Nectin-like molecule-1/TSLL1/SynCAM3: a neural tissue-specific immunoglobulin-like cell-cell adhesion molecule localizing at non-junctional contact sites of presynaptic nerve terminals, axons and glia cell processes

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Summary

Nectins are Ca^{2+}-independent immunoglobulin-like cell-cell adhesion molecules and comprise a family of four members. At the mossy fiber terminals of hippocampus, nectin-1 and nectin-3 localize at the presynaptic and postsynaptic sides of synaptic junctions, respectively, and their trans-interactions play a role in formation of synapses in cooperation with N-cadherin. Nectins are associated with the actin cytoskeleton through afadin, a nectin- and actin-filament-binding protein. Five nectin-like molecules (Necls) which have domain structures similar to those of nectins have been identified and here we characterize Necl-1/TSLL1/SynCAM3, from now on referred to as Necl-1. Tissue distribution analysis showed that Necl-1 was specifically expressed in the neural tissue. Immunofluorescence and immunoelectron microscopy revealed that Necl-1 localized at the contact sites among axons, their terminals, and glia cell processes that cooperatively formed synapses, axon bundles and myelinated axons. Necl-1 showed Ca^{2+}-independent homophilic cell-cell adhesion activity. It furthermore showed Ca^{2+}-independent heterophilic cell-cell adhesion activity with Necl-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 from now on referred to as Necl-2, nectin-1 and nectin-3, but not with Necl-5 or nectin-2. The C-terminal cytoplasmic region of Necl-1 did not bind afadin but bound membrane-associated guanylate kinase subfamily members that contain the L27 domain, including Dlg3, Pals2 and CASK. These results indicate that Necl-1 is a neural-tissue-specific Ca^{2+}-independent immunoglobulin-like cell-cell adhesion molecule which potentially has membrane-associated guanylate kinase subfamily member-binding activity and localizes at the non-junctional cell-cell contact sites.

Key words: Nectin, Necl-1, Axon, Glia, Myelin, Schwann cell

Introduction

The cell-cell adhesion systems are involved in neuronal cell migration, axon-bundle formation, target-cell recognition, activity-dependent plasticity of synapses, and formation of complex glial networks, which surround axons and synapses (for a review, see Redies and Takeichi, 1996). The cell-cell adhesion molecules implicated in brain function are classified into two superfamilies, the cadherin and immunoglobulin (Ig) superfamilies. Among the cadherin superfamily, N-cadherin localizes at both synaptic and puncta adherentia junctions of a certain set of neurons and plays a role in the formation and maintenance of these junctions (Yamagata et al., 1995; Fannon and Colman, 1996; Uchida et al., 1996). Synaptic junctions include presynaptic active zones, where synaptic vesicles and Ca^{2+} channels localize, and postsynaptic densities, where neurotransmitter receptors localize. Puncta adherentia junctions, by contrast, are ultrastructurally similar to adherens junctions of epithelial cells (Peters et al., 1976; Amaral and Dent, 1981). A novel family of Ca^{2+}-independent Ig-like cell-cell adhesion molecules named nectins has recently emerged (for reviews, see Takai and Nakanishi, 2003; Takai et al., 2003a). Nectins comprise a family of four members, nectin-1, nectin-2, nectin-3 and nectin-4 (Takai and Nakanishi, 2003). All nectins have one extracellular region with three Ig-like loops, one transmembrane region and one cytoplasmic region. Each nectin forms homo-cis-dimers, followed by formation of homo- and hetero-trans-dimers, causing cell-cell adhesion. Nectins are associated with the actin cytoskeleton through afadin, a nectin- and actin-filament-binding protein. Nectin-1 and nectin-3 localize asymmetrically and form hetero-trans-dimers at the presynaptic and postsynaptic side, respectively, of the plasma membrane of puncta adherentia junctions that

are located at the synapses between the mossy fiber terminals and the pyramidal cell dendrites in the CA3 area of the hippocampus (Mizoguchi et al., 2002). By contrast, N-cadherin, αN-catenin and afadin localize symmetrically at both sides of these synapses (Nishioka et al., 2000; Mizoguchi et al., 2002). Inhibition of the nectin-1- and nectin-3-based adhesion by inhibitors of nectin-1 in cultured rat hippocampal neurons impairs the formation of synapses (Mizoguchi et al., 2002). Together with cadherins, nectins are involved in the formation of adherens junctions in epithelial cells and fibroblasts (Takai and Nakanishi, 2003). Taken together, it is likely that nectins are also involved in the formation of synapses in cooperation with N-cadherin. This role of nectins is consistent with the finding that mutations in the nectin-1 gene are responsible for cleft lip/palate-ectodermal dysplasia (Zlotogora-Ogür syndrome), which is characterized by mental retardation and ectodermal dysplasia (Suzuki et al., 2000).

Five molecules with one extracellular region that contains three Ig-like loops, one transmembrane region and one cytoplasmic region have so far been identified and are called nectin-like molecules (Necls) (for a review, see Takai et al., 2003b). They include Necl-1 (Necl-1/TSLL1/SynCAM3), Necl-2 (Necl-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1), Necl-3 (Necl-3/ similar to Necl-3/SynCAM2), Necl-4 (TSLL2/SynCAM4) and Necl-5 (Tage4/PVR/CD155). Necl-1 was reported in GenBank [accession no. AF062733 (human), 1998; accession no. AF195662 (mouse), 1998]. *TSLL1* was identified to be one of the *TSLC1*-like genes (Fukuhara et al., 2001; Fukami et al., 2003), and SynCAM3 was identified to be one of the SynCAM family members (Biederer et al., 2002). Northern blotting has revealed that expression of the *TSLL1* occurs specifically in the brain and in neurogenic cells such as SW1783, NB41A3, N1E-115 and C-1300 (Fukuhara et al., 2001; Fukami et al., 2003), but its localization in the brain remains unknown. TSLL1 has been suggested to be a cell-cell adhesion molecule because it localizes at the cell-cell contact sites in N1E-115 cells (Fukami et al., 2003); however, it remains unknown whether it indeed shows cell-cell adhesion activity. We emphasize here that, SynCAM1 has been reported to be a brain-specific synaptic-adhesion molecule (Biederer et al., 2002), which is ubiquitously expressed and identical to Necl-2 but not Necl-1 (see Discussion). Therefore, we characterized Necl-1.

Materials and Methods

Construction of plasmids

Expression vectors were constructed in pFLAG-CMV1 (Sigma, St Louis, MO), pCAGIPuro (Miyahara et al., 2000), pCAGIZeo (Niwa et al., 1998), pGBD-C1 (James et al., 1996), pGEX4T-1 (Amersham Biosciences, Piscataway, NJ), pMAL-C2 (New England Biolabs Inc.), pGAD424-HA (Tachibana et al., 2000), pCMV-HA (Takaesu et al., 2000), and pEFBOS-Myc (Komuro et al., 1996). Various constructs of Necl-1 contained the following amino acid (aa) sequences: pFLAG-CMV1-Necl-1, aa 20-396 (deleting the signal peptide) (Shingai et al., 2003); pCAGIPuro-FLAG-Necl-1, aa 20-396 (deleting the signal peptide) (Shingai et al., 2003); pCAGIZeo-Necl-1, aa 1-396 (full-length); pFLAG-CMV1-Necl-1-ΔC, aa 20-392 (deleting the signal peptide and the C-terminal four aa); pCAGIPuro-FLAG-Necl-1-ΔC, aa 20-392 (deleting the signal peptide and the C-terminal four aa); pGBD-C1-Necl-1-ΔEC, aa 316-396 (deleting the extracellular region); and pGEX4T-1-Necl-1-CP, aa 350-396 (the cytoplasmic region). Various constructs of Necl-2, nectin-2α, nectin-3α, l-afadin, Pals2 and CASK contained the following aa sequences: pGEX4T-1-Necl-2-CP, aa 372-417 (the cytoplasmic region); pGBD-C1-Necl-2-ΔEC, aa 335-417 (deleting the extracellular region); pFLAG-CMV1-Necl-2, aa 43-417 (deleting the signal peptide); pFLAG-CMV1-Necl-2-ΔC, aa 43-413 (deleting the signal peptide and the C-terminal four aa); pGBD-C1-nectin-2-CP, aa 387-467 (the cytoplasmic region); pGEX4T-1-nectin-3-CP, aa 433-549; pGAD424-HA-l-afadin, aa 1-1829 (full-length); pMAL-C2-afadin-PDZ, aa 1007-1125 (PDZ domain); pGAD424-HA-Pals2, aa 1-539 (full length); pCMV-HA-Pals2, aa 1-539 (full-length); pGAD424-HA-CASK, aa 1-909 (full length); and pEFBOS-Myc-CASK, aa 1-909 (full-length) (Shingai et al., 2003; Satoh-Horikawa et al., 2000; Tachibana et al., 2000; Takahashi et al., 1999). The cDNA of mouse Pals2 was kindly provided by B. Margolis (University of Michigan Medical Center, Michigan, USA). pCMV-HA-Dlg3 was constructed using Dlg3 (lacking the N-terminal 1-5 aa) which was obtained as a positive clone from yeast two-hybrid screening (see below). The glutathione *S*-transferase (GST) and maltose-binding protein (MBP) fusion proteins were purified with glutathione-Sepharose beads (Amersham Biosciences) and amylose resin beads (New England Biolabs Inc.), respectively.

Cell culture and establishment of transfectants

Cocultures of mouse hippocampal neurons and glia cells were prepared as described (Abe et al., 2004). L cells were kindly supplied by S. Tsukita (Kyoto University, Kyoto, Japan). L and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. L-cell lines stably expressing human nectin-1α, mouse nectin-2α, mouse nectin-3α, mouse FLAG-tagged Necl-1, mouse FLAG-tagged Necl-2 or mouse FLAG-tagged Necl-5 (nectin-1-L, nectin-2-L, nectin-3-L, Necl-1-L, Necl-2-L or Necl-5-L cells, respectively) were prepared as described (Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Ikeda et al., 2003; Shingai et al., 2003). An L-cell line stably expressing full-length Necl-1 (non-tagged Necl-1-L cells) or FLAG-tagged Necl-1-ΔC (Necl-1-ΔC-L cells) was obtained by transfection with pCAGIZeo-Necl-1 or pCAGIPuro-FLAG-Necl-1-ΔC, respectively, using Lipofectamine Plus reagent (Invitrogen). For transient expression of hemagglutinin- (HA-) tagged Dlg3, HA-tagged Pals2, or Myc-tagged CASK, L-cell lines were transfected with pCMV-HA-Dlg3, pCMV-HA-Pals2 or pEFBOS-Myc-CASK. For the coimmunoprecipitation assay, HEK293 cells were co-transfected with pFLAG-CMV1-Necl-1, pFLAG-CMV1-Necl-1-ΔC, pFLAG-CMV1-Necl-2, or pFLAG-CMV1-Necl-2-ΔC and pCMV-HA-Dlg3, pCMV-HA-Pals2, or pEFBOS-Myc-CASK using LipofectAMINE 2000 reagent (Invitrogen). We mostly used Necl-1 L cells (FLAG-tagged Necl-1) in the present study, but the essentially similar results were obtained with non-tagged-Necl-1-L cells.

Antibodies

Rabbit antiserum against Necl-1 was raised against the cytoplasmic region of Necl-1 fused to GST (GST-Necl-1-CP). The antiserum was applied first to a GST-immobilized column and second to the cytoplasmic region of Necl-2 fused to a GST (GST-Necl-2-CP)-immobilized column for absorption of non-specific antibodies (Abs), and finally affinity purified with a GST-Necl-1-CP-immobilized column and used as polyclonal antibody (pAb). Columns with immobilized GST-fusion protein were prepared using Hi-Trap NHS-activated columns (Amersham Biosciences). Rat anti-Necl-2 monoclonal Ab (mAb) (clone 1C4-2), rabbit anti-nectin-1 and nectin-3 pAbs, rat anti-nectin-2 mAb and mouse anti-l-afadin mAb were prepared as described (Takahashi et al., 1999; Satoh-Horikawa et al., 2000; Shingai et al., 2003; Sakisaka et al., 1999). Mouse anti-FLAG mAb and mouse anti-glial fibrillary acidic protein (GFAP) mAb were

purchased from Sigma. Mouse anti-synaptophysin mAb was purchased from Chemicon (Temecula, CA). Mouse anti-HA mAb was purchased from BabCO (Richmond, CA). Rat anti-HA mAb was purchased from Roche (Basel, Switzerland). Rabbit anti-Myc pAb was purchased from Medical and Biological Laboratories (Nagoya, Japan). Hybridoma cells (9E10) expressing mouse anti-Myc mAb were purchased from the American Type Culture Collection (Rockville, MD). Affinity-purified rabbit anti-Dlg3 pAb was kindly provided by B. Margolis. Rabbit anti-Dlg3 antiserum was kindly provided by E. Peles (The Weizmann Institute of Science, Israel).

cis-Dimer formation assay

cis-Dimer formation assays were carried out as described (Satoh-Horikawa et al., 2000). Briefly, to obtain a single-cell suspension, cells were washed with PBS, incubated with 0.2% trypsin and 1 mM EDTA at 37°C for 5 minutes and dispersed by gentle pipetting. A single-cell suspension (1×10^6 cells/ml) was incubated in PBS with 1 mM bis (sulfosuccinimidyl) suberate (BS3) crosslinker (Pierce). After incubation at 14°C for 15 minutes, the reaction was stopped with the addition of 10 mM Tris-HCl at pH 7.5. The cells were washed with PBS and counted to confirm that there was no aggregation in the cell suspension by phase-contrast microscopy. Then, the cells were harvested with SDS sample buffer (60 mM Tris-HCl at pH 6.7, 3% SDS, 2% 2-mercaptoethanol and 5% glycerol), boiled for 5 minutes, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting.

Yeast two-hybrid screening

The yeast two-hybrid library constructed from a rat brain cDNA was purchased from Clontech (Palo Alto, CA), and Necl-1-binding protein(s) was screened using pGBD-C1-Necl-1-ΔEC as a bait. Two-hybrid screening using the yeast strain PJ69-4A (*MAT**a** trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*) was carried out as described (James et al., 1996).

Coimmunoprecipitation assay

The cerebellum was dissected from adult mice and the sample was homogenized in homogenization buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 10% glycerol, 10 μg/ml leupeptin, 1.5 μg/ml aprotinin and 10 μM *p*-amidinophenylmethanesulfonyl fluoride). An equal volume of homogenization buffer containing 2% Nonidet P-40 was added to the homogenate and subjected to centrifugation at 100,000 ***g*** for 20 minutes. The supernatant (2 mg of protein) was pre-incubated with protein G-Sepharose beads (Amersham Biosciences) at 4°C for 1 hour and then incubated with protein G-Sepharose beads coated with anti-Dlg3 antiserum or control non-immune serum at 4°C for 16 hours. After the beads were extensively washed with homogenization buffer containing 1% Nonidet P-40, bound proteins were eluted by boiling the beads in SDS sample buffer for 5 minutes, and subjected to SDS-PAGE, followed by western blotting. Coimmunoprecipitation assays using cultured cells were carried out as described (Takahashi et al., 1999).

Other procedures

Immunofluorescence microscopy of the brain was done as described (Kawabe et al., 1999). Immunofluorescence microscopy of cultured cells was done as described (Mandai et al., 1997). Immunoelectron microscopy of mouse brain was done using the silver-enhancement technique as described (Mizoguchi et al., 1994). The cell aggregation assay was done as described (Satoh-Horikawa et al., 2000). Affinity chromatography was done as described (Takekuni et al., 2003). SDS-PAGE was done as described (Laemmli, 1970). Protein concentrations were determined with BSA as a reference protein using the DC protein assay kit (BIORAD, Hercules, CA).

Results

Brain-specific expression of Necl-1

We first prepared a pAb against the cytoplasmic region of Necl-1. The cytoplasmic region of Necl-1 showed high homology to that of Necl-2 (aa sequences similarity 76%) (Fukuhara et al., 2001). Therefore, to obtain an Ab that is highly specific to Necl-1, antiserum against GST-Necl-1-CP was applied to GST- and GST-Necl-2-CP-immobilized columns to absorb non-specific Abs, and then affinity-purified with GST-Necl-1-CP-immobilized column. This Ab specifically recognized exogenously synthesized Necl-1 but not Necl-2 in L cells, as shown by western blotting (Fig. 1A). Thus, the anti-Necl-1 pAb obtained is specific for Necl-1 and does not cross-react with Necl-2.

TSLL1 has been shown by northern blotting to be expressed in the brain (Fukuhara et al., 2001; Fukami et al., 2003). We confirmed these earlier results by using our pAb in western blotting. The immunoreactive band with a molecular mass of about 48 kDa was detected in the brain, but not in other tissues including heart, spleen, lung, liver, kidney, skeletal muscle and testis (Fig. 1B). These results are consistent with the earlier observations (Fukuhara et al., 2001; Fukami et al., 2003) and indicate that Necl-1 is mainly expressed in the brain.

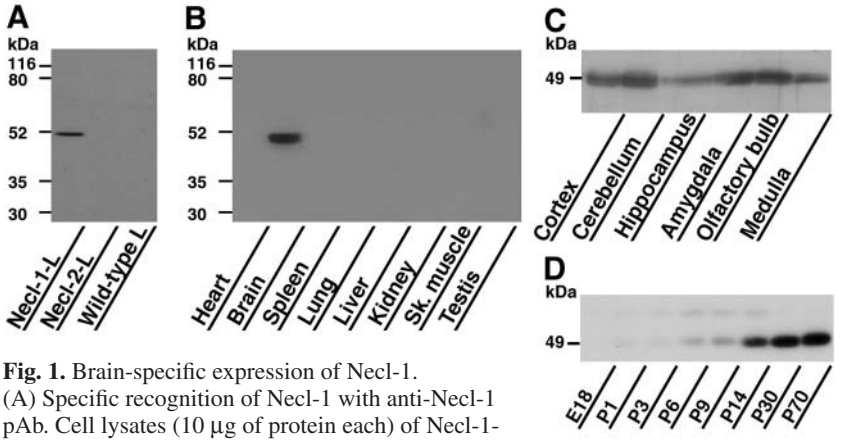

Fig. 1. Brain-specific expression of Necl-1. (A) Specific recognition of Necl-1 with anti-Necl-1 pAb. Cell lysates (10 μg of protein each) of Necl-1-L, Necl-2-L and wild-type L cells were subjected to SDS-PAGE (10%), followed by western blotting with anti-Necl-1 pAb. (B) Tissue distribution of Necl-1. Homogenates of various mouse tissues (20 μg of protein each) were subjected to SDS-PAGE (10%), followed by western blotting with anti-Necl-1 pAb. (C) Distribution of Necl-1 in the brain. Homogenates of various regions of rat brain (20 μg of protein each) were subjected to SDS-PAGE (10%), followed by western blotting with anti-Necl-1 pAb. (D) Developmental expression of Necl-1 in the brain. Homogenates of various developmental stages of total rat brain (20 μg of protein each) were subjected to SDS-PAGE (10%), followed by western blotting with anti-Necl-1 pAb. The results shown are representative of three independent experiments.

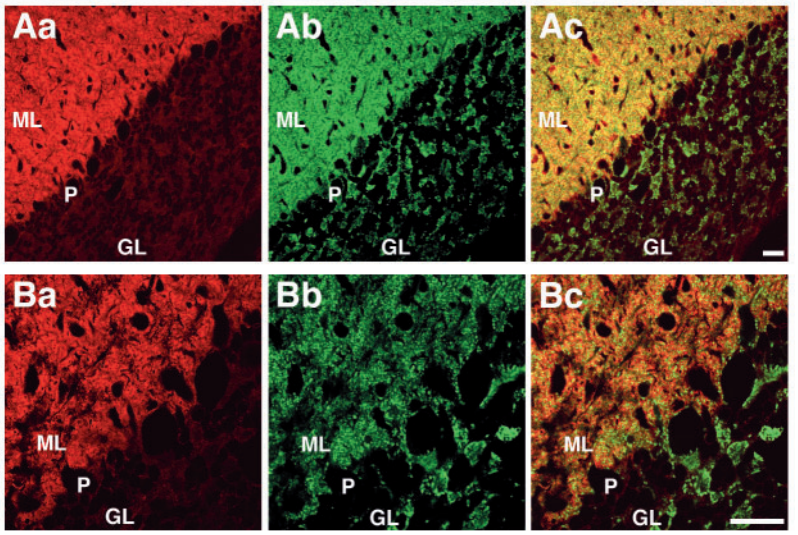

Fig. 2. Localization of Necl-1 in the molecular layer of the cerebellum. (A,B) Immunofluorescence images of mouse cerebellum. Frozen sections of mouse cerebellum were double-stained with anti-Necl-1 pAb and anti-synaptophysin mAb. Aa and Ba, Necl-1 (red); Ab and Bb, synaptophysin (green); Ac and Bc, merge. ML, molecular layer; P, Purkinje cells; GL, granular layer. Bars, 10 μm. The results shown are representative of three independent experiments.

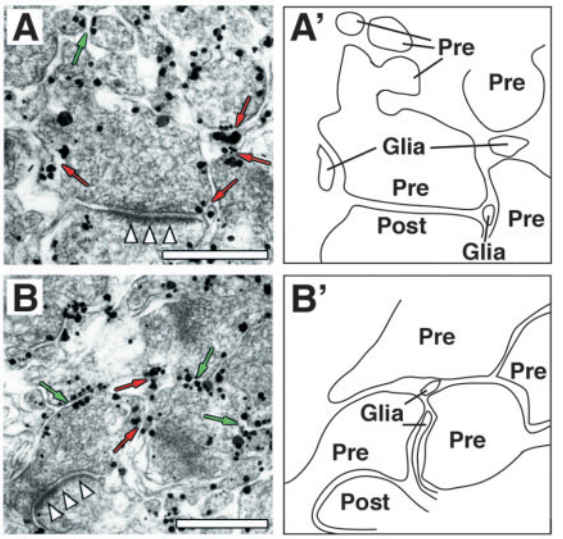

Fig. 3. Localization of Necl-1 at non-junctional cell-cell contact sites in the molecular layer of the cerebellum. Samples were stained with anti-Necl-1 pAb followed by immunogold electron microscopy with the silver-enhanced immunogold method. (A,B) The molecular layer in mouse cerebellum. Open arrowheads, synaptic junctions; red arrows, contact sites between a parallel fiber terminal or axon and a glia cell; green arrows, contact sites of two parallel fiber terminals. (A',B') Schematic drawings of A and B. Pre, parallel fiber terminals or axons of granular cells; Post, Purkinje cell dendrite synapses; Glia, glia cell processes. Bars, 250 nm. The results shown are representative of three independent experiments.

Localization of Necl-1 at non-junctional contact sites where presynaptic nerve terminals, axons and glia cell processes meet

Western blotting showed that Necl-1 was synthesized in various regions of rat brain, including the cortex, cerebellum, hippocampus, amygdala, olfactory bulb and medulla (Fig. 1C). A major band of Necl-1 with a molecular mass of 48 kDa was detected from postnatal day (P) 6 (Fig. 1D). Its expression level gradually increased until P30. A faint band of Necl-1 with a molecular mass of 60 kDa was detected during P6 to P14. The faint band may be owing to alternative splicing or posttranslational modification(s) such as glycosylation.

Immunofluorescence microscopy revealed that the signal for Necl-1 was highly concentrated at the molecular layer of the mouse cerebellum, although a faint signal for Necl-1 was also observed at the granular layer (Fig. 2Aa, Ac, Ba and Bc). The signal for Necl-1 did not colocalize with that of synaptophysin, a well-known synaptic protein (Wiedenmann and Franke, 1985) (Fig. 2Aa-Ac and Ba-Bc). Immunoelectron microscopy revealed that immunogold-labeled Necl-1 was concentrated at the contact sites of two parallel fiber terminals of granular cells, between a parallel fiber terminal and a parallel fiber axon, and between a parallel fiber terminal and a glia cell process surrounding the pre- and postsynaptic contact site (Fig. 3A, A', B and B'). Localization of nectin-1, nectin-3 and Necl-2 was also confirmed at the contact sites between parallel fiber terminals and Purkinje cell dendrites. The signal for nectin-1 was demonstrated in parallel-fiber terminals and the signal for nectin-3 was on the postsynaptic membrane (data not shown). The signal for Necl-2 was hardly detected at this area (data not shown).

Necl-1 was synthesized in both cultured hippocampal neurons and glia cells as detected by western blotting (data not shown). The signal for Necl-1 was a dot-like pattern at the contact sites of neurons (Fig. 4Aa) that, however, did not colocalize with that of synaptophysin, a marker for synapses (Fig. 4Aa-c). The signal for Necl-1 was also detected at the contact sites of glia cells (Fig. 4Ba-c) and was rather increased at the contact sites between neurons and glia cells (Fig. 4Ca-c, Da-c). These results are consistent with those of Figs 2 and 3.

In peripheral myelinated nerve fibers, the signal for Necl-1 was distributed homogeneously in the area of the axonal plasma membrane, where Schwann cells wrapped the axons with myelin sheath (Fig. 5A). At the nodes of Ranvier, the signal for Necl-1 was concentrated at the contact sites of cellular processes of Schwann cells (Fig. 5B,B'). These results indicate that Necl-1 is expressed not only in the central nervous system (CNS) but also in the peripheral nervous system, and that it localizes at the contact sites of axons, their terminals and glia cell processes that cooperatively form synapses, axon bundles and myelinated axons.

Ca^{2+}-independent homophilic cell-cell adhesion activity of Necl-1

To examine whether Necl-1 has cell-cell adhesion activity, we assayed the aggregation activity of Necl-1 using cadherin-deficient L cells stably expressing FLAG-tagged Necl-1 (Necl-1-L cells). As described previously, wild-type L cells slightly expressed endogenous nectin-1 and nectin-2

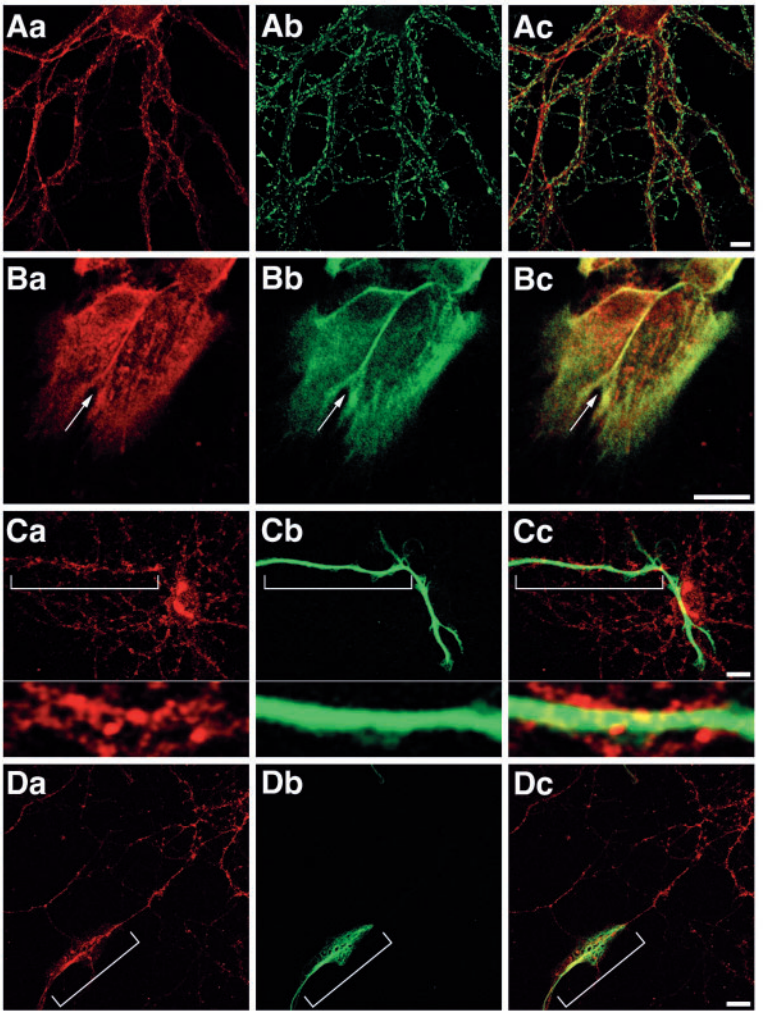

Fig. 4. Localization of Necl-1 in cocultured neurons and glia cells. Immunofluorescence images of mouse hippocampal cocultured neurons and glia cells at 24 days in vitro. (A) Contact sites of neurons. Cells were double stained with anti-Necl-1 pAb and anti-synaptophysin mAb. Aa, Necl-1 (red); Ab, synaptophysin (green); Ac, merge. (B) Contact sites of glia cells. Cells were double stained with anti-Necl-1 pAb and anti-GFAP mAb, a marker for glia cells. Ba, Necl-1 (red); Bb, GFAP (green); Bc, merge. Arrows indicate contact sites of glia cells. (C,D) Contact sites between a neuron and a glia cell. Cells were double-stained with anti-Necl-1 pAb and anti-GFAP mAb, a marker for glia cells. Ca and Da, Necl-1 (red); Cb and Db, GFAP (green); Cc and Dc, merge. Brackets indicate contact sites between a neuron and a glia cell. Inset in C, images at a higher magnification. Bars, 10 μm. The results shown are representative of three independent experiments.

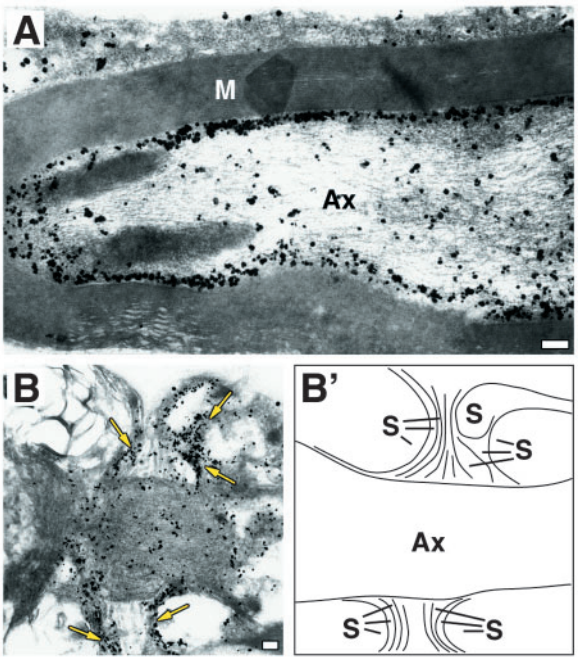

Fig. 5. Localization of Necl-1 at non-junctional cell-cell contact sites in peripheral myelinated nerve fibers. Samples were stained with anti-Necl-1 pAb followed by immunogold electron microscopy with the silver-enhanced immunogold method. (A) Axons with myelin sheath. (B) Nodes of Ranvier. (B') Schematic drawing of B. M, myelin sheath; Ax, axon of peripheral nerve fiber; S, cellular process of Schwann cells. Yellow arrows indicate contact sites of cellular processes of Schwann cells. Bars, 250 nm. The results shown are representative of three independent experiments.

(Miyahara et al., 2000; Sakisaka et al., 2001) but Necl-1 was not detected by western blotting (see Fig. 1A). Wild-type L cells did not form visible cell aggregates (Fig. 6Aa), whereas Necl-1-L cells did (Fig. 6Ab). The sizes of the aggregates formed in the presence of 1 mM $CaCl_2$ or 1 mM EDTA were not significantly different (data not shown), suggesting that Necl-1 has Ca^{2+}-independent homophilic cell-cell adhesion activity. The homophilic cell-cell adhesion activity of Necl-1 was confirmed by immunofluorescence microscopy. When Necl-1-L cells were stained using anti-FLAG mAb, the signal for Necl-1 was concentrated at cell-cell contact sites (Fig. 6B).

Each nectin first forms cis-dimers, and then forms trans-dimers, eventually causing cell-cell adhesion (Miyahara et al., 2000; Satoh-Horikawa et al., 2000). Similarly, Necl-1 formed cis-dimers (Fig. 6C). In addition, however, Necl-1 also formed a multimer. It is unknown whether the multimer is a homo- or hetero-multimer, but western blotting suggested that it does not include endogenous nectin-1 or nectin-2 (data not shown). By analogy with the mode of action of nectins, it is probable that Necl-1 first forms cis-dimers, and then forms trans-dimers, eventually causing cell-cell adhesion.

Ca^{2+}-independent heterophilic cell-cell adhesion activity of Necl-1

Nectins form both homophilic and heterophilic cell-cell adhesion activities (Takai et al., 2003b; Takai and Nakanishi, 2003). We next examined in a cell aggregation assay whether Necl-1 shows heterophilic cell-cell adhesion activity with other Necls and nectins. Necl-1-L cells formed heterologous cell aggregates with L cells that stably express Necl-2, nectin-1 or nectin-3 (Necl-2-L, nectin-1-L and nectin-3-L cells, respectively), but not with L cells that stably express Necl-5 or nectin-2 (Necl-5-L or nectin-2-L cells, respectively), or with

wild-type L cells (Fig. 7A-E and data not shown). These heterologous cell aggregates were similarly formed, irrespectively of the presence of 1 mM $CaCl_2$ or 1 mM EDTA (data not shown). Necl-2-L cells formed homologous cell aggregates (Masuda et al., 2002; Biederer et al., 2002; Shingai et al., 2003), whereas Necl-5-L cells did not form homologous cell aggregates as described (Aoki et al., 1997; Ikeda et al., 2003). Nectin-1-L, nectin-2-L and nectin-3-L cells formed homologous cell aggregates as described (Takahashi et al., 1999; Satoh-Horikawa et al., 2000). The size of the cell aggregates formed of nectin-1-L and nectin-3-L cells was the biggest among various combinations (Takai et al., 2003b; Takai and Nakanishi, 2003) (Fig. 7F). The sizes of the cell aggregates consisting of Necl-1-L and Necl-1-L cells, Necl-1-L and Necl-2-L cells, Necl-1-L and nectin-1-L cells, and Necl-1-L and nectin-3-L cells were about 5, 10, 15 and 15% of the size of the large nectin-1-L and nectin-3-L cell-aggregates. Taken together, these results indicate that Necl-1 has both Ca^{2+}-independent homophilic and heterophilic cell-cell adhesion activities.

Binding ability of Necl-1 to membrane-associated guanylate kinase subfamily members containing the L27 domain

All nectins except nectin-4 have a consensus motif of four aa (E/AXYV) at the extreme C-terminus for binding to the PDZ domain of afadin (Takai et al., 2003b; Takai and Nakanishi, 2003). Because Necl-1 also has a PDZ domain-binding motif, we examined whether Necl-1 can bind afadin. However, yeast two-hybrid assays showed that Necl-1 did not bind afadin under the conditions where nectin bound afadin (Fig. 8A). Coimmunoprecipitation assays and affinity chromatography yielded results that were essentially the same (Fig. 8B,C). Therefore, in the last set of experiments, we attempted to isolate a Necl-1-binding protein(s). As a bait for the yeast two-hybrid screening, we used a Necl-1 mutant that lacked the extracellular domain (Necl-1-ΔEC) and isolated one clone encoding Dlg3 (also called MPP3) from a rat brain library (Fig. 9A). Dlg3 was originally isolated as a novel membrane-associated guanylate kinase (MAGUK) family member (Smith et al., 1996). It consists of two Lin-2/-7 homology (L27) domains, one PDZ domain, one Src-homology3 (SH3) domain and one guanylate kinase domain. Recently, Dlg3 has been shown to be a subfamily of MAGUK that contains the L27

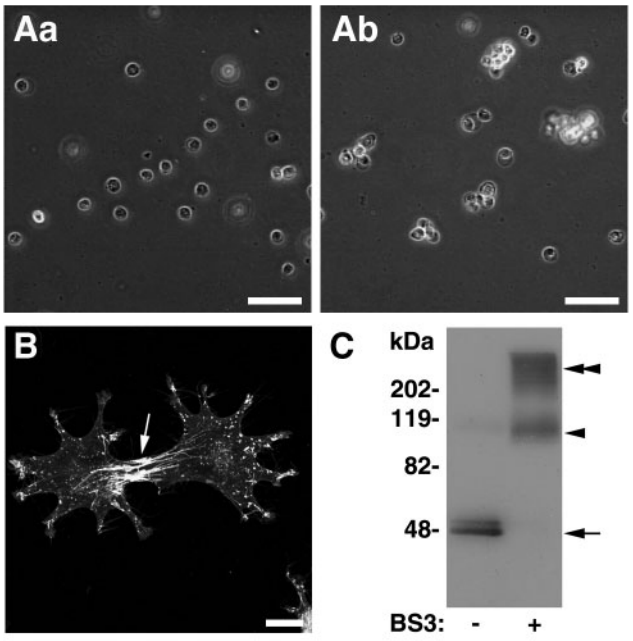

Fig. 6. Homophilic cell-cell adhesion activity of Necl-1. (A) Cell aggregation activity of Necl-1 when a single-cell suspension was rotated for 10 minutes. Aa, wild-type L cells; Ab, Necl-1-L cells. Bars, 100 μm. (B) Localization of Necl-1 at cell-cell contact sites of Necl-1-L cells. Necl-1-L cells were stained with anti-FLAG mAb. Arrow indicates cell-cell contact sites. Bar, 10 μm. (C) Homo-cis-dimer formation of Necl-1. A single-cell suspension of Necl-1-L cells was incubated in the presence or absence of BS3. The lysates (20 μg of protein each) were subjected to SDS-PAGE (10%), followed by western blotting with anti-FLAG mAb. Arrow, monomer; arrowhead, dimer; double-arrowhead, multimer. The results shown are representative of three independent experiments.

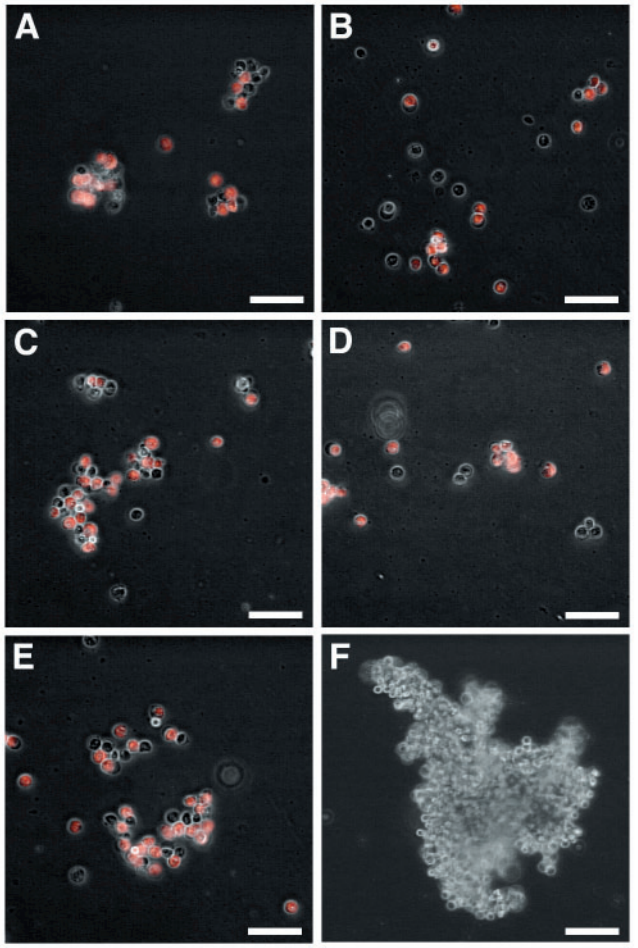

Fig. 7. Heterophilic cell-cell adhesion activity of Necl-1 when a single-cell suspension was rotated for 10 minutes. Heterophilic cell-aggregation activity between Dil-labeled Necl-1-L cells and (A) Necl-2-L cells, (B) Necl-5-L cells, (C) nectin-1-L cells, (D) nectin-2-L cells and (E) nectin-3-L cells. (F) Heterophilic cell aggregation activity between nectin-1-L and nectin-3-L cells. Bars, 100 μm. The results shown are representative of three independent experiments.

domain (Karnak et al., 2002). When FLAG-tagged Necl-1 and HA-tagged Dlg3 were coexpressed in HEK293 cells and FLAG-tagged Necl-1 was immunoprecipitated with anti-FLAG mAb, HA-tagged Dlg3 was coimmunoprecipitated with FLAG-tagged Necl-1 (Fig. 9B). However, when FLAG-tagged Necl-1-ΔC and HA-tagged Dlg3 were coexpressed in HEK293 cells and FLAG-tagged Necl-1-ΔC was immunoprecipitated, HA-tagged Dlg3 was not coimmunoprecipitated (Fig. 9B). Furthermore, when Dlg3 was immunoprecipitated with anti-Dlg3 antiserum from mouse-cerebellum extract, Necl-1 was coimmunoprecipitated with Dlg3 (Fig. 9C). When HA-tagged Dlg3 was overexpressed in Necl-1-L cells, the signal for Dlg3 colocalized with that for Necl-1 at the cell-cell contact sites (Fig. 9Da1-a3). However, when HA-tagged-Dlg3 was overexpressed in L cells that stably express Necl-1-ΔC, only the signal for Necl-1-ΔC concentrated at the cell-cell contact sites, but not the signal for Dlg3 (Fig. 9Db1-b3). These results indicate that Necl-1 has the potential to bind Dlg3 through the PDZ domain-binding motif in Necl-1 and the PDZ domain of Dlg3.

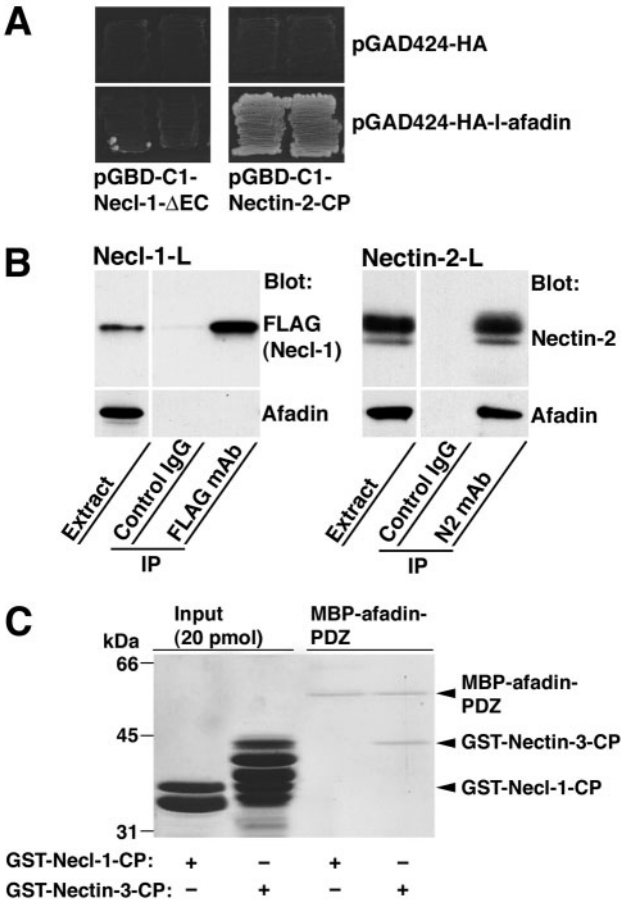

Fig. 8. Inability of Necl-1 to bind afadin. (A) Yeast two-hybrid assay. Yeast transformants with the indicated plasmids were streaked on synthetic complete medium lacking adenine to score the *ADE2*-reporter activity and incubated at 30°C for 3 days.
(B) Coimmunoprecipitation assay. FLAG-tagged Necl-1 was immnunoprecipitated with anti-FLAG mAb from the extract of Necl-1-L cells. For the control experiments, nectin-2 was immunoprecipitated with anti-nectin-2 mAb from the extract of nectin-2-L cells. Immunoprecipitates were then subjected to SDS-PAGE (10% polyacrylamide gel), followed by western blotting with anti-FLAG, anti-nectin-2 or anti-afadin mAbs. (C) Affinity chromatography. GST-Necl-1-CP or GST-nectin-3-CP (2 nmol of protein each) was applied to amylose resin beads on which MBP-afadin-PDZ (200 pmol of protein each) was immobilized. After the beads were extensively washed, the bound proteins were subjected to SDS-PAGE (13%), followed by protein staining with Coomassie brilliant blue. The results shown are representative of three independent experiments.

We have previously shown that Necl-2 binds Pals2 (also called MPP6), a MAGUK subfamily member containing the L27 domain (Shingai et al., 2003). Biederer et al. have shown that SynCAM1/Necl-2 binds CASK (also called mLin-2), a MAGUK subfamily member having L27 domain (Biederer et al., 2002). Fukuhara and colleagues have shown that TSLC1/Necl-2 binds Dlg3 (Fukuhara et al., 2003). The four aa (EYFI) in the C-terminal of Necl-1 are identical with those of Necl-2 (Fukuhara et al., 2001; Fukami et al., 2003). We therefore examined the binding specificity of Necl-1 and Necl-2 for these MAGUK subfamily members in the yeast two-hybrid system and coimmunoprecipitation assay. Under the same conditions in which Necl-2 bound Pals2, CASK and Dlg3, Necl-1 also bound all three proteins (Fig. 9A,B). Binding Pals2 and CASK to Necl-1 depended on the EYFI-sequence in its C-terminal (Fig. 9B). When Pals2 or CASK was overexpressed in Necl-1-L cells, their signal colocalized with that for Necl-1 at the cell-cell contact sites (Fig. 9Dc1-c3 and e1-e3). Recruitment of Pals2 or CASK to the cell-cell contact sites also depended on the EYFI-sequence of Necl-1 (Fig. 9Dd1-d3, f1-f3). Essentially similar results were obtained when these MAGUK subfamily members were overexpressed in Necl-2-L or Necl-2-ΔC-L cells (Shingai et al., 2003) (data not shown). These results indicate that the binding specificity of Necl-1 for MAGUK subfamily members in vitro is similar to that of Necl-2 and that Necl-1 has a broad binding potential to MAGUK subfamily members.

Discussion

We have shown by western blotting that Necl-1 is specifically synthesized in neural tissue, consistent with earlier observations (Fukuhara et al., 2001; Fukami et al., 2003). In the CNS, immunofluorescence and immunoelectron microscopic analyses have revealed that Necl-1 localizes at the contact sites between two axon terminals, between an axon terminal and an axonal shaft, and between an axon terminal and glia cell processes. In cultured neurons and glia cells, Necl-1 localizes at the contact sites of neurons, glia cells, and neurons and glia cells. We have shown here that, in aggregation assays of L cells expressing nectin and Necl family members, Necl-1 forms homo-cis-dimers and has both Ca^{2+}-independent homophilic cell-cell adhesion activity and Ca^{2+}-independent heterophilic cell-cell adhesion activity with Necl-2, nectin-1 and nectin-3 but not with Necl-5 or nectin-2.

Immunogold-labeled Necl-1 is detected on both sides of the contact sites of two parallel fiber terminals of granular cells, between a parallel fiber terminal and a parallel fiber axon, and between a parallel fiber terminal and glia cell processes. No signal for nectin-1, nectin-3, or Necl-2 is detected at these sites. These results suggest that, Necl-1 probably forms homo-trans-dimers and serves as a cell-cell adhesion molecule there.

In peripheral myelinated nerve fibers, immunoelectron-microscopy has revealed that Necl-1 localizes at the contact sites of the cellular processes of Schwann cells at the nodes of Ranvier. Necl-1 probably forms homo-trans-dimers and serves as a cell-cell adhesion molecule at these sites. However, because Necl-1 localizes at the axonal plasma membrane covered with Schwann cell plasma membrane, Necl-1 may form hetero-trans dimers with an unidentified cell adhesion molecule there.

Notice that, Necl-1 localizes at the cell-cell contact sites where axon terminals and glia cell processes contact. In the CNS, the processes of glia cells, astrocytes, surround pre- and

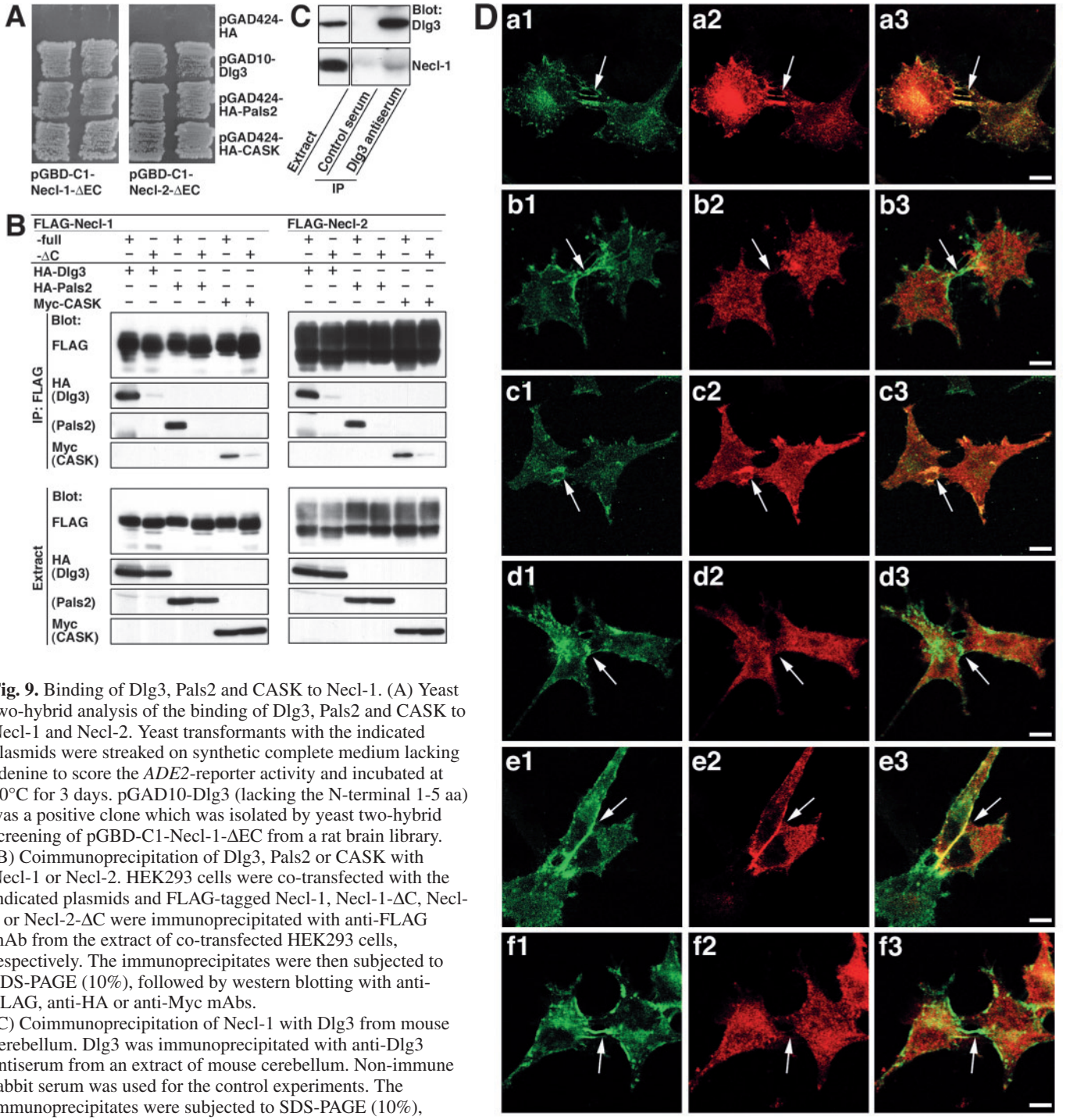

Fig. 9. Binding of Dlg3, Pals2 and CASK to Necl-1. (A) Yeast two-hybrid analysis of the binding of Dlg3, Pals2 and CASK to Necl-1 and Necl-2. Yeast transformants with the indicated plasmids were streaked on synthetic complete medium lacking adenine to score the *ADE2*-reporter activity and incubated at 30°C for 3 days. pGAD10-Dlg3 (lacking the N-terminal 1-5 aa) was a positive clone which was isolated by yeast two-hybrid screening of pGBD-C1-Necl-1-ΔEC from a rat brain library. (B) Coimmunoprecipitation of Dlg3, Pals2 or CASK with Necl-1 or Necl-2. HEK293 cells were co-transfected with the indicated plasmids and FLAG-tagged Necl-1, Necl-1-ΔC, Necl-2 or Necl-2-ΔC were immunoprecipitated with anti-FLAG mAb from the extract of co-transfected HEK293 cells, respectively. The immunoprecipitates were then subjected to SDS-PAGE (10%), followed by western blotting with anti-FLAG, anti-HA or anti-Myc mAbs. (C) Coimmunoprecipitation of Necl-1 with Dlg3 from mouse cerebellum. Dlg3 was immunoprecipitated with anti-Dlg3 antiserum from an extract of mouse cerebellum. Non-immune rabbit serum was used for the control experiments. The immunoprecipitates were subjected to SDS-PAGE (10%), followed by western blotting with affinity-purified anti-Dlg3 or anti-Necl-1 pAbs. (D) Necl-1-dependent recruitment of Dlg3, Pals2 or CASK to cell-cell contact sites. Necl-1-L (a,c,e) or Necl-1-ΔC-L cells (b,d,f) were transfected with pCMV-HA-Dlg3 (a,b), pCMV-HA-Pals2 (c,d) or pEFBOS-Myc-CASK (e,f), and double-stained with anti-FLAG mAb (green, a1-f1) and anti-HA mAb (red, a2-d2), or anti-Myc pAb (red, e2,f2). Arrows indicate cell-cell contact sites. Bars, 10 μm. The results shown are representative of three independent experiments.

post-synaptic components and segregate synapses from neighboring axons and synapses. The astrocytic processes have an important function to absorb neurotransmitters released from the excited synapses and prevent the released neurotransmitters from diffusing and stimulating the neighboring synapses. In the molecular layer of the cerebellum, each Purkinje cell forms approximately 100,000 synapses with parallel fiber terminals, which are all surrounded by astrocytic processes. Therefore, astrocytic processes form an extremely complex structure and the morphogenic process of this complex structure may be mediated by Necl-1. The fact, that the expression of Necl-1 increases rapidly on P14, when the formation of synapses between parallel fiber terminals and Purkinje cell dendrites is almost completed, suggests the involvement of Necl-1 in astrocytic process formation.

We also have shown that, Necl-1 does not bind afadin but has a potential activity to bind MAGUK subfamily members including Dlg3, Pals2 and CASK. Dlg3 was originally identified as a novel MAGUK family member (Smith et al., 1996) and is highly expressed in the brain (Lin et al., 1998). Pals2 was originally identified as a novel MAGUK family member that binds mLin-7 (also called Veli or Mals) (Kamberov et al., 2000). CASK was originally identified as a MAGUK family member with a calmodulin-dependent kinase-like domain (Hata et al., 1996) and binds mLin-7 (Borg et al., 1998). All three proteins have subsequently been shown to be a subfamily of MAGUK containing the L27 domain, including Dlg2 (also called MPP2), MPP4 and Pals1 (also called MPP5) (Karnak et al., 2002). Necl-2 also binds Dlg3, Pals2 and CASK (Biederer et al., 2002; Shingai et al., 2003; Fukuhara et al., 2003). Both Necl-1 and Necl-2 bind these three proteins to similar extents (Shingai et al., 2003; Biederer et al., 2002; Fukuhara et al., 2003), a result that is consistent with the fact that Necl-1 and Necl-2 have four identical aa at the C-terminal (Fukuhara et al., 2001). Dlg3, Pals2 or CASK bind to mLin-7 through their L27 domain and the L27 domain of mLin-7 (Kamberov et al., 2000; Karnak et al., 2002). mLin-7 is a PDZ-domain-containing protein and, in *C. elegans*, is implicated in the proper localization of the Let-23 protein, a homologue of the mammalian EGF receptor (Kaech et al., 1998). Therefore, Necl-1 may be involved in the localization of transmembrane proteins including membrane receptors. Furthermore, Dlg3 or CASK bind to SAP97 (also called hDlg) through the L27 domain of these two proteins and the MAGUK recruitment domain of SAP97 (Karnak et al., 2002). SAP97 belongs to the MAGUK family and was originally identified as a human homologue of *Drosophila* discs large tumor suppressor gene, *Dlg* (Lue et al., 1994). SAP97 consists of one MAGUK recruitment domain, three PDZ domains, one SH3 domain, one hook domain and one guanylate kinase-domain. SAP97 is highly expressed in the cerebellar molecular layer, and localizes at the axonal shafts and their terminals of parallel fibers, but not at postsynaptic structures (Muller et al., 1995). SAP97 is also expressed in cerebellar glia cells (Leonoudakis et al., 2001). SAP97 may localize at the cell-cell contact sites where Necl-1 localizes in the cerebellum. In addition, SAP97 binds to the strong inwardly-rectifying potassium channel Kir2.2, which is involved in maintenance and control of cell excitability; these two proteins colocalize in cerebellar glia (Leonoudakis et al., 2001). Recently, eight MAGUK family members including Dlg3, Pals2, CASK, SAP97 (others are: PSD-95, Chapsyn-110, SAP102 and Dlg2) and mLin-7 have been identified as being direct- or indirect-Kir2.2-associated proteins by affinity chromatography of brain extracts (Leonoudakis et al., 2004). Therefore, Necl-1 may be involved not only in the cell-cell contact at non-junctional sites in neural tissues but also in the localization of some membrane channels, such as Kir2.2, through the interactions with MAGUK subfamily members. Although Necl-1 shows potentially a broad binding activity to MAGUK subfamily members, further studies are necessary to understand the physiological role of their interaction.

Finally, we discuss the comparison of SynCAM1 and SynCAM3 by Biederer et al. (Biederer et al., 2002) with their respective supposedly identical molecules, Necl-2/IGSF4/RA175/SgIGSF/TSLC1 (Gomyo et al., 1999; Urase et al., 2001; Wakayama et al., 2001; Kuramochi et al., 2001) and Necl-1/TSLL1 (Fukuhara et al., 2001; Fukami et al., 2003). We did this because of the serious inconsistency between the results of Biederer et al. (Biederer et al., 2002), those of other groups (Wakayama et al., 2001; Fukami et al., 2002) and our present results. According to the DNA database, SynCAM1 is identical with Necl-2/IGSF4/RA175/SgIGSF/TSLC1 (Gomyo et al., 1999; Urase et al., 2001; Wakayama et al., 2001; Kuramochi et al., 2001); SynCAM3 is identical with Necl-1/TSLL1 (Fukuhara et al., 2001; Fukami et al., 2003). Necl-2 was directly submitted to GenBank [accession no. AF061260 (mouse), 1998; accession no. AF132811 (human), 1999]; IGSF4 was identified as a candidate for a tumor suppressor gene associated with loss of heterozygosity of chromosome 11q23.2 (Gomyo et al., 1999); the RA175 gene was identified to be highly expressed during neuronal differentiation of embryonic carcinoma cells (Urase et al., 2001); the SgIGSF gene was identified to be expressed in spermatogenic cells during earlier stages of spermatogenesis (Wakayama et al., 2001); TSLC1 was identified to be a tumor suppressor in human non-small cell lung cancer (Kuramochi et al., 2001). TSLC1 shows Ca^{2+}-independent homophilic cell-cell adhesion activity (Masuda et al., 2002); it has a band-4.1-binding motif at the juxtamembrane region and binds the tumor suppressor DAL-1, one of the band-4.1-family members, which connects TSLC1 to the actin cytoskeleton (Yageta et al., 2002). TSLC1 binds Dlg3 through its PDZ-binding motif at the C-terminus (Fukuhara et al., 2003). SynCAM1 is a brain-specific synaptic adhesion molecule and shows Ca^{2+}-independent homophilic cell-cell adhesion activity (Biederer et al., 2002). CASK, syntenin and Mint1 have been identified as SynCAM1-associated proteins by affinity chromatography from rat brain extracts (Biederer et al., 2002). SynCAM1 enables the formation of synapses between HEK293 cells expressing SynCAM1 exogenously and primary cultured hippocampal neurons in vitro (Biederer et al., 2002).

Biederer and co-workers detected by western blotting that SynCAM1 was specifically synthesized in mouse brain (Biederer et al., 2002), but northern blots showed that *TSLC1* and *SgIGSF* are expressed ubiquitously (Wakayama et al., 2001; Fukami et al., 2002). We have recently named this molecule with six different nomenclatures Necl-2 and have shown that Necl-2 has Ca^{2+}-independent homophilic cell-cell adhesion activity and Ca^{2+}-independent heterophilic cell-cell adhesion activity with Necl-1 and nectin-3 (Shingai et al., 2003). Western blotting has shown that Necl-2 is widely

synthesized in mouse tissues and that the protein localizes at the basolateral plasma membrane in epithelial cells of the gall bladder, but not at specialized cell-cell junctions, such as tight junctions, adherens junctions and desmosomes (Shingai et al., 2003). Necl-2 does not bind afadin but binds Pals2.

The major difference between the results of Biederer and co-workers (Biederer et al., 2002) and those of other groups (Wakayama et al., 2001; Fukami et al., 2002; Shingai et al., 2003), including ours, is: Biederer et al. showed SynCAM1 to be specifically synthesized in brain, whereas Necl-2 was found ubiquitously, as was the expression of *TSLC1* and *SgIGSF* (Shingai et al., 2003). In western blots published in the study by Biederer and colleagues, anti-SynCAM1 Ab recognized two proteins (48 kDa and 60 kDa) in the brain homogenates. In contrast to these results, Shingai and co-workers found anti-Necl-2 Ab to react with a 92 kDa band in the homogenate of the brain (Shingai et al., 2003). Therefore, anti-SynCAM1 Ab, used by Biederer et al., cannot have reacted with Necl-2.

If this is the case, which protein is recognized by anti-SynCAM1 Ab? Biederer et al. also reported a very unique distribution pattern of the major 48-kDa protein and the minor 60-kDa protein that are recognized by anti-SynCAM1 Ab: the 48-kDa protein is detected in brain homogenates of mice older than P10, whereas the 60-kDa protein is only seen in those of mice younger than P20 (Biederer et al., 2002). They, furthermore, showed that the bands correspond to the core-glycosylated and the highly-glycosylated (48 kDa and 60 kDa, respectively) form of the same protein (Biederer et al., 2002). With our anti-Necl-1 Ab, we observed a similar distribution pattern of the two proteins (Fig. 1D) to the one described by Biederer and co-workers. Because our anti-Necl-1 Ab is directed against the cytoplasmic domain of Necl-1, probably the cytoplasmic domain is recognized, and its extracellular domain can be either core-glycosilated or highly-glycosilated. However, in mouse cerebellum, anti-SynCAM1 Ab and anti-Necl-1 Ab result in a staining pattern of Necl-1 similar to that seen in adult mice (>P20): both anti-SynCAM1 Ab and our anti-Necl-1 Ab strongly stain the molecular layer and weakly stain the granular layer of the cerebellum. Taken together, anti-SynCAM1 Ab, used by Biederer and co-workers (Biederer et al., 2002), probably recognizes Necl-1. However, although it has been reported that SynCAM1 colocalizes with synaptophysin and localizes at synaptic junctions (Biederer et al., 2002), we could not repeat these results and the reason for this inconsistency remains unknown.

In summary, it is not Necl-2 but Necl-1 which is synthesized specifically in neural tissues (with one or two bands showing up in western blotting), localized at non-junctional contact sites (where presynaptic nerve terminals, axons and glia cell processes contact), and potentially has a MAGUK subfamily member-binding activity.

L cells were supplied from S. Tsukita (Kyoto University, Kyoto, Japan). Mouse Pals2 cDNA and rabbit anti-Dlg3 pAb were supplied from B. Margolis (University of Michigan Medical Center, Michigan, USA). Rabbit anti-Dlg3 antiserum was supplied from E. Peles (The Weizmann Institute of Science, Israel). We thank these researchers for their generous gifts. This investigation was supported by grants-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (2002, 2003).

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