The Postmitotic Growth Suppressor Necdin Interacts with a
Calcium-binding Protein (NEFA) in Neuronal Cytoplasm*

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Necdin, a growth suppressor expressed predominantly in postmitotic neurons, interacts with viral oncoproteins and cellular transcription factors E2F1 and p53. In search of other cellular targets of necdn, we screened cDNA libraries from neurally differentiated murine embryonal carcinoma P19 cells and adult rat brain by the yeast two-hybrid assay. We isolated cDNAs encoding partial sequences of mouse NEFA and rat nucleobindin (CALNUC), which are Ca\(^{2+}\)-binding proteins possessing similar domain structures. Necdin interacted with NEFA via a domain encompassing two EF hand motifs, which had Ca\(^{2+}\) binding activity as determined by \(^{45}\)Ca\(^{2+}\) overlay. NEFA was widely distributed in mouse organs, whereas necdn was expressed predominantly in the brain and skeletal muscle. In mouse brain in vivo, NEFA was localized in neuronal perikarya and dendrites. By immunoelectron microscopy, NEFA was localized to the cisternae of the endoplasmic reticulum and nuclear envelope in brain neurons. NEFA-green fluorescent protein (GFP) fusion protein expressed in neuroblastoma N1E-115 cells was retained in the cytoplasm and partly secreted into the culture medium. Necdin enhanced the cytoplasmic retention of NEFA-GFP and potentiated the effect of NEFA-GFP on caffeine-evoked elevation of cytosolic Ca\(^{2+}\) levels. Thus, necdn and NEFA might be involved in Ca\(^{2+}\) homeostasis in neuronal cytoplasm.

Neurons in the central nervous system withdraw permanently from the cell cycle after their differentiation from proliferative progenitors. The permanent arrest of cell division is the most fundamental feature displayed by terminally differentiated neurons. However, little is known about the molecular mechanisms whereby neurons become postmitotic. The murine embryonal carcinoma P19 cells differentiate into postmitotic neurons in response to retinoic acid treatment (1). We have isolated a 325-amino acid residue protein encoded in a cDNA sequence from a subtraction library of retinoic acid-treated P19 cells and termed this protein necdn for neurally differentiated embryonal carcinoma-derived protein (2). The necdn gene is expressed in postmitotic neurons derived from P19 cells but not in transformed cell lines originating from neuroblastomas and pheochromocytomas (3). The necdn gene is expressed in virtually all postmitotic neurons in mouse brain from early embryonic stages until adulthood, whereas necdn mRNA expression is undetectable in the proliferative progenitors (3, 4). Besides neurons in the central and peripheral nervous systems, the necdn gene is expressed in skeletal muscle (4), cartilage, and brown fat (5), all of which contain postmitotic cell populations. In man, necdn is expressed ubiquitously in both neuronal and non-neuronal tissues (6), in which many postmitotic cells are expected to exist.

Ectopic expression of necdn suppresses the growth of NIH3T3 fibroblasts (7) and SAOS-2 osteosarcoma cells (8). Necdin binds to the large T antigen at the NH\(_2\)-terminal region that encompasses the retinoblastoma protein (Rb)\(^1\)-binding domain (8). Necdin also binds to adenovirus E1A, another viral oncoprotein that targets Rb. More importantly, necdn, like Rb, interacts with the transactivation domain of E2F1 and represses E2F-driven transcription. Therefore, necdn is functionally similar to Rb, although the two growth suppressors are structurally dissimilar. Unlike Rb, necdn binds to NH\(_2\)-terminal transactivation domain of p53 and represses p53-dependent transcription of the p21 promoter (9). Necdin does not counteract p53-induced growth suppression but does inhibit p53-induced apoptosis of U2OS osteosarcoma cells. These findings suggest that necdn contributes to the maintenance of the postmitotic state of neurons as a unique growth suppressor that targets both E2F1 and p53.

Necdin has been characterized as a nuclear protein (2, 3). However, a recent study using a new anti-necdn antibody has demonstrated that a considerable amount of the necdn protein is present in the cytoplasm of differentiated neurons (10). These findings suggest that necdn not only acts as a transcriptional repressor in the nucleus but also exerts some functions in the cytoplasm of postmitotic neurons. In the present study, we have attempted to find out the cellular targets of necdn to elucidate the functional roles of this protein. We report that necdn interacts with NEFA and nucleobindin (Nuc), both of which are Ca\(^{2+}\)-binding proteins that contain two EF-hand motifs and a leucine zipper domain. We characterize physical

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1 The abbreviations used are: Rb, retinoblastoma protein; Nuc, nucleobindin; PAGE, polyacrylamide gel electrophoresis; P0, postnatal day 0; ER, endoplasmic reticulum; NE, nuclear envelope; GFP, green fluorescence protein; PWS, Prader-Willi syndrome; GST, glutathione S-transferase.
interactions between necdin and NEFA and demonstrate that necdin exerts modulatory effects on the distribution and function of NEFA.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—The GAL4 DNA-binding vector pGBT9, the GAL4 activation domain vector pGAD424, and adult rat brain cDNA library in pGAD10 vector were purchased from CLONTECH. cDNA was generated from poly(A)1 RNA isolated from neurally differentiated P19 cells using a cDNA synthesis kit (Amer sham Pharmacia Biotech) and inserted into pGAD424. Mouse necdin cDNA encoding amino acids 102–325 was cloned into pGBT9 (8). These vectors were introduced into yeast SFY526 strain, and transformants were grown on leucine- and tryptophan-deficient synthetic dropout medium plates. The colony lift assay for β-galactosidase activity was carried out as recommended by CLONTECH. Plasmid DNA from β-galactosidase positive colonies was recovered in Escherichia coli DH1 cells, and the sequence was determined with an automatic DNA sequencer (model 400; LI-COR, Lincoln, NE). For determination of binding sites, NEFA deletion mutants were generated by polymerase chain reaction using synthetic oligonucleotide primers, subcloned into pGAD424 vector, and co-transformed with pGBT9-necdin. Necdin deletion mutants in pGBT9 (8) were introduced into yeast cells with pGAD424-NEFA (amino acid 26–420) and interactions were scored for β-galactosidase activity by a colony lift assay. The reaction was evaluated three ranks with the time for appearance of blue colonies at 30 °C: ++ +, less than 2 h; ++, 2–6 h; +, 6–12 h; and −, remaining white over 12 h.

Immunoprecipitation—cDNAs encoding Myc-tagged NEFA (amino acids 26–420) and NEFA (amino acids 26–241) using a 6 × Myc tag plasmid (a gift from Dr. M. W. McBurney, University of Ottawa) were subcloned into pCMV expression vector (Invitrogen). Combinations of expression vectors of pRc-necdin (amino acids 1–325), pRc-mycNEFA (amino acids 26–420), and pRc-mycNEFACT (amino acids 26–241) were transfected into COS-1 cells and harvested 48 h after transfection (8). Cell extract (2.5 mg of protein) were incubated for 2 h at 4 °C with an anti-Myc antibody (9E10) (1:5) or an anti-necdin antibody C2 (1:125) (9) in a mixture for containing 1.5% BSA. Protein A-Sepharose (0.5 ml) bound to glutathione-agarose beads (Sigma) in phosphate-buffered saline for 1 h at 4 °C. Bound proteins were eluted with a buffer containing 20 mM glutathione and 50 mM Tris-HCl (pH 8.0), separated by 10% SDS-PAGE, transferred to Immobilon membrane, incubated with antibody C2 and peroxidase-conjugated anti-mouse (or anti-rabbit) IgG (Cappel) using chemiluminescence method (Renaissance; NEN Life Science Products). Protein concentrations were determined by the Bradford method (10). To detect an endogenous complex between NEFA and necdin, whole brain tissues from seven C57BL/6 mice at P0 were homogenized in phosphate-buffered saline containing 0.6% Nonidet P-40 and the protease inhibitors and ultracentrifuged at 100,000 × g for 1 h to obtain supernatant. The supernatant (1 mg of protein) was subjected to HitTrap NHS-activatedaffinity column (Amer sham Pharmacia Biotech) coupled with the IgG fractions of anti-necdin (C2) antisemur or anti-NEFA (NET1) antisemur, and bound proteins were eluted with 2 mM glycine HCl (pH 2.5). Fractions of flow-through and eluate were precipitated with 10% trichloroacetic acid, rinsed with cold acetone, separated by 10% SDS-PAGE, and immunoblotted with antibodies NET1 and NC243.

Subcellular Distribution of NEFA in Cultured Cells—P19 embryonal carcinoma cells were cultured and induced to differentiate by retinoic acid treatment as described previously (1). Postmitotic neurons were enriched by treatment with 5 mg/ml cytosine β-arabinofuranoside (Sigma) for 5 days (13). For immunocytochemistry, undifferentiated P19 cells and differentiated postmitotic neurons were stained with antibodies NET1 and NC243 by the avidin-biotin-peroxidase complex method (Vector Laboratories) (3). Undifferentiated P19 cells and enriched neurons were homogenized and dissolved in SDS-PAGE buffer. For subcellular fractionation, enriched neurons derived from P19 cells were homogenized in 0.2 M sucrose. The homogenate was pelleted by centrifugation at 1,000 × g for 10 min (P1 fraction), at 12,500 × g for 20 min (P2 fraction), and at 105,000 × g for 60 min (P3 fraction and supernatant). P1–P3 fractions were suspended in phosphate-buffered saline, homogenized, and dissolved in SDS-PAGE buffer. Cyttoplasmic and nuclear soluble fractions were prepared from enriched postmitotic neurons as described previously (14). Each extract (20 μg protein) was separated by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies NET1 and NC243.

Immunohistochemistry and Immunoelectron Microscopy—For immunohistochemistry, tissues in ether-anesthetized C57BL/6J mice at P0 were fixed by transcardial perfusion of 4% paraformaldehyde solution in phosphate-buffered saline (pH 7.4) for 10 min. Thin sections were prepared and observed by electron microscopy. Immunoreactive materials were detected by the avidin-biotin peroxidase complex method using a Histofine Kit (Nichirei, Tokyo, Japan). For electron microscopy, coronal sections of postnatal day 1 (P1) embryos were immersed in the same fixative followed by cryoprotection with 30% sucrose and cut coronally at a thickness of 20 μm by a cryostat. The sections were collected on glass slides coated with gelatin were treated with 0.3% H2O2 in methanol for 30 min, 3% normal goat serum for 1 h and antibody NET1 (1:2,000) overnight. Immunoreactive materials were detected by a peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch) coupled with the IgG fractions of anti-necdin (C2) antiserum or anti-NEFA (NET1) antisemur and the avidin-biotin peroxidase complex method as above. The stained sections were postfixed with 0.2% osmic acid, stained with 2% uranyl acetate, and embedded in epoxy resin. Ultrathin sections were prepared and observed by electron microscopy.

Expression of NEFA-GFP in NIE-115 Cells—NIE-115 cells (a gift from Dr. H. Higashida, Kanazawa University) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. cDNA encoding NEFA (amino acids 1–420) was subcloned into pEGFP-N1 expression vector to obtain pEGFP-NEFA (CLONTECH). NIE-115 cells were grown in 90-mm dishes and transfected with pEGFP-NEFA (negative control) (16) in combination with BSS buffer precipitated with 10% trichloroacetic acid. The conditioned medium (3 ml of BSS buffer precipitated with 10% trichloroacetic acid) was permeated with Fura-2 was performed as described (15).

The colony lift filter assay for β-galactosidase activity was carried out as recommended by CLONTECH. Plasmid DNA from β-galactosidase positive colonies was recovered in Escherichia coli DH1 cells, and the sequence was determined with an automatic DNA sequencer (model 400; LI-COR, Lincoln, NE). For determination of binding sites, NEFA deletion mutants were generated by polymerase chain reaction using synthetic oligonucleotide primers and inserted directionally in pGex5X.1 (Amer sham Pharmacia Biotech) to produce glutathione S-transferase (GST) fusion proteins. In vitro binding assay for interactions between NEFA mutants and His-tagged necdin was carried out as reported previously (9). Briefly, the necdin protein was mixed with GST fusion proteins (1 μg) bound to glutathione-agarose beads (Sigma) in phosphate-buffered saline for 1 h at 4 °C. Bound proteins were eluted with a buffer containing 20 mM glutathione and 50 mM Tris-HCl (pH 8.0), separated by 10% SDS-PAGE, transferred to Immobilon membrane, incubated with antibody C2 and peroxidase-conjugated anti-rabbit IgG, and detected by the chemiluminescence method. Protein concentrations were determined by the Bradford method (10). For 4Ca2+ overlay, GST-NEFA fusion proteins (10 μg each) were transfected from 10% SDS-PAGE gel onto Immobilon membrane, which was incubated with 4Ca2+ (calcium chloride in aqueous solution) (Amer sham Pharmacia Biotech) and autoradiographed (11).

Anti-NEFA Antibody—Anti-NEFA polyclonal antibody (NET1) was raised in a New Zealand rabbit against purified GST-NEFA (amino acid 26–420) fusion protein. Initial immunization was with Freund's complete adjuvant (Difco), and the three subsequent boosters were with Freund's incomplete adjuvant (Difco). Mouse Nuc cDNA used was prepared from P19 cell mRNA by reverse transcription-polymerase chain reaction and confirmed to be identical with the reported sequence (12). Expression vectors for mouse pGAD424 (amino acids 1–455) (pRc-Nuc) and Myc-tagged Nuc (amino acids 26–455) (pRc-mycNuc) were transfected into SAOS-2 cells (8). To test the cross-reactivity, extracts were separated by SDS-PAGE and immunoblotted with antibody NET1 and anti-Necdin antibody (8). Various mouse tissues from C57BL/6J mice at postnatal day 0 (P0) were homogenized in phosphate-buffered saline containing 0.6% Nonidet P-40 and protease inhibitors (Complete, Roche) and ultracentrifuged at 100,000 × g for 1 h at 4 °C to obtain the supernatant.

The equal amounts of protein (20 μg) were separated by 10% SDS-PAGE and blotted with antibody NET1 or anti-necdin antibody NC243 (10). To detect an endogenous complex between NEFA and necdin, whole brain tissues from seven C57BL/6 mice at P0 were homogenized in phosphate-buffered saline containing 0.6% Nonidet P-40 and the protease inhibitors and ultracentrifuged at 100,000 × g for 1 h to obtain the supernatant. The supernatant (1 mg of protein) was subjected to HitTrap NHS-activated affinity column (Amer sham Pharmacia Biotech) coupled with the IgG fractions of anti-necdin (C2) antisemur or anti-NEFA (NET1) antisemur, and bound proteins were eluted with 2 mM glycine HCl (pH 2.5). Fractions of flow-through and eluate were precipitated with 10% trichloroacetic acid, rinsed with cold acetone, separated by 10% SDS-PAGE, and immunoblotted with antibodies NET1 and NC243.
RESULTS

Isolation of Necdin-binding Proteins in the Yeast Two-hybrid System—We first screened cDNA libraries prepared from neurally differentiated P19 cells for necdin-binding proteins using the yeast two-hybrid assay. In this assay, an NH2-terminally truncated mutant of necdin (amino acids 102–325) in GAL4 DNA-binding pGBT9 vector was used because the NH2-terminal domain of necdin exerts transactivation in the two-hybrid system (8). Two positive clones were isolated from neurally differentiated P19 cells. One clone contained a sequence (NBP-1) identical with a COOH-terminal part of mouse NEFA (GenBank accession number AJ222586; Fig. 1). We then screened adult rat brain cDNA library and isolated one positive clone among 

\[105\] transformants from neurally differentiated P19 cells. This clone has a sequence (NBP-2) identical with rat Nuc (CALNUC) (12) (GenBank accession number Z36277). NEFA and Nuc are structurally similar Ca\(^{2+}\)-binding proteins (Fig. 1A). The interaction of necdin with NBP-1 or NBP-2 was as strong as that with SV40 large T antigen and was stronger than that with E2F1 (Fig. 2B). Although necdin functionally resembles Rb (8), neither NBP-1 nor NBP-2 bound to Rb.

Because little is known about expression and functions of NEFA, we attempted to elucidate the physiological implications of the interactions between NEFA and necdin. For the analyses of physical interactions between necdin and NEFA, we isolated the full-length mouse NEFA cDNA from a cDNA library of neurally differentiated P19 cells. The nucleotide sequence determined was identical with that deposited in the database (GenBank accession number AJ222586) except for two nucleotide substitutions in the protein coding region at positions 153 (T to C, Met to Thr at amino acid position 18) and 1942 (C to T, silent mutation at amino acid position 314) (data not shown). These discrepancies might reflect strain differences. The cDNA sequence encodes total 420 amino acids including a signal sequence at the NH2-terminal region, which can be cleaved to generate the mature NEFA protein (amino acids 26–420) (16). We used this NEFA cDNA for analyses of physical and functional interactions between NEFA and necdin in the following experiments.

Interactions in Vivo between Necdin and NEFA in Cultured Cells—To determine the necdin-binding site in NEFA, we constructed various NEFA deletion mutants and analyzed by the
neurons (amino acids 83–325 and 102–325) bound to NEFA. An
expression of Myc-tagged NEFA and Myc-tagged NEFAΔCT in
COS-1 cells. pRc/CMV vectors encoding Myc-tagged NEFA (amino acids 26–420, MycNEFA) and Myc-tagged NEFAΔCT (amino acids 26–241, MycNEFAΔCT) were transfected into COS-1 cells. Equal amounts (20 μg of protein) of transfected cell extracts were separated by 10% SDS-PAGE and immunoblotted with anti-Myc antibody (αMyc). pRc/CMV, empty vector. Molecular size markers (in kDa) are at the left. B, detection of necdin-NEFA complex in transfected COS-1 extracts. The extracts were immunoprecipitated (IP) with anti-Myc antibody (αMyc) and immunoblotted (IB) with anti-necdin antibody C2 (αnecdin) (upper panel) or conversely immunoprecipitated with antibody C2 and immunoblotted with anti-Myc antibody (lower panel).

We then determined the NEFA-binding domain of necdin by the same assay using various necdin deletion mutants (Fig. 2B). The necdin deletion mutants lacking NH₂-terminal regions (amino acids 83–325 and 102–325) bound to NEFA. An NH₂-terminally truncated form of necdin (amino acids 110–325 and 167–325) failed to bind to NEFA. Although a COOH-terminus truncated necdin (amino acids 83–292) interacted with NEFA, further COOH-terminal deletion to amino acids 83–279 lost the NEFA binding activity. These results suggest that the central region of necdin is indispensable for the interaction with NEFA. This region coincided with the region required for the interactions with the large T antigen (Fig. 2B), E2F1, and p53 (9).

To examine whether the interaction between NEFA and necdin occurs in cultured cells in vivo, expression vectors encoding Myc-tagged NEFA (amino acids 26–420) and Myc-tagged NEFAΔCT (amino acids 26–241, a negative control) were transfected with a necdin-expressing vector into COS-1 cells (Fig. 3A). When the cell lysates were treated with an anti-Myc antibody, necdin was co-immunoprecipitated with Myc-tagged NEFA but was hardly pulled down with Myc-tagged NEFAΔCT (Fig. 3B). Conversely, Myc-tagged NEFA was co-immunoprecipitated with necdin when the cell lysates were treated with anti-necdin antibody C2.

**Necdin-binding Domain of NEFA binds to Ca²⁺ in Vitro—**

The interaction between NEFA and necdin was analyzed by in vitro binding assay using GST fused to NEFA deletion mutants (Fig. 4). Purified His-tagged necdin protein was incubated with truncated GST-NEFA fusion proteins immobilized on glutathione-agarose beads (Fig. 4, A and B). Necdin bound to GST-NEFA deletion mutants (amino acids 26–420, 26–318, 26–241, and 214–358), all of which include the two EF-hand motifs and the intervening acidic region, bound to necdin with a strength similar to that of the intact form. Thus, the domain containing two EF hands (amino acids 214–358) was required for its interaction with necdin.

In vitro—protein binding assay and ⁴⁰Ca²⁺ overlay analysis for NEFA and its deletion mutants. A, GST-NEFA fusion proteins. Purified recombinant proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. GST, GST without fusion; GST-NEFA, GST fused to NEFA deletion mutants (amino acids 26–420, 26–318, 26–241, and 214–358). B, in vitro binding assay. His-tagged necdin was incubated with GST-NEFA fusion proteins bound to glutathione-agarose. Bound proteins were eluted, separated by SDS-PAGE, and immunoblotted with anti-necdin C2 antibody. C, ⁴⁰Ca²⁺ overlay assay. The GST-NEFA fusion proteins were separated by 10% SDS-PAGE, blotted onto Immobilon membrane, and incubated with ⁴⁰Ca²⁺. NEFA bound to ⁴⁰Ca²⁺ was located by autoradiography.
Cells. Cell extracts were separated by SDS-PAGE and immunoblotted 

Nuc (\textit{MycNuc}) and pRc-mycNuc (\textit{pRc-mycNuc}) were transfected into SAOS-2 

31678, pRc-mycNEFA (\textit{pRc-mycNEFA}), pRc-NEFA (\textit{pRc/CMV} 

ners. A and NEFA, respectively. E

E), flow-through (\textit{flow-through}), and eluate (\textit{eluate}).

\textit{B}, distribution of NEFA and necdin in neonatal mouse 

tissues. Homogenates of various organs from P0 mouse were separated 

by 10\% SDS-PAGE and immunoblotted with NET1 (\textit{NET1}). Homogenate (\textit{H}), flow-through (\textit{F}), and eluate (\textit{E}) fractions were separated by SDS-PAGE and immunoblotted with antibodies NC243 and NET1 for necdin and NEFA, respectively.

\textit{C}, detection of the NEFA-necdin complex in mouse brain extract. Mouse whole brain extract was applied to the immunoaffinity columns carrying antibodies NC243 (\textit{aNecdin column}) and NET1 (\textit{aNEFA column}). Homogenate (\textit{H}), flow-through (\textit{F}), and eluate (\textit{E}) fractions were separated by SDS-PAGE and immunoblotted with antibodies NC243 and NET1 for necdin and NEFA, respectively.

\textbf{Fig. 5.} Distribution of NEFA and necdin in neonatal mouse tissues. A, specificity of anti-NEFA antibody. A rabbit anti-GST-NEFA antibody (\textit{NET1}) was raised against GST-NEFA. Empty pRc/CMV vector (\textit{pRc/CMV}), pRc-NEFA (\textit{pRc-NEFA}), pRc-mycNEFA (\textit{pRc-mycNEFA}), pRc-Nuc (\textit{pRc-Nuc}), and pRc-mycNuc (\textit{pRc-mycNuc}) were transfected into SAOS-2 cells. Cell extracts were separated by SDS-PAGE and immunoblotted with anti-Myc (\textit{\alphaMyc}, \textit{left panel}) and anti-NEFA (\textit{\alphaNEFA}, \textit{right panel}) antibodies. B, distribution of NEFA and necdin in neonatal mouse organs. Homogenates of various organs from P0 mouse were separated by 10\% SDS-PAGE and immunoblotted with NET1 (\textit{upper panel}) and NC243 (\textit{lower panel}). Muscle, skeletal muscle (thigh). C, detection of the NEFA-necdin complex in mouse brain extract. Mouse whole brain extract was applied to the immunoaffinity columns carrying antibodies NC243 (\textit{aNecdin column}) and NET1 (\textit{aNEFA column}). Homogenate (\textit{H}), flow-through (\textit{F}), and eluate (\textit{E}) fractions were separated by SDS-PAGE and immunoblotted with antibodies NC243 and NET1 for necdin and NEFA, respectively.

\textbf{Distribution of NEFA in Mouse Tissues in Vivo and in Cultured P19 Cells—}To determine the distribution and localization of endogenous NEFA protein, a polyclonal rabbit antibody (\textit{NET1}) was raised against purified GST-NEFA (amino acids 26–420) fusion protein. Because mouse NEFA and Nuc have homologous sequences, the specificity of this antibody was tested by immunoblotting using SAOS-2 cells transfected with cDNAs for full-length NEFA (amino acids 1–420), Nuc (amino acids 1–455), Myc-tagged NEFA (amino acids 26–420), and Myc-tagged Nuc (amino acids 26–455) (Fig. 5A). The anti-Myc antibody detected myc-NEFA and myc-Nuc, whereas antibody \textit{NET1} recognized only NEFA and myc-NEFA, indicating that this antibody recognizes NEFA specifically.

Using this antibody, distribution of the NEFA protein in various organs of P0 mouse was examined by Western blotting (Fig. 5B, \textit{upper panel}). NEFA immunoreactive bands (55–kDa) were detected in all the tissues examined. Tissue levels of NEFA were high in the lung, brain, skeletal muscle, and spleen. On the other hand, necdin was enriched in the brain and skeletal muscle, and small amounts of necdin were detected in the kidney, lung, and heart (Fig. 5B, \textit{lower panel}). These results suggest that interactions between NEFA and necdin are of physiological significance particularly in the brain and skeletal muscle. We then attempted to demonstrate the NEFA-necdin complex in the brain of P0 mice by immunoaffinity chromatography using anti-NEFA and anti-necdin antibodies (Fig. 5C). Both necdin and NEFA were detected in the eluate from anti-necdin or anti-NEFA immunoaffinity column. The affinity chromatography using preimmune rabbit serum failed to bind to the necdin-NEFA complex (data not shown). These results suggest that necdin and NEFA are present as a complex in the soluble fraction of the mouse brain.

The intracellular distribution patterns of NEFA and necdin were examined by immunocytochemistry in cultured murine P19 cells, from which cDNAs for these proteins are isolated (Fig. 6). NEFA was localized to the cytoplasm near the nucleus in undifferentiated P19 cells, and its immunoreactivity in the cytoplasm was increased when P19 cells were induced to differentiate into neurons (Fig. 6A). On the other hand, the cytoplasm of undifferentiated cells was lightly stained for necdin, whose immunoreactivity was increased in both the cytoplasm and the nucleus of differentiated neurons. Western blot analysis revealed that levels of NEFA and necdin were elevated in postmitotic neurons (Fig. 6B). NEFA and necdin were distributed in all the subcellular fractions of crude nuclear (P1), mitochondrial (P2), microsomal (P3), and supernatant (S) (Fig. 6C). NEFA was barely detectable in the nuclear soluble fraction of P19-derived neurons, whereas necdin was present in the soluble fractions from the cytoplasm and nucleus.

\textbf{Distribution of NEFA in the Brain in Vivo—}We then determined the distribution of NEFA immunoreactivity in the neonatal mouse brain in \textit{vivo} by immunohistochemistry (Fig. 7). NEFA was distributed throughout the brain, including the cerebral cortex, hippocampal formation, hypothalamus, and medulla oblongata (Fig. 7). In the cerebral neocortex, apical dendrites and perikarya of neurons were immunopositive for NEFA, whereas the nuclei of these neurons were negatively stained (Fig. 7, A and B). In the hippocampus, the dendrites and perikarya of neurons in the pyramidal layer and dentate gyrus of the hippocampus (the CA1 and CA3 regions) were positively stained (Fig. 7, D and E). In the hypothalamus and medulla oblongata, the perikarya and proximal dendrites were intensely stained (Fig. 7, F–I). The staining patterns and regional distribution of NEFA-immunopositive neurons resemble those of necdin-immunopositive neurons as reported previously (10). To examine the distribution of NEFA immunoreactivity in intracellular organelles, we carried out immunoelectron microscopy for NEFA-immunoreactivity in the hypothalamus. NEFA was enriched in the cisternae of the endoplasmic reticulum (ER) and nuclear envelope (NE) but was not detected in the Golgi apparatus, mitochondria, or nucleoplasm (Fig. 8). In contrast, the necdin immunoreactivity showed no clear localization in the ER, NE, and Golgi apparatus by immunoelectron microscopy using antibody NC243 (data not shown).

\textbf{Necdin Increases Cytoplasmic Retention of NEFA—}It has been reported previously that NEFA is present in the membrane and cytosol fractions of cultured cells and is secreted into culture medium (16). To determine the cellular compartments to which the NEFA protein is distributed, we transfected cDNA encoding GFP fused to full-length NEFA (amino acids 1–420) into mouse neuroblastoma N1E-115 cells (Fig. 9), which contain undetectable levels of necdin and a small amount of NEFA
(data not shown). NEFA-GFP fluorescence was detected in the cytoplasm, and some cells contained intensely fluorescent areas near the nucleus (Fig. 9A, left panel). Co-expression of necdin increased the fluorescence of NEFA-GFP in the cytoplasm (Fig. 9A, middle panel), whereas necdinΔN (amino acids 110–325) lacking the NEFA-binding site failed to decrease the cytoplasmic accumulation of NEFA-GFP (Fig. 9A, right panel). We analyzed the levels of the NEFA-GFP protein in the cells and the culture medium by Western blotting with antibody NET1. Co-transfection of necdin increased the amount of NEFA-GFP within the cells and reduced the amount in the culture medium, whereas necdinΔN failed to alter the amounts of NEFA-GFP in the cells and the medium (Fig. 9B). These results suggest that necdin increases the cellular retention of NEFA by inhibiting the secretion of NEFA.

To examine the physiological implications of the interaction between NEFA and necdin, we measured endogenous steady state levels of Ca2+ by Fura-2 fluorescence analysis and found that transfection of NEFA-GFP exerted no appreciable effects on basal Ca2+ levels (data not shown). Instead, we measured the caffeine-evoked cytosolic Ca2+ levels, which are believed to reflect the Ca2+ pool in the ER (17), because the Ca2+-binding protein NEFA is localized in the cisterna of the ER (17), because the NEFA-binding site failed to decrease the cytoplasmic accumulation of NEFA-GFP. To examine the physiological implications of the interaction between NEFA and necdin, we measured endogenous steady state levels of Ca2+ by Fura-2 fluorescence analysis and found that transfection of NEFA-GFP exerted no appreciable effects on basal Ca2+ levels (data not shown). Instead, we measured the caffeine-evoked cytosolic Ca2+ levels, which are believed to reflect the Ca2+ pool in the ER (17), because the Ca2+-binding protein NEFA is localized in the cisterna of the ER (17), because the NEFA-binding site failed to decrease the cytoplasmic accumulation of NEFA-GFP. To examine the physiological implications of the interaction between NEFA and necdin, we measured endogenous steady state levels of Ca2+ by Fura-2 fluorescence analysis and found that transfection of NEFA-GFP exerted no appreciable effects on basal Ca2+ levels (data not shown). Instead, we measured the caffeine-evoked cytosolic Ca2+ levels, which are believed to reflect the Ca2+ pool in the ER (17), because the Ca2+-binding protein NEFA is localized in the cisterna of the ER (17), because the NEFA-binding site failed to decrease the cytoplasmic accumulation of NEFA-GFP.
Because Nuc binds to Ca\(^{2+}\) via its EF-hand domain and also interacts with DNA (12, 23), NEFA was initially thought to be a Ca\(^{2+}\)-binding protein that interacts with nuclear DNA (16). Although its DNA binding activity is still unclear, the Ca\(^{2+}\)-binding property of purified NEFA protein has been confirmed (Ref. 24 and Fig. 4C). Invertebrate homologs of mammalian Nuc and NEFA isolated from insects Spodoptera frugiperda and Drosophila melanogaster possess two EF-hand motifs and intervening acidic region but no Leu zipper (25, 26), indicating that the Ca\(^{2+}\)-binding domain is evolutionally conserved. Because necdin binds to the region encompassing the two EF-hand motifs (Fig. 2A), it seems likely that necdin binds to this conserved domain of Nuc.

Necdin is originally identified as a nuclear protein by immunohistochemistry (2, 3). The fact that necdin interacts with nuclear proteins such as E2F1 and p53 also supports the idea that necdin exerts its functions within the nucleus. However, our recent study using a new antibody (NC243) has shown that a considerable amount of necdin is present in the cytoplasm of differentiated neurons (10). Because necdin has no specific organelle-targeting signal sequences, intracellular distribution of necdin may depend on the localization of its target proteins. Necdin and NEFA are co-expressed in neurons and skeletal muscle cells after terminal differentiation (Ref. 4 and Fig. 5B). Thus, NEFA is likely to contribute, at least in part, to the sequestration of necdin in the cytoplasm of these postmitotic cells. It is noteworthy that p53, a target of necdin, stays in the nucleus during neuronal differentiation but is present in the cytoplasm of mature differentiated cells (27). Furthermore, E2F1, another target of necdin, is localized in the cytoplasm of terminally differentiated skeletal muscle (28). These findings suggest that necdin is sequestered in the cytoplasm of postmitotic cells by forming complexes with p53, E2F1, and NEFA.

We demonstrated that NEFA is distributed ubiquitously in the tissues of neonatal mice in vivo (Fig. 5B). In the brain, NEFA is localized in the perikarya and dendrites of virtually all neurons (Fig. 7). Although NEFA is distributed in all of the subcellular fractions of P19 cell-derived neurons (Fig. 6C), this protein is enriched in the cisternae of the ER and NE of neurons in vivo (Fig. 8). Recently, Nuc (CALNUC) has been iden-
tified as a Golgi resident protein (22). These results suggest that NEFA and Nuc are involved in Ca^{2+} homeostasis or Ca^{2+}-dependent physiological processes operative in intracellular organelles such as the ER, NE, and Golgi apparatus. Although it is unclear whether necdin is localized in the cisternae of the ER, NE, and Golgi apparatus, necdin may regulate the translocation of NEFA into the lumen of the ER. The present study has shown that necdin blocks the secretion of NEFA-GFP into extracellular space and induces intracellular retention of NEFA-GFP, which gives rise to the potentiation of caffeine-induced cytosolic Ca^{2+} elevation (Fig. 9). Because NEFA is a calcium-binding protein in the cisterna of the ER, the Ca^{2+} storage may be increased by accumulation of NEFA, which is enhanced by necdin. It is also possible that sensitivities of the ryanodine receptors to caffeine are increased by overexpressed NEFA whose effect is enhanced by necdin. Another possibility is that NEFA and necdin cooperatively increase the cytosolic levels of Ca^{2+}, which is rapidly taken up into the ER, resulting in the increase in the caffeine releasable Ca^{2+} storage.

The present findings that both necdin and NEFA are expressed in differentiated neurons and skeletal muscle raise the possibility that these proteins are involved in the regulation of Ca^{2+} homeostasis in postmitotic cells under physiological and pathological conditions. The Ca^{2+} release from internal stores such as the ER and the sarcoplasmic reticulum is important for physiological functions (e.g. neurotransmitter release, muscle contraction; reviewed in Ref. 29). In addition, high levels of Ca^{2+} in the ER are essential for the synthesis and processing of proteins. The decline in the Ca^{2+} levels in the ER would activate the genes associated with cell death (apoptosis). Furthermore, the NE is also regarded as a major intracellular storage site, and nuclear Ca^{2+} is suggested to modulate nuclear functions such as transcription and apoptosis (reviewed in Ref. 30). Thus, necdin and NEFA are likely to be involved in the regulation of survival and death of postmitotic cells by controlling Ca^{2+} homeostasis in the cytoplas.

The human necdin gene NDN is located on chromosome 15 q11.2-q12, which is known as the region deleted in the Prader-Willi syndrome (PWS) (31). NDN, a maternally imprinted gene, is transcribed only from the paternal allele, and necdin is not expressed in tissues of PWS patients (6, 32, 33). PWS is a neurogenetic disorder whose major symptoms such as feeding problems, gross obesity, and hypogonadism are consistent with a hypothalamic defect. Necdin and NEFA are present in the cytoplasm of postmitotic neurons and are specifically enriched in hypothalamic neurons (Refs. 3, 4, and Fig. 7, F and G). We assume that impaired Ca^{2+} homeostasis caused by necdin deficiency may induce neuronal dysfunction in the hypothalamus. Further studies on physiological implications of the interactions between necdin and NEFA provide insights into the involvement of necdin in the PWS pathogenesis.

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References

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