Coordinate Control of Muscle Cell Survival by Distinct Insulin-like Growth Factor Activated Signaling Pathways

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Abstract. Peptide growth factors control diverse cellular functions by regulating distinct signal transduction pathways. In cultured myoblasts, insulin-like growth factors (IGFs) stimulate differentiation and promote hypertrophy. IGFs also maintain muscle cell viability. We previously described C2 skeletal muscle lines lacking expression of IGF-II. These cells did not differentiate, but underwent progressive apoptotic death when incubated in differentiation medium. Viability could be sustained and differentiation enabled by IGF analogues that activated the IGF-I receptor; survival was dependent on stimulation of phosphatidylinositol 3-kinase (PI3-kinase). We now find that IGF action promotes myoblast survival through two distinguishable PI3-kinase–regulated pathways that culminate in expression of the cyclin-dependent kinase inhibitor, p21. Incubation with IGF-I or transfection with active PI3-kinase led to rapid induction of MyoD and p21, and forced expression of either protein maintained viability in the absence of growth factors. Ectopic expression of MyoD induced p21, and inhibition of p21 blocked MyoD-mediated survival, thus defining one PI3- kinase–dependent pathway as leading first to MyoD, and then to p21 and survival. Unexpectedly, loss of MyoD expression did not impede IGF-mediated survival, revealing a second pathway involving activation by PI3-kinase of Akt, and subsequent induction of p21. Since inhibition of p21 caused death even in the presence of IGF-I, these results establish a central role for p21 as a survival factor for muscle cells. Our observations also define a MyoD-independent pathway for regulating p21 in muscle, and demonstrate that distinct mechanisms help ensure appropriate expression of this key protein during differentiation.

Key words: insulin-like growth factors • p21 • MyoD • phosphatidylinositol 3-kinase • Akt

Introduction

The insulin-like growth factors (IGFs),1 IGF-I and IGF-II, comprise with insulin a structurally related family of peptides of fundamental importance for normal somatic growth, for intermediary metabolism, and for the survival, proliferation, and terminal differentiation of many different cell types (Jones and Clemmons, 1995; Stewart and Rotwein, 1996a; Baserga et al., 1997). The biological effects of these proteins are mediated by a pair of related receptors. Both the IGF-I and insulin receptors are heterotetrameric, membrane-spanning, tyrosine protein kinases that activate a number of shared intracellular signal transduction pathways upon ligand binding (LeRoith et al., 1995; Virkamaki et al., 1999). Insulin functions primarily as a hormone of intermediary metabolism, being synthesized in the beta cells of the endocrine pancreas, and reaching its sites of action in liver, muscle, fat, and other cell types through the circulation (Virkamaki et al., 1999). By contrast, IGFs function primarily as growth, survival, and differentiation factors, and reach target tissues through the circulation, or through local synthesis near sites of action (Jones and Clemmons, 1995; Stewart and Rotwein, 1996a).

IGF action plays key roles in the formation and maintenance of skeletal muscle. Mice engineered to lack the IGF-I receptor, or deficient in IGF-I and IGF-II, exhibit marked muscle hypoplasia and die in the neonatal period because of inadequate muscle mass to inflate their lungs (Liu et al., 1993; Powell-Braxton et al., 1993). Conversely, mice with enhanced expression of IGF-I in muscle develop enlarged myofibers (Coleman et al., 1995; Barton-Davis et al., 1998). In cultured skeletal muscle, activation of the IGF-I receptor stimulates terminal differentiation through an autocrine pathway dependent on expression of IGF-II (Florini et al., 1991; Montarras et al., 1996; Tollefsen et al., 1989a,b). Endogenously produced IGF-II also plays an important role in maintaining cell survival during the transition from proliferating to terminally differentiating myoblasts (Stewart...
and Rotwein, 1996b; Lawlor et al., 2000). The signal transduction pathways and mechanisms involved in IGF-mediated muscle cell survival and differentiation have not been completely elucidated. Recent studies have indicated that two classes of intracellular signaling molecules, phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein kinases, are involved in muscle differentiation (Kaliman et al., 1996, 1998; Bennett and Tonks, 1997; Coolican et al., 1997; Sarbassov et al., 1997; Gredinger et al., 1998; Jiang et al., 1998; Sarbassov and Peterson, 1998; Cuenda and Cohen, 1999; Musaro and Rosenthal, 1999; Rommel et al., 1999; Zetser et al., 1999; Tamir and Bengal, 2000; Wu et al., 2000), with the PI3-kinase pathway being considered more critical. At present, the mechanisms have not been established by which these signaling molecules or other pathways activated by the IGF-I receptor might collaborate with myogenic regulatory factors to regulate muscle cell viability or differentiation.

The muscle-specific basic helix-loop-helix (bHLH) transcription factors, MyoD, myogenin, MRF4, and Myf5, were initially identified as master regulators of cell fate because of their ability to confer a skeletal muscle phenotype on nonmuscle cells (Weintraub, 1993; Olson and Klein, 1994). These proteins function by activating genes that are required for muscle determination and/or differentiation through the formation of heterodimers with ubiquitous bHLH proteins, and subsequent binding to specific sequences termed E boxes in the promoter-regulatory regions of muscle-restricted target genes (Weintraub, 1993; Olson and Klein, 1994). One of the targets of MyoD is the gene encoding the cyclin-dependent protein kinase inhibitor, p21, also known as Waf1 and Cip1 (Ball, 1997; El-Deiry, 1998). This protein, and related molecules, p27/Kip1 and p57/Kip2 (Ball, 1997), act to block progression through the cell cycle by reversibly inhibiting complexes of several different cyclins and cyclin-dependent kinases (Elledge, 1996). In cultured muscle cells, p21 expression is induced as an early event during differentiation (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995; Mal et al., 2000). The increase in p21 is temporally associated with an ongoing decline in cyclin-dependent kinase activity as differentiation proceeds (Guo et al., 1995; Mal et al., 2000), and p21 has been found to become part of a complex containing cyclin E and cdk2 in differentiating C2 muscle cells (Mal et al., 2000). In addition, induction of p21 has been shown to correlate with development of an apoptosis-resistant phenotype during differentiation (Wang and Walsh, 1996). The significance of p21 action in skeletal muscle is underscored by observations that high levels of p21 mRNA are detected in early muscle fibers in the mouse embryo (Parker et al., 1995), and that mice deficient in both p21 and p57 have defective muscle formation and exhibit increased rates of myoblast apoptosis (Zhang et al., 1999). It generally has been accepted that MyoD is a key transcription factor regulating p21 gene expression during muscle differentiation. MyoD has been found to enhance activity of the p21 promoter in transient transfection experiments (Haley et al., 1995), and to stimulate p21 mRNA and protein accumulation in muscle cells and fibroblasts (Guo et al., 1995; Haley et al., 1995; Parker et al., 1995), including cells lacking p53, the tumor suppressor protein that also can activate the p21 gene (Haley et al., 1995).

In this manuscript, we address the question of how IGF action promotes muscle cell survival through study of C2 myoblasts and a derived cell line that lacks expression of IGF-II (Stewart and Rotwein, 1996b). These cells undergo rapid apoptotic death when incubated in low serum differentiation medium (Stewart and Rotwein, 1996b; Lawlor et al., 2000; Lawlor and Rotwein, 2000). Addition of IGF-I or analogues that activate the IGF-I receptor maintain cell viability, but this is reversed by inhibitors of PI3-kinase (Lawlor et al., 2000). We now find that IGF action promotes myoblast survival by two different PI3-kinase-dependent pathways that converge on p21. Incubation with IGF-I, or transfection with active PI3-kinase, leads to rapid induction of MyoD and p21, and ectopic expression of either protein sustains muscle cell survival in the absence of growth factors. Forced expression of MyoD induces p21, and inhibition of p21 expression blocks MyoD-mediated survival, thus defining one pathway as leading through PI3-kinase to MyoD, and then to p21 and survival. The second IGF-stimulated pathway involves activation of the serine-threonine kinase, Akt, a protein that has been shown to play a key role in the survival of many different cell types (Datta et al., 1999). We find that Akt induces p21 in myoblasts by a mechanism that does not require MyoD. Since inhibition of p21 causes apoptotic death even in the presence of IGF-I, our results establish a central role for p21 as a survival factor for muscle cells, and demonstrate that distinct but integrated mechanisms help ensure the appropriate expression of this key protein during myogenic differentiation.

Materials and Methods

Materials

Fetal calf serum, newborn calf serum, horse serum, Dulbecco's modified Eagle's medium, phosphate buffered saline, G418, PDGF-BB, and TRIZol were purchased from Life Technologies. The long-lasting IGF-I analogue, R1-IGF-I, was from Groeppe. Effectene was purchased from QIAGEN. Restriction enzymes, ligases, and polymerases were from New England Biolabs. The pEGFP-N3 plasmid was purchased from CLONTECH Laboratories, Inc. Protease inhibitor tablets were from Roche Molecular Biochemicals. The BCA protein assay kit was from Pierce Chemical Co. Antibodies to p21 and cdk4 were used for immunoblotting were purchased from Santa Cruz Biotechnology, Inc. The antibody to MyoD was a gift from Dr. Peter J. Houghton (St. Jude Children's Research Hospital, Memphis, TN). Secondary antibodies were from Sigma-Aldrich. Antibodies to MyoD and p21 used for immunocytochemistry were obtained from Santa Cruz Biotechnology, Inc. and PharMingen, respectively. The antibody to influenza hemagglutinin (HA) was from BabCO, and the anti-myc antibody (clone 9E-10) was from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Secondary antibodies conjugated to fluorophores were purchased from Molecular Probes. Nitrocellulose was from Schleicher & Schuell, and ECL reagents from Amersham-Pharmacia Biotech. X-ray film was purchased from Eastman Kodak Co. All other chemicals were reagent grade.

Cell Culture

C2 myoblasts stably transfected with the coding region of a mouse IGF-II cDNA in the antisense orientation (C2AS12 cells; Stewart and Rotwein, 1996b; Lawlor et al., 2000) were grown until >95% confluent on gelatin-coated tissue culture dishes in DMEM supplemented with 10% heat-inactivated FCS, 10% heat-inactivated newborn calf serum, 2 mM L-glutamine, and 400 μg/ml G418 (active drug) (growth medium). C2 myoblasts (Yaffe and Saxel, 1977) were grown until >95% confluent on gelatin-coated plates in growth medium minus G418. For both cell lines, differentiation was initiated after washing with PBS by incubating in differentia-
tion medium (DM) containing DMEM plus 2% horse serum, or in DM supplemented with 0.4 mM PDGF-BB or 2 nM IGF-I. At different intervals, adherent cells were trypsinized and counted by hemocytometer or by Coulter particle counter. Cos7 cells were grown in DMEM supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine.

RNA Isolation and Ribonuclease Protection Assays

Total RNA was isolated from cells using TRIzol and quantitated by spectrophotometry. RNA integrity was assessed by electrophoresis though 1% agarose-formaldehyde gels after staining with ethidium bromide. Solution hybridization ribonuclease protection assays were performed as described (Stewart et al., 1996), using single stranded [32P]CTP-labeled antisense riboprobes synthesized from linearized plasmid templates. Results were quantitated with a PhosphorImager (GS 525; Bio-Rad Laboratories).

Protein Isolation and Immunoblotting

Protein extracts were isolated after washing cells twice with cold PBS by incubating for 30 min at 4°C in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate) containing protease inhibitors, 1 mM PMSF, and 1 mM sodium orthovanadate. After removal of insoluble material by centrifugation at 14,000 rpm for 10 min at 4°C, protein concentration was determined by BCA assay. Protein extracts (60 μg) were separated by SDS-polyacrylamide gel electrophoresis under denaturing and reducing conditions before transfer to 0.2 μm nitrocellulose membranes at 18 V for 45 min using a semidy blotter. Membranes were blocked for 2 h at 25°C in TBST (Tris buffered saline plus 0.1% Tween-20) before being incubated with primary antibody (anti–MyoD undiluted supernatant, anti-p21 1:75, and anti-CDK4 1:500 in blocking buffer). After incubation with horseradish peroxidase–conjugated secondary antibodies (polyclonal anti–myc (1:500) in PBST plus 3% bovine serum albumin for 3–16 h being incubated with primary antibody (anti–MyoD undiluted supernatant, anti-p21 1:75, and anti-CDK4 1:500 in blocking buffer). After incubation with horseradish peroxidase–conjugated secondary antibodies (polyclonal anti–myc (1:500) in blocking buffer), proteins were detected by ECL, followed by exposure to x-ray film. Results were quantitated by densitometry (GS 700; Bio-Rad Laboratories).

Immunocytochemistry

Cells were washed twice with PBS before fixation in 1% paraformaldehyde for 10 min. Cells were then incubated for 5 min in PBS containing 0.2% Triton X-100 (PBST), washed twice with PBST, and incubated with primary antibodies: polyclonal rabbit anti–MyoD (1:500), polyclonal rabbit anti-p21 IgG (1:2,000), monoclonal anti–HA IgG (1:100), or monoclonal anti–myc (1:500) in PBST plus 3% bovine serum albumin for 3–16 h at 4°C. Cells were washed three times with PBST before incubation with polyclonal secondary antibodies, either Alexa 594-tagged goat anti–rabbit IgG (1:2,000), fluorescein-conjugated goat anti–rabbit IgG, or fluorescein-conjugated goat anti–mouse IgG (each 1:1,000), for 45 min in the dark. Images were captured with a fluorescence microscope (Eclipse TE 300; Nikon) and an CCD camera (Optronics) using Scion Image 1.62 software. Images were save in Photoshop 5.5 (Adobe Systems).

Construction of Bicistronic Expression Plasmids

The internal ribosome entry site (IRES) from mouse encephalomyocarditis virus (Ghatast et al., 1991) was subcloned into the polylinker of pEGFP-N3 to generate pIRES-EGFP. An Xhol-BamHI DNA fragment containing the coding region of murine MyoD in the sense orientation was engineered to lack IGF-II expression (C2AS12 cells) under control and Rotwein, 1996b; Lawlor et al., 2000; Lawlor and Rotwein, 2000). As seen in Fig. 1, C2 myoblasts engineered to lack IGF-II expression (C2AS12 cells) underwent rapid death when incubated in DM. Only 48 ± 1% of cells remained alive after 24 h, and only 34 ± 1% survived after 48 h. Viability continued to decline progressively with longer incubations in DM (Lawlor and Rotwein, 2000). Addition of IGF-I caused complete survival (102 ± 3% at 24 h, 98 ± 4% at 48 h). Other growth factors such as PDGF-BB also maintain viability of C2AS12 cells (Lawlor et al., 2000, and data not shown). Nontransfected C2 myoblasts also underwent apoptotic death when incubated in DM, with ∼30% of cells dying at 24 h (see Fig. 7; and Lawlor and Rotwein, 2000). No further death occurred upon longer incubation in DM, with survival correlating to the IRES to generate MyoD-IRES-EGFP. A BamHI-Sall DNA fragment containing the mouse MyoD coding region in the antisense orientation was used to generate MyoDAS-IRES-EGFP. The p21-IRES-EGFP, p21AS-IRES-EGFP, PI3-kinase, and iAkt plasmids have been described (Lawlor et al., 2000; Lawlor and Rotwein, 2000).

Transfections

Myoblasts were plated at 13,000 cells/cm2 onto 12-well tissue culture dishes and incubated for 24 h in growth medium. DNA-mediated gene transfer was performed using Effectene, following the protocol of the manufacturer. A total of 1 μg of DNA of sense plasmids was used per well. For antisense plasmids (MyoDAS-IRES-EGFP and p21AS-IRES-EGFP DNA), or control [EGFP], 2 μg of DNA were used, and for cotransfections a total of 2 μg of DNA was added to cells. After incubation with DNA for 16–18 h, fresh growth medium was added to the cells. When cells reached confluent density at ~48 h after transfection, they were incubated in DM without or with growth factors for an additional 24 or 48 h. Transfection efficiencies ranged from 12–20% (C2AS12 cells) to 19–23% (C2 myoblasts). Cos7 cells were grown in six-well dishes and were transfected with 2 μg of DNA using the calcium phosphate precipitation method (Stewart and Rotwein, 1996b). Expression of p21 and MyoD was analyzed by immunoblotting.

Survival Assays of Transfected Cells

Myoblasts were transfected as described above. When cells reached confluent density at ~48 h after transfection, two wells were harvested and both total cell number [Tt(total)] and transfection efficiency were assessed. The latter value was determined by averaging the fraction of cells expressing EGFP in 20 hemocytometer fields at a magnification of 200× [Ttransfected]. The remaining wells were incubated in DM without or with growth factors for 24 or 48 h. At each interval, cells were harvested and total and transfected cells were counted. Survival of transfected cells at 24 h was determined by the following formula: % survival = [Tt(transfected)/Tt(total)] × 100. Survival at 48 h was assessed similarly.

Statistical Analysis

Results are presented as the mean ± SEM. Statistical significance was determined using independent Student’s t test for paired samples. Results were considered statistically significant when P < 0.05.

Results

Prevention of Myoblast Death by IGF-I

We have shown previously that signaling through the IGF-I receptor by endogenously produced IGF-II is essential for myoblast survival as cells begin to differentiate (Stewart and Rotwein, 1996b; Lawlor et al., 2000; Lawlor and Rotwein, 2000). As seen in Fig. 1, C2 myoblasts engineered to lack IGF-II expression (C2AS12 cells) underwent rapid death when incubated in DM. Only 48 ± 1% of cells remained alive after 24 h, and only 34 ± 1% survived after 48 h. Viability continued to decline progressively with longer incubations in DM (Lawlor and Rotwein, 2000). Addition of IGF-I caused complete survival (102 ± 3% at 24 h, 98 ± 4% at 48 h). Other growth factors such as PDGF-BB also maintain viability of C2AS12 cells (Lawlor et al., 2000, and data not shown). Nontransfected C2 myoblasts also underwent apoptotic death when incubated in DM, with ∼30% of cells dying at 24 h (see Fig. 7; and Lawlor and Rotwein, 2000). No further death occurred upon longer incubation in DM, with survival correlating

![Figure 1](Image 308x151 to 524x252)

DM | IGF-I | DM | IGF-I
---|---|---|---
48 hr | 24 hr | Survival (% T0)
---|---|---|
100 | 100 | 100 | 100
98 | 98 | 98 | 98
95 | 95 | 95 | 95
92 | 92 | 92 | 92
89 | 89 | 89 | 89
86 | 86 | 86 | 86
83 | 83 | 83 | 83
80 | 80 | 80 | 80
77 | 77 | 77 | 77
74 | 74 | 74 | 74
71 | 71 | 71 | 71
68 | 68 | 68 | 68
65 | 65 | 65 | 65
62 | 62 | 62 | 62
59 | 59 | 59 | 59
56 | 56 | 56 | 56
53 | 53 | 53 | 53
50 | 50 | 50 | 50

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with expression of IGF-II (Tollefsen et al., 1989b; Lawlor and Rotwein, 2000).

Stimulation of MyoD and p21 mRNA and Protein Expression after IGF-I Treatment

The results in Fig. 1 prompted investigation of mechanisms of IGF-mediated muscle cell survival. The cyclin-dependent kinase inhibitor, p21, has been found to function as a survival factor for muscle and other cell types (Poluha et al., 1996; Wang and Walsh, 1996; Levkau et al., 1998), and MyoD has been shown to induce p21 expression (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). We thus asked whether IGF treatment stimulated expression of MyoD or p21 in C2AS12 cells. Results of time course studies are shown in Fig. 2. Treatment with IGF-I caused a sustained increase in the abundance of both MyoD and p21 mRNAs beginning at 8 h, the earliest time point examined, and lasting up to 48 h. Peak values of 7- and 10-fold above baseline were seen at 24 h for MyoD and p21, respectively. By contrast, PDGF-BB had a minimal effect. Similar results were seen when MyoD and p21 protein expression was examined. A progressive increase was seen in the abundance of both proteins after incubation with IGF-I, reaching fourfold above baseline levels or below within 8 h (Fig. 2 B). Additionally, as shown by immunocytochemistry, MyoD and p21 could be detected in nearly all myoblasts after a 24-h incubation with IGF-I, while few cells expressed either protein after treatment with PDGF (Fig. 2 C). Thus, IGF-I caused a progressive and sustained induction of MyoD and p21 mRNA and protein expression in C2AS12 cells.

Figure 2. Treatment with IGF-I stimulates expression of MyoD and p21. (A) Induction of MyoD and p21 mRNAs by IGF-I. The autoradiographs show results of representative ribonuclease protection assays performed using total RNA isolated from C2AS12 cells incubated with either R1IGF-I (2 nM) or PDGF-BB (0.4 nM) for the indicated times (top, MyoD; middle, p21). (Bottom) Photograph of an ethidium bromide–stained gel of the RNA used in these studies. Similar results were seen in three independent experiments. (B) Induction of MyoD and p21 proteins by IGF-I. Representative immunoblots are shown using whole-cell protein extracts from C2AS12 cells incubated with either R1IGF-I (2 nM) or PDGF-BB (0.4 nM) for the indicated times, and antibodies to MyoD (top), p21 (middle), and CDK-4 (bottom). Similar results were seen in three independent experiments. (C) Representative fluorescence micrographs of C2AS12 myoblasts treated with either R1IGF-I (2 nM) or PDGF-BB (0.4 nM) for 24 h, as described in Materials and Methods, and immunostained with antibodies to MyoD (top left), or p21 (top right), and incubated with Hoechst nuclear dye (bottom).
Forced Expression of MyoD or p21 Promotes Myoblast Survival

We next asked whether MyoD or p21 could maintain muscle cell survival in the absence of growth-factor treatment. We generated bicistronic expression plasmids using cDNAs for mouse MyoD or mouse p21, followed by a cassette containing an IRES derived from murine encephalomyocarditis virus and the marker protein EGFP. C2AS12 myoblasts were transiently transfected with these recombinant plasmids or with a control encoding EGFP alone, and cell survival was measured by cell counting after 24 h in DM. As depicted in Fig. 3, MyoD and p21 each promoted a significant increase in myoblast viability over results obtained with EGFP (MyoD: 84 ± 5% survival; p21; 98 ± 4%; EGFP: 52 ± 5%; P < 0.001 compared with EGFP). The latter value is similar to the 48 ± 1% survival seen in nontransfected myoblasts (Fig. 1). Incubation with IGF-I maintained complete cell viability, indicating that neither the recombinant plasmids nor the process of transfection were toxic. Comparable results were obtained after 48 h in DM (data not shown).

Figure 4. p21 is required for MyoD-stimulated muscle cell survival. (A) MyoD induces p21 protein expression. C2AS12 cells were transfected with either EGFP or MyoD-IRES-EGFP expression plasmids, and following a 24-h incubation in DM, fixed, and stained with an antibody to p21. (Top) Expression of EGFP, (middle) immunostaining for p21, and (bottom) nuclear staining with Hoechst dye. (B) The graph records the percent of transfected cells expressing p21. Significantly fewer cells are positive for p21 after transfection with EGFP than with MyoD-IRES-EGFP (*P < 0.005; mean ± SEM of three experiments, 100 transfected cells counted per experiment). (C) Inhibition of p21 prevents MyoD-stimulated muscle cell survival. C2AS12 cells were cotransfected with MyoD-IRES-EGFP (MyoD) and either EGFP or p21AS-IRES-EGFP (p21AS), as outlined in Materials and Methods. Cell counts of transfected myoblasts were performed after a 24-h incubation in DM. Results are presented as percent survival compared with T0 (mean ± SEM of three experiments, each performed in duplicate). *Survival was significantly less in myoblasts cotransfected with p21AS-IRES-EGFP than with EGFP (P < 0.014).

**Forced Expression of MyoD or p21 Promotes Myoblast Survival**

Based on published studies showing that MyoD stimulated p21 expression in muscle and fibroblasts (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995), we anticipated that forced expression of MyoD would promote accumulation of endogenous p21 in our cell line. C2AS12 myoblasts were transiently transfected with the MyoDAS-IRES-EGFP expression plasmid, as described in Materials and Methods. As shown in Fig. 4 A and graphed in B, MyoD induced p21 protein expression in the majority of transfected cells (75 ± 5%), compared with only 30% of cells transfected with EGFP (P < 0.005), a value similar to that seen in nontransfected myoblasts (data not shown). To determine whether p21 was necessary for MyoD-mediated muscle cell survival, we cotransfected C2AS12 myoblasts with MyoD and either a p21 antisense plasmid (p21AS) or a plasmid encoding EGFP. We have shown recently that this p21AS cDNA blocked IGF-I–stimulated MyoD expression. (A) C2AS12 cells were transiently transfected with the MyoDAS-IRES-EGFP expression plasmid, and incubated in DM plus R1IGF-I (2 nM) for 24 h, followed by fixation and staining for MyoD. (Left) Expression of EGFP, (center) immunostaining for MyoD, and (right) merged image. (B) The graph indicates the percentage of transfected cells expressing MyoD, as assessed by immunocytochemistry following IGF-I treatment. Significantly fewer cells are positive for MyoD after transfection with MyoDAS-IRES-EGFP than with EGFP (*P < 0.01; mean ± SEM of three experiments, counting 100 cells per experiment).

Figure 5. Forced expression of a MyoD antisense plasmid blocks IGF-I–stimulated MyoD expression. (A) C2AS12 cells were transiently transfected with the MyoDAS-IRES-EGFP expression plasmid, as described in Materials and Methods, and incubated in DM plus R1IGF-I (2 nM) for 24 h, followed by fixation and staining for MyoD. (Left) Expression of EGFP, (center) immunostaining for MyoD, and (right) merged image. (B) The graph indicates the percentage of transfected cells expressing MyoD, as assessed by immunocytochemistry following IGF-I treatment. Significantly fewer cells are positive for MyoD after transfection with MyoDAS-IRES-EGFP than with EGFP (*P < 0.01; mean ± SEM of three experiments, counting 100 cells per experiment).
These results indicate that MyoD promotes muscle cell survival by inducing p21.

**MyoD Is Not Required for IGF-mediated Myoblast Survival**

We have demonstrated that p21 is necessary for IGF-mediated survival of C2AS12 cells and wild-type C2 and L6 myoblasts (Lawlor and Rotwein, 2000). Since both MyoD and p21 are induced by IGF-I, and MyoD stimulates expression of p21, we next asked if MyoD was a necessary component of an IGF-stimulated muscle cell survival pathway. We first tested the effectiveness of a MyoD antisense cDNA (MyoD<sub>AS</sub>) to prevent IGF-stimulated MyoD protein expression. C2AS12 cells were transiently transfected with an expression plasmid containing a mouse MyoD cDNA in the antisense orientation (MyoD<sub>AS</sub>-IRES-EGFP), and were immunostained for MyoD after a 24-h incubation in DM plus IGF-I. As seen in Fig. 5, the MyoD<sub>AS</sub> plasmid caused a marked reduction in IGF-stimulated expression of MyoD. MyoD was detected in only 34±6% of transfected cells, compared with 78±6% of myoblasts transfected with p21<sub>AS</sub> (P<0.014). The MyoD<sub>AS</sub> plasmid did not reduce IGF-mediated muscle cell viability. As shown in Fig. 6A, IGF-I maintained complete survival of myoblasts transfected with the MyoD<sub>AS</sub> cDNA. In the absence of growth factor treatment, 49±5% of transfected cells remained alive after a 24-h incubation in DM, a level similar to that seen in non-transfected myoblasts (48±1%, see Fig. 1), and contrasting with the 82±4% viability of cells transfected with the MyoD sense vector (P<0.016). In contrast with our recent observations that a p21<sub>AS</sub> plasmid inhibited IGF-stimulated muscle cell survival (Lawlor and Rotwein, 2000), forced expression of the MyoD<sub>AS</sub> plasmid did not attenuate the ability of IGF-I to promote complete myoblast viability (100±3%). These results indicate that while MyoD can sustain muscle cell viability through p21, it is not necessary for IGF-regulated myoblast survival. Based on these observations, we next asked if IGF-I could stimulate p21 expression in cells transfected with the MyoD<sub>AS</sub> plasmid. As shown by immunocytochemistry in Fig. 6B, incubation with IGF-I equivalently induced p21 in myoblasts transfected with either plasmid expressed p21 after incubation with IGF-I.

**A MyoD Antisense cDNA Does Not Inhibit Survival of C2 Myoblasts**

Parental C2 myoblasts, which produce IGF-II during differentiation (Tollefsen et al., 1998), undergo limited apoptotic death during incubation in DM, with ~70% viability over the first 24 h, and little cell death subsequently (Lawlor and Rotwein, 2000). We next asked if MyoD was involved in the survival of these cells. C2 myoblasts were transfected with the MyoD<sub>AS</sub> plasmid or with a p21<sub>AS</sub> expression construct that we have shown diminishes C2 cell survival (Lawlor and Rotwein, 2000). Fig. 7 demonstrates that forced expression of MyoD<sub>AS</sub> had little effect on C2 muscle cell survival be-
Beyond what was seen in the nontransfected population (24 h viability: 72 ± 3% vs. 73 ± 3%; 48 h viability: 70 ± 2% vs. 69 ± 3%, respectively, *P = NS). By contrast, forced expression of p21AS resulted in a dramatic decrease in viability, with 42 ± 4% of transfected cells remaining alive after a 24-h incubation in DM, and only 17 ± 1% by 48 h. Taken together with results shown in Fig. 6, these observations indicate that induction of p21 expression by MyoD is not a critical pathway for IGF-mediated muscle cell survival.

**Differential Expression of MyoD and p21 by IGF-activated Signaling Pathways**

The results described above suggest that IGF-I induces the expression of MyoD and p21 by distinct mechanisms. In recent studies, we demonstrated that IGF-I treatment resulted in the sustained stimulation of both PI3-kinase and Akt kinase activities in muscle cells, and showed that a constitutively active PI3-kinase or an inducible Akt could maintain myoblast survival in the absence of growth factors (Lawlor et al., 2000). Based on these observations and on the results in Fig. 2, we next asked if either signaling molecule was involved in IGF-mediated stimulation of MyoD or p21. Transient transfection of an active PI3-kinase (p110*) into C2AS12 myoblasts led to the induction of MyoD and p21 in the absence of IGF-I treatment, as determined by immunocytochemistry after a 24-h incubation in DM (Fig. 8). Over 80% of cells positive for p110* also expressed both MyoD and p21. By contrast, in cells transfected with an inactive PI3-kinase (p110Δkin), the level of expression of MyoD or p21 (31–36%) did not exceed values detected in nontransfected myoblasts (data not shown). Thus, PI3-kinase can coordinate the expression of MyoD and p21 in muscle cells.

We next examined the ability of Akt to stimulate MyoD and p21 protein accumulation. Myoblasts were transfected with an expression plasmid encoding hydroxytamoxifen (HT)-inducible HA-tagged Akt (iAkt), and MyoD and p21 were detected by immunocytochemistry after incubation for 24 h in DM without or with HT. Upon transfection with the iAkt plasmid, Akt protein is expressed, but its enzymatic activity requires induction by HT (Lawlor et al., 2000). As shown in Fig. 9 A, HT treatment had little effect on the low level of MyoD protein accumulation observed (36 ± 2% vs. 33 ± 2% without HT, *P = NS). By contrast, addition of HT caused a significant increase in p21 protein expression (78 ± 4% vs. 24 ± 4%, *P < 0.01), as demonstrated previously (Lawlor and Rotwein, 2000). Based on these observations, and on the results in Fig. 6, we conclude that IGF-activated signal transduction pathways control muscle cell survival by at least two mechanisms, one involving induction of MyoD mRNA and protein...
through PI3-kinase, with the subsequent stimulation of p21 expression, and the other through induction of p21 by Akt.

**Discussion**

Muscle differentiation is a multi-step process involving permanent withdrawal from the cell cycle, expression of muscle-specific genes and proteins, and fusion of myoblasts into multinucleated myotubes (Lassar et al., 1994; Arnold and Winter, 1998). In this paper, we focus on steps involved in control of myoblast survival during early phases of differentiation. We show that the cyclin-dependent kinase inhibitor, p21, plays a key role in maintaining muscle cell viability and that its expression is regulated by two interdependent, yet distinguishable pathways. One pathway involves stimulation of MyoD expression by PI3-kinase, and subsequent induction of p21 by MyoD; the other involves activation of Akt by PI3-kinase, followed by stimulation of p21 expression. Both pathways collaborate to maintain survival and are connected through PI3-kinase, which itself is required for IGF-regulated muscle cell viability (Lawlor et al., 2000). The pathways diverge distal to PI3-kinase, as Akt induces expression of p21 but not MyoD, and inhibition of MyoD does not prevent IGF-stimulated production of p21 or IGF-mediated myoblast survival. A diagram outlining the interrelationships between these pathways is presented in Fig. 10.

It has been established in cultured muscle cells that p21 mRNA and protein expression increase dramatically with the onset of differentiation (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995), and that p21 contributes to the cell cycle arrest and resistance to apoptosis that occur early in differentiation (Andres and Walsh, 1996; Wang and Walsh, 1996). Although p21 null mice do not demonstrate abnormalities in skeletal muscle (Deng et al., 1995), mice deficient in both p21 and the related cyclin-dependent kinase inhibitor, p57, display marked defects in muscle differentiation and exhibit increased myoblast apoptosis during embryonic development (Zhang et al., 1999). These results confirm observations made with cultured muscle cells, but illustrate the complexity and functional redundancy within this family of cyclin-dependent kinase inhibitors in vivo. Little p57 can be detected in C2 myoblasts (Zhang et al., 1999), thus explaining why abrogation of p21 expression enhances cell death. A major function for p21 (and presumably p57) in muscle cells is to promote arrest in the G1 phase of the cell cycle by inhibiting activity of cyclin-dependent kinases, which otherwise would phosphorylate and inactivate the retinoblastoma protein, pRb (Elledge, 1996; Jacks and Weinberg, 1998). Active pRb in turn restrains E2F transcription factors, thus preventing induction of genes involved in cell-cycle progression (Weinberg, 1995). It remains to be established whether this is also the mechanism by which p21 sustains myoblast survival. Of interest, transgenic mice expressing low levels of pRb in an otherwise pRb null background have muscle defects similar to those of p21- and p57-deficient mice (Zackenhaus et al., 1996).

It has been shown by several investigators that MyoD induces p21 gene and protein expression in differentiating myoblasts (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995), in fibroblasts from intact and p53-deficient mice, and in other cell types by transcriptional pathways (Halevy et al., 1995), in fibroblasts from intact and p53-deficient mice, and in other cell types by transcriptional pathways (Ha...
tion (Jiang et al., 1998), although in no studies have the downstream signaling pathways been delineated. We now demonstrate that activation of PI3-kinase leads to induction of MyoD expression by a mechanism that does not appear to require Akt. Other downstream effectors of PI3-kinase have been identified in several cell types, including p70 S6 kinase, and atypical protein kinases C, δ, and ε (Chou et al., 1998; Le Good et al., 1998; Pullen et al., 1998). We have been unable to establish in preliminary experiments a role for either p70 S6 kinase or protein kinase Cε in myoblast survival (data not shown). It also has been found recently that PI3-kinase can induce the phosphorylation and enhance the activity of the MEF2 muscle transcription factors (Tamir and Bengal, 2000), although this has not been shown to be a direct effect, and it is not known if MEF2 proteins contribute to muscle cell survival. Additional signaling molecules potentially involved in regulating myoblast differentiation and downstream of PI3-kinase include members of the Rho family of small GTPases, RhoA, Rac, and Cdc42 (Nobes and Hall, 1994). Inhibition of activity of these proteins, either through use of dominant interfering mutants or overexpression of the GDP dissociation inhibitor, RalGDI, can block expression of muscle-specific genes and prevent differentiation (Carnac et al., 1998; Ramocki et al., 1998; Takano et al., 1998; Wei et al., 1998). To date, the Rho family has not been linked to muscle cell survival pathways controlled by the IGF-I receptor.

The signaling pathways and mechanisms by which Akt enhances p21 gene expression are similarly unknown. In some cell types, Akt has been shown to activate the transcription factor NF-κB by stimulating the kinases that phosphorylate its inhibitor, IκB, and target it for destruction (Kane et al., 1999; Romashkova and Makarov, 1999; Sizemore et al., 1999). Although NF-κB is expressed in C2 myoblasts (Kaliman et al., 1999), we have found that it is not induced by IGF-I in these cells (Lawlor and Rotwein, 2000), thus making it unlikely to be involved in Akt-mediated gene activation. Members of the p38 family of mitogen-activated protein (MAP) kinases have been demonstrated to phosphorylate and activate MEF2 proteins (Yang et al., 1999; Zhao et al., 1999), but p38 MAP kinases are not activated by Akt and have not been found to stimulate p21 gene expression or promote myoblast survival.

In summary, we have identified two distinct IGF-I receptor and PI3-kinase–activated signal transduction pathways that contribute to the maintenance of myoblast viability through induction of p21. This dual mechanism of p21 regulation may function to ensure the appropriate expression of this critical survival factor. It remains to be determined whether similarly multilayered interdependent pathways control p21 gene expression in vivo, where they potentially may act to modulate myoblast viability and muscle mass during embryonic and adult life.

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