Tricho-dento-osseous Syndrome Mutant Dlx3 Shows Lower Transactivation Potential but Has Longer Half-life than Wild-type Dlx3

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Dlx3 is a homeodomain protein and is known to play a role in development and differentiation of many tissues. Deletion of four base pairs in *DLX3* (NT3198) is causally related to tricho-dento-osseous (TDO) syndrome (OMIM #190320), a genetic disorder manifested by taurodontism, hair abnormalities, and increased bone density in the cranium. The molecular mechanisms that explain the phenotypic characteristics of TDO syndrome have not been clearly determined. In this study, we examined phenotypic characteristics of wild type *DLX3* (wtDlx3) and 4-BP DEL *DLX3* (TDO mtDlx3) in C2C12 cells. To investigate how wtDlx3 and TDO mtDlx3 differentially regulate osteoblastic differentiation, reporter assays were performed by using luciferase reporters containing the promoters of alkaline phosphatase, bone sialoprotein or osteocalcin. Both wtDlx3 and TDO mtDlx3 enhanced significantly all the reporter activities but the effect of mtDlx3 was much weaker than that of wtDlx3. In spite of these differences in reporter activity, electrophoretic mobility shift assay showed that both wtDlx3 and TDO mtDlx3 formed similar amounts of DNA binding complexes with Dlx3 binding consensus sequence or with ALP promoter oligonucleotide bearing the Dlx3 binding core sequence. TDO mtDlx3 exhibits a longer half-life than wtDlx3 and it corresponds to PESTfind analysis result showing that potential PEST sequence was missed in carboxy terminal of TDO mtDlx3. In addition, co-immunoprecipitation demonstrated that TDO mtDlx3 binds to Msx2 more strongly than wtDlx3. Taken together, though TDO mtDlx3 acted as a weaker transcriptional activator than wtDlx3 in osteoblastic cells, there is possibility that during *in vivo* osteoblast differentiation TDO mtDlx3 may antagonize transcriptional repressor activity of Msx2 more effectively and for longer period than wtDlx3, resulting in enhancement of osteoblast differentiation.

Key Words : Dlx3, TDO mutant Dlx3, Osteoblast differentiation, Transactivation potential, Protein stabilization, Msx2

Introduction

Homeodomain (HD) proteins comprise a large group of transcription factors and are essential regulators of many developmental processes, ranging from organization of the basic body to terminal differentiation of individual tissues (McGinnis and Krumlauf, 1992). The binding sites of HD proteins typically center around TAAT sequence motif and adjacent bases are thought to impart specificity to interactions between HD proteins and target genes (Scott et al., 1989; Laughon, 1991). The Distal-less family (Dlx) is one of the HD protein families. In vertebrates, there are six Dlx genes arranged in three clusters (Dlx1/Dlx2, Dlx3/Dlx4, Dlx5/Dlx6; Ghanem et al., 2003). Among these Dlx genes, the importance of Dlx3 during development arises from several observations of disease states. Mutations in *DLX3* are believed to be causally related to tricho-dento-osseous (TDO) syndrome (OMIM #190320), a genetic disorder manifested by taurodontism, hair abnormalities, and increased bone density in the cranium (Price et al., 1998a, 1998b; Wright et al., 1997). TDO syndrome is inherited as an autosomal dominant trait. The genetic defect leading to TDO syndrome appears to be associated with a four nucleotide deletion just downstream of the HD (4-BP DEL, NT3198), resulting in frame shift and premature truncation of the DLX3 protein.

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The promoter region of osteoblast differentiation marker genes, such as alkaline phosphatase (ALP), osteocalcin (OCN) and bone sialoprotein (BSP), contains multiple HD binding motifs (Benson et al., 2000; Hassan et al., 2004; Kim et al., 2004). In MC3T3-E1 cells, overexpression of Dlx3 induced the expression of osteoblast differentiation maker genes such as OCN and BSP while knockdown of Dlx3 by RNA interference down-regulated (Hassan et al., 2004). In addition, it has been suggested that the switching of HD proteins occupancy to Runx2 or OCN promoters regulates the transcription of these genes during osteoblast differentiation; that is, Msx2 occupancy is associated with repression or basal transcription but Dlx3 and/or Dlx5 occupancy is associated with active transcription (Hassan et al., 2004, 2006). These reports suggest that Dlx3 is an important osteogenic regulator. Since the phenotype of increased bone density appears in some of TDO syndrome patients, we examined the effects of wild type *DLX3* (wtDlx3) or 4-BP DEL *DLX3* (TDO mtDlx3) overexpression on osteoblastic differentiation using C2C12 cells. We demonstrated in this study that i) both wtDlx3 and TDO mtDlx3 enhanced osteoblast differentiation, ii) in spite of having similar DNA binding affinity, TDO mtDlx3 acted as a weaker transcriptional activator than wtDlx3, iii) TDO mtDlx3 had a longer half-life than wtDlx3, and iv) TDO mtDlx3 shows stronger interaction with Msx2.

Materials and Methods

Cell culture

C2C12 and NIH3T3 cells were maintained in Dulbecco's modified Eagles medium (DMEM; Hyclone; Logan, UT, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, Gibco BRL, Grand Island, NY, USA). All the cell cultures were performed at 37°C in a humidified atmosphere containing 5% CO_2.

Transient transfection

Human full length wild type DLX3 ORF expression clone (pReceiver-M12-wtDlx3) which contains N-terminal 3xFlag tag was purchased from Genecopoeia (Germantown, Maryland, USA) and sequence-verified. TDO mtDlx3 expression vector was prepared by polymerase chain reaction (PCR)-amplified site-directed mutagenesis cloning into pReceiver-M12 vector and sequence-verified as previously described (Cha et al., 2007).

For transient transfection, cells were plated at a density of 5×10^5 cells per 60 mm culture dish or 2×10^6 cells per 100 mm culture dish. After overnight incubation, cells were serum-starved. Plasmid DNA (8 μg per 60 mm dish, 16 μg per 100 mm dish) and Lipofectamine2000 (10 μl per 60 mm dish, 20 μl per 100 mm dish; Invitrogen; Carlsbad, CA, USA) were mixed and incubated for 20 minutes at room temperature. DNA-Lipofectamine complex was diluted in serum-free DMEM and added to serum-starved cells. After 4 hour incubation, culture medium was changed with DMEM supplemented with 5% FBS. Twenty-four hours after transfection, cells were used for experiments.

Immunoblot analysis and co-immunoprecipitation

After appropriate treatment, cells were washed with PBS and scraped into lysis buffer consisted of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin, and sonicated briefly. Protein concentrations were determined using a modified Bradford method (Bio-Rad Laboratories; Hercules, CA, USA). Samples containing equal amounts of protein were subjected to SDS-PAGE. The proteins separated in the gel were subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20. Exogenously expressed Dlx3 level was examined by using mouse anti-Flag M2-HRP (Sigma-Aldrich; St. Louis, MO, USA). Actin expression level was examined by using goat anti-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by rabbit anti-goat HRP-conjugated IgG (Zymed; San Francisco, CA, USA). Luminescence was detected by using Supex reagent in a LAS1000 (Fuji PhotoFilm; Tokyo, Japan).

To examine the protein-protein interactions between Msx2 and Dlx3, co-immunoprecipitation was performed. Twenty-four hours after transfection with HA-tagged Msx2 and Flag-tagged Dlx3 expression vectors, C2C12 cells were lysed with immunoprecipitation buffer consisted of 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM PMSF, 0.2 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μM leupetin, and 1 μM pepstatin for 30 minutes at 4°C and sonicated briefly. Samples containing equal amounts of protein were incubated with 4-6 μg of anti-HA antibody with gentle rocking overnight at 4°C. After adding protein G agarose beads, the samples were incubated for 4 hours at 4°C with gentle agitation. The bead pellet was washed five times with immunoprecipitation buffer, denatured by boiling in 2x SDS sample buffer including dithiothreitol, and then subjected to SDS-PAGE and immunoblot analysis.

In vivo Dlx3 protein stability

Twenty-four hours after transfection with Dlx3 expression plasmids, C2C12 cells were treated either with 5 μM MG132 (Calbiochem; La Jolla, CA, USA), a reversible proteasome inhibitor or with 10 μg/ml cycloheximide (CHX; Sigma-Aldrich), a protein synthesis inhibitor and incubated for the times indicated. Then cell lysates were prepared with RIPA buffer and immunoblot analysis was performed as described above.

Electrophoretic mobility shift assay (EMSA)

C2C12 and NIH3T3 cells were transiently transfected with Dlx3 expression plasmids. After 24 hour incubation in Opti-MEM supplemented with 5% FBS, nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE Biotechnology; Rockford, IL, USA) according to the manufacturer's instruction. Two sets of oligonucleotides were used for the EMSA; one is from DNA sequence containing the Dlx3 binding site of mouse ALP promoter (-511~-477) and the other is the Dlx3 consensus sequence. Oct-1 consensus sequence was used as a negative control. The nucleotide sequences are as follows; ALP promoter Dlx3 binding site sense strand 5'-GTCCC TGTTTCTTCAGATATTAGGGGAGGGGAGGGCCCTG-3', Dlx3 consensus sequence forward 5'-AGCTAGTGGTAA TTGCTCAGA-3' and Oct-1 consensus sequence forward 5'-TGTCGAATGCAAATCACTAGAA-3'. They were end labeled by using [^{32}P]γdATP and T4 polynucleotide kinase (Roche). Annealing of sense and antisense oligonucleotides was performed by boiling for 5 minutes and slow cooling to room temperature. Unincorporated nucleotides were removed using NICK column Sephadex G-50 DNA Grade (GE Health care; Buckinghamshire, UK). EMSA reaction mixtures were prepared by mixing of 2 pmol of probe, 25 mM HEPES, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.05% NP-40, 0.5 mM PMSF, 2 μg of poly(dI-dC) and 4 μg of nuclear protein. In some tubes, unlabeled double-stranded competitor oligonucleotides (50-fold molar excess over probe) were added to the reaction mixtures. After 20 minutes incubation at 22°C, reaction products were loaded onto 4% polyacrylamide gel in 0.5X Tris-borate EDTA buffer and ran for 1 hour at 200 V. Gels were vacuum-dried, exposed to Medical X-ray Film Blue (AGFA; Mechelen, Belgium) and incubated in the -80°C for 2 days. Then film was developed and band intensity was observed by autoradiography.

Reporter assay

C2C12 cells were transiently transfected with Dlx3 expression vector or empty vector (500 ng per 4×10^4 cells), luciferase reporter plasmid (1 μg per 4×10^4 cells) and Renilla luciferase plasmid (phRL-TK; Promega; 200 ng per 4×10^4 cells) by using Microporator MP-100 (Digital Bio Technology; Suwon, Korea) and microporation solution kit (Digital Bio Technology) following manufacturer's protocol. Each luciferase reporter plasmid contains one of following promoter region of mouse genes; 1.05 kb OCN promoter, 2.5 kb BSP promoter, 1.9 kb or 0.5 kb ALP promoter. BSP promoter was kindly provided by Dr. G. Xiao (University of Pittsburgh). Electroporation was performed at the condition of pulse voltage of 1650 V, pulse width of 10 msec and pulse number of 3. Then the cells were seeded at a density of 4×10^4 cells/well in 96-well plates with clear bottom (Corning Inc. Life Science; Lowell, MA, USA). After a twenty-four hours incubation, the luciferase activity was measured by using Dual-Glo Luciferase Assay System (Promega;

Madison, WI, USA). Relative luciferase activity was calculated after normalizing the transfection efficiency by Renilla luciferase expression level.

Results and Discussion

Both wtDlx3 and TDO mtDlx3 transactivated osteoblast differentiation marker genes expression but transactivational potential of TDO mtDlx3 was lower than that of wtDlx3

Previous studies have shown that the knock-down of Dlx3 causes blockade of mature osteoblast formation by suppressing bone related marker genes, indicating the critical role of Dlx3 in osteoblastogensis (Hassan et al., 2004, 2006). Since TDO mtDlx3 is known to be associated with autosomal dominant TDO syndrome, comparing the properties of wtDlx3 and TDO mtDlx3 would be important for understanding the molecular pathogenetic mechanism of TDO syndrome bone phenotype.

Osteoblast differentiation marker genes such as ALP, OCN and BSP are known to be regulated by HD transcription factors (Benson et al., 2000; Hassan et al., 2004; Kim et al., 2004). Promoter regions of ALP (1.9 kb), BSP and OCN used in this study, have five putative HD binding sites while ALP reporter of shorter size (553 bp) includes
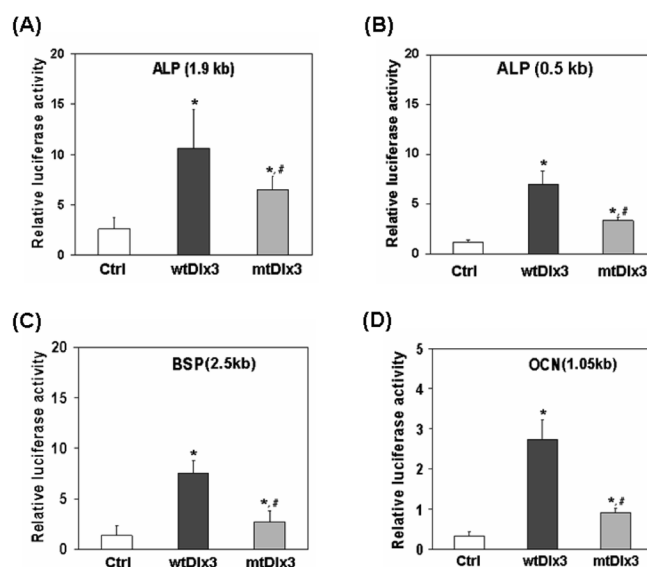

Fig. 1. Overexpression of wtDlx3 strongly enhanced the transcription of osteoblast differentiation marker genes while the effect of TDO mtDlx3 was much weaker than that of wtDlx3. C2C12 cells were transiently transfected with Dlx3 expression or pcDNA3.1 (Ctrl) vectors and luciferase reporter vectors containing 1.9 kb ALP promoter (A), 0.5 kb ALP promoter (B), 2.5 kb BSP promoter (C) or 1.05 kb OCN promoter (D). After 24 hour incubation, luciferase activity was measured and transfection efficiency was normalized by co-transfected Renilla luciferase activity. Data represent mean +S.D. (N = 10). * significant difference from the control (p < 0.01), # significant difference from wtDlx3 (p < 0.01).

one putative Dlx3 binding site. Both wtDlx3 and TDO mtDlx3 enhanced significantly ALP, BSP and OCN reporter activity (Fig. 1). However, wtDlx3 transactivated ALP promoters more strongly than TDO mtDlx3. These results suggest that the region carboxyl to the HD of Dlx3 that is frame-shifted in TDO mtDlx3, may be important to transactivation by Dlx3 of osteoblast differentiation marker genes.
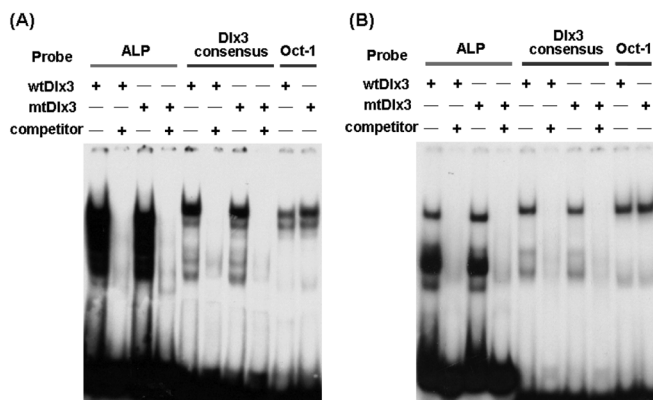
TDO mtDlx3 shows similar DNA binding affinity to wtDlx3

Since TDO mtDlx3 exhibited much lower reporter activity than wtDlx3, we examined whether there was any difference in DNA binding affinity between wtDlx3 and mtDlx3. We performed EMSA with oligonucleotides consisted of Dlx3 binding consensus sequence or of ALP promoter sequence including the core sequence (−492 and −487). Nuclear extracts from C2C12 or NIH3T3 cells which were transiently transfected with wtDlx3 or TDO mtDlx3 expression plasmids, were used as the sources of wtDlx3 and mtDlx3 proteins. NIH3T3 does not express endogenous Dlx3 (Park and Morasso, 1999). The radiolabeled ALP promoter probe as well as radiolabeled Dlx3 consensus probe formed binding complexes with the wtDlx3 and TDO mtDlx3 and these complexes disappeared in the presence of fifty-fold excess of unlabeled ALP probe and Dlx3 consensus probe (Fig. 2). As expected from the fact that TDO mtDlx3 contains intact HD, there was no remarkable difference in DNA binding affinity between wtDlx3 and TDO mtDlx3. This result indicates that reduced reporter activity in TDO mtDlx3-transfected cells is not due to reduced DNA binding but may be due to reduced transactivation potential of mtDlx3, and that carboxyl-terminal region to HD of Dlx3 is not related to DNA binding but may possess more important roles for transcriptional activation through protein-protein interactions. Outside of the HD domain, Dlx family proteins share

Fig. 2. wtDlx3 and TDO mtDlx3 showed similar DNA binding affinity. EMSA was performed using radiolabeled oligonucleotides consisted of ALP promoter sequence containing Dlx3 binding element or of Dlx3 consensus sequence and nuclear extracts of C2C12 (A) or NIH3T3 cells (B) overexpressing wtDlx3 and TDO mtDlx3. Competitor; Excess amounts of non-labeled probes (x 50) were added to the binding reaction.
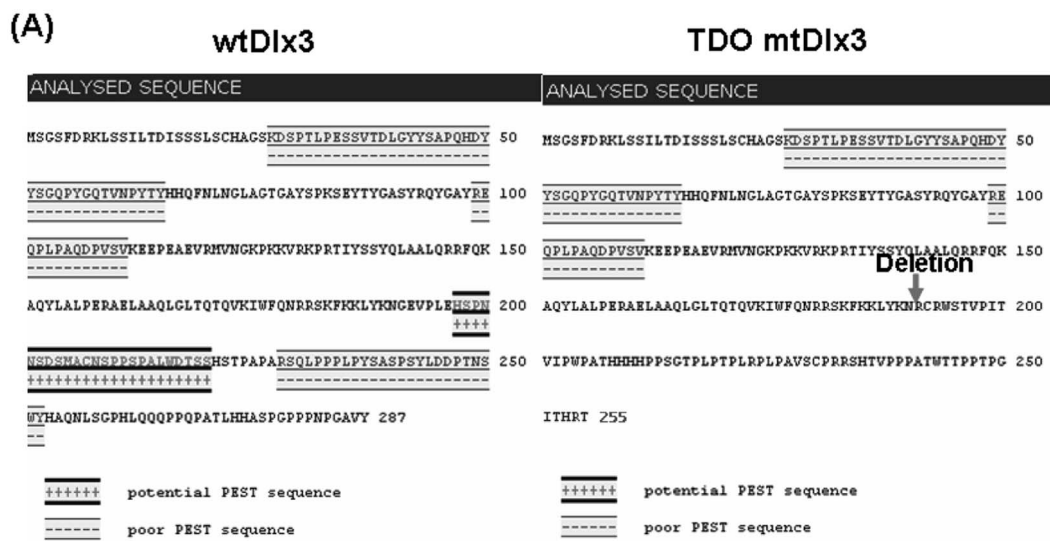
limited regions of similarity. For instance, all Dlx proteins possess at least two tryptophan residues that are carboxyl to HD and these tryptophans were suggested to be possible sites for interaction with other proteins such as PBC-family cofactors (Panganiban and Rubenstein, 2002). In addition, Dlx proteins are proline-rich both upstream and downstream of HD and these proline-rich domains have been implicated with transcriptional activation function of Dlx3 (Feledy et al., 1999). Therefore, lower transcriptional activation by TDO mtDlx3 might be related to change in carboxyl terminal amino acids that results in lack of these tryptophan residues and proline-rich domains.

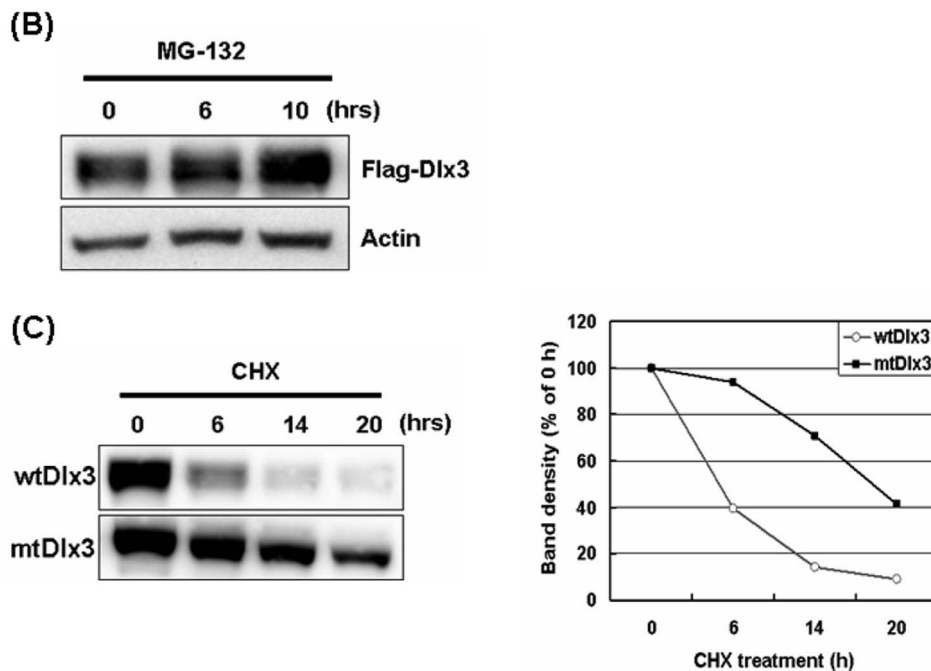### TDO mtDlx3 protein has a longer half-life than wtDlx3

Although overexpression of TDO mtDlx3 in C2C12 cells induced osteoblast differentiation marker genes expression, it was to a much lesser extent than that of wtDlx3 and it looks like the opposite of *in vivo* bone phenotype of TDO syndrome which exhibits increased bone density. Previously we reported that wtDlx3 overexpression enhanced expression of the receptor activator of nuclear factor-κB ligand (RANKL) in bone morphogenetic protein 2-treated C2C12 cells while TDO mtDlx3 did not (Cha et al., 2007). Since RANKL is a critical osteoclastogenic cytokine (Lacey et al., 1998), inability of TDO mtDlx3 to regulate expression of RANKL may contribute to increased bone density in TDO syndrome patients. Other possible explanation for this contradiction might be a longer half-life of TDO mtDlx3 *in vivo*. PEST domain is known as a proteolytic signal for degradation through 26S proteasome (Rogers et al., 1986; Rechsteiner and Rogers, 1996). PESTfind analysis has revealed that wtDlx3 contains one potential PEST sequence and several poor PEST sequences (Fig. 3A). To test whether Dlx3 protein is degraded by proteasome or not, C2C12 cells were transiently transfected with wtDlx3 expression vectors and incubated in the presence of MG132, a proteasome inhibitor. In the presence of MG132, the amount of wtDlx3 protein increased (Fig. 3B), indicating that Dlx3 proteins are degraded through proteasome-dependent pathway. Since PESTfind analysis result showed that potential PEST sequence and one of poor PEST sequences of wtDlx3 were lost in carboxyl terminal region of TDO mtDlx3 (Fig. 3A), it was expected that degradation rate of mtDlx3 might be slower than that of wtDlx3. Indeed, when the cells were incubated in the presence of CHX, an inhibitor of protein synthesis, the level of wtDlx3 protein diminished drastically while mtDlx3 decreased more slowly than wtDlx3 (Fig. 3C). Densitometry analysis showed that the half-life of wtDlx3 was much shorter than that of TDO mtDlx3. These results suggest that deleted PEST sequences in TDO mtDlx3 may play a role as a part of the regulatory sequences for proteasomal degradation of Dlx3 protein. Since Dlx3 expression is not constitutive but is temporally regulated during osteoblast differentiation (Hassan et al., 2004), TDO mtDlx3 may stimulate the expression of osteoblast differen-

Fig. 3. TDO mtDlx3 has longer half-life than wtDlx3. (A) Sequence analysis result of PEST domain finder shows that potential PEST sequence and one of poor PEST sequence of wtDlx3 were removed by frame shift in TDO mtDlx3. (B, C) C2C12 cells were transiently transfected with Dlx3 expression plasmids and incubated for 24 hours. Then cells were incubated in the presence of 5 μM MG132 (B) or 10 μg/ml cycloheximide (CHX) for the times indicated and Dlx3 protein level was determined by immuno blot analysis. Densitometry results showed that slope of wtDlx3 degradation was steeper than that of TDO mtDlx3 (C, *right panel*).

tiation marker genes for longer period than wtDlx3 *in vivo*, covering up the weak transactivational potential.

TDO mtDlx3 has higher binding affinity to Msx2 than wtDlx3

Another possibility is that mtDlx3 might antagonize the action of transcriptional repressors for longer period. One of possible transcriptional repressor candidates is Msx proteins.

Previous reports have shown that Dlx binds to Msx and antagonizes transcription regulatory activity of Msx (Zhang et al., 1997). In addition, it was reported that Msx2 binds competitively to the same DNA sites that Dlx3 binds and that Msx2 occupied the transcriptionally repressed promoters of ALP, Runx2 or OCN while Dlx3 and Dlx5 replaced Msx2 in transcriptionally active promoters (Kim et al., 2004; Hassan et al., 2004, 2006). Since EMSA results showed that
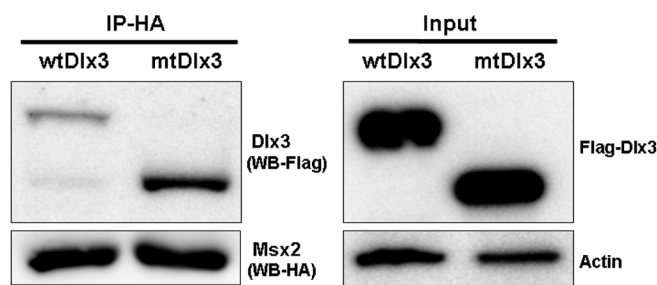
Fig. 4. Binding affinity of TDO mtDlx3 to Msx2 was higher than that of wtDlx3. Dlx3 and Msx2 expression plasmids were co-transfected into C2C12 cells and incubated for 24 h. Cell lysates were prepared and Dlx3-Msx2 complexes were immunoprecipitated (IP) by using anti-HA antibody. The immunoprecipitated complex were resolved in a 12% polyacrylamide gel and immunoblot analysis was performed by using antibodies indicated (*left panel*). The same cell lysates were used for immuno blot analysis (*right panel*).

wtDlx3 and TDO mtDlx3 had similar DNA binding affinity (Fig. 2), it is suggested that both Dlx3 proteins may show similar ability to compete with Msx2 for *cis*-acting elements binding in these promoters. Therefore, we examined whether there was any difference between wtDlx3 and mtDlx3 in binding affinity to Msx2. Co-immunoprecipitation result demonstrated that Dlx3 interacted with Msx2 (Fig. 4, left). Although the amounts of wtDlx3 and TDO mtDlx3 used for immunoprecipitation with anti-HA antibody were similar (Fig. 4, right), the amount of Dlx3 co-immunoprecipitated with Msx2 was larger in mtDlx3 than in wtDlx3. Though previous report had shown that Dlx and Msx dimerize through HDs (Bryan and Morasso, 2000), our data suggest that carboxyl terminal region to HD of Dlx3 may be involved in the regulation of dimerization. In addition, these results suggest that during *in vivo* osteoblast differentiation TDO mtDlx3 may antagonize transcriptional repressor activity of Msx2 more effectively and for longer period than wtDlx3, resulting in enhancement of osteoblast differentiation.

During the preparation of this manuscript, one report has been published showing that TDO mtDlx3 further enhanced osteoblastic differentiation and mineralized nodule formation in MC3T3-E1 and C2C12 cells than wtDlx3 did (Choi et al., 2007). In their report, TDO mtDlx3 induced significantly higher OCN promoter activity than wtDlx3 while the effect of mtDlx3 on type I collagen promoter were similar to or slightly lower than that of wtDlx3. The reason for the discrepancy between their results and ours is not clear now. One possibility is that they performed reporter assays with cloned cells that stably express wtDlx3 or mtDlx3 while we used transiently transfected cells. Further study will be needed to define the clear figure of *in vivo* behavior of TDO mtDlx3.

Acknowledgements

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