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Functional Domains of Necdin for Protein–Protein Interaction, Nuclear Matrix Targeting, and Cell Growth Suppression

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Abstract Necdin is a growth suppressor expressed predominantly in postmitotic neurons. The necdin gene is involved in the etiology of the genomic imprinting-associated neurodevelopmental disorder Prader–Willi syndrome and belongs to the MAGE gene family. All the MAGE family proteins contain a large homology domain termed the MAGE homology domain (MHD). We here characterize the regions of necdin required for the protein–protein interaction, nuclear matrix targeting, and cell growth suppression. The region including entire MHD (amino acids 116–280) of necdin was required for its interaction with p53, while the regions amino acids 144–184 and 191–222 within the MHD were required for both the nuclear matrix targeting and the cell growth suppression of osteosarcoma SAOS-2 cells. The amino-terminal proline-rich acidic region (amino acids 60–100) was also necessary for cell growth suppression. Tetracycline-regulatable overexpression of necdin induced growth arrest of SAOS-2 cells in a reversible manner, and the necdin-overexpressing cells showed a large, flattened morphology with double nuclei. In contrast, a necdin mutant lacking amino acids 191–222 did not induce such changes. These findings suggest that different functions of necdin are mediated via its distinct domains. J. Cell. Biochem. 94: 804–815, 2005.

Key words: MAGE homology domain; nuclear matrix; growth arrest; Prader–Willi syndrome

Necdin is identified as a neural differentiation-induced gene in embryonal carcinoma P19 cells [Maruyama et al., 1991]. The mouse necdin gene is highly expressed in postmitotic neurons from the early brain development [Uetsuki et al., 1996]. The human necdin gene is mapped to chromosome 15q11-q12, a region deleted in Prader–Willi syndrome (PWS) [Jay et al., 1997; MacDonald and Wevrick, 1997; Nakada et al., 1998]. PWS is a genomic imprinting-associated neurodevelopmental disorder and its major symptoms such as feeding problems, gross obesity, and hypogonadism are consistent with abnormalities in hypothalamic neurons. The necdin gene is maternally imprinted, transcribed only from the paternal allele, and not expressed in individuals with PWS. Disruption of the mouse necdin gene results in early postnatal lethality, reduction in specific groups of hypothalamic neurons, and behavioral alterations, which are reminiscent of PWS [Gerard et al., 1999; Muscatelli et al., 2000]. The most necdin deficient mice die of apparent respiratory insufficiency caused by abnormal neuronal activity within the putative respiratory center [Ren et al., 2003]. These findings suggest that necdin plays roles in differentiation and development of subsets of neurons in the brain.

Abbreviations used: MHD, MAGE homology domain; PWS, Prader–Willi syndrome; p75NTR, p75 neurotrophin receptor; RA, retinoic acid; tet, tetracycline.

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Necdin belongs to the MAGE superfamily proteins. A group of MAGE genes such as MAGE-A, B, and C are classified as Type I MAGE proteins expressed in the tumor cells. In contrast, Type II MAGE proteins such as MAGE-D, E, F, G, H, and necdin are expressed in the differentiated cells. The MAGE homology domain (MHD) is a 160∼170 amino acid motif of shared sequence similarity among Type II MAGE gene family [Barker and Salehi, 2002]. Mouse and human necdins are 325 and 321 amino acid residue proteins, respectively [Nakada et al., 1998]. The amino-terminal region (amino acids 1–100 of mouse necdin) is highly acidic and proline-rich and is less conserved between mouse and human necdin sequences. The rest of the region (amino acids 101–325 of mouse necdin) including the MHD is highly conserved between mouse and human. We have reported that necdin expression induces cell growth suppression [Hayashi et al., 1995] and characterized the interactions of necdin with various cytoplasmic and nuclear proteins such as E2F1, p53, hnRNP U, NEFA, and p75NTR [Taniura et al., 1998, 1999; Taniguchi et al., 2000; Taniura and Yoshikawa, 2002; Kuwako et al., 2004]. Among the binding partners of necdin, p75NTR binds to necdin-homologous MAGE proteins, MAGE-H1, MAGE-G1, and NRAGE/Dlxin [Salehi et al., 2000; Teherpakov et al., 2002; Kuwako et al., 2004]. Because of the strong conservation of the MHD sequence, the MHD could be involved in the protein–protein interaction. Here, we characterize the domains of necdin involved in the protein–protein interaction, subcellular localization, and cell growth suppression by using various deletion mutants, and found different domains of necdin are required for these functions.

MATERIALS AND METHODS

Plasmid Construction

Deletion constructs of necdin representing necdin amino acids Δ60–100, Δ100–110, Δ110–140, Δ144–184, Δ191–222, and Δ273–306 were made by two-step PCR using the inside primers containing the each deletions for the first round of amplifications. The primary PCR products were annealed and the second round of PCR were done with the outside primers for the full-length coding sequence of necdin designed with *Eco*RI and XbaI sites at their 5′ or 3′ end, respectively. For NH_2-terminal deletion of necdin, amino acid 35–325, DNA sequence was PCR amplified using primers with *Eco*RI and XbaI sites. The amplified cDNAs were digested with *Eco*RI and XbaI, then cloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA). p73 cDNA was purchased from Riken Gene bank and subcloned into pcDNA 3.1.

Two-Hybrid Assays

For yeast two-hybrid assay, GAL4 DNA binding vector, pGBT9 and GAL4 activation domain vector, pGAD424 were purchased from Clontech Laboratories (Alto, CA). The cDNAs encoding various NH_2-terminal and COOH-terminal deleted fragments of necdin in pGBT9 were described previously [Taniura et al., 1998]. Deletion constructs of necdin for the two-hybrid assay representing necdin amino acids 83–325 Δ83–101, Δ83–109, Δ100–110, Δ110–140, Δ144–184, Δ191–222, and Δ273–306 were derived from each pcDNA3.1-necdin plasmid by using oligonucleotide primers, and were cloned into pGBT9. For interaction assay, various pGBT9 necdin constructs described above were co-transformed with pGAD424-p53 (amino acids 1–393) or pTD1 encoding SV40 large T antigen into yeast strain SFY526. Interactions were scored for β-galactosidase activity by a colony-lift assay. The reaction was evaluated 4 ranks with the time for appearance of blue colonies at 30 min, +++; less than 2 h, ++; 2–6 h, +; 6–12 h, −; white over 12 h.

For two-hybrid assay using SAOS-2 cells, pBIND, pACT, and pG5 (luciferase reporter containing 5× GAL4 DNA binding sequence) were purchased from Promega (Madison, WI). Deletion constructs of necdin representing amino acid Δ60–100, Δ100–110, and Δ110–140 were derived from each pcDNA3.1-necdin plasmid by using oligonucleotide primers, and were cloned into pBIND. Various pBIND-necdin constructs were co-transfected with pACT-p53 (amino acids 1–393) and pG5 into SAOS-2 cells. Transfected cells were lysed in Passive Lysis Buffer (Promega) and assayed for firefly and Renilla luciferase activities (Lumat LB9501, Berthold, Bad Wildbad, Germany). Statistical significance was tested using Student's test. Expression of the proteins transfected with the plasmids as indicated was tested by immunoblot using anti-necdin serum (1:3,000) [Niinobe et al., 2000] or anti-p53 antibody (1:1,000) (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation

Combinations of the expression vectors for Myc-tagged p53 and necdin deletion mutant, necdin, Δ1–34, Δ60–100, Δ100–110, Δ191–222, or Δ273–306 were transfected into SAOS-2 cells and harvested 48 h after transfection. Cell extracts (200 μg of protein) were incubated for 2 h at 4°C with anti-necdin antiserum (1:100) [Niinobe et al., 2000] in 200 μl of a lysis buffer containing 10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$,

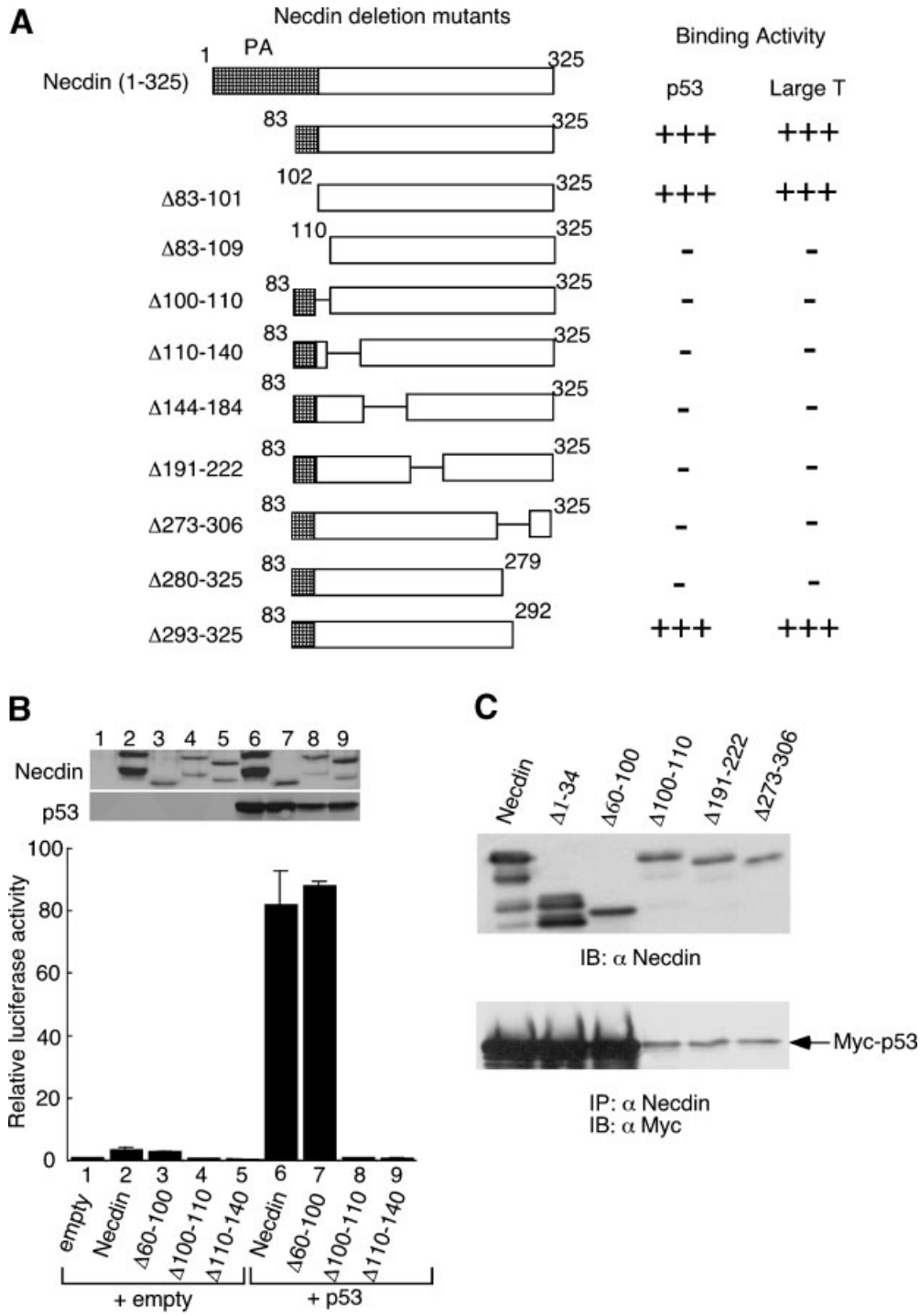


Fig. 1.

1 mM EGTA, 0.5% Triton X-100 and 1× protease inhibitor cocktail. The complexes were pelleted with protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ), separated by 10% SDS–PAGE, transferred to Immobilon membrane, and detected with anti-Myc antibody (9E10) (1:10).

Reporter Assay for p53-Driven Transactivation

Reporter assay for p53 or p73-driven transactivation using a 2.4-kilobase pair fragment of human p21/WAF1 promoter was described previously [Taniura et al., 1999]. p53 or p73 expression vector in combination with expression vector for various necdin deletion mutants were transfected into SAOS-2 cells for p53 and N1E-115 cells for p73 by the calcium phosphate method. Transfectants were harvested 36 h after transfection, and luciferase activities were measured with a luminometer. Transfection efficiency was normalized with co-transfected LacZ reporter plasmid. Statistical significance was tested using Student's test. Expression of the proteins transfected with the plasmids as indicated was tested by immunoblot using anti-necdin serum (1:3,000) [Niinobe et al., 2000], anti-Myc antibody (9E10) or anti-p73 antibody (1:500) (Santa Cruz Biotechnology).

Nuclear Matrix Preparation

The procedure used was essentially as described [He et al., 1990] for the preparation of RNA-containing nuclear matrix. For immunofluorescence studies, cells were grown on cover slips, washed in phosphate buffered saline (PBS) and extracted by adding and removing extraction solutions. For electrophoretic analyses, cells were extraction in suspension with centrifugation steps (600*g*, 3 min) between treatments. Cells were first extracted in cytoskeleton buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM vanadyl riboside complex) for 3 min at 4°C. DNA was digested with 25 U/ml RNase free DNaseI (RQ1, Life Technologies) for 50 min at 30°C in digestion buffer (10 mM PIPES pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM vanadyl riboside complex). Chromatin was then removed by three 10 min washes with 0.25M ammonium sulfate in digestion buffer to yield the nuclear matrix/intermediate filament structure. An additional high salt treatment was applied by washing the nuclear matrix with 2M NaCl in digestion buffer (three times for 5 min each). P19 embryonal carcinoma cells were cultured in minimum essential medium-alpha (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen). P19 cells were induced to differentiate by retinoic acid (RA) treatment as described previously [Taniguchi et al., 2000]. P19 cells were aggregated in bacteria-grade dishes in the presence of 5×10^{-7}M all-trans RA (Sigma) for 3 days. The aggregates were then trypsinized and plated into poly-L-lysine (Sigma) coated culture-grade dishes. The RA-treated cells were cultured in serum-free medium (Opti-MEM I, Life Technologies) for enriching the neuronal population for 4 days. For electrophoretic analyses, an equal aliquot of the extracts at each step was subjected to SDS–PAGE and immunoblotted with anti-necdin serum (1:3,000).

Fig. 1. Interaction between necdin and SV40 large T antigen or p53. **A**: Yeast two-hybrid assay. cDNAs for necdin and necdin deletion mutants were cloned into pGBT9 and cotransformed into yeast cells with pGAD424 carrying SV40 large T antigen or p53 cDNA. The β-galactosidase activities were semi-quantified as described under "Materials and Methods." Deletion constructs of necdin for the two-hybrid assay represent necdin amino acids 83–325, Δ83–101, Δ83–109, Δ100–110, Δ110–140, Δ144–184, Δ191–222, Δ273–306, Δ280–325, and Δ293–325. PA, a domain rich in proline and acidic amino acids. MHD, MAGE homology domain. **B**: Two-hybrid assay in SAOS-2 cells. cDNAs for necdin and necdin deletion mutants and p53 cDNA were cloned into pBIND and pACT, respectively. Combinations of expression vectors for necdin and p53 were transfected into SAOS-2 cells along with the reporter vector pG5, and cell lysates were assayed for firefly and Renilla luciferase activities. Transfection efficiency was normalized with Renilla luciferase activity. The total amount of plasmids was adjusted to 2 μg/assay by adding empty vector. Each value represents the mean ± SE (n = 3). The immunoblot analysis of cell lysates was also shown using anti-necdin serum or anti-p53 antibody. The lane numbers were corresponding to the lane numbers in the assay. **C**: Immunoprecipitation. SAOS-2 cells were transfected with expression vectors for necdin, Δ1–34, Δ60–110, Δ100–110, Δ191–222, or Δ273–306, and Myc-tagged p53 in combination. **Top**: Immunoblot to detect the expression of necdin, Δ1–34, Δ60–100, Δ100–110, Δ191–222, and Δ273–306 with anti-necdin serum (IB). **Bottom**: Cell lysates were immunoprecipitated with anti-necdin serum (IP) and immunoblotted with anti-Myc antibody (IB).

Colony Formation Assay

The colony formation assay using SAOS-2 cells was carried out as described previously [Taniura et al., 1998]. SAOS-2 cells grown in 35 mm dishes were transfected with the expression vectors encoding various necdin deletion mutants by the calcium phosphate method. G418 (500 μg/ml) was added to the culture medium 48 h after transfection. The cells were incubated for 14 days, fixed with 10% acetate/10% methanol for 15 min, and stained with 0.4% crystal violet in 20% ethanol for 15 min to visualize the colonies. Images were processed using Photoshop software (Adobe Systems, San Jose, CA), and the integrated density was quantified using the NIH image software.

Immunocytochemistry

SAOS-2 cells seeded onto glass cover slips were transfected with the expression plasmids encoding various necdin deletion mutants by the calcium phosphate method. The cells were fixed in 4% formaldehyde (pH 7.4) for 20 min at 4°C 48 h after transfection, and then permeabilized in methanol for 20 min at room temperature. For in situ isolation of nuclear matrix, the cells were extracted directly on the coverslips described above, and then fixed in 4% formaldehyde (pH 7.4). In all cases, the cover slips were incubated for 1 h at room temperature with anti-necdin serum (1:3,000) in 5% goat serum/PBS. Fluorescein-conjugated anti-rabbit antibodies (Cappel, Aurora, OH) were used for detection. The preparations were observed with a fluorescence microscope (BX 50-34-FLAD1, Olympus, Tokyo, Japan). The immunocytochemical staining for P19 cells was performed by the avidin–biotin peroxidase complex method (Vector laboratory, Burlingame, CA) using anti-necdin serum (1:3,000). Images were processed using Photoshop software.

Inducible Expression of Necdin in SAOS-2 Cells

Necdin and necdin Δ91–222 cDNAs were subcloned downstream of tetracycline (tet)-regulated promoter into pcDNA4/TO (Invitrogen). SAOS-2 cells were transfected with pcDNA4/TO-necdin or necdin Δ191–222 and pcDNA6/TR (tet repressor expression vector, Invitrogen), and doubly selected clonal lines in the presence of Zeocin and Blasticidin. Cells were cultured in the presence of 1 mg/ml tet to induce necdin or necdin Δ191–222. Cells were cultured in the presence or absence of tet and used for cell growth analysis, immunoblot analysis, and immunocytochemistry.

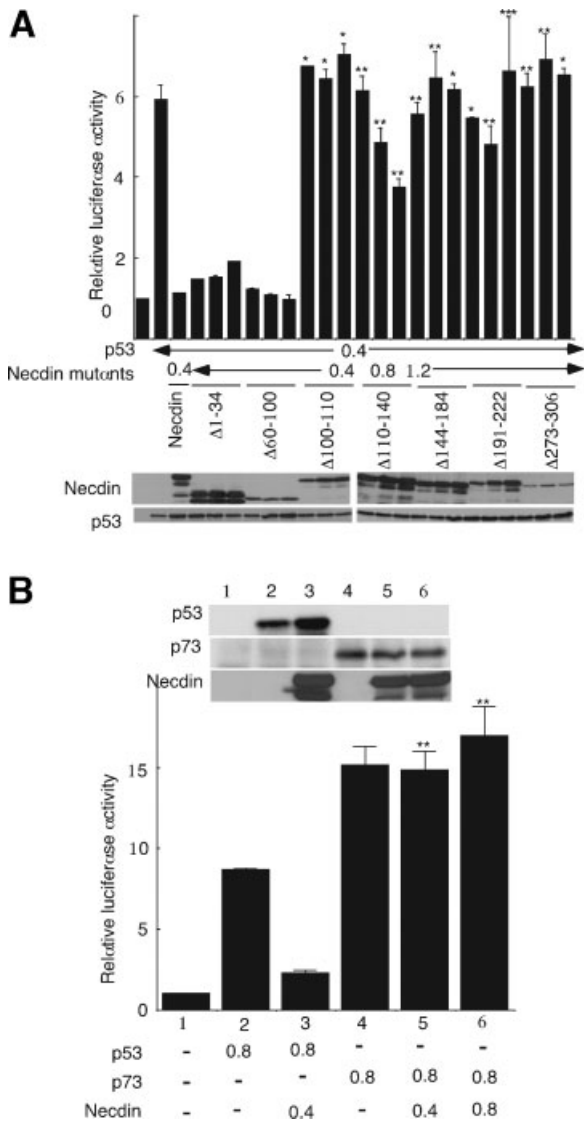


Fig. 2. Necdin suppresses the p53-driven, but not the p73-driven transcription. Combinations of expression vectors for necdin or necdin mutants and p53 (**A**) or p73 (**B**) were transfected into SAOS-2 cells (A) or N1E-115 cells (B) along with the reporter vector containing p21/WAF1 promoter. The amount of each plasmid is shown in μg/assay (0.4, 0.8, and 1.2). The total amount of plasmids was adjusted to 4 μg/assay by adding empty vector. Transfection efficiency was normalized with co-expressed β-galactosidase activity. Each value represents the mean ± SE (n = 3). *, **, and *** significant at <0.001, <0.01, and <0.05 compared with the value of the combination of full-length necdin and p53, respectively in A and B. The immunoblot analyses of cell lysates were also shown using anti-necdin, anti-Myc, or anti-p73 antibody. The lane numbers in B are corresponding to the lane numbers in the assay.

About 10 μg of cellular protein were subjected to 10% SDS–PAGE and immunoblotted with anti-necdin serum (1:3,000). Cells grown on coverslips were fixed and stained with anti-necdin serum and Hoechst dye 33342 (3.3 mM).

RESULTS

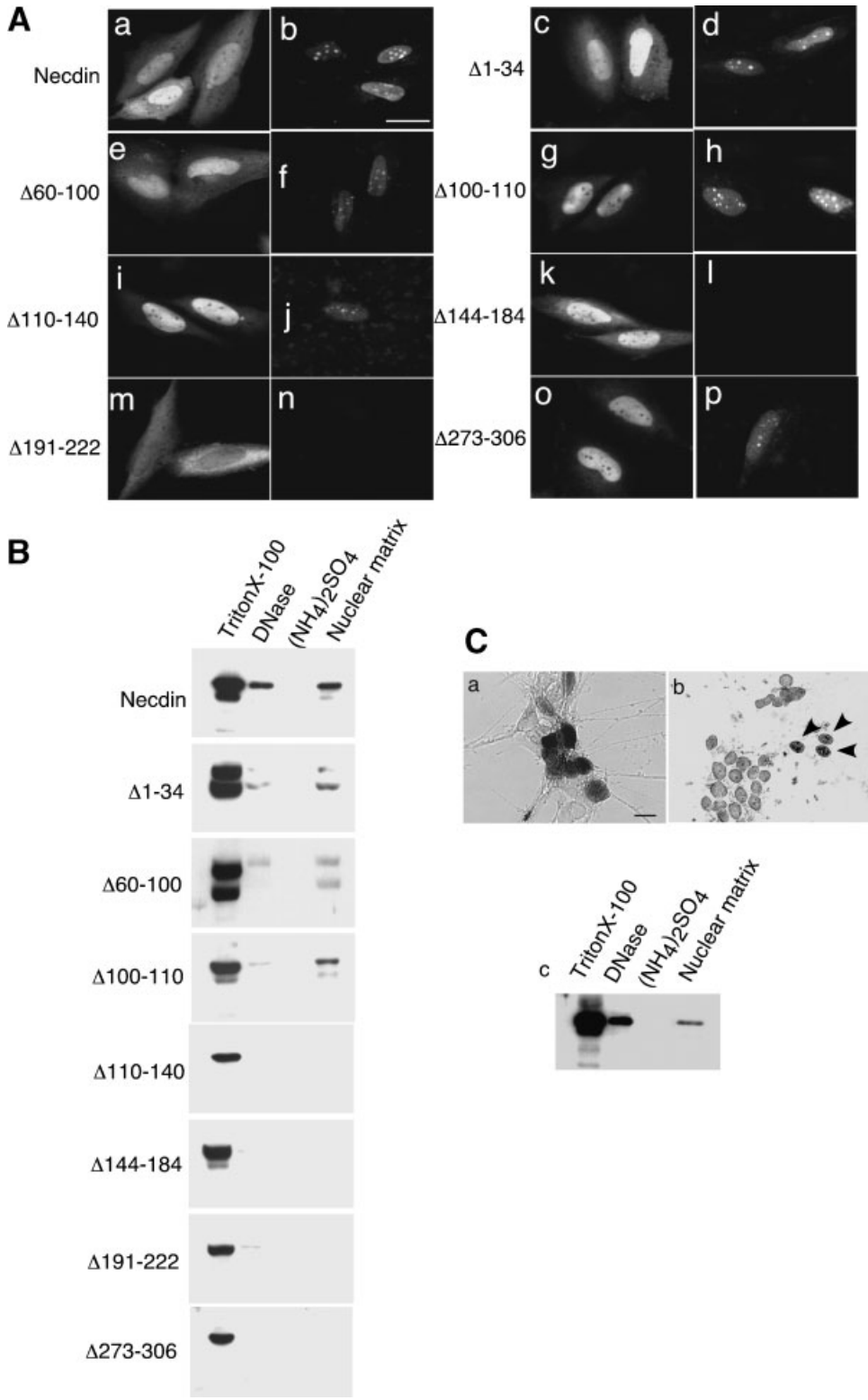
Determination of the Regions Required for the Protein–Protein Interaction

We have previously reported that the large T antigen and p53 bind to necdin [Taniura et al., 1998, 1999]. To clarify the regions of necdin necessary for the binding, we expressed various necdin deletion mutants as fusion products of GAL4 DNA binding domain in the yeast two-hybrid system (Fig. 1A). Because full-length necdin has a pseudo-positive activity [Taniura et al., 1998], we generated the necdin mutants on the basis of necdin amino acids 83–325. NH_2-terminal truncations of both necdin (amino acids 83–325) and necdin (amino acids 102–325) strongly bound to p53 and the large T antigen, but a necdin mutant (amino acids 110–325) failed to interact. On the other hand, COOH-terminal truncation of necdin (amino acids 83–292) retained the ability to bind to these proteins, but further deletion of necdin (amino acids 83–279) eliminated the activity. Furthermore, internally deleted constructs of necdin (amino acids 83–325 Δ100–110, Δ110–140, Δ144–184, Δ191–222, and Δ273–306) also resulted in a failure of binding. We also tested the binding activity of several necdin deletion mutants expressed in SAOS-2 cells by the two-hybrid assay (Fig. 1B). Full-length necdin expression increased the luciferase activity about 3-fold and the combination of necdin and p53 increased about 80-fold as compared with that of empty vectors alone. A mutant Δ60–100 deleted in the NH_2-terminal region retained the same activity, but mutants Δ100–110 and Δ110–140 deleted adjacent or within MHD (amino acids 116–280) lost the activities. To confirm the requirement of the MHD for interaction between necdin and p53, necdin, Δ1–34, Δ60–100, Δ100–110, Δ191–222, or Δ273–306 was co-expressed with Myc-tagged p53 in SAOS-2 cells (Fig. 1C). Myc-tagged p53 was co-immunoprecipitated efficiently with full-length necdin, Δ1–34 or Δ60–100 compared with Δ100–110, Δ191–222, or Δ273–306. These data suggest that the region including entire MHD of necdin is indispensable for the protein–protein interactions.

Necdin binds to the NH_2-terminal activation domain of p53 and leads to the suppression of the p53-driven transcriptional activity [Taniura et al., 1999]. We transfected full-length and series of necdin mutants with p53 into SAOS-2 cells with a luciferase reporter vector driven by the p21/WAF1 promoter. As shown in Figure 2A, the p53-stimulated activity was suppressed by full-length necdin. Necdin Δ1–34 and Δ60–100 also suppressed the activity, whereas other deletions Δ100–110, Δ110–140, Δ144–184, Δ191–222, and Δ273–306) adjacent or within the MHD had no suppressive effect. These results are consistent with those of the binding activity of necdin to p53. Recently, several members of the p53 family have been identified. To test the specificity of necdin–p53 interaction, we transfected full-length necdin and p73, a member of p53 family, into N1E-115 neuroblastoma cells with a luciferase reporter vector containing the p21/WAF1 promoter. As shown in Figure 2B, necdin suppressed the activity of p53, but had no effects on p73-driven activity.

Regulation of the Subcellular Distribution of Necdin

Necdin is localized to both the nucleus and the cytoplasm, and a fraction is also localized in the nuclear matrix [Taniura and Yoshikawa, 2002; Kuwako et al., 2004]. We then determined which regions of necdin are responsible for the differential distribution. SAOS-2 cells were transfected with cDNAs for a series of necdin deletion mutants, and indirect immunofluorescence was observed using anti-necdin serum. Expression of all the necdin protein resulted in the distribution of diffuse fluorescence throughout the cells, but relatively abundant in the nucleus except Δ191–222 (Fig. 3A (a,c,e,g,i,k,m,o)). Expression of necdin Δ191–222 showed a variable distribution in the cytoplasm and nucleus (Fig. 3A(m)). Although the necdin sequence do not contain any NLS consensus, the region amino acids 191–222 may mediate to confer nuclear localization. After cells were treated by sequential extraction to remove soluble nuclear components, necdin immunoreactivity remained as nuclear speckles, and a low level of nucleoplasmic staining was also observed. A series of necdin deletions demonstrated that the regions amino acids 1–34, 60–100, 100–110, and 273–306 were not


A
a
b
Necdin
c
d
Δ1-34
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f
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n
Δ191-222
o
p
Δ273-306
B
TritonX-100
DNase
(NH4)2SO4
Nuclear matrix
Necdin
Δ1-34
Δ60-100
Δ100-110
Δ110-140
Δ144-184
Δ191-222
Δ273-306
C
a
b
c
TritonX-100
DNase
(NH4)2SO4
Nuclear matrix

Fig. 3.

required for the nuclear matrix association (Fig. 3A (b,d,f,h)), but the speckles of both necdin Δ110–140 and Δ273–306 were less apparent (Fig. 3A (j,p)). By contrast, fluorescence of both necdin Δ144–184 and Δ191–222 were completely removed after the extraction (Fig. 3A(l,n)). As shown in Figure 4B, ~20% of the expressed necdin protein was also detected in immunoblot analysis of the nuclear matrix preparations of transfected SAOS-2 cells. We could not see the speckled distribution of necdin protein in whole cell preparation because of only minor fraction of total necdin protein ditributed in the nuclear matrix. We observed the speckled structure with or without final high salt extraction. Consistent with the results of the immunofluorescence analysis, necdins Δ1–34, Δ60–100, and Δ100–110 were also found in the insoluble nuclear matrix pellet, but necdins Δ110–140 and Δ273–306 were not. We also found that necdins Δ144–184 and Δ191–222 were released with the extraction. The immunoblot seems to be less sensitive compared with the detection by immunocytochemistry, because the necdin protein was highly concentrated in the nuclear speckles. The regions amino acids 110–140 and 273–306 are just adjacent to the critical region amino acids 144–222 and may influence the nuclear matrix targeting. Taken together, these data suggest that a part of the necdin protein tightly associates with the nuclear matrix, and the regions of amino acids 144–184 and 191–222 are required for targeting the nuclear matrix. We also examined subcellular localization of necdin proteins in neurally differentiated P19 cells (Fig. 3C). By indirect immunostaining, necdin showed a nucleoplasmic staining accompanied by several speckled structures after extraction (Fig. 3C(b)). Although most of the necdin protein was released in the Triton X-100 extraction, less than 10% was found in the insoluble nuclear matrix pellet by immunoblotting (Fig. 3C).

Regions of Necdin Required for Cell Growth Suppression

We next examined the regions of necdin necessary for the cell growth suppression by the colony formation assay (Fig. 4A,B). Full-length necdin expression reduced the colony formation of SAOS-2 cells by 22%. Expression of necdin mutants Δ100–110, Δ110–140, and Δ273–306 showed a similar reduction of the colony formation to ~30% of the control level, while mutants Δ60–100, Δ144–184, and Δ191–222 showed less reduction to 60%–70% of the control level, suggesting that the NH_2-terminal proline-rich region amino acids 60–100, and amino acids 144–184 and 191–222 within the MHD are required for the cell growth suppression.

To confirm the requirement of the region of necdin for the cell growth suppression, we generated the tet-regulatable expression system. SAOS-2 cells, which contain the tet-repressor with a Blasticidin-resistant marker, were transfected with either expression vector encoding full-length necdin or necdin Δ191–222 under the tet-operator and a Zeocin-resistant marker. Stable clones were isolated by double selection. As shown in Figure 5A, within 1 day after adding 1 mg/ml tet in the medium, necdin and necdin Δ191–222 were induced and became readily detectable by immunoblot analysis. In response to necdin induction in SAOS-2 cells, profound alterations in both cell proliferative capacity and morphology were observed. Whereas SAOS-2 cells expressing necdin Δ191–222 grew in the presence of tet to an extent similar to that of the cells in the absence of tet, the induction of full-length necdin expression caused cells to stop growing almost completely

Fig. 3. Subcellular distribution of necdin mutants. **A**: In situ extraction of the nuclear matrix. SAOS-2 cells were transfected with expression vectors for necdin and necdin mutants as indicated. Cells were fixed and stained for necdin (**a**, **c**, **e**, **g**, **i**, **k**, **m**, and **o**). After cells were treated with in situ sequential extraction, the association of necdin with the nuclear matrix was detected by immunostaining for necdin (**b**, **d**, **f**, **h**, **j**, **l**, **n** and **p**). Scale bar, 20 μm in b. **B**: Immunoblot of sequential extraction to prepare the nuclear matrix. An equivalent aliquot of extracts at each step of the nuclear matrix preparation was analyzed by immunoblotting using anti-necdin serum. Lanes represent the proteins extracted with Triton X-100, DNase, 0.25M ammonium sulfate and the final nuclear pellet (nuclear matrix) after 2M NaCl wash. **C**: Endogenous necdin protein in the nuclear matrix. Neurally differentiated P19 cells were treated with in situ sequential extraction to prepare the nuclear matrix. The association of necdin with the nuclear matrix was detected by immunostaining using anti-necdin serum. Scale bar, 25 μm in **a**. (a, whole cell preparation and **b**, nuclear matrix preparation). Arrowheads in b point to speckled distribution of the necdin protein in neurally differentiated P19 cells. An equivalent aliquot of each step of the extraction to prepare the nuclear matrix from differentiated P19 cells was analyzed by immunoblotting using anti-necdin serum. Lanes represent the proteins extracted with Triton X-100, DNase, 0.25M ammonium sulfate and the final nuclear pellet (nuclear matrix) after 2M NaCl wash (**c**).

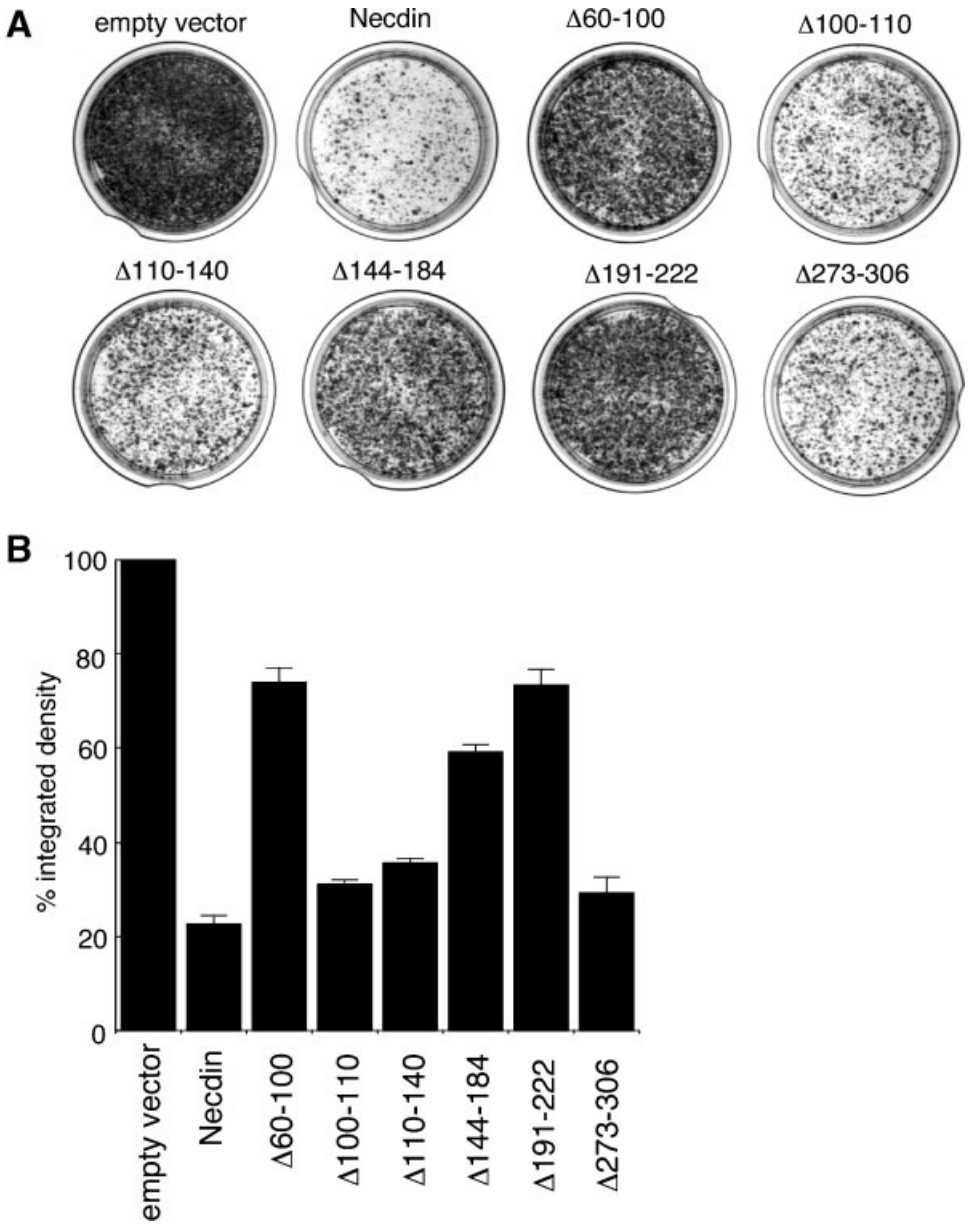

Fig. 4. Colony formation assay. **A**: Colony formation. SAOS-2 cells were transfected with empty vector or expression vectors for necdin and necdin mutants as indicated. Cells were cultured in the presence of G418 for 14 days, and the drug-resistant colonies were visualized by crystal violet staining. **B**: Quantification of the colony formation. Integrated densities of stained colonies of transfected SAOS-2 cells are presented. The mean values are presented (n = 3).

(Fig. 5B). To test whether necdin expression is reversibly controlled, tet was removed from the medium after induction for 2 days, and necdin protein levels were analyzed. The necdin expression returned to the basal level (compare lanes 1–2 and lanes 3–7 in Fig. 5C), indicating that necdin expression was fully reversible. To examine the reversibility of necdin-induced growth arrest in SAOS-2 cells, the cells were maintained in the presence of tet for 2 days and subsequently maintained for 5 days in the absence of tet. As shown in Figure 5D, induced expression of necdin in SAOS-2 cells caused growth arrest in a reversible manner. When the cells were examined microscopically by double-labeling with anti-necdin antibody and Hoechst dye 2, 4, and 6 days after necdin induction, we found that the cells expressing necdin had a large, flattened shape (Fig. 6). Most of these flat cells contained double nuclei after 6 days.

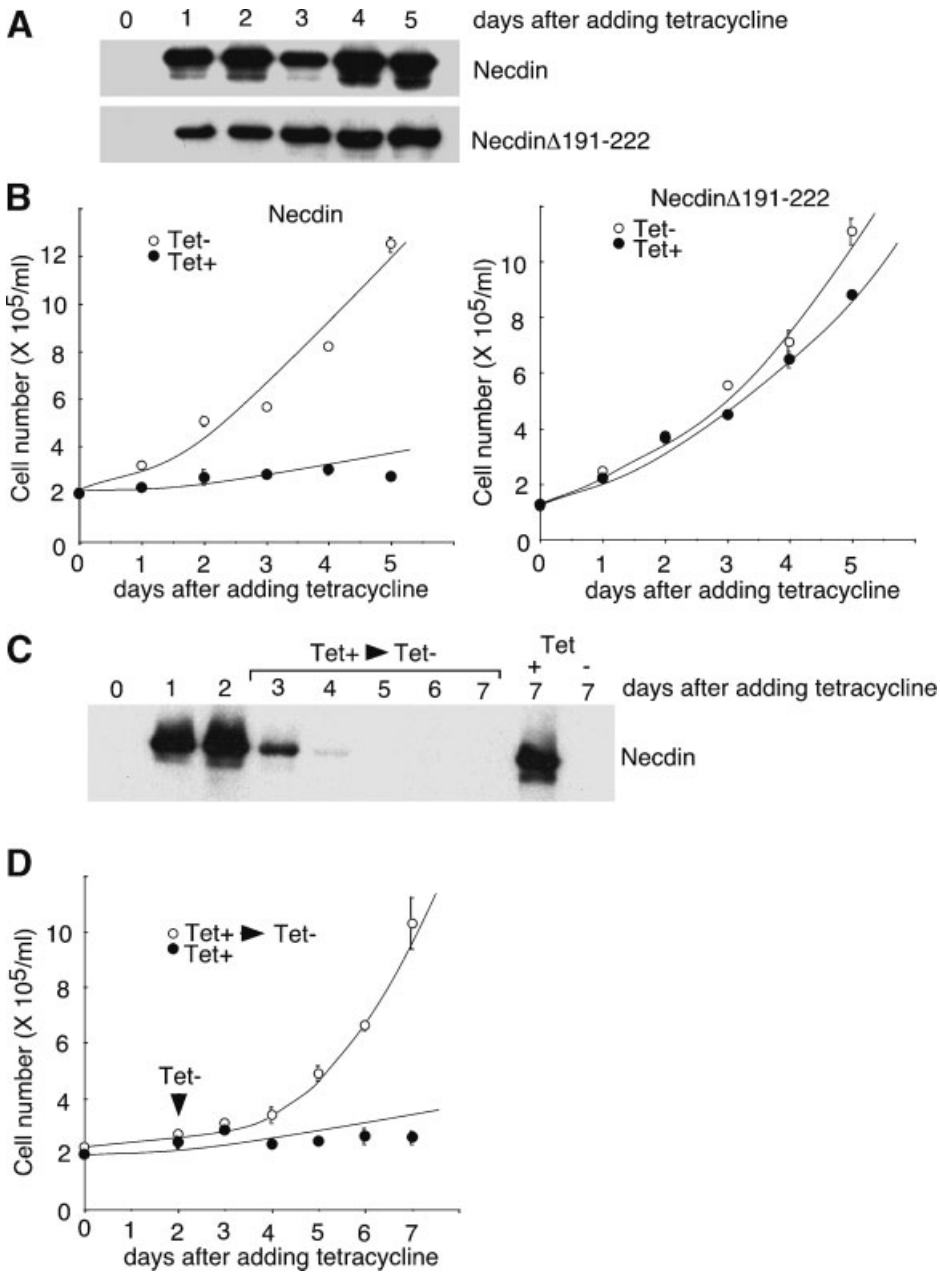

Fig. 5. Necdin induces growth arrest. **A**: Tet regulation of necdin and Δ191–222 expression. Immunoblot analysis of necdin and Δ191–222 expression from the cells grown in tet (–) as 0 day or tet (+) for 1, 2, 3, 4, and 5 days. **B**: Cell growth suppression by necdin. Cells expressing necdin or Δ191–222 were grown for the days indicated in the presence or absence of tet. The Cell number was counted and plotted at the time point indicated. Each time point is triplicate. **C**: Reversible expression of necdin. Immunoblot analysis of necdin expression from the cells grown in tet (+) for 2 days followed by the culture in tet (–) for another 5 days (3, 4, 5, 6, and 7 days after adding tet). Necdin expression in the cells grown in tet (–) or tet (+) for 7 days were also shown in lanes. **D**: Reversible growth suppression by necdin. Necdin-expressing cells were grown in tet (+) for 2 days followed by the culture in tet (–) for 5 days (3, 4, 5, 6, and 7 days after adding tet) or grown in tet (+) for 7 days. The cell number was counted and plotted at the time point as indicated. Each time point is triplicate.

SAOS-2 cells expressing necdin Δ191–222 showed no morphological alterations looking the same as the cells in the absence of tet (data not shown).

DISCUSSION

Here, we characterized the regions of necdin required for protein–protein interaction,

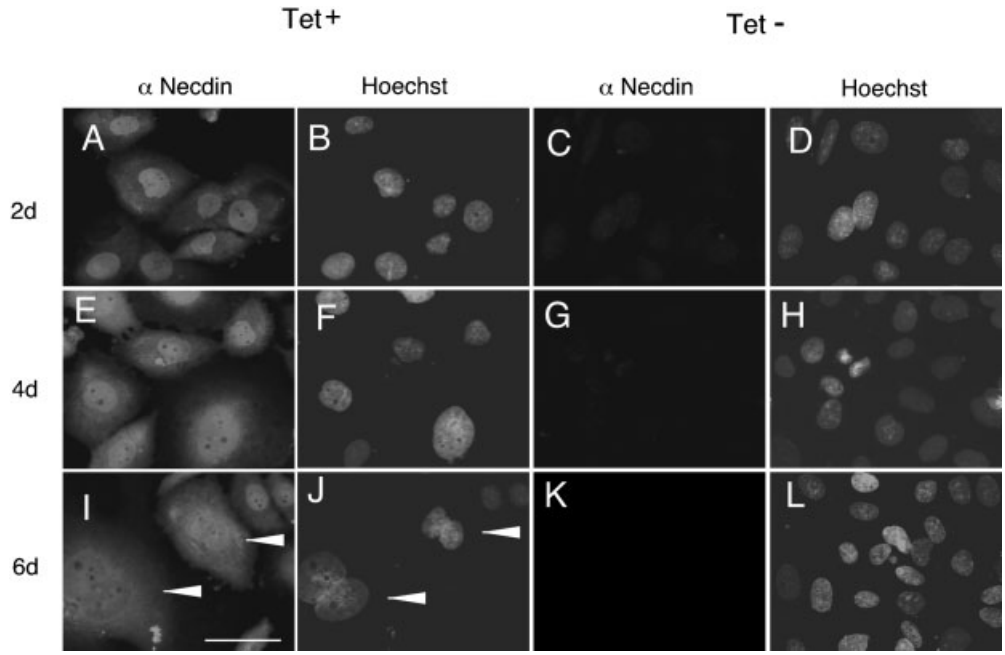

Fig. 6. Necdin expression leads to endoreduplication. Necdin-expressing cells were grown in the presence (**A**, **B**, **E**, **F**, **I**, and **J**) or absence (**C**, **D**, **G**, **H**, **K**, and **L**) of tet. Cells were fixed at 2, 4, and 6 days and stained for necdin (A, C, E, G, I, and K) and chromosomal DNA (Hoechst) (B, D, F, H, J, and L). Arrowheads in I and J point to double nuclei in necdin-overexpressing cells. Scale bar, 50 μm in I.

nuclear matrix targeting, and cell growth suppression. We found that the region containing entire MHD was required for the necdin-p53 binding, while the regions amino acids 144–184 and 191–222, only a part of the MHD, were required for both of nuclear matrix targeting and cell growth suppression, and also the amino-terminal proline-rich acidic region (amino acids 60–100) for cell growth suppression. Distinct subdomain requirement suggests the different mechanisms operative in these functions of necdin. Because necdin can bind to DNA directly [Matsumoto et al., 2001] and amino-terminal sequence of necdin shows a characteristic for the activation domain among transcription factors, necdin may induce specific genes involving in cell cycle regulation. The MHD is a 160∼170 amino acid motif of shared sequence similarity among MAGE gene family including necdin [Barker and Salehi, 2002]. Although the MHD is a predominant feature in the MAGE gene family, the sequence shows no significant homology to other proteins and its roles remains unclear. We and others reported some of the binding protein could be shared between the MAGE family proteins. NRAGE, a rat homolog of MAGE-D1, is identified as a p75 NTR binding protein [Salehi et al., 2000] and p75 NTR also binds to necdin, MAGE-H1, and MAGE-G1 [Teherpakov et al., 2002; Kuwako et al., 2004]. E2F1 binds to necdin and MAGE-G1 [Kuwako et al., 2004]. Praja1 identified as a Dlxin (a mouse homolog of MAGE-D1) binding protein can also bind to necdin [Sasaki et al., 2002]. These findings suggest that one of the function of the MHD is involved in a protein-protein interaction. Our data also support that the MHD of necdin is a crucial region for the protein-protein interaction. Barker and Salehi [2002] describe the subdomain structure of the MHD and divide five distinct regions that represent distinct areas of conservation. Domains 1, 3, and 5 are very similar, whereas the domains linking them represent less well conserved portions of the MHD. The region amino acids 191–222, a crucial region for nuclear matrix targeting and cell growth suppression, is corresponding to the well-conserved domain 3. Saburi et al. [2001] showed that overexpression of magphinin, a mouse homolog of MAGE-D3, suppresses the cell growth. There are three major forms of magphinins, magphinin-α, -β, and -γ, in the mouse and magphinin-βΔ22, a minor transcript, which is deleted 22 amino-acid residues within the MHD, has no cell growth suppressive activity. A part of the region

within the MHD among some of the MAGE family proteins may be essential for the cell growth suppression. Interestingly, necdin deleted in amino acids 144–184 or 191–222 lost the activities of both the nuclear matrix targeting and the cell growth suppression. The interaction of necdin with the nuclear matrix through these regions may contribute to the regulation of cell growth. Although the region encompassing amino acids 144–222 is critical for nuclear matrix association, it remains to be determined whether this region can function autonomously and target an unrelated, non-nuclear matrix protein to the nuclear matrix. We also found that the cells expressing necdin showed large and flattened morphology contained double nuclei after 6 days in the tet-regulatable expression system. It is unclear whether necdin also has a function in regulating a late step in mitosis or cytokinesis. Further studies are necessary for clarifying the mechanisms in regulating cell cycle by necdin. The distinct subdomain requirement suggests the different mechanisms operative in these functions of necdin.

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