A Novel NE-dlg/SAP102-associated Protein, p51-nedasin, Related to the Amidohydrolase Superfamily, Interferes with the Association between NE-dlg/SAP102 and N-Methyl-D-aspartate Receptor*

(Received for publication, February 24, 1999, and in revised form, August 6, 1999)

Hiroaki Kuwahara‡§, Norie Araki‡, Keishi Makino‡, Norio Masuko‡, Shinobu Honda‡§, Kozo Kibuchi‡, Kohji Fukunaga‡, Eishichi Miyamoto‡, Michio Ogawa‡, and Hideyuki Saya‡**

From the Department of Tumor Genetics and Biology, the Department of Surgery II, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan and the Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma 630-0101, and the Department of Pharmacology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan

The membrane-associated guanylate kinase proteins have been known to interact with various membrane receptors with their N-terminal segments designated the PDZ domains and to cluster these receptors at the target site of the cell membrane. NE-dlg/SAP102, a neuronal and endocrine tissue-specific MAGUK family protein, was found to be expressed in both dendrites and cell bodies in neuronal cells. Although NE-dlg/SAP102 localized at dendrites was shown to interact with N-methyl-D-aspartate receptor 2B via the PDZ domains to compose postsynaptic density, the binding proteins existing in the cell body of the neuron are still unknown. Here we report the isolation of a novel NE-dlg/SAP102-associated protein, p51-nedasin. Nedasin has a significant homology with amidohydrolase superfamily proteins and shows identical sequences to a recently identified protein that has guanine aminohydrolase activity. Nedasin has four alternative splice variants (S, V1, V2, and V3) that exhibited different C-terminal structures. NE-dlg/SAP102 is shown to interact with only the S form of nedasin which is predominantly expressed in brain. The expression of nedasin in neuronal cells increases in parallel with the progress of synaptogenesis and is mainly detected in cell bodies where it co-localizes with NE-dlg/SAP102. Furthermore, nedasin interferes with the association between NE-dlg/SAP102 and NMDA receptor 2B in vitro. These findings suggest that alternative splicing of nedasin may play a role in the formation and/or structural change in synapses during neuronal development by modifying clustering of neurotransmitter receptors at the synaptic sites.

At the sites of cell-cell contacts of epithelial cells or the synaptic junctions of neuronal cells, several membrane receptors and channels are clustered into multiprotein complexes linked to the cytoskeleton via interactions of their C-terminal cytoplasmic tails with a novel protein family called membrane-associated guanylate kinase homologues (MAGUKs) (1). The MAGUK family proteins contain three distinct domains as follows: an N-terminal segment comprised of one or three copies of an 80–90-amino acid motif called the PDZ (PSD-95/Dlg/ZO-1) domain, an src homology 3 (SH3) domain, and a region with high similarity to guanylate kinases (GK) (2, 3). The PDZ domain is utilized as a module for interacting with the C-terminal Xaa-(Ser/Thr)-Xaa-Val (X(S/T)XV) motif of various proteins and generating multiprotein complexes (4, 5).

Each MAGUK protein is thought to perform a distinct function depending upon its tissue distribution, cellular localization, and associated molecules. For instance, PSD-95/SAP90, which is one of the MAGUK proteins, is predominantly expressed in the brain and localizes at the postsynaptic membrane and presynaptic axonal terminals of inhibitory neurons (6–8). PSD-95/SAP90 binds to the cytoplasmic tail of both Shaker-type voltage-gated K⁺ channels and the 2B subunit of N-methyl-D-aspartate (NMDA)-type glutamate receptors (7, 9–11). PSD-95/SAP90 expressed in neuronal cells is therefore thought to contribute to clustering ion channels and neurotransmitter receptors at the synaptic membranes. Mutations of the lethal (1)-discs large (dlg) gene in Drosophila, which also encodes a MAGUK family protein, was shown to cause postsynaptic structural defects (12, 13), suggesting that the dlg protein and its associated proteins are involved in the maturation of neuronal cells. These lines of evidence indicate that some MAGUKs play a role in synaptic organization in neuronal cells by linking interacting receptors to downstream signal molecules and regulating the structure of the synaptic junction.

We recently identified a novel member of human MAGUK protein, NE-dlg (neuronal and endocrine dlg) (14). NE-dlg is considered to be a human homologue of the rat postsynaptic protein SAP102 (10, 15), since the two proteins share 86% amino acid identity. NE-dlg/SAP102 contains three PDZ domains, an SH3 domain and a GK domain as do PSD-95/SAP90, and is highly expressed in neuronal and endocrine tissues. In the neurons, NE-dlg/SAP102 has been shown to be expressed in axons and dendrites (10, 14) and to bind to NMDA receptor...
subunit 2B (NR2B) at the synaptic membrane sites (15). Furthermore, NE-dlg/SAP102 has been found to interact with PSD-95/SAP90 in the presence of calmodulin and Ca\(^2+\) and is speculated to regulate the clustering of NMDA receptors to form the synapses at the specific site of membrane (16). However, the NE-dlg/SAP102 is abundantly expressed also in cytoplasm of the matured neuron, which is not co-localized with NR2B (16). Therefore, it is possible that NE-dlg/SAP102 has some interactive molecules in the cytoplasm of the neuron, and it may modulate the NE-dlg/SAP102-related signaling in neuronal cells.

In this study, we tried to identify a cytoplasmic NE-dlg/SAP102-interacting protein using GST-NE-dlg/SAP102 affinity column chromatography. From a bovine brain cytosol, we purified and determined a novel amidohydrolase superfamily protein, termed nedasin, that interacts with the PDZ domains of NE-dlg/SAP102 both in vitro and in vivo. Immunolocalization study shows that nedasin and NE-dlg/SAP102 co-localize at cell bodies of neuronal cells. Nedasin was shown to have four alternative splicing isoforms that have diversity at their C-terminal tails, and one isoform, called nedasin S, specifically binds to NE-dlg/SAP102. We also found that the nedasin S isoform competitively inhibits the binding between the NR2B subunit of NMDA receptors and the PDZ domains of NE-dlg/SAP102. These results suggest that nedasin modifies the dlg-related molecular clustering at the synaptic sites during development of neuronal cells and that alternative splicing of the nedasin transcript may affect this interaction.

**EXPERIMENTAL PROCEDURES**

**Construction of Various GST Fusion Proteins**—The cDNA fragments coding full-length NE-dlg/SAP102 and six deletion variants ΔGR, PDZ1 + 2 + 3, PDZ1 + 2, PDZ2, and PDZ1, as illustrated in Fig. 4, were amplified by PCR, subcloned into a pCR2 TA cloning vector (Invitrogen, San Diego, CA), digested with EcoRI or with both EcoRI and HindIII, excised an inserted cDNA, and subcloned into a pGEX-2TH bacterial expression vector. The cDNA fragments coding full-length nedasin S and nedasin V1 were also amplified by PCR and subcloned into a pGEX-2TH vector. The expression and purification of GST fusion proteins were performed previously (17).

**Cytosol Preparation of Bovine Brain and Rat Brain**—Cytosol of bovine brain was prepared as described (18). In brief, bovine brain gray matter was cut into small pieces and suspended in homogenizing buffer A (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl\(_2\), 10% sucrose). The suspension was homogenized with a Potter-Elvehjem Teflon glass homogenizer and filtered through gauze. The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C. Solid ammonium sulfate was added to the supernatant to a final concentration of 40% saturation. After being stirred for 1 h, the precipitate was collected by centrifugation and dissolved in 4 ml of buffer A, diazylated against buffer A three times, and stored at −80 °C as the 0–40% cytosolic fraction. Subsequently, the supernatant was saturated by adding solid ammonium sulfate to a final concentration of 80%. The precipitate was collected, dissolved in buffer A, diazylated as described above, and stored as the 40–80% cytosol fraction. All procedures were performed at 4 °C.

Cytosol of rat brain was prepared as described (19, 20) with minor modifications. In brief, adult rat brain was homogenized in 5 volumes of buffer B (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 1 mM sodium orthovanadate, 1 mM monoethylbenzenesulfonyl fluoride, 10 mM pepstatin, 3% aprotinin, and 10 mg/ml leupeptin with a Potter-Elvehjem Teflon glass homogenizer and centrifuged at 1,000 rpm for 10 min. The supernatant was added to Nonidet P-40 to a final concentration of 1%, lysed for 60 min, and then centrifuged at 14,000 rpm for 60 min. The resulting supernatant was used as the crude cytosol of rat brain. All procedures were performed at 4 °C.

**GST-NE-dlg Affinity Chromatography**—The GST-NE-dlgAGK fusion protein (400 µg) was immobilized on GSH-agarose, which was packed into a column. The column was equilibrated with buffer B (30 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl\(_2\), 1 mM dithiothreitol). Bovine brain cytosolic fraction (800 µl) was first precleared by passing it through a GSH column and then was loaded onto the GST-NE-dlgAGK affinity column. The column was washed with 2 ml of buffer B, and the protein bound to the column was eluted by the addition of 5 ml of buffer C (buffer A containing 0.5 M NaCl), and fractions of 1 ml each were collected. The second and third fractions were mixed and loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Purification of p51 and Determination of Peptide Sequence**—To purify p51, 4 ml of the cytosol fraction of bovine brain was applied to 2 ml of GST-NE-dlgAGK immobilized to GSH beads at a half-slurry with phosphate-buffered saline (PBS). Eluates were collected and dialyzed against distilled water. After being concentrated to a 100-µl solution by freeze-drying, the sample was loaded on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was stained with Ponceau S, and immobilized p51 was cut out from membrane. After being reduced and carboxymethylated, p51 was digested by Acromobacter protease I. After sonication, the supernatant was loaded into a C20 chromatography column. Fractionated samples were subjected to amino acid sequencing using the 492 Procise protein sequencing system (Perkin-Elmer).

**PCR-based Full-length Nedasin cDNA Cloning**—The PCR-based full-length nedasin cDNA cloning was performed as previously reported (14). Primers for cloning (A1, 5'-TGGTTGCAATCCAGGCCCATT-3'; A2, 5'-CTTATACCCAGTTATAAAACCTA-3'; A3, 5'-CATCACGTGCATTATTGGTCAAAAG-3'; A4, 5'-CACAGATGTGGATGTATTTTAATACCTA-3') were designed based on the sequence information of the EST clone R34820 whose open reading frames contained two peptide sequences of the purified p51 protein. For amplification of the 5′ region, the first PCR was performed using only primer A1 to amplify single-strand cDNA from a HeLa cell cDNA library (Marathon Ready\(^\text{TM}\) cDNA, CLONTECH, Palo Alto, CA). The first PCR product was used as a template in the second run, where the A2 primer and adaptor primer 1 (5'-CCATCTAATGACTCATATAGGGC-3'), based on the sequence of the Marathon cDNA adaptor, were utilized as primers to amplify the 5′ region cDNA. Amplification of the 3′ region was also performed by the same two-step PCR procedure. A3 primer was used for amplification of genomic PCR to amplify the 3′ region, and A4 and adaptor primer 1 were used for the second-round PCR. The PCR fragments were ligated into a pGEM-T Easy cloning vector (Promega, Madison, WI) and sequenced. All PCR procedures were performed by using Tth DNA polymerase (Perkin-Elmer), which has proof-reading activity. The nucleotide sequence was confirmed by sequencing several clones that were generated by independent PCR to avoid errors introduced during the PCR reaction.

**PCR Mapping of nedasin Gene**—PCR was performed to detect nedasin sequences in the GeneBridge 4 Radiation Hybrid Screening Panel (Research Genetics, Huntsville, AL) using a set of primers (B1, 5'-ATTGAAGGGTTTATGTGGGC-3'; B2, 5'-CAAGGGAGATGGCACACCC-3') that were designed based on a partial genomic sequence of the nedasin gene. PCR was carried out as described previously (14), and the PCR results were sent to the Whitehead Institute/MIT Center for Genome Research for the mapping of the gene (21).

**Northern Blotting**—A Northern blot derived from various human tissues (CLONTECH) was probed with an 848-bp cDNA fragment of nedasin that had been labeled with \([\alpha-\text{32P}]/\text{dCTP as described previously}\) (14).

**mRNA Extraction and cDNA Synthesis**—Tissue samples were frozen.
Characteristics of p51-nedasin.

A. Deduced amino acid sequence of nedasin and comparison of sequence identities in proteins encoded by the S. pombe SPAC2C6.08 and S. cerevisiae YDL238c genes with nedasin. The GenBank data base accession number of nedasin is AF019638.

B.

C.

D.

E.

Fig. 2. Characteristics of p51-nedasin. A, deduced amino acid sequence of nedasin and comparison of sequence identities in proteins encoded by the S. pombe SPAC2C6.08 and S. cerevisiae YDL238c genes with nedasin. The GenBank data base accession number of nedasin is AF019638.
immediately after surgical resection and stored at −80 °C. Poly(A)+ mRNA was extracted using a Micro Fast Track kit (Invitrogen). First-strand cDNA was synthesized from mRNA with Superscript II reverse transcriptase and random primers. The cDNA was used for the subsequent RT-PCR reaction. Human fetal brain and HeLa cell cDNA libraries (Marathon-Ready™ cDNA) were purchased from CLONTECH.

RT-PCR—The sequences of the primers for RT-PCR were P1 (5’-ATTGAGGTTTGGGTTGCCC-3’) and P2 (5’-CAAGGGAGATCGACAACCCACGCTA-3’). The PCR reaction was performed in a volume of 15 μl containing 0.5 μl of cDNA, 10 pm primers, 10× PCR mixture, 1.5 μl of 25 mM MgCl₂, 1.5 μl of GeneAmp dNTP mixture, and 0.5 units of AmpliTaq Gold DNA polymerase. The PCR cycle sequence consisted of an initial denaturation at 96 °C for 90 s and 40 cycles of 96 °C/30 s, 60 °C/30 s, and 72 °C/30 s and a final extension at 72 °C/7 min using the Perkin-Elmer PCR thermocycler 2400. Amplified products (5 μl) were resolved on 2.5% ethidium bromide-stained TAE-agarose gels.

Production of Anti-nedasin Polyclonal Antibody—An antibody against the C-terminal region of nedasin was raised by the subcutaneous immunization of a rabbit with a synthetic peptide (RNIEEVYVGCQVFPSSSV) coupled to the keyhole limpet hemocyanin.

Construction of the Nedasin Expression Plasmids—Both the S form and V1 constructs were amplified by PCR from the HeLa cDNA library using rTth DNA polymerase and a set of primers, P3 (5’-TGGCGGAATTGGGACCAGATTGTGGCGCTAGATAAGCGG-3’) and P4 (5’-AACATAGTCTCAAGAATTTAAGGAAATCGTGGAACGATGG-3’). The PCR products were then digested with BamHI and ligated into pBj-Myc vector, which has the Cre-mediated gene activation unit, to form pCALNL5/nedasin S or pCALNL5/nedasin V1. The S and V1 cDNA were then ligated into pBj-Myc/nedasin S or pBj-Myc/nedasin V1. The HA-tagged full-length NE-dlg/SAP102 and nedasin S were then digested with BamHI and ligated into pBj-Myc to construct pBj-Myc/nedasin S or pBj-Myc/nedasin V1. The S and V1 cDNA were then ligated into pCALNL5 vector, which has a Cre-recombinase mediated activation unit (22, 23), to construct pCALNL5/nedasin S or pCALNL5/nedasin V1. The HA-tagged full-length NE-dlg/SAP102-expressing plasmid, pCALNL5/nedasin S, was constructed as described previously (14). The pCGN/NE-dlg/ΔGK construct lacking a guanylate kinase domain was obtained by digesting the pCGN/full-length NE-dlg plasmid with BamHI and then self-ligated. The plasmids were transfected into COS-7 cells by the liposome-mediated gene transfer method.

Interaction of NE-dlg/SAP102 with Nedasin in Vitro—Various deletion derivatives of NE-dlg/SAP102 fused to GST or GST immobilized to GSH-agarose beads were incubated with the lysates of COS-7 cells, which were transfected with pBj-Myc/nedasin S or pBj-Myc/nedasin V1, for 2 h at 4 °C. After washing with TNN buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Nonidet P-40) 3 times, the precipitates were probed with the anti-Myc antibody.

Production of Anti-Nedasin Antibody—Both the S form and V1 constructs were amplified by PCR from the human chromosomal contents of all hybrids, determined by the Whitehead Institute/MIT technology, Inc.) or rabbit antin nedasin antibody. Western blot analysis of GST-NE-dlg affinity-purified p51 from bovine brain extract. The eluted fraction was immunoblotted with anti-nedasin antibody (lane 1) and preimmune serum (lane 2). The molecular mass (kDa) is indicated on the left. D, digestion of nedasin gene by PCR-based radiation hybrid mapping. Screening of the Nedasin GeneBridge 4 radiation-hybrid mapping panel and linkage analysis based on comparison with the human chromosomal contents of all hybrids, determined by the Whitehead Institute/MIT Center for Genome Research, revealed that nedasin localized at 7.5 cR (cR) from D9S166 on chromosome 9. The surrounding markers and the interlocus distances for the markers are shown on the right. E, expression of nedasin transcripts. Northern blot derived from various human tissues (Human Multiple Tissue Northern blot III, CLONTECH, Palo Alto, CA) was hybridized with an 848-bp cDNA fragment of nedasin gene. The arrowhead indicates the position of the nedasin transcript. The size marker (kilobase pairs) is indicated on the left.
Isolation of the p51 cDNA—To clarify the molecular identity of p51, the purified protein was subjected to amino acid sequencing, and 10 peptide sequences derived from p51 were determined. These peptide sequences did not match any previously identified molecules, but two peptide sequences (NLVPSYK and NYTSVYD) were found in one of the open reading frames of a human EST clone, R34820. Based on the sequence of this EST clone, we performed a two-step polymerase.

**FIG. 3.** Four variant forms of nedasin generated by alternative splicing. A, a schematic representation of the splice variants of nedasin. The putative tetrapeptide sequences at the C-terminal end of each isoform is shown in bold. Arrows indicate the positions of primers for RT-PCR analysis shown in C. White bars indicate the nedasin cDNA. The hatched, gray, and black boxes shown in A and B indicate the exons alternatively spliced at the C-terminal region of nedasin. Nedasin V1 transcript lacks the sequence indicated by the black box. The V2 transcript lacks the sequence indicated by the gray and black boxes. The V3 transcript lacks the sequence indicated by the hatched, gray, and black boxes. aa, amino acid.

**B,** comparison of 3′-terminal cDNA sequences of nedasin splice variants. Arrows (P1 and P2) indicate the positions of primers for the RT-PCR analysis shown in C. TAA in the box indicates the position of the putative translation stop codon of nedasin S. Nucleotide numbers from the first nucleic acid of the start codon are shown on the right.

**C,** expression of nedasin splice variants. Amplification of the C-terminal region of nedasin transcripts by RT-PCR using extracted mRNA from various human normal tissues and cancer cell lines. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was amplified to ensure the successful completion of cDNA synthesis and PCR reaction. HFB, human fetal brain.
Nedasin, a Novel NE-dlg/SAP102-binding Protein

A.

**Probe: α-Myc**

1. **Myc-nedasin V1**
2. **Myc-nedasin S**
3. Input
4. GST
5. Input
6. GST

B.

**NE-dlg full**

1. PDZ1
2. PDZ2
3. PDZ3
4. SH3
5. GK

**NE-dlg ΔGK**

1. PDZ1
2. PDZ2
3. PDZ3
4. SH3
5. GK

**NE-dlg PDZ1+2**

1. PDZ1
2. PDZ2
3. SH3
4. GK

**NE-dlg PDZ1**

1. PDZ1
2. SH3
3. GK

**NE-dlg PDZ2**

1. PDZ2
2. SH3
3. GK

C.

**Probe: α-GST**

1. Input
2. PDZ1+2+3
3. PDZ1+2
4. PDZ2
5. PDZ1
6. GST
7. Input
8. GST

**Probe: α-Myc**

1. Input
2. PDZ1+2+3
3. PDZ1+2
4. PDZ2
5. PDZ1
6. GST
7. Input
8. GST

**Fig. 4. Interaction of NE-dlg/SAP102 with nedasin.**

A. *In vitro* association of NE-dlg/SAP102 with nedasin S but not with nedasin V1. After the lysates of COS-7 cells expressing Myc-nedasin V1 (lanes 1–3) or Myc-nedasin S (lanes 4–6) were incubated with full-length NE-dlg/SAP102 fused with GST (full, lanes 2 and 5) or GST (lanes 3 and 6) for 2 h, the samples were precipitated with GSH-agarose. The precipitates were probed with the anti-Myc antibody. The arrow indicates the position of Myc-nedasin V1 or Myc-nedasin S. The neighbors of NE-dlg/SAP102 deletion mutants with nedasin S. The lysates of COS-7 cells expressing Myc-nedasin S were incubated with NE-dlg/SAP102 deletion mutants, which are illustrated in Fig. 3B, fused with GST (lanes 2–7) or GST (lane 8) for 2 h. The samples were then precipitated with GSH-agarose, and the precipitates were probed with the anti-Myc (upper panel) and anti-GST (lower panel) antibodies. The arrow and arrowheads indicate the position of Myc-nedasin S and NE-dlg/SAP102 deletion mutants fused with GST, respectively.

We identified a 2,040-bp cDNA that contains one large open reading frame encoding a polypeptide of 454 amino acids (GenBank accession number AF019638). The calculated molecular mass was 51,003 Da, which is close to the apparent molecular mass of p51 estimated by SDS-PAGE. The deduced amino acid sequences are shown in Fig. 2A. All 10 of the peptide sequences determined above were found within the deduced amino acid sequences. The neighboring sequence around the putative initiation codon was consistent with the translation initiation start site proposed by Kozak, and we found an in-frame termination codon in the preceding region. We designated p51 as nedasin (NE-dlg associated protein). It is noteworthy that nedasin has a serine-serine-valine sequence at its C-terminal end, which is known as a consensus motif (X(S/T)(XV-COOH) utilized for binding to the PDZ domain of the MAGUKs.

The predicted amino acid sequence of nedasin showed 40%
Nedasin, a Novel NE-dlg/SAP102-binding Protein

Identification of Splice Variants of Nedasin—In the course of the cDNA cloning of nedasin, we found that nedasin had 4 alternative splicing variants that generate different C-terminal sequences. As shown in Fig. 2A, the initially identified form, designated the S (standard) form, consists of 454 amino acid residues that have an SSSV (Ser-Ser-Ser-Val) sequence at the C-terminal end. The other three variants, designated V1, V2, and V3, were found to consist of 471, 505, and 460 amino acids, respectively (Fig. 3, A and B). These variant forms do not possess an XIS/TXV motif at their C-terminal ends. The V1 transcript was found to lack a 68-bp exon of the S transcript, which causes a frameshift of the translation, resulting in the addition of 17 amino acids at the C-terminal end of nedasin S (Fig. 3B). The V2 transcript lacks an additional 23-bp exon of the V1 transcript, which leads to an extension of translation, generating a 505-amino acid protein. The V3 transcript further lacks a 44-bp exon of V2 transcript, which causes a frameshift resulting in the generation of a 460-amino acid protein (Fig. 3B). To examine the expression patterns of these 4 splice variants in various human tissues and cancer cell lines, we performed RT-PCR analysis using a set of primers, P1 and P2, which are capable of amplifying all 4 forms of nedasin transcripts. Most of the normal tissues and cancer cell lines were found to express the S and V1 transcripts (Fig. 3C).

In Vitro Interaction between NE-dlg/SAP102 and Nedasin—The RT-PCR analysis of nedasin transcripts revealed that the S and V1 isoforms are expressed in human brain tissues, in which NE-dlg/SAP102 is also highly expressed. To address which isoforms of nedasin interact with NE-dlg/SAP102, immobilized GST-NE-dlg was mixed with the lysate of COS-7 cells that were transfected with Myc-tagged nedasin S (Myc-nedasin S) expression plasmid or Myc-tagged nedasin V1 (Myc-nedasin V1) expression plasmid. The Myc-nedasin S was co-precipitated with GST-NE-dlg, whereas the Myc-nedasin V1 was not (Fig. 4A, lanes 2 and 5).

To determine which region of NE-dlg/SAP102 interacts with nedasin, various deletion derivatives of NE-dlg/SAP102 fused to GST (Fig. 4B) were immobilized to glutathione-agarose beads and were mixed with COS-7 cell lysates which were transiently transfected with Myc-nedasin S expression plasmid. Co-precipitated Myc-nedasin S was detected with an anti-Myc monoclonal antibody. Nedasin S was co-precipitated with both the PDZ1 and PDZ2 domains of NE-dlg/SAP102 (Fig. 4C, lanes 6 and 7). Interestingly, this association was enhanced when GST fusions contained both a PDZ1 and PDZ2 domain (Fig. 4C, lanes 2–4). Nedasin did not bind to the PDZ3 domain alone (Fig. 4C, lane 5). These findings indicated that the C-
terminal X(S/T)XV motif, which exists in the S form but not in the V1 form, interacts with the PDZ1 and PDZ2 domains of NE-dlg/SAP102. We therefore suggest that the interaction between NE-dlg/SAP102 and nedasin is regulated by an alternative splicing mechanism of the nedasin transcript.

**Intracellular Association between NE-dlg/SAP102 and Nedasin**—To examine whether NE-dlg/SAP102 interacts with nedasin in intact cells, we co-expressed HA-tagged NE-dlg/SAP102 lacking the GK domain (NE-dlgΔGK) with Myc-tagged nedasin S or V1 in COS-7 cells. The HA-tagged human NF2 protein (72 kDa) expression plasmid was used as a negative control. The cell lysates were immunoprecipitated with an anti-Myc monoclonal antibody (9E10), and the precipitates were immunoblotted with an anti-HA monoclonal antibody (12CA5). NE-dlg/SAP102 was co-immunoprecipitated with nedasin S but not with nedasin V1 (Fig. 5, left panel). Conversely, nedasin S was co-immunoprecipitated with NE-dlgΔGK when the same lysates were immunoprecipitated with the anti-HA monoclonal antibody (Fig. 5, right panel).

**Detection of the Endogenous Nedasin Protein and Its in Vivo Interaction with NE-dlg/SAP102**—To detect the endogenous nedasin protein, we performed a Western blot analysis using the anti-nedasin antibody. A band of about 51 and 52 kDa was detected in lysates of nedasin S- and V1-expressing COS-7 cells.
which were transiently transfected with pCALNL5/nedasin S or pCALNL5/nedasin V1, respectively. This finding indicates that the antibody can detect both the S and V1 isoforms. By using this antibody, we performed Western blot analysis to detect endogenous nedasin in human and rat brain tissues, HeLa cells, and MCF-7 cells. The 51-kDa band, which corre-
sponds to the nedasin S form, was detected in lysates from human and rat brain but not from MCF-7 cells, in which the nedasin transcript was also not detected. Both S and V1 forms were found in HeLa cells (Fig. 6A). Moreover, nedasin was detected in both cytosolic and nuclear fractions of HeLa cells, indicating that nedasin is not only the nucleus but also the cytoplasmic protein (data not shown).

Next, we investigated the changes in expression of nedasin in the cultured neonatal rat neuronal cells by Western blot analysis. Nedasin protein was not detected in the 1st day of cultured neonatal neuronal cells and slowly increased until 7 days in culture. However, the expression level of nedasin began to increase from 14 days and continued to elevate at 21 days (Fig. 6B). In contrast, NE-dlg/SAP102 protein began to elevate a few days earlier than nedasin and reached a maximum at 14 days of culture (16).

By employing the anti-nedasin antibody and the adult rat brain which abundantly expresses both nedasin and NE-dlg/SAP102, we examined in vivo interaction between endogenous nedasin and NE-dlg/SAP102. As shown Fig. 6C, nedasin was co-immunoprecipitated with NE-dlg/SAP102 from the detergent-solubilized extract of adult rat brain. These results strongly suggest that NE-dlg/SAP102 interacts with nedasin in neural cells as well as in vitro.

**Immunolocalization of Nedasin in Cultured Neuronal Cells—**
To examine the subcellular localization of nedasin and NE-dlg/SAP102, cultured human neural cells were immunostained with anti-nedasin and anti-NE-dlg/SAP102 antibodies. In the cells for inducing neuronal differentiation, the antibody against NE-dlg/SAP102 gave punctate immunoreactivity in cell bodies and the dendrites (Fig. 7A), and the anti-nedasin antibody gave a similar staining in the cell bodies but not along dendrites (Fig. 7B). As shown in Fig. 7C, nedasin co-localized closely with NE-dlg/SAP102 in neuronal cell bodies.

**Effect of Nedasin S on the Interaction between NE-dlg/SAP102 and C-terminal Peptides of NMDA Receptor Subunit 2B—**
The PDZ1 and PDZ2 domains of MAGUK family proteins are known to preferentially bind to the C-terminal end of NMDA receptor subunit 2B (NR2B) and Shaker-type K⁺ channels. Thus, we examined whether nedasin S interfered with these interactions by a surface plasmon resonance analysis. We immobilized two peptides on BIAcore sensor chips as follows: a peptide containing the C-terminal 15 residues of NR2B (PEP7154), and the same peptide lacking the last valine (PEP7153) as a negative control. The superfusion of the immobilized peptides with a GST fusion protein from NE-dlg/SAP102 that contained the first two PDZ domains (GST-NE-dlg PDZ1 PDZ2) resulted in a strong signal for PEP7154 peptide (Kₐ = 6.5 ± 1.1 nM) but not for PEP7153 peptide. Superfusion with the GST protein alone failed to elicit a binding signal (data not shown). The interaction between GST-NE-dlg PDZ1 + 2 and PEP7154 was competitively inhibited by GST-nedasin S in a dose-dependent manner (Fig. 8A) but not by GST-nedasin V1 (Fig. 8B).

**DISCUSSION**

In the present study, by means of GST-NE-dlg affinity col-
umn chromatography, we have purified a protein that specifi-
cally interacts with the NE-dlg/SAP102 protein. We deter-
dined its primary structure and named it nedasin. The homology analysis revealed that nedasin has a significant sim-
ilarity to amidohydrolase superfamily proteins such as atzA, atzB, dihydroorotases, ureases, adenine deaminases, and cyto-
sine deaminases. These proteins have a histidine-aspartic acid signature which is required for metal binding (27) and are known to catalyze hydrolytic reactions with nitrogen heterocy-
clic ring substrates (29). During the preparation of this manu-

![Fig. 7. Co-localization of nedasin and NE-dlg/SAP102 in the cultured neuronal cells.](image-url)
script, cDNA encoding a human protein having guanine amidohydrolase (GAH) activity was reported (28), and its deduced amino acid sequence is identical to nedasin. GAH catalyzes the hydrolytic deamination of guanine, yielding xanthine and ammonium, and is considered to be involved in a major pathway for producing uric acid (30). As consistent with our findings, GAH was shown to be abundant in brain, and its activity was detected in both nuclear and cytosolic fractions (31–33), although the biological significance has been largely unknown.

Recent observations suggest that some of the amidohydrolase superfamily proteins no longer function as enzymes but rather re-use the fold for another purpose, presumably another type of biological function. C. elegans UNC-33 protein, which belongs to this protein family, has been shown to be required for appropriately directed axonal extension (34, 35). Genetic studies revealed that a mutation in unc-33 causes severely uncoordinated movement and abnormalities in the elaboration of neuronal axons in nematode (34). Additionally, CRMP-62, a chicken homologue of UNC-33, was proposed to mediate collapsin-induced growth cone collapse during neuronal development (36). These findings raised the interesting possibility that some of the amidohydrolase superfamily proteins function as signal molecules required for various biological events such as neuronal development. It can therefore be speculated that nedasin modulates NE-dlg/SAP102-dependent cellular signaling by interacting with NE-dlg/SAP102.

The nedasin gene is mapped at 7.6 cR from D9S166 on chromosome 9. Genome-wide linkage analyses identified the hereditary inclusion body myopathy (hIBM) locus at D9S166 (maximum load score = 5.32, θ = 0.0) (37). The hIBM is a severe and progressive muscle disease of unknown etiology, characterized pathologically by vacuolated muscle fibers that contain 15–18-nm cytoplasmic tubulofilaments (38). The close proximity of nedasin gene to the hIBM locus raises the possibility that hIBM may be a related neurogenic disorder.

Nedasin was found to have four alternative splicing isoforms that have diversity at their C-terminal tails. Although nedasin S, which is an isoform dominantly expressed in neuronal tissues, possesses the X/S/T/XV consensus motif at its C-terminal tail for binding to PDZ domains, the other isoforms (V1, V2, and V3) do not have the PDZ-binding motif. We have shown that NE-dlg/SAP102 interacts with nedasin S both in vitro and in vivo and that the first two PDZ domains of NE-dlg/SAP102 are responsible for the interaction. However, this association has not been observed with the nedasin V1 isoform, which is predominantly expressed in non-neuronal tissues. These findings clearly indicate that the C-terminal sequence motif in nedasin S is required for binding to the first and second PDZ domains of NE-dlg/SAP102 and that the alterations in the C-terminal amino acids of nedasin caused by alternative splicing abolish this binding. An alternative splicing mechanism has been reported to affect the function, subcellular localization, and interaction of various proteins. A recent study demonstrated that the C-terminal tail of the membrane protein Ca\(^{2+}\) pumps is altered by alternative splicing and that one of the splicing isoforms (b type), which has the X/S/T/XV motif at its C-terminal tail, specifically interacts with PDZ domains of MAGUKs (39). This observation, together with our present findings, suggests that alternative splicing occurring at the C-terminal tails of the PDZ-binding proteins such as nedasin and Ca\(^{2+}\) pumps may regulate their specific interaction with MAGUK family proteins, potentially influencing their localization and function.

The PDZ domains of MAGUK family proteins were shown to interact with the C-terminal X/S/T/XV motif of various membrane and cytosolic proteins including the NR2B subunit of NMDA receptors (9, 10), Shaker-type K\(^+\) channels (7), tumor suppressor protein APC (the adenomatous polyposis coli gene...
product (14, 40), and oncoprotein E6 (41). Thus, the MAGUK proteins are thought to play an important role in cellular morphogenesis, adhesion, the regulation of ion density and cell growth by clustering various molecules via their PDZ domains. Nedasin S has been shown to associate with the PDZ1 and PDZ2 domains in NE-dlg/SAP102. Since NMDA receptors and K⁺ channels are known to interact strongly with the first and second PDZ domains of MAGUKs (42), we tested whether nedasin S interferes with the association between NE-dlg/SAP102 and the C-terminal oligopeptides of NR2B. The surface plasmon resonance analysis revealed that this interaction was competitively inhibited by nedasin S but not by nedasin V1. Nedasin might therefore modulate the receptor clustering function of the PDZ domains of MAGUKs, and this modulation is regulated by alternative splicing of nedasin transcript.

In the present study, we have demonstrated that the expression of nedasin increases in parallel with the progress of synaptogenesis and is abundantly detected in mature neuron. Furthermore, nedasin has been found to interact with NE-dlg/SAP102 in vivo, and they co-localize mainly at cell bodies in cultured human neuronal cells. In contrast, we previously showed that NE-dlg/SAP102 and NR2B co-localize in dendritic spines, at presumed synaptic sites, of cultured rat neuronal cells (16). Furthermore, NE-dlg/SAP102 was found to interact with PSD-95/SAP90 in the presence of Ca²⁺ and calmodulin, suggesting that Ca²⁺/calmodulin-dependent heteromeric complex formation of MAGUK proteins contributes to clustering of NR2B at membrane and plays a role in formation and/or structural change in synapses. Based on these findings, we speculate that the association of nedasin with NE-dlg/SAP102 may interfere with the clustering of NMDA receptors, resulting in alteration of intracellular distribution of the receptors and leading to appropriate assembly of the central synapses during neuronal development.

Some MAGUKs expressed in non-neuronal cells have been demonstrated to be associated with the signaling pathway of the cellular proliferation and/or tumorigenicity. In Drosophila, the recessive mutation at dig locus leads to not only defect of the postsynaptic structure as noted above but also disruption of normal cell-cell adhesion and the neoplastic overgrowth of the imaginal disc epithelium (2). In mammals, PDZ domains of a human homologue of dig gene product (HDLG-1) was found to interact with the tumor suppressor protein APC (40), and colon cancers frequently express truncated APC proteins that cannot interact with HDLG-1 (43). Moreover, the high risk human papilloma virus oncoprotein E6 was also found to bind to PDZ domains of HDLG-1 (44, 41). Thus, the MAGUKs expressed in non-neuronal cells have been demonstrated to be associated with the signaling pathway of the cellular proliferation and/or tumorigenicity.