Necdin Interacts with the Msx2 Homeodomain Protein via MAGE-D1 to Promote Myogenic Differentiation of C2C12 Cells*

Received for publication, April 14, 2004, and in revised form, June 24, 2004 Published, JBC Papers in Press, July 21, 2004, DOI 10.1074/jbc.M404143200

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Necdin is a potent growth suppressor that is expressed predominantly in postmitotic cells such as neurons and skeletal muscle cells. Necdin shows a significant homology to MAGE (melanoma antigen) family proteins, all of which contain a large homology domain. MAGE-D1 (NRAGE, Dlxin-1) interacts with the Dlx/Msx family homeodomain proteins via an interspersed hexapeptide repeat domain distinct from the homology domain. Here we report that necdin associates with the Msx homeodomain proteins via MAGE-D1 to modulate their function. In vitro binding and co-immunoprecipitation analyses revealed that MAGE-D1 directly interacted with necdin via the homology domain and Msx1 (or Msx2) via the repeat domain. A ternary complex of necdin, MAGE-D1, and Msx2 was formed in vitro, and an endogenous complex containing necdin, MAGE-D1, and Msx2 proteins was detected in differentiating embryonal carcinoma cells. Co-expression of necdin and MAGE-D1 released Msx-dependent transcriptional repression. C2C12 myoblast cells that were stably transfected with Msx2 CDNA showed a marked reduction in myogenic differentiation, and co-expression of necdin and MAGE-D1 canceled the Msx2-dependent repression. These results suggest that necdin and MAGE-D1 cooperate to modulate the function of Dlx/Msx homeodomain proteins in cellular differentiation.

Necdin is a 325-amino acid residue protein encoded in a cDNA sequence isolated from the library of neurally differentiated P19 embryonal carcinoma cells (1). The necdin gene is expressed predominantly in postmitotic neurons (2, 3). Necdin is also expressed in non-neuronal cells such as skeletal myocytes, chondrocytes, adipocytes, and skin fibroblasts (4–7). Ecotropic expression of necdin strongly suppresses the growth of proliferative cells (8–10) and promotes differentiation of neuronal, myocytic, chondrocytic, and adipocytic cells (11). In primary cultures of dorsal root ganglia from mouse embryos, down-regulation of endogenous necdin expression results in a severe impairment of neuronal maturation and an increase in the number of apoptotic cells (12). Necdin knockout mice show a phenotype resembling Prader-Willi syndrome, a genomic imprinting-associated neurological disorder, suggesting that the absence of necdin impairs neuronal differentiation or maturation (5, 13, 14). These findings suggest that necdin facilitates terminal differentiation and prevents apoptosis in neurons and that necdin has a similar function in terminally differentiated non-neuronal cells.

Necdin shows a significant homology to MAGE family proteins, the remarkable feature of which is a large central region termed MAGE homology domain (MHD).1 MHD is consistent with the functional region through which necdin interacts with various proteins such as SV40 large T antigen, adenovirus E1A, E2F1, E2F4, p53, NEFA, heterogeneous nuclear ribonucleoprotein U, and p75 neurotrophin receptor (4, 9–11, 15–17). Furthermore, the MHDs of necdin/MAGE family proteins are thought to be responsible for their functions. For example, necdin, MAGE-D1 (18), MAGE-G1 (17), and magphinin (MAGE-D4) (19), whose MHDs have close similarities to necdin, suppress cell proliferation. The fact that most of the necdin interactors are related to cell cycle regulation, differentiation, and apoptosis suggests that necdin exerts its diverse biological activities through its MHD.

Recently, the necdin homologous protein MAGE-D1(also designated NRAGE or Dlxin-1) has been characterized as a regulator of apoptosis and transcriptional modulators. NRAGE, a rat homolog of human MAGE-D1, binds to p75 neurotrophin receptor via the MHD and confers nerve growth factor-dependent apoptosis through a c-Jun N-terminal kinase-dependent mitochondrial pathway (18, 20). NRAGE also interacts with inhibitors of apoptosis proteins (21) and the axon guidance receptor UNC5H which mediates apoptosis (22). Dlxin-1, a mouse homolog of MAGE-D1, interacts with Dlx/Msx homeodomain proteins (23) and Ror receptor kinases (24). Because necdin forms a homodimer (16), it seems likely that necdin and MAGE-D1 form a complex via their MHDs, and that these two proteins cooperate to modulate the activities of MAGE-D1 interactors.

In this study, we attempted to examine the association between necdin and MAGE-D1 in vivo and in vitro. We demonstrate that necdin interacts with Msx homeodomain proteins via MAGE-D1, releases MxA-dependent transcriptional repression, and promotes muscle differentiation by canceling Msx-induced repression. These indicate that two types of MAGE proteins cooperate to regulate the function of Msx homeodomain proteins in cellular differentiation.

EXPERIMENTAL PROCEDURES

Immunoblotting—Tissues of ICR mouse embryos were homogenized with a lysis buffer containing 10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 5 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, and 1× protease inhibitor mixture (Complete, Roche Applied Science) and ul-

1 The abbreviations used are: MHD, MAGE homology domain; MBP, maltose-binding protein; GST, glutathione S-transferase; MHC, myosin heavy chain; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; E, embryonic day.
transiently transfected at 100,000 × g for 1 h at 4°C to obtain the supernatant. C2C12, COS-7, and N1E-115 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, harvested, and suspended in the lysis buffer. P19 embryonal carcinoma cells were cultured and induced to differentiate as described (25). The equal amounts of proteins (20 μg) were separated by 10% SDS-PAGE, blotted onto Immobilon membrane (Millipore), and incubated with anti-MAGE-D1 (amino acids 1–106) or anti-necdin antibody (NC243) (26). Anti-mouse MAGE-D1 polyclonal antibody was raised in New Zealand rabbit against purified maltose-binding protein (MBP)-MAGE-D1 (amino acids 1–775) fusion protein. After incubation with peroxidase-conjugated anti-rabbit IgG (Cappel), necdin and MAGE-D1 were detected by the chemiluminescence method (Renaissance, PerkinElmer Life Sciences). The protein concentration was determined by the Bradford method (Bio-Rad).

Fluorescence Immunohistochemistry—Tissues in ICR mice at E12.5 and E14.5 were fixed by transcardial perfusion of 4% paraformaldehyde solution in phosphate buffer (pH 7.4). The whole embryos were immersed in the same fixative followed by cryoprotection with 20% sucrose and cut at a thickness of 20 μm by a cryostat. The sections mounted on glass slides coated with gelatin were incubated with anti-necdin antibody (NC243) (1:500) and anti-MAGE-D1 antibody (1:300) in PBS containing 0.05% Tween 20 and 5% normal goat serum at room temperature for 3 h. The tissues were then incubated at room temperature for 2 h with anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:500) (Cappel). Fluorescent images were observed with a fluorescence microscope (BX60–FLA1, Olympus), taken by CCD camera system (MICRO1, Olympus), and processed using Adobe Photoshop 5.0 software.

In Vitro Binding—cDNAs encoding MAGE-D1 (amino acids 1–775) and MAGE-D1 deletion mutants were generated by PCR using synthetic oligonucleotide primers and inserted directionally into pMALC2 (New England Biolabs). MBP-MAGE-D1 fusion proteins were affinity-purified with amylose resin (New England Biolabs). Purified fusion proteins (5 μg) bound to amylose resin (40 μl) were incubated with purified His-tagged necdin (amino acids 1–325) (50 ng) at 4°C for 30 min in 0.5 ml of the binding buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM EDTA (10), and the bound proteins were eluted with 20 mM maltose. His-tagged necdin was separated by 10% SDS-PAGE, blotted, and detected with an anti-necdin antibody (C2) (1:1,000) (1) and peroxidase-conjugated goat anti-rabbit IgG (Cappel) by the chemiluminescence method. For detection of the complex of MAGE-D1 and Msx, cDNAs encoding mouse Msx1 (amino acids 1–297) (a gift from Dr. C. Abate-Shen, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School) and Msx2 (amino acids 1–267), which was isolated from the library of neurally differentiated P19 embryonal carcinoma cells, were cloned into pGEX-5X-1 (Amersham Life Sciences) to produce glutathione S-transferase-MAGE-D1 (GST–MAGE-D1) fusion proteins. MBP-MAGE-D1 fusion proteins (5 μg) bound to amylose resin (40 μl) were incubated with GST-Msx1 or GST-Msx2 fusion proteins (1 μg) at 4°C for 30 min in 0.5 ml of the binding buffer. Bound proteins were eluted and detected by Western blotting using an anti-GST antibody (1:2,000) (Sigma) as above.

Co-immunoprecipitation—cDNAs encoding Myc-tagged MAGE-D1 and its deletion mutants were subcloned into pcDNA3.1 (Invitrogen) as described previously (10). Combinations of the expression vectors p3xFLAG-necdin (amino acids 1–325), Myc-tagged MAGE-D1, and Myc-tagged MAGE-D1 deletion mutants were transfected into COS-7 cells 48 h after transfection. Cell extracts (400 μg) were incubated at 4°C for 2 h with an anti-Myc antibody (9E10) (1:5) or antibody NC243 (1:100) in 200 μl of a lysis buffer containing 10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, and 1× protease inhibitor mixture. The complexes were pelleted with protein A-Sepharose (Amersham Biosciences), separated by 10% SDS-PAGE, transferred to Immobilon membrane, and blotted with antibody 9E10 (1:10) or antibody NC243 (1:3,000) and peroxidase-conjugated goat anti-rabbit IgG (Cappel) or peroxidase-conjugated goat anti-mouse IgG (Cappel). For detection of the complexes containing Max1 (or Max2), cDNAs encoding Max1 and Max2 were inserted into p3xFLAG expression vector (Sigma). Combinations of the expression vectors encoding FLAG-tagged Max1 and Max2 were transfected into COS-7 cells, and cell extracts containing complexes were immunoprecipitated and detected with anti-FLAG antibody M2 and anti-MAGE-D1 antibody. For analysis of the ternary complex of necdin, MAGE-D1, and Max1 (Max2), combinations of expression vectors for FLAG-tagged necdin, Myc-tagged MAGE-D1, and Myc-tagged Max1 (Max2) were transfected into COS-7. The complex was precipitated with antibody NC243 (1:100) and detected with antibody 9E10 (1:10) as above.

Immunoadfinity Purification—To detect the complex containing necdin, MAGE-D1, and Max2, P19 embryonal carcinoma cells were cultured and induced to differentiate by retinoic acid treatment for 4 days as described previously (25). Cell extracts were applied to HiTrap N-Hydroxysuccinimide-activated affinity column (Amersham Biosciences) coupled with IgG fractions of anti-necdin (NC243) antibody, anti-MAGE-D1 antibody, or premuamine antiserum. Bound proteins were eluted with 2 μl glycine-HCl (pH 2.5). Fractions were precipitated with 10% trichloroacetic acid, rinsed with cold acetone, separated by 10% SDSPAGE, and immunoblotted with antibody NC243 (1:3,000), anti-MAGE-D1 antibody (1:1,000), and anti-Msx2 polyclonal antibody (1:1,000) (Sigma). Integrated signal intensities were quantified as described previously (25). The bands were visualized with an ultraviolet camera system (LAS-1000, FujiFilm).

Mdx-dependent Transcription—N1E-115 neuroblastoma cells were cultured and transfected as described (11). For Gal4-mediated luciferase assay, cDNAs encoding Max1 (amino acids 1–297) and Max2 (amino acids 1–267) were inserted into PBIND (Promega) to make Gal4-Max1 (PBIND-Max1) and Gal4-Max2 (PBIND-Max2), respectively. Combinations of PBIND-Max1, PBIND-Max2, Myc-MAGE-D1, and FLAG-necdin were transfected into N1E-115 cells along with pG5Luc reporter vector (Promega). For Wnt1 enhancer luciferase reporter assay, combinations of expression vectors encoding FLAG-Max1 (FLAG-Max2), Myc-MAGE-D1, and FLAG-necdin were infected into N1E-115 cells along with the Wnt1 luciferase reporter construct (pGL2-WIP) (provided by Dr. C. Abate-Shen) (27). Luciferase activity was measured with a luminometer (Lumat LB9501, Berthold) using a reagent kit (Toyo Ink, Tokyo, Japan). Transfection efficiency was normalized with co-transfected LacZ reporter plasmid (pRL-CMV).

Immunocytochemistry—C2C12 myoblast cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum as a growth medium. p3xFLAG-Msx2 and p3xFLAG (4 μg each) were transfected into C2C12 cells by LipofectAMINE Plus reagent (Invitrogen). G418-resistant colonies were obtained after a 14-day incubation. C2C12 cells expressing FLAG-Msx2 and FLAG were subcultured in the growth medium for 7–14 days. Myogenic differentiation was initiated by incubating confluent cloned cells for 3 days in Dulbecco’s modified Eagle’s medium containing 2% horse serum as a differentiation medium (28). Cells were fixed with 4% formaldehyde in PBS (pH 7.4) at 4°C for 20 min and permeabilized with methanol/acetone (1:1) at −20°C for 20 min. Fixed cells were incubated with anti-FLAG M2 antibody (1:300) and an antibody against MHC (MF-20) (1:2) (a gift from Dr. T. Komiya, Osaka City University) in PBS containing 0.05% Tween 20 and 5% normal goat serum at 4°C overnight. The cells were then incubated at room temperature for 120 min with anti-mouse IgG conjugated with fluorescein isothiocyanate (1:500), treated with 3.3 μg Hoechst 33342 for chromosomal DNA detections, and observed with the fluorescence microscope.

Recombinant Adenoviruses—Adenoviruses expressing LacZ, necdin (amino acids 1–325), and Myc-tagged MAGE-D1 (amino acids 1–775) were constructed using AdEasy adenoviral system (Strategene). cDNAs encoding LacZ, necdin, and Myc-MAGE-D1 were inserted into pShuttle-CMV, amplified, and purified according to the manufacturer’s instructions. Cloned C2C12 cells were infected with each adenovirus at 1.5 × 108 plaque-forming units/ml and cultured in the growth medium for 24 h and then in differentiation medium for 72 h. Efficiency of muscle differentiation was judged by MHC expression with antibody MF-20 (1:2) and MyoD (M-318, Santa Cruz Biotechnology) (1:500). MHC expression levels relative to tubulin expression levels were quantified with the image analyzer.

RESULTS

Interactions between Necdin and MAGE-D1 in Vivo and in Vitro—We first analyzed the expression patterns of necdin and MAGE-D1 in mouse embryo by Western blotting and immunohistochemistry (Fig. 1). Endogenous ~43-kDa necdin protein was detected almost exclusively in the brain and skeletal muscle at embryonic day (E) 18.5 (Fig. 1A). In contrast, endogenous ~85-kDa MAGE-D1 protein was expressed in a ubiquitous manner, although the levels in the brain, skeletal muscle, heart, lung, and kidney were higher than those in the small intestine and liver. In various cell lines, necdin was detected only in neurally differentiated P19 embryonal carcinoma cells, whereas MAGE-D1 was expressed in C2C12 myoblasts, COS-7 kidney cells, N1E-115 neuroblastoma cells, and P19
cells (Fig. 1B). Immunohistochemistry revealed that necdin and MAGE-D1 were concentrated in the preplate (i.e. a structure composed of early differentiated postmitotic cells) of the forebrain at E12.5 and skeletal muscle tissues in the hind limb at E14.5 (Fig. 1C). In developing neural tube, MAGE-D1 immunoreactivity was distributed in the ventricular zone containing a mixed population of neuroepithelial stem cells and neuronal progenitors as well as in postmitotic cells at the marginal zone. Because expression of necdin and MAGE-D1 overlapped in these regions, we speculated that necdin and MAGE-D1 potentially form a heterodimer in vivo.

Interactions between Necdin and MAGE-D1—To investigate whether necdin and MAGE-D1 form a heterodimer in vitro, we constructed various MAGE-D1 deletion mutants fused to MBP and examined their interactions with His-tagged necdin (Fig. 2, A–C). Full-length MAGE-D1 (amino acids 1–775) and deletion mutants encompassing the MHD (amino acids 466–775 and 553–688) interacted with His-tagged necdin, indicating that necdin and MAGE-D1 form a heterodimer via the MHD. We then examined the interaction between necdin and MAGE-D1 in vivo by co-immunoprecipitation analysis (Fig. 2, D and E). Full-length MAGE-D1 and MAGE-D1ΔN was co-immunoprecipitated with necdin, but MAGE-D1ΔC failed to bind necdin (Fig. 2E, upper panel). Necdin was also co-immunoprecipitated with MAGE-D1 (Fig. 2E, lower panel). These results together suggest that necdin complexes with MAGE-D1 in vitro and in vivo.

Interactions of MAGE-D1 with Msx1 and Msx2—Because MAGE-D1 (Dlxin-1) possesses a specific domain that interacts with Dlx/Msx family homeodomain proteins (23), we examined whether MAGE-D1 interacts in vitro with Msx1 and Msx2 (Fig. 3, A–C). Msx1 and Msx2 bound to MBP-MAGE-D1 fusion pro-
proteins containing the WQXPXX repeats (amino acids 1–775, 1–479, and 291–441), but the deletion mutants lacking the repeat domain (amino acids 466–775 and 1–291) failed to interact with Mxs1 and Mxs2. We next examined whether MAGE-D1 binds to Mxs1 and Mxs2 in vitro by co-immunoprecipitation assay using COS-7 cells, which express endogenous
MAGE-D1 (Fig. 3D). FLAG-Msx1 and FLAG-Msx2 were co-immunoprecipitated with endogenous MAGE-D1. Conversely, endogenous MAGE-D1 was co-immunoprecipitated with FLAG-Msx1 and FLAG-Msx2. These data indicate that MAGE-D1 directly binds to Msx1 and Msx2.

Formation of a Complex Containing Necdin, MAGE-D1, and Msx—We next examined the formation of a complex containing necdin, MAGE-D1, and Msx in vitro by co-immunoprecipitation assay (Fig. 4). Msx1 was co-immunoprecipitated with necdin in the presence and absence of MAGE-D1 (Fig. 4A). The complex formation between necdin and Msx1 in the absence of MAGE-D1 is likely because of the presence of endogenous MAGE-D1 in COS-7 cells. Similarly, Msx2 was co-immunoprecipitated with necdin (Fig. 4B). Neither Myc-Msx1 nor Myc-Msx2 was co-immunoprecipitated with a necdin deletion mutant (necdinΔ243–306), which was defective in MAGE-D1 binding activity, when co-expressed with MAGE-D1 in this assay system (data not shown). These results suggest that necdin interacts with Msx1 and Msx2 via MAGE-D1.

We then attempted to detect an endogenous complex containing necdin, MAGE-D1, and Msx2 in tissue lysates by immunoprecipitation. However, we were unable to find normal tissues or primary cells that contain high levels of Msx and necdin for biochemical analyses. Furthermore, we failed to detect the Msx protein in skeletal muscle tissues of the hind limb at E14.5 and E18.5 by Western blotting (data not shown).
We alternatively used P19 embryonal carcinoma cells, whose endogenous Msx2 expression is up-regulated upon cell aggregation (29). Expression of necdin and Msx2 was up-regulated in retinoic acid-treated P19 cells, but MAGE-D1 expression levels remained almost unchanged during the course of neural differentiation (Fig. 4C). For detection of the endogenous complex, extracts of retinoic acid-treated P19 cells were subjected to the immunoaffinity purification using antibodies against necdin.
and MAGE-D1 (Fig. 4, D and E). Both MAGE-D1 and Mxs2 bound to immunopurified necdn, and both necdn and Mxs2 bound to immunopurified MAGE-D1. About 2% of the amounts of necdn and MAGE-D1 in the lysate were recovered after immunoaffinity purification. Under these conditions, 0.9 and 0.3% of the amounts of Mxs2 and MAGE-D1, respectively, in the lysate bound to necdn, whereas 0.7 and 0.5% of the amounts of Mxs2 and necdn, respectively, in the lysate bound to MAGE-D1. These results suggest that the endogenous complex containing necdn, MAGE-D1, and Mxs2 is present in neurally differentiating P19 cells in vivo.

Release from Mxs-dependent Transcriptional Repression by Necdn and MAGE-D1—Because Mxs1 and Mxs2 are potent repressors of the Wnt1 promoter (30), we examined whether a complex of necdn and MAGE-D1 modulates the Mxs-dependent transcriptional activity (Fig. 5). We carried out the Mxs-dependent transcriptional assay in N1E-115 neuroblastoma cells, which express endogenous MAGE-D1 but not necdn as shown in Fig. 1B. Transfection of expression vectors for Gal4-Mxs1 and Gal4-Mxs2 resulted in the repression (44 and 32% of control values, respectively) of GAL4 site-containing reporter, whereas co-transfection with necdn and MAGE-D1 released the repression to 95 and 97% of the control levels, respectively (Fig. 5A). Necdn alone released the repression by Gal4-Mxs1 and Gal4-Mxs2 to 70 and 80%, respectively, presumably due to endogenous MAGE-D1 present in N1E-115 cells. We next examined the effects of these proteins on the transcription of the Wnt1 promoter (WIP) containing the Mxs-binding site (30) (Fig. 5B). Mxs1 and Mxs2 also repressed the transcription of the WIP promoter (41 and 30% of the control level, respectively), whereas the activity was recovered to 80 and 67% of the control level, respectively, by co-expression of necdn and MAGE-D1. Necdn alone released the repression by Mxs1 and Mxs2 to 65 and 50%, respectively. These observations indicate that necdn releases Mxs-dependent transcriptional repression via MAGE-D1.

Release from Mxs2-induced Myogenic Repression by Necdn and MAGE-D1—Because Mxs2 inhibits myogenic differentiation of C2C12 myoblasts (28, 31), we used this cell line as a model system to examine the effects of necdn and MAGE-D1 on Mxs2-dependent myogenic repression. We established C2C12 myoblast cells that stably express FLAG-Mxs2. In wild-type C2C12 cells, MHC (i.e. a marker for skeletal muscle differentiation) and MAGE-D1 were distributed predominantly in the cytosol of multinucleated differentiated C2C12 cells (Fig. 6A). Western blot analysis revealed that differentiated C2C12 cells expressed MHC and MAGE-D1, whereas necdn was undetected in C2C12 cells even under differentiated conditions (Fig. 6B). We then analyzed myogenic differentiation of FLAG-Mxs2-expressing C2C12 clones. Under differentiation conditions, only a few MHC-positive mononucleated cells appeared in Mxs2-expressing C2C12 cultures, indicating an impairment in myotube formation (Fig. 6C). C2C12 cells expressing FLAG-Mxs2 differentiated into myocytes at much lower efficiency than control C2C12 cells as judged by MHC expression levels (Fig. 6D).

We then examined the effects of necdn and MAGE-D1 on myogenic differentiation of Mxs2-expressing C2C12 cells (Fig. 7). Expression of necdn by adenovirus vector-mediated gene transfer up-regulated MHC expression and promoted myotube formation, whereas infection of adenoviruses expressing LacZ and MAGE-D1 had little effect on MHC expression (Fig. 7A). Necdn and MAGE-D1 increased MHC expression ~9 times the control level (Fig. 7B, C). Necdn alone up-regulated the MHC expression 3 times the control level, presumably because of the presence of endogenous MAGE-D1. Similarly, necdn and MAGE-D1 up-regulated the expression of the myogenic transcription factor MyoD ~5 times the control level, and necdn alone increased the MyoD level ~3 times the control level (Fig. 7B). The complex of necdn, MAGE-D1, and Mxs2 in the nuclear fraction was detected by co-immunoprecipitation assay (Fig. 7D). Co-expression of necdn and Myc-tagged MAGE-D1 formed the complex efficiently. This may be because exogenous MAGE-D1, when co-expressed with necdn, tends to accumulate preferentially in the nucleus. Expression of MAGE-D1,
necdin, or both in control C2C12 cells, which express no endogenous Msx2, failed to promote the myogenesis (data not shown). These data indicate that necdin and MAGE-D1 released Msx2-induced myogenic repression through formation of the complex with Msx2.

**DISCUSSION**

The present study has shown that MAGE family members cooperate to modulate the function of Msx homeodomain proteins, which are key transcription factors for cellular differentiation (32). The MAGE family proteins are grouped into two types based on their sequence similarities in the MHDs (33). Type I MAGE members (MAGE-A–C subfamilies) are expressed in undifferentiated cells such as transformed cells and testicular germ cells, whereas type II MAGE members are expressed in differentiated cells such as neurons. Necdin and MAGE-D1, whose functions have been best documented among MAGE proteins, are classified as type II MAGE proteins. Type II MAGE proteins may be divided further into two subgroups based on the similarities in the MHD sequences and molecular sizes; A subgroup (“necdin subgroup”) of type II MAGE proteins consists of relatively short proteins (<350 amino acid residues) whose MHD sequences are more homologous to that of necdin. This subgroup includes necdin, MAGE-F1, G1, and H1 in mammals, *Drosophila* MAGE (34), and zebrafish MAGE (35). The other subgroup (“MAGE-D subgroup”) consists of larger proteins (>650 amino acid residues) bearing N-terminal extensions. This subgroup includes MAGE-D1-D4, MAGE-E1 (DAMAGE) (36), and MAGEL2 (NDNL1). Earlier studies have shown that MAGE-D1 interacts, via its MHD, with the death domain receptors p75NTR (18) and UNC5H1 (22), the receptor tyrosine kinase Ror2 (24), and the RING finger protein Praja1 (37). The present study has shown that necdin is another MAGE-D1 MHD interactor. Because MAGE-D1 also associates with Msx/Dlx homeodomain proteins via the interspersed repeat domain district from the MHD (Ref 23 and present study), MAGE-D1 serves as an adapter protein that links these MHD interactors with Msx/Dlx homeodomain proteins.

Msx1 and Msx2 (previous Hox-7 and Hox-8, respectively) are

![Figure 6](http://www.jbc.org/)
two homologs of *Drosophila* muscle segment homeobox (*msh*) genes that are expressed in mouse embryos at critical stages of neural tube, neural crest, and craniofacial development (32). Therefore, Msx1 and Msx2 are thought to play important roles in organogenesis and cellular differentiation. For example, forced expression of Msx1 in myoblasts blocks terminal differentiation, and Msx1-expressing cells acquire a transformed phenotype (38). Msx1 inhibits transcription of the myogenic transcription factor MyoD, which is a target for homeobox gene regulation (39). Msx proteins serve as transcriptional repressors and negative regulators of differentiation by preventing cell cycle exit and blocking terminal differentiation of mesenchymal progenitor cells (28). Furthermore, Msx2 prevents differentiation and stimulates cell proliferation at the extreme ends of osteogenesis (40). These findings suggest that Msx homeodomain proteins generally repress terminal differentiation of mesenchymal cells. The present findings showed that necdin releases Msx2-induced repression of myogenic differentiation in C2C12 myoblasts (Fig. 7). This supports the notion that necdin promotes terminal differentiation of postmitotic
MAGE proteins form a heteromeric complex developing mouse brain. These observations suggest that these plate, which consists of early differentiated postmitotic cells, in overlapping expression of necdin and MAGE-D1 in the pre­

present study, we confirmed that MAGE-D1 is expressed in ral progenitors and young postmitotic neurons (18, 42). In the postmitotic neurons, whereas MAGE-D1 is in proliferative neu­

eration of mesoangioblast stem cells. Furthermore, these raise MAGE-D1, cooperate to promote the smooth muscle differenti­

Msx2 and necdin, presumably in the presence of endogenous (41) that Msx2 and necdin combined activities are required for

interactions of MAGE proteins with Msx/Dlx homeodo­

gic neurons (44). These observations suggest that the complex expression of Dlx2 and Dlx5 induces the phenotype of GABAer­

In the nervous system, necdin is expressed predominantly in

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Acknowledgments—We thank Dr. C. Abate-Shen for the generous provision of research materials and Drs. M. Niinobe and T. Uetsuki for helpful discussions.
