Selective Control of Skeletal Muscle Differentiation by Akt1*

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The phosphatidylinositol 3-kinase-Akt pathway plays a central role in growth, development, and metabolism in both normal and neoplastic cells. In skeletal muscle, Akt has been implicated in regulating regeneration and hypertrophy and in countering atrophy. Here we provide evidence that Akt1 and not Akt2 is essential for muscle differentiation. Using a robust model of MyoD-mediated muscle development, in which dominant-negative Akt blocked differentiation, we show that targeted loss of Akt1 was equally inhibitory. Selective elimination of Akt1 had no effect on myoblast viability or proliferation but prevented differentiation by impairing the transcriptional actions of MyoD. In contrast, knockdown of Akt2 had no effect on myoblast survival or differentiation and minimally inhibited MyoD-regulated transcription. Our results define isoform-specific Akt-regulated signaling pathways in muscle cells that act through Akt1 to sustain muscle gene activation and promote differentiation.

The serine-threonine protein kinases of the Akt family have been subjects of intense scrutiny since their discovery in the early 1990s (1, 2). The three Akts share a common structure and ~85% amino acid identity and are activated by growth factors and hormones through similar biochemical mechanisms that are initiated by production of the lipid signaling molecule, phosphatidylinositol-3,4,5 trisphosphate (1, 2). Initial studies of the effects of these proteins established roles for them in cell proliferation, survival, and metabolism (1, 2), whereas more recent analyses in mice revealed distinct developmental functions for each Akt. Akt1 deficiency led to a reduction in somatic growth (3); targeted loss of Akt2 caused insulin resistance and diabetes mellitus and growth impairment (4); and knock-out of Akt3 led to reduced brain size (5). Combined deficiency of Akt1 had no effect on myoblast viability or proliferation but prevented differentiation by impairing the transcriptional actions of MyoD. In contrast, knockdown of Akt2 had no effect on myoblast survival or differentiation and minimally inhibited MyoD-regulated transcription. Our results define isoform-specific Akt-regulated signaling pathways in muscle cells that act through Akt1 to sustain muscle gene activation and promote differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—C3H10T1/2 mouse embryonic fibroblasts (ATCC number CCL226) were incubated on gelatin-coated tissue culture dishes (16). Differentiation was initiated at ~95% of confluent density by addition of differentiation medium (DM)2 (Dulbecco’s modified Eagle’s medium plus 2% horse serum (16)).

Construction and Use of Recombinant Adenoviruses—The following have been described: mouse MyoD (Ad-MyoD), dominant-negative Akt (Ad-AktDN), mouse IGF-II in the antisense orientation (Ad-IGF-IIAS), tetracycline-inhibited transcriptional activator (Ad-tTA), β-galactosidase (Ad-β-Gal) (16, 17). Adenoviruses expressing short hairpin inhibitor RNAs targeting mouse Akt1 or Akt2 mRNAs were prepared after testing three sets of double-stranded oligonucleotides for each Akt. Akt-selective hairpin oligonucleotides plus a RNA polymerase III termination signal were cloned into an adenoviral shuttle plasmid 3 to the human H1 RNA promoter (18). The sense strand is as follows: mouse Akt1, 5′-CTAGTGTAGGGTTGACAGAGGAATCCCTAACAGACGGATATCTCACACTTTTGTGATCA-3′; mouse Akt2, 5′-CTAGTCCCAACCTTGCTGTTACTACAGAACGAGATGTAACAGCCAAGGCTGCTTTTGGATCCA-3′. Akt-specific sequences are underlined. Adenoviruses were generated, purified on discontinuous cesium chloride gradients, and titrated by optical density (16).

2 The abbreviations used are: DM, differentiation medium; PI3, phosphatidylinositol 3; IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin; Ad, adenovirus; Dox, doxycycline.

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Infections with Ad-MyoD, Ad-IGF-II<sup>AS</sup>, and Ad-Akt<sup>DN</sup> were performed as described (19). For infections with Ad-β-gal, Ad-shAkt<sub>1</sub>, or Ad-shAkt<sub>2</sub>, viruses were added to cells at ~25% of confluent density (multiplicities of infection of 500) followed 24 h later by infection with Ad-MyoD (multiplicity of infection of 125). After an additional 24 h in growth medium, cells were washed, and DM was added.

**Immunoblotting**—Immunoblots were performed as described (19). Primary antibodies were obtained from Cell Signaling (Beverly, MA) unless otherwise indicated and were used at the following dilutions: anti-Akt (1:2000), anti-phospho-Akt<sup>Ser473</sup> (1:1000), anti-Akt<sup>2</sup>-I-β (1:1000), anti-phospho-FoxO3a (1:1000), anti-phospho-JNK (1:5000), anti-JNK (1:1000), anti-Akt (1:300), anti-Akt<sup>2</sup> (1:1000), anti-Akt<sup>1</sup> (1:2000), anti-Akt<sup>2</sup> (1:1000), anti-Akt<sup>1</sup> (1:2000), anti-Akt<sup>2</sup> (1:1000), anti-Akt<sup>1</sup> (1:3000), anti-Akt<sup>2</sup> (1:1000), anti-Akt<sup>1</sup> (1:3000), and anti-tubulin (Sigma-Aldrich, 1:5000), anti-myogenin (F5D, 1:100), anti-myogenin (F5D, 1:100), anti-Akt<sup>1</sup> (1:2000), and anti-Akt<sup>2</sup> (1:1000), anti-Akt<sup>1</sup> (1:2000), and anti-Akt<sup>2</sup> (1:1000), and anti-tubulin (Sigma-Aldrich, 1:5000), anti-myogenin (F5D, 1:100), anti-myogenin (F5D, 1:100), and anti-tronopin T (CT3, 1:1000, Developmental Studies Hybridoma Bank, Iowa City, IA). Conjugated secondary antibodies were from Molecular Probes (Eugene, OR, 1:5000). Akt enzymatic assays were performed as described (16).

**Immunocytochemistry**—All steps have been described (16), as have calculation of the cell number, fusion index, and myotube area (17).

**Luciferase Reporter Gene Assays**—Reporter plasmids and luciferase assays have been described (19). Cells were seeded at 1 × 10<sup>5</sup>/12-well dish, and the next day, they were transfected with 0.2 μg of plasmid DNA using TransIT LT-1 (Mirus Corp, Madison, WI). Cells were infected sequentially 16 h later with Ad-β-gal, Ad-shAkt<sub>1</sub>, or Ad-shAkt<sub>2</sub> or both Ad-shAkt<sub>1</sub> and Ad-shAkt<sub>2</sub> followed by Ad-MyoD, as above.

**RNA Isolation and Analysis**—Reverse transcription-PCR was performed with whole cell RNA (16) and the following primers: mouse Akt1 sense strand, 5′-GTCTCTAGGGTCCAGGGCC- AAAGTC-3′, and antisense, 5′-CATCTAAAGGAAGCAAGT- CTAAAGGAG-3′; mouse Akt2 sense, 5′-CCAAGACATATT- GGGCCCCCTTGA-3′, and antisense, 5′-AGCTTGGAGTG- CTCACGACTGGTC-3′; mouse Akt3 sense, 5′-ATCCCAAGCAAG- AAGCTTGGAGTG- CTCACGACTGGTC-3′; S17 sense, 5′-ATCCCAAGCAAG- AAGCTTGGAGTG- CTCACGACTGGTC-3′; S17 sense, 5′-ATCCCAAGCAAG- AAGCTTGGAGTG- CTCACGACTGGTC-3′; S17 sense, 5′-ATCCCAAGCAAG- AAGCTTGGAGTG- CTCACGACTGGTC-3′. Results were quantified by densitometry after agarose gel electrophoresis.

**Statistical Analysis**—Data are presented as mean ± S.D. Statistical significance was determined by paired Student’s t test (p < 0.05).

**RESULTS AND DISCUSSION**

**Inhibition of Akt Activity Blocks MyoD-mediated Muscle Differentiation**—We previously defined an autocrine growth factor circuit involving IGF-II, the IGF-1 receptor, and the PI3-kinase-Akt pathway that was necessary for differentiation of muscle cell lines (16, 17) and mesenchymal stem cells acutely converted to myoblasts by MyoD (16). The importance of Akt was assessed in Fig. 1, which shows that muscle differentiation can be reversibly blocked by inhibition of IGF-II production or by dominant-negative Akt (Akt<sup>DN</sup>). Adenoviruses encoding IGF-II<sup>AS</sup> or Akt<sup>DN</sup> not only led to loss of induction of Akt phosphorylation at serine 473 but also prevented accumulation of muscle proteins, including...
myogenin and troponin-T, yet had no effect on adenoviral-derived MyoD (Fig. 1A). Akt\textsuperscript{DN} blocked the normal increase in the enzymatic activity of Akt (Fig. 1B) and completely inhibited morphological differentiation (Fig. 1C), as was seen in myoblasts lacking IGF-II (16, 19).

To address mechanisms by which Akt drives muscle differentiation, we assessed the effects of Akt\textsuperscript{DN} on transcriptional actions of MyoD, using a promoter-reporter gene containing four copies of a MyoD-responsive E-box. We found recently that stimulation of the IGF-I receptor by myoblast-derived IGF-II was required for sustained MyoD-mediated muscle gene activation (19). E-box-dependent promoter activity was reduced by 85% by Akt\textsuperscript{DN} or IGF-II\textsuperscript{AS} but increased to control values when doxycycline inhibited production of these molecules (Fig. 1D). Thus, interference with Akt-mediated signaling blocks muscle differentiation by impairing transcriptional actions of MyoD.

Akt1 Is Critical for Initiation and Maintenance of Muscle Differentiation—To address the roles of individual Akt isoforms in differentiation, we developed adenoviruses encoding short hairpin interfering RNAs targeting Akt1 or Akt2 mRNAs. Ad-shAkt1 specifically reduced levels of Akt1 mRNA in MyoD-expressing stem cells by 90% but had no effect on transcripts for Akt2 or S17, and Ad-shAkt2 specifically decreased Akt2 mRNA levels (Fig. 2A). When cells were infected with Ad-shAkt1 and Ad-shAkt2, transcripts for both Akts were eliminated (Fig. 2A).

Knockdown of Akt1 mRNA caused a 90% reduction of Akt1 protein levels but did not prevent the normal increase in Akt2 seen during differentiation (10–12) and did not change the amount of adenoviral-derived MyoD (Fig. 2B). Deficiency of Akt1 led to a marked reduction in myogenin and troponin-T (Fig. 2B) and caused an 80% decrease in myotube formation (Fig. 2, C and D) and a 90% decline in myocyte fusion index when compared with controls (Fig. 2E). It is likely that this residual muscle differentiation reflects the ~15% of cells that were not infected with Ad-shAkt1. Knockdown of Akt2 mRNA caused the near disappearance of Akt2 protein but had little effect on Akt1 or MyoD and did not inhibit induction of myogenin or troponin-T (Fig. 2B). Loss of Akt2 also did not reduce myofiber formation or fusion index (Fig. 2, C–E). The combination of Ad-shAkt1 and Ad-shAkt2 led to the near elimination of both Akt proteins and blocked differentiation to the same extent as Ad-shAkt1 alone (Fig. 2B). We observed identical results in the C2 muscle cell line, where Akt1 deficiency inhibited differentiation at its earliest stages and knockdown of Akt2 did not (supplemental Fig. 1).

Deficiency of either Akt1 or Akt2 did not diminish cell proliferation during incubation in growth medium and did not increase myoblast death during 48 h in DM (Fig. 2F). Only loss of both Akts led to reduction in total cell number after 24 or 48 h in DM (17–20%; Fig. 2F). Overall, the minimal cell death probably reflects the effectiveness of MyoD in sustaining myoblast survival when overexpressed (20), although Akt has been shown to be a key agent in maintaining muscle cell viability (20, 21).

Assessing Signaling Pathways Activated by Akt1 and Akt2 in Muscle Cells—To address mechanisms by which Akt1 and Akt2 differentially influenced differentiation, we examined the effects of reduction of each protein on selected Akt target proteins. Akt1 deficiency led to a greater decline in total Akt and phosphorylation at serine 473 than did loss of Akt2 (Fig. 3A). Deficiency of Akt1 or Akt2 led to reductions in inhibitory phosphorylation of GSK3\textalpha and \beta on serine residues 21 and 9. Akt1 loss caused a greater decline in abundance of p70S6 kinase and in its activating phosphorylation by mTOR than did loss of Akt2. Reduction of Akt1 led to a slight rise in inhibitory phosphorylation of FoxO3a (23), although neither Akt appeared to modify the decline in abundance of FoxO3a seen during incubation in DM. Similar alterations in Akt target proteins were observed in C2 cells (data not shown). Thus, loss of each Akt appears to lead to analogous changes in some signaling proteins and different effects on others.

Lack of Akt1 Impairs Transcriptional Functions of MyoD—We next asked whether Akt1 deficiency inhibited MyoD-mediated transactivation. Myogenin promoter activity was reduced by...
50% decline (Fig. 3B). Loss of both Akts yielded results similar to lack of Akt1 (Fig. 3B). Thus, Akt1 and Akt2 exert distinct effects on muscle gene activation. Akt1 appears to be needed for induction of muscle gene expression early in differentiation, thus explaining why its loss prevents differentiation. By contrast, Akt2 is dispensable for early muscle gene expression but may contribute to regulation of later genes.

**Akts in Cell Fate and Function**—Our results add to a perplexing literature on specific actions of individual Akts in different cell lineages. For example, viability of suprabasal keratinoctyes and their subsequent differentiation was reduced by loss of Akt1 but not by lack of Akt2 and could be restored to normal by preventing cell death with a broad spectrum caspase inhibitor (24). We find that the same inhibitor did not reverse the lack of muscle differentiation seen with Akt1 deficiency (data not shown), indicating that different mechanisms operate in keratinoctyes and myoblasts. By contrast, osteoclast differentiation appears to require Akt1 and Akt2 as knockdown of either protein was inhibitory, without reducing cell viability or proliferation (25). In mature adipocytes or myocytes, Akt2 is essential for normal glucose metabolism, whereas Akt1 was dispensable (26, 27). In breast cancer cells, Akt1 appears to control motility and invasiveness, with overexpression being inhibitory and its loss facilitating, as long as Akt2 also was expressed (28). The mechanisms by which Akt1 negatively regulates motility in these cells are not defined. In one study, Akt1 deficiency led to increased Erk activity, which was required for enhanced cell migration (28), whereas in another, Akt1 stimulation degraded the transcription factor NFAT1 by the E3 ubiquitin-protein isopeptide ligase, MDM2 (29). Thus, depending on the cell or tissue type, Akt1 or Akt2 exert either similar or distinct effects on differentiation, survival, intermediary metabolism, or motility. The steps by which individual Akts couple selectively with specific downstream effector pathways in different cell types yield unique outcomes remain an enigma.

**Akts and Skeletal Muscle**—A growing literature supports roles for Akt proteins in muscle growth and regeneration after injury (8, 9). Akts inhibit FoxO transcription factors by direct phosphorylation, thereby blocking expression of FoxO-activated genes (23). In muscle, this prevents induction of atrogin-1/MAFbx and MuRF1, E3 ubiquitin ligases involved in promoting atrophy (30, 31). In transgenic mice, overexpression of active Akt led to extensive hypertrophy (14), as did injection of individual mouse or rat muscles with a similar expression vector (13, 15). Overexpression of active Akt in rodents also reduced muscle atrophy after denervation and enhanced repair after injury, effects blocked by the mTor inhibitor, rapamycin (13, 15), implying that selective pathways downstream of Akt are critical to regeneration. Myofiber hypertrophy was seen in transgenic mice overexpressing IGF-I in muscle (32), and muscle damage was diminished in mice with muscular dystrophy by an IGF-I transgene, coincident with sustained Akt phosphorylation (32). Thus, Akts mediate many actions of IGF-I in muscle, but the specific responsible Akt remains an open question.

We recently established that autocrine signaling mediated by the IGF-I receptor, P13-kinase, and Akt, was necessary for sustained transcriptional actions of MyoD shortly after onset of muscle differentiation (19). Inhibition of this pathway blocked
myogenin gene transcription without altering MyoD or its DNA binding partner E12/E47. Rather, inhibition of IGF signaling reduced accumulation of transcriptional co-activators p300 and P/CAF (p300/CBP-associated factor) on chromatin at the myogenin promoter, thereby preventing histone acetylation and impairing recruitment and activation of RNA polymerase II (19). Taken together with current observations, these results implicate Akt1 as a key intermediate in MyoD-mediated transcriptional pathways in muscle cells, although the mechanisms by which Akt1 sustains activity of MyoD remain to be elucidated. A model outlining our findings is shown in Fig. 3C.

Previous observations indicating that Akt2 increases in abundance during muscle differentiation (10–12) and that its neutralization can inhibit differentiation (10) have led to the idea that Akt2 rather than Akt1 plays a central role in muscle. In contrast, our results demonstrate clearly that Akt1 is critical for induction and maintenance of muscle differentiation, although we did note subtle deficits in Akt2-deficient myotubes. In MyoD-converted 10T1/2 cells, lack of Akt2 led to reduction in overall myofiber length without a decrease in fusion index (Fig. 2, C and E), whereas in C2 cells, its loss was associated with thinner myotubes (supplemental Fig. 1). Two molecules that act downstream of Akt have been linked to myofiber formation, although the mechanisms are unknown. The protein kinase mTor regulates protein synthesis and is activated indirectly by Akt (22). C2C12 myocytes overexpressing an enzymatically inert mTor developed smaller myotubes than did cells with wild-type protein (33). The transcription factor FoxO1 was found to accelerate fusion and myotube formation when overexpressed (34), although other results showed that FoxO1 inhibited differentiation (35).

Active signaling through the IGF-I receptor has been associated with increased cancer risk and progression (36) and accelerated aging in experimental animals (37). At the same time, treatment with IGF-I has been proposed for muscle disorders and to counteract sarcopenia in aging humans (8, 9). Optimal therapeutic use of this growth factor in muscle or other tissues will require a fuller understanding of its key signaling molecules and their mechanisms of action.

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