Densin-180, a protein purified from the postsynaptic density fraction of the rat forebrain, is the founding member of a newly described family of proteins termed the LAP (leucine-rich repeats and PSD-95/Dlg-AZO-1 (PDZ) domains) family that plays essential roles in establishment of cell polarity. To identify Densin-180-binding proteins, we screened a yeast two-hybrid library using the carboxyl-terminal fragment of Densin-180 containing PDZ domain as bait, and we isolated δ-catenin/neural plakophilin-related armadillo repeat protein (NPRAP) as a Densin-180-interacting protein. δ-catenin/NPRAP, a member of the armadillo repeat family, is a nervous system-specific adherens junction protein originally discovered as an interactor with presenilin-1, a protein involved in Alzheimer’s disease. Densin-180 PDZ domain binds the COOH terminus of δ-catenin/NPRAP containing the PDZ domain-binding sequence. Endogenous Densin-180 was co-immunoprecipitated with δ-catenin/NPRAP and N-cadherin. Although Densin-180 was reported to be a transmembrane protein, Densin-180 was not accessible to surface biotinylation in dissociated hippocampal neurons; hence Densin-180 may be a cytosolic protein. Densin-180 co-localized with δ-catenin/NPRAP at synapses in dissociated hippocampal neurons. We propose that Densin-180 is associated in vivo with δ-catenin/NPRAP and may be involved in organization of the synaptic cell-cell junction through interaction with the δ-catenin/NPRAP-N-cadherin complex.

Neurotransmission takes place at synapses that are highly specialized sites of cell-cell contact between neurons (1). When viewed electron-microscopically, excitatory synapses have an electron-dense thickening of postsynaptic membrane termed postsynaptic density (PSD). PSD has specific receptors for glutamate as well as numerous receptor-associated proteins; hence PSD can be regarded as a proteinaceous organelle specialized for postsynaptic signal transduction (4).

Densin-180 was identified as a transmembrane protein tightly associated with PSD in central nervous system neurons and was postulated to function as a synaptic adhesion molecule (5, 6). The sequence of Densin-180 contains 16 leucine-rich repeats, a putative transmembrane domain, and a PSD-95/Dlg-AZO-1 (PDZ) domain. Four splice variants (A–D) of the putative cytosolic tail of Densin-180 are differentially expressed during development of the rat brain (7). Densin-180 is phosphorylated by Ca2+/calmodulin-dependent kinase II (CaMKII) (5) and binds CaMKII (6, 7), thereby suggesting a possible role for Densin-180 in the localization of CaMKII at PSD (6, 7). It was also reported that Densin-180 forms a ternary complex with the α-subunit of CaMKII and α-actinin (6). Densin-180 belongs to a recently identified family of PDZ domain-containing proteins. These proteins include Scribble, a Drosophila protein essential for epithelial integrity (8) with tumor suppressive function (9); ERBIN, an ErbB2-interacting protein (10); LET-413, an ERBIN ortholog in Caenorhabditis elegans (11). In addition to the PDZ domain in the COOH termini, members of this family have 16 leucine-rich repeats in the NH2 termini and have thus been named LAP (for leucine-rich repeats and PDZ) proteins (10).

To identify proteins interacting with Densin-180, we screened a human brain cDNA library using the yeast two-hybrid procedure and the putative intracellular domain of Densin-180 as bait. We isolated δ-catenin/neural plakophilin-related armadillo repeat protein (NPRAP) (12, 13) as a potential binding partner for Densin-180. δ-catenin was identified based on its ability to bind to the loop region of presenilin-1, which is encoded by the gene most commonly mutated in familial Alzheimer’s disease (12). Independently, the same gene was identified as a protein homologous to plakophilin 1 and was termed NPRAP (13). δ-catenin/NPRAP is a member of the p120 catenin subfamily, defined as proteins with 10 armadillo repeats (in contrast to the 13 armadillo repeats of β-catenin) in characteristic spacing and often with quite diverse NH2- and COOH-terminal sequences that flank the repeats (14). δ-catenin is reported to co-localize and interact with N-cadherin in the mouse brain (15) and to undergo dynamic relocalization during brain development (16). It was recently reported that δ-catenin/NPRAP binds to PDZ domains of the synaptic scaffolding molecule (S-SCAM) (17) and PAPIN (18). We have now obtained evidence for the in vivo association of Densin-180 with δ-catenin/NPRAP. We propose that Densin-180 may possibly be involved in the organization of synaptic cell-cell adhesion.
**Association of Densin-180 with δ-catenin/NPRAP**

**In Vitro Translation, Pull-down Assays, and Immunoprecipitation**

The cDNAs encoding amino acid residues 1014–1225 (C-SWV) and 1014–1222 (C-ASWV) of human δ-catenin/NPRAP were insert into the pCMV-Tag3B (Myc) expression vector (Stratagene, La Jolla, CA). The C-SWA fragment, which contains a mutation of the COOH-terminal valine to alanine in C-SWV, was also cloned into the pCMV-Tag3B plasmid. These pCMV-Tag3B vectors harboring various constructs of δ-catenin/NPRAP COOH termini were used for coupled in vitro transcription/translation in rabbit reticulocyte lysates using the TNT kit (Promega, Madison, WI). Labeled δ-catenin/NPRAP C-SWV, C-SWA, and C-ASWV polypeptides in lysates were detected using Trans-Act
directive transcription/translation systems (Promega). crude synaptosomes were prepared as described by Hirao et al. (22). In brief, one adult rat brain was homogenized in 8 ml of 0.32 m sucrose containing 4 mM Hepes/NaOH at pH 7.4 and centrifuged at 800 x g for 10 min at 4 °C. The supernatant (S1) was centrifuged at 9,200 x g for 15 min at 4 °C to collect the pellet. The pellet was resuspended in 8 ml of 0.32 m sucrose containing 4 mM Hepes/NaOH at pH 7.4 and centrifuged 10,200 x g for 15 min at 4 °C. The pellet (P2; the crude synaptosomal fraction) was resuspended with 8 ml of a buffer containing 20 mM Hepes/NaOH (pH 8.0), 100 mM NaCl, 5 mM EDTA, and 1% (v/v) Triton X-100 and centrifuged at 100,000 x g for 30 min at 4 °C. The supernatant was used in the pull-down assay and immunoprecipitation.

**Equal amounts of GST or GST-Densin-180 fusion protein beads (~50 µl) were incubated with 1% Triton X-100 soluble lysates at room temperature.** To label surface proteins, rat hippocampal neurons were incubated overnight at 4 °C with anti-Densin-Ext polyclonal antibody, anti-δ-catenin monoclonal antibody (BD Transduction Laboratory, Lexington, KY), anti-N-cadherin monoclonal antibody (BD Transduction Laboratory), control rabbit IgG, or control mouse IgG, and immunocomplexes were immobilized on protein G-agarose beads (Sigma). Immunoprecipitation was analyzed by SDS-PAGE and immunoblotting. Anti-N-cadherin antibody (BD Transduction Laboratory) or anti-N-cadherin monoclonal antibody (BD Transduction Laboratory).

**Labeling of Surface Proteins**—To label surface proteins, rat hippocampal neurons were cultured on a 10-cm dish for 4 days, washed with cold phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂, and incubated with 0.5% paraformaldehyde and 0.1% Triton X-100 in the same buffer at room temperature for 30 min. The labeling reaction was quenched by incubation with 100 mM glycine for 10 min at room temperature. Cells were then lysed in a buffer containing 1% Triton X-100 and 1% deoxycholate. Lysates were clarified by centrifugation at 15 rpm for 30 min, and the supernatants were incubated with streptavidin-agarose beads overnight at 4 °C. Bound proteins were subjected to SDS-PAGE and immunoblotting.

**Immunofluorescence**—Rat hippocampal neurons dissociated at E18 were grown in culture on coverslips for 14 days and fixed by incubation 4% paraformaldehyde in phosphate-buffered saline for 10 min, followed by treatment with 0.2% methanol for 10 min at room temperature. Cells were then lysed in a buffer containing 1% Triton X-100 and 1% deoxycholate. Lysates were clarified by centrifugation at 15 rpm for 30 min, and the supernatants were incubated with streptavidin-agarose beads overnight at 4 °C. Bound proteins were subjected to SDS-PAGE and immunoblotting.

**RESULTS**

**Identification of δ-catenin/NPRAP As a Densin-180-interacting Protein**—We first obtained the full-length cDNA for human Densin-180 (AF434715), as described under “Experimental Procedures.” To identify interaction proteins of Densin-180, we screened a human brain cDNA library, using the yeast two-hybrid technique and the putative cytosolic domain of human Densin-180 as bait. From 1.7 x 10⁷ transformants, 30 positive clones were obtained. Among them, eight positive clones encoded human δ-catenin/NPRAP (12, 13) (Fig. 1A), and two clones were p0071 (23). In addition, the positive clones con-
Association of Densin-180 with δ-catenin/NPRAP

FIG. 1. Identification of δ-catenin/NPRAP as a Densin-180-binding protein. A, schematic description of eight positive clones encoding δ-catenin/NPRAP. The region of δ-catenin/NPRAP encoded by these clones is indicated (bars). B, schematic description of various constructs of Densin-180 and δ-catenin/NPRAP. The cDNAs encoding amino acid residues 1242–1537 (C-SWV) of human δ-catenin/NPRAP were inserted into the yeast prey vector (pGAD-C1). The C-SWA fragment, which contains a mutation of the COOH-terminal valine to alanine in human δ-catenin/NPRAP, was also cloned into the pGAD-C1 vector. C, schematic description of various constructs of Densin-180 and δ-catenin/NPRAP containing a binding site for a PDZ domain. Yeast strain Y190 co-transformed with the bait and the prey vectors, harboring various cDNA fragments shown in B, were plated on Trp−/Leu−/His− medium. A plus sign (+) indicates growth on selective medium and positive β-galactosidase activity; a minus sign (−) indicates no growth on selective medium and negative β-galactosidase activity.

tained one clone of the α-subunit of CaMKII and one clone of α-actinin reported to be Densin-180-binding proteins (6, 7). δ-catenin/NPRAP has a structure similar to that of p0071 and is considered to be a neural isoform of p0071, which is expressed ubiquitously (18). Because Densin-180 has been detected only in the brain, we further examined the interaction of δ-catenin/NPRAP with Densin-180.

Densin-180 PDZ Domain Binds to the Carboxyl Terminus of δ-catenin/NPRAP—PDZ domains of mammalian LAP proteins belong to class I domains that interact with peptides containing a carboxyl-terminal (S/T)XV (in single letter amino acid code, where X is any residue) motif (24, 25). δ-catenin/NPRAP contains a COOH-terminal SWV motif matching a canonical class I PDZ domain binding site. Therefore, the PDZ domain of Densin-180 was speculated to bind the COOH-terminal PDZ-binding motif of δ-catenin/NPRAP. We next examined interactions between several constructs of Densin-180 and δ-catenin/NPRAP, using yeast two-hybrid methods (Fig. 1, B and C). The Densin-180 intracellular domain lacking the PDZ domain (C-ΔPDZ) did not bind wild-type δ-catenin/NPRAP COOH terminus (C-SWV). Conversely, mutation of the carboxyl-terminal valine to alanine in δ-catenin/NPRAP (C-SWA) or deletion of the COOH-terminal three amino acids in δ-catenin/NPRAP (C-ΔSWV) abrogated the interaction (Fig. 1C). These results show that Densin-180 interacts with the carboxyl terminus of δ-catenin/NPRAP via its PDZ domain.

To confirm the validity of the interaction, we next examined the binding of δ-catenin/NPRAP to an immobilized GST fusion protein containing the putative intracellular domain of Densin-180 (GST-Densin-C) in three sets of GST pull-down experiments (Fig. 2). GST-Densin-C binds specifically with in vitro-translated δ-catenin/NPRAP COOH terminus (C-SWV), but not with δ-catenin/NPRAP C-SWA or C-ΔSWV (Fig. 2A). We also noted the interaction of GST-Densin-C and overexpressed Myc-tagged δ-catenin/NPRAP in COS7 cells (Fig. 2B). Furthermore, endogenous δ-catenin/NPRAP in crude synaptosomal fractions of rat brain could be precipitated by GST-Densin-C (Fig. 2C). Because δ-catenin/NPRAP was reported to bind N-cadherin in the brain (15), we asked if precipitates by GST-Densin-C contained N-cadherin. Indeed, N-cadherin and δ-catenin/NPRAP were co-precipitated by GST-Densin-C (Fig. 2C). These results show that the bacterially produced Densin-180 PDZ domain can bind in vitro-translated COOH terminus of δ-catenin/NPRAP, overexpressed Myc-tagged δ-catenin/NPRAP in COS7 cells, and endogenous δ-catenin/NPRAP in crude synaptosomal fractions of the rat brain.
**Fig. 2.** Interaction of GST fusion proteins containing the COOH terminus of Densin-180 with δ-catenin/NPRAP. A, interaction of the COOH terminus of Densin-180 requires the COOH terminus of δ-catenin/NPRAP. cDNAs encoding δ-catenin/NPRAP C-SWV (wild-type), C-SWA, and C-ΔSWV fragments (Fig. 1B) were ligated into the pcMV-Tag3B (Myc) expression vector for coupled in vitro transcription/translation in rabbit reticulocyte lysates using the TNT kit (Promega). Labeled δ-catenin/NPRAP C-SWV (wild-type), C-SWA, and C-ΔSWV polypeptides in lysates were detected using Transcard non-radioactive translation detection systems (Promega) (lane 1). In vitro-translated products were incubated with GST (lane 2) or the Densin-180 COOH terminus protein produced as GST fusion (GST-Densin-C) (lane 3), and precipitated using glutathione beads. GST-Densin-C interacted with δ-catenin/NPRAP C-SWV (wild-type), but not with C-SWA or C-ΔSWV.

B, interaction of overexpressed Myc-δ-catenin/NPRAP with the COOH terminus of Densin-180. The extract of COS7 cells expressing Myc-δ-catenin/NPRAP (containing amino acid residues 434–1225) was incubated with either GST or GST-Densin-C fixed on glutathione beads, and proteins attached to the beads were detected by anti-Myc antibody. Lane 1, input; lane 2, the precipitate with GST; lane 3, the precipitate with GST-Densin-C. C, the pull-down experiment of endogenous δ-catenin/NPRAP and N-cadherin with GST-Densin-C, using crude extracts of rat brain synaptosomes. The 1% Triton X-100 extract of rat crude synaptosomes was incubated with either GST or GST-Densin-C fixed on glutathione beads, and proteins bound to the beads were immunoblotted with anti-δ-catenin antibody or anti-N-cadherin antibody. Lanes 1 and 4, the input; lanes 2 and 5, the precipitate with GST; lanes 3 and 6, the precipitate with GST-Densin-C.

**Fig. 3.** Association of Densin-180 with δ-catenin/NPRAP in vivo. A, characterization of anti-Densin-Ext polyclonal antibody. To explore the interaction between native DENSIIN-180 and δ-catenin, antibodies were raised against human Densin-180. Affinity-purified rabbit anti-Densin-Ext antibody specifically recognized a polypeptide with a relative molecular mass of ~180 kDa in lysates of rat brain (lane 1). The preincubation of anti-Densin-Ext antibody with the antigen specifically inhibited immunoreactivity (lane 2). B, endogenous Densin-180 is associated with δ-catenin/NPRAP and N-cadherin. Immunoprecipitates were prepared from 1% Triton X-100 extracts of crude synaptosomal fractions of rat brain (lane 1), using control rabbit IgG (lane 2), anti-Densin-Ext antibody (lane 3), control mouse IgG (lanes 4 and 6), anti-δ-catenin antibody (lane 5), and anti-N-cadherin antibody (lane 7). Each precipitate, separated into three parts, was subjected to immunoblotting with anti-N-cadherin antibody, anti-δ-catenin antibody, or anti-N-cadherin antibody. C, Densin-180 may be a cytosolic protein. We biotinylated surface proteins in rat dissociated hippocampal neurons. These proteins were lysed in 1% deoxycholate/1% Triton X-100 buffer and then precipitated using streptavidin beads. The precipitates were subjected to immunoblotting with anti-N-cadherin antibody (lanes 1–3) and anti-Densin-Ext antibody (lanes 4–6). Lanes 1 and 4, homogenates; lanes 2 and 5, 1% deoxycholate/1% Triton X-100 soluble fraction; and lanes 3 and 6, the precipitate with streptavidin beads. D, characterization of Densin-180-δ-catenin/NPRAP-N-cadherin complex. Y190 cells co-transformed with pGBD-δ-catenin/NPRAP (amino acids 434–1225), and pGAD-Densin-180 full-length or pGAD-N-cadherin intracellular domain vectors were selected in -Trp-Leu media and subjected to β-galactosidase filter assay. pGBD-N-cadherin intracellular domain and pGAD-Densin-180 full-length vectors were also co-transformed. The interactions were determined as described in the legend to Fig. 1C. N.D., not determined.

**Densin-180 Is Associated with δ-catenin/NPRAP and N-cadherin in Vivo**—To explore the interaction between native Densin-180 and δ-catenin/NPRAP, polyclonal antibodies were raised against Densin-180, using as the immunogen purified His<sub>6</sub>-tagged protein containing amino acids 474–754 of human Densin-180 (Densin-Ext). Affinity-purified rabbit anti-Densin-Ext antibody specifically recognized a polypeptide with a relative molecular mass of ~180 kDa in lysates of rat brain (lane 1). The preincubation of anti-Densin-Ext antibody with the antigen specifically inhibited immunoreactivity (lane 2). B, endogenous Densin-180 is associated with δ-catenin/NPRAP and N-cadherin. Immunoprecipitates were prepared from 1% Triton X-100 extracts of crude synaptosomal fractions of rat brain (lane 1), using control rabbit IgG (lane 2), anti-Densin-Ext antibody (lane 3), control mouse IgG (lanes 4 and 6), anti-δ-catenin antibody (lane 5), and anti-N-cadherin antibody (lane 7). Each precipitate, separated into three parts, was subjected to immunoblotting with anti-N-cadherin antibody, anti-δ-catenin antibody, or anti-N-cadherin antibody. C, Densin-180 may be a cytosolic protein. We biotinylated surface proteins in rat dissociated hippocampal neurons. These proteins were lysed in 1% deoxycholate/1% Triton X-100 buffer and then precipitated using streptavidin beads. The precipitates were subjected to immunoblotting with anti-N-cadherin antibody (lanes 1–3) and anti-Densin-Ext antibody (lanes 4–6). Lanes 1 and 4, homogenates; lanes 2 and 5, 1% deoxycholate/1% Triton X-100 soluble fraction; and lanes 3 and 6, the precipitate with streptavidin beads. D, characterization of Densin-180-δ-catenin/NPRAP-N-cadherin complex. Y190 cells co-transformed with pGBD-δ-catenin/NPRAP (amino acids 434–1225), and pGAD-Densin-180 full-length or pGAD-N-cadherin intracellular domain vectors were selected in -Trp-Leu media and subjected to β-galactosidase filter assay. pGBD-N-cadherin intracellular domain and pGAD-Densin-180 full-length vectors were also co-transformed. The interactions were determined as described in the legend to Fig. 1C. N.D., not determined.
As shown in Fig. 3, otinylated N-cadherin, cells were solubilized with 1% Triton X-100 with streptavidin-agarose beads through association with biotinylated proteins. To address more precisely the nature of the interaction, we attempted to identify new Densin-180 binding partners and characterize protein complexes important for function. Using the two-hybrid system, GST pull-down assay, co-immunoprecipitation analysis, and immunocytochemical staining of dissociated hippocampal neurons, we found that Densin-180 interacts with δ-catenin/NPRAP. The Densin-180 PDZ domain binds to the SWV motif present in the carboxyl terminus of δ-catenin/NPRAP. Densin-180 was immunoprecipitated not only with δ-catenin/NPRAP but also with N-cadherin. Although Densin-180 was reported to be an integral protein, Densin-180 was not accessible to surface biotinylation in dissociated hippocampal neurons, which suggests that Densin-180 may be a cytosolic protein (Fig. 5). In rat hippocampal neurons, Densin-180 co-localizes with δ-catenin/NPRAP and N-cadherin at synapses. These results suggest that Densin-180 may be involved in organization of the synaptic cell-cell junction through association with δ-catenin/NPRAP and N-cadherin. At synapses, cadherin-catenin complexes show both presynaptic and postsynaptic locations (27). In contrast, Densin-180 was originally reported to be enriched in PSD, although it has not yet been determined whether Densin-180 is presynaptic, postsynaptic, or both. Therefore, it is yet unclear if Densin-180 interacts with δ-catenin/NPRAP and N-cadherin at synapses.

**DISCUSSION**

In the present study, we attempted to identify new Densin-180 binding partners and characterize protein complexes important for function. Using the two-hybrid system, GST pull-down assay, co-immunoprecipitation analysis, and immunocytochemical staining of dissociated hippocampal neurons, we found that Densin-180 interacts with δ-catenin/NPRAP. The Densin-180 PDZ domain binds to the SWV motif present in the carboxyl terminus of δ-catenin/NPRAP. Densin-180 was immunoprecipitated not only with δ-catenin/NPRAP but also with N-cadherin. Although Densin-180 was reported to be an integral protein (5), Densin-180 was not accessible to surface biotinylation in dissociated hippocampal neurons, which suggests that Densin-180 may be a cytosolic protein (Fig. 5). In rat hippocampal neurons, Densin-180 co-localizes with δ-catenin/NPRAP and N-cadherin at synapses. These results suggest that Densin-180 may be involved in organization of the synaptic cell-cell junction through association with δ-catenin/NPRAP and N-cadherin. At synapses, cadherin-catenin complexes show both presynaptic and postsynaptic locations (27). In contrast, Densin-180 was originally reported to be enriched in PSD, although it has not yet been determined whether Densin-180 is presynaptic, postsynaptic, or both. Therefore, it is yet unclear if Densin-180 interacts with δ-catenin/NPRAP and N-cadherin at synapses.

By way of summary, we provide evidence that Densin-180 is associated in vivo with an armadillo protein, δ-catenin/NPRAP, and we suggest that Densin-180 may be involved in the organization of synaptic cell-cell contacts. As other LAP proteins regulate cell polarity in epithelial cells, it may be that brain-specific Densin-180 can affect cell polarity in neurons through interactions with an armadillo protein. Identification of proteins binding to other domains (e.g., leucine-rich repeats) of Densin-180 will be necessary to unravel the entire function of Densin-180.
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