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Akt phosphorylates and regulates the osteogenic activity of Osterix

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ABSTRACT

Osterix (Osx), a zinc-finger transcription factor is required for osteoblast differentiation and new bone formation during embryonic development. Akt is a member of the serine/threonine-specific protein kinase and plays important roles in osteoblast differentiation. The function of Osterix can be also modulated by post-translational modification. But, the precise molecular signaling mechanisms between Osterix and Akt are not known. In this study, we investigated the potential regulation of Osterix function by Akt in osteoblast differentiation. We found that Akt phosphorylates Osterix and that Akt activation increases protein stability, osteogenic activity and transcriptional activity of Osterix. We also found that BMP-2 increases the protein level of Osterix in an Akt activity-dependent manner. These results suggest that Akt activity enhances the osteogenic function of Osterix, at least in part, through protein stabilization and that BMP-2 regulates the osteogenic function of Osterix, at least in part, through Akt.

1. Introduction

Bone is a highly dynamic tissue that is constantly remodeled throughout life. 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]. The activities of osteoclasts and osteoblasts can be regulated at the level of differentiation by various regulatory signals. Also, bone remodeling is regulated by various anabolic factors including Wnt, insulin, bone morphogenetic proteins (BMPs), insulin growth factor-I, members of the TGF-β family and kinases such as Akt. Bone formation is a complex developmental process involving the differentiation of mesenchymal stem cells to osteoblasts. The regulation of osteoblast differentiation is mediated by BMPs and various transcription factors such as Runx2, Osterix and several homeodomain (HD) proteins [2–8].

The expression of many osteogenic homeodomain transcription factors, including Osterix genes, is induced by BMPs during early embryogenesis and adult skeletogenesis. Osterix (also known as Sp7) is a novel zinc finger – containing osteoblast-specific transcription factor that is essential for osteoblast differentiation, proliferation and bone formation [9–13]. The DNA-binding domain of Osterix is located at its C-terminus and contains three C2H2-type zinc finger domains that share a high degree of identity with similar motifs to Sp1, Sp3, and Sp4. There is a proline-rich region (RRR) close to the N-terminus. The subcellular localization of Osterix is restricted to the nucleus. The RRR region is responsible for the Osterix inhibitory effect on the Wnt signaling pathway. Osterix is necessary for the osteoblast lineage [14,15]. Osterix proteins regulate the expression of many osteogenic factors including Runx2, osteonectin, osteopontin, osteocalcin and alkaline phosphatase (ALP) [9,14–17]. A serine-threonine protein kinase, Akt is one of the key players in the signaling of potent bone anabolic factors. Akt1 in osteoblasts and osteoclasts controls bone remodeling [1]. Akt enhances transcription factor-dependent osteoblast differentiation, function and transcriptional activity. However, the precise regulatory mechanism between Osterix and Akt function is still under investigation.

In this study, we examined whether Akt plays a role in regulating Osterix function during osteogenesis. We found that Akt phosphorylates Osterix and that Akt activation increases protein stability, osteogenic activity and transcriptional activity of Osterix. We also found that BMP-2 increases the protein level of Osterix in an Akt activity-dependent manner. These results suggest that Akt enhances the osteogenic function of Osterix by increasing protein stability and transcriptional activity.

2. Materials and methods

2.1. Plasmids and antibodies

Myc-tagged Osterix, HA-tagged Akt WT and Akt KD were constructed in a CMV promoter-derived mammalian expression vector (pCS4-3Myc,-3HA). Antibodies against Myc (9E10) and GFP (Santa Cruz Biotechnology), HA (12CA5, Roche Applied Science),

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phospho-threonine (PTR-8) and phospho-serine (PSR-45, Sigma–Aldrich), and phospho-Akt substrate motif (RXXS*/T*) (110B7E, Cell Signaling Technology) were used.

2.2. Cell culture and transient transfection

HEK 293 human embryonic kidney epithelial cell line and 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 calcium phosphate-mediated method. Total amounts of transfected plasmids in each group were equalized by the addition of 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 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 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.

2.5. Luciferase reporter assay

Cells were seeded on 24-well plates the day before transfection. C2C12 cells were transfected with ALP (ALP-Luc), Osteocalcin (OC-Luc), Bone sialoprotein (BSP-Luc) reporter plasmid, pCMV-β-gal, and combinations of Osterix and Akt WT expression vectors or Akt inhibitor on 24 well plates and 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 Osterix or HA-tagged Akt (WT/KD) expression vectors. After 24 h, cells were exposed 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. The levels of protein were analyzed by immunoblotting using the anti-Myc or anti-HA antibodies.

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

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 sec, annealing at a temperature optimized for each primer pair for 30 sec, and extension at 72 °C for 30 sec; 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′; 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′; 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 affects on expression levels of Osterix protein

During osteogenesis, the expression of Osterix is dependent on several signaling pathways. We examined whether Akt affects the protein levels of Osterix. HEK 293 cells were transfected with Osterix, Akt WT or KD. The protein levels of Osterix were determined by western blotting. The levels of Osterix were dramatically increased by Akt WT and decreased by Akt KD-inactive form. Also, the protein levels of Osterix were strongly regulated by increasing amounts of Akt WT or KD in a dose-dependent manner (Fig. 1A). But, inhibition of Akt by the Akt inhibitor decreased the protein levels of Osterix (Fig. 1B). In previous reports, BMP2 induces osteoblast differentiation [4,6–8]. Therefore, we analyzed whether Osterix is regulated by endogenous Akt. The expression of Osterix induced by BMP2 and decreased by Akt inhibitor is endogenous (Fig. C). These results suggest that the Akt pathway may regulate the protein levels of Osterix.

3.2. Exogenous and endogenous Akt interacts with Osterix; also Osterix is phosphorylated by Akt

Given the results above, we asked whether Osterix might interact with Akt. To confirm this hypothesis, HEK 293 cells were transfected with Myc- or HA-Osterix and HA-Akt WT or KD and then performed immunoprecipitation (IP). We found that Osterix bound to exogenous Akt WT and Akt KD (Fig. 2A). Also, for the interaction between Osterix and endogenous Akt, we performed by immunoprecipitation (IP). We determined that Osterix bound to endogenous Akt (Fig. 2B and C). To investigate the potential regulation of Osterix activity by Akt, we next examined whether Akt can phosphorylate Osterix using the phospho-Akt substrates, phospho-threonine and phospho-serine. Myc-tagged Osterix was subjected to an immunoprecipitation (IP) with or without active Akt. Osterix was phosphorylated by Akt substrate (RXXS*/T*) in presence of Akt (Fig. 2D, first panel). Also, Akt induced the phosphorylation of threonine residues (Fig. 2D, second panel) but not serine residues (Fig. 2D, third panel) of Osterix. These results indicate that Akt interacts with Osterix and also phosphorylates Osterix at threonine residues.

3.3. Akt regulates the protein stability of Osterix

Akt may regulate the expression of Osterix at the level of transcription, translation or by protein stability. The protein levels of Osterix are increased by Akt (Fig. 1). To identify the molecular mechanism for Akt-induced increase of Osterix protein levels, we examined whether Akt affects the protein stability of Osterix by using cycloheximide (CHX). HEK 293 cells were transfected with Osterix with or without Akt WT/Akt KD. To estimate the Osterix stability by Akt, transfected cell were treated with 40 μM of cycloheximide for the indicated times and then harvested. The protein levels of Osterix were determined by western blotting. Osterix