Biochemical and Biophysical Research Communications 425 (2012) 800–805

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Akt1 regulates phosphorylation and osteogenic activity of Dlx3

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ARTICLE INFO

Article history:
Received 25 July 2012
Available online 2 August 2012

Keywords:
Dlx3
Akt1
Osteoblast
Differentiation
Phosphorylation

ABSTRACT

Distal-less 3 (DLX3) is a highly conserved homeobox containing transcription factor. DLX3 is specifically expressed in osteoblasts and osteocytes of all developing bones. DLX3 is essential for osteoblast differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression. Akt can be activated by several osteogenic signaling molecules, but its precise function and downstream targets in bone development are unknown. In this report, we investigated a potential regulation of Dlx3 function by Akt1. We found that Akt1 phosphorylates Dlx3 and Akt1 activation increases protein stability, osteogenic activity and transcriptional activity of Dlx3. Also, BMP2 was shown to increase the protein level of Dlx3 in an Akt1 activity-dependent manner. Conversely, inhibition of Akt1 by the Akt inhibitor decreases the protein levels of Dlx3. These results suggest that Dlx3 is a novel target of Akt1 and the activity of Dlx3 could be modulated by a novel mechanism involving Akt1 during osteoblast differentiation.

1. Introduction

Bone is maintained by the coordinated balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoblasts and osteoclasts play important roles in bone remodeling [1]. Osteoblasts mainly regulate bone deposition whereas osteoclasts mainly regulate bone resorption. The activities of osteoclasts and osteoblasts can be regulated at the level of differentiation by various regulatory signals. Bone remodeling is regulated by various anabolic factors including Wnt, insulin, bone morphogenetic proteins (BMPs), insulin growth factor-I and kinases such as Akt.

Akt proteins play integral roles in mediating the effects of signaling molecules in numerous biological processes [2]. Akt proteins are serine/threonine protein kinases, and vertebrates express this in three isoforms: Akt1/PKBδ, Akt2/PKBβ and Akt3/PKBγ. Genetic studies indicate that Akt isoforms play essential roles in bone development. Akt1 and Akt2 double knockout mice display severe defects in bone development and die shortly after birth [3]. Mice lacking Akt1, the major isoform in bone tissue, exhibit osteopenia [4,5]. Akt1 also has important functions in the late stages of endochondral bone formation [6]. Akt activity enhances the osteogenic function of Osterix and Dlx5, at least in part, through protein stabilization, transcriptional activity and phosphorylation [7,8].

Skeletal development is a complex process whereby multiple signaling pathways converge to induce the formation of bone structures. The BMP2/4/7 members of the transforming growth factor superfamily of signaling polypeptides are essential for commitment and differentiation of mesenchymal cells to the osteoblast lineage for bone tissue formation. BMP2 target genes include a cohort of transcription factors with specialized activities for induction of cell type-specific lineages [9]. The regulation of osteoblast differentiation is mediated by BMPs with various transcription factors such as Runx2, Osterix and several homeodomain (HD) proteins [10–15].

Homeodomain proteins play a key role in skeletal development. The families of homeobox genes are classical repressor proteins that are expressed in neural crest cells and limb tissues to regulate skeletal patterning and the number of osteoprogenitor cells [16–18]. Many vertebrate homeobox-containing genes have been identified on the basis of their sequence similarity with Drosophila developmental genes. Members of the Dlx gene family contain a homeobox that is related to that of Distal-less (Dll), a gene expressed in the head and limbs of the developing fruit fly. The Distal-less (Dlx) family of genes comprises at least 6 different members, Dlx1-Dlx6. Dlx2 and Dlx3, that contribute to craniofacial development but with distinct temporal and spatial patterns of expression [19,20]. Dlx2 also has an important role in mesenchymal condensation. Dlx5 and Dlx6 are essential for the development of jaw, axial, and appendicular bone. Although Dlx3 null mice are embryonically lethal prior to the skeletal development due to placental failure [21,22], recent studies showed that DLX3 is expressed in periosteum, osteoblasts, and chondrocytes of the developing limb and in osteogenic lineage cells. The transcriptional regulation of DLX3 affects Osteoblast differentiation [23–25].

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http://dx.doi.org/10.1016/j.bbrc.2012.07.155

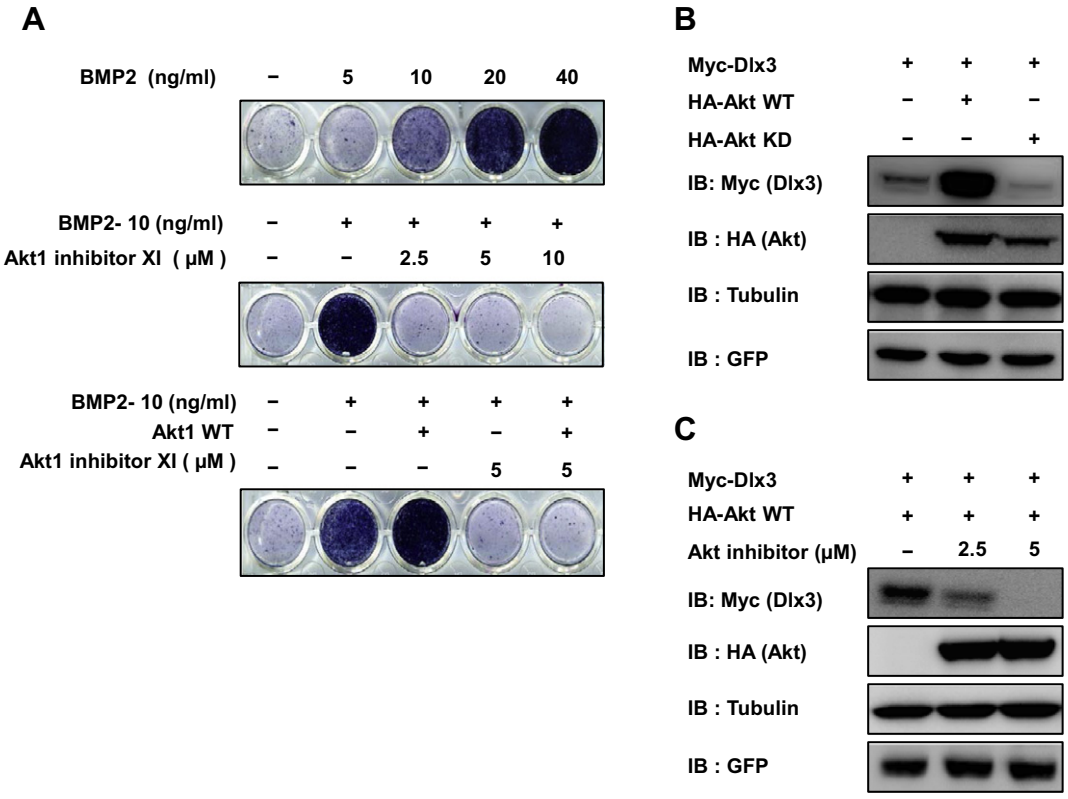

Fig. 1. Akt1 activation is induced in BMP2-stimulated osteoblast differentiation and increases the protein levels of Dlx3. (A) C2C12 myoblast cells were cultured with 10% FBS containing DMEM supplemented medium. After 24 h, growth media were changed to DMEM supplemented with 2% FBS. Cells were transfected with HA-tagged Akt1 WT and treated with the Akt inhibitor at the indicated concentrations in the absence or presence of BMP2. After 3 days, the extent of osteoblast differentiation was compared by ALP staining. ALP positive cells stained blue/purple and examined under a fluorescent microscope. Similar results were obtained from three independent experiments. (B) HEK 293 cells were transfected with Myc-tagged Dlx3 (0.5 μg, IB: Myc) and HA-tagged Akt1 WT/ KD (0.5 μg, IB: HA) or a control vector along with GFP. GFP was used as a transfection control (IB: GFP) and Tubulin was used as a loading control (IB: Tubulin). Protein levels were determined by immunoblotting using anti-Myc antibody. (C) HEK 293 cells were transfected with Myc-tagged Dlx3 (0.5 μg, IB: Myc), HA-tagged Akt1 WT (0.5 μg, IB: HA) and were treated with Akt inhibitors (2.5, 5 μM) or untreated vehicle as a control (DMSO). GFP was used as a transfection control (IB: GFP) and Tubulin was used as a loading control (IB: Tubulin). Protein levels were determined by immunoblotting using anti-Myc antibody.

In this study, Akt1 proteins regulate the activities of target proteins primarily through the post-translational modifications of target proteins or their upstream effectors. We examined whether Akt1 plays a role in the regulation of DLX3 function during osteogenesis. We found that Akt1 phosphorylates DLX3. In addition, Akt1 kinase activity is important for the regulation of protein stability and transcriptional activity of DLX3. We also discovered that BMP2 increases the protein level of DLX3 via Akt1 activity. These results suggest that Akt1 enhances the osteogenic function of DLX3 by increasing the stability and transcriptional activity of proteins.

2. Materials and methods

2.1. Plasmids and antibodies

Myc-tagged Dlx3, HA-tagged Akt1 WT and Akt1 KD were constructed in a CMV promoter-derived mammalian expression vector (pCS4-3Myc,-3HA). Antibodies against Myc (9E10, Roche Applied Science) and GFP (G1544, Santa Cruz Biotechnology), HA (12CA5, Roche Applied Science), phospho-threonine (PTR-8) and phospho-serine (PSR-45, Sigma–Aldrich), and phospho-Akt substrate motif (RXXS//T/) (110B7E, Cell Signaling Technology), Dlx3 (3B8, Abfrontier), and α-Tubulin (B-5-1-2, Sigma–Aldrich) were used.

2.2. Cell culture and transient transfection

The HEK 293 human embryonic kidney epithelial cell line and the C2C12 mouse myoblast cell line were cultured at 37 °C, 5% CO_2 in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 5% or 10% fetal bovine serum (FBS) with 100 units/ml penicillin and 100 μg/ml streptomycin. DMEM, FBS and antibiotics were purchased from Invitrogen. Transient transfection was performed using the Effectene (QIAGEN) or by the calcium phosphate-mediated method. Total amounts of transfected plasmids in each group were equalized by adding an empty vector.

2.3. Immunoblotting

Cells were rinsed twice with ice-cold PBS and lysed in an ice-cold lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na3VO4, 250 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. After centrifugation, supernatants containing 30 μg of total protein were subjected to SDS–PAGE. Proteins were transferred to a PVDF membrane and visualized using appropriate primary antibodies, HRP-conjugated secondary antibodies and ECL reagent.

2.4. Immunoprecipitation

Supernatants of cell lysates, prepared in the same way as immunoblotting analysis, were subjected to immunoprecipitation using appropriate antibodies and protein A or G-Sepharose beads. The immunoprecipitated proteins were separated by SDS–PAGE and visualized by immunoblotting.

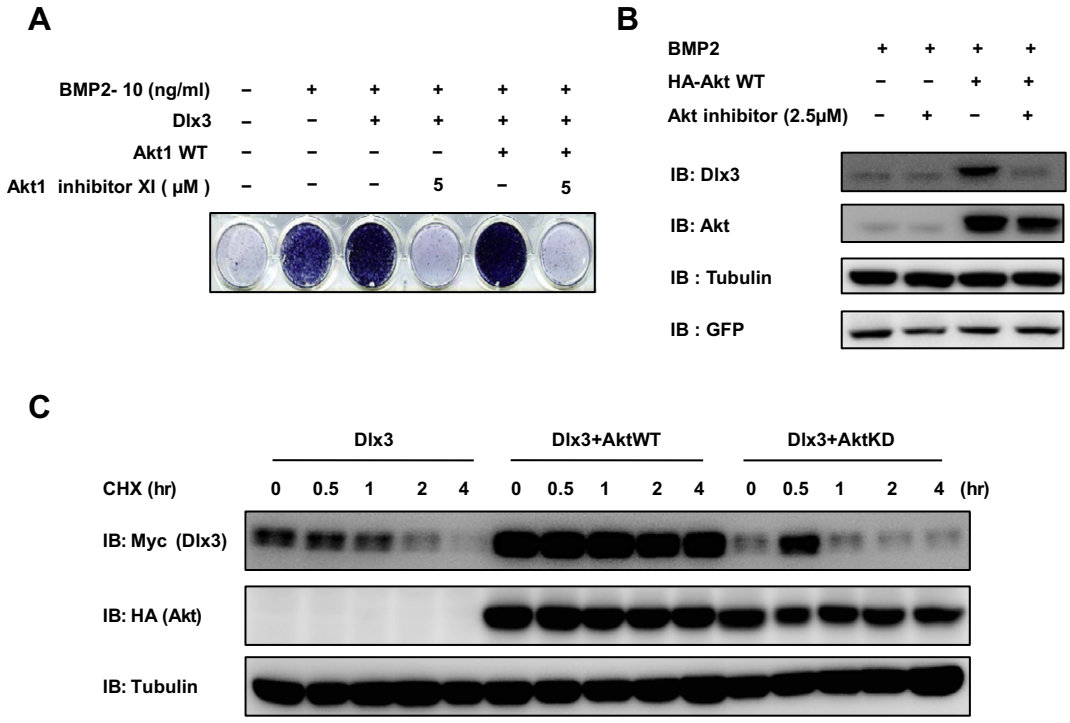

Fig. 2. Akt1 activation increased endogenous Dlx3 expression and protein stability of Dlx3. (A) BMP2 induced osteoblast differentiation in C2C12 myoblast cells were transfected with Myc-tagged Dlx3, HA-tagged Akt1 WT and treated with the Akt inhibitor at the indicated concentrations in the absence or presence of BMP2. After 3 days, the extent of osteoblast differentiation was compared by ALP staining. ALP positive cells stained blue/purple and examined under a fluorescent microscope. Similar results were obtained from three independent experiments. (B) BMP2 induced osteoblast differentiation in C2C12 cells and C2C12 cells were transfected with HA-tagged Akt1 WT (0.5 μg, IB: HA) and were treated with Akt inhibitors (5 μM) or untreated vehicle as a control (DMSO). GFP was used as a transfection control (IB: GFP) and Tubulin was used as a loading control (IB: Tubulin). Protein levels were determined by immunoblotting using anti-Dlx3 antibody. (C) HEK 293 cells were co-transfected with Myc-tagged Dlx3 (0.5 μg, IB: Myc), Akt1 WT/KD (0.5 μg, IB: HA) and treated with cycloheximide (40 μM). After 24 h, transfected cells were harvested at the indicated times. The experiment was repeated three times and the average and standard deviations are shown.

2.5. Luciferase reporter assay

Cells were seeded on 24-well plates the day before transfection. C2C12 cells were transfected with ALP (ALP-Luc), Bone sialoprotein (BSP-Luc) reporter plasmid, pCMV-β-gal, and combinations of Dlx3 and Akt1 WT expression vectors or Akt inhibitor on 24 well plates and were lysed 36 h after transfection. Luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized with corresponding β-galactosidase activities for transfection efficiency. Experiments were performed in triplicate and were repeated at least three times.

2.6. Protein stability assay

HEK 293 cells were co-transfected with Myc-tagged Dlx3 or HA-tagged Akt1 (WT/KD) expression vectors. After 24 h, the cells were exposed to fresh media. Transfected cells were incubated for the indicated times and were treated with 40 μM of cycloheximide (CHX) and harvested with lysis buffer as described above. Protein levels were analyzed by immunoblotting using anti-Myc or anti-HA antibodies.

2.7. Total RNA extraction, reverse transcribed RT-PCR analysis

Total cellular RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Random primed cDNAs were synthesized from 1 μg of total RNA using Super- Script III First-Strand Synthesis System (Invitrogen). The following conditions were used for amplification by PCR: initial denaturation at 94 °C for 1 min; followed by 28–30 cycles of denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 30 s; final extension at 72 °C for 5 min. The following PCR primers were used: ALP Forward 5′-GAT CAT TCC CAC GTT TTC AC-3′ and Reverse 5′-TGC GGG CTT GTG GGA CCT GC-3′; Colla1 Forward 5′-TCT CCA CTC TTC TAG GTT CCT-3′ and Reverse 5′-TTG GGT CAT TTC CAC ATG C-3′; BSP Forward 5′-ACA CTT ACC GAG CTT ATG AGG-3′ and Reverse 5′-TTG CGC AGT TAGCAA TAG CAC-3′;GAPDH Forward 5′-ACC ACA GTC CATGCC ATC AC-3′ and Reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′. Dlx3 Forward 5′-TTC TTC TTC ACC GAC ACT G-3′ and Reverse 5′-TCT GGT TCC AGA ACC GCC GCT-3′.

2.8. Alkaline phosphatase (ALP) staining

C2C12 cells in 24-well plates were transfected using Effectene (QIAGEN). C2C12 cells were induced by stimulating the cells with BMP2. Cells were pretreated with BMP2 for 3 days. These cellswere cultured at 5% CO_2, 37 °C. Transfected C2C12 cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT), washed with PBS and stained with 300 μg/ml BCIP/NBT (5-bro-mo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) solution (Sigma–Aldrich) for 20 min at RT. The alkaline phosphatase-positive cells were stained blue/purple.

3. Results

3.1. Akt1 affects Dlx3 in BMP2-stimulated osteoblast differentiation

BMP2 stimulation induces osteoblast differentiation in C2C12 myoblast cells [26]. In a previous study, it has been reported that Akt1 in osteoblasts and osteoclasts controls bone remodeling [1]. To investigate the role of activated Dlx3 in BMP2-induced

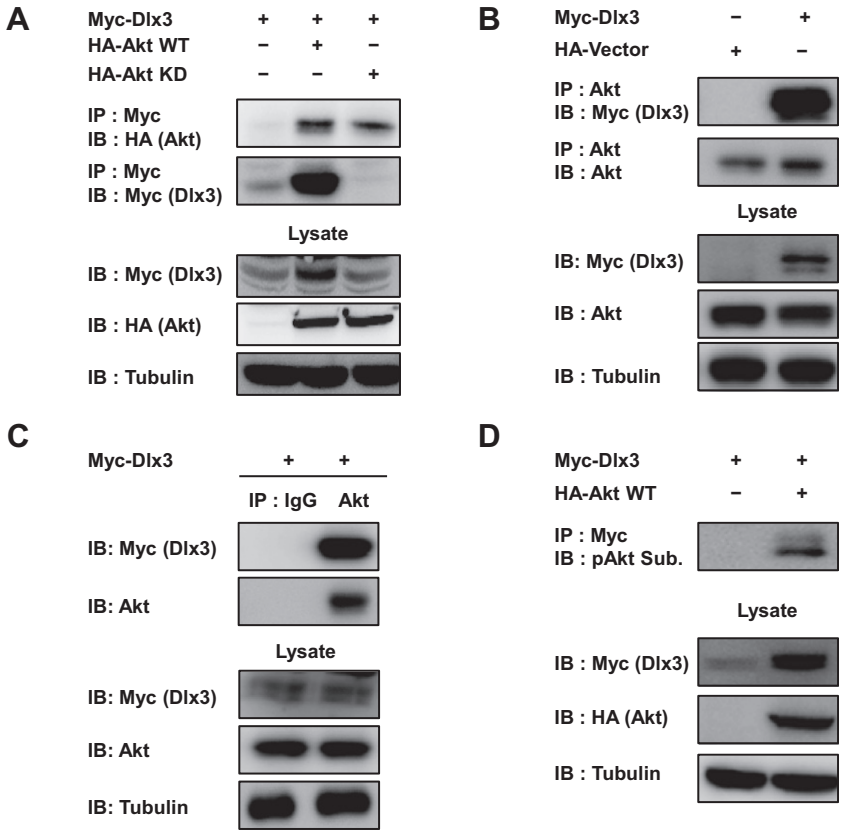

Fig. 3. Akt1 interacts with and induces the phosphorylation of Dlx3. (A) HEK 293 cells were transfected with Myc-tagged Dlx3, HA-tagged Akt WT, and Akt KD. To examine the binding of Dlx3, Akt WT and Akt KD, immunoprecipitation (IP) was performed using an anti-Myc antibody followed by western blot analysis using an anti-HA antibody. (B) HEK 293 cells were transfected with Myc-tagged Dlx3 and HA-tagged Vector. For the interaction of Dlx3 and Akt1, immunoprecipitation was performed using an Akt Ab and western blotting with Myc Ab. (C) Myc-tagged Dlx3 was transfected to examine whether the binding of Dlx3 and Akt is endogenous. The binding of Dlx3 in HEK 293 cells was performed by immunoprecipitation using IgG or Akt Ab and western blotting with Myc Ab. (D) Myc-tagged Dlx3 and HA-tagged Akt1 WT were transfected to examine the phosphorylation of Dlx3 in HEK 293 cells. The phosphorylation of Dlx3 was determined by immunoprecipitation using the specific phospho-Akt substrate antibody.

osteoblast differentiation, we used mesenchymal C2C12 cells that can be differentiated into osteoblasts by BMP2 treatment [13,14,27]. Osteoblast differention was measured by ALP staining. First, we examined whether BMP2 induced Akt1 activation in C2C12 cells. When the cells were pretreated with BMP2 or increasing amounts of Akt inhibitors, at the indicated concentrations, BMP2 increased ALP activity in a dose-dependent manner (Fig. 1A). ALP staining showed that blocking Akt1 activation with Akt inhibitors strongly suppressed ALP activity in a dose-dependent manner (Fig. 1A). Second, we examined whether Akt affected the protein levels of Dlx3. HEK 293 cells were transfected with combinations of Dlx3, Akt1 WT (active form) and Akt1 KD (inactive form). The protein levels of Dlx3 were compared by immunoblotting. The levels of Dlx3 were increased by Akt1 WT and decreased by Akt1 KD (Fig. 1B top panel). However, the inhibition of Akt1 by the Akt inhibitor decreased the protein levels of Dlx3 (Fig. 1C). These results suggest that Akt1 regulates Dlx3-induced osteoblastogenesis.

3.2. Akt1 regulates expression levels and stability of the osteoblast-specific transcription factor Dlx3

We confirmed that Akt1 activation is involved in BMP2-induced osteoblast differentiation (Fig. 1). In order to understand the mechanism of the transcription factor-Dlx3, we first examined whether Akt1 affected Dlx3-induced osteoblast differentiation. C2C12 cells were transfected with Dlx3, Akt1 WT and treated with Akt inhibitors. Dlx3 induced the expression of ALP, an osteoblast-specific marker, in the presence of BMP2. Akt inhibitors significantly decreased ALP staining induced by Dlx3 with BMP2 (Fig. 2A). Second, the protein level of endogenous Dlx3 was increased by BMP2 and decreased by endogenous Akt inhibitors (Fig. 2B). Third, Akt1 may regulate the expression of Dlx3 at the level of transcription, translation or by protein stability. The protein levels of Dlx3 are increased by Akt1 (Figs. 1B, C and 2B). To identify the molecular mechanism for the Akt1-induced increase of Dlx3 protein levels, we examined whether Akt1 affected the protein stability of Dlx3 using cycloheximide (CHX). HEK 293 cells were transfected with Dlx3 with or without Akt1 WT/Akt1 KD. To estimate the stability of Dlx3 by Akt1, transfected cells were treated with 40 μM of cycloheximide for the indicated times and then harvested. The protein levels of Dlx3 were determined by western blotting. Dlx3 protein was degraded in the absence of Akt1 with a half- life of approximately 2 h. However, Akt1 significantly blocked Dlx3 degradation and prolonged the half-life of Dlx3 protein (Fig. 2C). Akt1 extended the half-life of the Dlx3 protein and enhanced the accumulation of Dlx3. These results suggest that Akt1 increases the protein levels of Dlx3 by increasing the protein stability of Dlx3.

3.3. Akt1 interacts with and phosphorylates Dlx3

Given the results above, we examined a possible interaction betweenDlx3 and Akt1. To confirm this hypothesis, HEK 293 cells were transfected with Myc-Dlx3 and HA-Akt1 WT or KD and then underwent immunoprecipitation (IP). We found that Dlx3 was bound to

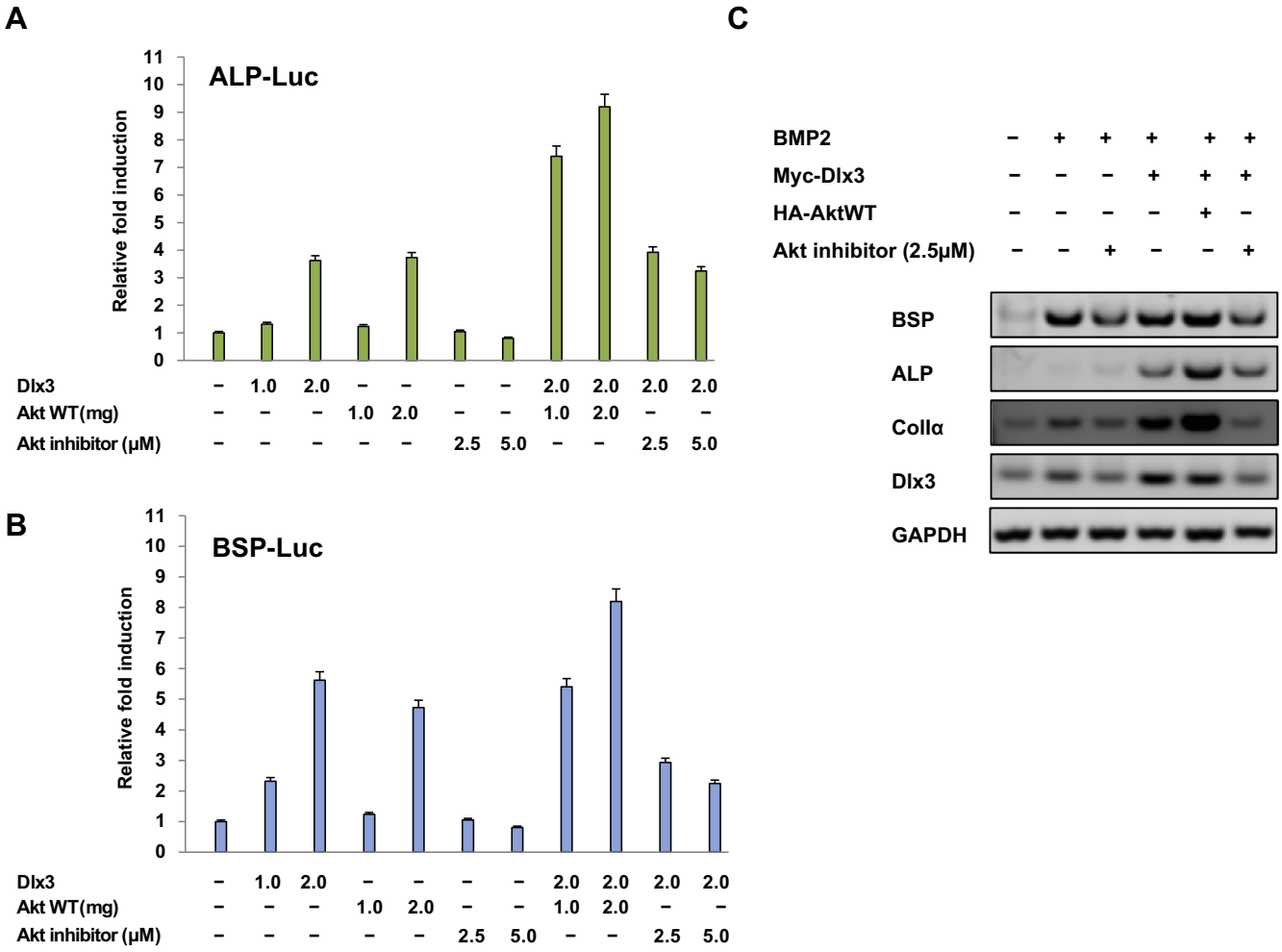

Fig. 4. Akt1 enhances the transcriptional activity of Dlx3. (A,B) C2C12cells were transfected with pCMV-β-gal, BSP-Luc, and ALP-Luc reporter vector along with the indicated combinations of Dlx3 and Akt1 WT expression vectors or were treated with Akt inhibitors. Reporter activity was then measured. Data are expressed as relative induction ratios to an internal control, and relative luciferase activities with S.D. are shown. The experiment was performed in triplicate. (C) C2C12 cells were transfected for 24 h with the indicated combinations of Dlx3 and Akt1 or were treated with Akt inhibitors. Cells were then treated with BMP2 (5 ng/ml) for 24 h. The expression levels of bone sialoprotein (BSP), collagen type I α (Collα), alkaline phosphatase (ALP), and Dlx3 were compared by semi-quantitative RT-PCR. GAPDH was used as an internal control.

exogenous Akt1 WT and Akt1 KD (Fig. 3A). Furthermore, immunoprecipitation (IP) was performed again for the interaction between Dlx3 and endogenous Akt. We determined that Dlx3 was bound to endogenous Akt (Fig. 3B and C). To investigate the potential regulation of Dlx3 activity by Akt, we next examined whether Akt1 can phosphorylate Dlx3 using the anti-phospho-Akt substrate motif antibody. HEK 293 cells were transfected with Akt1 expressing plasmid or control plasmid. Dlx3 proteins were immunoprecipitated and immunoblotted using the anti-phospho-Akt substrate motif antibody. Akt1 was found to induce the phosphorylation of Dlx3 (Fig. 3D). These results indicate that Akt1 interacts with and phosphorylates Dlx3.

3.4. Akt1 enhances the transcriptional activity of Dlx3

To analyze whether Akt1 can modulate the transcriptional activity of Dlx3, C2C12 cells were transfected with alkaline phosphatase (ALP)-Luc, and bone sialoprotein (BSP)-Luc osteoblast reporter genes which are known to be markers of osteoblast differentiation. C2C12 cells were transfected with an alkaline phosphatase promoter-luciferase reporter (ALP-Luc) along with combinations of Dlx3 and Akt1 WT or were treated with Akt inhibitors using the indicated concentrations. Dlx3 or Akt1 WT by itself increased the reporter expression above the basal level, and co-transfection of them further increased the reporter expression (Fig. 4A). However, transfection of Dlx3 with Akt inhibitor treatment failed to increase the reporter expression. These results indicate that Akt1 increases the transcriptional activity of Dlx3.

Next, we examined whether Akt1 affected Dlx3-induced activation of a bone sialoprotein promoter-luciferase reporter (BSP-Luc). Bone sialoprotein is an osteoblast marker induced in the early stages of osteoblast differentiation. C2C12 cells were transfected with the bone sialoprotein promoter-luciferase reporter (BSP-Luc) along with combinations of Dlx3 and Akt1 WT and treated with the Akt inhibitor at the indicated concentrations. Dlx3 or Akt1 WT significantly increased the BSP-Luc activation above the basal level (Fig. 4B). Co-transfection with Dlx3 and Akt1 WT increased the reporter activation in a dose-dependent manner, whereas treatment with the Akt inhibitor failed to do so. These results suggest that Akt1 activity enhances the Dlx3-mediated activation of the BSP promoter.

We then examined the effect of Akt1 activity on the BMP2- and Dlx3-induced expression of osteoblast marker genes. C2C12 cells were transfected with combinations of Dlx3 and Akt1 WT, and then treated with the Akt inhibitor and BMP2. The transcriptional levels of the following osteoblast markers were examined: alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen type I α (ColIα) and Dlx3. Dlx3 was increased by the BMP2-induced

expression of the osteoblast markers (Fig. 4C). Akt1 further increased the expression of the markers. Interestingly, the Akt inhibitor decreased the expression of BSP, ALP, Dlx3 and CoIIα below the levels seen with Osterix alone or with BMP2. These results indicate that Akt1 activity regulates the transcriptional activity of Dlx3 and is critical for the Dlx3-induced expression of at least a subset of osteoblast markers.

4. Discussion

The bone regulation system involves coupling between bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoblasts and Osteoclasts play important roles in bone remodeling. The Dlx gene family, related to the Distal-less homeobox gene in Drosophila, encodes for proteins that act as transcription activators [23]. Dlx genes are the key-players in the skeletal development [17,22,28]. *[Editor's note: Incomplete sentence.]* Dlx genes have also been postulated to regulate cell differentiation in the skeleton including bone [29], cartilage and tooth. Also, Akt controls osteoblast and osteoclast bone remodeling [1]. However, BMP2 regulates the transcription of Dlx3 and Akt signaling pathway [9,30]. We investigated the effects of Akt1 on Dlx3 function during osteoblast differentiation. We found evidence for a regulatory mechanism whereby Akt1 controls osteoblast differentiation. Akt1 increases the protein level, protein stability and transcriptional activity of Dlx3. First, the protein levels of Dlx3 were increased by Akt1 and this increase was abolished by Akt inhibitors. Second, endogenous and exogenous Akt 1 interacts with Dlx3. Third, Akt1 phosphorylates Dlx3, and Akt1 activation prolonged the half-life of the Dlx3 protein. Fourth, the transcriptional activity of Dlx3 was enhanced by Akt1 but it was decreased by Akt inhibitors. Akt1 affected the levels of Dlx3 mRNA. These results indicate that osteoblast differentiation is regulated through the enhancement of the protein stability and transcriptional activity of Dlx3 by Akt1 signaling. The function of Dlx3 can be regulated post-translationally by protein kinase-mediated ostogenesis. In previous reports, Phosphorylation of murine homeodomain protein Dlx3 was shown to be regulated by protein kinase C [31]. Our current results suggest that Akt1 regulates the function of Dlx3. We found that the activation of Akt1 induces the phosphorylation on Dlx3. Akt mostly phosphorylates serine or threonine residues within the consensus target sequence of (RXXS∗/T∗). However, sequence analysis did not reveal any residues corresponding to a consensus Akt1 phosphorylation target site. Identification of Akt1 phosphorylation site(s) on Dlx3 and the precise mechanisms of modification will enhance our understanding of the regulatory mechanisms of Dlx3 osteogenic function and how Akt1 regulates osteoblast differentiation. Therefore, further research is needed to determine the phosphorylation site(s) to understand the significance such regulation between Dlx3 and Akt1. Our work provides a basis for understanding the roles of Akt1 during osteoblast differentiation. Akt1 modulates Dlx3 function through the regulation of protein stabilization, transcriptional activity and phosphorylation.

Acknowledgments

This work was supported by a Grant from the Next-Generation BioGreen 21 Program (No. PJ0081472011), Rural Development Administration, Republic of Korea.

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