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Protein Kinase A Phosphorylates Dlx3 and Regulates the Function of Dlx3 During Osteoblast Differentiation

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ABSTRACT

Protein kinase A (PKA), a serine/threonine kinase, regulates bone formation, and enhances Bone morphogenetic protein (BMP)-induced osteoblast differentiation. However, the mechanisms of how PKA controls the cellular response to BMP are not well known. We investigated the effects of modulating PKA activity during BMP2-induced osteoblast differentiation, and found that PKA regulates the function of Dlx3. *Dlx3* plays crucial roles in osteoblast differentiation and it is expressed in most skeletal elements during development. We found that PKA activation increases BMP2-induced expression of Dlx3 protein, and enhances the protein stability, DNA binding, and transcriptional activity of Dlx3. In addition, PKA activation induces the phosphorylation of Dlx3 at consensus PKA phosphorylation target site(s). Lastly, substitution of serine 10 in Dlx3 to alanine significantly reduces, if not completely abolishes, the phosphorylation of Dlx3 and the regulation of Dlx3 function by PKA. These results suggest that Dlx3 is a novel target of PKA, and that PKA mediates BMP signaling during osteoblast differentiation, at least in part, by phosphorylating Dlx3 and modulating the protein stability and function of Dlx3. J. Cell. Biochem. 115: 2004–2011, 2014.

KEY WORDS: PROTEIN KINASE A; Dlx3; OSTEOBLAST DIFFERENTIATION; PROTEIN STABILITY; TRANSCRIPTIONAL ACTIVITY

Constant homeostatic balance mediated by bone resorption and bone formation is crucial for bone development and remodeling. Activities of bone resorption mediated by osteoclasts and bone formation mediated by osteoblasts are regulated by various regulatory factors including cytokines, growth factors, hormones, and transcription factors [Karsenty, 2000]. Bone morphogenetic proteins (BMPs) function as critical regulators during osteoblast differentiation by inducing the expression of several osteogenic transcription factors such as Runx2, Osterix, homeobox proteins, and helix-loop-helix proteins [Lee et al., 2002; Chen et al., 2004; Hassan et al., 2006].

Members of Dlx transcription factor family play critical roles during bone development and adult skeletogenesis [Luo et al., 2001; Hassan et al., 2004b]. Dlx family consists of 6 members, Dlx1-Dlx6, and they all have conserved homeobox domains related to *Drosophila* Distal-less. *Dlx2* is involved in craniofacial development and mesenchymal condensation [Robinson and Mahon, 1994]. *Dlx5* is essential for the development of the jaw, axial, and appendicular

Hongyan Li and Hyung Min Jeong contributed equally to this work.

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bones [Luo et al., 2001]. *Dlx3* plays important roles in tooth development and the differentiation of keratinocytes and hair follicle cells [Hwang et al., 2008; Duverger et al., 2012; Viale-Bouroncle et al., 2012]. *Dlx3* is also important for bone development, and it is expressed in periosteum, chondrocytes and osteogenic lineages [Beanan and Sargent, 2000]. In human, a 4 bp deletion mutation in *DLX3* is associated with an autosomal dominant condition called tricho-dento-osseous syndrome, which is characterized by variable clinical conditions including kinky/curly hair, taurodontism, amelogenesis imperfect, and increased bone thickness [Nieminen et al., 2011]. Dlx3 promotes osteoblast differentiation by inducing the expression of osteogenic factors including *Runx2*, *Osterix*, *Osteocalcin*, and *Alkaline phosphatase* [Hassan et al., 2004a; Li et al., 2008].

Protein kinase A (PKA), also known as cyclic AMP-dependent protein kinase, regulates numerous cellular processes including osteoblast differentiation [Pasqualucci et al., 2006; Tseng et al., 2010]. PKA activation, using various compounds that directly or indirectly modulate PKA activity, enhances osteoblast differentiation of mesenchymal stem cells and BMP-induced osteogenesis [Wu et al., 2007; Siddappa et al., 2008; Lo et al., 2012]. In addition, PKA activation enhances osteogenic differentiation of vascular cells and increases the risk of vascular sclerosis [Tintut et al., 1998].

We investigated the mechanisms of how PKA regulates osteoblast differentiation, and found evidences that PKA modulates Dlx3 function through post-translational modification. In this study, we demonstrated that PKA induces the phosphorylation of Dlx3 which is likely stimulated by BMP2, and that PKA increases the protein stability and transcriptional activity of Dlx3. In addition, we found evidences that serine 10 of Dlx3 is a major PKA phosphorylation target site, and it is important for the regulation of Dlx3 function by PKA.

MATERIALS AND METHODS

CELL CULTURE

C2C12 mouse pre-myoblast cells and 293 human embryonic kidney epithelial cells were maintained at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. For BMP2, forskolin, and H89 treatment, C2C12 cells were incubated in differentiation medium (DMEM supplemented with 2% FBS and antibiotics), pre-treated with forskolin and H89 for 1 h and then treated with BMP2 (20 ng/ml), forskolin and H89 for 3 days unless indicated otherwise. Cell culture reagents were purchased from Life Technologies (Grand Island, NY).

PLASMIDS, DNA TRANSFECTION, ANTIBODIES, AND CHEMICALS

Myc-and HA-tagged wild type and S10A, S276A, T223A mutants Dlx3, Myc, or GFP-tagged PP2A plasmids were constructed in a CMV promoter-derived mammalian expression vector (pCS4+). 0.5 μg of each plasmids (unless otherwise noted) were transiently transfection using the calcium phosphate-mediated method or polyethyleneimine (PEI) (Polysciences, Inc., Warrington, PA) mediated method. Antibodies against Dlx3 (YF-PA11375, Ab Frontier, Seoul, Korea), GFP (sc-8334, Santa Cruz Biotechnology, Dallas, TX), Myc (9E10, Roche Applied Science, Indianapolis, IN), HA (12CA5, Roche Applied Science, Indianapolis, IN), phospho-PKA substrate (100G7E, Cell Signaling Technology, Danvers, MA), and α-tubulin (B-5-1-2, Sigma–Aldrich, St. Louis, MO) were used. Cycloheximide (Sigma–Aldrich), forskolin (Tocris Bioscience, Bristol, UK), H89 (Tocris Bioscience), and recombinant human BMP2 protein (355-BM, R&D Systems, Minneapolis, MN) were used.

ALKALINE PHOSPHATASE (ALP) STAINING

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT), washed several times with PBS, and stained with NBT/BCIP solution (Sigma–Aldrich) for 20 min at RT. ALP-positive cells stain blue/purple.

ONE-STEP REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total cellular RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instruction. Random hexamer-primed cDNAs were synthesized from 1 μg of total RNA using Super-Script III First-Strand Synthesis System (Life Technologies). 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; and 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′; *GAPDH* forward 5′-ACC ACA GTC CATGCC ATC AC-3′ and reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′; *Runx2* forward 5′-CCA CCA CTC ACT ACC ACA CG-3′ and reverse 5′-TAT GGA GTG CTG CTG GTC TG-3′.

LUCIFERASE REPORTER ASSAY AND STATISTICAL ANALYSIS

Cells were seeded on 24-well plates the day before transfection, and then transfected with pCMV-β-Gal (0.05 μg), ALP or DRE luciferase reporter (0.2 μg), and indicated plasmids. 12 h later, cells were treated with BMP2, forskolin, and H89 for 24 h. Luciferase activities were measured using a Luciferase Reporter Assay Kit (Promega, Madison, WI), and normalized with corresponding β-galactosidase activities for transfection efficiency. Experiments were performed in triplicates and repeated at least three times. Student's *t*-test was used to assess statistical significance with $P < 0.05$.

IMMUNOBLOTTING (IB) AND IMMUNOPRECIPITATION (IP)

48 h after transfection, cells were 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]. Lysates were cleared by centrifugation, and the supernatants were used for subsequent analysis. For IP, cell lysates were incubated with appropriate antibodies and protein A or G-sepharose beads. Cell lysates and immunoprecipitated proteins were subjected to SDS-PAGE, and proteins were transferred to PVDF membrane. Proteins were visualized using appropriate primary antibodies, HRP-conjugated secondary antibodies and ECL reagent (EMD Millipore, Billerica, MA).

DNA AFFINITY BINDING ASSAY

Cell lysates were incubated with streptavidin beads and a biotin-labeled, double-stranded oligonucleotide containing three tandem repeats of Dlx3 responsive element (DRE; 5′-GCG ATA ATT GCG GCG ATA ATT GCG GCG ATA ATT GCG-3′, DRE underlined). Affinity precipitated proteins were subjected to anti-Dlx3 IB.

RESULTS

PKA ACTIVATION ENHANCES BMP2-INDUCED OSTEOBLAST DIFFERENTIATION

BMP2 stimulation induces C2C12 myoblast cells to differentiate into osteoblasts [Yamaguchi et al., 2000], and PKA activation induces osteoblast differentiation of mesenchymal stem cells [Wu et al., 2007; Siddappa et al., 2008; Lo et al., 2012]. We examined the effect of PKA activation or inhibition on BMP2-induced osteoblast differentiation of C2C12 cells using alkaline phosphatase (ALP) staining. ALP is expressed from early stages of osteoblast differentiation, and ALP staining has been used extensively to measure the extent of osteoblast differentiation. Forskolin, a PKA activator, enhanced BMP2-induced ALP staining in a dose-dependent manner (Fig. 1A). Conversely, a PKA inhibitor H89 reduced ALP staining in a dose-dependent manner. When examined by RT-PCR, forskolin also increased BMP2-induced expression of osteoblast markers *ALP* and *Runx2* whereas H89 reduced it in dose-dependent manners (Fig. 1B).

PKA ACTIVATION ENHANCES THE TRANSCRIPTIONAL ACTIVITY AND DNA BINDING OF Dlx3

BMP2 stimulates osteoblast differentiation by inducing the expression of several osteogenic transcription factors including *Dlx3*, *Dlx5*, *Osterix*, and *Runx2* [Lee et al., 2002; Chen et al., 2004; Hassan et al., 2006]. The activities of these transcription factors have been reported to be modulated by post-translational modifications, and we found a consensus PKA phosphorylation target sequence (RXXS/T) in Dlx3. Therefore, we examined whether activation or inhibition of PKA affects the transcriptional activity of Dlx3 using luciferase reporter assay. ALP-Luc reporter contains the *cis*-regulatory sequence of *ALP*, and DRE-Luc reporter contains 3 tandem copies of Dlx3 responsive element (DRE) [Park et al., 2001; Duverger et al., 2011; Choi et al., 2012]. Dlx3 induced the expression of ALP-Luc and DRE-Luc (Figs. 2A and B). Forskolin increased Dlx3-induced reporter expression in a dose-dependent manner, and H89 reversed the effect of forskolin on reporter expression in a dose-dependent manner. However, H89 had only minimal effect, if any, on reporter expression induced by Dlx3 alone. Next, we examined the effect of PKA activation on DNA binding of Dlx3 by DNA affinity binding assay. Forskolin alone as well as BMP2 co-treated increased the amount of Dlx3 protein bound to DNA (Fig. 2C). These results indicate that PKA activation enhances the transcriptional activity and DNA binding of Dlx3.

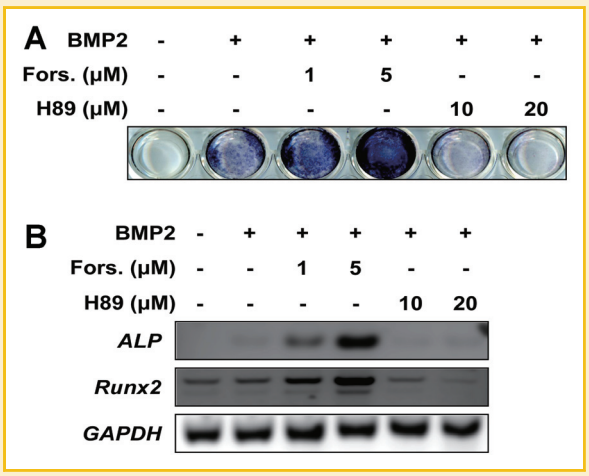

Fig. 1. PKA enhances BMP2-induced osteoblast differentiation in C2C12 cells. C2C12 cells were treated with indicated combinations of BMP2 (20 ng/ml), a PKA activator forskolin (Fors.) and a PKA inhibitor H89 for 3 days. (A) Cells are stained for alkaline phosphatase (ALP). ALP-positive cells stain blue/purple. (B) mRNA levels of osteoblast markers *ALP* and *Runx2* are compared by RT-PCR. *GAPDH* is used as a control.

PKA PHOSPHORYLATES Dlx3 AND INCREASES Dlx3 PROTEIN LEVEL

PKA regulates the function of its target proteins through phosphorylation. We examined whether PKA activation induces the phosphorylation of Dlx3 by immunoprecipitation using an anti-phospho-PKA substrate antibody. Forskolin increased the protein level and enhanced the phosphorylation of Dlx3 in a dose-dependent manner (Fig. 3A). Studies have shown that the function of osteogenic transcription factors can be modulated by regulating their degradation. Therefore, we examined if PKA activity affects Dlx3 protein level. In 293 cells, forskolin increased the level of overexpressed Dlx3, and H89 suppressed the effect of forskolin on Dlx3 protein level in dose-dependent manners (Fig. 3B). We then examined whether PKA-induced phosphorylation of Dlx3 is important for the increase of Dlx3 protein level. Protein phosphatase PP2A suppressed the effect of forskolin on Dlx3 protein level (Fig. 3C). Next, we examined if PKA inhibition affects the level of endogenous Dlx3 protein in C2C12 cells. BMP2 stimulation increased Dlx3 protein level, but H89 abolished BMP2-induced increase of Dlx3 protein level in a dose-dependent manner (Fig. 3D). These results indicate that PKA-induced phosphorylation of Dlx3, possibly stimulated by BMP2, is important to maintain or increase the level of Dlx3 protein.

PKA INCREASES THE PROTEIN STABILITY OF Dlx3

Protein level can be regulated at various steps including transcription, translation, RNA stability, and protein stability. PKA activation did not alter the level of *Dlx3* mRNA significantly when examined by RT-PCR (data not shown). Therefore, we examined whether PKA activation influences the protein stability of Dlx3 using a translation inhibitor cycloheximide. In the absence of PKA activation, the half-life of Dlx3 protein was approximately 1.5 h (Fig. 4). Forskolin significantly prolonged the half-life of Dlx3. These results indicate that PKA increases Dlx3 protein level by enhancing the protein stability of Dlx3.

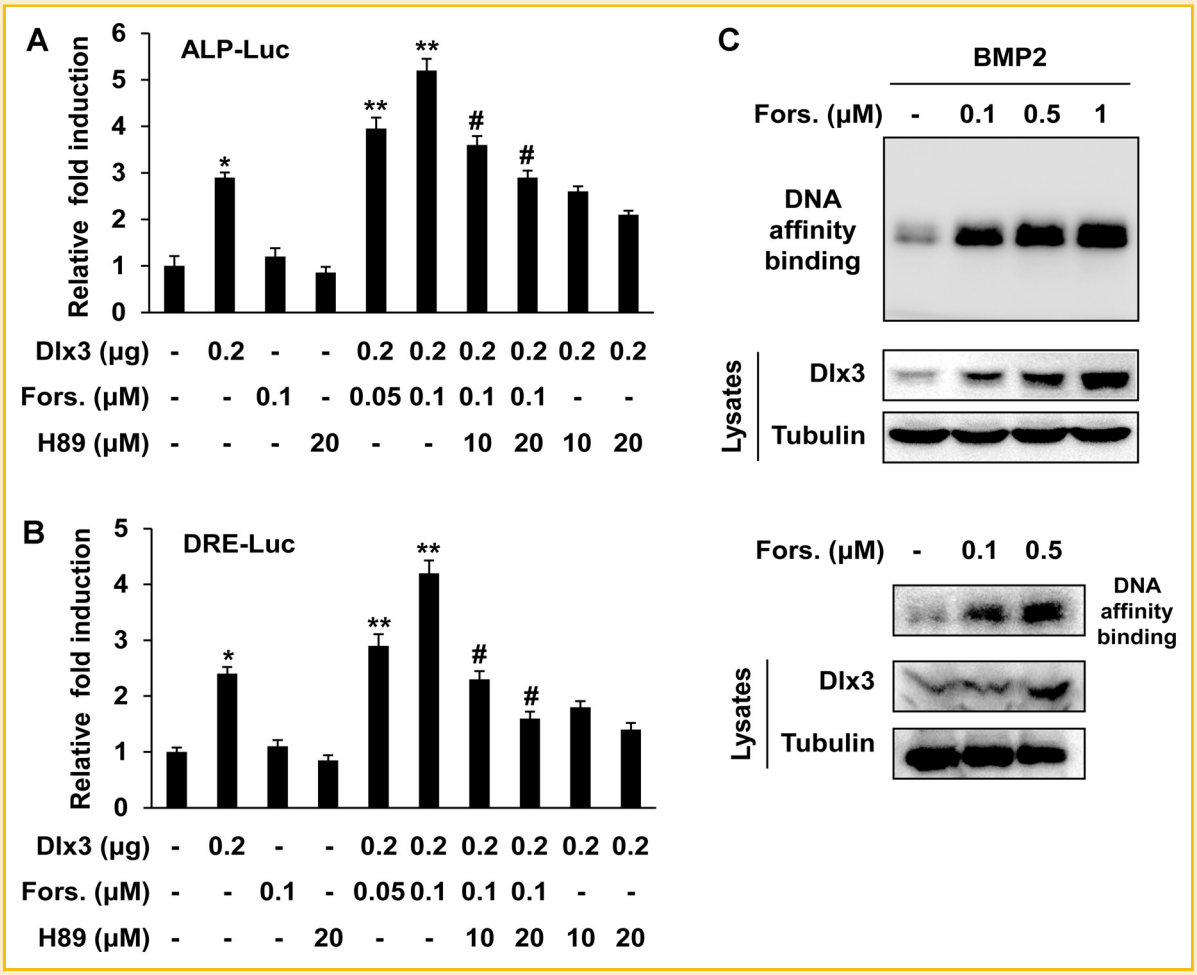

Fig. 2. PKA increases the transcriptional activity and DNA binding of Dlx3. (A, B) C2C12 cells were transfected for Dlx3 along with ALP-Luc (A) or DRE-Luc (B). Cells were then treated with indicated combinations of forskolin (Fors.) and H89 for 12 h. Luciferase activities were measured, and the averages and standard deviations of triplicate samples are shown. *, **, and # indicate that the difference is significant ($P < 0.05$) compared to control, Dlx3 alone, or Dlx3 + forskolin (0.1 μM), respectively. (C) C2C12 cells were treated with indicated concentrations of forskolin alone or along with BMP2 (20 ng/ml) for 3 days. Cell lysates were incubated with a biotin-labeled oligonucleotide containing Dlx3 responsive elements, and the DNA-bound proteins are subjected to anti-Dlx3 immunoblotting (DNA affinity binding). The levels of Dlx3 protein in cell lysates are also compared. Tubulin is used as a loading control.

SERINE 10 OF Dlx3 IS IMPORTANT FOR THE REGULATION OF Dlx3 BY PKA

Sequence analysis indicates that serine 10, 276, and threonine 223 of Dlx3 are a potential PKA phosphorylation target sites. We generated Dlx3 mutants, Dlx3 (S10A, S276A, T223A), in which serine and threonine aresubstituted to alanine. Among them serine 10 is a best PKA phosphorylation target site (Fig. S1 of Supporting information). First, we tested if PKA activation induces the phosphorylation of Dlx3 (S10A). Forskolin increased the protein level and phosphorylation of wild type Dlx3 as previously shown, whereas forskolin increased those of Dlx3 (S10A) mutant with significantly lower efficiency compared to wild type (Fig. 5A). However, PKA activation still increased the protein level and phosphorylation of Dlx3 (S10A), suggesting that there could be other PKA target site(s) in Dlx3 beside serine 10. Nevertheless, these results suggest that serine 10 of Dlx3 is a major PKA phosphorylation target site.

Next, we examined whether PKA-induced phosphorylation of Dlx3 (S10A) is important for PKA-induced increase of Dlx3 protein level. Proteosome-mediated degradation is a major protein degradation process in the cell, therefore, we investigated the effects of a proteasome inhibitor (MG132). Dlx3 (S10A) mutant decreased the protein level compare with wild type Dlx3, and this decreased protein was incresed in the presence of a proteasome inhibitor MG132 (Fig. 5B). PP2A abolished forskolin-induced increase of wild type Dlx3 protein level, but it failed to do so for Dlx3 (S10A) mutant (Fig. 5C). We also examined whether BMP2 stimulation increases Dlx3 (S10A) protein level. BMP2 stimulation did not affect Dlx3 (S10A) protein level whereas it increased the level of endogenous Dlx3 protein in a dose-dependent manner (Fig. 5D). In addition, PKA inhibition by H89 did not alter Dlx3 (S10A) level in BMP2-stimulated cells, whereas H89 reduced the level of endogenous Dlx3 in a dose-dependent manner (Fig. 5E). S10A mutation significantly

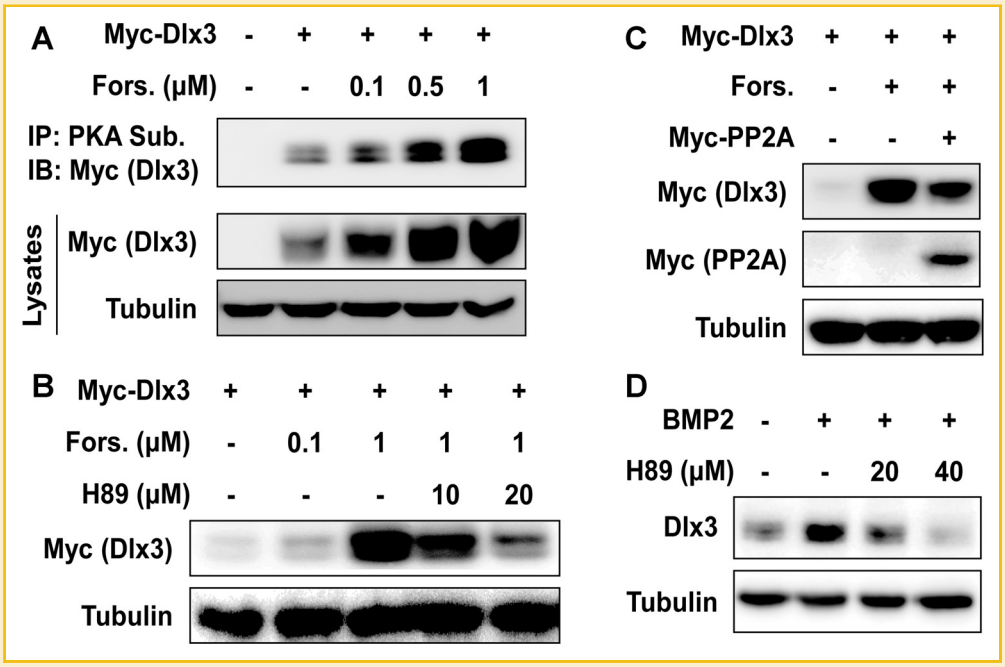

Fig. 3. PKA induces the phosphorylation of Dlx3, and increases the protein level of Dlx3. (A, B) 293 cells were transfected with Myc-Dlx3, and then treated with indicated concentrations of forskolin (Fors.), H89 and vehicle DMSO for 12 h. (A) The levels of Dlx3 phosphorylation are examined by immunoprecipitation (IP) using antibody against phospho-PKA substrate (IP: PKA Sub.) followed by anti-Myc immunoblotting [IB: Myc (Dlx3)]. (B) The levels of Dlx3 in cell lysates are compared by anti-Myc IB [Myc (Dlx3)]. (C) 293 cells were transfected with indicated combinations of Myc-Dlx3 and Myc-PP2A, and then treated with forskolin (0.5 μM). The levels of Dlx3 are compared by anti-Myc IB. (D) C2C12 cells were treated with indicated combinations of BMP2 (20 ng/ml) and H89 for 24 h. The levels of endogenous Dlx3 are determined by anti-Dlx3 IB.

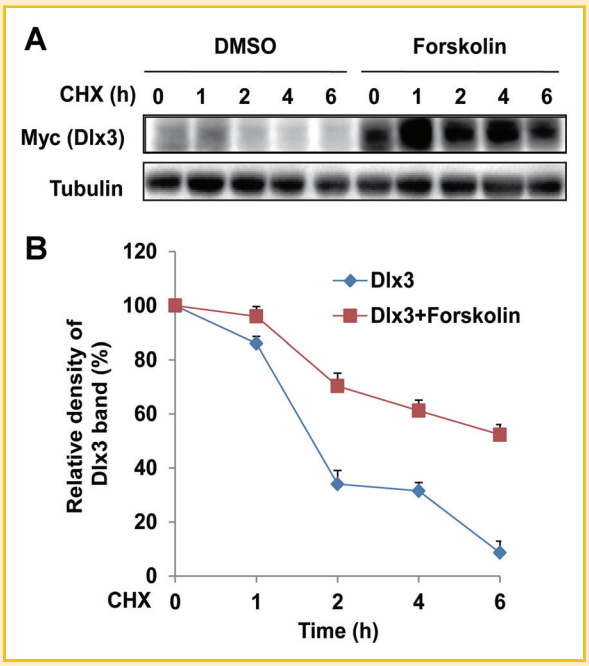

Fig. 4. PKA enhances the protein stability of Dlx3. (A) 293 cells were transfected with Myc-Dlx3 and then treated with forskolin (1 μM) or DMSO for 12 h, and then treated with a translation inhibitor cycloheximide (CHX, 40 μM) for indicated amounts of time. The levels of Dlx3 are determined by anti-Myc IB. (B) The intensities of Dlx3 bands in panel A were determined by densitometry. The level of Dlx3 in CHX-untreated cells (0 h) is considered as 100%. The experiment was repeated three times, and the averages and standard deviations are shown.

shortened the half-life of Dlx3 (Fig. 5F). These results indicate that serine 10 of Dlx3 is important for PKA-induced increase of Dlx3 protein level, which is likely stimulated by BMP2.

We then investigated if the transcriptional activity of Dlx3 (S10A) is modulated by PKA activity. Wild type Dlx3 induced the expression DRE-Luc reporter. Reporter expression was further increased by BMP2 stimulation, and H89 abolished BMP2-induced increase of reporter expression (Fig. 6A). However, Dlx3 (S10A)-induced reporter expression was neither increased by BMP2 stimulation nor decreased by H89. We also examined if DNA binding of Dlx3 (S10A) is modulated by PKA activation. Although forskolin increased the amount of DNA-bound Dlx3 (S10A), it did so considerably less for Dlx3 (S10A) when compared to wild type Dlx3 (Fig. 6B). These results indicate that serine 10 of Dlx3 is important for the regulation of Dlx3 function by PKA. It is also interesting to note that the DNA binding ability of Dlx3 (S10A) mutant was significantly reduced compared to wild type Dlx3 even when they were expressed at comparable levels (Fig. 6B). This suggest that serine 10 of Dlx3, and possible the phosphorylation at this residue by PKA, may be important for the regulation of Dlx3 DNA binding ability in addition to the PKA-induced increase of Dlx3 protein stability.

DISCUSSION

In the present study, we provide evidences that PKA enhances BMP2-induced osteoblast differentiation, at least in part, by modulating the function of homeobox transcription factor Dlx3

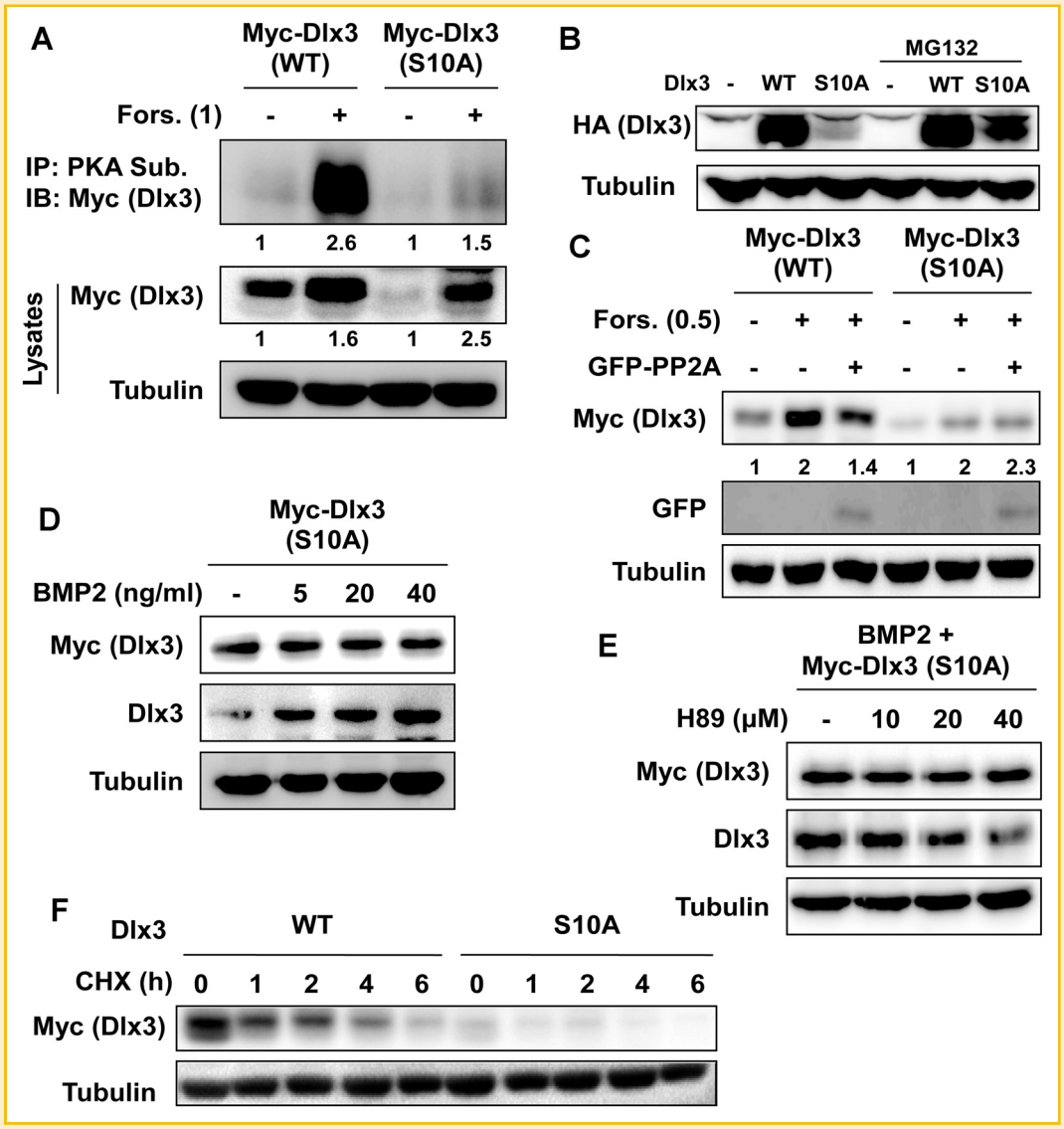

Fig. 5. PKA regulates Dlx3 through phosphorylation at serine 10 of Dlx3. (A–C) 293 cells were transfected with indicated combinations of wild type Myc-Dlx3 (WT), Myc-Dlx3 (S10A) mutant and GFP-PP2A, and then treated with indicated concentration of forskolin (μM) for 12 h or MG132 (2 μM) for 8 h. (A) The levels of Dlx3 phosphorylation are examined by anti-phospho-PKA substrate IP (IP: PKA Sub.) followed by anti-Myc IB [IB: Myc (Dlx3)]. (B,C) The levels of Dlx3 are examined by anti-Myc IB. (D) C2C12 cells were transfected with Myc-Dlx3 (S10A) for 24 h, and then treated with indicated concentrations of BMP2 for 24 h. The levels of Myc-Dlx3 (S10A) and endogenous Dlx3 are examined by anti-Myc IB and anti-Dlx3 IB, respectively. (E) C2C12 cells were transfected with Myc-Dlx3 (S10A) for 24 h, and then treated with BMP2 (20 ng/ml) and indicated concentrations of H89 for 24 h. The levels of Myc-Dlx3 (S10A) and endogenous Dlx3 are examined by anti-Myc IB and anti-Dlx3 IB, respectively. (F) 293 cells were transfected with Myc-tagged wild type or S10A Dlx3. Two days later, cells were treated with cyclohexamide (CHX, 40 μM) for indicated amounts of time. The levels of overexpressed Dlx3 are compared by anti-Myc IB [IB: Myc (Dlx3)].

through the phosphorylation of Dlx3 at serine 10. First, PKA activation enhanced BMP2-induced osteoblast differentiation of C2C12 cells. Second, PKA activation increased BMP2-induced expression of Dlx3 protein. Third, PKA activation increased the protein stability, DNA binding and transcriptional activity of Dlx3. Fourth, PKA activation induced the phosphorylation of Dlx3 at site(s) recognized by an anti-phospho-PKA substrate antibody. Lastly, substitution of serine 10 in Dlx3 to alanine significantly reduced, if not completely abolished, the PKA-induced phosphorylation of Dlx3 and the regulation of Dlx3 function by PKA.

The function of Dlx3 can be modulated by various post-translational modifications. Protein kinase B/AKT phosphorylates Dlx3 and upregulates its transcriptional activity [Choi et al., 2012]. SUMOylation of Dlx3 by SUMO1 also enhances its transcriptional activity [Duverger et al., 2011]. Conversely, phosphorylation of Dlx3 by PKC results in downregulation of Dlx3 DNA binding affinity [Park et al., 2001]. We showed that PKA induces phosphorylation of Dlx3 enhancing its protein stability and transcriptional activity. PKA primarily phosphorylates serine or threonine residue within the consensus target sequence of RXXS/T or RRXS/T. Sequence analysis

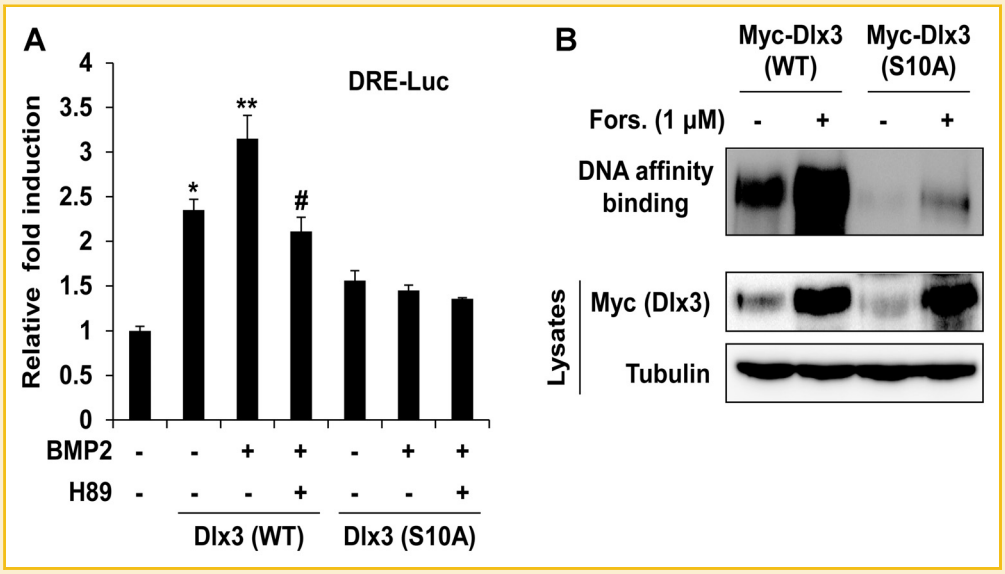

Fig. 6. Serine 10 of Dlx3 is important for PKA-mediated regulation of Dlx3 transcriptional activity and DNA binding. (A) C2C12 cells were transfected for DRE-Luc (0.2 μg) along with wild type or S10A mutant Dlx3 (0.2 μg each), and then treated with indicated combinations of BMP2 (20 ng/ml) and H89 (20 μM) for 24 h. Luciferase activities were measured, and the averages and standard deviations of triplicate samples are shown. *, **, and # indicate that the difference is significant ($P < 0.05$) compared to control, Dlx3 alone, and Dlx3 + BMP2, respectively. (B) C2C12 cells were transfected with wild type (0.5 μg) or S10A mutant (1.5 μg) Myc-Dlx3, and then treated with forskolin or DMSO for 24 h. Cell lysates were incubated with a biotin-labeled oligonucleotide containing Dlx3 responsive elements, and the DNA-bound proteins are subjected to anti-Dlx3 IB (DNA affinity binding).

revealed that serine 10 of Dlx3 (MSGSFDRKLSSILTD) is a potential PKA phosphorylation target site. Dlx3 (S10A) mutant did not response to the modulation of PKA activity including BMP2-induced increase of protein level and transcriptional activity. These results indicate that PKA is a novel regulator of Dlx3 during osteoblast differentiation, and Dlx3 is a novel target of PKA. Our finding also suggests that serine 10 of Dlx3 is the major target site of PKA.

PKA-induced phosphorylation of serine 10 in Dlx3 may modulate the DNA binding activity of Dlx3 in addition to its protein stability. DNA binding ability of Dlx3 (S10A) mutant was significantly reduced compared to wild type Dlx3 even when they were expressed at comparable levels (Fig. 6B). This suggests that serine 10 of Dlx3, and possible the phosphorylation at this residue by PKA, may be important for the DNA binding ability of Dlx3. Although serine 10 is located outside of the DNA binding domain of Dlx3, we cannot rule out the possibility that this residue itself, not its phosphorylation by PKA, is important for DNA binding. PKA may also regulate the function of Dlx3 by other mechanisms besides phosphorylation at serine 10. The level of Dlx3 (S10A) was increased by forskolin, but this increase was not affected by PP2A (Fig. 5B). In addition, PKA activation slightly increased the phosphorylation of Dlx3 (S10A) mutant at residue(s) recognized by anti-phospho-PKA substrate antibody (Fig. 5A). These results suggest that PKA may modulate the protein stability of Dlx3 through the modification of other regions, which are insensitive to PP2A, in addition to serine 10 phosphorylation.

PKA modulates BMP signaling during osteoblast differentiation. BMP-induced gene expression is primarily regulated by Smad-transcription factor complexes [Lee et al., 2002; Chen et al., 2004; Hassan et al., 2006]. PKA regulates gene expression by recruiting CREB (cyclic AMP response element-binding protein) transcription factor. Cyclic AMP, the endogenous PKA activator, has been shown to enhance Smad-mediated BMP signaling through PKA-CREB pathway [Ohta et al., 2008]. We show that BMP2-induced expression of Dlx3 protein is reduced by PKA inhibition (Figs. 3D and 5C). These results suggest that PKA may function as a mediator of BMP signaling during osteoblast differentiation.

In conclusion, our results suggest that PKA mediates BMP signaling during osteoblast differentiation, at least in part, by phosphorylating Dlx3 and modulating the protein stability and function of Dlx3.

ACKNOWLEDGEMENTS

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REFERENCES

Beanan MJ, Sargent TD. 2000. Regulation and function of Dlx3 in vertebrate development. Dev Dyn 218:545–553.

Chen D, Zhao M, Mundy GR. 2004. Bone morphogenetic proteins. Growth Factors 22:233–241.

Choi YH, Choi HJ, Lee KY, Oh JW. 2012. Akt1 regulates phosphorylation and osteogenic activity of Dlx3. Biochem Biophys Res Commun 425:800–805.

Duverger O, Chen SX, Lee D, Li T, Chock PB, Morasso MI. 2011. SUMOylation of DLX3 by SUMO1 promotes its transcriptional activity. J Cell Biochem 112:445–452.

Duverger O, Zah A, Isaac J, Sun HW, Bartels AK, Lian JB, Berdal A, Hwang J, Morasso MI. 2012. Neural crest deletion of Dlx3 leads to major dentin defects through down-regulation of Dspp. J Biol Chem 287:12230–12240.

Hassan MQ, Javed A, Morasso MI, Karlin J, Montecino M, van Wijnen AJ, Stein GS, Stein JL, Lian JB. 2004a. Dlx3 is a transcriptional regulator of osteoblast differentiation and increases expression and promoter activity of Runx2. Journal of Bone and Mineral Research 19:S37–S37.

Hassan MQ, Javed A, Morasso MI, Karlin J, Montecino M, van Wijnen AJ, Stein GS, Stein JL, Lian JB. 2004b. Dlx3 transcriptional regulation of osteoblast differentiation: Temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. Mol Cell Biol 24:9248–9261.

Hassan MQ, Tare RS, Lee SH, Mandeville M, Morasso MI, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2006. BMP2 commitment to the osteogenic lineage involves activation of Runx2 by DLX3 and a homeodomain transcriptional network. J Biol Chem 281:40515–40526.

Hwang J, Mehrani T, Millar SE, Morasso MI. 2008. Dlx3 is a crucial regulator of hair follicle differentiation and cycling. Development 135:3149–3159.

Karsenty G. 2000. Bone formation and factors affecting this process. Matrix Biol 19:85–89.

Lee M, Kim Y, Sung J, Wozney JM, Kim H, Ryoo H. 2002. Dlx5 is a direct and specific target of BMP-signaling, which in turn, triggers downstream transcrption factors, Runx2 and Osterix. J Bone Miner Res 17:S245–S245.

Li H, Marijanovic I, Kronenberg MS, Erceg I, Stover ML, Velonis D, Mina M, Heinrich JG, Harris SE, Upholt WB, Kalajzic I, Lichtler AC. 2008. Expression and function of Dlx genes in the osteoblast lineage. Dev Biol 316:458–470.

Lo KW, Kan HM, Ashe KM, Laurencin CT. 2012. The small molecule PKA-specific cyclic AMP analogue as an inducer of osteoblast-like cells differentiation and mineralization. J Tissue Eng Regen Med 6:40–48.

Luo T, Matsuo-Takasaki M, Sargent TD. 2001. Distinct roles for Distal-less genes Dlx3 and Dlx5 in regulating ectodermal development in Xenopus. Mol Reprod Dev 60:331–337.

Nieminen P, Lukinmaa PL, Alapulli H, Methuen M, Suojarvi T, Kivirikko S, Peltola J, Asikainen M, Alaluusua S. 2011. DLX3 homeodomain mutations cause tricho-dento-osseous syndrome with novel phenotypes. Cells Tissues Organs 194:49–59.

Ohta Y, Nakagawa K, Imai Y, Katagiri T, Koike T, Takaoka K. 2008. Cyclic AMP enhances smad-mediated BMP signaling through PKA-CREB pathway. J Bone Mineral Metab 26:478–484.

Park GT, Denning MF, Morasso MI. 2001. Phosphorylation of murine homeodomain protein Dlx3 by protein kinase C. FEBS Lett 496:60–65.

Pasqualucci L, Kitaura Y, Gu H, Dalla-Favera R. 2006. PKA-mediated phosphorylation regulates the function of activation-induced deaminase (AID) in B cells. Proc Natl Acad Sci USA 103:395–400.

Robinson GW, Mahon KA. 1994. Differential and overlapping expression domains of Dlx-2 and Dlx-3 suggest distinct roles for Distal-less homeobox genes in craniofacial development. Mech Dev 48:199–215.

Siddappa R, Martens A, Doorn J, Leusink A, Olivo C, Licht R, van Rijn L, Gaspar C, Fodde R, Janssen F, van Blitterswijk C, de Boer J. 2008. CAMP/PKA pathway activation in human mesenchymal stem cells in vitro results in robust bone formation in vivo. Proc Natl Acad Sci USA 105:7281–7286.

Tintut Y, Parhami F, Bostrom K, Jackson SM, Demer LL. 1998. CAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. J Biol Chem 273:7547–7553.

Tseng W, Graham LS, Geng Y, Reddy A, Lu J, Effros RB, Demer L, Tintut Y. 2010. PKA-induced receptor activator of NF-kappaB ligand (RANKL) expression in vascular cells mediates osteoclastogenesis but not matrix calcification. J Biol Chem 285:29925–29931.

Viale-Bouroncle S, Felthaus O, Schmalz G, Brockhoff G, Reichert TE, Morsczeck C. 2012. The transcription factor DLX3 regulates the osteogenic differentiation of human dental follicle precursor cells. Stem Cells Dev 21:1936–1947.

Wu X, Zeng LH, Taniguchi T, Xie QM. 2007. Activation of PKA and phosphorylation of sodium-dependent vitamin C transporter 2 by prostaglandin E2 promote osteoblast-like differentiation in MC3T3-E1 cells. Cell Death Differ 14:1792–1801.

Yamaguchi A, Komori T, Suda T. 2000. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev 21:393–411.

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