A RING Finger Protein Praja1 Regulates Dlx5-dependent Transcription through Its Ubiquitin Ligase Activity for the Dlx/Msx-interacting MAGE/Necdin Family Protein, Dlxin-1*

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Mxs2 and Dlx5 are homeodomain proteins that play an important role in osteoblast differentiation and whose expression is induced by bone morphogenetic proteins. Recently we have identified a novel protein, Dlxin-1, that associates with these homeodomain proteins and regulates Dlx5-dependent transcriptional function (Masuda, Y., Sasaki, A., Shibuya, H., Ueno, N., Ikeda, K., and Watanabe, K. (2001) J. Biol. Chem. 276, 5331–5338). In an attempt to elucidate the molecular function of Dlxin-1, two closely related RING finger proteins, Praja1 and Neurodap-1, were isolated by yeast two-hybrid screening using the C-terminal necdin homology domain of Dlxin-1 as bait. Glutathione S-transferase pull-down and immunoprecipitation/Western blotting assays following co-transfection of Dlxin-1 and Praja1 revealed that Praja1 binds to the C-terminal necdin homology domain of Dlxin-1 in vitro and in vivo, respectively. Overexpression of Praja1 caused a decrease in Dlxin-1 protein level, which was reversed when a proteasome inhibitor was added. Overexpression of Praja1 with a mutation in the RING finger inhibited the decrease in Dlxin-1 protein, pointing to the importance of ubiquitin-protein isopeptide ligase (E3) activity associated with RING finger. Wild-type Praja1, but not its RING finger mutant, promoted ubiquitination of Dlxin-1 in vivo. Finally, expression of Praja1 down-regulated Dlx5-dependent transcriptional activity in a GAL4-dependent assay. These results suggest that Praja1 regulates the transcription function of the homeodomain protein Dlx5 by controlling the stability of Dlxin-1 via an ubiquitin-dependent degradation pathway.

Dlxin-1 has been isolated as a novel Dlx/Msx-binding protein that contains MAGE/Necdin family protein homology domain (1). Dlxin-1 binds to the homeoprotein Dlx5 and regulates its transcriptional activity when overexpressed in HT1080 human fibroblast cells (1). Dlxin-1 binds not only to Dlx5 but also to other Dlx/Msx family proteins, suggesting that Dlxin-1 is a common transcriptional regulator for Dlx/Msx family proteins (1). Dlxin-1 binds to the homeoprotein Dlx5 and regulates its transcriptional activity when overexpressed in HT1080 human fibroblast cells (1). Dlxin-1 binds not only to Dlx5 but also to other Dlx/Msx family proteins, suggesting that Dlxin-1 is a common transcriptional regulator for Dlx/Msx family proteins (1).

MAGE family protein, classically subdivided into three clusters, MAGE-A, -B, and -C, has first been identified as a gene coding for a tumor antigen on melanoma cells (2). Although many tumors express MAGE mRNA, the expression is usually restricted to male germ lines. In tumor cells, MAGE gene products are processed and presented as antigenic peptides in the context of major histocompatiblity complex class I molecules (3). However, the physiological role of MAGE-A, -B, and -C proteins in germ line cells and the significance in tumorigenesis are poorly understood. MAGE-D1, a novel class D molecule of the family (4), is the human ortholog of Dlxin-1, which is expressed ubiquitously in adult tissues and has a peculiar amino acid repeat sequence adjacent to the MAGE/Necdin family protein homology domain, suggesting its unique function distinct from other MAGE family members (5, 6).

Among the MAGE family the most characterized protein is Necdin, which is highly expressed in post-mitotic neurons. Necdin binds to E2F1 and inhibits cellular proliferation when overexpressed in SAOS-2 cells that lack Rb protein, mimicking a Rb-like function (7). Necdin also associates with p53 and inhibits p53-dependent apoptosis and gene expression (8). It has been reported that Necdin interacts with NEFA, a calcium-binding protein, and regulates its subcellular localization and cytosolic Ca2+ homeostasis (9). The chromosome locus including Necdin gene is maternally imprinted, and deletion of the paternal allele or disomy of the maternal allele causes Prader-Willi syndrome, a congenital disease with various neurological abnormalities (10–12).

Recently, characterization of rat Dlxin-1 homologues has been reported independently from two groups. Salehi et al. (13) have shown that NRAGE, a rat homologue of Dlxin-1, binds to p75 neurotropin receptor (p75NTR) and confers NGF1-dependent apoptosis of developing neuronal cells (13). Hennuy et al. (14) showed that Dlxin-1 mRNA is expressed in Sertoli cells in the testis and down-regulated by follicle-stimulating hormone (FSH) (14). Although Dlxin-1 is a ubiquitously expressed protein, the restricted expression of their binding partners or regulator proteins, such as Dlx/Msx family proteins or p75NTR, to certain cell types and developmental stages points to the possibility that Dlxin-1 may function in cell survival and differentiation, depending on cell types.

Ubiquitination plays crucial roles in gene transcription, signal transduction, and cell cycle via regulation of protein stability (15). Ubiquitination of target proteins is achieved in three enzymatic steps. First, ubiquitin forms a thioester bond to the ubiquitin-activating enzyme (E1) in an ATP-dependent man-
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In the presence of an E3 ubiquitin protein ligase, E2 transfers ubiquitin to a specific substrate. Among the molecules involved in ubiquitin conjugation reactions, E3 ubiquitin ligases play a pivotal role in substrate recognition. There are a wide variety of E3 proteins to recognize diverse target protein substrates. E3 proteins or E3 protein complexes have either the HECT domain or the RING finger domain as an E2-recognition module (16–19). Conversely, most RING finger proteins have been shown to possess E3 activity (20, 21).

In this study, we identified two RING finger proteins, Praja1 (22) and Neurodap1 (23), as Dlxin-1-interacting proteins. Praja1 acts as an ubiquitin protein ligase against Dlxin-1 and mediates its ubiquitination and subsequent degradation, thereby down-regulating Dlx5-dependent transcription. Thus, our results suggest that Dlxin-1 is an adapter molecule regulating Dlx5-dependent transcription via ubiquitin/proteasome-dependent degradation by Praja1.

MATERIALS AND METHODS

Plasmid Construction—The BamHI fragment of Dlxin-1, which contains the whole Necdin/MAGE homology domain (NHD), was subcloned into pGBT9 (CLONTECH, Palo Alto, CA) and used as a bait plasmid (pGBT9-DXN/ Bam).

The full-length Dlxin-1 cDNA was subcloned into pcDNA3 (Invitrogen) with HA or FLAG epitope tagging at the N terminus to generate pHA-DXN and pF-DXN, respectively. The BamHI fragment of Dlxin-1 that contains NHD was subcloned as above to generate pHA-DXN-NHD or pF-DXN-NHD. pHA-DXN or pF-DXN-NHD was generated by deletion of BamHI fragment from pHA-DXN or pF-DXN, respectively. pHA-DXN-W encodes Dlxin-1 containing 18 of 25 WQ repeats, which was derived from a positive clone in the yeast two-hybrid screening using Dlxin-1 as a bait. pHA-DXN-N encodes N-terminal deletion of the C-terminal Dlxin-1 fragment after the first Ets site.

Wild-type and a RING finger mutant (Cyts222 to Ala) Praja1 were generated by PCR and subcloned into pcDNA3 with HA or Myc epitope tagging at the N terminus. For the construction of RING finger-deleted Praja1 (PrajαΔC), a C-terminal fragment after the PslI site was deleted from the wild-type Praja1 cDNA. The Praja1 cDNA fragment was also subcloned into pGEX-SX-1 (Amersham Biosciences, Uppsala, Sweden) for expression of GST fusion protein in Escherichia coli strain BL21.

Human ubiquitin cDNA was amplified by reverse transcriptase-PCR and subcloned into pcDNA3 with HA or FLAG epitope tag at the N terminus to generate HA or FLAG-tagged ubiquitin constructs (pHA-Ub and pF-Ub).

All cDNAs and the constructs amplified by PCR were fully sequenced (ABI PRISM™ 310 Genetic Analyzer, Applied Biosystems, Foster City, CA).

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed as described previously (1). Briefly, a yeast strain Y153 was co-transformed with pGBT9-DXN/Bam (described above) and the mouse embryo (day 11) cDNA library (CLONTECH). The transformants were scored in the selection media plates in the absence of Trp, Leu, and His, and in the presence of 60 mM 3-amino-1,2,4-triazole. Colonies grown on the selection media were then selected for luciferase activity in filter assay. Prey plasmids were recovered from β-galactosidase-positive colonies and were subjected to sequence analysis.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 9% fetal bovine serum. LipofectAMINE (Invitrogen) reagent was used for transfection.

In Vitro and in Vivo Binding—For in vitro binding, GST pull-down assay was performed as described previously (1). Briefly, [35S]methionine-labeled Dlxin-1 and its deletion mutant proteins were generated by in vitro transcription/translation system (Promega, Madison, WI). Aliquots were incubated with GST or GST-Prajα1 in binding buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% bovine serum albumin) followed by precipitation with glutathione-Sepharose beads (Amersham Biosciences). Bound proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE, and visualized by autoradiography.

For in vivo association, HEK293 cells were transiently transfected with various plasmids. 24 h after transfection, cells were lysed in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate) supplemented with a mixture of protease inhibitors (Complete™, Roche Diagnostics, Mannheim, Germany). Pre-cleared lysates were subjected to immunoprecipitation with anti-FLAG M2 antibody (Sigma) and protein G adsorption (Amer sham Biosciences). Precipitated proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Amer sham Biosciences). Immunoblotting was performed using anti-FLAG M5 antibody (Sigma), anti-HA 3F10 antibody (Roche Biosciences), or anti-Myc PL14 antibody (Medical & Biological Laboratories, Nagoya, Japan) and visualized by ECL Plus reagents (Amersham Biosciences).

In Vivo Protein Stability—A proteasome inhibitor, MG-132, was purchased from Sigma. HEK293 cells were transfected with plasmids with or without MG-132 treatment (0.5 mM). 24 h after transfection, cells were lysed in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Aliquots of lysates were separated by SDS-PAGE and immunoblotted. Affinity-purified polyclonal rabbit anti-DNX-NHD Ab was used to detect endogenous Dlxin-1. Anti-α-tubulin antibody (Sigma) was used for control of the protein amount.

In Vivo Ubiquitination—HEK293 cells were transfected with ubiquitin constructs (pHA-Ub or pF-Ub) and Dlxin-1 constructs (pF-DXN-NHD or pHA-DXN/NHD) with or without wild-type Praja1 or its RING finger mutants. 24 h after transfection, cells were harvested and lysed by sonication. 200 μl of 25% SDS in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) at 100 °C for 10 min. Subsequently, 800 μl of 1% Triton X-100 in TBS were added, and lysates were sonicated. The lysates were subjected to immunoprecipitation of FLAG-tagged NHD of Dlxin-1 (F-DXN-NHD) or HA-tagged Dlxin-1 (HA-DXN) using anti-FLAG antibody (M2) or anti-HA antibody, respectively, separated on SDS-PAGE and analyzed by immunoblotting to detect ubiquitinated and nonubiquitinated Dlxin-1 proteins.

Reporter Gene Assay—Gal4-mediated reporter gene assays for evaluating Dlx5-dependent transcription was performed as described (1). HEK293 cells were transfected with pG5-luc, pBIND-Dlx5/AC, and Dlxin-1-expressing plasmids (1). In all transfection experiments, Renilla luciferase expression vector pCMV-RL (Promega) was used as an internal control for normalization of transfection efficiency. 24 h after transfection, cells were lysed in Passive Lysis Buffer (Promega) and assayed for firefly and Renilla luciferase activities (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). Each assay was carried out at least in triplicates.

RESULTS

Identification of RING Finger Proteins as Dlxin-1-binding Proteins—Dlxin-1 has a domain homologous to other MAGE/Necdin family proteins in its C-terminal portion. In fact, Necdin associates with its binding partners, such as E2F1 and p53, through the NHD in exhibiting its function. To investigate the function of Dlxin-1 achieved by this domain and to identify Dlxin-1-binding partners, we performed yeast two-hybrid screening using a fragment including NHD as bait. Screening of 3 million transformants resulted in isolation of three independent clones encoding Praja1 and another clone encoding the mouse orthologue of rat Neurodap1. Praja1 and Neurodap1 share a highly homologous C-terminal region, including a RING finger domain (Fig. 1). All four prey plasmids covered a homologous region just proximal to the RING finger domain, suggesting that the overlapping C-terminal region may represent a Dlxin-1-binding domain (Fig. 1).

Prajα1 Binds to Dlxin1 in Vitro and in Vivo—To determine whether Praja1 directly binds to Dlxin-1, GST pull-down assay was performed using GST-Prajα1 fusion protein and 35S-labeled Dlxin-1. As shown in Fig. 2A, the NHD of Dlxin-1 (DXX-NHD) specifically bound to GST-Prajα1, which is consistent with the result of yeast two-hybrid screening. The full-length Dlxin-1 protein also bound to Praja1, although weakly. Dlxin-1 forms a homodimer or multimer (1), and DXX-NHD is co-immunoprecipitated with DXX-NHD, a construct lacking NHD (Fig. 2B), suggesting that full-length Dlxin-1 tends to bind to Dlxin-1 via inter-or intramolecular interaction rather

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than to Praja1. DXN-ΔNHD and DXN-W, both lacking the NHD but containing WQxPxx repeats, failed to bind to GST-Praja1. These results suggest that the Praja1-binding domain of Dlxin-1 resides in the NHD (Fig. 2B). Necdin also bound to Praja1, but much less efficiently than did Dlxin-1 (Fig. 2A, two panels on the right).

The RING finger motif of Praja1 is suggested to be the recognition module for E2 and therefore necessary for E3 activity (24). RING finger proteins, such as Mdm2, lose E3 activity by substitution of the conserved cysteine residues within RING finger domain to other amino acids (16, 21, 25). Thus, two Praja1 mutants, one that lacks almost all of the RING finger motif (Praja-ΔC) and the other that carries substitution of the cysteine at the residue 353 to alanine (Praja-C353A), were constructed to test its E3 activity for Dlxin-1 (Fig. 3B). Wild-type Praja1 bound to Dlxin-1 in vivo when overexpressed in HEK293 cells (Fig. 3B, lanes 1 and 2). Association of Dlxin-1 to two mutants, PrajaΔC and Praja-C353A, was also detected,

FIG. 1. Identification of cDNA clones encoding RING finger proteins in yeast two-hybrid screening with Dlxin-1 as bait. Schematic presentation of the primary structure of Praja1 and rat Neurodap1 and the clones isolated by yeast two-hybrid screening above and below each molecule, respectively. RING finger domains located in the C-terminal region are indicated by dark gray boxes. Praja1 and Neurodap1 share homologous regions (amino acids 62–398 for Praja1 and amino acids 341–681) indicated by gray boxes. Note that all three Praja1-encoding clones (cl.4, cl.13-2, and cl.34) and one Neurodap1-encoding clone (cl.17) contain a highly homologous region (amino acids 191–398 for Praja1 and amino acids 473–681 for Neurodap1) indicated by gray boxes (putative Dlxin-1-binding domain).

FIG. 2. Direct binding of Praja1 to Dlxin-1 in vitro. A, in vitro binding of Dlxin-1 deletion mutants, Dlx/Msx, and Necdin with Praja1. HA-tagged Dlxin-1, its deletion mutant proteins, and Necdin or FLAG-tagged Dlx5 and Mox2 were synthesized in vitro with 35S labeling and incubated with GST (G) or GST-Praja1 (P), followed by precipitation with glutathione-Sepharose beads. Precipitated proteins were separated by SDS-PAGE (GST pull-down). 10% of 35S-labeled proteins were separated without pull-down to evaluate protein synthesis (10% input). B, schematic diagram of the structure of Dlxin-1 (DXN) deletion mutants and summary of GST pull-down assay shown in A.

FIG. 3. Interaction of Praja1 with Dlxin-1 in mammalian cells. A, schematic presentation of the structure of wild-type Praja1 and its RING finger mutants. Almost all of RING finger motif is deleted in Praja1-ΔC, and the cysteine residue at 353 within the RING finger is substituted to alanine in Praja-C353A. B, wild-type Praja1 as well as its RING finger mutants bind to Dlxin-1 in vitro. HEK293 cells were transiently transfected with the indicated plasmids (0.3 μg each/well for pHA-Praja1, pHA-Praja-ΔC, and pHA-Praja-C353A and 2.0 μg/well for pF-DXN). 24 h after transfection, cells were lysed, and cell lysates were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were resolved by SDS-PAGE, and precipitated proteins were detected by immunoblotting with anti-HA antibody or anti-FLAG antibody. Aliquots of cellular extracts were immunoblotted without immunoprecipitation to evaluate protein expression (lower panels). A, Praja1 forms complexes with Dlxin-1 and Mox2. Transfection (0.5 μg for pF-Mox2, 1.5 μg for Dlxin-1 expression, and 0.5 μg for Praja1 expression), protein extraction, immunoprecipitation, and Western-blotting were performed as described in the legend to B.
indicating that the RING finger motif is dispensable for binding to Dlxin-1 (Fig. 3B, from lane 3 to lane 6). These Praja1 mutants migrate slower than expected from the molecular weight of wild-type Praja1. Taken together with the observation by Fang et al. (16) that RING finger mutants of Mdm2 migrate slower than wild-type protein on SDS-PAGE (16), it is conceivable that the RING finger motif adopts a tight conformation even in the presence of detergent and that destruction of this motif may reduce mobility of proteins on SDS-PAGE.

Next, immunoprecipitation of Msx2 was performed to determine whether Praja1 forms molecular complexes with Msx2 and Dlxin-1. As shown in Fig. 3C, Praja1 was co-precipitated with Msx2 either with (lane 5) or without (lane 4) Dlxin-1 transfection. These results suggest that Msx2, Praja1, and Dlxin-1 form molecular complexes in the cells and that endogenous Dlxin-1 may participate in the formation of this complex, because direct interaction between Msx2 and Praja1 was not observed (Fig. 2A).

**Praja1 Causes Degradation of Dlxin-1 and Msx2**—Although Praja1 has been shown to possess E3 activity for self-ubiquitination (21), its substrate for ubiquitination remains to be determined. The finding that Dlxin-1 associates with a RING finger protein Praja1 prompted us to hypothesize that Dlxin-1 may be a candidate for the substrate of Praja1. The protein level of ectopically expressed HA-Dlxin-1 (HA-DXN) was markedly decreased when wild-type Praja1, but not RING finger mutants (HA-Praja-C or HA-Praja-C353A), was co-expressed (Fig. 4A, left panel). This reduction of the Dlxin-1 protein in the presence of wild-type Praja1 was reversed by treatment with a proteasome inhibitor MG-132, suggesting that it is because of degradation of Dlxin-1 through proteasomes. Endogenous Dlxin-1 protein was also degraded upon introducing wild-type Praja1, but not its RING finger mutants, when assessed by immunoblotting using anti-Dlxin-1 polyclonal antibody (Fig. 4A, right panel).

Similarly, the protein level of Msx2, which forms complexes with Dlxin-1 and Praja1, was reduced by overexpression of wild-type Praja1, but not the RING finger mutants, in a proteasome-dependent degradation pathway (Fig. 4B).

**Praja1 Is a Ubiquitin Ligase for Dlxin-1**—Next, the E3 activity of Praja1 for Dlxin-1 was examined in vivo. HEK293 cells introduced with HA-tagged Dlxin-1 (HA-DXN) and FLAG-tagged ubiquitin (F-Ub) were lysed in SDS-containing buffer, and the cell lysates were boiled to dissociate noncovalent protein-protein interactions. The lysates were then immunoprecipitated with anti-HA antibody, separated on SDS-PAGE, and analyzed by immunoblotting using the indicated antibody. Note that bands corresponding to ubiquitinated DXN-NHD migrated slower than DXN-NHD itself.

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**Fig. 4.** Dlxin-1 as well as Msx2 is degraded through a ubiquitin/proteasome pathway in a RING finger-dependent manner. A and B, HEK293 cells were transiently transfected with the indicated plasmids with or without MG-132 treatment. 24 h after transfection, cells were lysed, and aliquots of cellular extracts were immunoblotted with anti-HA antibody or anti-Dlxin-1 polyclonal antibody. The membrane was re-blotted with anti-α-tubulin antibody to evaluate the amounts of proteins (bottom panel).

**Fig. 5.** Dlxin-1 is ubiquitinated by Praja1 in vivo. A and B, HEK293 cells were transiently transfected with the indicated plasmids with or without MG-132. 24 h after transfection, cells were lysed in 2% SDS buffer and boiled at 100 °C for 10 min. Diluted lysates were subjected to immunoprecipitation with anti-FLAG antibody (M2), separated on SDS-PAGE, and analyzed by immunoblotting using the indicated antibody. Note that bands corresponding to ubiquitinated DXN-NHD migrated slower than DXN-NHD itself.
transfection of wild-type Praja1 in the presence of MG-132 (compare lanes 6 and 8). In contrast, ubiquitination of Dlxin-1 decreased with co-transfection of Praja1 RING finger mutants, suggesting that they act in a dominant-negative fashion (lanes 9–12). These results indicate that Dlxin-1 is ubiquitinated in mammalian cells and that Praja1 is involved in this ubiquitination process via its RING finger.

As shown in Fig. 2, A and B, a Praja1-binding domain resides in NHD of Dlxin-1. To determine whether ubiquitination occurs within this domain, the same experiment was performed using FLAG-tagged NHD of Dlxin-1 (F-DXN-NHD) and HA-tagged ubiquitin (HA-Ub). In addition to smears of polyubiquitinated DXN-NHD, bands corresponding to DXN-NHD conjugated with one, two, or three ubiquitin molecules were observed (Fig. 5B, upper panel), indicating that ubiquitination takes place within the NHD of Dlxin-1. In the presence of MG-132, ubiquitination of DXN-NHD increased with co-transfection of wild-type Praja1 (compare lanes 6 and 8, Fig. 5B), but not of its RING finger mutants (lanes 9–12).

Praja1 Regulates Dlx5-dependent Transcriptional Activity—We have previously reported that the N-terminal domain of Dlx5 (Dlx5ΔC), which lacks homeodomain, possesses transcriptional activity (1). When Dlx5ΔC cDNA was fused to GAL4-DNA-binding domain (pBIND-Dlx5ΔC) and co-transfected into 293 cells with pG5-luc reporter plasmid, which contains GAL4-binding sites and luciferase reporter gene, reporter activity was increased dependently on the amount of GAL4-Dlx5ΔC (1). This assay was utilized to determine whether Praja1 is involved in Dlxin-1-mediated stimulation of Dlx5-dependent transcription.

When DXN-W, the Dlx5-binding domain of Dlxin-1, was overexpressed in HEK293 cells, the transcriptional activity of GAL4-Dlx5ΔC decreased in a dose-dependent manner (data not shown). The construct DXN-W is expected to block the association of Dlx5ΔC with Dlxin-1, thereby acting in a dominant-negative fashion (1). These results indicate that endogenous Dlxin-1 regulates the transcriptional activity of GAL4-Dlx5ΔC in HEK293 cells.

To examine whether Praja1 may modulate the transcriptional activity of Dlx5, wild-type Praja1 or its mutants were co-transfected with pBIND-Dlx5ΔC and pG5-luc. As shown in Fig. 6, co-expression of Praja1 reduced the transcriptional activity mediated by GAL4-Dlx5ΔC dose-dependently, whereas the RING finger mutants of Praja1 failed to inhibit the transcriptional activity. It is to be noted that MG-132 treatment only partially rescued the inhibition of the transcription (Fig. 6). These results suggest that ubiquitination of Dlxin-1 per se, even without its proteasome-dependent degradation, can reduce Dlx5-dependent transcriptional activity, possibly by spatial redistribution and/or conformational changes of Dlxin-1 molecule.

DISCUSSION

The ubiquitin-proteasome pathway is an important system for modifying protein function and regulating a wide variety of biological processes as diverse as cell cycling, signal transduction, and gene transcription. In this paper we presented evidence suggesting that the RING finger protein Praja1 binds to Dlxin-1 and acts as an E3 ubiquitin ligase for Dlxin-1 (Figs. 2, 3, and 5). Dlxin-1 is recognized and ubiquitinated by Praja1, with subsequent degradation by proteasome (Fig. 4A). Mx2, which forms complexes with Praja1 and Dlxin-1, is also degraded in the presence of Praja1 (Fig. 4B). Although it remains to be determined whether ubiquitination of Dlx/Msx family proteins takes place, Dlxin-1 may serve as an adapter molecule for Dlx/Msx family proteins to be recognized by proteasome. Recently, anti-differentiation activity of Mxs has been demonstrated (27–29). Constitutive or ectopic expression of Mxs proteins inhibit terminal differentiation not only of myogenesis but also of osteogenesis and chondrogenesis (27, 29). Furthermore, overexpression of Msx1 leads to dedifferentiation of myotubes to proliferating myoblasts, which keep the potential to differentiate into osteoblasts or chondrocytes (28). Hu et al. (27) have shown that the anti-differentiation activity of Msx proteins is coupled to up-regulation of cyclin D1 by Msx (27). These findings suggest that tight regulation of Msx protein is required for cell differentiation process. The regulation of the stability of Msx proteins, as demonstrated in this study, may play a role in the precise execution of differentiation program.

In this study we demonstrated that Praja1 inhibits Dlx5-dependent transcription in a RING finger-dependent manner (Fig. 6), suggesting that ubiquitination of Dlxin-1 is important for repressing Dlx5-mediated transcription. In view of the results that Mx2 was degraded when Praja1 was overexpressed, the mechanism of transcriptional repression may involve degradation of Dlx5. However, there remains the possibility that Praja1 or ubiquitinated Dlxin-1 may recruit a transcriptional repressor protein(s) to the complex, because transcriptional repression by Praja1 was only partially rescued by treatment with a proteasome inhibitor, and because certain RING finger proteins have been shown to function as a transcriptional repressor (30–32). Alternatively, nonubiquitinated Dlxin-1 may recruit a transcriptional co-activator(s), and when ubiquitination of Dlxin-1 takes place, the transcriptional co-activator may dissociate from Dlxin-1.

The concept that Dlxin-1 acts as an adapter molecule for transcriptional repression apparently contradicts with the conclusion of our previous publication that Dlxin-1 functions as transcriptional activator on Dlx5-dependent transcription (1). However, ubiquitination of Dlxin-1 may be strictly regulated in a physiological state. Whereas ubiquitination and degradation of Dlxin-1 take place constantly in artificial experimental systems employing overexpression of proteins, some triggering events may be required for ubiquitination in a physiological context. One of the candidates is phosphorylation of Dlxin-1, because Dlxin-1 contains potential target sequences of protein kinases (data not shown). In light of the findings that the stability of p53 increases with its acetylation because of pro-
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tection from ubiquitination (33), acetylation may also stabilize Dlxin-1 protein through protection against degradation by Praja1. It remains to be determined whether post-translational modifications, such as phosphorylation and acetylation, of Dlxin-1 play a role in the regulation of its stability. Recently, it has been shown that sumoylation (conjugation of SUMO-1) of the RING finger protein Mdm2, an E3 enzyme for p53, stabilizes Mdm2, thereby protecting self-ubiquitination of Mdm2 and accelerating ubiquitination and degradation of p53 (34). It is possible that the E3 activity of Praja1 is also regulated by sumoylation, because a lysine residue required for both sumolation and ubiquitination is conserved among these RING finger proteins.

The association of Praja1 with the Necdin homology domain of Dlxin-1 suggests that Praja1 may also bind to other MAGE/Necdin family proteins and function as a common E3 enzyme. In fact, we obtained evidence that Praja1 bound to Necdin in vitro (Fig. 2A). Moreover, some potential ubiquitination sites are present in the NHD of Dlxin-1 (Fig. 5B). It is widely accepted that most antigenic peptides are generated by proteasomes in the pathway of the presentation on major histocompatibility complex class I molecules (35). The MAGE gene products are processed into 8–10-residue peptides and presented as antigenic complexes (3). If Praja1 acts as a common E3 for MAGE/Necdin family proteins, Praja1 may be a potentially useful tool for gene therapy for MAGE-expressing tumors. For example, when Praja1 is overexpressed in MAGE-expressing tumor cells, the increased rate of degradation of MAGE gene products is expected to accelerate the processing of antigen peptides derived from MAGE gene products, thereby augmenting tumor rejection by host immune system.

Recently, Dlxin-1 has been shown to associate with p75 neurotropin receptor (p75NTR) and confer NGF-dependent apoptosis of developing neuronal cells (13). In view of the reported findings that Dlxin-1 accumulates to plasma membrane concomitantly with NGF treatment of PC12 rat pheochromocytoma cells (13), it is tempting to speculate that Praja1 may also be recruited to p75NTR via association with Dlxin-1. Because cell surface expression and activity of certain growth factor receptors are regulated by ubiquitination (17, 26), Praja1 has the potential to modulate NGF signaling by ubiquitinating p75NTR-associated Dlxin-1.

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