Activation of Akt2 Inhibits anoikis and apoptosis induced by myogenic differentiation

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Abstract

***Akt2*, a homolog of *Akt1*, encodes a serine/threonine protein kinase that is amplified in ovarian and pancreatic cancers. The antiapoptotic activities of the Akt1 proto-oncogene product have been well documented, but the role of Akt2 in cellular survival is poorly understood. Here, we demonstrate that Akt2 mRNA, protein and kinase activity are upregulated during serum deprivation-induced C2C12 cell myogenic differentiation, a process that is associated with the acquisition of an apoptosis-resistant phenotype. Transient transfection of plasmids encoding wild-type and constitutively-active *Akt2* conferred resistance against apoptosis in differentiating C2C12 cells, while a kinase-negative *Akt2* construct did not. Adenovirus-mediated transfer of the constitutively-active *Akt2* cDNA also suppressed apoptosis during serum deprivation-induced myogenic differentiation and it protected cells from apoptosis induced by cell detachment. These data indicate that *Akt2* functions as an anti-apoptotic gene during cellular differentiation, a property that may contribute to its oncogenicity.** *Cell Death and Differentiation* (2001) **8,** 1207 – 1212.

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Abbreviations: PKB, protein kinase B; GM, growth medium; DM, differentiation medium; GFP, green fluorescence protein; DMEM, Dulbecco's modified Eagle medium; IGF, insulin-like growth factor; HA, hemagglutinin; PBS, phosphate-buffered saline; CMV, cytomegalovirus; MOI, multiplicity of infection

Introduction

Akt2, a serine-threonine protein kinase, is a member of *Akt/ protein kinase B (PKB)* proto-oncogene family.[1] Both Akt1 and Akt2 function as signal transducers downstream of phosphatidylinositol 3-kinase (PI 3-kinase).[2,3] Akt1 and Akt2 are encoded by separate genes and are most divergent at their carboxy-terminal regions.[3] Although it is well established that Akt1 functions as a regulator of glycogen metabolism, growth and survival,[4] much less is known about the cellular functions of Akt2. Previously, we demonstrated that *Akt1* mRNA is expressed ubiquitously, while *Akt2* is expressed in a tissue specific manner and is abundant in striated muscles and brown fat.[5 – 7] Of particular note, the *Akt2* gene is amplified in 10 – 20% of ovarian and pancreatic cancers,[8,9] while recurrent amplification of *Akt1* has not been observed, suggesting that overexpression of Akt2 contributes to development of some human malignancies. In transformed cells, Akt2 is activated by growth factors, v-ras, and v-src through a PI 3-kinase-mediated mechanism, promoting cell cycle activity and oncogenicity in this context.[10,11] Deletion of the carboxy-terminal tail in Akt2 abolishes its oncogenic activity.[11]

In contrast to Akt2, it is well recognized that Akt1 inhibits apoptotic cell death in diverse cell types through its ability to phosphorylate and modulate the activities of apoptosis-regulatory molecules including Bad, caspase 9, a forkhead transcription factor and IkB.[1] Previously, we reported that differentiating myocytes which fail to exit the cell cycle under serum-deprivation conditions will initiate programmed cell death, whereas myocytes that successfully exit the cell cycle will acquire an apoptosis-resistant phenotype and go on to form mature myotubes.[12] Related studies showed that the myogenic induction of the cell cycle inhibitor p21 and the retinoblastoma protein are essential for successful cell cycle exit, and hence, cellular survival.[13 – 16] More recently, we demonstrated that cell cycle exit is essential for efficient Akt1 induction during myogenesis and that the induction of Akt1 is important for myocyte survival.[17]

Antisense RNA to *Akt2*[9] and drugs that interfere with Akt2 signaling[18] inhibit the tumorigenic phenotype and induce apoptosis in cancer cells that exhibit *Akt2* over-expression, consistent with the hypothesis that *Akt2* gene amplification promotes cellular survival in tumors. However, nothing is known about the functions of Akt2 in non-tumorigenic cells. Here, we report that Akt2 protein and kinase activity are markedly upregulated during serum deprivation-induced myogenic differentiation in C2C12 cells. Under these conditions, transient transfection of plasmids expressing wild-type or constitutively-active *Akt2* promoted myocyte survival. Furthermore, overexpression of Akt2 by adenovirus-mediated gene transfer protected against both differentiation-associated apoptosis and anoikis, cell death that results from the disruption of cell-matrix contacts.[19] These data provide evidence to suggest that Akt2 induction can promote survival in nontransformed cells in response to diverse cellular stresses.

Results

Akt2 protein and kinase activity are upregulated during myogenic differentiation

Initial experiments examined Akt2 regulation during myogenic differentiation in C2C12 cells. Like Akt1,[17] Akt2 protein and histone-kinase activity was expressed at low levels in cycling myoblast cultures in growth medium (GM) (Figure 1A). Two days after cultures were shifted to differentiation media (DM), there was a marked induction of Akt2 protein relative to cdk4, which does not change upon differentiation.[20,21] Akt1 and Akt2 activity was assessed by determining the histone H2B kinase activity in anti-Akt1 and anti-Akt2 immunoprecipitates from extracts prepared from C2C12 cultures in GM or DM for 2 days (Figure 1B). Corresponding to the induction of protein, kinase activities for both Akt1 and Akt2 were induced to similar levels upon myogenic differentiation. Upregulation of *Akt2* transcript was also observed at both day 1 and 2 after C2C12 cells were switched to differentiation media (Figure
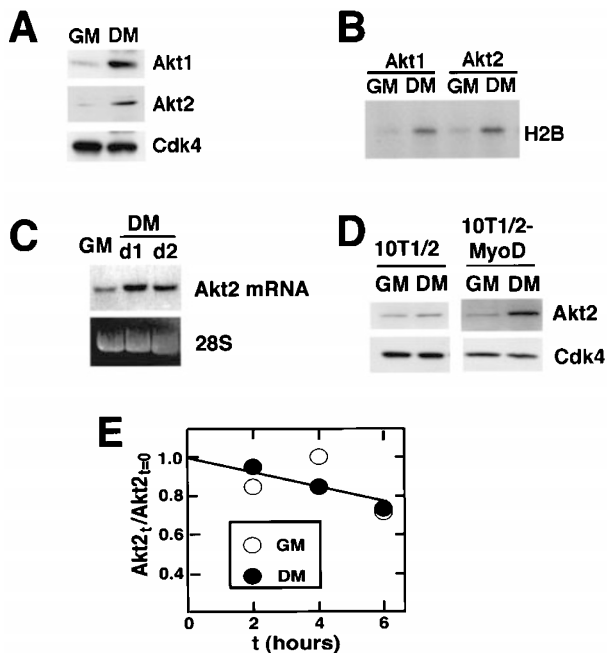
1C). Densiometric scanning indicated a fourfold upregulation by 1 day.

To examine myogenic Akt2 protein induction in greater detail, we analyzed its expression in a 10T1/2 cell line that is stably transformed with MyoD[15]. These MyoD-transformed cells differ from the parental 10T1/2 line in that they form multinucleated myotubes when exposed to differentiation medium. Differentiating 10T1/2-MyoD cells upregulated Akt2 protein, whereas Akt2 was induced when parental 10T1/2 cells were exposed to differentiation media (Figure 1D). Thus, Akt2 induction requires myogenic bHLH factor-induced commitment to differentiation.

Finally, Akt2 protein half-life was examined in C2C12 cells cultured in the presence of cyclohexamide in growth medium or differentiation medium (Figure 1E). In contrast to Akt1,[17] no changes in Akt2 protein stability were detected. Collectively, these data show that the induction of Akt2 protein during myogenesis is due to changes in the level of its transcript.

Transient transfection of Akt2 promotes cell survival in C2C12 cell: As reported previously,[12,14,17] a portion of C2C12 cells will undergo apoptotic cell death prior to cell cycle withdrawal when exposed to DM. To analyze the effects of Akt2 on cell survival, plasmid vector alone (pcDNA3) or vector expressing wild-type and mutated forms of Akt2 were co-transfected with a GFP expression vector to mark transfected cells. After cells were cultured in GM overnight, the medium was changed to serum-free DMEM. The fraction of GFP-positive cells with pyknotic nuclei, a feature of apoptosis, was determined at 24 h (Figure 2A). As shown in Figure 2B, transfection with wild-type Akt2 or myrAkt2 resulted in a significantly lower frequency of GFP-positive cells with pyknotic nuclei compared with vector alone. Furthermore, a vector expressing a kinase-negative form of Akt2 (knAkt2), with a mutation in the active site at amino acid 299 which converts a glutamate residue to lysine, displayed no protective effect during serum deprivation-induced myogenic differentiation.

Adenoviral transfer of constitutively-active Akt2 confers resistance to anoikis and apoptosis induced by myogenic differentiation: To further analyze the anti-apoptotic properties of Akt2, replication-defective adenoviral vectors expressing HA-tagged kinase-negative (Adeno-knAkt2) and constitutively-active Akt2 (Adeno-myrAkt2) were constructed (Figure 3A). C2C12 cells infected with Adeno-knAkt2 or Adeno-myrAkt2 expressed comparable levels of transgene as determined by immunoblot analysis with anti-HA antibody (Figure 3B). No immunoblot signal was detected in C2C12 cells infected with a control adenovirus vector that expresses β-galactosidase (Adeno-βgal) from the CMV promoter. The expression levels of knAkt2 and myrAkt2 adenovirus transgenes relative to endogenous Akt2 protein is shown in Figure 3C.

To analyze the anti-apoptotic effects of Akt2 during mitogen deprivation-induced myogenesis, myoblast cultures were mock-infected or infected with Adeno-βgal, Adeno-knAkt2, or Adeno-myrAkt2 and then induced to differentiate in serum-free DMEM. Flow cytometric analysis revealed a large fraction of cells containing hypodiploid DNA, an indicator of apoptotic cell death, in the mock, Adeno-βgal
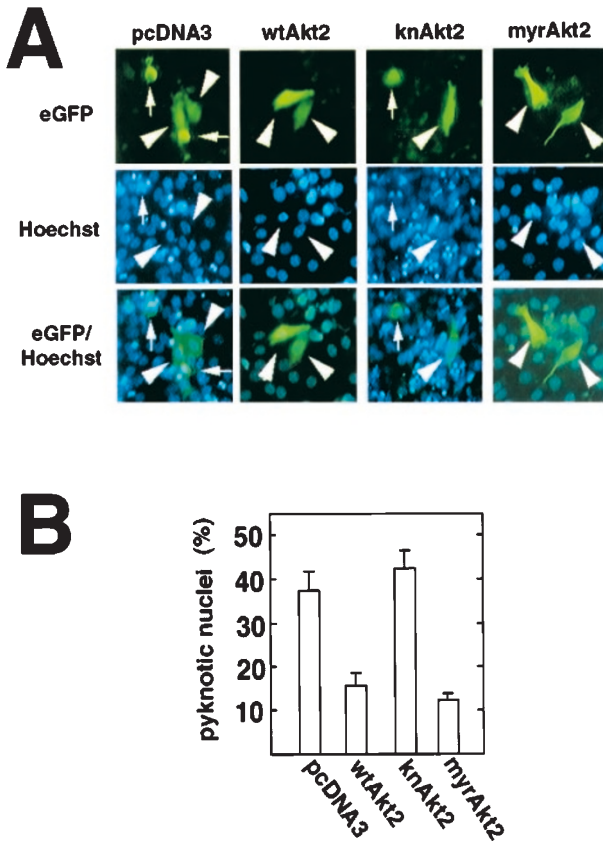

Figure 1 Akt2 upregulation during myogenic differentiation. C2C12 cells were cultured in growth medium (GM) (**A, B, C** and **E**). To induce differentiation, the medium was changed to differentiation medium (DM) for 2 days. (**A**) Cell lysates were immunoblotted with anti-Akt1, anti-Akt2, or anti-Cdk4 antibody. (**B**) Cell lysates were immunoprecipitated with anti-Akt1 or anti-Akt2 antibody, and kinase activities in the immunoprecipitate were measured using histone H2B as a substrate. (**C**) Total RNA was prepared from C2C12 cells cultured in growth medium (GM) or differentiation medium (DM) for 1 day (1d) or 2 days (2d). Total RNA (25 μg) was analyzed by Northern blotting using the *Akt2* probe. Ribosomal 28S RNA indicates equal loading. (**D**) 10T1/2 cells and 10T1/2 cells expressing MyoD were cultured in growth medium (GM) or differentiation medium (DM) for 2 days. Cell lysates were prepared and immunoblotted with anti-Akt2 or anti-Cdk4 antibody. (**E**) C2C12 cells were cultured in GM or DM for 2 days and exposed to cyclohexamide to inhibit protein synthesis. At 2, 4 and 6 h lysates were prepared from parallel cultures and immunoblotted for Akt2 and cdc2 expression. Cdc2 levels do not change upon myogenic differentiation.[30] The ratio of Akt2 to cdc2 signal in the absence of cyclohexamide (T=0) is assigned a value of 1.0. Data from two independent experiments are shown

Figure 2 Effect of Akt2 on myocyte apoptosis during serum deprivation-induced differentiation. C2C12 cells were co-transfected with a plasmid expressing green fluorescent protein (GFP), as a transfection marker, and plasmids encoding wild-type Akt2 (wtAkt2), kinase-negative Akt2 (knAkt2), or constitutively-active Akt2 (myrAkt2). Control cultures were transfected with the GFP-encoding plasmid and empty vector (pcDNA3). Apoptosis was assessed following incubation in serum-free medium for 24 hours. Cells were fixed and stained with Hoechst 33342 and fluorescence was observed in microscopic fields. (**A**) Representative photomicrographs showing myocytes co-transfected with the GFP expression vector and empty vector (pcDNA3) or expression vectors encoding knAkt2 or myrAkt2. Note that myrAkt2 or wtAkt2 co-transfected cells display normal nuclei (arrowheads), while some cells co-transfected with knAkt2 or empty vector become rounded and display pyknotic nuclei (arrows). (**B**) Quantitation of GFP-positive cells that display condensed nuclei. Data were shown as mean +/− S.E.M. (wtAkt2 and myrAkt2 are $P < 0.05$ relative to pcDNA3 condition)

and Adeno-knAkt2 experimental conditions (Figure 4). In contrast cells infected with Adeno-myrAkt2 displayed a reduced fraction of hypodiploid DNA indicating a reduction in apoptosis.

Previously, it has been reported that Akt1 protects against anoikis in endothelial and epithelial cells.[20,22] To test whether Akt2 has a similar function in muscle cells, that also undergo anoikis,[23] myoblast cultures were transfected with Adeno-βgal or Adeno-myrAkt2 and cultured in BSA-coated bacterial plates to prevent adhesion. After 24 h in suspension culture, DNA content was analyzed to assess apoptosis. As shown in Figure 5, a large fraction of the total DNA was hypodiploid in the mock- and Adeno-βgal-transduced cultures. In contrast, infection with Adeno-myrAkt markedly reduced the fraction of the DNA in the sub-G1 population, indicating
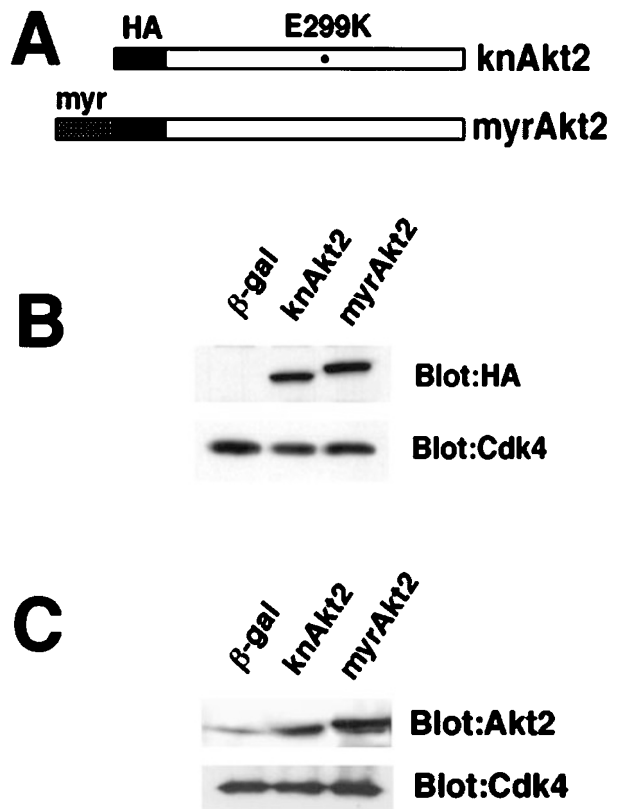

Figure 3 Structures and expression of Akt2 cDNAs from adenoviral vectors. (**A**) The knAkt2 construct has glutamate substituted for lysine at amino acid position 299. The myrAkt2 construct has the wild-type-Akt2 protein coding sequence fused to the c-*src* myristoylation sequence. Both proteins are fused to the HA tag for immunoblot analysis. (**B** and **C**) C2C12 myoblasts were infected with Adeno-βgal, Adeno-knAkt2 or Adeno-myrAkt2 for 15 h. Cell lysates were prepared and immunoblotted with anti-HA antibody (**B**) or anti-Akt2 antibody (**C**). Immunoblots for Cdk4 indicate equal loading

that constitutive activation of Akt2 is sufficient to promote survival in the absence of cellular adhesion. Infection with Adeno-knAkt2 had no detectable effect on the fraction of cells undergoing anoikis relative to mock- and Adeno-βgal-transduced cultures (data not shown).

Discussion

In this study we demonstrate that Akt2 can promote survival under conditions where non-tumorigenic cells are deprived of mitogens or proper matrix associations. Transient transfection of plasmids encoding either wild-type or constitutively-active Akt2 was found to inhibit apoptosis in C2C12 myocytes that are induced to differentiate under low serum culture conditions. These data were corroborated by the observation that adenovirus-mediated expression of constitutively-active Akt2 also promotes survival in differentiating myocyte cultures. Constitutively-active Akt2 also inhibits anoikis, apoptotic cell death that occurs in non-transformed cells when their substratum-cell adhesions are disrupted.[19,23] Collectively, these data suggest that Akt2 can modulate both growth factor- and matrix attachment-mediated survival signals.
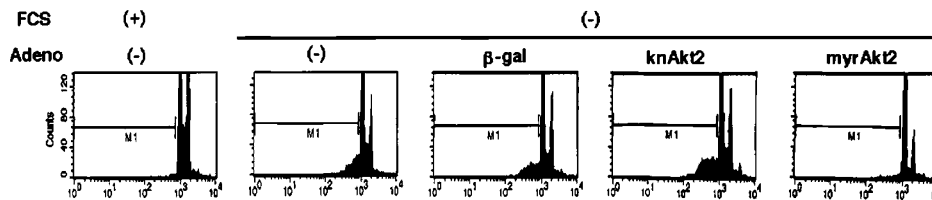
Figure 4 Adenovirus-mediated transfer of constitutively-active Akt promotes cellular survival during mitogen deprivation-induced myogenesis. Representative experiment showing adenoviral transfer of myrAkt2 confers resistance to apoptosis induced by serum-deprivation. C2C12 cells were mock-infected or infected with Adeno-βgal, Adeno-knAkt2 or Adeno-myrAkt2 in GM overnight. The media was then changed to serum-free DMEM 24 hours. Alternatively, a mock-infected culture was maintained in GM. Cells were harvested and stained with propidium iodide, and DNA content was analyzed with a flow cytometer. M1 indicates region of hypodiploid DNA
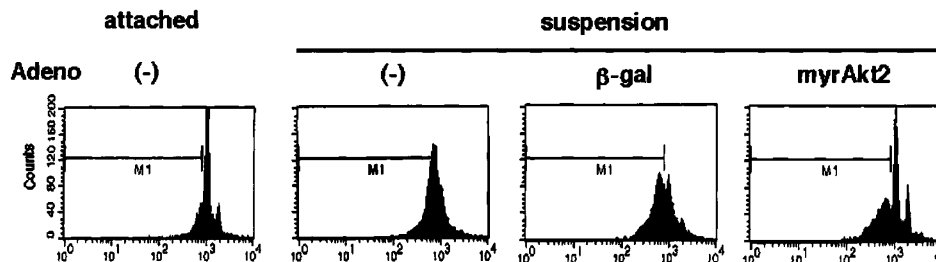

Figure 5 Adenovirus-mediated transfer of constitutively-active Akt2 inhibits anoikis in C2C12 cells. Representative experiment showing constitutively-active Akt2 protects against cell death induced by cell detachment. Cells were mock-infected or infected with Adeno-βgal or Adeno-myrAkt2 overnight. Cells were then harvested with EDTA and incubated in suspension culture in GM for 24 h prior to DNA content analysis. Parallel mock-infected cultures were maintained in monolayer conditions (attached) for comparison. M1 indicates region of hypodiploid DNA

This study also demonstrates that both Akt2 protein and protein kinase activity are upregulated during myogenic differentiation in C2C12 cells and in 10T1/2 cells that are transformed with MyoD. These data are consistent with the observation that *Akt2* mRNA is induced in myogenic cell lines upon differentiation.[5,6] At the levels of activity and protein, the upregulation of Akt2 is similar to that of Akt1 (see Figure 1). However, the mechanism of the upregulation is different for Akt1 and Akt2. Myogenic induction of Akt2 is mediated by an increase in transcript,[5,6] while induction of Akt1 occurs through stabilization of the protein with no accompanying change in mRNA levels.[17] Data from the current study also suggest that Akt2 induction may function to protect differentiating myocytes from apoptosis. Overexpression of wild-type Akt2 suppressed apoptosis to a similar degree as constitutively-active Akt2. Presumably, the wild-type transgene is activated by insulin-like growth factor-II (IGF-II) which is produced by differentiating myocytes.[24–26] In this regard, it has been shown that IGF-II functions as an autocrine survival factor during myoblast differentiation.[27,28]

Finally, the anti-apoptotic activities of Akt2 described here may contribute to its role in malignancy and explain why *Akt2* gene amplification is observed in pancreatic and ovarian cancers.[8,9] It is well recognized that anti-apoptotic genes contribute to malignancy. In particular, anoikis prevents detached cells from colonizing in inappropriate locations, thereby protecting against metastasis.[29] Thus, our observation that enhanced Akt2 signaling can confer resistance to anoikis suggests that the amplification and/or

overexpression of this factor may contribute to tumor formation and invasiveness. Therefore, as an anti-apoptotic signal transducer, Akt2 could have a role in controlling cell survival during normal differentiation as well as contribute to the development of human malignancies.

Materials and Methods

Cell Culture

C2C12 cells were cultured as described previously.[13] Cells were maintained in growth medium (GM) which consists of Dulbecco's modified Eagle medium (DMEM) containing 20% fetal calf serum. Differentiation was induced by switching GM to differentiation medium (DM) which consists of serum-free DMEM or DMEM plus 2% heat-inactivated horse serum. Suspension cultures were obtained as described in.[20] Briefly, cells were washed with phosphate-buffered saline (PBS) twice and harvested with PBS containing 0.5% EDTA. Cells were collected by centrifugation, resuspended in DMEM, and cultured on bacterial culture dishes precoated with 20 mg/ml bovine serum albumin (BSA). 10T1/2 cells and MyoD-transformed 10T1/2 cells were cultured as described previously.[15] Cells were maintained in GM, and switched to DM for 2 days.

Northern blot analyses

Northern blotting was performed as described previously.[17] Briefly, total RNA was prepared with the acid guanidium thiocyanate-phenol-chloroform method.[29] Total RNA (25 μg) was separated in formaldehyde gel and transferred to a Hybond N membrane. The cDNA

fragment corresponding to the pleckstrin homology domain of mouse Akt was used as a probe. After hybridization and washing, membranes were exposed to X-ray film overnight.

Immunoblot analyses

Immunoblot analyses were performed as described in.[17] In brief, cells were washed twice in PBS on ice and extracted with cell lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl [pH 7.4], 20 mM NaF, 1 mM phenylmethyl-sulfonyl fluoride) for 15 min. at 4°C, followed by centrifugation at 15 000 r.p.m. for 15 min. Twenty micrograms of protein were separated on SDS polyacrylamide gel. Proteins were transferred onto a polyvinylidine difluoride membrane (Millipore). After blocking with 5% nonfat milk protein, the membranes were incubated with anti-Akt1 (Santa Cruz), anti-Akt2 (Upstate), anti-cdc2 (Santa Cruz), anti-hemagglutinin (HA) (Boehringer), or anti-Cdk4 antibody (Santa Cruz). Blots were incubated with the secondary antibody conjugated with horse-radish peroxidase (donkey anti-goat (Santa Cruz), donkey anti-rabbit (Amersham), or rabbit anti-sheep (Pierce). An enhanced chemiluminescence protocol was used for detection according to the directions of the manufacturer (Amersham Life Science).

Akt2 half-life determinations

Cultures of C2C12 cells were incubated in growth medium or differentiation media for two days. Parallel cultures were then exposed to 35 μM cyclohexamide and lysates were prepared after 2, 4 and 6 h. Western immunoblots were performed to determine Akt2 levels relative to the level of cdc2, which remains constant under these conditions.[30]

Akt protein kinase assays

Cell lysates were prepared as described above. Cell lysates (200 μg per assay) were immunoprecipitated with anti-Akt1 or Akt2 antibody using protein G agarose (Boehringer). After two washes with lysis buffer, one wash with H_2O, and two washes with kinase buffer (20 mM HEPES [pH 7.2], 10 mM $MgCl_2$, 10 mM $MnCl_2$), kinase assays were performed as described previously[17] using histone H2B as a substrate.

Transient transfection and cell viability analysis

Plasmids included vector (pcDNA3) with no insert or encoding wild-type Akt2, kinase-negative Akt2 (knAkt2), or constitutively-active Akt2 (myrAkt2) expressed from the cytomegalovirus (CMV) promoter/enhancer.[31] Plasmids were cotransfected with an expression vector encoding enhanced green fluorescent protein (GFP) (Clontech) with LipofectAmine as described in.[17] After 18 h incubation in GM, apoptosis was induced by changing GM to serum-free medium. After incubated for 24 h, cells were fixed with 3.7% formaldehyde in PBS and stained with Hoechst 33342. GFP-positive cells with pyknotic nuclei were determined by visual examination.

Adenovirus construction and transfection

Replication-defective adenovirus vectors were constructed according to.[32] Briefly, knAkt2 or myrAkt2 cDNAs, fused at their N-terminus to the hemagglutinin epitope (HA), were inserted into the multicloning site of pACCMVpLpA. The resulting plasmids were cotransfected with pJM17 into 293 cells to allow for homologous recombination. An adenovirus construct expressing β-galactosidase from the CMV promoter/enhancer was used as a control.[33] All constructs were amplified in 293 cells and purified by ultracentrifugation in the presence of CsCl. For transfection, C2C12 cells were incubated with adenovirus vectors in GM for 15 h. C2C12 cells were infected at a multiplicity of infection (MOI) of 250, which leads to > 95% efficiency of transgene transduction.[17]

Flow cytometry

DNA content of adenovirus construct-infected or mock-infected cells was measured with a FACScan flow cytometer (Becton Dickson) as described in.[14]

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