Positive Feedback Regulation between Akt2 and MyoD during Muscle Differentiation

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Akt2 is a member of the Akt/PKB family, which is involved in a variety of cellular events including cell survival, proliferation, and differentiation. During skeletal muscle differentiation, the Akt2 but not Akt1 expression was significantly increased. Microinjection of anti-Akt2 but not anti-Akt1 antibody efficiently abrogated myogenesis, indicating that Akt2 plays a specific role in muscle differentiation. It has been well documented that Akt2 can induce myoblasts to enter the differentiation program is driven by the expression of MyoD family of transcription factors and the myocyte enhancer binding factor-2 (MEF2) family members (1). The MyoD family (also called myogenic regulatory factors) of basic helix-loop-helix proteins includes MyoD, myogenin, Myf5, and myogenic regulatory factor-4. Forced expression of MyoD transcription factor can inhibit cell cycle progression and induce muscle differentiation. The transcription of many muscle-specific genes is activated by the consensus regulatory DNA sequence termed E-box, present in regulatory regions of many muscle-specific genes. The MEF2 family of transcription factors includes MEF2A, MEF2B, MEF2C, and MEF2D, which belongs to the MADS (MCM1, agamous, deficiens, serum response factor) box transcription factors. Evidence for the positive feedback regulation between Akt2 and MyoD-MEF2, during muscle differentiation, is essential for MyoD-induced myogenesis.

Skeletal muscle differentiation requires an ordered multiple step process in which myoblasts irreversibly exit from the cell cycle, elongate, and fuse into multinucleated myotubes. This program is driven by the expression of the MyoD family of transcription factors and the myocyte enhancer binding factor-2 (MEF2) family members (1). The MyoD family (also called myogenic regulatory factors) of basic helix-loop-helix proteins includes MyoD, myogenin, Myf5, and myogenic regulatory factor-4. Forced expression of MyoD transcription factor can inhibit cell cycle progression and induce muscle differentiation. The transcription of many muscle-specific genes is activated by the consensus regulatory DNA sequence termed E-box, present in regulatory regions of many muscle-specific genes. The MEF2 family of transcription factors includes MEF2A, MEF2B, MEF2C, and MEF2D, which belongs to the MADS (MCM1, agamous, deficiens, serum response factor) box transcription factors. Evidence for the positive feedback regulation between Akt2 and MyoD-MEF2, during muscle differentiation, is essential for MyoD-induced myogenesis.

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This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, lanes 2 and 3 from the RNA panel from 10T1/2 were reused in lanes 1 and 2 from the RNA panel from MyoD-10T1/2 in Fig. 2C. The authors state that they stand by the overall conclusions of the study.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF452411.

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† The abbreviations used are: MEF2, myocyte enhancer binding factor-2; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; HER, human embryonic kidney; Luc, luciferase; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP-response element-binding protein; HDAC, histone deacetylase; PI3K, phosphatidylinositol 3-kinase; IGF, insulin-like growth factor.
The serine/threonine protein kinase Akt (also named PKB) is a major downstream target of PI3K and has been implicated in muscle differentiation (9). Three different isoforms of Akt have been identified including Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors in a PI3K-dependent manner. The full activation of the Akt requires phosphorylation at Thr308 (Akt1), Thr209 (Akt2), or Thr308 (Akt3) in the activation loop and Ser473 (Akt1), Ser473 (Akt2), or Ser472 (Akt3) in the C-terminal activation domain (10). The most studied isofrom is Akt1, which mediates IGF signaling to regulate cell survival, cell growth, GLUT4 translocation, and muscle differentiation. It has been shown that ectopic expression of constitutively activated Akt1 can promote extensive differentiation in different myoblast cell lines in the absence of IGF-I and can reverse the inhibitory effects of PI3K inhibitors LY294002 and wortmannin on myogenic differentiation (5, 9, 11). However, several studies including ours show that both the mRNA and protein levels of the endogenous Akt1 were not changed, whereas Akt2 was elevated during muscle differentiation, suggesting that Akt2 but not Akt1 plays a specific role in myogenesis under physiological condition (12–14). A recent study provides compelling supporting evidence by showing that microinjection of Akt2 antibody inhibited the differentiation of muscle cells, whereas anti-Akt1 antibody did not inhibit cell differentiation (15). However, the mechanism by which Akt2 is involved in myogenesis is currently unknown. In this study, we cloned the Akt2 promoter and demonstrated that MyoD transcriptionally regulates Akt2. During muscle differentiation, elevated Akt2 in turn activated MyoD-MEF2 transactivating activity resulting in myogenin expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Materials**—Human fetal myoblasts (HEK293 cells) were grown in Dulbeccos medium containing 10% fetal bovine serum, grown in Dulbeccos modified Eagle medium containing 10% horse serum (differentiation medium). pCSA-MyoD was kindly provided by Dr. Lassar (Harvard). The FLAG-tagged MyoD was constructed by subcloning MyoD into p3XFLAG-CMV10 (Sigma). MEF2 and myogenin-Luc plasmids are described elsewhere (6). The antibodies to Akt1 and Akt2 were purchased from Cell Signaling Technology (Cambridge, MA) and Alexis (San Diego, CA), respectively. The anti-Myc antibody was provided by GenScript (Piscataway, NJ). MEF2 and myogenin-Luc plasmids are described elsewhere (6). The antibodies to Akt1 and Akt2 were purchased from Cell Signaling Technology (Cambridge, MA) and Alexis (San Diego, CA), respectively. The anti-Myc antibody was provided by GenScript (Piscataway, NJ).

**Cloning and Analysis of the Human Akt2 Promoter**—The 5′-flanking region of Akt2 gene, which is considered the putative promoter, revealed multiple binding sites for MyoD, Oct1, and p300 and single sites for AP1, C/EBPβ, C/EBP, CREB, and SP1 (Fig. 1A). The transcription factor that has the most binding sites in Akt2 promoter is MyoD (nine putative MyoD binding sites at positions 220 containing nine putative MyoD binding sites, 1852–1861, 1502–1512, 1483–1493, 981–991, 754–764, 432–442, 405–415, 250–260, and 1722–1742). A MyoD binding site is also called an E-box and its consensus sequence is CANNTG (Fig. 1B).

**Defining the MyoD Binding Sites (s) in the Akt2 Promoter**—To determine the MyoD binding elements, we carried out the electrophoretic mobility shift analysis. Nine double-stranded oligonucleotides, each containing an E-box from the Akt2 promoter, were labeled with 32P and incubated with the nuclear extract from FLAG-tagged MyoD-transfected HEK293 cells. The quality of the nuclear extracts was examined with oligo(dT)-cellulose chromatography. The mobility shift was observed in −1861 to −1832, −1750 to −1722, −1512 to −1483, −754 to −726, −432 to −403, and −415 to −386, −260 to −231, and −51 to −22 fragments (Fig. 1C). The formation of the electrophoretically retarded complexes was inhibited when an excess of unlabeled oligonucleotides (competitor) were introduced (middle lane of each E-box). Moreover, an addition of an anti-FLAG antibody to the reaction mixtures induced the supershift of the protein-DNA complex as appearing in −1861 to −1832, −1750 to −1722, −1512 to −1483, −754 to −726, −432 to −403, −415 to −386, −260 to −231, and −51 to −22 fragments. These results indicate that eight of the Akt2 promoter-derived E-box oligonucleotides can specifically bind MyoD.

**MyoD Transactivates the Akt2 Promoter**—To investigate whether MyoD regulates the transcription of Akt2, a 3.1-kilo base genomic fragment corresponding to the region from bases −2898 to +220 containing nine putative MyoD binding sites,
**FIG. 1. Human Akt2 promoter contains multiple MyoD binding sites.**

**A.** Schematic representation of the human Akt2 genomic locus. The exons are shown as boxes 1–14.

**B.** Akt2 promoter sequence. Putative transcription factor binding sites are boxed.

**C.** MyoD binding to the DNA element from the Akt2 promoter. The electrophoretic mobility shift analysis of double-stranded oligonucleotides containing each MyoD binding site as indicated at the top. Equal amounts of 32P-labeled oligonucleotides were incubated with nuclear extract prepared from FLAG-MyoD-transfected HEK293 cells in the presence or absence of a 100-molar excess of the unlabeled oligonucleotides (competitor). Supershift was examined by incubation of the reactions with anti-FLAG antibody.
was subcloned upstream of the luciferase gene in pGL3 basic vector (pGL3-AKT2/3.1). A co-transfection of pGL3-Akt2/3.1 with MyoD into HEK293 cells resulted in a significant increase in reporter activity compared with the control sample co-transfected with the reporter and an empty vector (pcDNA3). Moreover, Akt2 promoter is regulated by MyoD in a dose-dependent manner (Fig. 2A). A similar level of induction of Akt2 reporter activity was also observed upon the transfection of 10T1/2 cells, which lack the endogenous MyoD (data not shown).

To define the MyoD-responsive regions in this promoter, we constructed a group of deletion reporters containing the Akt2 promoter serially deleted from the 5’ to 3’ fragment (Fig. 2B). The deletion of −2898 to −1351 significantly increased the MyoD responsiveness by ~40%, even though two potential myoD binding sites were eliminated, suggesting the presence of inhibitory elements for MyoD responsiveness within this region. The further deletion of E-box 7 reduced MyoD responsiveness by 15%. The deletion of the region from −642 to −86 (pGL3-AKT2−0.3), containing a cluster of three E-boxes, decreased the MyoD responsiveness by ~16%. Nevertheless, pGL3-AKT2−0.3, which contains only one E-box, was still induced by MyoD >4.5-fold (Fig. 2B), suggesting that the E-box 1 could be a major MyoD response site within the promoter.

Akt2 Is Induced by MyoD during the Muscle Differentiation—We next examined whether MyoD induces mRNA of Akt2. Because 10T1/2 myoblast do not express MyoD and are unable to differentiate to myotubes, we have established a 10T1/2 cell line, which was stably transformed with a MyoD expression vector. These MyoD-transformed cells expressing myocyte-specific markers form multinucleated myotubes when exposed to mitogen-poor differentiation medium (17, 18). The levels of Akt2 mRNA were evaluated in parental and MyoD-transfected 10T1/2 cells in both growth medium and differentiation medium. Akt2 mRNA was significantly increased in the 10T1/2-MyoD cells, but this induction did not occur in the parental 10T1/2 fibroblasts when exposed to the differentiation culture medium (Fig. 2C).

We further investigated the status of Akt1 and Akt2 in C2C12 cells, which express endogenous MyoD, during differentiation. Western immunoblot analysis revealed that Akt1 protein is stably expressed at a relative high level prior to and during differentiation (Fig. 2E). However, both mRNA and protein levels of Akt2 were very low in C2C12 myoblasts cultured in high mitogen growth medium but progressively increased following exposure of cultures to differentiation medium (Fig. 2, D and E). Moreover, Akt2 kinase activity was induced after switching the culture to differentiation medium.
B. Akt2 activates the MyoD and MEF2 activities and induces myogenin expression

Fig. 3. Akt2 activates the MyoD and MEF2 activites and induces myogenin expression. C2C12 cells were transfected along with either 4RE-Luc, which contains G133-Luc having both MEF2 and MyoD binding sites. After 16 h of incubation, cell lysates were subjected to luciferase assay analysis. The expression of myogenin and transfected Akt2 was analyzed by Western blot analysis.

(Fig. 2E), suggesting an important role for Akt2 in myogenesis.

Akt2 Induces Myogenin and Activates MyoD-dependent Reporter Genes—The induction of mRNA/protein and kinase activity of Akt2 during muscle differentiation suggests that it regulates muscle-specific gene(s) that controls differentiation. In fact, a previous report shows that ectopic expression of Akt1 and Akt2 could induce muscle-specific gene muscle creatine kinase, and Akt2 was more effective than Akt1 (15). However, the mechanism of Akt induction of muscle-specific gene expression has not been well documented. To explore this hypothesis further, we tested the effects of Akt2 on myogenin expression and MyoD transcriptional activity. G133-Luc, which is a 133-bp myogenin proximal promoter containing MyoD and MEF2 binding sites or 4RE-Luc, which is a MyoD-dependent reporter gene containing four MyoD binding sites, was co-transfected into C2C12 myoblasts with either wild type Akt2 or constitutively active Akt2. The expression of the wild type Akt2 induced G133-Luc and 4RE-Luc reporter activities at 1.6- and 1.5-folds, respectively, whereas the levels of G133-Luc and 4RE-Luc reporter activities were significantly increased (4.2- and 2.5-fold) in the cells transfected with the constitutively active Akt2 (Fig. 3A). Consistent with the reporter results, myogenin expression was induced by both wild type and constitutively active Akt2 in C2C12 cells after 16 h of exposure to differentiation medium (Fig. 3B). These data suggest that Akt2 can up-regulate the endogenous myogenin expression and promote the MyoD transcriptional activity during muscle differentiation.

DISCUSSION

Previous studies have shown that IGF1-induced muscle differentiation is mediated by the PI3K/Akt pathway (5–9). Among Akt family, Akt2 is highly expressed in heart (12, 20, 21). However, the ability of Akt2 to up-regulate the differentiation and abrogate differentiation by anti-Akt2 antibody (15). However, the mechanism by which Akt2 is up-regulated during the differentiation and stimulates myotube formation are currently unclear. In this report, we provide evidence showing that Akt2 promoter possesses multiple MyoD binding sites, the expression of Akt2 was induced by MyoD through stimulation of its promoter activity, and that the elevated Akt2 activated MyoD transactivation and induced muscle-specific gene myogenin expression to trigger muscle cell differentiation. Our data indicate a positive feedback regulation loop between Akt2 and MyoD during skeletal muscle differentiation (Fig. 4).

In ectopic expression systems, three isoforms of Akt display very similar functions including muscle differentiation. In fact, previous studies have mostly focused on Akt1 and demonstrated that Akt1 is a critical intermediate in IGF1-induced muscle differentiation hypertrophy and muscle survival (5–9). Among the three isoforms of Akt family, Akt2 is expressed in skeletal muscle (12) and plays a specific role in differentiated muscle cells and promotes the MyoD transcriptional activity during muscle differentiation hypertrophy and muscle survival (12, 20, 21). (a) The inhibition of Akt2 but not Akt1 and Akt3 during differentiation and abrogated differentiation (15). (b) The inhibition of Akt2 but not Akt1 expression abrogates IGF1-induced muscle differentiation (15). (c) Akt2 and Akt3 but not Akt1 are amplified and/or up-regulated in certain types of human cancer (22). (d) NIH3T3 cells are transformed by wild type Akt2 but not Akt1 and Akt3 (23). (e) Akt2- and Akt3-deficient mice displayed different phenotypes. Akt2 knock-out mice exhibited a typical type 2 diabetic phenotype that cannot be compensated by the presence of Akt1 and Akt3 (24). In contrast, Akt1−/− mice exhibited no diabetic phenotype (25, 26) but showed an impairment in organismal growth, i.e. smaller when compared with wild type littermates.
Such relatively subtle phenotype of Akt1−/− mice suggests that Akt2 and Akt3 may substitute to some extent for Akt1 (25). Nevertheless, these data indicate that there are non-redundant functions between three isoforms of Akt in certain tissue and/or cell types. In this study, we cloned Akt2 promoter and identified multiple MyoD binding sites in Akt2 (Fig. 1) but not in Akt1 promoter,2 further indicating the different transcriptional regulation between Akt1 and Akt2.

Previous studies have shown that MyoD and MEF2 transcription factors interact with each other to synergistically induce muscle-restricted target gene expression resulting in muscle differentiation (2), and that class II histone deacetylase (HDAC) 4 and 5 bind to MEF2 and inhibit MEF2/MyoD transactivation activity and muscle differentiation (27). Calcium/calmodulin-dependent protein kinase induces muscle differentiation by phosphorylation of HDACs 4 and 5 and shuttling of the phosphorylated HDACs from nuclear MEF2 complex to the cytoplasm (28). It has been also shown that class II HDACs-repressed muscle differentiation can be overcome by treating cells with IGF1, which induces HDACs export from the nucleus (29). However, the mechanism by which IGF1 regulates HDACs 4 and 5 has not been well characterized. In this report, we have shown that the protein level and kinase activity of Akt2 were elevated during muscle differentiation. The up-regulation of Akt2 was because of MyoD induction of Akt2 promoter activity, whereas activated Akt2 might result from autocrine production of IGFs by myoblasts under differentiation medium condition (4). Nevertheless, elevated Akt2 during muscle differentiation could mediate IGFs signals to regulate HDACs 4 and 5 functions, even though a previous study showed that Akt1 did not phosphorylate HDACs 4 and 5 (28).

Additional studies are required to define Akt2 activation of MyoD-MEF2 transcription factors.

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