We have identified a novel epidermal growth factor (EGF)-like repeat-containing single-pass transmembrane protein that is specifically expressed in the developing and mature central nervous system. Sequence analysis revealed that the 10 EGF-like repeats in the extracellular domain are closely related to those of the developmentally important receptor Notch and its ligand Delta. We thus named the molecule Delta/Notch-like EGF-related receptor (DNER). DNER protein is strongly expressed in several types of post-mitotic neurons, including cortical and hippocampal pyramidal neurons, cerebellar granule cells, and Purkinje cells. DNER protein is localized to the dendritic plasma membrane and endosomes and is excluded from the axons, even when overexpressed. The tyrosine-based sorting motif in the cytoplasmic domain is required for dendritic targeting of DNER. Direct in vivo binding of DNER to the coat-associated protein complex AP-1 strongly suggests that DNER undergoes AP-1-dependent sorting to the somatodendritic compartments from the trans-Golgi network and subsequent passage through the endosomal system.

Neurons are highly polarized cells with two types of structurally and functionally distinct processes, axons and dendrites. Axons are long thin processes that are specialized for the fast conduction of electrical impulses, whereas dendrites are short tapering processes subdivided into numerous branches and spines that receive and integrate chemical signals at synapses. The distinct properties of axons and dendrites are generated by specific localization of membrane proteins such as ion channels and transmitter and cytokine receptors as well as signaling molecules that decode the signal input and cytoskeletal components that scaffold the membrane and cytoplasmic proteins (1, 2). Hence, the precise targeting and anchoring of proteins are crucial for establishment of neuronal polarity.

The targeting of plasma membrane proteins to specific domains of neurons shares common mechanisms with that in polarized epithelial cells (3–5). The neuronal somatodendritic compartment is basically analogous to the epithelial basolateral domain, whereas the axonal membrane is analogous to the epithelial apical surface. Both targeting processes are initiated at the trans-Golgi network (TGN),1 where proteins are packaged into specific transport vesicles. Basolateral/somatodendritic targeting of transmembrane proteins is mediated by cytoplasmic sorting signals that can be grouped into two major classes (6–11). The first class is characterized by an essential tyrosine found in the context of NPXY or YXXØ (where X represents any amino acid and Ø is a bulky hydrophobic residue). The second class contains a dileucine sequence, in which one of the leucines is sometimes substituted by isoleucine, valine, alanine, or methionine (12). These motifs are thought to be responsible for interaction with the coat-associated protein complex AP-1, which mediates packaging of proteins into clathrin-coated transport vesicles targeted to the basolateral/somatodendritic membrane (13–16).

We have been interested in the molecular mechanisms underlyng polarization of developing central nervous system (CNS) neurons. Toward this goal, we performed PCR-based subtractive hybridization and identified genes that are differentially expressed in cerebellar granule cells at distinct developmental stages. This study reports a novel single-pass transmembrane protein with 10 extracellular repeats homologous to the epidermal growth factor (EGF). It has a short cytoplasmic tail with tyrosine-based and dileucine-type sorting motifs. We demonstrate that this novel transmembrane protein binds to the adaptor binding complex AP-1 by the tyrosine-based motif and is specifically sorted to the dendrites and cell bodies of developing and mature CNS neurons.

EXPERIMENTAL PROCEDURES

Subtractive Hybridization—PCR-based subtractive hybridization was performed using the PCR-Select cDNA subtraction kit (CLON-TECH) according to the manufacturer’s instructions. Total RNA was isolated from microexplant cultures of P4 mouse cerebellar external germinal layer, in which cellular morphogenesis of granule cells can be reconstituted (17). The mRNA from 3-day cultures of cerebellar granule cells was used to synthesize the tester cDNA, whereas the driver cDNA was made from those cells cultured for 2 days.

1 The abbreviations used are: TGN, trans-Golgi network; CNS, central nervous system; EGF, epidermal growth factor; DNER, Delta/Notch-like EGF-related receptor; P4, for example, postnatal day 4; E14.5, for example, embryonic day 14.5; HA, hemagglutinin; MAP2, microtubule-associated protein-2; contig, group of overlapping clones; CA, cornu ammonis.

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Cloning of Mouse and Human Delta/Notch-like EGF-related Receptor (DNER) cDNAs—A 800-bp fragment of DNER obtained by subtractive hybridization was used as a probe to screen a AZAP1 cDNA library (Strategene) prepared from P0–P10 mouse brains. The cDNA inserts of positive clones were subcloned into pBluescript (Strategene) and sequenced. The human homologue was then identified and the full-length cDNA was used as a probe to screen a human brain cDNA library (Strategene).

Northern Blotting—A mouse multiple-tissue Northern blot (CLONTECH) was probed with a riboprobe prepared from nucleotides 2577–3280 of DNER cDNA according to the manufacturer’s instructions. The membrane was hybridized in ExpressHyb solution (CLONTECH) at 65 °C overnight and washed under high-stringency conditions. The blot was then washed with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000) for 1 h at room temperature and incubated in CSPD solution (Roche Molecular Biochemicals). Membranes were then exposed to Kodak X-Omat AR films.

In Situ Hybridization—In situ hybridization was performed using 12-μm fresh-frozen cryosections of E14.5 whole embryos. Sections were fixed with 4% paraformaldehyde for 15 min at room temperature, permeated with 1 μg/ml proteinase K for 1 min, and then probed with a digoxigenin-labeled riboprobe of DNER in hybridization solution (50% formamide, 2× SSC, 10 mM Tris, 1× Denhardt’s solution, 0.2% SDS, and 0.5 mg/ml yeast tRNA) at 60 °C overnight. To remove excess probes in a series of wash buffers, sections were immersed in saline phosphate buffered saline-phosphatase-conjugated anti-digoxigenin antibody and then stained with anti-mouse alkaline phosphatase-conjugated antibody and 5-bromo-4-chloro-3-indolyl phosphate color substrate.

Anti-DNER Polyclonal Antibody—A polyclonal antibody against DNER protein was raised in rabbits by immunization with an 18-mer polypeptide corresponding to the mouse and human DNER carboxy terminus (N-DYSDPKVLVTLDKTL). The affinity-purified antibodies were used at 1:1000 for Western blot analysis and 1:300 for immunohistochemistry.

Cell Cultures—Primary cultures of rat Purkinje neurons were performed as previously described (18). Low-density cultures of hippocampal neurons were prepared by the method described by Goslin et al. (19).

Immunohistochemistry and Fluorescent Microscopy—Cultures and fresh-frozen cryosections (12 μm) were fixed with 4% paraformaldehyde for 15 min at room temperature. To specifically label the endocytosed signal, a preimmune IgG or anti-DNER antibody for 1 h at 4 °C, washed with ice-cold acidic solution (0.5 M NaCl and 0.2 M acetic acid) for 1 min prior to fixation to strip the surface protein. After permeabilization in 1.0% Tween 20 in phosphate-buffered saline, samples were processed for immunofluorescence. Primary antibodies or secondary antibodies used for staining were as follows: mouse monoclonal antibodies against calbindin-28 (1:1000; Swant), EEA1 (1:1000; Transduction Laboratories), MAP2 (1:200; Upstate Biotechnology, Inc.), Tau1 (1:200; Chemicon International, Inc.), and γ-adaptin (AP-1) (1:200, Transduction Laboratories); rabbit polyclonal antibody against the HA epitope (1:100; Upstate Biotechnology, Inc.); and Alexa 488- or Alexa 568-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:500; Molecular Probes, Inc.). After washing, slides were mounted in the glycerol-based medium SlowFade (Molecular Probes, Inc.) and analyzed using a Nikon E800 microscope equipped with Yokogawa CSU10 confocal scanner unit.

Western Blotting and Immunoprecipitation—Brains from P7 mice were homogenized in ice-cold homogenization buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 2% EDTA, 1% Nonidet P-40, 0.5% Triton X-100, and Complete protease inhibitor mixture (Roche Molecular Biochemicals)) and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was then washed under high-stringency conditions. The blot was incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000) for 1 h at room temperature and incubated in CSPD solution (Roche Molecular Biochemicals). Membranes were then exposed to Kodak X-Omat AR films.

RESULTS

Identification of Human and Mouse DNER cDNAs—In an attempt to identify the molecules that are involved in polarization of developing CNS neurons, we searched for molecules that are strongly expressed in cerebellar granule neurons during formation of dendrites and axons. A sensitive PCR-based differential subtractive hybridization followed by in situ hybridization revealed seven different cDNA fragments, the expression of which was significantly stronger in the differentiating granule cells than in mitotic cells in the external granular layer of the developing cerebellum (data not shown). One of these cDNA fragments (B-22, 733 bp) encoding an unknown cDNA sequence was used to screen a oligo(dT)-primed cDNA library from P0–P10 mouse cerebellum to obtain full-length cDNA. We isolated three cDNA clones and characterized a cDNA contig of 3294 bp (data not shown). A putative initiation codon that fulfills the criteria of Kozak (20) was identified at nucleotide 150. The first in-frame stop codon, followed by a putative polyadenylation signal (AATAAA) in the 3′-side, was at nucleotide 2356, predicting a protein product of 737 amino acids (Fig. 1A).

To identify the human homolog, the mouse full-length cDNA was used as a probe against a human brain cDNA library. We obtained the human full-length cDNA contig of 2854 bp. The molecule has been highly conserved during evolution, with overall identities of 85 and 90% at the nucleotide and protein levels, respectively. The human clone was mapped to chromosome 2q37 utilizing the human genome resources at NCBI. The N-terminal portion of the predicted protein contains a conventional signal peptide sequence with a putative cleavage site. The following stretch is characterized by 10 distinct EGF-like motifs, each of which is defined by pairing of cysteine residues (Fig. 1B). The EGF-like repeats are thought to mediate protein-protein interactions of various secreted and transmembrane proteins (21, 22). The tenth motif displays the typical signature of the calcium-binding EGF-like domain that is believed to be important for orientation of the neighboring modules to exert biological activities (23, 24). Over 300 types of EGF-like motifs have been classified by computer analysis, depending on the number and type of amino acids in the variable subdomains between the conserved cysteines (25). The EGF motifs of the protein demonstrate significant homology to those of Delta and Notch, the developmentally important ligand and receptor, respectively, with a single transmembrane segment (Fig. 1C). The predicted protein products of human and mouse cDNAs are also likely to have a single membrane-spanning region with a hydrophobic stretch of 30 amino acids. The intracellular carboxyl terminus comprises 70 amino acids, with a potential phosphorylation site by tyrosine kinases. The intracellular segment also contains a typical tyrosine-based sorting signal (YEEF) proximal to the membrane and a dileucine-type signal (LI) near the C terminus (Fig. 1, A and B). These motifs would bind to the coat-associated protein complexes AP-1, AP-2, and AP-3 (26–28). From these results, we named the novel transcript Delta/Notch-like EGF-related receptor (DNER) (Mouse Genome Database Nomenclature Committee symbol).

DNER Is Expressed in Developing and Adult CNS Neurons—As an initial step in exploring the activity of DNER, the distribution of mRNA was determined. Northern blot analysis...
FIG. 1. Domain structures of predicted protein products of mouse and human DNER. A, alignment of deduced amino acid sequences of mouse (m) and human (h) DNER. Conserved residues are shaded. The putative signal sequence is italicized. The EGF-like repeats (numbered) and the calcium-binding EGF domain (C-EGF) are boxed. The dashed underlined sequence represents the predicted transmembrane domain. The solid underlined sequence is the putative target site for tyrosine kinases. Boldface sequences represent the tyrosine-based sorting signal (YXXØ) and the dileucine-type signal. B, schematic illustration of the DNER protein structure. C, multiple alignment of EGF-like repeats of various proteins. The EGF-like repeats of DNER (exemplified by the sixth repeat (E6)) are highly homologous to those of mouse Notch1 (60%), human Delta1 (60%), mouse Slit1 (56%), and mouse Jagged1 (54%), but not to those of human EGFL6 (31%). Residues identical to DNER are shaded.
with various adult mouse tissues revealed a single RNA species of ~3.7 kb, which was predominantly expressed in the brain (Fig. 2A). In situ hybridization revealed that DNER mRNA was almost exclusively expressed in the CNS as early as E14.5 in mouse embryos (Fig. 2B).

To determine the site of DNER action within the CNS, we raised a rabbit polyclonal antibody against the C-terminal tail of DNER. The affinity-purified anti-DNER antibody recognized a major band of the size expected from the molecular mass (~90 kDa) in lysates of P7 mouse brain (Fig. 3A). Two additional bands with higher molecular masses, presumably the glycosylated forms of DNER, were also occasionally detected. The antibody also recognized a protein product in extracts of HEK293 cells transfected with DNER cDNA, but not with vector alone (data not shown).

We next analyzed the cellular and subcellular localization of DNER in the developing CNS by indirect immunofluorescent staining against anti-DNER antibody. Prominent immunoreactivity demarcated dendrites and cell bodies of cerebellar Purkinje cells throughout pre- and postnatal development. At P7, a relatively low level of expression was observed in the deeper portion of the external germinal layer and in the emerging internal granular layer, where post-mitotic differentiating granule cells were located (Fig. 3B). DNER expression in the cerebellum was decreased in mature granule cells in the internal granular layer and mostly confined to Purkinje cells at P20 (Fig. 3C). On the other hand, DNER expression increased in the hippocampus during the course of postnatal development. At P7, weak signals were observed in the pyramidal cell layers of CA1–CA3, with the strongest level in CA3 (Fig. 3D). By P20, the DNER-positive zone was broadened to include the granule cell layer of the dentate gyrus and the strata radiatum and lucidum of CA1–CA3, where the apical dendrites of the pyramidal cells are located (Fig. 3E). It was notable that some strongly positive cells were scattered in the polymorphic layer of the dentate gyrus and the stratum radiatum. DNER was also expressed in some neurons in the cortical plate. At P7, little or
no expression was observed in the ventricular zones populated with actively dividing neuroblasts (Fig. 3F). At P20, DNER-positive signals were observed in the apical dendrites and cell bodies of pyramidal neurons throughout all six layers of the neocortex (Fig. 3G). We found that the signal intensity was not uniform, but was stronger in a subset of neurons scattered in each layer of the cortex.

DNER Protein Is Localized in the Somatodendritic Compartments of Developing CNS Neurons—To obtain higher subcellular resolution, we stained for DNER in cultured hippocampal neurons. Approximately one-fifth of the pyramidal neurons endogenously expressed DNER protein in culture. Endogenous DNER was seen throughout the MAP2-positive dendrites in a punctate pattern, but was not detectable in the MAP2-negative axons (Fig. 4A). We next analyzed the subcellular localization of DNER in cultured Purkinje cells. Purkinje cells possess massive, highly branched dendrites that are clearly distinguishable from single axons with a small and uniform diameter. We found that DNER expression was mostly confined to the dendrites and cell bodies and was excluded from the axons in Purkinje cells (Fig. 4B). Confocal microscopy revealed that immunoreactivity for DNER was on the plasma membrane and intracellular puncta of the somatodendritic compartments of Purkinje cells (Fig. 4C). We found that some DNER-positive puncta in dendrites colocalized with EEA1 (Fig. 4D), which has been shown to specifically localize in the early sorting endosomes in the somatodendritic region (29). We next examined whether DNER protein was internalized from the plasma membrane to the EEA1-positive endosomal compartment. Cultured hippocampal neurons were transfected with DNER cDNA tagged with an HA epitope in the extracellular domain and incubated with anti-HA antibody for 2 h to allow endocytosis of the surface DNER bound to the antibody. To specifically label the internalized protein, cells were washed with acid solution to strip the surface protein and then fixed, permeated, and double-stained for DNER and EEA1. We found a considerable number of HA-positive puncta in the cytoplasm, some of which colocalized with EEA1 (Fig. 4E), indicating that DNER was endocytosed from the plasma membrane and delivered to the early sorting endosomes. Taken together, these results demonstrate that DNER protein is distributed to the plasma membrane and sorting endosomal compartment of dendrites and cell bodies in several types of CNS neurons.

The Cytoplasmic Domain of DNER Mediates Dendritic Targeting and Endocytosis—We next asked which structural domain of DNER was required for its somatodendritic targeting. We utilized low-density cultured hippocampal neurons and misexpressed wild-type and deletion mutant protein constructs in polarized pyramidal neurons. Full-length DNER tagged with an HA epitope displayed a distribution similar to that of the endogenous protein (Fig. 5B). Deletion of the extracellular domain of DNER (DdE) did not affect the polarized distribution in the somatodendritic compartment, suggesting that the sorting signal must be present in either the transmembrane or cyto-
plasmic domain of the protein (Fig. 5C). In contrast, deletion of the DNER cytoplasmic domain (DdI) allowed entry of the mutant protein into the axons as well as the dendrites (Fig. 5D). These results indicate that the cytoplasmic domain of DNER contains the signal(s) responsible for the dendritic targeting.

**The YXXØ Motif Is Required for Somatodendritic Targeting of DNER**—Sequence analysis showed that the cytoplasmic tail of DNER bears putative motifs implicated in sorting to the epithelial basolateral domain and neuronal somatodendritic compartment (YEEF matching in the context of YXXØ and LI matching the dileucine-type sequence) (Fig. 1). To test whether these potential sorting motifs in the cytoplasmic tail are required for somatodendritic targeting of DNER, we misexpressed mutant constructs lacking these motifs in hippocampal neurons. We found that mutation in the tyrosine-based sorting motif significantly disrupted polarized trafficking of DNER to the somatodendritic compartment. The protein product of the DY677A construct, with a point mutation of Tyr-677 to Ala, was found in the axon as well as in the dendrite (Fig. 5E). In contrast, deletion of the dileucine motif (DdL1) did not alter the localization to the somatodendritic compartment (Fig. 5F). These results indicate that the tyrosine-based sorting motif, but not the dileucine-type motif, is required for somatodendritic targeting of DNER.
DNER puncta in the cell bodies exactly overlapped with AP-1 immunoreactivity. We next analyzed whether AP-1 binding depends on the sorting motifs in the cytoplasmic tail of DNER. Full-length DNER tagged with an HA epitope colocalized with AP-1 (data not shown). Similarly, the DdLI mutant (lacking the dileucine-type sorting motif) was distributed in AP-1-positive puncta (Fig. 6, E and F). In contrast, the DY677A mutant (missing the tyrosine-based motif) was completely segregated from AP-1-positive puncta (Fig. 6, C and D). It is striking that the mutant construct that failed to colocalize with AP-1 was also unable to localize in dendrites. These results strongly suggest that DNER is targeted to the somatodendritic compartments in neurons through the TGN via an AP-1-dependent sorting pathway mediated by the cytoplasmic YXXO sorting motif.

DISCUSSION

This study has described the identification and characterization of a novel EGF-like repeat-containing protein that is specifically expressed in developing and adult CNS neurons. Sequence analysis revealed that DNER is a transmembrane protein containing 10 EGF-like repeats. The proximal-most EGF repeat from the transmembrane segment fulfills the consensus of the calcium-binding EGF repeat, which is thought to mediate protein-protein interaction. The human and mouse clones display a significant degree of identity, implying a fundamental role of DNER in the mammalian CNS. From its molecular characteristics and expression pattern, DNER is likely to function as a receptor and/or a ligand in the dendrites of CNS neurons.

Expression Patterns of DNER mRNA and Protein—DNER mRNA was almost exclusively expressed in the developing and adult CNS. Immunohistochemistry revealed that DNER protein was prominently expressed in a subpopulation of CNS neurons, including cortical and hippocampal pyramidal neurons, cerebellar granule cells, and Purkinje neurons. Little or no expression was detected in mitotic neuroblasts in the ventricular zones. Overall expression levels peaked during postnatal development and declined in the adult brain, suggesting that DNER might be involved in development of post-mitotic neurons. Notably, DNER expression was maintained at a high level in a fraction of cells in the neocortex and hippocampus in adult mice. This raised an intriguing possibility that DNER might function during specific phases of neuronal activity in mature neural circuits.

Somatodendritic Targeting of DNER—DNER protein was specifically expressed in dendrites and cell bodies of CNS neurons. Overexpression of DNER protein in hippocampal neurons was not sufficient to cause entry into the axon, suggesting that DNER undergoes active targeting to the somatodendritic compartments. In mammalian epithelial cells, AP-1 is thought to mediate polarized targeting of proteins in clathrin-coated transport vesicles from the TGN to the basolateral surface through the endosomal system (15, 31). The immunoprecipitation and double immunofluorescence studies strongly suggested that AP-1 directly binds to DNER. As far as we know, this is the first indication of the direct in vivo binding of AP-1 with a somatodendritic protein. Mismatch of various mutant forms of DNER revealed that the cytoplasmic tyrosine-based sorting motif is essential for the somatodendritic targeting as well as AP-1 binding of DNER. These results strongly support the idea that AP-1 recognizes the YEEF sorting motif in the DNER cytoplasmic tail and targets the protein en route to the somatodendritic compartment through the TGN/endosomal system.

Both endogenous and exogenous expression of DNER were observed in the plasma membrane and intracellular puncta in
dendrites. Significant uptake of anti-HA antibody in the neurons transfected with HA-tagged DNER indicates that DNER is endocytosed from the plasma membrane and delivered to the EEA1-positive early endosomes. It is thought that another class of the adaptor binding complex AP-2 also recognizes the tyrosine-based and dileucine-type sorting motifs and mediates the endocytosis of membrane proteins. Active endocytosis of DNER from the plasma membrane might occur via an AP-2-dependent pathway.

Possible Role of DNER in Development and Function of the CNS—EGF-like repeat-containing proteins exert a myriad of biological activities during various aspects of neural development, including early neural patterning (32), laminar formation of cortices (33), axon and cell migration (34), and synaptogenesis at neuromuscular junctions (35, 36). Among hundreds of variations, the EGF modules of DNER exhibit the highest degree of homology to those of Notch, which might give a clue toward elucidating DNER function in normal development. Notch is a large single-pass transmembrane protein with 36 tandem EGF-like repeats in its extracellular domain. Notch functions as a receptor for other related EGF repeat-containing transmembrane proteins (Delta and Serrate) to transduce signals that prevent neuronal determination of proneural cells (37, 38). Taking into account that DNER is specifically expressed in dendrites of post-mitotic neurons, it is unlikely that DNER is a regular component of Notch signaling during early neurogenesis. Interestingly, it has recently been shown that Notch signaling is also involved in branching formation of dendrites in post-mitotic neurons (39–41). This raises an intriguing possibility that DNER exerts a similar regulatory role in dendritic patterning of the CNS neurons, maybe in concert with Notch signaling. Misexpression of various mutant forms of DNER in combination with Notch signaling components is required to clarify this point.

Alternatively, it is also possible that DNER interacts with other signaling components as a receptor or a ligand. The human DNER gene was mapped to chromosome 2q37, where a few causative mutations for neurological genetic disorders have been located (42, 43). Detailed linkage mapping is needed to clarify whether mutation in the DNER gene causes these disorders. On the other hand, to gain further insight into the function of the gene, analysis of mice with targeted disruption of DNER is in progress to reveal the biological activity of DNER in development and function of the mammalian CNS.

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Delta/Notch-like Epidermal Growth Factor (EGF)-related Receptor, a Novel EGF-like Repeat-containing Protein Targeted to Dendrites of Developing and Adult Central Nervous System Neurons
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