient expression to determine whether the phosphomimetic p21 construct could act as a survival factor in myoblasts at the time of plating at very low cell density for colony formation assays (Fig. 10B). The same transfection protocol was used, except that a vector control was included. Transient expression of the phosphomimetic p21-S153D construct, and to a lesser extent the wild-type p21, increased myoblast colony formation (Fig. 10B), a reflection of increased survival during the initial day on which cells were plated.

**Fig. 6.** When Mirk is up-regulated as a part of the myoblast differentiation program, both wild-type p21, as well as the Mirk-phosphorylation site phosphomimetic mutant p21-S153D, are translocated to the cytoplasm as shown by fluorescence microscopy. In contrast, the nonphosphorylatable p21-S153A is localized exclusively in the nuclei of the multinucleated myotubes. GFP-p21-wild-type, GFP-p21-S153A, and GFP-p21-S153D were transfected into C2C12 cells, which were then allowed to differentiate for 3 days. Similar data were seen in each of four experiments.

**Fig. 7.** Endogenous p21 is exclusively nuclear in cycling C2C12 cells in growth medium, but a portion of the endogenous p21 protein is translocated to the cytoplasm when C2C12 cells are treated for 48 h with differentiation medium. Endogenous p21 was visualized with the F-5 monoclonal antibody and goat anti-mouse antibody conjugated to Alexa Fluor 488; the nuclei were stained with DAPI, and actin within the entire cell body was visualized with phalloidin conjugated to Alexa Fluor 594. Identical fields were photographed for each fluorochrome (1st three rows) and then merged with SPOT RT software. Identical results were obtained with the C-19 polyclonal antibody. Scale bar = 50 μm.

**Fig. 8.** p21 localizes in both the cytoplasm and nucleus of adult skeletal muscle. Frozen sections of human muscle were analyzed for the localization of p21 by immunofluorescence using the polyclonal C-19 antibody (1:200) and goat anti-rabbit IgG Alexa Fluor 594 (1:500); nuclei were counterstained with DAPI. Scale bars = 50 μm.
plated. Transient expression of p21-S153D for only 1–2 days (Fig. 10A) thus inhibits caspase 3 activation during the initial plating period when cells are at very low density and susceptible to apoptosis. Loss of expression of the p21 construct after this period allows the cells to re-enter the cell cycle and grow to form a discernible colony. In contrast, the phosphorylation site mutant p21-S153A had no survival effect compared with the vector (Fig. 10B). Thus phosphorylation of p21 by Mirk enables p21 to oppose apoptosis and to enhance myoblast survival.

**DISCUSSION**

In the current study we have demonstrated that the kinase Mirk mediates cell survival of myoblasts, during both growth and differentiation. These observations complement the known survival function of Mirk in colon carcinoma cells (20). We also show that at least some of the pro-survival functions of Mirk can be ascribed to its phosphorylation of the CDK inhibitor p21 and subsequent increased localization of p21 in the cytoplasm. Cytoplasmic p21 has a role in pro-survival signaling in differentiating myoblasts (Fig. 9) and is maintained in the cytoplasm of mature myofibrils. The anti-apoptotic function of p21 in myogenesis is well documented. In C2 myoblasts, ectopic expression of p21 has been shown to block apoptosis, whereas depletion of p21 by antisense oligonucleotides induces apoptosis (15). A role for cytosolic p21 has also been demonstrated in neuronal differentiation. After functioning in the nucleus to block cell proliferation, cytoplasmic p21 promotes neuronal differentiation where it regulates Rho-induced actin remodeling leading to neurite outgrowth (38). Cytoplasmic p21 has also been shown recently (39) to enhance axonal regeneration and functional recovery after spinal cord injury.

Cell cycle progression is mediated by the sequential activation of members of the CDK protein kinase family. Cell cycling is blocked when cyclin-CDK complexes cannot form or when the catalytic activity of these complexes is blocked through binding of a CDK inhibitor molecule. One of these inhibitors is p21Cip1/Waf1 (40), which was originally identified as a PCNA-binding protein (41). p21 inhibits DNA replication by preventing PCNA from contributing to DNA polymerase δ and ε function (42). The p21 gene is induced by the tumor suppressor p53 (43) and by cellular senescence (44). p21 is a major mediator of the G1 growth arrest induced by activation of the tumor suppressor p53 in response to DNA damage (reviewed in Ref. 45). Other major functions for p21 are to promote assembly of cyclin D-CDK4 complexes and to increase cyclin D1 accumulation by direct inhibition of GSK3β-triggered nuclear export (46, 47).

The cell cycle modulatory activity of p21 is tightly correlated with its nuclear localization. Movement of p21 from the nucleus to the cytoplasm blocks its cell cycle inhibitory activity and contributes to the cell growth induced by the oncogene Her-2/neu (21). Akt activated by Her-2/neu phosphorylates Thr-145 and Ser-146 within the nuclear localization signal of p21 (21, 30, 48). Phosphorylation of p21 by Akt has been shown to promote association with 14-3-3 proteins, which function as nuclear export proteins (49). In the current study, we have
documented that the kinase Mirk, like Akt, also promotes translocation of p21 to the cytoplasm by phosphorylation within the nuclear localization signal of p21. In the case of Mirk, the phosphorylation site is serine 153, close to the Akt phosphorylation site. Moreover, differentiation of myoblasts do remain in G0 arrest, suggesting that the elevated levels of endogenous p21 seen in myotubes are sufficient to enable adequate nuclear levels of p21 to be maintained to keep CDK2 activity in check. These elevated p21 are sufficient to enable adequate nuclear levels of p21 to be

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