Necdin, A Postmitotic Neuron-specific Growth Suppressor, Interacts with Viral Transforming Proteins and Cellular Transcription Factor E2F1*

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Necdin is a nuclear protein expressed in virtually all postmitotic neurons, and ectopic expression of this protein strongly suppresses the proliferation of NIH3T3 cells. Simian virus 40 large T antigen targets both p53 and the retinoblastoma protein (Rb) for cellular transformation. By analogy with the interactions of the large T antigen with these nuclear growth suppressors, we examined the ability of necdin to bind to the large T antigen. Necdin was co-immunoprecipitated with the large T antigen from the nuclear extract of necdin cDNA-transfected COS-1 cells. Yeast two-hybrid and in vitro binding analyses revealed that necdin bound to an amino-terminal region of the large T antigen, which encompasses the Rb-binding domain. Moreover, necdin bound to adenovirus E1A, another viral oncoprotein that forms a specific complex with Rb. We then examined the ability of necdin to bind to the transcription factor E2F1, a cellular Rb-binding factor involved in cell-cycle progression. Intriguingly, necdin, like Rb, bound to a carboxyl-terminal domain of E2F1, and repressed E2F-dependent transactivation in vivo. In addition, necdin suppressed the colony formation of Rb-deficient SAOS-2 osteosarcoma cells. These results suggest that necdin is a postmitotic neuron-specific growth suppressor that is functionally similar to Rb.

In the vertebrate central nervous system, neurons withdraw from the cell cycle immediately after differentiation from their proliferative precursors, and remain in the postmitotic state all of their lives. Differentiated neurons are absolutely incapable of dividing even in the presence of chemical and physical stimuli that promote cell-cycle progression of proliferative cells. However, little is known about molecular mechanisms underlying the permanent quiescence displayed by all neurons. Several previous studies have suggested that the retinoblastoma protein (Rb),1 a well characterized growth suppressor protein, is involved in neuronal differentiation-associated growth arrest. In the brain of Rb-deficient mouse embryos, aberrant mitotic figures accompanied by massive neuronal death are observed particularly in the hindbrain, spinal cord, and sensory ganglia (1–3). In cultured murine embryonal carcinoma cells, Rb is markedly induced during neural differentiation (4). Expression of adenovirus E1A, an oncoprotein that suppresses Rb functions, impairs neuronal differentiation and induces cell death (5). These findings raise the possibility that Rb plays a critical role in cell-cycle arrest of certain types of neurons during differentiation. However, the fact that many neurons still differentiate properly in Rb-deficient mice (1–3) suggests that other growth-suppressive proteins compensate the loss of Rb functions in neurogenesis.

We have previously isolated a novel cDNA sequence encoding a 325-amino acid residue protein, termed necdin, from a subtraction cDNA library of neurally differentiated murine embryonal carcinoma cells (6). Necdin is a nuclear protein, whose gene is expressed in virtually all postmitotic neurons in the central and peripheral nervous systems of mice (7, 8). The necdin gene is expressed in postmitotic neurons derived from embryonal carcinoma cells, but not in transformed cell lines originating from neuroblastomas and pheochromocytomas even after they are induced to differentiate (7). In developing mouse brain, the necdin gene is constitutively expressed in neurons from early embryonal stages (e.g. embryonic day 10 at the forebrain) until adulthood, whereas necdin mRNA is undetectable in neuronal precursor cells (i.e. neuroepithelial stem cells) in the neural tube (8). These observations suggest that necdin is expressed in postmitotic neurons that are differentiated from their precursor cells in an irreversible manner. Furthermore, the fact that ectopic expression of necdin strongly suppresses the growth of proliferative NIH3T3 cells (9) leads to the speculation that necdin acts as a growth suppressor in postmitotic neurons.

The tumor suppressor gene products Rb and p53 are nuclear proteins that interact with transforming proteins encoded by small DNA tumor viruses such as simian virus 40 (SV40), adenovirus, and human papillomavirus (10). For example, SV40 large T antigen binds to both Rb and p53, whereas adenovirus E1A and E1B form specific complexes with Rb and p53, respectively. These viral transforming proteins target cellular growth suppressors that are operative in normal cells. Here we demonstrate that necdin binds to SV40 large T antigen and adenovirus E1A, both of which interact with Rb. Moreover, we found that necdin, like Rb, interacts with the transcription factor E2F1, which promotes cell-cycle progression. Necdin functionally replaces Rb as a growth suppressor in Rb-deficient SAOS-2 cells, leading to the suggestion that necdin is a neuron-specific growth suppressor that is functionally similar to Rb.

EXPERIMENTAL PROCEDURES

Western Blotting and Immunoprecipitation—Necdin cDNA-carrying p94BFL was transfected into COS-1 cells by DEAE-dextran method as
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SAOS-2 cells in a 60-mm dish by the calcium phosphate method (13). Nuclear extracts and their immunoprecipitates of cDNA-transfected SAOS-2 cells were prepared, and the proteins were detected by antibodies C2 and anti-FLAG M2 (Kodak) as described above.

Two-hybrid Assay—GAL4 DNA-binding domain vector (pGBT9), GAL4 activation domain vector (pGAD424), pTD1 encoding SV40 large T antigen, and pVA3 encoding mouse p53 were purchased from CLONTECH. Rb cDNA and adenovirus type 5 E1A gene were provided by Dr. T. Akiyama (Osaka University) and Dr. K. Shiroki (University of Tokyo), respectively. DNA fragments for hybrid proteins were generated by polymerase chain reaction using synthetic oligonucleotide primers with restriction sequences at both ends. After treatment with respective restriction enzymes, the fragments were directionally inserted in pGAD424 and pGAD424, and introduced into Saccharomyces cerevisiae SFY 526. Transformants were spread onto a 100-mm dish, and selected for both leucine and tryptophan requirements. The above procedure and colony lift filter assay for β-galactosidase activity were carried out as recommended by CLONTECH. The reaction was evaluated 4 ranks with the time for the appearance of blue colonies at 30 °C: ++ + +, less than 2 h; ++, 2–6 h; +, 6–12 h; –, remaining white over 12 h.

In Vitro Binding Assay—EcoRI-BamH1 fragments were excised from inserted cDNAs in pGAD424, and subcloned directionally into pMOLC2 (New England Biolabs) to make maltose-binding protein (MBP) fusion proteins, which were purified as recommended by New England Biolabs. RNA was synthesized in vitro by transcribing linearized Blue.
script II (Stratagene) carrying cDNA for necdin (amino acids 1–325) or Rb (amino acids 379–928) with T7 RNA polymerase (New England Biolabs), and translated in the rabbit reticulocyte lysate system (Promega) supplemented with [35S]methionine (Amersham). For the large T antigen binding, 10 μl of the translation reaction mixture was incubated for 2 h at 4 °C with 150 μl of binding buffer A (20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 0.2 mM EDTA, 2% bovine serum albumin, 0.2 mM phenylmethylsulfonyl fluoride) containing MBP fusion proteins bound to amylose resin (5 μg). For E1A binding, 10 μl of the translation reaction mixture was incubated for 30 min at 4 °C with 150 μl of binding buffer B (50 mM Hepes (pH 7.0), 500 mM NaCl, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride) containing MBP fusion proteins bound to amylose resin (5 μg). After washing three times with binding buffer A, bound 35S-labeled proteins were eluted with 20 mM maltose, separated by 10% SDS-PAGE, and visualized by fluorography. Lanes 1, in vitro translated products (input).

Fig. 3. Necdin interacts with SV40 large T antigen. A, yeast two-hybrid assay. Combinations of GAL4 DNA-binding domain fusions (pGBT9 inserts: necdin (amino acids 83–325), Rb (amino acids 379–928), p53 (amino acids 72–390)) and GAL4 activation domain fusions (pGAD424 inserts: large T antigen (amino acids 84–708, T) and NH2-terminally truncated T (amino acids 121–708, TAN)) were introduced into yeast cells. The Rb- and p53-binding domains are indicated. B, in vitro binding assay. 35S-Labeled necdin (amino acids 1–325) (left panel) and Rb (amino acids 379–928) (right panel) were incubated with immobilized MBP (lanes 2), MBP-TAN (lanes 3), and MBP-T (lanes 4). The bound proteins were separated by 10% SDS-PAGE, and detected by fluorography. Lanes 1, in vitro translated products (input).

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RESULTS

Necdin Interacts with SV40 Large T Antigen—COS-1 cells, a monkey kidney cell line transformed by SV40, constitutively express the large T antigen, which forms stable complexes with Rb and p53 (17). We have previously found that ectopic necdin is accumulated in the nucleus of cDNA-transfected COS-1 cells...
To examine whether necdin interacts with SV40 large T antigen in the nucleus in vivo, necdin cDNA was transiently transfected into COS-1 cells. By Western blot analysis, similar levels of the large T antigen were present in untransfected and necdin cDNA-transfected COS-1 cells (Fig. 1A, lanes 1 and 2), and a 45-kDa band of necdin was detected in the cDNA-transfected cells (Fig. 1A, lane 4). The large T antigen was co-immunoprecipitated with necdin from the nuclear extract of the cDNA-transfected cells (Fig. 1B, lane 4, compared with negative controls in lanes 2 and 3), suggesting that ectopic necdin forms a specific complex with the large T antigen in the nucleus in vivo.

We then examined the interaction between necdin and the large T antigen by the yeast two-hybrid assay. We first examined whether necdin fused to GAL4 DNA-binding protein stimulates the transcription in this assay even in the absence of GAL4 activation domain fusions (Fig. 2). Full-length necdin (amino acids 1–325) showed a high reporter activity, suggesting that necdin possesses a transactivation domain. The amino (N)-terminal region (amino acids 1–100), which is highly acidic (calculated pI = 3.9) and proline-rich (21%) (6), was deleted to make three truncated forms, which showed reduced reporter activities. The truncated form of amino acids 83–325 exhibited no longer transactivation. Therefore, we used this truncated form as a DNA-binding domain fusion in the following two-hybrid assay. Necdin strongly bound to the large T antigen (amino acids 84–708, T) with which both Rb and p53 interacted (Fig. 3A). The Rb-binding domain of the large T antigen comprises the region exhibiting cellular transforming activity (18), and is distinct from the p53-binding domains (17). Therefore, the NH2 terminus of T (amino acids 84–120) including the Rb-binding domain was deleted to make TΔN (amino acids 121–708) for testing whether necdin interacts with this region. Neither necdin nor Rb bound to TΔN, but p53 still interacted with the truncated form. This suggests that necdin and Rb share a specific binding site of the large T antigen. We then examined the direct interaction between...
necdin and the large T antigen by \textit{in vitro} binding assay (Fig. 3B). Like Rb, necdin bound to T fused to maltose-binding protein (MBP-T), but not to MBP-T\textsubscript{D}, suggesting that necdin directly binds to the NH\textsubscript{2} terminus of the large T antigen, which comprises Rb-binding region.

\textit{Necdin Interacts with Adenovirus E1A—}Rb forms a specific complex with adenovirus oncoproteins E1A, whereas p53 interacts with E1B (10). Rb-binding sites of the large T antigen and E1A contain the L\textsubscript{X}C\textsubscript{X}E motif (\(X = \text{any amino acid}\)) (19). Our data that necdin and Rb share the ability to bind to the NH\textsubscript{2} terminus of the large T antigen led us to examine whether necdin interacts with adenovirus E1A. We used part of the E1A gene (amino acids 1–185) including three functional domains designated conserved regions (CR) 1–3, of which CR1 and CR2 possess transforming activities (20). Both necdin and Rb bound to E1A (amino acids 1–185), with which p53 failed to interact (Fig. 4A). We then tested whether necdin binds to three types of deletion mutants; E1A\textsubscript{D}CR2 (lacking CR2), E1A\textsubscript{D}CR2/3 (lacking CR2 and CR3), and E1A-CR3 (containing only CR3). Necdin bound weakly but significantly to both E1A\textsubscript{D}CR2 and E1A\textsubscript{D}CR2/3, but failed to bind to E1A-CR3. On the other hand, Rb failed to bind to the three deletion mutants. It was confirmed, by \textit{in vitro} binding assay, that both necdin and Rb bound to E1A (Fig. 4B). Necdin bound, albeit weakly, to E1A\textsubscript{D}CR2 (Fig. 4B, left panel, lane 3) and E1A\textsubscript{D}CR2/3 (data not shown). Since CR2 contains the L\textsubscript{X}C\textsubscript{X}E motif (19), it is suggested that this motif is less requisite for binding to necdin.

\textit{Necdin Interacts with E2F1—}Since necdin and Rb show similar, if not identical, binding characteristics toward the large T antigen and E1A, we then examined whether necdin interacts with cellular Rb-binding factor E2F, which directly regulates the transcription of a diverse set of genes involved in DNA replication and cell growth control (19). We first analyzed the binding of necdin to E2F1 by the two-hybrid assay (Fig. 5A). Both necdin and Rb bound to p2F1 (amino acids 55–430), which covers the domains for cyclin A binding, DNA binding,
and transcriptional activation that comproises the Rb-binding domain (19). Necdin bound weakly to a carboxyl (COOH)-terminally truncated form lacking the Rb-binding domain (21) (E2F1ΔRb), but failed to interact with a form devoid of the entire transactivation domain (22) (E2F1ΔTa). These results suggest that neither binds to the transactivation domain of E2F1 to modulate E2F-driven transcription.

Since SAOS-2 cells are established osteosarcoma cells lacking functional Rb, we chose this cell line to test the ability of necdin to form a nuclear complex with E2F1 in the absence of interference with Rb. We examined the interaction between necdin and E2F1 in vivo by immunoprecipitating the nuclear extracts from cDNA-transfected SAOS-2 cells (Fig. 5B). Expression vectors carrying cDNAs for FLAG-tagged E2F1 and necdin were transfected into SAOS-2 cells, in which FLAG-E2F1 and necdin showed single bands at ~60 and ~45 kDa, respectively (upper panels). FLAG-E2F1 was co-precipitated with necdin from the nuclear extract of SAOS-2 cells (Fig. 5B). Necdin (amino acids 102–325) is unable to bind to large T antigen and E2F1, but failed to interact with E1A, suggesting that amino acids 83–101 are requisite for binding to E1A. Further deletion of the NH2 terminus of necdin (amino acids 110–325 and 167–325) completely eliminated the binding activity. These data suggest that the central region of amino acids 83–292 is important for the interactions with these three proteins.

We determined the functional domain of necdin required for the interactions with the large T antigen, E1A, and E2F1 using various NH2- and COOH-terminal deletion mutants in the two-hybrid system (Fig. 6A). Necdin (amino acids 102–325) retained the ability to bind to the large T antigen and E2F1, but failed to interact with E1A, suggesting that amino acids 83–101 are requisite for binding to E1A. Further deletion of the NH2 terminus of necdin (amino acids 110–325 and 167–325) resulted in a failure of binding to these proteins. On the other hand, a COOH-terminally truncated form (amino acids 83–292) of necdin retained the ability to bind to these proteins, but further COOH-terminal deletion (amino acid 83–279) completely eliminated the binding activity. These data suggest that the central region of amino acids 83–292 is important for the interactions with these three proteins.

We then examined whether the NH2-terminally truncated form of necdin (necdinN, amino acids 110–325) is unable to interact with E2F1 in SAOS-2 cells in vivo (Fig. 6B). NecdinN, like full-length necdin, was stably expressed in the transfected cells (upper panel), but failed to interact with E2F1 (lower panel) in agreement with the finding in the two-hybrid system. Since SV40 large T antigen and E2F1 share the binding domain of necdin, the competition between the large T antigen and E2F1 for binding to necdin was examined in SAOS-2 cells in vivo (Fig. 6C). The large T antigen was stably expressed (upper panel), and reduced the E2F1 content in the immunoprecipitates (lower panel), implying that the large T antigen antagonizes the formation of necdin-E2F1 complex in the nuclei of SAOS-2 cells in vivo.
Necdin Represses E2F Site-dependent Transcription—We then examined whether necdin represses E2F-driven transcription in vivo using SAOS-2 cells (Fig. 7A). Necdin and Rb reduced the basal (intrinic) E2F site-dependent transcription to 57 and 40%, respectively. E2F1 increased 13-fold the basal transcriptional activity, and necdin and Rb repressed the activation to 41 and 52%, respectively. Necdin had no effect on SV40 early promoter activity. In this analysis, the suppression of E2F site-dependent transcription by the truncated form of Rb (amino acids 379–928) was weaker than the previous report in which full-length Rb was used (23). We analyzed the expression of Rb in transfected SAOS-2 cells by Western blotting, and found that a considerable amount of Rb immunoreactivity underwent degradation (data not shown), inferring that the weak suppression by Rb is attributable, at least in part, to its metabolic instability. The inhibition of E2F1-driven transcription activity by necdin was concentration-dependent, and the maximum suppression was 30% of the control value (Fig. 7B, left panel). On the other hand, the NH$_2$-terminally truncated necdin (necdinΔN, amino acids 110–325) exerted little or no growth suppression of SAOS-2 cells. To rule out the possibility that the repressed colony formation by necdin is attributable to its cytotoxicity, we analyzed the levels of necdin expressed in these transfectants. Similar amounts of necdin were detected in cell lysates from the 4- and 18-day cultures (Fig. 8B), suggesting that the suppression by necdin is due to its constitutive expression and not to the cytotoxic effect. Immunoreactive necdin was localized to the nuclei of transfected SAOS-2 cells (Fig. 8C). Moreover, the necdin-positive cells exhibited a severalfold increase in size, a typical phenotype displayed by Rb cDNA-transfected SAOS-2 cells (16). These results suggest that necdin serves as a substitute for Rb in these Rb-deficient cells.
DISCUSSION

This study has shown that necdin interacts with SV40 large T antigen and adenovirus E1A, both of which possess cellular transforming activities. Necdin bound to the NH₂-terminal region of the large T antigen (amino acids 84–120) (see Fig. 3). The NH₂-terminal fragment of the large T antigen (amino acids 1–120) induces cellular transformation, and missense mutations and short deletions in this region are defective in transformation (17). Thus, necdin potentially inhibits the transforming activity of the large T antigen by binding to the NH₂-terminal region. Moreover, necdin bound to the region encompassing two conserved regions, CR1 and CR2 of E1A (see Fig. 4), both of which are responsible for cellular transformation and induction of cellular DNA synthesis (20). We failed to obtain replication-defective recombinant adenovirus carrying necdin cDNA from 293 cells, a cell line constitutively expressing adenovirus oncoproteins, suggesting that ectopic necdin inactivates endogenous E1A that promotes the replication of adenovirus DNA. These findings raise the possibility that necdin modifies or neutralizes the transforming activities of these viral oncoproteins. Conversely, it seems likely that these viral oncoproteins target necdin for cellular transformation because this nuclear protein is normally involved in growth-suppressive mechanisms.

Necdin is functionally similar, but structurally dissimilar to Rb. Besides necdin and Rb, two Rb-related proteins, p107 and p130, interact with the large T antigen and E1A (24). These Rb family proteins are structurally similar, and comprise the binding region designated “pocket domain,” with

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**FIG. 8.** Colony formation of Rb-deficient SAOS-2 cells is suppressed by necdin. A, colony formation of transfectants. SAOS-2 cells were transfected with empty pRc/CMV, pRc-necdin (amino acids 1–325), pRc-necdinΔN (amino acids 110–325), or pRc-Rb (amino acids 379–928), and grown in the presence of G418 for 14 days. The G418-resistant colonies were visualized by crystal violet staining. B, immunoblot analysis of necdin. SAOS-2 cells were transfected with empty pRc/CMV (−) and pRc-necdin (+), and incubated for 4 and 18 days. Necdin in 20% of the total cell lysate per dish was detected by immunoblotting with antibody C2. C, immunocytochemistry of necdin cDNA-transfectants. Necdin cDNA-transfected SAOS-2 cells were incubated for 18 days, and immunostained with antibody C2. Scale bar = 100 μm.

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2 T. Uetsuki and K. Yoshikawa, unpublished observations.
which various factors such as viral oncoproteins and cellular transcription factors interact (25). Necdin consists of 325 amino acid residues (6), much smaller than Rb family proteins, and no homologous sequences are found between necdin and Rb family proteins. We found recently that amino acid sequences of the core functional domain of necdin (amino acids 83–292, see Fig. 6) are highly conserved (91% identity) between human and mouse, whereas the sequences of the NH2-terminal region (amino acids 1–82) are less conserved (60% identity). Therefore, this region may be evolutionally conserved because of its functional importance.

The biological significance of the formation of necdin-E2F1 complex in postmitotic neurons remained to be elucidated: postmitotic neurons differentiated from P19 cells contain high levels of mRNAs for E2F1 (5), necdin (6), and Rb (4), leading to speculation that E2F1 activities in these neurons are regulated by Rb and necdin in combination. As shown presently, necdin binds to the transactivation domain of E2F1, and represses E2F1-induced transactivation in vivo. E2F-binding sites exist within the promoters of a number of cellular genes involved in cell-cycle progression (19). Thus, it is tempting to speculate that necdin, in cooperation with Rb, suppresses the expression of a battery of genes required for DNA replication by silencing E2F1 in postmitotic neurons. We are currently investigating whether necdin substantially controls E2F1 functions in postmitotic neurons by transferring cDNAs encoding necdin and E2F1 using recombinant adenovirus vectors.

Rb is expressed in both neuroepithelial stem cells and postmitotic neurons (26), and thus potentially induces growth arrest of the stem cells at the very beginning of neuronal differentiation. On the other hand, necdin is expressed only in postmitotic neurons (7, 8), suggesting that necdin has a function to keep differentiated neurons staying in the postmitotic state. Rb loses its growth inhibitory functions upon phosphorylation by D-type cyclins and cyclin-dependent kinases (27). Interestingly, these cyclins and kinases physiologically coexist with the Rb-E2F system in postmitotic neurons (5, 28). If Rb is the sole molecule that arrests the cell cycle of differentiated neurons, then pathological or accidental phosphorylation of Rb would lead quiescent neurons to undergo abortive mitosis and death. Thus, necdin might be involved in a “fail-safe” mechanism, complementing Rb to prevent postmitotic neurons from resuming cell division. Studies using necdin-defective animal models are currently under way in our laboratory to clarify the implications of necdin in neuronal postmitotic phenotype in vivo. Further information about interactions between necdin and cell-cycle regulatory machinery will lead to a better understanding of molecular mechanisms underlying permanent quiescence displayed by all nerve cells.

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