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Akt phosphorylates and regulates the function of Dlx5

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ABSTRACT

Akt, a phosphoinositide-dependent serine/threonine protein kinase, acts as a key regulator in bone formation. Akt can be activated by several osteogenic signaling molecules, but its precise function and downstream targets in bone development are unknown. Dlx5 transcription factor plays important roles during bone development and osteoblast differentiation. Its expression is regulated by several osteogenic signals. In addition, Dlx5 function is also regulated through post-translational modification by several kinases. In this report, we have investigated a potential regulation of Dlx5 function by Akt. Our results indicate that Akt interacts with and phosphorylates Dlx5. In addition, we provide evidences that Akt kinase activity is important for Akt to enhance the protein stability and transcriptional activity of Dlx5. These results suggest that Dlx5 is a novel target of Akt and that the activity of Dlx5 could be modulated by a novel mechanism involving Akt during osteoblast differentiation.

1. Introduction

Bone formation and homeostasis are orchestrated by coordinated actions of osteoblasts and osteoclasts. Osteoblasts mainly regulate bone deposition whereas osteoclasts mainly regulate bone absorption. These dynamic and intricate processes are governed by various signaling molecules and transcriptional regulators, which exert their effects primarily by regulating the differentiation of osteoblasts and osteoclasts [1,2].

Akt proteins play integral roles in mediating the effects of signaling molecules in numerous biological processes. Akt proteins are serine/threonine protein kinases and vertebrates express three isoforms: Akt1/PKBα, 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 during late stages of endochondral bone formation [6]. Cell line-based studies indicate that Akt plays important roles in mediating the actions of osteogenic signaling molecules. Phosphoinositide-3 kinase/Akt signaling promotes osteoblast differentiation induced by IGF or BMP2 [7]. However, the precise mechanism of how Akt isoforms regulate bone formation and the downstream effectors of Akt proteins in bone formation are largely unknown.

Dlx5 transcription factor plays important roles during bone development and its activity is regulated by several osteogenic signaling effectors. Dlx5 is a member of the vertebrate Dlx family homologous to the Drosophila Distal-less homeodomain protein [8]. *Dlx5* is expressed in most skeletal tissue and its expression is up-regulated during osteoblast differentiation [9–11]. *Dlx5* null mice exhibit defects in axial, appendicular and craniofacial bone formation [12–14]. *In vivo* and *in vitro* studies indicate that Dlx5 is sufficient to induce the differentiation of chondrocytes and osteoblasts [15–17]. Recent studies revealed that, in addition to its transcription, the function of *Dlx5* is regulated by post-translational modification. CaMKII, p38 MAPK and PKA have been shown to regulate the activity of Dlx5 protein [18–20], suggesting that osteogenic signaling molecules may exert their function partly by regulating the function of Dlx5 through post-translational modification.

Akt proteins regulate the activities of target proteins primarily through the post-translational modifications of target proteins or their upstream effectors. Because the activity of Dlx5 can be modulated by various signaling effectors, we postulated that Akt may regulate the activity of Dlx5. In this report, we examined and provide evidences that Akt interacts with and directly phosphorylates Dlx5. In addition, Akt kinase activity is important for the regulation of protein stability and transcriptional activity of Dlx5. Taken together, our results suggest that Akt is a novel regulator of Dlx5.

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2. Materials and methods

2.1. Cell culture and transfection

C2C12 mouse myoblast cell line and 293 human embryonic kidney cell line were maintained at 5% CO_2, 37 °C in DMEM supplemented with 10% FBS and antibiotics–antimycotics. For transient transfection, cells were seeded to 6-well (for immunoprecipitation and immunoblotting assays) or 12-well (for luciferase assay) plates, and transfected using calcium phosphate-mediated method or Effectene (Qiagen) according to the manufacturer's recommendation.

2.2. Plasmids, inhibitors and antibodies

Following CMV promoter driven expression plasmids were used (amounts used for transfection): pCMV–GFP (0.25 μg), pCS4 + 6Myc-Dlx5 (0.5 μg for immunoblotting, 0.25 μg for luciferase assay), pCS4 + 3HA-Akt1(WT) and pCS4 + 3HA-Akt1(KD) (0.5 μg for immunoblotting, 0.1–0.25 μg for luciferase assay). Following reporter plasmids were used (amount used for transfection): an osteocalcin promoter luciferase reporter (OC-Luc) (0.1 μg), an alkaline phosphatase promoter luciferase reporter (ALP-Luc) (0.1 μg), and a CMV promoter driven β-galactosidase reporter (pCMV-β-Gal) (0.05 μg). For inhibition of Akt activity, 5 mM stock solution of Akt inhibitor XI (Calbiochem) was used at 5 μM final concentration. Following antibodies were used: Akt (pan) (11E7, Cell Signaling Technology), Dlx5 (O-25, Santa Cruz Biotechnology), GFP (G1544, Sigma–Aldrich), HA (12CA5, Roche Applied Science), Myc (9E10, Roche Applied Science), phospho-Akt substrate motif (RXXS*/T*) (110B7E, Cell Signaling Technology), α-Tubulin (B-5-1-2, Sigma–Aldrich).

2.3. Immunoblotting analysis

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 Na_3VO_4, 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 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 bead. The immunoprecipitated proteins were separated by SDS–PAGE and visualized by immunoblotting.

2.5. In vitro kinase assay

Myc-tagged Dlx5 was immunoprecipitated from transfected 293 cells. Immunoprecipitates were resuspended in a kinase buffer [20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP]. They were then incubated for 30 min at 37 °C in the presence of γ-[^{32}P]-ATP with or without recombinant active Akt1 protein (Akt1 kinase, Cell Signaling Technology). The reactions were terminated by adding SDS-sample buffer. The phosphorylation status of Dlx5 was determined by autoradiography.

2.6. Luciferase reporter assay

Luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized with corresponding β-galactosidase activities for transfection efficiency.

2.7. Semi-quantitative RT-PCR

Total cellular RNA was prepared using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Random-primed cDNAs were synthesized from 1 μg of total RNA using SuperScript 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. 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′; Collα1 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 TAG CAA TAG CAC-3′; Runx2 Forward 5′-AGC AAC AGC AAC AAC AGC AG-3′ and Reverse 5′-GTA ATC TGA CTC TGT CCT TG-3′; GAPDH Forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and Reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′.

3. Results

3.1. Akt interacts with and phosphorylates Dlx5

To examine the potential regulation of Dlx5 by Akt, we first examined the interaction between Akt and Dlx5 in C2C12 cells. C2C12 cells possess osteogenic potential and express Dlx5. Dlx5 interacted with Akt (Fig. 1A). Next, we examined whether Akt induced the phosphorylation of Dlx5. C2C12 cells were transfected with Akt1 expressing plasmid or control plasmid. Dlx5 proteins were immunoprecipitated and immunoblotted using anti-phospho-Akt substrate motif antibody. Akt1 induced the phosphorylation of Dlx5 (Fig. 1B). We then examined whether Akt can phosphorylate Dlx5 directly. Dlx5 proteins, immunoprecipitated from transfected 293 cells, were subjected to an *in vitro* kinase assay with or without active Akt1 protein. Akt1 phosphorylated Dlx5 directly (Fig. 1C). These results indicate that Akt interacts with Dlx5 and phosphorylates Dlx5.

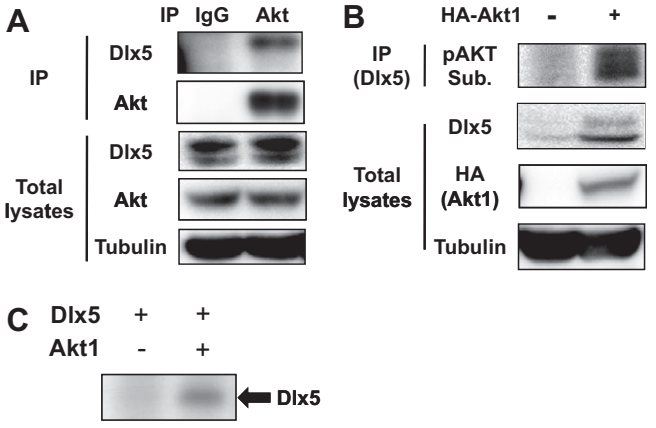

Fig. 1. Akt interacts with and phosphorylates Dlx5. (A) Lysates from C2C12 cells were immunoprecipitated (IP) with anti-Akt (pan) antibody or control IgG. Immunoprecipitates were analyzed for Dlx5 and Akt by immunoblotting. Levels of Dlx5 and Akt in total lysates were also compared. Tubulin was used as a loading control. (B) C2C12 cells were transfected with control or HA-tagged Akt1 plasmid for 24 h, and lysates were immunoprecipitated for Dlx5 [IP (Dlx5)]. Phosphorylation of Dlx5 by Akt1 was examined by immunoblotting using anti-phospho-Akt substrate motif antibody (pAkt sub.). Levels of Dlx5 and Akt1 [HA (Akt1)] in total lysates were also compared. (C) Dlx5 proteins, immunoprecipitated from transfected 293 cells, were incubated with or without recombinant active Akt1 protein in *in vitro* kinase reactions. Phosphorylated Dlx5 was visualized by autoradiography.