Ectopic Expression of Necdin Induces Differentiation of Mouse Neuroblastoma Cells*

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Necdin is expressed predominantly in postmitotic neurons, and ectopic expression of this protein strongly suppresses cell growth. Necdin has been implicated in the pathogenesis of Prader-Willi syndrome, a human neurodevelopmental disorder associated with genomic imprinting. Here we demonstrate that ectopic expression of neddin induces a neuronal phenotype in neuroblastoma cells. Necdin was undetectable in mouse neuroblastoma N1E-115 cells under undifferentiated and differentiated conditions. N1E-115 cells transfected with neddin cDNA showed morphological differentiation such as neurite outgrowth and expression of the synaptic marker proteins synaptotagmin and synaptophysin. In addition, Western blot analysis of the retinoblastoma protein (Rb) family members Rb, p130, and p107 revealed that neddin cDNA transfectants contained an increased level of p130 and a reduced level of p107, a pattern seen in differentiated G0 cells. The transcription factors E2F1 and E2F4 physically interacted with neddin via their carboxyl-terminal transactivation domains, but only E2F1 abrogated neddin-induced growth arrest and neurite outgrowth of neuroblastoma cells. Overexpression of E2F1 in differentiated N1E-115 cells induced apoptosis, which was antagonized by co-expression of neddin. These results suggest that neddin promotes the differentiation and survival of neurons through its antagonistic interactions with E2F1.

Necdin is a 325-amino acid protein encoded in a cDNA sequence that has been isolated from a subtraction library of neurally differentiated mouse embryonal carcinoma P19 cells (1). The neddin gene is expressed in most of the terminally differentiated neurons, although its expression levels vary among neuronal cell types, being the highest in the hypothalamus and brain stem (2, 3). The human neddin gene is mapped to chromosome 15q11.2-q12, a region deleted in Prader-Willi syndrome (PWS)1 (4–6). PWS is a neurodevelopmental disorder associated with genomic imprinting, and its major symptoms such as feeding problems, gross obesity, and hypogonadism are consistent with a hypothalamic defect. Human and mouse neddin genes are maternally imprinted and transcribed only from the paternal allele (4, 5, 7). Necdin is not expressed in the cells prepared from PWS patients whose chromosome 15q11.2-q13 region in the paternal allele is deleted. Disruption of the paternal allele of mouse neddin gene results in early postnatal lethality (8), reduction in specific hypothalamic neurons such as luteinizing hormone-releasing hormone- and oxytocin-containing neurons, and behavioral alternations, which are reminiscent of human PWS (9). Therefore, it is likely that a defect in neddin expression is responsible, at least in part, for various clinical symptoms of PWS. However, little is known about the functional roles of neddin in neuronal differentiation and development.

Accumulating evidence has suggested that neddin is a growth suppressor expressed in terminally differentiated cells. Ectopic expression of neddin suppresses the proliferation of several cell lines (10–12). Furthermore, neddin interacts with viral oncoproteins such as SV40 large T antigen, adenovirus E1A, and the transcription factor E2F1 (11). These characteristics of neddin resemble those of the retinoblastoma gene product (Rb), a major growth suppressor protein that is mutated in many cancer cells. Rb is believed to play a pivotal role in terminal mitosis and subsequent differentiation during neurogenesis (13). These findings prompted us to examine whether neddin also promotes neuronal differentiation through its growth inhibitory property.

N1E-115 neuroblastoma cells show differentiated characteristics in response to forced expression of growth suppressors such as Rb and its family members p107 and p130 (14, 15). Furthermore, p73, a p53-related growth suppressor, induces differentiation of N1E-115 cells (16). Therefore, we have attempted to examine whether ectopic expression of neddin induces a neuronal phenotype in N1E-115 neuroblastoma cells. We here demonstrate that neddin induces morphological and biochemical markers of neurons in N1E-115 neuroblastoma cells. Furthermore, we show that neddin and its binding partner E2F1 act antagonistically in the differentiation and apoptosis of neuroblastoma cells. The present gain-of-function study provides valuable information about the functional roles of neddin in the induction and maintenance of the terminally differentiated state of neurons.

EXPERIMENTAL PROCEDURES

Cell Culture and cDNA Transfections—Mouse neuroblastoma N1E-115 cells (a gift from Dr. H. Higashida, Kanazawa University, Kanazawa, Japan) were cloned by limited dilution to obtain the subclone N1E-115(sc2). The cloned cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO2. Cells were plated at 1 × 105 cells in 35-mm dishes, cultured in growth medium for 16 h, and transfected with expression vectors by the calcium phosphate method (17). For neurite outgrowth assay, the expression vectors pBc-necdin (11), pBc-necdinAN (11), pBc-E2F1 (11), and pDNA3-RA-E2F4 (a gift from Dr. D. M. Livingston, Dana-Farber Cancer Institute, Boston, MA) were transfected in combi-
Induction of neuronal markers by ectopic expression of necdin in N1E-115 cells. A, morphology of necdin cDNA-transfected N1E-115 cells. N1E-115 cells were transfected with the LacZ-expression vector (0.8 μg) and the expression vector (0.2 μg) for necdin (Ndn) or necdinΔN (NdnΔN). Cells were cultured for 72 h in the growth medium, fixed, stained for β-galactosidase activity in situ, and observed with a phase-contrast microscope. CT, cells transfected with the empty vector. Scale bar, 50 μm. B, quantification of cells carrying extended neurites. Cells were transfected as above. Transfected N1E-115 cells carrying extended neurites (>2 times the cell body diameter) among >100 β-galactosidase-positive cells were counted. Each value represents the mean ± S.E. (n = 4). C, immunocytochemistry of synaptic marker proteins. Transfected cells were cultured in the growth medium for 60 h, fixed, and double-stained for β-galactosidase (βGal) and synaptophysin (SP) or synaptotagmin (ST). Scale bar, 50 μm. D, Western blot analysis of necdin and synaptic marker proteins. Cells were treated with 2% Me2SO (DMSO treatment) and harvested at the time points indicated. Whole cell lysates were prepared and analyzed by immunoblotting using antibodies against necdin (Necdin), synaptophysin (SP), synaptotagmin (ST), and tubulin (Tubulin). Ndn, positive control (lysate of necdin cDNA-transfected cells). E, Western blot analysis of synaptic marker proteins. Cells were transfected with each expression vector in combination with pcDNA6/TR encoding blasticidin-resistant gene and cultured in blasticidin-containing growth medium for 60 h. The lysates of selected cells were analyzed by Western blotting as in D. UD, undifferentiated N1E-115 cells (0 h control); DF, cells treated with Me2SO for 72 h.

Western Blot Analysis—Cells were morphologically differentiated by culturing in the medium containing 2.5% FBS in the presence of 2% dimethyl sulfoxide (Me2SO) for 3 days. Me2SO-treated or cDNA-transfected N1E-115 cells were collected by centrifugation at 150 × g for 5 min, homogenized by vortexing at 4 °C for 3 min in CSM buffer (10 mM HEPES (pH 6.8), 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 0.5% Triton X-100, and 1× complete protease inhibitor mixture (Roche Molecular Biochemicals)), and centrifuged at 12,000 × g at 4 °C for 15 min to obtain the supernatant. The cell lysates (20 μg of protein/ lane) were separated by 7.5–15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon membrane (Millipore) by electroblotting. The membrane was blocked with 4% nonfat milk in PBS with 0.05% Tween 20 and incubated for 1 h at room temperature with primary antibodies against necdin (NC243) (1:1,000), synaptophysin (1:200), synaptotagmin (1:500), and β-tubulin (65-095, ICN Biomedicals) (1:1,000). The following antibodies against cell cycle-related proteins were purchased from Santa Cruz Biotechnology: anti-Rb (sc-50) (1:500), anti-p107 antibody (sc-318) (1:500), anti-p130 (sc-317) (1:500), anti-E2F1 (sc-193) (1:500), anti-E2F2 (sc-193) (1:500), anti-E2F4 (sc-866) (1:500), anti-cdc2 antibody (sc-54) (1:500), and anti-cyclin A1 antibody (sc-586-G) (1:200). The membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel), goat anti-rabbit IgG (Cappel), or bovine anti-goat IgG (sc-2350, Santa Cruz Biotechnology) at room temperature for 1 h. Proteins were visualized using chemiluminescence method (Renaissance, PerkinElmer Life Sciences). The protein concentration was determined by the Bradford method (Bio-Rad).

In Vitro Binding Assay—DNAs encoding various deletion mutants of E2F1 and E2F4 were generated from full-length mouse E2F1 or human E2F4 cDNA (pcDNA3-HA-E2F4) by polymerase chain reaction using synthetic oligonucleotide primers and inserted directionally in pMAL-c2 (New England Biolabs) to produce maltose-binding protein.
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(MBP) fusion proteins. MBP-E2F1 fusion proteins (∼500 ng) or MBP-E2F4 fusion proteins (1 μg bound to amylese resin (20 μl of 50% slurry) were incubated with purified His-tagged necdin (50 ng) (12) for 30 min at 4 °C in 0.5 ml of binding buffer (20 mM Tris-HCl (pH 7.8), 200 mM NaCl, 1 mM EDTA). His-tagged necdin was eluted with 20 mM maltose and detected by Western blotting using an anti-necdin antibody (12).

Co-immunoprecipitation Assay—The Myc tag was added to E2F1 and E2F4 by subcloning their cDNAs into a 6×His tag plasmid (a gift from Dr. M. W. McBurney, University of Ottawa, Ottawa, Canada). cDNAs encoding Myc-tagged E2F1 (amino acids 1–430) and its COOH-terminal deletion mutant E2F1ΔC (amino acids 1–337) were inserted into pRC-CMV (Invitrogen) to construct pRC-MycE2F1 and pRC-MycE2F1ΔC, respectively. cDNAs encoding Myc-tagged E2F4 (amino acids 1–417) and its COOH-terminal deletion mutant E2F4ΔC (amino acids 1–360) were also inserted into pRC-CMV to construct pRC-MycE2F4 and pRC-MycE2F4ΔC, respectively. Combinations of these expression vectors were transiently transfected into N1E-115 cells by the calcium phosphate method, and whole cell extracts were prepared 48 h after transfection. The cell extracts were incubated for 2 h at 4 °C with anti-Myc antibody (9E10) or anti-necdin antibody C2 in CSK buffer (12). The complexes were precipitated with Protein A-Sepharose (Amersham Biosciences), eluted with SDS-PAGE buffer, separated by 10% SDS-PAGE, and analyzed by immunoblotting using anti-necdin antibody NC243 and anti-Myc antibody.

Reporter Assay for E2F-dependent Transactivation—N1E-115 cells were plated at a density of 1 × 104 cells in 35-mm dishes, cultured overnight in growth medium, and transfected with the luciferase reporter plasmid containing the promoter sequence (−241 to +45) of mouse DNA polymerase α catalytic subunit (20) (a gift from Dr. F. Hanaoka, Osaka University, Osaka, Japan) in PGV-B (Toy Ink, Tokyo, Japan) (0.8 μg). E2F expression vector (0.4 μg of pRC-E2F1 or pcDNA3-HA-E2F4), necdin expression vector (0.2, 0.4, or 0.8 μg of pRC-necdin or pRC-necdinΔN) and pRC-LacZ (0.4 μg). The amount of plasmid DNA was adjusted to 4 μg by adding empty pRC-CMV. Cells were cultured for 48 h and harvested to measure luciferase activity with a luminometer (Lumat LB9501, Berthold) using a reagent kit (Toy Ink). Transfection efficiency was normalized by β-galactosidase activity.

Bromodeoxyuridine Incorporation Assay—N1E-115 cells were plated at 1 × 104 cells in 35-mm dishes and cultured in growth medium overnight. Cells were transfected with pRC-necdin, pRC-necdinΔN, pRC-E2F1, pC20A/E2F4, pCDNA3-HA-E2F4 (0.4 μg each), and pRC-LacZ (1.2 μg). Total amount of DNA was adjusted to 4 μg by adding empty pRC-CMV. Cells were cultured in the medium containing 2.5% FBS for 48 h and incubated in the presence of bromodeoxyuridine (BrdUrd) for 5 h. Cells were fixed and double-stained with anti-BrdUrd antibody (Roche Diagnostics) and the anti-β-galactosidase antibody (1:300).

Apoptosis Assay—N1E-115 cells were grown on coverslips in 35-mm dishes and transfected with combinations of pRC-LacZ, pRC-E2F1, pCDNA3-HA-E2F4, p3xFLAG-necdin, and p3xFLAG-necdinΔN (1.3 μg each) using LipofectAMINE Plus reagent (Invitrogen). p3xFLAG-necdin and p3xFLAG-necdinΔN were constructed by inserting respective cDNAs into p3xFLAG-CMV-14 expression vector (Sigma). The cells were treated with Me2SO 24 h after transfection, fixed, and permeabilized as above. The cells were incubated with the anti-Flag M2 monoclonal antibody (1:300) (Sigma) and the anti-E2F1 antibody (1:500) or the anti-E2F4 antibody (1:500) for 1 h at room temperature, and expressed proteins were visualized with FITC-conjugated anti-mouse IgG and rhodamine B-conjugated anti-rabbit IgG. Nuclear morphology was examined by staining with 3.3 mM Hoechst 33342 (Molecular Probes) for 15 min at room temperature and observed by fluorescence microscopy. Nuclear DNA fragmentation was analyzed by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) method (21) combined with immunostaining for Flag-tagged necdin, E2F1, or E2F4. TUNEL reactivity and each expressed protein were visualized with Texas Red and FITC, respectively.

RESULTS

Ectopic Expression of Necdin Induces Neuronal Markers in N1E-115 Cells—We first examined whether ectopic expression of necdin induces morphological markers of differentiated neurons in N1E-115 cells. Some N1E-115 cells transfected with necdin cDNA showed morphological characteristics of neurons such as neurite outgrowth and flattened cell body, whereas cells expressing necdinΔN (amino acids 110–325), a mutant defective in binding to E2F1 (11), did not show these characteristics (Fig. 1A). Ectopic expression of necdin increased the number of neuron-like cells to 35% of total transfected cells, whereas 10 and 14% of cells transfected with the empty vector (control) and necdinΔN cDNA, respectively, showed morphological differentiation (Fig. 1B).

We analyzed the expression of the synaptic markers synaptophysin and synaptotagmin in necdin cDNA-transfected cells by immunocytochemistry. Both synaptophysin and synaptotagmin were induced in N1E-115 cells transfected with necdin cDNA, whereas cells transfected with the empty vector (control) or necdinΔN cDNA contained very small amounts of these proteins (Fig. 1C). By Western blot analysis, necdin was undetected in undifferentiated and differentiated N1E-115 cells, whereas the levels of synaptophysin and synaptotagmin were gradually increased by Me2SO treatment (Fig. 1D). Necdin cDNA transfectants selected by blastidin resistance contained increased levels of synaptotagmin and synaptophysin (Fig. 1E), suggesting that necdin promotes synaptic differentiation.

Fig. 2. Cell cycle-related proteins in N1E-115 cells transfected with necdin cDNA. A, Western blot analysis of Rb family proteins and E2Fs in Me2SO-treated N1E-115 cells. Cells were treated with Me2SO (DMSO treatment) and harvested at the time points indicated. Rb, p107, p130, E2F1, E2F4, and tubulin were analyzed by Western blotting. B, Western blot analysis of cell cycle-related proteins in necdin cDNA transfectants. Cells were transfected with the expression vector for necdin (Ndn) or necdinΔN (NdnΔN) in combination with pcDNA6/TR, and cultured in blasticidin-containing growth medium for 60 h. Total lysates of selected cells were analyzed by Western blotting using antibodies against necdin, Rb, p107, p130, cdc2, cyclin A, and tubulin. UD, 0 h control; CT, control cells transfected with the empty vector; DF, differentiated cells treated with Me2SO for 72 h. Double arrows (in A and B) point to hyperphosphorylated Rb (upper arrow) and hypophosphorylated Rb (lower arrow).
Necdin Alters the Expression of Cell Cycle-related Proteins

We analyzed the amounts of cell cycle-related proteins in Me2SO-treated N1E-115 cells by Western blotting (Fig. 2A). The level of the hypophosphorylated form of Rb was higher than that of hyperphosphorylated Rb at 0 and 72 h, but the levels of hyperphosphorylated Rb remained almost consistent during the course of neuronal differentiation. We also examined the levels of other Rb family members p107 and p130, which are believed to be involved in the cell cycle control during neuronal differentiation (22–24). We found that the p107 level was greatly reduced, whereas the p130 level increased in necdin cDNA transfectants. Expression of E2F-regulated proteins cdc2 and cyclin A1 was strikingly reduced in necdin cDNA transfectants, and the expression levels of these proteins in undifferentiated cells and cells transfected with the empty vector or necdinΔN cDNA remained high. The E2F1 and E2F4 levels in necdin cDNA transfectants were similar to those of differentiated N1E-115 cells (data not shown). These results suggest that necdin cDNA transfectants resemble differentiated N1E-115 cells in the expression pattern of cell cycle-regulatory proteins.

Necdin Directly Interacts with E2F1 and E2F4—We have previously demonstrated that necdin binds to E2F1 by yeast

![Fig. 3. Physical interactions of necdin with E2Fs and their deletion mutants. A, expression of MBP-E2F mutant proteins. Purified E2F1 deletion mutant proteins fused to MBP (MBP-E2F1, left panel) or E2F4 deletion mutant proteins fused to MBP (MBP-E2F4, right panel) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular size markers (kDa) are at the left. B, in vitro binding assay. His-tagged necdin was incubated with MBP-E2F1 mutant proteins (left panel) or MBP-E2F4 mutant proteins (right panel). Bound His-tagged necdin was eluted and detected by Western blotting using anti-necdin antibody. C, summary of E2F1 and E2F4 deletion mutants capable of binding to necdin. The results shown in B were schematized for deletion mutants of E2F1 (left panel) and E2F4 (right panel). The hatched box represents the Rb-binding domain in E2F1 and E2F4.](image-url)
two-hybrid assay (11). We examined in vitro binding ability of necdin with E2F1 and E2F4 by using their deletion mutants fused to MBP (Fig. 3A). Necdin bound to the COOH-terminal regions of both E2F1 and E2F4 (Fig. 3B). By this assay, we were able to narrow the necdin binding regions to E2F1 (amino acids 388–430) and E2F4 (amino acids 360–417), which encompass Rb binding region as schematized in Fig. 3C. We then examined the interactions of necdin with E2F1 and E2F4 in N1E-115 neuroblastoma cells by the co-immunoprecipitation assay (Fig. 4). Expression vectors encoding Myc-tagged E2F and necdin were co-transfected into N1E-115 cells. Major bands of expressed polypeptides were detected at the positions of their predicted sizes (necdin, 43 kDa; necdinΔN, 35 kDa; Myc-E2F1 and Myc-E2F4, 75 kDa; Myc-E2F1ΔC and Myc-E2F4ΔC, 65 kDa) (Fig. 4A). In N1E-115 cells, necdin was co-immunoprecipitated with Myc-E2F1 or Myc-E2F4, but not with Myc-E2F1ΔC or Myc-E2F4ΔC (Fig. 4, B and C, upper panels). Conversely, Myc-E2F1 was co-immunoprecipitated with necdin, but not with necdinΔN (Fig. 4, B and C, lower panels). These results suggest that necdin binds to the COOH-terminal transactivation domains of E2F1 and E2F4 in N1E-115 cells.

**E2F1 Abrogates Necdin-induced Growth Arrest of N1E-115 Cells**—To examine functional interactions of necdin with E2Fs, we carried out E2F site-dependent transcription assay in N1E-115 neuroblastosomas cells using a luciferase reporter carrying a promoter region of mouse DNA polymerase α catalytic subunit (20) (Fig. 5A). In this assay, E2F1 and E2F4 induced 15- and 4.5-fold activation, respectively. Co-expressed necdin reduced E2F-dependent transcription by necdin. N1E-115 cells were transfected with DNA polymerase α promoter-luciferase reporter plasmid (0.4 μg) and combinations of necdin expression vectors (Ndn and NdnΔN) (0.1, 0.2, and 0.4 μg), E2F expression vectors (E2F1 and E2F4) (0.2 μg), and pRc-LacZ (0.2 μg). After incubation for 48 h, luciferase activity in cell lysates was measured with a luminometer. B, BrdUrd incorporation assay. Cells were transfected with expression vectors (0.4 μg each) and pRc-LacZ (0.8 μg), cultured for 60 h, and incubated with BrdUrd for 8 h. Transfected cells were fixed and double-stained for β-galactosidase and BrdUrd. BrdUrd-positive cells among >100 β-galactosidase-positive cells were counted. Each value represents the mean ± S.E. (n = 3) with Myc-E2F1ΔC or Myc-E2F4ΔC (Fig. 4, B and C, lower panels). Conversely, Myc-E2F1 was co-immunoprecipitated with necdin, but not with necdinΔN (Fig. 4, B and C, upper panels). These results suggest that necdin binds to the COOH-terminal transactivation domains of E2F1 and E2F4 in N1E-115 cells.

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transfectants, whereas those among transfectants expressing E2F1 and E2F4 were 42 and 40%, respectively, which were slightly greater than the values of control and necdinΔN (both 34%). The necdin-induced growth suppression was abrogated by E2F1 (36%), but not by E2F4 (19%). Neither E2F1 nor E2F4 altered the population of BrdUrd-positive cells in necdinΔN cDNA transfectants. These results suggest that E2F1 and E2F4 exert different effects on necdin-induced growth suppression of N1E-115 cells.

E2F1 Antagonizes Necdin-induced Neurite Outgrowth of N1E-115 Cells—Several lines of evidence have suggested that E2F1 and E2F4 have distinct functions in cell cycle regulation and differentiation (25). Thus, we examined whether necdin-induced morphological differentiation of N1E-115 cells is modulated by E2F1 or E2F4 (Fig. 6A). E2F1-transfected cells showed no apparent morphological changes, but co-transfected E2F1 suppressed necdin-induced neurite outgrowth. On the other hand, E2F4 per se induced neurite outgrowth, and cells transfected with both necdin and E2F4 showed morphological changes similar to those transfected with necdin alone. We then quantified the modulatory effects of E2F1 and E2F4 on necdin-induced neurite outgrowth. E2F1 slightly increased the number of cells carrying extended neurites, but it antagonized the effect of necdin in a dose-dependent manner (Fig. 6B). In contrast, E2F4 alone induced neurite outgrowth, and further increased the number of cells carrying extended neurites in combination with necdin (Fig. 6C). These results indicate that E2F1 and E2F4 exert opposite effects on necdin-induced morphological differentiation of N1E-115 cells.

Necdin Suppresses E2F1-induced Death of N1E-115 Cells—We have previously reported that ectopic expression of E2F1 induces apoptosis in postmitotic neurons, in which endogenous E2F1 levels are markedly down-regulated (26). We thus examined whether necdin has modulatory effects on E2F1-induced apoptosis in differentiated N1E-115 cells. N1E-115 cells were transfected with each expression vector alone or two vectors in combination, and subsequently treated with MeSO. Necdin was accumulated in the nucleus and exerted no apparent effects on the nuclear morphology (Fig. 7A). In contrast, E2F1 induced a severe shrinkage of the nucleus, in which DNA was fragmented as detected by TUNEL (Fig. 7, A and B). Co-expression of necdin prevented differentiated N1E-115 cells from E2F1-induced apoptosis. On the other hand, overexpressed E2F4, either alone or in combination with necdin, was distributed primarily in the cytoplasm and had no apparent effect on the nuclear morphology. Quantification of apoptotic and TUNEL-positive cells revealed that necdin suppressed E2F1-induced apoptosis almost completely, whereas the E2F1 binding-defective mutant necdinΔN had little or no anti-apoptotic effect (Fig. 7, C and D). These results indicate that necdin prevents differentiated neuroblastoma cells from E2F1-induced apoptosis.

DISCUSSION

We have recently found that primary dorsal root ganglion neurons, which express high levels of necdin, fail to differentiate properly even in the presence of nerve growth factor when endogenous necdin is down-regulated by necdin antisense oligonucleotide (27). The antisense oligonucleotide-treated cells eventually undergo caspase-3-dependent apoptosis, suggesting that necdin is required for the terminal differentiation and survival of nerve growth factor-dependent sensory neurons. Because necdin is not expressed in neural stem cells or transformed cell lines derived from pheochromocytoma and neuroblastoma, we speculated that ectopic expression of necdin induces a neuronal phenotype in these necdin-deficient cells. We initially transferred necdin cDNA into multipotent neural stem cells prepared from the neural tube and found that necdin-overexpressing stem cells failed to undergo neuronal differentiation. We thus used necdin-defective N1E-115 neuroblastoma cells, which have been often used to examine the effects of exogenous genes encoding growth suppressor proteins such as E2F1-A.

These results suggest that E2F1 and E2F4 exert different effects on necdin-induced growth suppression of N1E-115 cells.

Fig. 6. Suppression of necdin-induced neurite outgrowth by E2F1-A. A, morphology of transfected N1E-115 cells. N1E-115 cells were transfected with expression vectors for LacZ (0.2 μg), E2F1 (0.4 μg), E2F4 (0.4 μg), and necdin (0.2 μg) for −Ndn, 0 μg for +Ndn). Cells were cultured for 72 h, fixed, and stained for β-galactosidase activity. Scale bar, 50 μm. B, effects of necdin and E2F1 on neurite outgrowth. N1E-115 cells were transfected with expression vectors for necdin (Ndn) (0.2 μg) and E2F1 (E2F1) (0.02, 0.1, and 0.4 μg) in combination. Cells with extended neurites (>2 times the cell body diameter) among >100 β-galactosidase-positive cells were counted. Each value represents the mean ± S.E. (n = 4). C, effects of necdin and E2F4 on neurite outgrowth. N1E-115 cells were transfected with expression vectors for necdin (Ndn) (0.2 μg) and E2F4 (E2F4) (0.02, 0.1, and 0.4 μg) in combination. Cells carrying extended neurites were counted as in B.

E2F1 antagonizes necdin-induced neurite outgrowth of N1E-115 cells.

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Rb, p130, p107, p27KIP1, and p73 (14–16). Using this cell line as a gain-of-function model, we were able to demonstrate that necdin induces characteristics of differentiated neurons such as neurite outgrowth (Fig. 1, A and B), changes of expression levels of synaptic marker proteins (Fig. 1, C and E) and cell cycle regulatory proteins (Fig. 2B), and cell cycle repression (Fig. 5B). We infer that necdin requires some complementary factors expressed in the neuronal lineage-committed cells (or partially differentiated cells in a neuronal direction) but not in multipotent stem cells.

Necdin is initially characterized as a nuclear protein (1, 2) and is partly associated with the nuclear matrix (28). Necdin binds to the typical nuclear proteins E2F and p53 (11, 12). Recent studies have shown that necdin is distributed in the cytoplasm of differentiated neurons and skeletal muscles (19, 29). Similarly, E2F1 is primarily cytoplasmic in terminally differentiated skeletal muscles, whereas E2F4 is partitioned between cytoplasmic and nuclear compartments (30). Although p53 appears in the nucleus during neuronal differentiation, it is present mainly in the cytoplasm in mature differentiated neurons.

**Fig. 7. Suppression of E2F1-induced apoptosis by necdin.** A, immunocytochemistry for transfected N1E-115 cells. N1E-115 cells were transfected with expression vectors (1.5 μg each) for FLAG-tagged necdin (FlagNdn), E2F1 (E2F1), E2F4 (E2F4), or their combinations (FlagNdn/E2F1 and FlagNdn/E2F4). The cells were induced to differentiate by MeSO treatment, fixed at 48 h, and stained with antibodies against FLAG (Flag), E2F1 (E2F1), and E2F4 (E2F4) for fluorescence immunocytochemistry. The nuclei were stained with 3.3 μM Hoechst 33342 (Hoechst). Scale bar, 20 μm. B, TUNEL reactivity in transfected N1E-115 cells. Cells carrying fragmented DNA were visualized by TUNEL (TUNEL). After TUNEL reaction, the cells were immunostained for FLAG, E2F1, or E2F4. Scale bar, 20 μm. C, quantification of apoptotic cells. N1E-115 cells were transfected with combinations of expression vectors for FLAG-tagged necdin (FlagNdn), FLAG-tagged necdinΔN (FlagNdnΔN), E2F1 (E2F1), and E2F4 (E2F4). Apoptotic cells with shrunken or fragmented nuclei among >100 transfected immunopositive cells were counted. Each value represents mean ± S.E. (n = 3). D, quantification of TUNEL-positive cells. Transfected cells were labeled by TUNEL, and positive cells among >100 transfected immunopositive cells were counted. Each value represents mean ± S.E. (n = 3). pRc-LacZ was used as a negative control (leftmost bars in C and D).
neurons (31). These observations raise the possibility that nuclear proteins involved in cell cycle regulation are sequestered in the cytoplasm after accomplishment of terminal differentiation. We infer that necdin-induced growth arrest is attributable, at least in part, to the sequestration of E2F’s from their target sites to suppress E2F-dependent transcription, and that the suppression of E2F1-responsive genes triggers the differentiation program in neuroblastoma cells.

We have confirmed the previous finding that MeSO-treated N1E-115 cells express reduced p107 and increased p130 levels (15). A similar expression pattern of these Rb family proteins was observed in necdin cDNA transfectants (Fig. 2A). These are consistent with the idea that p130 is found in quiescent cells but not in growing cells, whereas p107 is inversely regulated (32). Such changes in the expression pattern of Rb family proteins are observed during the course of neuronal differentiation of P19 embryonal carcinoma cells (30, 33) and neuronal progenitor cells (23). These findings suggest that changes in the predominance of Rb family proteins from p107 to p130 are correlated with the transition from proliferative state of neuronal progenitor cells to the postmitotic state of differentiated neurons. In contrast to the striking changes in p107 and p130 levels during neuronal differentiation, only a small change in the ratio between hypophosphorylated and hyperphosphorylated Rb was observed in differentiated N1E-115 cells (Fig. 2B). We observed that differentiated N1E-115 cells and necdin cDNA transfectants contained very low levels of cdc2 and cyclin A1, both of which are up-regulated by E2F (Fig. 2, A and B), suggesting that E2F is silenced in differentiated N1E-115 cells. Thus, the phosphorylation status of Rb may exert little or no influence on the mitotic quiescence in differentiated neuroblastoma cells expressing high levels of p130 and low levels of E2F1.

The present study has shown that necdin interacts with E2F4 as well as E2F1 (11). E2F1 is thought to promote the exit from G0 phase (34), and its overexpression induces apoptosis in quiescent cells (35) and postmitotic neurons (26, 36). We found that E2F1 abrogated necdin-induced growth arrest and morphological differentiation of N1E-115 cells (Figs. 5 and 6). In addition, differentiated N1E-115 cells overexpressing E2F1 underwent apoptosis, which was antagonized by co-expression of necdin (Fig. 7). These findings suggest that necdin plays a role in the blockade of cell cycle reentry of postmitotic neurons. This idea is also supported by the present findings that necdin suppresses the S phase entry (Fig. 5B) and antagonizes E2F1-induced apoptosis (Fig. 7). These results suggest that necdin is a unique growth suppressor that blocks cell cycle reentry and promotes survival of postmitotic neurons. Further studies on the functions of necdin will provide insights into the mechanisms underlying terminal differentiation of postmitotic neurons.

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