Permissive Roles of Phosphatidyl Inositol 3-Kinase and Akt in Skeletal Myocyte Maturation

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Skeletal muscle differentiation, maturation, and regeneration are regulated by interactions between signaling pathways activated by hormones and growth factors, and intrinsic genetic programs controlled by myogenic transcription factors, including members of the MyoD and myocyte enhancer factor 2 (MEF2) families. Insulin-like growth factors (IGFs) play key roles in muscle development in the embryo, and in the maintenance and hypertrophy of mature muscle in the adult, but the precise signaling pathways responsible for these effects remain incompletely defined. To study mechanisms of IGF action in muscle, we have developed a mouse myoblast cell line termed C2BP5 that is dependent on activation of the IGF-I receptor and the phosphatidyl inositol 3-kinase (PI3-kinase)–Akt pathway for initiation of differentiation. Here, we show that differentiation of C2BP5 myoblasts could be induced in the absence of IGF action by recombinant adenoviruses expressing MyoD or myogenin, but it was reversibly impaired by the PI3-kinase inhibitor LY294002. Similar results were observed using a dominant-negative version of Akt, a key downstream component of PI3-kinase signaling, and also were seen in C3H 10T1/2 fibroblasts. Inhibition of PI3-kinase did not prevent accumulation of muscle differentiation-specific proteins (myogenin, troponin T, or myosin heavy chain), did not block transcriptional activation of E-box containing muscle reporter genes by MyoD or myogenin, and did not inhibit the expression or function of endogenous MEF2C or MEF2D. An adenovirus encoding active Akt could partially restore terminal differentiation of MyoD-expressing and LY294002-treated myoblasts, but the resultant myofibers contained fewer nuclei and were smaller and thinner than normal, indicating that another PI3-kinase–stimulated pathway in addition to Akt is required for full myocyte maturation. Our results support the idea that an IGF-regulated PI3-kinase pathway functions downstream of or in parallel with MyoD, myogenin, and MEF2 in muscle development to govern the late steps of differentiation that lead to multinucleated myotubes.

INTRODUCTION

The determination, differentiation, maturation, and regeneration of skeletal muscle comprise a multistage process that involves functional interactions between intrinsic muscle-restricted genetic programs and extrinsic pathways regulated by hormones and growth factors. The muscle-specific basic helix-loop-helix transcription factors, MyoD, myf5, MRF4, and myogenin, play critical roles in myoblast specification and differentiation through their ability to activate muscle genes (Buckingham, 2001; Sabourin and Rudnicki, 2000). These proteins bind as heterodimers with other ubiquitous basic helix-loop-helix transcription factors to control regions termed E-boxes found often in the promoters of muscle-specific genes (Sabourin and Rudnicki, 2000; Buckingham, 2001). Subsequent interactions with transcriptional coactivators such as p300 and P/CAF seem necessary for gene activation (Sartorelli *et al.*, 1999; Poleskaya *et al.*, 2001). Some muscle genes additionally contain binding sites for transcription factors of the MEF2 family, which have been

shown to function as accessory regulators of muscle gene expression and differentiation (Black and Olson, 1998).

Many hormones and growth factors are able to promote myoblast proliferation through activation of signal transduction pathways initiated after binding to specific cell-surface receptors. The Ras-Raf-Mek-Erk pathway has been shown to mediate growth factor-stimulated cell proliferation and co-ordinately to inhibit differentiation (Bennett and Tonks, 1997; Coolican *et al.*, 1997; Rommel *et al.*, 1999), in part by impairing the activity of MyoD (Perry *et al.*, 2001). Unlike most other growth factors, the insulin-like growth factors IGF-I and IGF-II are able to enhance differentiation of muscle cells in tissue culture (Engert *et al.*, 1996; Montarras *et al.*, 1996; Stewart *et al.*, 1996; Coolican *et al.*, 1997; Musaro and Rosenthal, 1999; Musaro *et al.*, 1999; Rommel *et al.*, 1999; Semsarian *et al.*, 1999) and also to positively influence muscle growth, maintenance, and regeneration in vivo (Coleman *et al.*, 1995; Barton-Davis *et al.*, 1998; Musaro *et al.*, 2001; Barton *et al.*, 2002; Paul and Rosenthal, 2002). The actions of IGF-I and IGF-II in muscle are mediated primarily by the IGF-I receptor, a growth factor-activated transmembrane tyrosine protein kinase (Nakae *et al.*, 2001). On ligand binding, the IGF-I receptor engages distinct intracellular adaptor molecules, including IRS-1, IRS-2, Gab-1, and others, which then activate a series of signal transduction pathways (Dupont and LeRoith, 2001; Nakae *et al.*, 2001). IGF action is also modulated in muscle and other tissues through a family of high-affinity IGF binding proteins (IGFBPs; Clemmons, 1998), which not only limit access to cell-surface IGF-I re-

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Abbreviations used: DM, differentiation medium; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MEF2, myocyte enhancer factor 2; MHC, myosin heavy chain; PI3-kinase, phosphatidyl inositol 3-kinase.

ceptors but also extend growth factor half-life in the extracellular environment (Clemmons, 1998).

Several laboratories have demonstrated various roles for IGF-activated PI3-kinase and Akt signaling pathways in muscle differentiation (Engert *et al.*, 1996; Kaliman *et al.*, 1996; Coolican *et al.*, 1997; Jiang *et al.*, 1999; Rommel *et al.*, 1999, 2001; Tamir and Bengal, 2000; Xu and Wu, 2000; Bodine *et al.*, 2001; Vandromme *et al.*, 2001; Takahashi *et al.*, 2002). We have found in cultured muscle cells that endogenously produced IGF-II stimulates the IGF-I receptor, phosphatidylinositol 3-kinase (PI3-kinase), and Akt to induce expression of the cyclin-dependent kinase inhibitor p21/waf-1 and, through this autocrine mechanism, maintains myoblast viability during the earliest phases of differentiation (Lawlor and Rotwein, 2000a,b; Lawlor *et al.*, 2000). In subsequent studies, we showed that the same pathway could initiate differentiation by inducing expression of myogenin (Tureckova *et al.*, 2001). The current experiments were based on our observations that the PI3-kinase inhibitor LY294002, which blocked IGF-mediated myoblast differentiation by inhibiting myogenin production, also prevented myogenin-stimulated differentiation (Tureckova *et al.*, 2001), indicating an additional role for PI3-kinase signaling downstream of this myogenic transcription factor. Our new results support a model in which IGF-regulated PI3-kinase pathways have multiple functions in muscle development, including facilitating the steps leading to myocyte fusion into multinucleated myofibers.

MATERIALS AND METHODS

Materials

Trypsin, fetal calf serum, newborn calf serum, and horse serum were purchased from Invitrogen (Carlsbad, CA). DMEM and phosphate-buffered saline were obtained from Mediatech (Herndon, VA). R₃IGF-I was from Gro-Pep (Adelaide, Australia). G418 was purchased from Fisher Scientific (Pittsburgh, PA). LY294002 was from BIOMOL Research Laboratories (Plymouth Meeting, PA) and was dissolved in dimethyl sulfoxide at a concentration of 20 mM and stored at -20°C until use; 4-hydroxytamoxifen (HT) was from Sigma-Aldrich (St. Louis, MO) and was dissolved in ethanol at a concentration of 50 mM and stored at -20°C until use. Effectene was from QIAGEN (Valencia, CA) and TransIT-LT-1 was from Mirus (Madison, WI). Protease inhibitor tablets were obtained from Roche Diagnostics (Indianapolis, IN), okadaic acid was from Alexis Biochemicals (San Diego, CA), and sodium orthovanadate from Sigma-Aldrich. The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). Nitrocellulose was from Osmonics (Westborough, MA). Reagents for enhanced chemifluorescence were obtained from Amersham Biosciences (Piscataway, NJ). Restriction enzymes, buffers, ligases, and polymerases were purchased from BD Biosciences Clontech (Palo Alto, CA) or Fermentas (Hanover, MD). Hoechst 33258 nuclear dye was from Polysciences (Warrington, PA). The following monoclonal antibodies were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA): F5D (anti-myogenin; W.E. Wright), MF20 (anti-myosin heavy chain [MHC]; D.A. Fischman), and CT3 (anti-troponin T; J.J.-C. Lin). A monoclonal antibody to MyoD was from BD Pharmingen (San Diego, CA) and a monoclonal antibody to MEF3D from BD Biosciences (San Diego, CA). Polyclonal antibodies to Akt, phospho-Akt (Ser473), Akt substrates, and MEF2C were obtained from Cell Signaling Technology (Beverly, MA). The following antibody conjugates were purchased from Molecular Probes (Eugene, OR): goat anti-mouse IgG1-Alexa 488, goat anti-mouse IgG_{2b}-Alexa 594, anti-mouse IgG-alkaline phosphatase, and anti-rabbit IgG-alkaline phosphatase. The AdEasy adenoviral recombinant kit was from Q-BIO Gene (Carlsbad, CA). All other chemicals were reagent grade and purchased from commercial suppliers.

Cell Culture

C2BP5 cells are C2 myoblasts stably expressing a mouse IGFBP-5 cDNA (James *et al.*, 1996). Cells were incubated on gelatin-coated tissue culture dishes in growth media (DMEM containing 10% fetal bovine serum, 10% newborn calf serum, and G418 at 400 µg/ml) at 37°C in humidified air with 5% CO₂, until they reached 50% of confluent density for infection with recombinant adenoviruses, and 95% for studies of differentiation. C3H10T1/2 mouse embryonic fibroblasts were plated on gelatin-coated tissue culture dishes and incubated under similar conditions in growth media (DMEM

containing 10% fetal bovine serum). For both cell lines, differentiation was initiated after washing cells with phosphate-buffered saline by addition of differentiation medium (DM), consisting of DMEM plus 2% horse serum, as described previously (James *et al.*, 1996; Tureckova *et al.*, 2001).

Luciferase Reporter Gene Assays

The 4X E-box reporter plasmid containing four copies of the right hand E-box element from the mouse muscle creatine kinase promoter (Weintraub *et al.*, 1990) was a gift from Dr. Matthew Thayer (Oregon Health and Science University, Portland, OR). The 1.7-kb proximal fragment of the mouse muscle creatine kinase promoter was isolated after restriction enzyme digestion of the 3.3-kb promoter (Apone and Hauschka, 1995) with BglIII and SmaI endonucleases. After the BglIII site was made blunt with Klenow fragment of DNA polymerase I, the DNA fragment was ligated into the SmaI site of pGL3 Basic (Promega, Madison, WI), and recombinant plasmids in the correct orientation were identified by restriction endonuclease analysis. The 3X MEF2 reporter plasmid was prepared by ligating annealed complementary oligonucleotides containing three copies of the mouse muscle creatine kinase MEF2 element (Edmondson *et al.*, 1992) into pTK:Luc (a gift from Dr. Susan Berry, University of Minnesota, Minneapolis, MN) via 5' HindIII and 3' SalI sites. The oligonucleotides are as follows (MEF2 sites are underlined): top strand, 5' agctt-GGCTCTAAAAATAACCCCGGCTCTAAAAATAACCCCGGCTCTAAAAATAACCCCGG 3'; and bottom strand, 5' tgcacGGGGGTATTTTTATGAGCCGCGGGGGTTAT TTTTATGAGCCGCGGGGTATTTTTATGAGCCG 3'. For transfection experiments C2BP5 cells were seeded at 1 × 10⁵/well of 12-well tissue culture dishes. The following day, each well was transfected with 0.5 µg of a promoter-reporter plasmid by using either Effectene (C2BP5 cells) or TransIT LT-1 (C3H10T1/2 cells) and following protocols from each supplier. The day after transfection, cells were infected with recombinant adenoviruses as described in the figure legends. Twenty-four hours later, cells were washed and incubated in DM ± 20 µM LY294002 for an additional 18–24 h. Luciferase values were measured using an assay kit from Promega and an PerkinElmer Life Sciences (Boston, MA) luminometer. Enzymatic activity was normalized to protein concentration.

Construction and Use of Recombinant Adenovirus

Using polymerase chain reaction, a FLAG epitope tag followed by a stop codon and XbaI site was added to the 3' end of the coding regions of mouse MyoD and mouse myogenin. Each modified cDNA was sequenced, digested with SalI and XbaI restriction endonucleases, and ligated into the pShuttle:CMV vector. The adenoviral plasmid containing enhanced green fluorescent protein (EGFP) (Ad:EGFP) was engineered by digestion of pEGFPN1 (BD Biosciences Clontech) with SalI and XbaI restriction endonucleases, followed by ligation of the 680-nucleotide coding region into a modified pShuttle plasmid containing a tetracycline-regulated promoter. To generate a recombinant adenovirus encoding dominant-negative Akt (Ad:Akt^{DN}), a human Akt-1 cDNA with a T7 epitope tag at the carboxy terminus (a gift from Dr. Rich Roth, Stanford University School of Medicine, Stanford, CA) was first modified by independently mutating codons for lysine 179, threonine 308, and serine 473 to alanines by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Each mutation was verified by restriction endonuclease mapping and by DNA sequencing. The Akt^{DN} cDNA was then subcloned via SalI and XbaI restriction endonuclease sites into the modified pShuttle plasmid containing a tetracycline-regulated promoter. All recombinant adenoviruses were generated and isolated by following a protocol supplied by Q-BIO Gene. Adenoviruses encoding inducible Akt (Ad:iAkt) and tetracycline-inhibited transactivator (Ad:tTA) have been described previously (Tureckova *et al.*, 2001). Ad:β-galactosidase (Ad:β-Gal) was a gift from Dr. J. Molkenstein (University of Cincinnati School of Medicine, Cincinnati, CA). All recombinant viruses were purified on discontinuous cesium chloride gradients and titered by optical density.

For infections, recombinant adenoviruses were diluted in DMEM containing 2% fetal bovine serum, filtered through a Gelman syringe filter (0.45 µm), and added to cells at 37°C for 90–120 min. After addition of an equal volume of DMEM with 20% fetal bovine serum and 20% newborn calf serum, cells were incubated for a further 24 h. For C2BP5 cells, Ad:tTA was used at a multiplicity of infection (MOI) of 1000 and all other viruses at an MOI of 2000. For C3H10T1/2 cells, all viruses were used at an MOI of 200. Using these conditions, ~90% of cells were infected. For studies of differentiation, cells were rinsed with phosphate-buffered saline and incubated at 37°C in DM with the indicated additives for 1–4 d.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 15 min at 20°C and permeabilized with a 50:50 mixture of ethanol and acetone for 2 min before blocking in 0.25% normal goat serum for >1 h at 20°C. Primary antibodies diluted in blocking buffer were added for 16 h at 4°C (anti-MHC, 1:250 dilution; anti-myogenin, 1:250 dilution). After a washing step, cells were incubated for 2 h at 20°C in goat anti-mouse IgG_{2b}-Alexa 594 (red) and goat anti-mouse IgG1-Alexa 488 (green), each diluted to 1:1000 in blocking buffer. Images were captured with a Roper Scientific Cool Snap FX charge-coupled device camera attached to a Nikon Eclipse T300 fluorescent microscope by using IP Labs 3.5

Figure 1. MyoD promotes differentiation of C2BP5 myoblasts. Results are shown of time course experiments using C2BP5 cells infected with Ad:MyoD or Ad:EGFP and incubated in DM for 1 or 2 d. (A) Immunocytochemistry of Ad:MyoD-infected cells for MHC (gray) and myogenin (white). Magnification, 200 $\times$. (B) Immunoblots for MyoD, myogenin, troponin T, and Akt by using whole cell protein lysates from C2BP5 cells infected with Ad:MyoD or Ad:EGFP.

software. Myotube area was calculated from MHC staining by using the computer program NIH Image. Fusion index was determined by calculating the fraction of cells with two or more nuclei in 10 microscopic fields under 200 $\times$ magnification.

Immunoblotting

Whole cell protein lysates were prepared after washing cells with phosphate-buffered saline and incubating on ice for 15 min in extraction buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, and 1% IGEPAL CA-630 [a nonionic detergent]). Lysates were passed through a 22-gauge needle and centrifuged at 15,000 rpm at 4 $^{\circ}$ C to remove insoluble material, and protein concentrations were determined using the BCA protein assay kit. Protein samples (30 μ g each) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with primary and secondary antibodies as described previously (Lawlor and Rotwein, 2000b; Tureckova *et al.*, 2001). Antibodies were used at the following dilutions: anti-MHC (1:500), anti-myogenin (1:500), anti-troponin T (1:1000), anti-MyoD (1:2000), anti-Akt (1:2000), anti-phospho-Akt (Ser473) (1:1000), anti-Akt substrates (1:1000), anti-MEF2C (1:1000), and anti-MEF2D (1:3000).

RESULTS

Inhibition of PI3-Kinase Activity Prevents Differentiation by MyoD or Myogenin

We previously established myogenic cell lines from C2 myoblasts after stable transfection with a mouse IGFBP-5 cDNA (James *et al.*, 1996). These C2BP5 cells did not undergo morphological or biochemical differentiation in low serum DM unless IGF-I or analogs that activated the IGF-I receptor were included (James *et al.*, 1996). In recent studies, we found that overexpression of myogenin could induce differentiation in the absence of added IGF-I, but that this effect on myogenesis was blocked by the PI3-kinase inhibitor LY294002 (Tureckova *et al.*, 2001). The current experiments were designed to define the potential relationships between the actions of myogenic transcription factors and signaling through the IGF-initiated PI3-kinase pathway.

As seen in Figure 1, infection with a recombinant adenovirus encoding mouse MyoD (Ad:MyoD) was able to induce robust differentiation of C2BP5 cells incubated in DM in the absence of IGF-I, as measured by expression of myogenin and troponin T and by extensive myofiber formation, but infection with Ad:EGFP had no effect. As shown in Figure 2, addition of LY294002 (20 μ M) was able to block Ad:MyoD-induced differentiation, as indicated by a marked decline in the fusion index from 85 to 25% after 2 d in DM, and in the size and abundance of myotubes (from 55 ± 5.3 to $15 \pm 2.4\%$ of total surface area; Figure 2B). Treatment with LY294002

did not appreciably inhibit MyoD expression (Figure 2C), when corrected for protein loading by using Akt levels, and did not reduce induction of myogenin (Figure 2, B and C), MHC, or troponin T (Figure 2C), implying little alteration in MyoD function by the drug, although it completely inhibited phosphorylation of both Akt and Akt substrates (Figure 2C), documenting effectiveness in blocking PI3-kinase activity. The inhibitory effects of LY294002 were reversible, with complete differentiation being restored within 2 d of its removal from Ad:MyoD-infected myoblasts, indicating that the drug did not cause permanent toxicity to the cells (Figure 3). Inhibition of differentiation by LY294002 additionally was observed in C3H 10T1/2 fetal fibroblasts infected with Ad:MyoD (Figure 4), showing that the requirement for PI3-kinase activity for myotube formation was not limited to C2BP5 myoblasts. Similar results were seen in C2BP5 cells with a recombinant adenovirus encoding myogenin, although MHC expression was decreased by LY294002 (Figure 5). In addition, a recombinant adenovirus encoding a dominant-negative version of human Akt-1, in which the ATP acceptor site and the regulatory phosphorylation sites were changed to alanine residues, potentially inhibited MyoD-stimulated differentiation of C2BP5 cells, as evidenced by reduction in myocyte fusion (Figure 6). Together, these observations demonstrate functional collaboration between the actions of the myogenic regulatory factors MyoD and myogenin, and PI3-kinase-dependent signaling pathways in muscle differentiation but do not define the mechanisms of cooperation.

Transcriptional Activity of MyoD or Myogenin Is Not Inhibited by LY294002

Incubation of Ad:MyoD-infected C2BP5 cells with LY294002 did not prevent induction of myogenin, MHC, or troponin T (Figure 2), indicating little impairment of MyoD function. However, to investigate this question directly, we examined the transcriptional activity of MyoD on both simple and complex muscle gene promoters. Expression of a promoter-reporter gene containing four copies of the right E-box derived from the mouse muscle creatine kinase promoter was enhanced nearly 100-fold in transfected C2BP5 myoblasts by Ad:MyoD infection, compared with cells infected with an adenovirus encoding β -Gal (Figure 7A). Reporter gene activity was not reduced by LY294002. Similar results were observed with the more complicated mouse muscle creatine kinase promoter (Figure 7B) and also were seen with both promoter-reporter genes in C2BP5 cells infected with Ad:myogenin (Figure 7, C and D). We thus conclude that the functions of MyoD and myogenin were not impaired by LY294002, implying that inhibition of PI3-kinase activity blocked differentiation independent of the actions of both transcription factors.

MEF2 Expression and Activity Are Not Regulated by LY294002

The MEF2 family of transcriptional activators plays a secondary but significant role in controlling muscle gene expression and differentiation through functional and biochemical collaboration with MyoD and myogenin (Black and Olson, 1998). Morphological and gene regulatory studies were performed to determine whether MEF2 factors were targets of inhibition of PI3-kinase in C2BP5 myoblasts. Infection of cells with Ad:MyoD and incubation in DM led to a slight increase in accumulation of MEF2C and MEF2D over time compared with cells infected with Ad: β -Gal. Addition of LY294002 had little effect when corrected for protein loading by abundance of Akt (Figure 8A). Promoter-

Figure 2. LY294002 inhibits MyoD-mediated differentiation. Results of time-course experiments are shown for C2BP5 cells infected with Ad:MyoD and incubated in DM with vehicle (dimethyl sulfoxide, DMSO) or the PI3-kinase inhibitor LY294002 in DMSO (LY, 20 μ M) for

Figure 3. Inhibition of differentiation by LY294002 is reversible. C2BP5 cells were infected with Ad:MyoD and incubated in DM with dimethyl sulfoxide (DMSO) (veh) or LY294002 in DMSO (LY, 20 μ M) for up to 3 d as shown. (A) Results of immunocytochemistry for MHC (gray) and myogenin (dark gray). Magnification, 200 $\times$. (B) Quantification of myotube area by using MHC staining after incubation of cells for 3 d in DM with vehicle (black bars), LY (white bars), or LY for 24 h and then vehicle (+/-; gray bars). Each bar graph represents the mean $\pm$ SEM of 18 fields from three experiments at 200 $\times$ magnification.

reporter gene assays were used to assess the transcriptional actions of endogenous MEF2 proteins. Enzymatic activity of a luciferase reporter gene containing three tandem copies of a MEF2 binding site from the mouse muscle creatine kinase gene cloned 5' to a basal promoter was enhanced >50-fold

1 or 2 d. (A) Results of immunocytochemistry for MHC (red) and myogenin (green). Magnification, 200 $\times$. (B) Top, quantification of myotube area by using MHC staining (mean $\pm$ SEM of three experiments counting 20 fields at 200 $\times$ magnification). Bottom, quantification of myogenin expression (mean $\pm$ SEM of 12 fields from two experiments at 200 $\times$ magnification). (C) Immunoblots for MyoD, myogenin, MHC, troponin T, Akt, phospho-Akt (Ser 473) (pAkt), and Akt substrates (Akt sub) by using whole cell protein lysates.

Figure 4. MyoD-mediated differentiation of 10T1/2 fibroblasts is impaired by LY294002. 10T1/2 fetal fibroblasts were infected with Ad:MyoD and incubated in DM with vehicle or LY294002 (LY, 20 μ M) for 1 or 2 d. (A) Results of immunocytochemistry for MHC (gray). Nuclei have been stained with Hoechst dye (light gray). Magnification, 200 $\times$. (B) Quantification of myotube area by using MHC staining (mean $\pm$ SEM of 12 fields from three experiments at 200 $\times$ magnification).

in Ad:MyoD-infected C2BP5 cells compared with myoblasts infected with Ad: β -Gal. Addition of LY294002 caused a twofold increase in reporter gene activity in cells transduced with Ad:MyoD. Thus, inhibition of PI3-kinase did not block either the expression or actions of MEF2C or MEF2D. Together with results shown in Figure 7, these observations indicate that PI3-kinase-dependent signaling pathways function either downstream of or in parallel with both MyoD/myogenin and MEF2 to permit later events in muscle differentiation, including myocyte maturation into multinucleated myotubes.

Sustained Akt Phosphorylation during MyoD-induced Differentiation

The serine-threonine kinase Akt is activated in a PI3-kinase-dependent manner in many cell types, including muscle (Brazil and Hemmings, 2001), and has been shown to play a

Figure 5. LY294002 inhibits myogenin-induced myoblast differentiation. Results are shown of C2BP5 myoblasts infected with Ad:myogenin and incubated in DM with vehicle or LY294002 (LY, 20 μ M) for 1 or 2 d. (A) Immunocytochemistry of Ad:myogenin-infected cells for MHC (red) and myogenin (green). Magnification, 200 $\times$. (B) Quantification of myotube area by using MHC staining (mean $\pm$ SEM of 12 fields from three independent experiments at 200 $\times$ magnification). (C) Immunoblots for myogenin, MHC, troponin T, Akt, and phospho-Akt (p-Akt) by using whole cell protein lysates from Ad:myogenin-infected C2BP5 cells.

facilitating role in early muscle differentiation (Jiang *et al.*, 1999; Xu and Wu, 2000; Tureckova *et al.*, 2001; Vandromme *et al.*, 2001) and to potentially mediate myofiber hypertrophy (Bodine *et al.*, 2001; Pallafacchina *et al.*, 2002; Takahashi *et al.*, 2002). To determine whether Akt is involved in MyoD-stimulated myoblast differentiation, we first looked for induction of Akt activity in Ad:MyoD-infected C2BP5 cells. Cell extracts were isolated for immunoblotting by using an antibody that recognizes phosphorylation at serine 473, the second step in the kinase pathway that activates Akt (Brazil and Hemmings, 2001). As seen in Figure 9, in Ad:MyoD-infected myoblasts, phosphorylation of Akt is increased at the onset of incubation in DM compared with cells infected with Ad: β -Gal. In Ad:MyoD myoblasts, Akt phosphorylation was maintained at these levels, $\sim$ 50% of the values seen in C2BP5 cells incubated with IGF-I for 1 h, whereas in myoblasts infected with Ad: β -Gal, Akt phosphorylation declined rapidly during incubation in DM, being $\sim$ 15-fold lower by 24 h (Figure 9). There was no change in abundance

Figure 6. Dominant-negative Akt blocks MyoD-stimulated muscle differentiation. Results are shown of immunocytochemistry of C2BP5 myoblasts infected with Ad:MyoD and Ad:Akt^{DN} and incubated in DM for 1 or 2 d in the absence (-) or presence (+) of doxycycline (Dox). MHC staining is red and myogenin is green.

Figure 7. LY294002 does not block MyoD- or myogenin-stimulated gene transcription. C2BP5 cells were transfected with luciferase reporter plasmids; infected with Ad:MyoD, Ad:myogenin (Ad:myog), or Ad:β-galactosidase (Ad:β-Gal); and incubated with vehicle or LY294002 (LY, 20 μ M) for 1 d, as described in MATERIALS AND METHODS. (A and C) Results with a promoter-reporter gene containing four copies of the right hand E-box element from the mouse muscle creatine kinase gene (4X E-Box). (B and D) Results with the 1.7-kb proximal promoter of the mouse muscle creatine kinase gene (MCK). Results for all panels have been normalized, with values from MyoD-expressing or myogenin-expressing cells incubated in DM being set to 100. The mean $\pm$ SEM of duplicate samples from three independent experiments is shown. For cells infected with Ad:β-Gal, the error bars are too small to be seen in the graph.

of total Akt during this interval in either Ad:MyoD or Ad:β-Gal-infected cells. Thus, MyoD promotes sustained phosphorylation of Akt during a time period when extensive myofibers begin to form (Figure 1A).

Akt Partially Restores Myocyte Maturation

Because Akt was induced in Ad:MyoD-infected C2BP5 cells incubated in DM and dominant-negative Akt blocked later events in differentiation (Figure 6), we next asked whether its ectopic expression could reverse the impaired myotube formation caused by LY294002. To address this question, Ad:MyoD-infected C2BP5 cells were coinfecting with two recombinant adenoviruses, one expressing a tetracycline-inhibited transcriptional activator (Ad:tTA), and the other a tTA-regulated modified human Akt1 with a membrane-targeting sequence replacing the PH domain at the NH₂ terminus, and an HT-inducible ligand binding domain from the mouse estrogen receptor at the COOH terminus (Ad:iAkt; Tureckova *et al.*, 2001). On coinfection in the absence of tetracycline, a membrane-targeted Akt protein is produced (Figure 10C), but its full enzymatic activity is achieved only after addition of HT (Kohn *et al.*, 1998).

Coinfection of Ad:MyoD-infected C2BP5 myoblasts with Ad:tTA and Ad:iAkt resulted in an increase in the number of multinucleated myotubes even in the presence of LY294002, which was further enhanced by HT (Figure 10, A and B). The stimulation of myotube formation by Ad:iAkt correlated

Figure 8. LY294002 does not alter the expression or transcriptional activity of MEF2 proteins. C2BP5 cells were infected with Ad:MyoD or Ad:β-galactosidase (Ad:β-Gal) and treated with vehicle or LY294002 (LY, 20 μ M) for 1 or 2 d. (A) Results of immunoblots for MEF2C, MEF2D, and Akt by using whole cell protein lysates. (B) Results of promoter-reporter gene experiments by using a luciferase fusion gene containing three copies of the MEF2 site from the mouse muscle creatine kinase gene. Values have been normalized with results obtained from MyoD-expressing cells incubated in DM being set to 100. The mean $\pm$ SEM of duplicate samples from three independent experiments is shown.

with induction of phosphorylated iAkt but was not accompanied by significant increases in expression of MHC, MEF2C, or MEF2D (Figure 10C). Although activated iAkt enhanced myotube formation, the individual myofibers seemed shorter and thinner compared with C2BP5 cells infected with Ad:MyoD in the absence of LY294002. The cells expressing iAkt also contained fewer nuclei (compare Figures 1A and 10A). Thus, ectopic expression of Akt was able to reverse the inhibition of myofiber formation caused by blocking PI3-kinase activity, but it did not fully restore the normal pattern of extensive multinucleated myotubes induced by Ad:MyoD or Ad:myogenin.

DISCUSSION

Muscle differentiation is a multistep process that involves permanent withdrawal from the cell cycle, expression of muscle-specific genes and proteins, and a later series of biochemical and morphological steps that lead to formation of multinucleated myotubes, and to assembly of the contractile apparatus and other specialized subcellular structures (Sabourin and Rudnicki, 2000; Buckingham, 2001). In this report, using a model of MyoD- or myogenin-induced differentiation, we have determined that an active PI3-kinase-Akt pathway is needed for myocyte maturation.

Figure 9. Early and sustained phosphorylation of Akt during MyoD-induced muscle differentiation. Results are shown of C2BP5 cells infected with Ad:MyoD or Ad:β-Gal and incubated in DM for up to 24 h. (A) Immunoblots are pictured for p-Akt or total Akt in a representative experiment in which noninfected myoblasts were incubated with IGF-I (2 nM concentration of the R₃ analog) for 1 h. (B) The experiment shown in A has been plotted in a bar graph. Results from noninfected myoblasts incubated in DM for 1 h (small white bar) have arbitrarily been assigned a value of 1.

In previous studies, we demonstrated that IGF-stimulated activation of PI3-kinase and Akt promoted myoblast survival during the initial stages of differentiation by inducing expression of the cyclin-dependent kinase inhibitor p21 (Lawlor and Rotwein, 2000a; Lawlor *et al.*, 2000). We subsequently showed that sustained activation of PI3-kinase and Akt was required for IGF-mediated initiation of differentiation, in part acting to stimulate myogenin accumulation (Tureckova *et al.*, 2001), and that forced expression of active versions of either of these molecules could substitute for IGF signaling (Tureckova *et al.*, 2001). Other investigators have reached similar conclusions, demonstrating that pharmacological inhibition by using LY294002 or wortmannin blocked induction of myogenin and other muscle genes, and impaired differentiation in a variety of myogenic cell lines (Kaliman *et al.*, 1996; Montarras *et al.*, 1996; Coolican *et al.*, 1997; Jiang *et al.*, 1999). Comparable results also were observed with a dominant-negative regulatory subunit of PI3-kinase (Tamir and Bengal, 2000), and conversely, forced expression of active PI3-kinase was shown to enhance the rate and extent of both biochemical and morphological differentiation (Xu and Wu, 2000). Similar findings were noted with active Akt (Jiang *et al.*, 1999; Rommel *et al.*, 2001; Vandromme *et al.*, 2001; Takahashi *et al.*, 2002).

In the current experiments, by using a model of differentiation induced by myogenic regulatory factors, inhibition of PI3-kinase activity impaired morphological differentiation but did not block the activity of either MyoD or myogenin to stimulate endogenous muscle genes encoding myogenin, MHC, or troponin T, or to activate simple and complex E-box-containing muscle gene promoters. These latter results are in agreement with observations of Tamir and Bengal (2000), who found in cotransfected 10T1/2 fibroblasts that neither LY294002 nor a dominant-negative PI3-kinase regulatory subunit prevented induction of an E-box-containing reporter gene by MyoD. In contrast, they concluded that inhibition of PI3-kinase activity blocked differentiation by impairing the transcriptional functions of MEF2C, with-

Figure 10. Akt restores myocyte maturation after inhibition by LY294002. Results are shown of C2BP5 cells infected with Ad:MyoD or with both Ad:MyoD and Ad:iAkt. Cells were incubated in DM $\pm$ LY294002 (LY, 20 μ M) and $\pm$ HT (1 μ M) for 1 or 2 d. (A) Results of immunocytochemistry for MHC (red) and myogenin (green) after 2 d in DM. Magnification, 200 $\times$. (B) Quantification of myotube area by using MHC staining (mean $\pm$ SEM of three experiments counting a total of 12 fields at 200 $\times$ magnification). (C) Immunoblots for myogenin, MHC, MEF2C, MEF2D, Akt, and phospho-Akt (Ser473) (p-Akt) by using whole cell protein lysates.

out blunting its ability to bind to its cognate DNA element (Tamir and Bengal, 2000). Using the identical DNA binding site in a similar reporter gene, but examining endogenous rather than transfected MEF2 proteins, we find no impairment by LY294002 on the transcriptional activity of MEF2. In fact, reporter gene activity increased in the presence of inhibitor, for unknown reasons. Because LY294002 also did not alter the abundance of either MEF2C or MEF2D, we conclude that in addition to a role in early myoblast differentiation PI3-kinase provides a necessary signal acting either in parallel with or downstream of MyoD, myogenin, and MEF2 to govern the later stages of muscle differentiation that culminate in myocyte fusion.

The serine-threonine kinase Akt is a key component of the actions of PI3-kinase in multiple cell types (Brazil and Hemmings, 2001) and is activated by the protein kinases PDK-1 and PDK-2 when targeted to the cell membrane through association of its pleckstrin-homology domain with phosphatidyl inositol tris-phosphate (Brazil and Hemmings, 2001). In skeletal muscle cells, Akt expression and activity is induced during differentiation in vitro and during regeneration in vivo, particularly in models of muscle hypertrophy (Fujio *et al.*, 1999; Rommel *et al.*, 1999; Bodine *et al.*, 2001; Tureckova *et al.*, 2001; Vandromme *et al.*, 2001; Barton *et al.*, 2002). Similarly, we now observe an increase in Akt phosphorylation in Ad:MyoD-infected cells incubated in DM. Akt has been implicated as an agent of IGF-mediated myofiber hypertrophy in cell culture models and in vivo (Rommel *et al.*, 1999, 2001; Barton *et al.*, 2002), acting in part by inhibiting the Raf—Mek—Erk pathway at the level of Raf (Rommel *et al.*, 1999), and by blocking glycogen-synthase kinase-3 (Rommel *et al.*, 2001), because dominant-negative versions of Raf-1 or GSK-3 β have been shown to mimic the effects of activated Akt (Rommel *et al.*, 1999, 2001). Our results argue for a role for Akt in myocyte maturation, because infection with Ad:iAkt could overcome the inhibitory actions of LY294002 on myotube formation. However, in contrast to published studies, in our model forced expression of Akt did not lead to myofiber hypertrophy. Rather, the individual myotubes were smaller and thinner than normal, suggesting that another PI3-kinase-dependent pathway, inhibited here by LY294002, is required for complete myotube formation and may be permissive for hypertrophy. The individual components of this putative pathway are unknown but could include mammalian target of rapamycin, which in some studies has been shown to be required for muscle differentiation (Erbay and Chen, 2001; Rommel *et al.*, 2001; Shu *et al.*, 2002). However, we find that rapamycin, a specific mammalian target of rapamycin inhibitor, had no effect on MyoD- or myogenin-induced differentiation of C2BP-5 myoblasts (our unpublished data). Alternatively, other protein kinases potentially involved in muscle differentiation, including Erk-5 and p38 MAPK (Wu *et al.*, 2000; Dinev *et al.*, 2001), or as yet unknown signaling proteins, could interact with a PI3-kinase-dependent signal to regulate the later stages of differentiation in collaboration with Akt.

The three isoforms of Akt found in mammalian cells are products of distinct but highly related genes (Brazil and Hemmings, 2001). Recent studies have indicated that Akt-2 may be more critical for muscle development than Akt-1, at least in vitro. Akt-2 is induced during muscle differentiation in tissue culture, but expression of Akt-1 is constant, and Akt-3 is not detectable (Vandromme *et al.*, 2001). Akt-2 is found in both nuclear and cytoplasmic compartments of muscle cells, whereas Akt-1 is cytoplasmic (Vandromme *et al.*, 2001) and is more potent than Akt-1 in activating muscle-

specific reporter genes such as myogenin (Vandromme *et al.*, 2001). In contrast to these observations, gene knockout studies have not detected an impairment in muscle development or function in mice lacking either Akt-1 or Akt-2, although Akt-1-deficient animals showed generalized growth deficiency (Cho *et al.*, 2001a,b).

Although a few specific targets of Akt have been identified to date (Brazil and Hemmings, 2001), none are unique to skeletal muscle. Little is known about the physiological mechanisms through which Akt or other parallel pathways mediate IGF-regulated myofiber hypertrophy or muscle regeneration, or maintain muscle mass during aging (Barton-Davis *et al.*, 1998; Bodine *et al.*, 2001; Musaro *et al.*, 2001; Barton *et al.*, 2002; Pallafacchina *et al.*, 2002; Paul and Rosenthal, 2002). Further characterization of components of IGF-stimulated PI3-kinase and Akt signaling pathways in muscle, and their interactions with myogenic genetic programs, should lead to new insights with implications for treatment of muscle diseases.

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