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Characterization of Densin-180, a New Brain-Specific Synaptic Protein of the *O*-Sialoglycoprotein Family

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We purified an abundant protein of apparent molecular mass 180 kDa from the postsynaptic density fraction of rat forebrain and obtained amino acid sequences of three tryptic peptides generated from the protein. The sequences were used to design a strategy for cloning the cDNA encoding the protein by polymerase chain reaction. The open reading frame of the cDNA encodes a novel protein of predicted molecular mass 167 kDa. We have named the protein densin-180. Antibodies raised against the predicted amino and carboxyl sequences of densin-180 recognize a 180 kDa band on immunoblots that is enriched in the postsynaptic density fraction. Immunocytochemical localization of densin-180 in dissociated hippocampal neuronal cultures shows that the protein is highly concentrated at synapses along dendrites. The message encoding densin-180 is brain specific and is more abundant in forebrain than in cerebellum. The sequence of densin-180 contains 17 leucine-rich repeats, a sialomucin domain, an apparent transmembrane domain, and a PDZ domain. This arrangement of domains is similar to that of several adhesion molecules, in particular GPIbα, which mediates binding of platelets to von Willebrand factor. We propose that densin-180 participates in specific adhesion between presynaptic and postsynaptic membranes at glutamatergic synapses.

Key words: postsynaptic density; adhesion molecule; synapse development; synaptic cleft; microsequencing; polymerase chain reaction

Glutamatergic synapses are crucial for information processing and storage in the brain, yet, until recently, little was known about the protein machinery at the postsynaptic membrane that functions in adhesion to the presynaptic terminal, neurotransmitter receptor clustering, and signal transduction. We reasoned that at least some of the molecules important for these functions are likely to be part of the postsynaptic density (PSD), an electron-dense thickening just beneath the postsynaptic membrane (Palay, 1956). Our lab and others have focused on the characterization of proteins found in the PSD fraction. This subcellular fraction is prepared after detergent extraction of synaptosomes (Cotman et al., 1974; Cohen et al., 1977; Carlin et al., 1980). A common criticism of the strategy of characterizing proteins associated with this fraction is that non-PSD proteins may adhere to the PSD during homogenization or detergent extraction. To minimize this possibility, we have concentrated on studying proteins that remain associated with the PSD fraction after extraction with the relatively harsh detergent *N*-lauroyl sarcosinate (sarcosyl). We refer to the proteins that remain in the insoluble pellet after sarcosyl extraction as "core" PSD proteins.

Our lab previously identified three core PSD proteins that have potentially important functions at the synapse. First, the α subunit of the type II calcium/calmodulin-dependent protein kinase (CaMKII) is enriched in the core fraction and has been localized to the PSD by immunoelectron microscopy (Kennedy et al., 1983, 1990). CaMKII mediates signal transduction in response to calcium influx at the synapse and is important for synaptic plasticity (Silva et al., 1992). A second PSD protein characterized in our laboratory is PSD-95 (Cho et al., 1992), a novel brain-specific protein with significant homology to the *Drosophila* disks-large protein (dlg; Woods and Bryant, 1991). Cho et al. identified three repeats in PSD-95 and dlg that are now called PDZ domains. Like the α subunit of CaMKII, PSD-95 has been localized to the PSD by immunoelectron microscopy of synaptosomes (Hunt et al., 1996). A third core PSD protein that we identified is the 2B subunit of the NMDA receptor (NR2B), which is the major tyrosine-phosphorylated protein in the PSD fraction (Moon et al., 1994). Recently, PSD-95 has been shown to bind directly to NR2B *in vitro* and to colocalize with NR2B at synapses in dissociated hippocampal neuronal cultures (Kornau et al., 1995). The association occurs via the second of three repeat domains, identified by Cho et al. (1992), that are now called PDZ domains. Protein associations formed by PDZ domains may reflect a mechanism for clustering NMDA receptors and other molecules in the postsynaptic membrane.

One potential function of the proteins associated with the PSD is adhesion between the pre- and postsynaptic membranes. A dense material that is coextensive with the PSD fills the synaptic cleft and has been proposed to contain adhesion and extracellular matrix molecules. Furthermore, the tight linkage between sites of vesicle docking at the presynaptic membrane and sites of thick postsynaptic densities beneath the postsynaptic membrane is likely to be mediated by adhesion molecules. Here, we describe the cloning of densin-180, a core PSD protein that has characteristics of a synaptic adhesion molecule.

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MATERIALS AND METHODS

Purification of densin-180 and sequencing of tryptic peptides. The crude PSD fraction was prepared as described previously (Cho et al., 1992) by a modification of the method developed by Carlin et al. (1980). The densin-180 protein (previously termed PSD-up180) was purified as described in Moon et al. (1994). Briefly, detergent-extracted, deglycosylated PSD proteins (63 mg) were fractionated by electrophoresis on 60 preparative 6% SDS-PAGE gels. The densin-180 protein band was cut from each gel. Gel pieces were pooled, chopped into 5 mm pieces, and electroeluted into 25 mM *N*-ethylmorpholine, pH 8.5, and 0.1% SDS at 250 V in an Elutrap device (Schleicher & Schuell, Keene, NH). The electroeluted protein (1.2 mg) was fractionated on a second set of eight preparative 6% SDS-PAGE gels, transferred to nitrocellulose, and trypsinized as described previously (Aebersold et al., 1987). The trypsinized densin-180 protein was concentrated to 0.4 ml and fractionated on a C4 high performance liquid chromatography (HPLC) column with a gradient of 3.5–73.5% acetonitrile in 0.1% trifluoroacetic acid. We hand-collected fractions of 0.1–1.0 ml corresponding to the elution of major peaks of absorbance at 280 nm. Most of the major peaks were not single peptides and were further fractionated on a second C18 HPLC column. Peak fractions again were collected by hand, flash-frozen in liquid nitrogen, concentrated to 50–100 μl, and submitted to the Caltech Biopolymer Analysis Facility for peptide sequencing on an ABI automated gas phase sequencer. Amino acid sequences were obtained from seven of these samples with initial yields of 1–25 pmol.

Molecular cloning of densin-180. Degenerate oligonucleotide primers were designed on the basis of the three unique peptide sequences, synthesized on an ABI automated oligonucleotide synthesizer, and used as primers to amplify 5-week-old rat forebrain cDNA by polymerase chain reaction (PCR; Saiki et al., 1988). The cDNA was prepared from mRNA with the First Strand cDNA kit purchased from Clontech (Palo Alto, CA). The PCR reactions contained 0.2 μM each of sense and antisense primer; 2.5 mM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Indianapolis, IN); 3.75 ng/μl cDNA; 125 mU/μl *Taq* polymerase (Boehringer Mannheim); 1× *Taq* polymerase buffer (supplied with enzyme); and 0.5 mM extra $MgCl_2$. PCR products from large-scale reactions (100 μl) were purified by agarose gel electrophoresis and inserted into the TA plasmid supplied with the TA cloning kit (Invitrogen, San Diego, CA), and the plasmid was amplified by growth in *Escherichia coli*. We sequenced the ends of each cloned PCR product by priming with oligonucleotides complementary to the M13 and T7 promoter sites in the TA plasmid. This permitted us to check which of the PCR products encoded the entire sequence of the original pair of peptides, including those amino acids that were not encoded in the original PCR primers. The sequence of the ends of one 1.2 kb product encoded the complete sequences of peptides 1 and 3. This product was purified by agarose gel electrophoresis, labeled with ^{32}P according to the Random Primed DNA Labeling Kit (USB), and used to screen a λZapII cDNA library prepared from 13- to 16-d-old rat brains (Snutch et al., 1990; generously provided by T. Snutch, University of British Columbia). Positive cDNA clones were plaque-purified and then excised from λZapII with the ExAssist/SOLR system (Stratagene, La Jolla, CA). The cDNA inserts were aligned and classified by restriction mapping. The cDNAs were ligated into the pBluescript plasmid (Stratagene) and sequenced by the method of Sanger (Sanger et al., 1977), according to the instructions supplied with the Sequenase kit (USB). Initial sequencing from primers complementary to the pBluescript vector and to the PCR product revealed that clone 1.1 (5.2 kb) contained a 5′ ribosome-binding site and initiation codon as well as a long open reading frame, including sequences encoding peptides 1 and 3. We sequenced exonuclease digests of clone 1.1 generated with the Erase-a-Base System (Promega, Madison, WI) in both directions. Gaps in the sequence were filled in with the use of oligonucleotide sequence primers. These primers were also used for the partial sequencing of other clones. Programs of the Wisconsin Package (Genetics Computer Group) and local programs at the Caltech Sequence Analysis Facility were used for sequence assembly, motif searches, and hydrophobicity analysis.

Preparation of antibodies against densin-180. We amplified two regions of the densin-180 cDNA encoding amino acids 466–958 and 1374–1495 by PCR and then cloned the products into the pGEX2T vector (Pharmacia Biotech, Piscataway, NJ) to create glutathione sulfotransferase (GST) fusion proteins. The PCR products were sequenced to ensure that no mutations were introduced during the PCR reaction. The recombinant pGEX2T plasmids were grown in protease-deficient (lon^-) *E. Coli* cultures at 30°C to an optical density of 0.5 at 600 nm wavelength. A 1 l culture was induced with 0.1 mM isopropyl-β-Dthiogalacytopyranoside (IPTG) for 5 hr at 30°C, and cells were pelleted by centrifugation at 5000 × *g* for 10 min. Pellets were resuspended in 40 ml of lysis buffer [20 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 1× protease inhibitor cocktail (Boehringer Mannheim), 0.5 mM DTT, and 10 U/ml DNase (Boehringer Mannheim)]. The cells were lysed by sonication (2 min, level 6, 50% pulse with Branson Sonifier 450), Triton X-100 was added to 1%, and the solution was mixed well. Lysates were cleared by centrifugation for 10 min at 10,000 × *g*. The supernatant fractions were applied to a washed column containing 100 mg of glutathione-agarose beads (Sigma Chemical, St. Louis, MO). The column was washed twice with 40 ml of PBS (20 mM sodium phosphate, pH 7.4, and 0.15 M NaCl), and the GST fusion proteins were eluted with 10 mM reduced glutathione (Sigma Chemical), 50 mM Tris-Cl, pH 8.0, and 1% Triton X-100. The purity and concentration of the proteins in each eluted fraction were estimated by SDS-PAGE and by staining with Coomassie blue.

The fusion protein containing amino acids 466–958 of densin-180 was further purified by electrophoresis on 6% SDS-PAGE gels. The full-length fusion protein was visualized by soaking in 0.25 M KCl and cut from the gel for injection into Swiss–Webster mice as an antigen for production of polyclonal ascites fluid (Ou et al., 1993). One mouse (M2) produced antibodies specific for densin-180 when used for immunoblots, immunoprecipitations, or immunostaining. This M2 ascites fluid (3 ml) was purified by 50% ammonium sulfate precipitation overnight at 4°C, followed by centrifugation at 10,000 × *g* for 10 min. The protein pellet was resuspended in 1 ml of 25 mM Tris-HCl, pH 7.5, and dialyzed against two changes of the same buffer overnight. Purified M2 Ascites fluid was used for immunoblots of PSD fraction (at 1:3000 dilution), immunoprecipitation from PSD fraction (at 1:10 dilution), and immunofluorescent staining (at 1:150 to 1:300 dilution).

The fusion protein containing residues 1374–1495 of densin-180, corresponding to the C terminus containing the PDZ domain, eluted from the glutathione column as 95% full-length fusion protein and was dialyzed against PBS, diluted to 1 mg/ml in PBS, and used to immunize rabbits (Cocalico Biologicals). The rabbit polyclonal antibodies (termed CT245) were highly specific for densin-180 on immunoblots and could be used for immunocytochemistry. CT245 serum was used for immunoblots of the PSD fraction (at 1:25,000 to 1:50,000 dilution) and for immunofluorescent staining (at 1:2500 to 1:5000 dilution).

Immunoblots. Proteins were separated by SDS-PAGE under reducing conditions, electrophoretically transferred to nitrocellulose, and blocked from 2 hr to overnight in 5% normal goat serum (NGS) diluted in TTBS (0.2% Tween-20, 10 mM Tris-Cl, pH 7.5, and 0.2 M NaCl). After one wash in TTBS for 10 min, blots were incubated in primary antibodies diluted in TTBS plus 1% NGS from 3 hr to overnight. Blots were washed three times in TTBS and then incubated for 1 hr in alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies as appropriate (Boehringer Mannheim) diluted in TTBS plus 1% NGS. After three 10 min washes with TTBS, blots were developed according to the suppliers' instructions.

Subcellular fractions of rat brain. Forebrain homogenates, synaptosomes, and detergent-extracted PSD fractions were prepared from Sprague Dawley rats exactly as described in Cho et al. (1992).

For the membrane extraction experiments, we prepared a crude membrane fraction by homogenizing two rat forebrains in 20 ml of buffer A containing (in mM): 0.32 sucrose, 1 sodium bicarbonate, 1 $MgCl_2$, 0.5 $CaCl_2$, 0.1 PMSF, and 1 mg/ml leupeptin at 4°C with six strokes of a Teflon/glass homogenizer rotating at 900 rpm. The homogenate was cleared by centrifugation at 2500 × *g* for 10 min, and the supernatant was divided into 10 separate tubes containing 2 ml each. Membranes were pelleted by centrifugation at 170,000 × *g* for 45 min, and the crude membrane pellets were resuspended in 2 ml of each test extraction buffer by five up-and-down strokes in a Teflon/glass homogenizer. The extractions were incubated at 4°C for 30 min, and the membrane residue was pelleted by centrifugation at 170,000 × *g* for 45 min. Supernatants were collected, and the pellets were resuspended in HKA buffer containing (in mM): 10 HEPES-KOH, pH 7.5, 140 potassium acetate, 1 $MgCl_2$/0.1 EGTA, 0.1 PMSF, and 5 mg/ml leupeptin. The pellet and supernatant fractions were frozen in aliquots at −80°C for use in immunoblots. We used the following extraction buffers: 1 M NaCl, 2% CHAPS, 2% Triton X-100, 1 M NaCl + 2% CHAPS or 1 M NaCl + 2% Triton X-100, all in HKA buffer, or 0.2 M sodium bicarbonate buffer, pH 11. The presence of densin-180 in each fraction was assessed by immunoblotting. Immunoblots with antibody specific for synapsin I were used for comparison. Approximately 90% of synapsin was solubilized in 2% CHAPS, 1 M NaCl

+ 2% CHAPS, 2% Triton X-100, 1 M NaCl + 2% Triton X-100, and pH 11 buffers, but only 30% was solubilized in 1 M NaCl.

Isolation of mRNA and Northern blotting. Total RNA was isolated from rat tissues (frozen in liquid nitrogen and purchased from Pel-Freeze Biologicals, Rogers, AR, or Harlan Bioproducts, Indianapolis, IN) with the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) and purified on CsCl gradients. Poly(A^+) RNA was isolated with the Poly(A) Tract mRNA Isolation System (Promega). RNA from different tissues was fractionated on 1% agarose gels and transferred to Zeta-Probe membranes (Bio-Rad, Richmond, CA) overnight in 20× SSC (3 M NaCl/0.3 M trisodium citrate). RNA transfer was confirmed by staining with methylene blue. A cDNA probe corresponding to nucleotides 1950–2400 of the densin-180 cDNA was amplified by PCR. The PCR product and human β-actin cDNA (Clontech) were radiolabeled by random priming (Random Primed DNA Labeling Kit from USB) to specific activities of 10^9 and 10^7 cpm/μg, respectively. The RNA blots were probed with the labeled cDNAs according to the protocol suggested for use with the Zeta-Probe membrane. Labeled bands were detected by autoradiography.

Deglycosylation with neuraminidase. Aliquots of PSD protein (40 NOG μg) were denatured by boiling for 3 min in 0.8% SDS. *N*-octyl glucoside was added to a final concentration of 3%. Deglycosylation reactions were prepared containing denatured PSD protein (0.8 mg/ml), 0.2 M sodium phosphate buffer, pH 7.2, and Complete Protease inhibitor cocktail (Boehringer Mannheim). Neuraminidase (40 mU) from *Arthrobacter ureafasciens* (Boehringer Mannheim) was added in two aliquots, and the reaction proceeded for a total of 24 hr at 37°C. Control reactions contained no added neuraminidase or included the neuraminidase inhibitor *N*-bromosuccinimide at 10 mM, added in two aliquots with the neuraminidase. Fresh protease inhibitors were added to all tubes twice during the reaction. Reactions were terminated by boiling in SDS-PAGE sample buffer for 3 min. Proteins were fractionated by SDS-gel electrophoresis, and densin-180 was detected by immunoblotting.

Digestion of densin-180 with O-*sialoglycoprotease.* Nondenatured PSD protein (24 μg) was incubated with 36 μg of *O*-sialoglycoprotein endoprotease from *Pasteurella haemolytica* (Accurate Chemical & Scientific, Westbury, NY) in 20 mM sodium phosphate, pH 7.4, 0.15 M NaCl, and 0.2 mM PMSF in a final volume of 60 μl. Protease reactions were incubated at 37°C for 15 min, 1 hr, or 3 hr. Control reactions without added protease were incubated for 3 hr. Reactions were terminated by boiling in SDS-PAGE sample buffer for 3 min, and proteolytic products were fractionated by SDS-PAGE. Fragments of densin-180 were detected on immunoblots probed with either M2 or CT245 anti-densin-180 antibodies. Control immunoblots were probed with anti-PSD-95 (1:10,000; Cho et al., 1992) or anti-NMDA receptor 2B antibodies (1:80,000; Kornau et al., 1995).

Phosphorylation and immunoprecipitation of densin-180. Phosphorylation reactions contained 24 μg of PSD protein, 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 0.4 mM EGTA, 10 mM DTT, and 10 μg/ml added calmodulin in a final volume of 50 μl. Some reactions also contained 0.6 mM calcium and/or a mixture of 4A11 (0.3 μg/μl) and 6E9 (0.4 μg/μl) anti-CaMKII-inhibiting monoclonal antibodies (Molloy and Kennedy, 1991). After a 3 min preincubation at 30°C, ^{32}P-ATP (10,000 cpm/pmol) was added to a final concentration of 25 μM, and the reaction was incubated for 2 min at 30°C. Phosphorylation was terminated by addition of SDS (0.2% final), followed by boiling for 3 min. For immunoprecipitation, the phosphorylated protein was brought to a final concentration of 0.28 mg/ml phosphorylated PSD protein in 1× SDS-RIPA buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) in a final volume of 85 μl. The solutions were precleared by incubation with 50 μg of washed protein A-Sepharose beads (Pierce, Rockford, IL) at 4°C for 2 hr. Precleared supernatant was collected and incubated with M2 antibody (10 μl) overnight at 4°C. This solution was added to 100 μg of washed protein A beads and incubated at 4°C for 2 hr. After three washes in 1× SDS-RIPA buffer, the beads were boiled for 5 min in 50 μl of 1.5× SDS-gel buffer and applied to a 7.5% SDS-PAGE minigel. After electrophoresis, the gel was stained with Coomassie R-250, dried, and subjected to autoradiography. The amount of densin-180 protein was estimated by comparing the Coomassie-stained densin-180 band with stained bovine serum albumin standards. Bands corresponding to densin-180 were cut from the gel, and their radioactivity was determined in a Beckman liquid scintillation counter. The stoichiometry of densin phosphorylation was estimated at 1 mol/mol by calculating a calcium-induced incorporation of 0.21 of pmol ^{32}P- phosphate into 40 ng (0.22 pmol) of densin-180 protein.

Immunocytochemical labeling of dissociated hippocampal neurons. Hippocampi from E18 rats were dissociated by trypsinization, and cells were plated on laminin-coated coverslips (15 mm in diameter) at a density of ~200/mm^2. Cultures were plated and maintained in the B27 media described by Brewer et al. (1993). After 2–4 weeks *in vitro*, the coverslips were removed from the culture wells and placed cell-side up into wells containing ice-cold PBS. After being washed briefly in ice-cold methanol, cultures were fixed with −20°C methanol for 20 min, washed once with PBS for 15 min, and then preblocked in 5% NGS, 0.05% Triton X-100, 450 mM NaCl, and 20 mM phosphate buffer, pH 7.4, for 1 hr at 4°C. Next, primary antibodies were added in the preblock buffer at appropriate dilutions and incubated overnight at 4°C. In addition to the M2 and CT245 anti-densin-180 antibodies, the following antibodies were also used for immunofluorescent staining: anti-synapsin I rabbit antiserum at a 1:1000 dilution, affinity-purified anti-PSD-95 rabbit antiserum at 60 μg/ml final concentration (both described in Cho et al., 1992), and 6G9 anti-αCaMKII at 20 μg/ml (Erondu and Kennedy, 1985). After three washes in the preblock solution, the coverslips were incubated in goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or Cy3 fluorophores (diluted 1:100 in preblock) at room temperature. Coverslips were washed once in the preblock buffer for 15 min, twice with PBS for 15 min, post-fixed for 5 min with 2% paraformaldehyde, washed twice with PBS for 10 min, and washed twice with 0.1 M sodium bicarbonate, pH 9.2, for 5 min. Coverslips were then mounted on slides in 80% glycerol, 4 mg/ml *p*-phenylenediamine, and 0.1 M sodium bicarbonate buffer, pH 9.2, left at room temperature for 2 hr, and then either viewed immediately or stored at −20°C for no longer than 1 week. Cultures were viewed in a fluorescence laser-scanning confocal microscope (Zeiss LSM310, Oberkochen, Germany). A 63× oil immersion objective was used at electronic zoom factors of 1 or 2. Images were scanned at 64 sec. Fluorescein was excited at 488 nm and Cy3 at 543 nm. Images were collected through filters appropriate for the two fluorophores. The contrast and brightness settings were optimized to spread the data over the 8 bit range. Contrast settings were 360–410, and brightness settings were 6000–6800. Double images were colorized and aligned in Adobe Photoshop without adjusting the original data. Final images were printed at 300 dpi resolution on a Kodak XLS 8300 printer.

The concentrations of the antibodies were estimated by comparison to IgG standards on SDS-PAGE gels after staining with Coomassie blue R-250. The M2 mouse IgG was ~0.3 mg/ml, and the CT245 rabbit IgG was ~5 mg/ml. Preabsorption of anti-densin-180 antibodies with antigen at a 1:3 molar ratio entirely blocked staining at the contrast and brightness settings above.

RESULTS

PCR cloning based on tryptic peptide sequences

The purification of densin-180 was described previously (Moon et al., 1994). Briefly, the crude PSD fraction was isolated from rat forebrain, extracted with 1% NOG, and applied to 6% preparative SDS polyacrylamide gels. Densin-180 was then electroeluted and trypsinized as described under Materials and Methods. Seven tryptic peptides were purified sufficiently for automated sequencing. A search of the GenBank database performed with the BLAST network service revealed that three of these peptide sequences are not homologous to any known protein.

Initial attempts to select cDNA clones by screening several libraries with radiolabeled "guessmer" oligonucleotides on the basis of the sequence of peptide 1 proved unsuccessful. Therefore, we used a PCR-based approach similar to that of Saiki et al. (1988). Several sets of degenerate 29 base sense and antisense oligonucleotide primers were designed on the basis of the sequences of the three peptides (Fig. 1*A*). The neutral base inosine was included at eight or fewer positions to reduce primer degeneracy to no more than 32-fold. All possible combinations of sense and antisense primers were used for PCR amplification of rat brain cDNA. Of all possible primer combinations, only the (1Sense + 3Antisense) reaction produced a 1.2 kb PCR product that was absent in control reactions containing only the primers (Fig. 1*C*). This product was cloned and sequenced. The strategy for obtaining and analyzing this clone is diagrammed in Figure

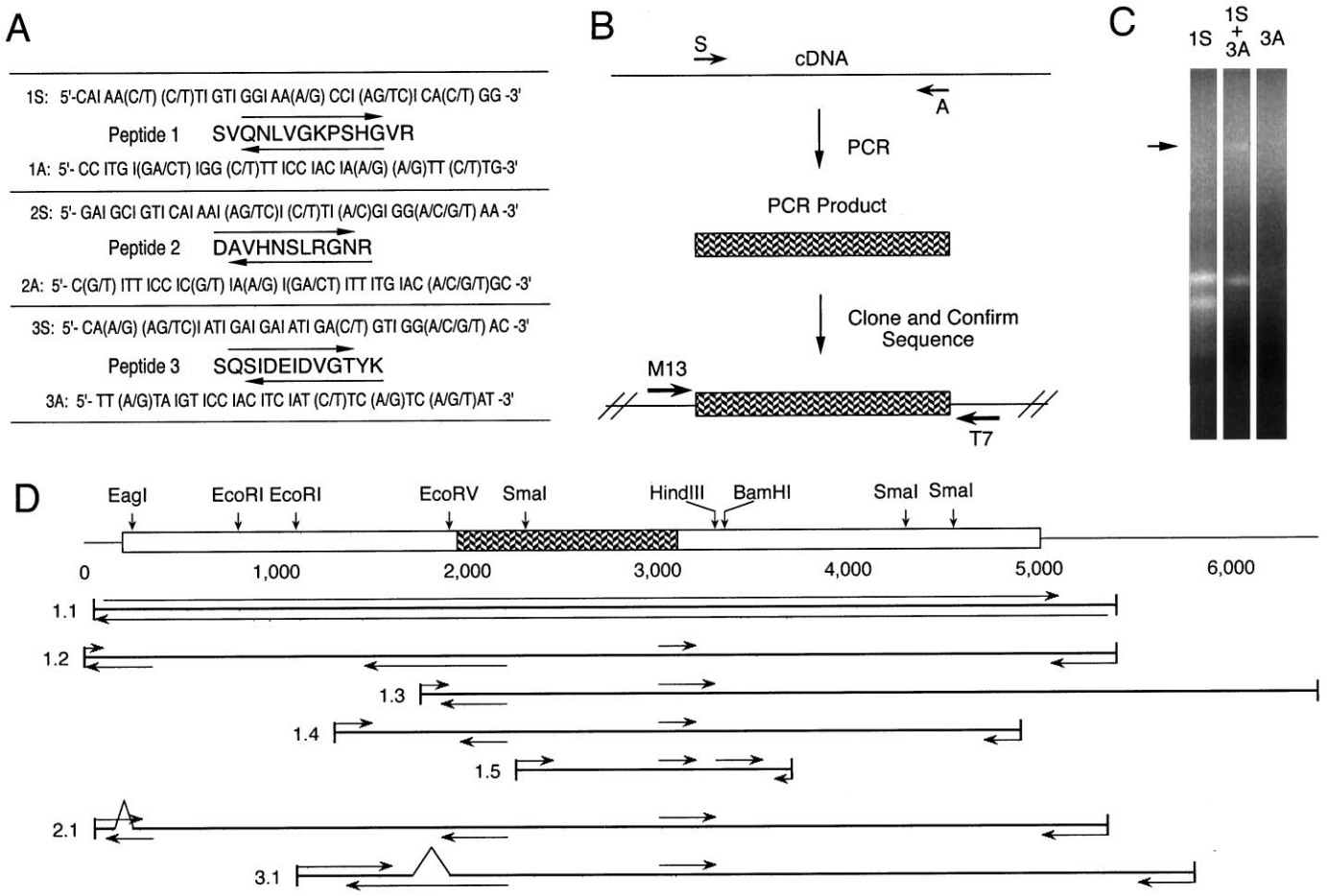

Figure 1. Tryptic peptide sequences and PCR cloning of densin-180. *A*, Amino acid sequences of three tryptic peptides from densin-180 were used to design sense (*S*) and antisense (*A*) degenerate 29 mer oligonucleotide primers: *A*, adenosine; *C*, cytosine; *G*, guanosine; *I*, inosine; *T*, thymidine. Degenerate nucleotide positions are enclosed in *parentheses*. The 10 amino acids used to design the sense (*right-pointing arrows*) and antisense (*left-pointing arrows*) are indicated *above* and *below* the peptide sequences, respectively. *B*, PCR cloning strategy. Combinations of sense and antisense primers (*arrows*) were used for amplification of sequences from rat forebrain cDNA by PCR. A PCR product (represented by *hatched box*) was cloned into a vector, and its nucleotide sequence was determined by dideoxy sequencing from the M13 and T7 primer sites of the vector (*bold arrows*). *C*, DNA from PCR reactions was fractionated on a 1.2% agarose gel, and the DNA was visualized by ethidium bromide staining. The size of the PCR product was estimated by comparison with DNA molecular weight markers. The 1S + 3A primer combination produced a 1.2 kb PCR product (*arrow*) that was absent in reactions containing 1S or 3A alone. *D*, Restriction map and sequencing strategy for cDNA clones encoding densin-180. Clone 1.1 was sequenced in its entirety, and the coding region (*open box*), the region of hybridization with the PCR product (*hatched box*), and the 5′ and 3′ noncoding regions (*horizontal lines*) are indicated on the restriction map (map units are in base pairs). The locations of cDNA clones determined by restriction mapping and sequencing are shown *below* the restriction map. The extent and directionality of overlapping cDNA sequences are depicted as *arrows* for each cDNA clone. Clone 2.1 lacks nucleotides 111–186 of the densin-180 sequence (*broken line*) containing the ribosome-binding site and part of the initiation codon. Clone 3.1 lacks a 249 base pair sequence spanning nucleotides 1632–1880 (*broken line*) encoding amino acids 483–565 of the densin-180 sequence.

1*B*. The sequence immediately 3′ downstream of the 1Sense primer encoded the amino acids Val-Arg, matching the corresponding amino acid sequence of peptide 1. In addition, the complementary sequence immediately 3′ downstream of the 3Antisense primer encoded the amino acids Ser-Gln-Ser, corresponding to the amino acid sequence of peptide 3. We found that the 1.2 kb fragment could be amplified from rat brain cDNA but not from cDNA made from other tissues (data not shown), suggesting that the encoded protein is brain specific. Partial sequences of the PCR product were compared with the database and found to encode a novel protein.

Cloning and sequencing of full-length densin-180 cDNAs

The PCR product was labeled by random priming and used to screen a λZap II rat brain cDNA library (kindly provided by Dr. Terry Snutch). Five independent positive cDNA clones spanning 6.8 kb were aligned by restriction mapping and partial sequencing (Fig. 1*D*). The entire 5.2 kb cDNA clone 1.1 was sequenced; we found that it contains the complete densin-180 open reading frame. It includes an initiation codon at position 186 preceded by a stop codon at 177, which fits the consensus for translation initiation sites (Kozak, 1989). There is also a purine-rich Shine–Dalgarno ribosome-binding consensus motif beginning ~10 nucleotides upstream of the initiation codon (Shine and Dalgarno, 1974). This initiation codon is followed by a single 4485 bp open reading frame encoding a 1495 residue protein with a molecular weight of 167,499. The complete nucleotide sequence has been deposited in the GenBank database and assigned accession number U66707. The amino acid sequence that it encodes is shown in Figure 2. All three of the original tryptic peptide sequences (Fig.

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   1  MQCLEMTTKR KLIGRLVPCR CFRGEEEIIS VLDYSHCSLQ QVPKEVFNFE RTLEELYLDA NQIEELPKQL
  71  FNCQALRKLS IPDNDLSSLP TSIASLVNLK ELDISKNGVQ EFPENIKCCK CLTIIEASVN PISKLPDGFT
 141  QLLNLTQLYL NDAFLEFLPA NFGRLVKLRI LELRENHLKT LPKSMHKLAQ LERLDLGNNE FSELPEVLDQ
 211  IQNLRELWMD NNALQVLPGS IGKLKMLVYL DMSKNRIETV DMDISGCEAL EDLLLSSNML QQLPDSIGLL
 281  KKLTTLKVDD NQLTMLPNTI GNLSLLEEFD CSCNELESLP PTIGYLHSLR TLAVDENFLP ELPREIGSCK
 351  NVTVMSLRSN KLEFLPEEIG QMQRLRVLNL SDNRLKNLPF SFTKLKELAA LWLSDNQSKA LIPLQTEAHP
 421  ETKQRVLTNY MFPQQPRGDE DFQSDSDSFN PTLWEEQRQQ RMTVAFEFED KKEDDESAGK VKALSCQAPW
 491  DRGQRGITLQ PARLSGDCCT PWARCDQQIQ DMPVPQSDPQ LAWGCISGLQ QERSMCAPLP VAAQSTTLPS
 561  LSGRQVEINL KRYPTPYPED LKNMVKSVQN LVGKPSHGVR VENANPTANT EQTVKEKFEH KWPVAPKEIT
 631  VEDSFVHPAN EMRIGELHPS LAETPLYPPK LVLLGKDKKE STDESEVDKT HCLNNSVSSG TYSDYSPSQA
 701  SSASSNTRVK VGSLQPTTKD AVHNSLWGNR IAPPFPQPLD AKPLLTQREA VPPGNLPQRP DRLPMSDAFP
 771  DNWTDGSHYD NTGFVSEEAT GENANNNPLL SSKARSVPAH GRRPLIRQER IVGVPLELEQ STHRHTPETE
 841  VPPSNPWQNW TRTPSPFEDR TAFPSKLETT PTTSPLPERK DHMKEPTETP GPFSPGVPWE YHDPTPNRSL
 911  GNVFSQIHCR PDSSKGVIAI SKSTERLSPL MKDIKSNKFK KSQSIDEIDV GTYKVYNIPL ENYASGSDHL
 981  GSHERPDKFL GPEHGMSSMS RSQSVPMLDD EMLMYGSSKG PPQQKASMTK KVYQFDQSFN PQGAVEVKAE
1051  KRIPPPFAHN SEYVQQPGKN IAKDLVSPRA YRGYPPMEQM FSFSQPSVNE DAMVNAQFAS QGPRAGFLRR
1121  ADSLASSTEM AMFRRVSEPH ELPPGDRYGR AAYRGGLEGQ SSVSMTDPQF LKRNGRYEDE HPSYQEVKAQ
1191  AGSFPAKNLT QRRPLSARSY STESYGASQT RPVSARPTMA ALLEKIPSDY NLGNYGDKTS DNSDIKTRPT
1261  PVKGEESCGK MPADWRQQLL RHIEARRLDR TPSQQSNILD NGQEDVSPSG QWNPYPLGRR DVPPDTITKK
1331  AGSHIQTLMG SQSLQHRSRE QQPYEGNINK VTIQQFQSPL PIQIPSSQAT RGPQPGRCLI QTKGQRSMDG
1401  YPEQFCVRIE KNPGLGFSIS GGISGQGNPF KPSDKGIFVT RVQPDGPASN LLQPGDKILQ ANGHSFVHME
1471  HEKAVLLLKS FQNTVDLVIQ RELTV
```

Figure 2. Protein sequence translated from the densin-180 cDNA. The DNA sequence of clone 1.1, containing the entire coding region, was determined by sequencing both strands. It has been deposited in the GenBank database and assigned accession number U66707. The protein translation is shown in the figure. Protein sequences of tryptic peptides 1, 2, and 3 are *underlined*. Potential N-linked glycosylation sites, CaMKII phosphorylation sites (*bold*), and RGD cell attachment motif are shown as *boxed* residues. The potential transmembrane domain is *underlined* (*gray bar*), and the 16 leucine-rich repeats are contained in amino acids 53–420. The amino- and carboxy-flanking cysteine-rich domains span amino acids 19–37 and 486–546, respectively. The mucin homology domain spans amino acids 825–915, and the PDZ domain spans amino acids 1405–1492.

1) are present in the amino acid sequence. Sequences 1 and 3 match exactly, and sequence 2 has one mismatch (Arg at position 8 corresponds to a Trp in densin-180). This mismatch most likely results from an ambiguous call during the peptide sequencing and explains the absence of specific PCR products from the 1Sense/2Antisense and 2Sense/3Antisense primer combinations.

The message encoding densin-180 may be alternatively spliced. Partial sequencing of two more of the cDNAs (2.1 and 3.1; Fig. 1*D*) revealed possible splice variants. A 76 nucleotide sequence is missing at the 5′ end of clone 2.1 when compared with clone 1.1 (*underlined* in Fig. 2). This sequence spans nucleotides 111–186 of the densin-180 sequence and includes the ribosome-binding site and the adenosine of the ATG initiation codon. Clone 3.1 contains a 249 bp deletion between nucleotides 1631 and 1881 of clone 1.1 that does not shift the reading frame and deletes amino acids 483–565 (*underlined* in Fig. 2), including the second cysteine-rich domain (see below).

Domain structure of densin-180

A search of the GenBank database performed with the BLAST network service through the National Center for Biotechnology Information revealed significant homology in the N terminus of

A

```
                    1                                   23
LRR1   53-LEELYLDAN QIEELPKQLF NCQA
LRR2      LRKLSIPDN DLSSLPTSIA SLVN
LRR3      LKELDISKN GVQEIPENIK CCKC
LRR4      LTIIEASVN PISKLPDGIT QLLN
LRR5      LTQLYLNDA FLEFLPANIG RLVK
LRR6      LRILELREN HLKTLPKSVH KLAQ
LRR7      LERLDLGNN EISELPEVLD QIQN
LRR8      LRELWIDNN ALQVLPGSIG KLKM
LRR9      LVYLDISKN RIETVDMDIS GCEA
LRR10     LEDLLLSSN MLQQLPDSIG LLKK
LRR11     LTTLKVDDN QLTMLPNTIG NLSL
LRR12     LEEIDCSCN ELESLPPTIG YLHS
LRR13     LRTLAVDEN FLPELPREIG SCKN
LRR14     VTVISLRSN KLEFLPEEIG QIQR
LRR15     LRVLNLSDN RLKNLPFSIT KLKE
LRR16     LAALWLSDN QSKALIPLQT EIHP-420

Consensus L--L-L--N -L--LP--IG -L--
```

B

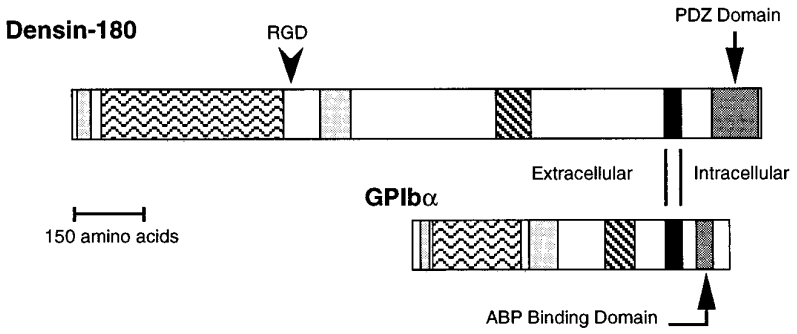
```
                    1                                                  50
DENSIN  1400 GYPEQICVRI EKNPGLGFSI SGGISGQGNP FKPSDKGIFV TRVQPDGPA.
1PSD95       ....EITLER GNS.GLGFSI AGGTDNP..H I.GDDPSIFI TKIIPGGAAA
2PSD95       ....EIKLIK GPK.GLGFSI AGGVGNQ..H I.PGDNSIYV TKIIEGGAAH
3PSD95       ....RIVIHR GST.GLGFNI VGGEDGE... ......GIFI SFILAGGPAD
1DLG         ....DIQLER GNS.GLGFSI AGGTDNP..H I.GTDTSIYI TKLISGGAAA
2DLG         ....EIDLVK GGK.GLGFSI AGGIGNQ..H I.PGDNGIYV TKLTDGGRAQ
3DLG         ....TITIQK GPQ.GLGFNI VGGEDGQ... ......GIYV SFILAGGPAD
1ZO1         ....TVTLHR APGFGIGIAI SGGRDNP..H FQSGFTSIVI SDVLKGGPA.
2ZO1         ...KVTLVKS RKNEEIGIRP ASH..... .......IFV KEISQDSLAA
3ZO 1        .....KLVKF RKGDSVGIRL AG..... ..GNDVGIFV AGVLEDSPAA
NOS          .....VRLFK RKVGGLGFLV K.ERVS.... ....KPPVID SDLIRGGAAE

Consensus    -----I-L-K G---GLGFSI AGG-DN---- ----D-GIYV T-IL-GGPAA

                    51                                                 100
DENSIN       .SNLLQPGDK ILQANGHS.. ..IVHMEHEK AVLLLKSFQN TVDLVIQRELTV*
1PSD95       QDGRLRVNDS ILFVNEVD.. ..VREVTHSA AVEALKEAGS IVRLYVMRRR.
2PSD95       KDGRLQIGDK ILAVNSVG.. ..LEDVMHED AVAALKNTYD VVYLKVA KP.
3PSD95       LSGELRKGDQ ILSVNGVD.. ..LRNASHEQ AAIALKNAGQ TVTIIAQYK.
1DLG         ADGRLSINDI ISVNDVS.. ..VDVPHAS AVDALKKAGN VVKLHVKRK.
2DLG         VDGRLSIGDK LAVRTNGSE KNLENVTHEL AVATLKSIT. ..........
3DLG         LGSELKRGDQ LLSVNNVN.. ..LTHATHEE AAQALKTSGG VVTLIAQYR.
1ZO1         .EGQLQENDR VAMVNGVS.. ..MINVEHAF AVQQLKKSGK NAKITIRRKK
2ZO1         RDGDIQEGDV VLKINGTVT. ...ENMSLTD AKTLIERSKG KLKIVVQRD.
3ZO1         KEG.LEEGDQ ILRVNNVD.. ..ITNIIREE AVLFLLDLPK GEEVTLLAQK
NOS          QSGLIQAGDI ILAVNDRP.. ..LVDLSYDS AIEVLRGIAS ETHVVILR.

Consensus    -DGRL--GD- IL-VN-V--- --LENV-HE- AV-ALK--G- -V-LVV-RK-
```

C



densin-180 with the superfamily of leucine-rich repeat (LRR)-containing proteins. Alignment of the 16 contiguous LRRs in densin-180 reveals a repeating 23 residue consensus sequence (Fig. 3*A*) that fits the general consensus defined for LRRs from a variety of transmembrane and secreted proteins, including adhesion molecules (for review of LRR-containing proteins, see Kobe and Deisenhofer, 1994, 1995b). LRRs vary from 20 to 29 residues in length, with 24 residues most common. Clusters of cysteine residues are found immediately flanking the LRRs in densin-180. At the N terminus, three cysteine residues are found between amino acids 19 and 37, and on the carboxyl side six cysteine residues are found between residues 486 and 546. Cysteine-rich domains typically flank the LRRs of adhesion molecules, but the densin-180 cysteine clusters are of a different type, because they do not match the consensus described in Kobe and Deisenhofer (1993).

Amino acids 825–915 define a region rich in serine, threonine, and proline residues similar to repeats found in mucin. Mucin-like repeats are thought to serve as sites of attachment of O-linked sugars in mucin and many other proteins, including the platelet protein GPIbα (for review, see Strous and Dekker, 1992; Van Klinken et al., 1995).

The BLAST search identified a clear PDZ domain consensus at the C terminus, spanning residues 1405–1492. (Fig. 3*B*). The PDZ motif was first defined in PSD-95, another PSD protein identified in our laboratory. The motif mediates protein–protein interactions and is present in a variety of other proteins associated with intracellular junctions, including the *Drosophila* dlg protein (Woods and Bryant, 1991) and the human tight junction protein ZO-1 (Itoh et al., 1993).

Initial analysis of the densin-180 sequence failed to reveal a hydrophobic signal sequence expected in a transmembrane protein. However, the SIGCLEAVE program identified an embedded signal sequence spanning amino acids 28–40 with cleavage at residue 41 and a score of 3.6. The SIGCLEAVE program uses the Von Heijne (Von Heijne, 1986, 1987) method to locate signal sequences and is 95% accurate with a score of 3.5 or higher. Using the method of Kyte and Doolittle to predict regions of high hydrophobicity in the sequence, we have assigned a transmembrane domain from residues 1223 to 1246, placing the PDZ domain on the cytosolic side. The 24 residue putative transmembrane domain is atypical, because it contains nine charged and polar amino acids. Helical wheel projections of this region using the HELICALWHEEL program reveal an amphipathic helix-like structure, with one face of the α-helical surface containing exclusively hydrophobic residues and the rest of the surface containing a mixture of hydrophobic, charged, and polar residues (data not shown). The sequence contains two proline residues that would produce a kinked α helix. It is possible that the transmembrane domain is a β sheet, as has been proposed for a number of transmembrane proteins, including the nicotinic receptor (Unwin, 1993).

Analysis of the densin-180 sequence with the MOTIFS program identified an Arg-Gly-Asp (RGD) tripeptide sequence between the LRR domain and the cysteine-rich repeats (amino acids 437–439 in Fig. 2). The RGD tripeptide was originally identified as a sequence in fibronectin that mediates cell attachment. RGD sequences from fibronectin and a number of other proteins have been found to mediate adhesion via binding to integrins (for review, see D'Souza et al., 1991). Finally, we identified two consensus sequences that are potential sites of phosphorylation by CaMKII (Fig. 2; see below).

The arrangement of domains in densin-180 is similar to that of the family of LRR-containing glycoproteins, although there is little significant primary sequence homology with any of them. One of the most well characterized of the LRR-containing glycoproteins is the platelet adhesion molecule GPIbα (Lopez et al., 1987). Both densin-180 and GPIbα contain LRRs flanked by cysteine-rich domains and mucin homology domains in the putative extracellular portion of the proteins (Fig. 3*C*). The PDZ domain of densin-180, which likely represents a protein-binding site (Kim et al., 1995; Kornau et al., 1995), is in a position analogous to the cytosolic actin-binding protein (ABP)-binding domain of GPIbα. GPIbα is part of a protein complex that mediates binding of von Willebrand factor (vWF) via its LRR domain and flanking cysteine-rich domain. The binding induces adhesion of platelets to blood vessel walls (for review, see Williams et al., 1995). The C terminus of GPIbα has been shown to interact with actin-binding protein (ABP) to mediate association with the cytoskeleton (Andrews and Fox, 1992).

Densin-180 is highly enriched in the PSD fraction

One criterion that we have used to check the specificity of the association of a protein with the PSD fraction is its enrichment in the PSD fraction, as compared with other subcellular fractions. CaMKII (Kennedy et al., 1983), PSD-95 (Cho et al., 1992), and the 2B subunit of the NMDA receptor (NR2B; Moon et al., 1994) are all ~10- to 30-fold enriched in PSD fractions prepared by successive extraction with detergents. We raised antibodies against fusion proteins containing sequences from the putative extracellular domain (mouse, M2) and the putative intracellular C-terminal domain (rabbit, CT245), as described under Materials and Methods. These antibodies react strongly with a 180 kDa band that migrates at the position of the densin-180 protein on SDS gels (Fig. 4*A*; data not shown). We prepared immunoblots of rat brain homogenates, synaptosomes, and three different PSD fractions extracted with successively harsher detergent (Fig. 4*A*). The 180 kDa densin-180 band is highly enriched in synaptosomes, as compared with the crude homogenate, and is further enriched in all of the detergent-extracted PSD fractions, as compared with synaptosomes. Densin-180 remained associated with the PSD fraction even after extraction with *N*-lauroyl sarcosinate and therefore can be considered a core PSD protein.

←

Figure 3. Domain structure of densin-180. *A*, Alignment of the 16 densin-180 leucine-rich repeats reveals a 23 residue consensus shown at the *bottom*. Corresponding amino acid numbers of densin-180 are indicated to the *left* of the first repeat and to the *right* of the 16th repeat. *B*, Identification of a PDZ domain in densin-180. Alignment of amino acids 1400–1493 of densin-180 with 10 PDZ domains from four other proteins [3 PDZ domains from *PSD95* (Cho et al., 1992); 3 from *Drosophila* disks-large protein (*DLG*; Woods and Bryant, 1991); 3 from the human zona occludens protein (*ZO-1*; Itoh et al., 1993); 1 from neuronal nitric oxide synthase (*nNOS*; Bredt et al., 1991)]. *C*, The domain structure of densin-180 as compared with the LRR-containing glycoprotein GPIbα. The leucine-rich repeats (16 in densin-180 and 7 in GPIbα; *wavy lines*) with N-terminal and C-terminal cysteine-rich flanking regions (*lightly stippled*) and mucin-like domains (*diagonal lines*) are indicated for both proteins. Potential transmembrane domains are depicted in *black*. The ABP-binding protein domain of GPIbα and the PDZ domain of densin-180 at the C-terminal regions of the proteins are represented in *gray*. The position of the RGD sequence is indicated with an *arrowhead*. Scale bar, 150 amino acids.

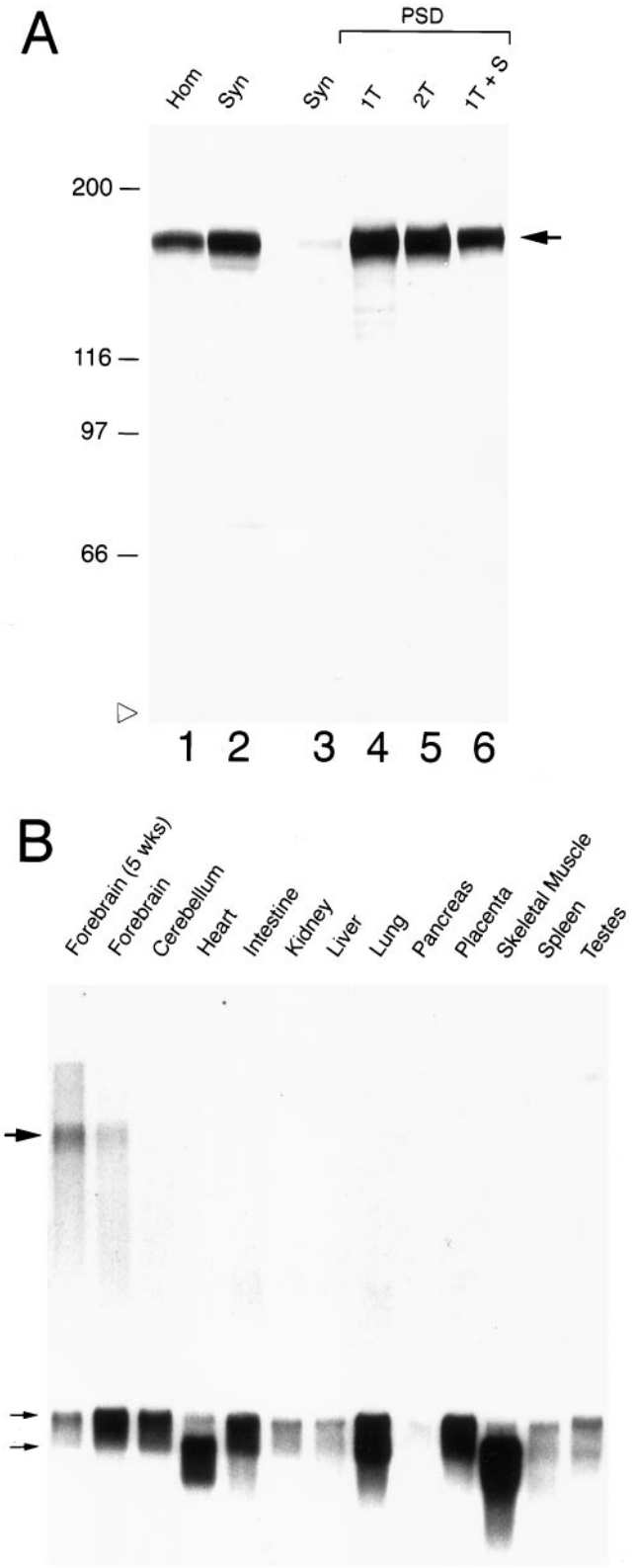


Figure 4. Densin-180 protein is enriched in PSD fractions, and its mRNA expression is brain-specific. *A*, Enrichment of densin-180 protein in detergent-extracted PSD fractions. Immunoblots were prepared with 50 μg (*lanes 1*, *2*) of rat brain homogenate (*Hom*) and synaptosome fractions (*Syn*) and 7.5 μg (*lanes 3–6*) each of synaptosome (*Syn*), once Triton X-100-extracted PSD (*1T*), twice Triton X-100-extracted PSD (*2T*), and once Triton X-100 and then sarcosyl-extracted PSD (*1T + S*; Cho et al., 1992). Densin-180 protein band (*arrow*) is visualized with antibody M2 against densin-180. Molecular weight markers and position of the dye front (*open arrowhead*) are shown at *left*. *B*, Densin-180 Northern blot. Poly(A)$^+$ RNA (5 μg) from 13 different tissue samples was electrophoresed on a 1% agarose gel. The mRNA was transferred to Zeta-Probe blotting membrane (Bio-Rad), and all lanes were determined to have equal amounts of RNA by methylene blue staining. Blots were probed with a random prime-labeled PCR-amplified DNA fragment of densin-180 spanning nucleotides 1100–2170 (specific activity, 10^9 cpm/μg). A single broad band at 7.4 kb was detected (*large arrow*) on autoradiographs exposed for 14 d with an intensification screen. The blot was then stripped and reprobed with the 2 kb random prime-labeled human β-actin cDNA (specific activity, 10^7 cpm/μg). The autoradiograph of an 8 hr exposure with an intensification screen is shown in the *bottom panel*. The two forms of β-actin message are indicated (*small arrows*).

Densin-180 mRNA is detected only in brain

Northern blots prepared with Poly(A)$^+$ RNA from several tissues of 10-week-old rats and from forebrain of 5-week-old rats were probed for messages encoding densin-180. A single 7.4 kb message was detected in forebrain and faintly in cerebellum (Fig. 4*B*). There was no detectable densin-180 mRNA in any of the other tissues. Densin-180 mRNA was expressed at a higher level in the forebrain of 5-week-old than of 10-week-old rats (*far left lane*, Fig. 4*B*), suggesting age-dependent regulation of expression of the mRNA.

Densin-180 is a sialoglycoprotein

The predicted amino acid sequence of densin-180 implies a molecular weight of 167 kDa, but the densin-180 protein migrates at an apparent molecular weight of 188 kDa on a 7.5% SDS polyacrylamide gel. One explanation for this discrepancy could be that it is glycosylated, as would be expected for a transmembrane protein. We tested whether densin-180 is glycosylated by treating the PSD fraction with various glycosidases. Only very slight shifts in the mobility of densin-180 were observed after treatment with *N*-glycosidase F or *O*-glycosidase. However, a shift in its apparent molecular weight from ~188 to 148 kDa was evident after treatment with neuraminidase from *Arthrobacter ureafasciens* (Fig. 5*A*). The shift in molecular weight was attributable to the neuraminidase activity and not to contaminating protease activity, because addition of a specific inhibitor of the *Arthrobacter ureafasciens* neuraminidase, 10 mM *N*-bromosuccinimide, inhibited the shift in molecular weight. Thus, the densin-180 protein is heavily glycosylated with sialic acid residues.

An *O*-sialoglycoprotein endoprotease recently has been identified from *Pasteurella haemolytica* that specifically cleaves proteins containing mucin-like O-linked glycans (Abdullah et al., 1992; reviewed, Mellors and Sutherland, 1994). Substrates that have been identified contain 15 or more closely spaced O-linked glycosylation sites along the protein backbone (Norgard et al., 1993). These substrates include GPIbα (Yeo and Sutherland, 1993), cranin (Smalheiser and Kim, 1995), glycophorin A (Abdullah et al., 1992), CD34 and CD43 (Sutherland et al., 1992a,b), and epiglycanin (Kemperman et al., 1994). To test the sensitivity of densin-180 to this protease, an aliquot of the nondenatured PSD fraction was incubated with protease for varying times (Fig. 5*B,C*). PMSF was included in all incubations to inhibit serine proteases. The digested PSD proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies M2 and CT245, which are specific for the extracellular and intracellular portions of densin-180, respectively. After 3 hr of incubation with protease, complete loss of the 185 kDa densin-180 band was evident in both immunoblots. The pattern of proteolysis detected

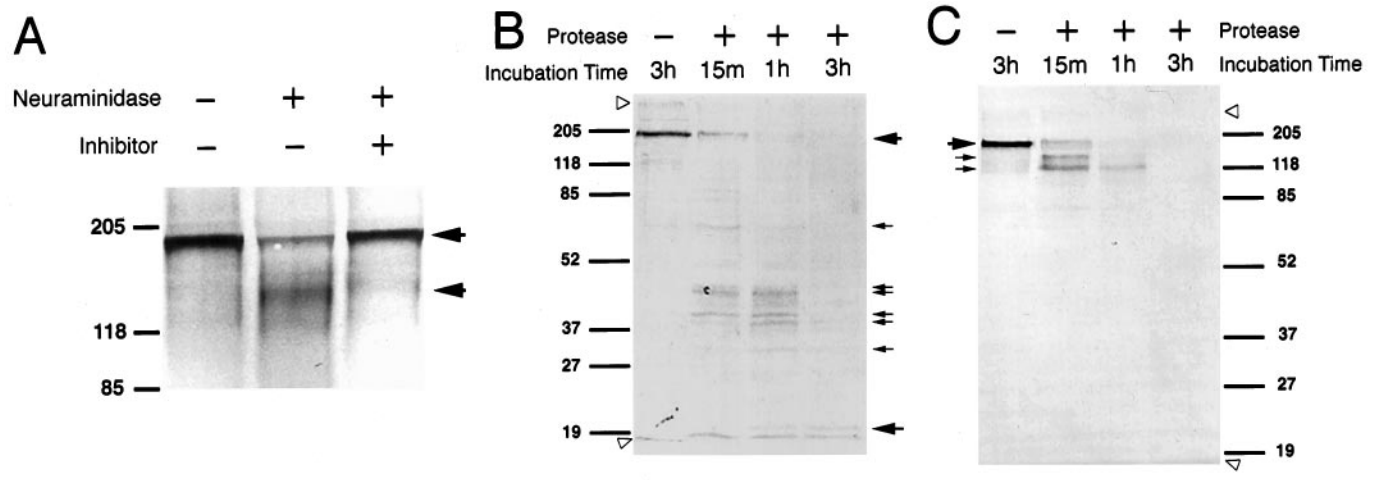


Figure 5. Densin-180 is a mucin-like sialoglycoprotein. *A*, Densin-180 is heavily glycosylated with sialic acid. Twenty micrograms of denatured protein from the PSD fraction were incubated overnight at 37°C under each of the following conditions: control reaction with no added neuraminidase (*lane 1*), with added neuraminidase (*lane 2*), and with added neuraminidase plus 10 mM *N*-bromosuccinimide (*lane 3*). Digested protein was fractionated by SDS-PAGE and probed with antibody against densin-180, as described under Materials and Methods. The 188 kDa undigested (*top*) and 148 kDa digested (*bottom*) densin-180 protein bands are indicated by *arrows*. The positions of 205, 118, and 87 kDa molecular weight markers are shown at *left*. *B, C,* Proteolysis of densin-180 by *O*-sialoglycoprotein endoprotease. Nondenatured PSD fraction (24 μg) was incubated with 0.4 mg/ml final volume of *O*-sialoglycoprotein endoprotease, as described under Materials and Methods. All incubations were performed in the presence of 0.2 mM PMSF to inhibit endogenous proteases in the PSD fraction. Protease reactions were incubated at 37°C for 15 min (*15m*), 1 hr (*1h*) and 3 hr (*3h*). Control reactions with no protease added were incubated for 3 hr at 37°C. Reactions were terminated by adding gel sample buffer and boiling for 3 min. Digested protein was fractionated by SDS-PAGE and probed with two different antibodies against densin-180. The immunoblot shown in *B* was probed with CT245, a rabbit polyclonal serum that reacts with epitopes in the potential cytoplasmic domain spanning residues 1374–1495 of densin-180. The CT245 antibody detects the undigested 188 kDa densin-180 protein band (*large black arrow*) and a complex pattern of proteolytic fragments (*gray arrows*) of densin-180. These proteolytic fragments include major bands at ~70, 45 (doublet), 40 (doublet), 30, and 20 kDa. The 20 kDa band (*large gray arrow*) is resistant to proteolysis after 3 hr at 37°C. The immunoblot shown in *C* was prepared with M2, a mouse polyclonal ascites that reacts with epitopes contained in amino acids 466–958 of the putative extracellular domain of densin-180. This antibody detects the undigested 188 kDa densin-180 protein band (*large black arrow*) and 140 and 120 kDa proteolytic fragments (*gray arrows*). The positions of molecular weight standards are shown at the *right* side with *open arrowheads* indicating the origin of the gel (*top*) and dye front (*bottom*).

with each antibody was consistent with initial proteolysis at a site near the mucin homology domain of densin-180, producing extracellular fragments with approximate molecular weights of 140 and 120 kDa detected by the M2 antibody. The largest major breakdown product detected with the CT245 antibody had a molecular weight of ~65 kDa, corresponding to a site of initial proteolysis in the mucin homology domain of densin-180. The CT245 antibody also detected a 21 kDa protease-resistant fragment, even after 3 hr incubations (Fig. 5*B*), suggesting that the C-terminal region of densin-180 may be inaccessible to the protease because of tight interactions with other PSD proteins. Epitopes of other sialoglycoproteins have been shown to resist proteolysis (Mellors and Sutherland, 1994). Control blots of PSD digests detected no *O*-sialoglycoprotein sensitivity for either NR2B or PSD-95, even after 3 hr incubations (data not shown).

Nature of association of densin-180 with the membrane fraction

The domain structure of densin-180 places it in the family of LRR-containing glycoproteins that span the membrane, yet the putative transmembrane domain contains several charged and polar amino acids. To test how tightly densin-180 associates with membrane fractions, we extracted crude membranes from rat forebrain with detergent and/or salt. Immunoblots of the soluble and particulate fractions were prepared and probed with specific mouse polyclonal antibodies raised against recombinant densin-180 protein (Fig. 6). Densin-180 is not solubilized by extraction with 2% Triton X-100 or 2% CHAPS, conditions that solubilize many membrane proteins but do not solubilize proteins tightly bound to the PSD fraction. When the membranes were extracted with 1 M NaCl to disrupt protein interactions, densin-180 also remained in the pellet fraction. However, when the membranes were extracted with a combination of 1 M NaCl and 2% Triton X-100 or of 1 M NaCl and 2% CHAPS, approximately one-half of the densin-180 was solubilized. Taken together, the solubility profile is consistent with anchoring of densin-180 in the membrane fraction by a combination of lipid and protein interactions.

Extraction with sodium bicarbonate buffer, pH 11, also solubilized approximately one-half of the densin-180. It is generally assumed that high pH buffers extract mainly peripheral membrane proteins, yet its sequence and biochemical characteristics

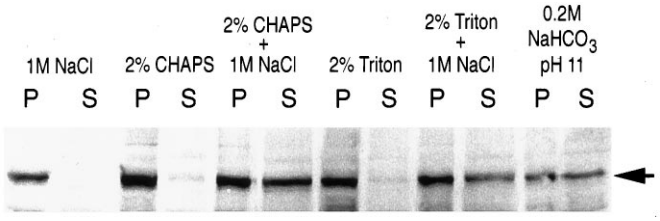


Figure 6. Solubility of densin-180 in brain membrane fractions. Crude membrane fractions were isolated from rat brain. Pellet (*P*) and supernatant (*S*) fractions were separated by centrifugation at 170,000 × *g* after extraction of membranes with 1 M NaCl, 2% CHAPS, 1 M NaCl + 2% CHAPS, 2% Triton X-100, 1 M NaCl + 2% Triton X-100, or 0.2 M sodium bicarbonate, pH 11, for 1 hr at 4°C. Proteins were fractionated by SDS-PAGE and probed with antibody against densin-180. The position of the densin-180 band is indicated with an *arrow*.

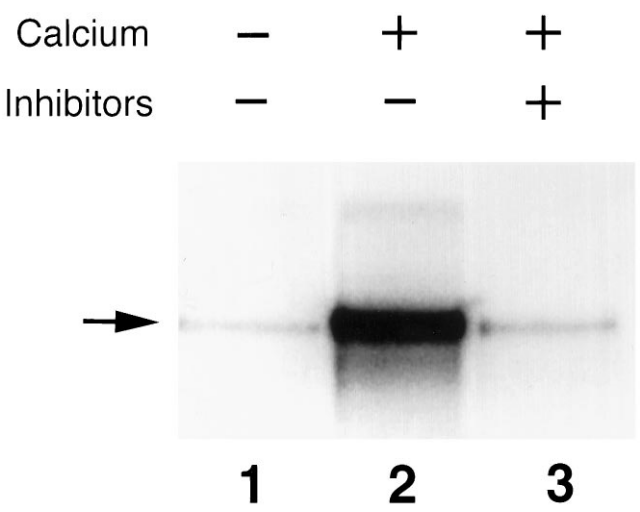


Figure 7. Densin-180 is phosphorylated by endogenous CaMKII in the PSD fraction. Phosphorylation reactions containing ^{32}P-labeled ATP and 24 μg of protein from the PSD fraction were performed in the absence (*lane 1*) and presence (*lane 2*) of calcium and in the presence of calcium plus inhibiting antibodies to CaMKII (*lane 3*), as described under Materials and Methods. Reactions were terminated by adding SDS to a final concentration of 1% and boiling for 5 min. Densin-180 was immunoprecipitated from the denatured phosphorylation reactions with M2 antibody to densin-180, as described under Materials and Methods, and applied to a 6% SDS- polyacrylamide gel. A section of the autoradiograph of a 16 hr exposure of the dried gel is shown. The position of densin-180 is indicated by an *arrow*.

(see below) suggest that densin-180 is a transmembrane protein. This unusual extraction profile could reflect the atypical sequence of the putative transmembrane domain of densin-180. The ability of the two positive (R, K) and two negative (D, E) residues in this domain to form salt bridges with other transmembrane proteins may explain the sensitivity of densin-180 to extraction in pH 11 buffers.

Densin-180 is phosphorylated by CaM kinase II

CaMKII is highly concentrated in the PSD, as determined by both biochemical and immunocytochemical experiments (Kennedy et al., 1983, 1990) and can be activated *in vitro* in the PSD fraction. We labeled substrates of CaMKII in the PSD fraction by performing a phosphorylation reaction for 2 min at 30°C in the presence of calcium, calmodulin, and ^{32}P-ATP. After phosphorylation, densin-180 was immunoprecipitated from the PSD fraction. Figure 7 shows an autoradiogram of the immunoprecipitates. Phosphorylation was stimulated by calcium and reached a stoichiometry of ~1 pmol of phosphate per picomole of protein, estimated as described under Materials and Methods. The reaction was inhibited ~90% by addition of two antibodies against CaMKII that have been shown to inhibit kinase activity (Fig. 6, *lane 3*; Molloy and Kennedy, 1991). Thus, densin-180 is specifically phosphorylated by endogenous CaMKII in the PSD fraction. This phosphorylation and the extensive glycosylation of densin-180 are consistent with the transmembrane orientation proposed in Figure 3*C*.

Densin-180 is located at synapses in dissociated hippocampal neurons

Antibodies to densin-180 were used for immunocytochemical staining of dissociated rat brain hippocampal cell cultures. Hippocampal neurons plated at E18 were grown in culture for 2 to 4 weeks (Brewer et al., 1993). Cells were stained as described under Materials and Methods with antibodies against densin-180, synapsin I, PSD-95, and the α subunit of CaMKII. Confocal imaging revealed that the staining for densin-180 was membrane-associated and punctate along dendrites, with little cytoplasmic staining above background (Fig. 8). Staining of the axon initial segment was also frequently observed (Fig. 8*B*). The pattern of staining was identical for the M2 and CT245 antibodies, and staining with both antibodies was completely blocked by overnight preabsorption with their antigens (data not shown).

We double-labeled cultures with anti-densin-180 antibodies and antibodies against other synaptic markers. Staining for synapsin I, a presynaptic vesicle marker, overlaps significantly with densin-180 staining (Fig. 8*A*) and thus confirms that densin-180 is located at synapses. At higher magnification, it is evident that the synapsin I is present over a larger area than densin-180, extending away from the dendrite. The larger structure stained by synapsin I likely corresponds to the presynaptic terminal (Fig. 8*A*, *inset*).

Double-labeling with antibodies to densin-180 and PSD-95 revealed a stricter correlation of the extent of staining (Fig. 8*B*, *inset*). Finally, double-labeling with densin-180 and the α subunit of CaMKII resulted in correlated punctate staining along dendrites. However, densin-180 is not found in so large quantities in the cytoplasm of dendritic shafts or cell bodies as is CaMKII (Fig. 8*C*). Images of this staining at high magnification reveal that densin-180 is located along dendrites at what seem to be spines (Fig. 8*C*, *inset*). The high degree of colocalization of densin-180 with the postsynaptic density proteins PSD-95 and CaMKII pro-

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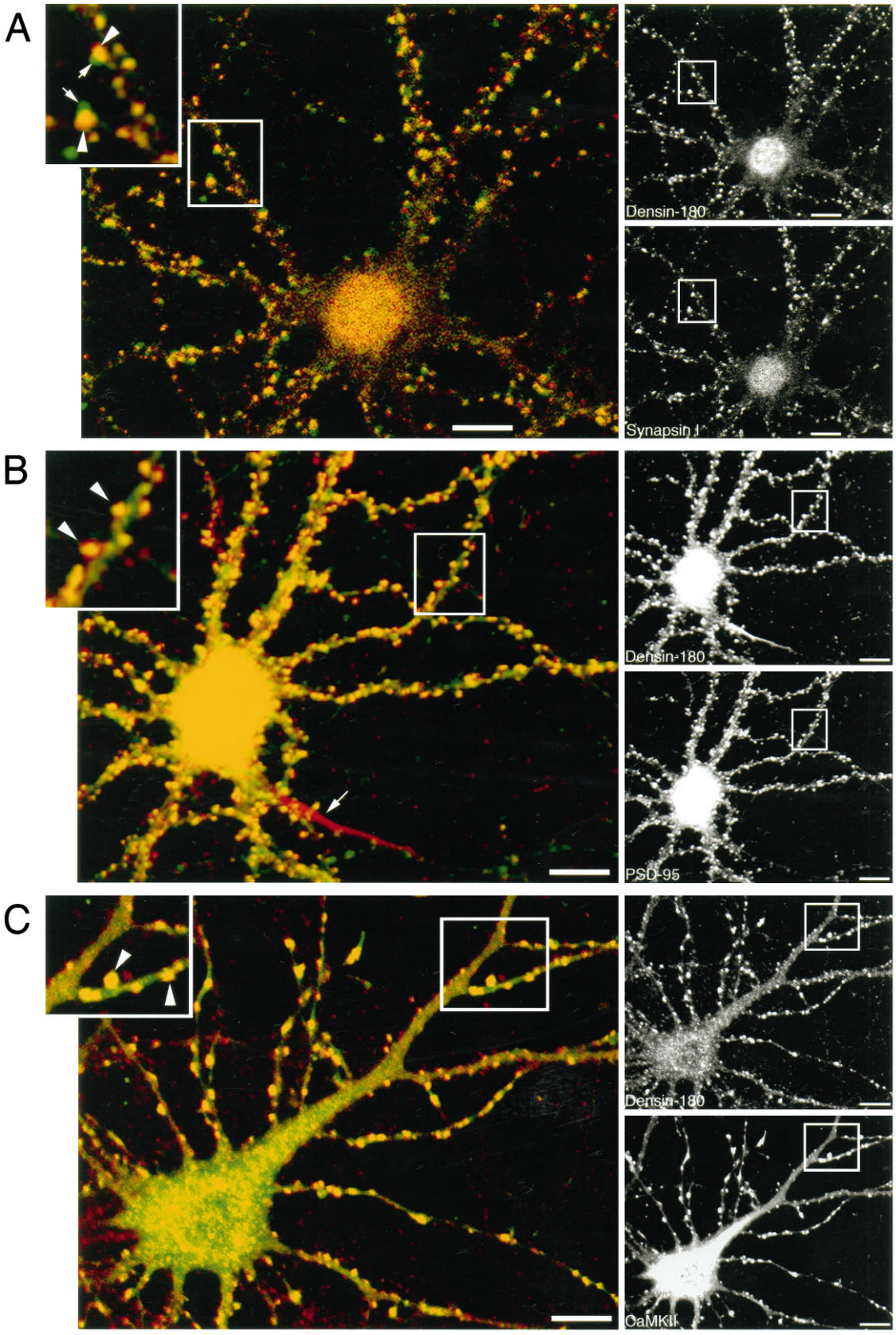
Figure 8. Immunocytochemical localization of densin-180 at synapses in dissociated hippocampal neurons. *A–C*, Hippocampal neurons dissociated at E18 were grown in culture on coverslips for 14–21 d and fixed with ice-cold methanol. After coverslips were incubated for 1 hr in preblock and overnight with the indicated pairs of primary antibodies, cultures were washed three times with preblock and incubated with Cy3-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit secondary antibodies. The coverslips then were washed and mounted on slides. Procedures are described in detail under Materials and Methods. Images were taken with a Zeiss laser-scanning fluorescence confocal microscope, and images of double-labeled cells were combined and colorized with Adobe Photoshop software. *Red* pseudocolor represents Cy3 staining, and *green* represents FITC staining. Regions of overlap are *yellow*. *A*, Double-staining for synapsin I and densin-180. Cultures grown for 21 d *in vitro* were double-labeled with anti-synapsin I (1:1000; *green*) and anti-densin-180 (M2, 1:150; *red*). A combined image taken with a 63× objective is shown. The *inset* at *left* is a 3× zoom of the area included in the *white box*. Note the overlap in staining for densin-180 (*large arrowheads*) and synapsin I (*small arrows*). At *right* are the single images of densin-180 (*top*) and synapsin I (*bottom*). *B*, Double-staining for PSD-95 and densin-180. Cultures grown for 17 d *in vitro* were double-labeled with anti-PSD-95 (affinity-pure, 1:100; *green*) and anti-densin-180 (M2, 1:150; *red*). A combined image taken with a 63× objective at Zoom 1.5 is shown. The axon initial segment stained for densin-180 is indicated with an *arrow*. The *inset* at *left* is a 2× zoom of the area included in the *white box*. Note the precise colocalization of PSD-95 staining and densin-180 staining at spine-like structures along dendrites (*large arrowheads*). At *right* are the single images of densin-180 (*top*) and PSD-95 (*bottom*). *C*, Double-staining for αCaMKII and densin-180. Cultures grown for 14 d *in vitro* were double-labeled with anti-αCaMKII (6G9, 1:500; *green*) and anti-densin-180 (CT245, 1:3000; *red*). A combined image taken with a 63× objective at Zoom 2 is shown. The *inset* at *left* is a 2× zoom of the area included in the *white box*. Note examples of colocalization of αCaMKII staining and densin-180 staining at spine-like structures along dendrites (*large arrowheads*). At *right* are the single images of densin-180 (*top*) and αCaMKII (*bottom*).


A
Densin-180
Synapsin I
B
Densin-180
PSD-95
C
Densin-180
CaMKII

vides additional evidence for localization of densin-180 at the synaptic junction. We have not yet determined whether densin-180 is presynaptic, postsynaptic, or both.

DISCUSSION

The relatively uniform dimensions of the disk-shaped structures present in the PSD fraction suggests that they contain proteins that form a tight complex *in vivo* at postsynaptic membranes (Cotman et al., 1974; Cohen et al., 1977). Because the highly insoluble nature of the PSD renders traditional biochemical purification schemes inadequate, we have used direct microsequencing of protein bands from the PSD fraction, followed by molecular cloning, to characterize proteins associated with the PSD. Three major proteins from the PSD fraction characterized in our lab have been shown by several criteria to be concentrated in the PSD *in situ*, confirming the hypothesis that the PSD fraction represents a physiological structure (Kennedy et al., 1983; Cho et al., 1992; Moon et al., 1994). Here we report the characterization of a fourth protein from the PSD fraction, densin-180, that is highly concentrated at synapses and colocalizes with the other PSD proteins in hippocampal neurons.

In this study, the sequencing strategy was complicated by the existence of more than one comigrating protein in the region of the densin-180 band (Moon et al., 1994). Because of their insolubility in detergents other than SDS, PSD proteins are not amenable to separation by two-dimensional gel electrophoresis. Therefore, to circumvent the problem of multiple protein bands, we used a PCR strategy that allowed us to confirm the presence of DNA encoding at least two of our peptide sequences in PCR clones before screening cDNA libraries, reducing the risk of cloning a minor contaminant of the PSD fraction (Fig. 1). The domain structure of densin-180 revealed in the sequence of the clones places it in the LRR-containing family of proteins and suggests that it is an adhesion molecule (Fig. 3). Two cDNA variants, in addition to the one described in detail here, were characterized. One lacks the ribosome-binding domain of the 5′-untranslated region, suggesting that alternative splicing might regulate the expression of densin-180. The second lacks the carboxy-flanking cysteine-rich domain that might be important in ligand binding and is found in most LRR glycoproteins.

The LRR-containing family of proteins has a wide range of functions, including cell adhesion and signal transduction. The crystal structure of one member of the family, porcine ribonuclease inhibitor protein (RI) bound to its ligand, has been reported (Kobe and Deisenhofer, 1993, 1995a). The largest group of LRR-containing proteins are adhesion molecules. Proteins in this group often contain cysteine-rich domains flanking the LRR on the N- and C-terminal sides. They include many proteins involved in *Drosophila* development (Hortsch and Goodman, 1991). *Drosophila* LRRs that mediate homotypic adhesion include chaoptin, important for eye development (Krantz and Zipursky, 1990), connectin, involved in axon pathfinding and formation of neuromuscular connections (Nose et al., 1992; Meadows et al., 1994), and toll, which is required for formation of dorsal/ventral polarity in the embryo (Hashimoto et al., 1988; Keith and Gay, 1990). In mammalian platelets, all four members of the GPIb complex contain LRRs, but only GPIBα binds directly to vWF, inducing adhesion of platelets to blood vessels. In the mammalian brain, densin-180 is a new member of a growing family of LRR glycoproteins that include trk (Martin-Zanca et al., 1989), trkB (Klein et al., 1989; Schneider and Schweiger, 1991), oligodendrocyte myelin glycoprotein (Mikol et al., 1990), and NLRR-3 (Taniguchi et al., 1996).

Densin-180 contains an RGD cell attachment motif between the last LRR and the C-terminal flanking cysteine-rich domain (Fig. 2). RGD motifs have been shown to mediate intercellular interaction by binding to integrins (D'Souza et al., 1991). Integrins may be present at the synapse, because RGD peptides block long-term potentiation (Xiao et al., 1991), and a 55 kDa RGD-binding protein purified from synaptic membranes cross-reacts with anti-$\alpha_5\beta_1$ integrin antibodies (Bahr and Lynch, 1992). Thus, densin-180 may interact with synaptic integrins.

We identified densin-180 as a sialomucin by two criteria: a large shift in its apparent molecular weight on SDS-gels after neuraminidase treatment (Fig. 5*A*) and its sensitivity to proteolysis by *O*-sialoglycoprotein endopeptidase (Fig. 5*B,C*), which is highly specific for sialomucins (Mellors and Sutherland, 1994). GPIbα (Yeo and Sutherland, 1993) and cranin, recently identified as the brain form of α-dystroglycan (Smalheiser and Kim, 1995), are also sensitive to this protease. The *O*-glycosylated domain in sialomucins forms an extended filamentous conformation, 2.5 angstroms per residue in length (Strous and Dekker, 1992), surrounded by a cloud of negative charges associated with the sialic acid residues (Jentoft, 1990). The negative charges can play a protective role by repelling adhesion molecules on other cells, or they can mediate specific binding to lectin domains of selectins (Cummings and Smith, 1992). Finally, the filamentous domain can act as a stiff rod to extend a ligand-binding domain for interaction with other cells or with the extracellular matrix (Van der Merwe and Barclay, 1994).

The solubility properties of densin-180 are unusual and are reminiscent of those of α-dystroglycan, which has been reported as an integral membrane protein (Ma et al., 1993), a peripheral protein (Ervasti and Campbell, 1993), and is now recognized to be a membrane-associated extracellular protein (Fallon and Hall, 1994). Densin-180 is solubilized most effectively either by a combination of nonionic detergent and high salt or by pH 11 buffers (Fig. 6). A portion of densin-180 is extracellular, as evidenced by its glycosylation. We have identified a possible membrane-spanning domain near the C terminus, followed by a PDZ protein interaction domain (Fig. 3*B*). It seems most likely that the PDZ domain is located in the cytosol where it would associate with intracellular proteins. However, a definitive model of the membrane orientation of densin-180 remains to be established.

The cytoplasmic domains of transmembrane proteins often have important functions in signal transduction across the membrane. Examples in the LRR family include toll, which contains an interleukin-1-like cytoplasmic domain (Hashimoto et al., 1988), and gp150, which contains a receptor protein tyrosine phosphatase-binding domain that is phosphorylated on a tyrosine residue *in vitro* (Tian and Zinn, 1994). In addition, the ABP-binding domain at the C terminus of GPIbα is likely to mediate cytoskeletal rearrangement in response to ligand binding (Andrews and Fox, 1992). Densin-180 contains a PDZ domain at its C terminus that may participate in binding to cytoplasmic elements. PDZ domains are protein-binding motifs and seem to play a role in the association of proteins in signal transduction complexes, in particular at cellular junctions. For example, the second PDZ domain (PDZ2) of PSD-95 interacts with a short sequence SDV*, termed tSXV, at the extreme C terminus of subunits of the NMDA-type glutamate receptor, and this interaction has been proposed to anchor NR2B in the PSD (Kornau et al., 1995). Additionally, the PDZ2 domain from PSD-95 can bind to tSXV motifs in a subset of potassium channels (Kim et al., 1995) and

directly to the PDZ domain of nNOS (Brenman et al., 1996). The identification of a 21 kDa protease-resistant C-terminal fragment of densin-180 (Fig. 5*B*) suggests that this putative cytoplasmic domain may be tightly embedded in the PSD via the PDZ domain. Notably, there is a consensus CaMKII phosphorylation site only two amino acids from the densin-180 C terminus, immediately after the PDZ domain (Fig. 2). It is possible that the phosphorylation of densin-180 by CaMKII (Fig. 7) regulates the association of densin-180 with binding partners in the PSD.

Immunocytochemical studies suggest that densin-180 is located at the synaptic membrane (Fig. 8). Double immunofluorescence labeling of densin-180 and synapsin I, a synaptic vesicle marker, reveals that the two molecules are colocalized. However, at higher magnification a slight shift of the synapsin I staining away from the dendrite relative to the densin-180 staining is apparent (Fig. 8*A*), suggesting that densin-180 is more closely associated with the junctional membrane than is synapsin. We have not yet determined at the electron microscopic level whether densin-180 is concentrated on the postsynaptic side of the junction. However, double immunofluorescence labeling of densin-180 and PSD-95, a PSD-marker, reveals that the two molecules precisely colocalize at synapses in mature cultured neurons within the limit of resolution of the laser-scanning confocal microscopy. Furthermore, densin-180 is expressed predominantly in dendrites of developing neurons in culture (data not shown) and seems restricted to the axon hillock of more mature neurons (Fig. 8*B*), suggesting that it may be principally a postsynaptic protein.

Densin-180 and the platelet surface protein GPIbα contain an assembly of similar domains that suggest they may function in a similar way. GPIbα mediates adhesion of platelets to vWF that is exposed in the extracellular matrix of injured blood vessels. This adhesion is characterized by fast association and dissociation rates, as well as by high resistance to tensile stress, functioning to bind platelets to the vessel wall in the presence of high shear forces (Savage et al., 1996). The GPIbα association with vWF facilitates binding of $\alpha_{IIb}\beta_3$ integrins on the platelet surface to the RGD domain of vWF. We hypothesize that a similar type of adhesion may be mediated by densin-180 at the synapse.

The location of densin-180 at the synapse and its domain structure suggest several hypotheses concerning a role for densin-180 in the adhesion between pre- and postsynaptic membranes. First, the sialomucin region of densin-180 may form an extended conformation across the synaptic cleft to present the LRR-containing ligand-binding domain to the apposing synaptic membrane. Second, the presence of an RGD sequence near the LRR domain suggests that a synaptic membrane ligand may be an integrin-like protein. Third, the O-linked sugars could mediate selective adhesion through selectin-like molecules. Together, these extracellular motifs have the potential for the tight yet flexible adhesion that may be important in synapse formation, maintenance, and plasticity. Fourth, on the cytoplasmic face, densin-180 may participate in assembly and maintenance of the PSD structure through its PDZ domain. Finally, densin-180 function may be regulated by CaMKII-mediated signal transduction. We are presently testing these hypotheses.

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