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Calmodulin-dependent kinase II regulates Dlx5 during osteoblast differentiation

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ABSTRACT

Calmodulin-dependent kinase II (CaMKII) acts as a key regulator of osteoblast differentiation. CaMKII is a Ca^{2+}-activated serine/threonine kinase and it regulates the activity of target proteins by phosphorylation. Dlx5 transcription factor plays crucial roles in osteoblast differentiation. The expression of Dlx5 is regulated by several osteogenic signaling pathways from early stages of osteoblastogenesis. In addition, Dlx5 can be phosphorylated and activated by p38, suggesting that the function of Dlx5 can be also modulated by post-translational modification. Although CaMKII and Dlx5 both play crucial roles during osteoblast differentiation, the interaction between CaMKII and Dlx5 has not been investigated. In the current study, we examined the effects CamKII on the function of Dlx5. We found that CaMKII phosphorylates Dlx5, and that CaMKII increases the protein stability and the osteoblastogenic transactivation activity of Dlx5. Conversely, a CaMKII inhibitor KN-93 decreased the osteogenic and transactivation activities of Dlx5. These results indicate that CaMKII regulates osteoblast differentiation, at least in part, by increasing the protein stability and the transcriptional activity of Dlx5.

Introduction

Bone is a dynamic tissue that undergoes continuous remodeling throughout life. Bone remodeling and homeostasis are largely the result of a coordinated action of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation while osteoclasts are responsible for bone absorption. The proper balance between osteoblasts and osteoclasts is essential for maintaining the proper bone function.

Osteoblasts are differentiated from mesenchymal stem cells [1]. Several transcription factors- including homeodomain-containing Dlx proteins, Runx2 (Cbfa1/AML3) and Osterix- regulate the differentiation of osteoblasts [2–7]. Dlx proteins were originally identified as homologs of the *Drosophila* Distal-less [8]. They can be divided to two groups based on their sequence homology and function: one group includes *Dlx1*, *-4*, *-6*, *-7*, and the other includes *Dlx2*, *-3*, *-5* [9]. Among them, *Dlx5* is expressed in almost every skeletal tissue from early stages of osteoblast differentiation, and it plays important roles in osteoblast differentiation [10]. Overexpression of Dlx5 leads to the expression of several osteoblast markers and accelerates osteoblast differentiation in chicken calvarial cells [11,12]. In addition, Dlx5 mediates the transcriptional control by many osteoblastogenic signaling pathways. The bone morphogenetic protein-2 (BMP-2) signaling pathway induces the expression of Runx2 and Osterix through the up-regulation of Dlx5 [13–15]. The function of Dlx5 is also regulated by post-translational modification, such that p38 can phosphorylate and increase the transactivation ability of Dlx5 [16,17]. However, the mechanisms for the regulation of Dlx5 function are still under investigation.

Ca^{2+} is one of the critical second messengers that regulate a variety of cellular responses including osteoblast differentiation [18]. Ca^{2+} signaling is mediated mainly by a Ca^{2+} binding protein calmodulin (CaM). Upon binding to Ca^{2+}, CaM interacts and activates various target proteins including calmodulin-dependent protein kinases (CaMKs), the major targets of CaM. CaMKs are multifunctional serine/threonine kinases, and the CaMK family includes CaMK I, II, and IV. Among them, CaMKII plays important roles in regulating osteoblast differentiation [19,20]. CaMKII also regulates the growth of osteosarcoma cells by controlling the progression of cell cycle and by modulating the expression of collagenases [21,22]. Although these results indicate that CaMKII is

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involved in many aspects of bone development, its exact functions in osteoblastogenesis are still unclear.

In this study, we examined whether CaMKII regulates osteoblastogenesis through the regulation of Dlx5. We found that CaMKII phosphorylates and increases the protein stability of Dlx5. Furthermore, we provide evidences that the transactivation activity of Dlx5 is enhanced by CaMKII and repressed by a CaMKII inhibitor KN-93. Our results indicate that CaMKII regulates osteoblast differentiation, at least in part, by up-regulating the function of Dlx5.

Materials and methods

Cell cultures and transient transfection. HEK 293T human embryonic kidney cells and C2C12 murine myoblast cells were cultured in DMEM supplemented with 5% or 15% fetal bovine serum (FBS), respectively. Cells were transfected using Effectene (QIAGEN) or calcium phosphate. Osteoblast differentiation of C2C12 cells was induced by stimulating the cells with BMP-2 in fresh DMEM supplemented with 2% FBS.

Alkaline phosphatase staining. C2C12 cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT), washed with PBS and stained with BCIP/NBT solution (SIGMA) for 15 min at RT. The alkaline phosphatase positive cells stain blue/purple.

Western blot analysis. Cell lysates containing 30 μg of total proteins were subjected to SDS–PAGE, and proteins were transferred to PVDF membrane. The membrane was probed with appropriate primary antibodies and HRP-conjugated secondary antibodies. Proteins were visualized using ECL reagents.

In vitro kinase assay. Anti-Myc immunoprecipitates of 293T cells, transfected with Myc-tagged CaMKII, were suspended in a kinase buffer [20 mM Tris–Cl(pH7.4), 10 mM $MgCl_2$, 50 μM ATP]. They were then mixed with anti-HA immunoprecipitates of 293T cells transfected with HA-tagged Dlx5 expression vector or a control vector. The reactions were incubated at 37 °C in the presence of 5 μCi of γ-[^{32}P]-ATP. After 20 min, the reactions were stopped by adding SDS-sample buffer. The samples were subjected to SDS–PAGE and phosphorylated Dlx5 was visualized by autoradiography.

Luciferase assay. HEK 293T cells were transfected with ALP (ALP-Luc) or osteocalcin (OC-Luc) luciferase reporter plasmid, pCMV-β-gal, and combinations of Dlx5 and CaMKII expression vectors. Luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized with corresponding β-gal activities for transfection efficiency. Experiments were performed in triplicate and repeated at least three times.

Results

CaMKII affects Dlx5-induced osteoblastogenesis

BMP-2 stimulation of C2C12 myoblast cells induces them to differentiate to osteoblasts and to express Dlx5 [23,24]. Dlx5 in turn induces the expression of alkaline phosphatase (ALP), an osteoblast-specific marker, directly by binding to the ALP promoter and/or indirectly by activating Runx2 expression [16,25]. We examined whether CaMKII affects Dlx5-induced osteoblast differentiation. C2C12 cells were transfected with CaMKII and/or Dlx5, and cultured in the presence or absence of BMP-2. The extents of osteoblast differention were measured by ALP staining. In the absence of BMP-2 stimulation, Dlx5 or CaMKII alone did not cause any significant change in ALP staining (Fig. 1). In the presence of BMP-2 stimulation, Dlx5 and/or CaMKII significantly increased ALP staining. However, a CaMKII inhibitor KN-93, but not an inactive analogue KN-92, abolished the Dlx5-induced as well as the CaMKII-induced increase of ALP staining (Fig. 1). These results indicate that CaMKII regulates Dlx5-induced osteoblastogenesis.

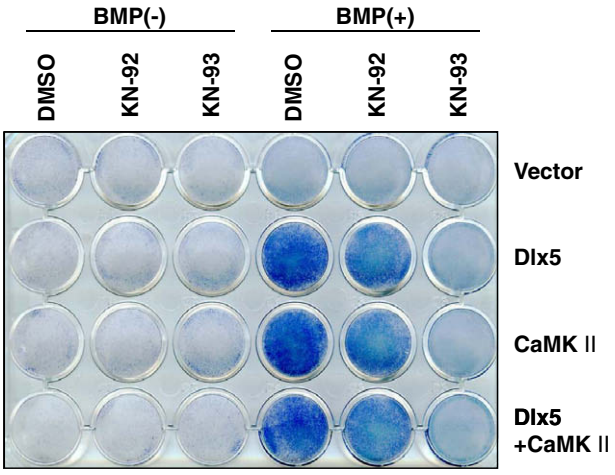

Fig. 1. CaMKII regulates Dlx5-induced osteoblast differentiation. C2C12 cells were cultured in DMEM supplemented with 15% FBS, and transfected with Dlx5 and/or CaMKII expression vectors (0.2 μg each). After 24 h, growth media were changed to DMEM supplemented with 2% FBS. Cells were then treated with a CaMKII inhibitor KN-93 (1 mM) or an inactive analog KN-92 (1 mM) in the absence or presence of BMP-2 (30 ng/ml). After 3 days, the extents of osteoblast differentiation are compared by ALP staining. ALP positive cells stain blue/purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CaMKII phosphorylates Dlx5 and increases the protein levels of Dlx5

p38 can phosphorylate Dlx5 at Ser-34 and Ser-217 leading to the increase of Dlx5 transactivation activity and Dlx5-induced Osterix expression [17]. We postulated that CaMKII may also regulate Dlx5 through phosphorylation. We first analyzed whether Dlx5 could be a target of CaMKII by an *in vitro* kinase assay. In the presence of γ-[^{32}P]-ATP, immunoprecipitated CaMKII was incubated with anti-HA immunoprecipitates of cells transfected with HA-tagged Dlx5 or control vector. CaMKII phosphorylated Dlx5 (Fig. 2A), suggesting that Dlx5 is a novel substrate of CaMKII.

We next examined whether CaMKII affects the protein levels of Dlx5. 293T cells were transfected with Dlx5 and increasing amounts of CaMKII. The levels of Dlx5 protein were determined by Western blotting. The levels of Dlx5 were dramatically increased by CaMKII in a dose-dependent manner (Fig. 2B).

CaMKII increases the protein stability of Dlx5

During osteoblast differentiation, several signaling pathways can affect the expression of Dlx5. BMP-2 and p38 can induce Dlx5 expression while TGF-β can suppress Dlx5 expression [14,26–28]. CaMKII may increase the protein levels of Dlx5 by regulating the transcription, translation or protein stability of Dlx5. To identify the mechanism of how CaMKII increases the protein levels of Dlx5, we first examined whether CaMKII affects the transcription of Dlx5. CaMKII did not change the levels of Dlx5 mRNA significantly when examined by RT-PCR (data not shown). Therefore, we next examined whether CaMKII affects the protein stability of Dlx5. 293T cells were transfected with Dlx5 alone or with CaMKII. To examine the patterns of Dlx5 protein turnover, transfected cells were treated with cycloheximide, a translation inhibitor, for increasing amounts of time. The levels of Dlx5 protein were then determined by Western blotting. Dlx5 protein was gradually degraded in the absence of CaMKII (Fig. 3A and B). However, CaMKII significantly prolonged the half-life of Dlx5 protein. These results

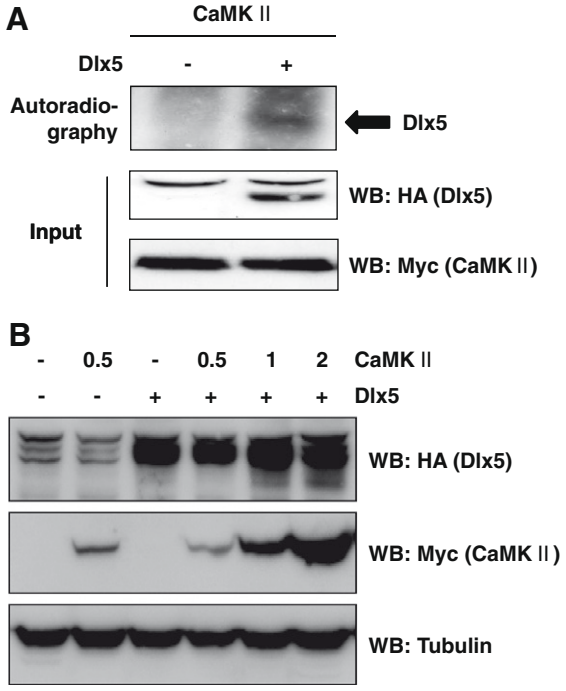

Fig. 2. CaMKII phosphorylates DLx5 and increases the protein levels of Dlx5. (A) CaMKII phosphorylates Dlx5. 293T cells were transfected with Myc-tagged CaMKII expression vector. Anti-Myc immunoprecipitates were then incubated, in the presence of γ-[^{32}P-ATP, with anti-HA immunoprecipitates of 293T cells transfected with HA-tagged Dlx5 expression vector or a control vector. Proteins were separated by SDS–PAGE and phosphorylated Dlx5 is visualized by autoradiography. Levels of Dlx5 [WB: HA (Dlx5)] and CaMKII [WB: Myc (CaMKII)] proteins in inputs are also compared by Western blotting. (B) CaMKII increases the protein levels of Dlx5. 293T cells were transfected with HA-tagged Dlx5 expression vector (0.5 μg) and increasing amounts of Myc-tagged CaMKII expression vector (0.5, 1, or 2 μg). After 36 h, cell lysates were subjected to SDS–PAGE and the levels of Dlx5 protein are examined by Western blotting [WB: HA (Dlx5)]. The levels of CaMKII are also compared [WB: Myc (CaMKII)], and tubulin is used as a loading control (WB: Tubulin).

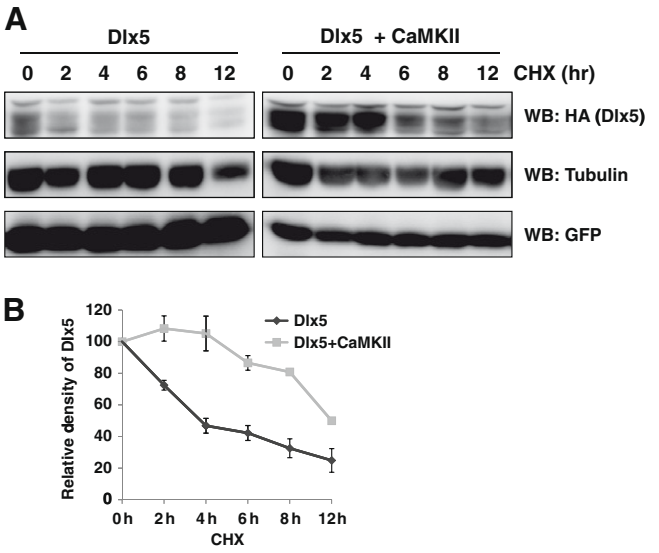

Fig. 3. CaMKII increases the protein stability of Dlx5. (A) 293T cells were transfected with HA-tagged Dlx5 (0.5 μg) and/or CaMKII (1 μg) expression vectors along with GFP expression vector (0.2 μg). After 36 h, cells were treated with cycloheximide (CHX, 40 μg/ml) and harvested at indicated times. Cell lysates were subjected to SDS–PAGE, and the levels of Dlx5 protein are examined by Western blotting [WB: HA (Dlx5)]. The levels of GFP are compared for transfection efficiency (WB: GFP), and tubulin is used as a loading control (WB: Tubulin). (B) The levels of Dlx5 in panel A are determined by densitometry. The levels of Dlx5 protein in CHX untreated cells (0 h) are considered as 100%. The experiment was repeated three times and a representative result is shown.

suggest that CaMKII increases the levels of Dlx5 protein by increasing the protein stability of Dlx5, but not the transcription.

CaMKII increases the transactivation activity of Dlx5

We next examined whether CaMKII affects the transactivation activity of Dlx5. 293T cells were transfected with an ALP (ALP-Luc) or osteocalcin (OC-Luc) luciferase reporter construct. ALP and osteocalcin are markers of osteoblast differentiation. Cells were then transfected with combinations of Dlx5 and/or CaMKII in the presence or absence of CaMKII inhibitors. Dlx5 increased the transcription of both ALP-Luc and OC-Luc reporters (Fig. 4). CaMKII further increased the Dlx5-induced transactivation of ALP-Luc and OC-Luc in a dose-dependent manner. However, KN-93, but not KN-92, significantly diminished the CaMKII-induced increase of Dlx5 transactivation activity. These results indicate that CaMKII increases the transactivation ability of Dlx5.

Discussion

We examined the effects of CaMKII on Dlx5 function during osteoblast differentiation. We found evidences for a novel regulatory mechanism that CaMKII regulates osteoblast differentiation, at least in part, through direct phosphorylation of Dlx5. First, when assessed by ALP staining, Dlx5/BMP-2-induced osteoblast differentiation is inhibited by a CaMKII inhibitor KN-93, but not by an inactive analog KN-92. Second, CaMKII phosphorylates Dlx5 *in vitro*. Third, CaMKII increases the protein stability of Dlx5. Lastly, CaMKII increases transactivation ability of Dlx5 while this increase is abolished by KN-93.

In adult, bone is constantly removed by osteoclasts and replenished by osteoblasts. The coordinated action of osteoblasts and osteoclasts maintains the proper bone volume and calcium homeostasis. While Ca^{2+} homeostasis is under the control of osteoblasts and osteoclasts, Ca^{2+} also regulates bone remodeling as an important intracellular second messenger. The increase of intracellular Ca^{2+} levels is considered to promote osteoblast proliferation and/or differentiation during bone remodeling [29,30]. Ca^{2+} signaling is mainly mediated by calmodulin (CaM) and calmodulin-dependent kinases (CaMKs) [18]. Several evidences strongly suggest that CaMKII plays an essential role in osteoblast differentiation [31]. The role of CaMKII in osteoblast differentiation is an interesting subject as Ca^{2+} signaling is also important for bone homeostasis. For this aspect, the regulation of Dlx5 function by CaMKII may play a significant role in osteoblast differentiation.

The activity of Dlx5 is regulated at the levels of transcription and post-translational modification. BMP-2 regulates the transcription of Dlx5 [14,32], while p38 regulates the transactivation activity of Dlx5 by phosphorylation [17]. Our results indicate that, during osteoblast differentiation, CaMKII regulates Dlx5 by phosphorylation.

Our work provides a basis for understanding the roles of CaMKII during osteoblast differentiation. We showed that CaMKII modulates Dlx5 function by suppressing protein degradation, not by up-regulating protein synthesis. Further study is needed to understand the mechanism of Dlx5 protein degradation, and to understand the significance such regulation in osteoblast differentiation. Ubiquitination and sumoylation are the two main pathways for protein degradation. Elucidation of the upstream and downstream regulatory components for the regulation of Dlx5 by CaMKII will help understanding the significance of this novel interaction.

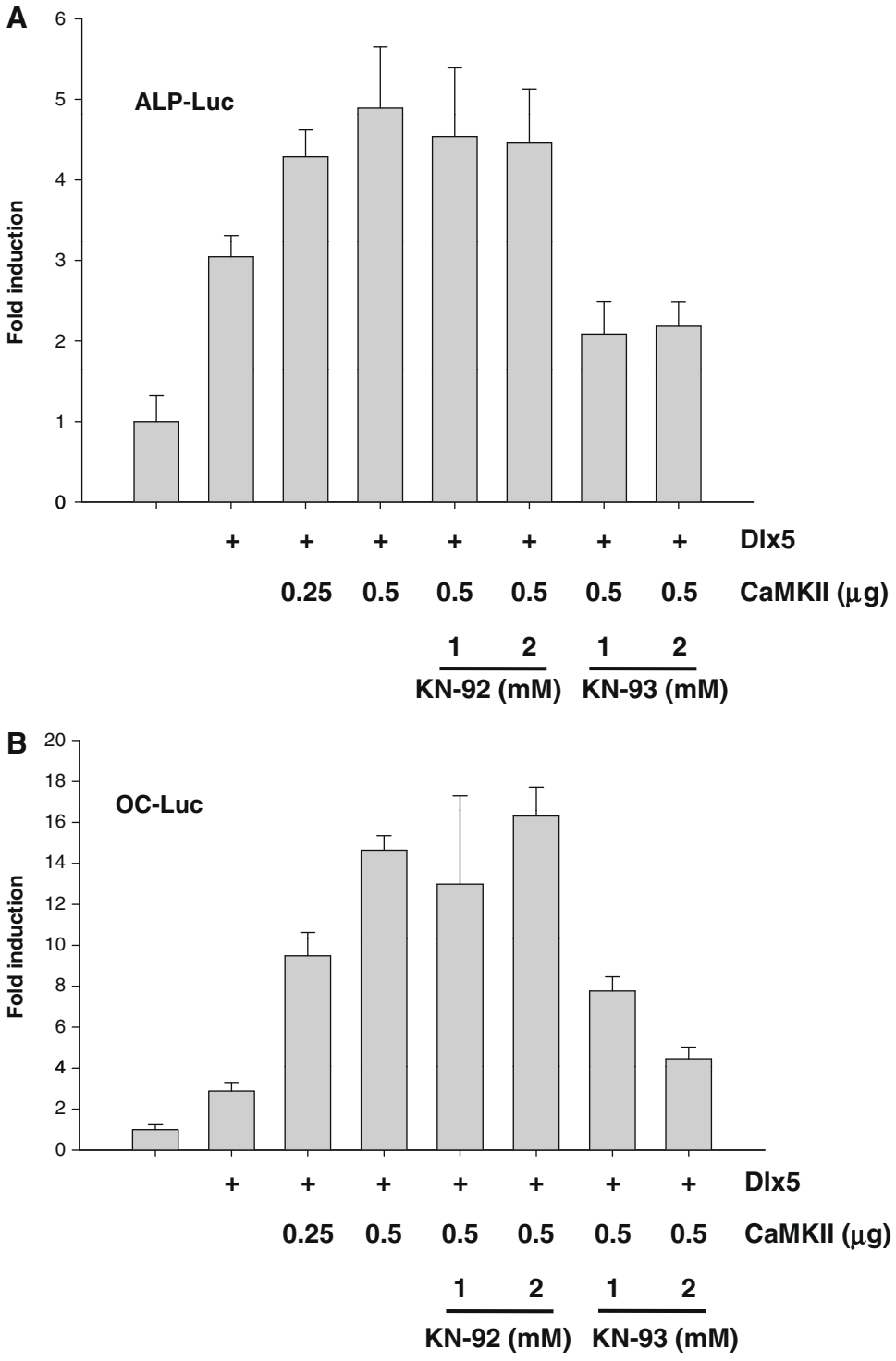

Fig. 4. CaMKII increases the transactivation activity of Dlx5. 293T cells were transfected with pCMV-β-gal (0.05 μg), ALP-Luc (A) or OC-Luc (B) reporter vector (0.1 μg each) along with indicated combinations of Dlx5 (0.25 μg) and CaMKII (0.25 or 0.5 μg) expression vectors. After 24 h, cells were treated with KN-93 or KN-92 (1 or 2 mM) in fresh media for 12 h. Luciferase activities are normalized with corresponding β-galactosidase activities. The experiment was performed in triplicates, and the average relative luciferase activities with SD are shown.

Acknowledgments

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