Characterization of the δ2 Glutamate Receptor-binding Protein Delphilin

SPlicing Variants With Differential Palmitoylation and an Additional PDZ Domain*

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The glutamate receptor δ2 (GluRδ2) is predominantly expressed at parallel fiber-Purkinje cell postsynapses and plays crucial roles in synaptogenesis and synaptic plasticity. Although the mechanism by which GluRδ2 functions remains unclear, its lack of channel activity and its role in controlling the endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors have suggested that GluRδ2 may convey signals by interacting with intracellular signaling molecules. Among several proteins that interact with GluRδ2, delphilin is unique in that it is selectively expressed at parallel fiber-Purkinje cell synapses and that, in addition to a single PDZ domain, it contains a formin homology domain that is thought to regulate actin dynamics. Here, we report a new isoform of delphilin, designated as L-delphilin, which has alternatively spliced N-terminal exons encoding an additional PDZ domain. Although original delphilin, designated S-delphilin, was palmitoylated at the N terminus, this region was spliced out in L-delphilin. As a result, S-delphilin was associated with plasma membranes in COS cells and dendritic spines in hippocampal neurons, whereas L-delphilin formed clusters in soma and dendritic shafts. In addition, S-delphilin, but not L-delphilin, facilitated the expression of GluRδ2 on the cell surface. These results indicate that, like PSD-95 and GRIP/ABP, delphilin isoforms with differential palmitoylation and clustering capabilities may provide two separate intracellular and surface GluRδ2 pools and may control GluRδ2 signaling in Purkinje cells.

Numerous spontaneous ataxic mutant mice occur as a result of null mutations in the gene encoding glutamate receptor δ2 (GluRδ2), a member of the ionotropic glutamate receptor family that is predominantly expressed at parallel fiber (PF)-Purkinje cell synapses in the cerebellum (1). Detailed morphological and electrophysiological analyses on GluRδ2-null mice suggest that GluRδ2 plays a crucial role in aligning and maintaining the postsynaptic density (PSD) with the presynaptic element at PF-Purkinje cell synapses (for reviews, see Ref. 2). In addition, long-term depression (LTD) of PF-Purkinje cell transmission, which is thought to underlie a form of information storage in the cerebellum (3), is completely blunted in GluRδ2-null Purkinje cells (4). Several lines of evidence indicate that LTD is caused by an activity-dependent decrease in the number of postsynaptic AMPA receptors (5, 6). Interestingly, the application of an antibody specific for the extracellular region of GluRδ2 to cultured Purkinje cells induced the endocytosis of AMPA receptors in Purkinje cells and abrogated subsequent LTD (7). These results indicate that GluRδ2 plays another unique role at PF-Purkinje cell synapses, the regulation of postsynaptic AMPA receptor endocytosis. However, the mechanisms by which GluRδ2 plays these roles remain unclear; GluRδ2 does not contribute to normal excitatory postsynaptic currents (8).

Excitatory synapses, in particular their PSDs, contain many PDZ domain-containing proteins that interact with postsynaptic receptor channels. It is becoming increasingly clear that PDZ proteins control not only the spatial localization of the receptors but also their intracellular signaling pathways by accumulating specific signaling complexes near the receptors. For example, N-methyl-D-aspartate (NMDA) receptor-induced excitotoxicity was greatly reduced without affecting NMDA receptor-mediated currents by disrupting the neuronal NR2B-PSD-95-nitric oxide synthase interaction (9). Thus, the analysis of PDZ proteins that interact with GluRδ2, such as PSD-93 (10), PTPMEG (11), shank (12), and delphilin (13), is expected to provide clues to the function of GluRδ2. Of these PDZ proteins, delphilin is unique in that it is selectively expressed at PF-Purkinje cells (13) and that in addition to a PDZ domain it contains a formin homology domain, which is thought to control actin dynamics with profilin, and a tyrosine kinase Src. Here, we report a new isoform of delphilin, designated as L-delphilin, which we found while analyzing delphilin exons encoding an additional PDZ domain but lacking the

kidney 293 cells; PSD, postsynaptic density; LTD, long-term depression; GST, glutathione S-transferase; PF, parallel fiber.
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N-terminal palmitoylation site found in the original delphilin (designated as S-delphilin). L-delphilin was localized differently from S-delphilin in heterologous cells and neurons. In addition, the two isoforms showed different capabilities of clustering and maintaining GluR82 on the cell surface. The presence of isoforms with differential palmitoylation and clustering capabilities is analogous to other important PDZ proteins, such as ABP/GRIP for AMPA receptors (14) and PSD-95 (15), and may point to the central role of delphilin in modulating GluR82 signaling in Purkinje cells.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequence Analysis—Exon-intron boundaries and splicing variants of delphilin were examined by applying NetGene2 (The Technical University of Denmark, Lyngby, Denmark) to the mouse genome data base. The predicted cDNA for L-delphilin was cloned as two fragments that overlapped by 450 bp. The primers used for reverse transcription PCR were as follows: 5'-ATG CCA GCC ACC AAT CAA GGC TG-3' and 5'-AAG AGG CCC TCA GGG AAC TC-3' for the first fragment and 5'-CAC CCC CCG TCT TAG TAT AGA C-3' and 5'-GCA GTA AAC ACC TGC TCC TTG G-3' for the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mini kit (Qiagen, Valencia, CA); the second fragment. Total RNA from adult mouse cerebellum was prepared using an RNeasy mi...
rose beads. Cell lysate from transfected HEK293 cells with the indicated delphilin-HA expression vectors were incubated overnight with these GST-82-Sepharose beads. After being washed with TNE buffer and eluted with SDS-PAGE sample buffer, the bound delphilin-HA was detected using Western blot analysis.

**Metabolic Labeling for Analysis of Palmitoylation**—At 36 h after transfection, the HEK293 cells were pretreated with serum-free Dulbecco's modified Eagle's medium containing 3 μg/ml cerulein and 10 mg/ml fatty acid-free bovine serum albumin (Sigma) for 30 min. [%H]Palmitic acid at 0.5 mCi/ml (50 Ci/mmol) was added to the culture, and the cells were incubated at 37 °C for 5 h. The cells were then washed with phosphate-buffered saline, collected in tubes, and lysed with TNE buffer. The soluble fraction was immunoprecipitated with anti-HA (Roche Applied Science) or anti-PSD-95 (UpState, Charlottesville, VA) antibody as previously described (17). The immunoprecipitates were boiled in SDS-PAGE sample buffer without 2-mercaptoethanol but containing 1 mM diithiothreitol. After SDS-PAGE, the gels were fixed in 40% methanol and 10% acetic acid for 20 min, dried in a heat vacuum, and exposed to an imaging plate (Fujifilm Life Science) for 3 days. One-twentieth of each sample was used in an immunoblot analysis to examine the protein expression level. For hydroxylamine treatment, duplicate gels were incubated with 1 M hydroxylamine or Tris·HCl, pH 7.0, for 18 h before exposure to the imaging plate.

**Cell Surface Biotinylation Assays**—pCAGGS vectors expressing NT-FLAG-82 and delphilin-HA (or EGFP) were co-transfected at the ratio of 1:4 into HEK293 cells. 36–48 h later, cell surface biotinylation was carried out by using EZ-link sulfo-NHS-LC-Biotin (Pierce) at 4 °C for 15 min as previously described (16). After solubilization with TNE buffer, cell lysates were precipitated by NeutrAvidin beads (Pierce) and subjected to the immunoblot analysis using anti-GluR82 antibody (Chemicon International, Temecula, CA). 20% of surface proteins (captured with NeutrAvidin beads) and 2% of total lysates were used to calculate the surface GluR82 ratio.

**Immunocytochemical Analysis of Transfected Cells and Neurons**—COS cells expressing delphilin and/or GluR82 were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature 24–36 h after transfection. Cultured hippocampal neurons (16) were transfected at 3 days in vitro and fixed at 14–21 days in vitro. To visualize the transfected neurons, pCAGGS expressing delphilin cDNA and pCAGGS expressing EGFP (pCAGGS-EGFP) were transfected at a ratio of 5:1. Sindbis virus was also used to express proteins in a large number of hippocampal neurons in culture. The cDNA encoding HA-tagged delphilin was cloned into a pSin-Rep5 vector (Invitrogen), and virus particles were produced according to the manufacturer’s instructions. One day after the addition of the virus particles, the hippocampal neurons were fixed with 4% paraformaldehyde. After fixation, the cells were incubated with blocking solution (2% bovine serum albumin, 0.4% Triton X-100, and 2% normal goat serum in phosphate-buffered saline) for 1 h at room temperature and then incubated with the primary antibodies for an additional 1 h at the following dilutions: rat anti-HA, 1:1000 (Roche Applied Science); mouse anti-PSD-95, 1:1000 (UpState); rabbit anti-MAP2, 1:1000 (Chemicon); chicken anti-GFP, 1:5000 (Chemicon); rabbit anti-GluR82, 1:1000 (Chemicon); and mouse anti-FLAG, 1:1000 (Sigma). For visualization, appropriate secondary antibodies conjugated to Alexa 546 or 488 (diluted at 1:2000; Invitrogen) were used. For cell surface staining, the cells were cotransfected with pCAGGS-delphilin-FLAG and pCAGGS-NT-HA-82 at a ratio of 4:1. After fixation for 10 min at 4 °C, the cells were incubated with blocking buffer without Triton X-100 and then incubated with mouse anti-HA antibody (1:1000; Covance Research Products Inc., Denver, PA). An HA tag on the N terminus of GluR82 on the cell surface was visualized by incubating the cells with Alexa 546 anti-mouse IgG antibody (1:2000; Invitrogen) for 1 h. The cells were then permeabilized with blocking buffer containing 0.4% TritonX-100 for 30 min at room temperature and incubated with anti-L-delphilin (1:500). L-delphilin was visualized by appropriate secondary antibodies conjugated to Alexa 488 (1:2000; Invitrogen). No fluorescence was observed when each of the secondary antibodies was omitted, confirming the specific excitation of each fluorescent substance. To determine the number of cells that formed intracellular clusters of delphilin isoforms or its mutants (see Fig. 5A), a total of 20–30 randomly chosen fields/cover slips were counted using ×200 magnification under a fluorescence microscope in a double-blind manner.

**RESULTS**

**Cloning of Alternatively Spliced N-terminal Form of Delphilin**—Previous studies have suggested that the functions of several PDZ proteins, like ABP and PSD-95, are crucially controlled by alternative splicing at their N termini (14, 15). Because delphilin transcription results in a major (~9 kb) band and a minor (~4 kb) band when examined using Northern blot analysis (13), we examined the possibility of alternative splicing at the extreme N terminus of delphilin. An analysis of mouse genomic data bases revealed that delphilin is encoded by 21 exons and that all the exon-intron junctions are identical to canonical splice donor and acceptor sites (Fig. 1A). In addition, at 5,500 bp upstream of the original first exon (Fig. 1A,SJ), we found an additional putative exon (Fig. 1A, LJ) that could potentially encode a PDZ domain. To examine whether a transcript with an additional PDZ domain was expressed in vivo, we performed reverse transcription PCR on mouse cerebellar cDNA using a primer set designed at exons L1 and 8 (Fig. 1A, arrows). We obtained a single PCR amplicon (~1.5 kb) whose sequence was coded by exon L1 and exons 2 through 8 (Fig. 1A). The transcript lacked the original exon SJ; instead, it included another short exon L1’ (Fig. 1A). Exons L1 and L1’ also encoded a leucine-rich short sequence, which we have referred to as a linker (Fig. 1C). We detected a translation initiation signal in exon L1 compatible with the Kozak sequence and an in-frame termination codon at 200 bp upstream of this initiation signal. The exon-intron junctions of exons L1 and L1’ were identical to canonical splice donor and acceptor sites (Fig. 1B). These results indicated that the delphilin gene was alternatively spliced at the N terminus to generate a variant transcript from exons L1 and L1’, instead of exon SJ. As a result, the new transcript contained an additional 184 amino acids at its N terminus and encoded a new PDZ domain, designated PDZ1, plus a
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A schematic drawing of the genome structure of delphilin. An unshaded line (S1) indicates the first exon of the original delphilin, S-delphilin. Filled boxes (L1 and L1') indicate the exons of newly identified delphilin, L-delphilin. These exons encode 184 L-delphilin-specific amino acids at the N terminus. Common exons are indicated by shaded boxes (2, 3, to 21). Start and stop codons are indicated by black and inverse triangle, respectively. The arrows correspond to the reverse transcription PCR primers. B, exon-intron boundary deduced by comparing genomic DNA and cDNA sequences. The bold letters correspond to exons; predicted donors and acceptors for splicing are underlined. C, domain structure of each isoform. Both delphilin isoforms contain PDZ (PDZ2), FH, and coiled-coil domains. In addition, L-delphilin contains an additional PDZ domain (PDZ1) and a 120-amino acid “linker” region at its N terminus. Instead, S-delphilin has 5 specific amino acids, MSCLG, at its N terminus.

linker domain in addition to the original PDZ domain, designated PDZ2 (Fig. 1C). We designated this longer form of delphilin as L-delphilin and the original form as S-delphilin.

Analysis of human, dog, and rat genomic DNA data bases revealed that similar putative exons encoding a PDZ domain were conserved in the corresponding 5’ upstream regions of the delphilin genes (Fig. 2A), suggesting that the PDZ1 domain encoded by exons L1 and L1’ was functionally important for delphilin. The PDZ1 domain of delphilin contained a conserved PDZ ligand binding motif (Φ-G-Φ, where Φ is a hydrophobic residue) between the putative βα and ββ strands (Fig. 2B). In addition, the first residue of the putative helix αβ of the PDZ1 domain, as well as that of the PDZ2 domain, was arginine (Fig. 2B), which is conserved among class I PDZ domains (18). Indeed, of the known PDZ proteins, the PDZ1 domain was most similar to the PDZ domain of regulators of G-protein signaling12 (RGS12; 42% identical and 32% similar amino acids by BLAST), followed by the PDZ2 domain of delphilin itself (33% identical and 53% similar amino acids; Fig. 2B). The PDZ domain of RGS12 and the PDZ2 domain of delphilin have been reported to bind to the C terminus of the G-protein-coupled receptor CXCR2 (19) and the C terminus of GluR62 (13), respectively, via class I interactions. Similarly, we found that L-delphilin specifically interacted with the C terminus of GluR62 (Fig. 2C) via both PDZ1 and PDZ2 domains (Fig. 2, D and E). These results suggested that the PDZ1 domain of L-delphilin encodes a functional class I PDZ domain.

Expression of L-delphilin in the Cerebellum—To confirm the expression of L-delphilin in vivo, we performed a Northern blot analysis on mRNAs prepared from adult cerebral cortex and cerebellum using specific probes. A probe common to L-delphilin and S-delphilin detected a major ~9-kb band (black arrow heads) and a minor ~4-kb band (open circle) in the cerebellum (Fig. 3A), consistent with earlier reports (13). Similarly, a probe specific to L-delphilin (exons L1 and L1’) detected a major ~9-kb band and a minor ~4-kb band in the cerebellum, but not in the cerebral cortex (Fig. 3A). In contrast, although a probe specific to S-delphilin (exon S1) also reacted with a ~9-kb band, the signal in the cerebral cortex was stronger than that in the cerebellum (Fig. 3A). An additional ~6-kb band detected by this probe may reflect a nonspecific hybridization, as this band was not detected by the common probe. These results indicated that L-delphilin was indeed expressed in vivo and that L-delphilin did not contain exon S1. In addition, because S-delphilin did not exhibit the cerebellum-dominant expression pattern observed with the common probe, L-delphilin may be the predominant isoform. Alternatively, other major splicing variants at the N terminus that could not be detected with the probe used for S-delphilin may exist.

To further confirm the expression of the L-delphilin protein in the cerebellum, we generated a specific anti-L-delphilin antibody using a synthetic peptide corresponding to the unique region of L-delphilin. An immunoblot analysis of HEK293 cells expressing L-delphilin-HA or S-delphilin-HA showed that this anti-L-delphilin antibody recognized a protein of ~150 kDa, which agrees with the predicted molecular mass of L-delphilin (Fig. 3B, upper left panel). After preabsorption with the antigen peptide, the anti-L-delphilin antibody no longer recognized this band (data not shown). In addition, the antibody did not react with S-delphilin-HA protein, whereas anti-HA antibody detected both S- and L-delphilin proteins (Fig. 3B, upper right panel). These results confirmed the specificity of the anti-L-delphilin antibody.
Immunoblot analysis of cerebellar lysates immunoprecipitated by this antibody, but not by IgG, also specifically reacted with an 150-kDa protein (Fig. 3B, lower left panel). After pre-absorption with the antigen peptide, the anti-L-delphilin antibody no longer recognized this band (Fig. 3B, lower right panel). These results indicated that L-delphilin was indeed translated from mRNA and associated with native GluR2 in adult mouse cerebellum.

Palmitoylation of S-delphilin Is Necessary for the Cell Surface Expression of GluR2—Exon S1, which is spliced out in L-delphilin, encodes an S-delphilin-specific peptide, MSCLG (Fig. 1C), at the extreme N terminus. As the cysteine residue at the

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FIGURE 2. Characterization of an additional PDZ domain of L-delphilin. A, alignment of predicted L-delphilin-like amino acid sequences coding the PDZ domain found in genomic data bases of various species. The white letters in the gray background indicate completely conserved identical amino acids. B, alignment of PDZ domains of selected proteins. Various genome data bases were searched using the amino acid sequence encoding the first PDZ domain (PDZ1) of mouse L-delphilin as a query. Alignment of the first PDZ domain of the putative human L-delphilin (Del PDZ1 human), the second PDZ domain of mouse S- and L-delphilin (Del PDZ2 mouse), a PDZ domain of rat RGS-12 PDZ, and the third PDZ domain of rat PSD-95 (PSD95–3) were performed using the ClustalW program. The secondary structure of PSD-95, consisting of six β sheets and two α helices, is indicated as boxes (40). Letters are shaded according to the ClustalW program in the Biology WorkBench suites. Dots under the characters indicate a highly conserved PDZ ligand binding motif (Φ-G-Φ, where Φ is a hydrophobic residue) necessary for ligand binding. The arginine or histidine residue at the beginning of the helix αB is marked with an asterisk. C, coimmunoprecipitation assay. Wild-type GluR2 (GluR2 WT) or GluR2 whose 4 most extreme C-terminal amino acids were deleted (GluR2 Δ4) were coexpressed with HA-tagged L-delphilin (L-del-HA) in HEK293 cells. The cells were solubilized with TNE buffer, and the cell lysates were immunoprecipitated with anti-HA antibody. The amount of L-delphilin in each lysate was confirmed by immunoblotting with anti-HA antibody. These results indicate that L-delphilin interacted with GluR2, dependent on the C-terminal 4 amino acids of GluR2. D, pulldown assay using GST fusion protein with the C terminus region of GluR2 (GST-Δ2-CT), which was coupled to glutathione-Sepharose beads. HEK293 cells expressing L-delphilin-HA, L-delphilin lacking the second PDZ domain (PDZ2-HA), L-delphilin lacking the first PDZ domain (PDZ1-HA), and S-delphilin lacking the second PDZ domain (ΔPDZ1-ΔPDZ2-HA) were solubilized with TNE buffer, the cell lysates were incubated with GST-Δ2-CT beads, and bound protein was collected. The total protein amount of each L-delphilin construct in the cell lysates (lower panel) and the amount of delphilin mutants bound to GST-Δ2-CT Sepharose beads (upper panel) were examined by immunoblotting with anti-HA antibody. E, pulldown assay using glutathione-Sepharose beads coupled to GST-Δ2-CT or GST only. HEK293 cells expressing the PDZ1 and the linker domain of L-delphilin (PDZ1-FLAG) or the PDZ2 domain of L-delphilin (PDZ2-FLAG) was solubilized as above and incubated with GST-Δ2- or GST-Sepharose beads. The total protein amount of each L-delphilin construct in the cell lysates (lower panel) and the amount of delphilin mutants bound to GST-Δ2-Sepharose beads or GST-Sepharose beads (upper panel) were examined by immunoblotting with anti-FLAG antibody.
third position is flanked by hydrophobic residues (Fig. 4A), this sequence may constitute the palmitoylation site, which regulates the binding of adaptor proteins to the plasma membrane and trafficking to postsynaptic regions (14, 20, 21). Indeed, the sequence around this region is reminiscent of the N terminus of PSD-95α and GAP-43 (Fig. 4A), which are known to undergo palmitoylation (14, 22). In contrast, L-delphilin does not contain the corresponding cysteine residue for the putative palmitoylation site. To test the hypothesis that delphilin isoforms are differentially palmitoylated, HEK293 cells expressing L-delphilin-HA or S-delphilin-HA were incubated with [3H]palmitate and the cellular lysates were immunoprecipitated with anti-HA antibody. The level of palmitoylation was visualized by fluorography, and the amount of each protein was assessed by immunoblot analysis with anti-HA antibody. Like PSD-95α, S-delphilin was clearly palmitoylated, whereas S-delphilin whose third cysteine residue was replaced by alanine (S-del-C3A) and L-delphilin were not (Fig. 4B). When duplicate gels were treated with 1 M Tris-HCl or 1 M hydroxylamine overnight, the [3H]palmitate signals of PSD-95α and S-delphilin disappeared (Fig. 4C). These results further confirmed that PSD-95α and S-delphilin were palmitoylated via the thioester bond.

Although delphilin is reported to associate with GluR82, its function remains unclear. Because one of the roles of PDZ domain proteins is to enhance the expression of its associated membrane receptors on the cell surface, we expressed GluR82 with or without delphilin in HEK293 cells. 24–36 h after transfection, HEK293 cells were treated with membrane-impermeable biotinylation reagents. After solubilization, the biotinylated proteins were captured with NeutrAvidin beads. The amounts of total GluR82 and biotinylated surface GluR82 were then compared using an immunoblot analysis (Fig. 4D). The specificity of the biotinylated surface proteins was confirmed by the absence of labeled actin, which is localized in the cytosol (data not shown). Although some GluR82 proteins were expressed on the cell surface when expressed alone (23), GluR82 was more densely located on the cell surface when it was coexpressed with S-delphilin (Fig. 4D). This effect was not observed with L-delphilin or S-del-C3A, indicating that the palmitoylation of S-delphilin is necessary for the enhanced cell surface expression of GluR82.

**L-delphilin Forms Intracellular Clusters via Its Specific Domains**—Instead of a palmitoylation site, L-delphilin contains an additional PDZ domain, PDZ1, and a linker domain at the N terminus (Fig. 5A). When expressed in COS cells and visualized under a confocal microscope, L-delphilin immunoreactivity showed a punctate distribution pattern throughout the cytoplasm (Fig. 5B). In contrast, S-delphilin was enriched at the edges of the plasma membranes (Fig. 5C), as expected based on the results of the palmitoylation assay (Fig. 4B). To examine the contribution of each domain of L-delphilin to the punctate pattern of distribution, we created several mutant delphilin proteins and expressed them in COS cells. When both PDZ1 and linker domains were deleted (Fig. 5, A and D, C-del) or when the coiled-coil domain was deleted (Fig. 5, A and E, L-del-ΔCC), L-delphilin was no longer capable of forming clusters. When the coiled-coil domain was present, either the PDZ1 domain (L-del-Δlinker) or the linker domain (L-del-ΔPDZ1) was sufficient to cause L-delphilin clustering in COS cells (Fig. 5, F and G). These results indicate that the unique N-terminal region of L-delphilin, which works in concert with the coiled-coil domain, is responsible for the clustering of L-delphilin.

When GluR82 was expressed alone in COS cells, it diffusely distributed throughout the cytoplasm with enrichment around the perinuclear region, which corresponded to the endoplasmic...
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The expression of each protein was examined by immunoblot analysis using anti-HA or anti-PSD-95 antibody (B, left panel). The expression of each protein was examined by immunoblot analysis using anti-HA or anti-PSD-95 antibody, respectively, subjected to SDS-PAGE, and analyzed by fluorography (B, left panel). The expression of each protein was examined by immunoblot analysis using anti-HA or anti-PSD-95 antibody (B, right panel). The protein markers correspond to 195, 116, and 95 kDa. Duplicated gels were treated with 1 M Tris-HCl (+Tris) and 1 M hydroxylamine (+NH₄OH) before fluorography. The expression of each protein was examined by immunoblot analysis using anti-HA or anti-PSD-95 antibody (C, middle panel) before fluorography. The expression of each protein was examined by immunoblot analysis using anti-HA or anti-PSD-95 antibody (C, right panel). D, effects of delphilin isoforms on expression of GluR62 on the cell surface. Surface proteins of HEK293 cells expressing GluR62 and delphilin isoforms were biotinylated as previously described (17). Surface GluR62 proteins (upper panel), which were precipitated by streptavidin-coupled beads, and total GluR62 proteins in the lysates (middle panel) were immunoblotted by anti-GluR62 antibodies. The total amount of delphilin proteins in each lysate was immunoblotted by anti-HA antibodies (lower panel). Quantitative analysis of the mean amount of surface GluR62 was also shown in bar graphs (left graph, n = 7; right graph, n = 3 for each construct). The amount of biotinylated GluR62 in cells expressing EGFP was arbitrarily established as 100%. Error bars indicate S.E. **, p < 0.01.

reticulum, and at the edges of the plasma membrane (17). On the other hand, when GluR62 was coexpressed with L-delphilin, it was partially colocalized with L-delphilin in a punctate pattern (Fig. 5H). When GluR62 coexpressed with L-delphilin was stained under nonpermeabilizing conditions, GluR62 at the edges of the plasma membrane did not exhibit a punctate pattern (Fig. 5I). In addition, L-delphilin did not colocalize with a cyan fluorescence protein that had endoplasmic reticulum retention signal (data not shown). These results suggested that L-delphilin formed cytosolic protein clusters with some population of GluR62 in the non-endoplasmic reticulum intracellular compartment.

Differential Localization of L- and S-delphilin in Cultured Neurons—Next, we examined whether delphilin isoforms were differentially localized in neurons, as observed in heterologous cells. For this purpose, to exclude the possibility of endogenous delphilin or GluR62 modifying the location of exogenously expressed delphilin, we expressed L- or S-delphilin in addition to EGFP in cultured hippocampal neurons, where no endogenous GluR62 or delphilin is expressed (13, 24). Immunohistochemical analysis revealed that L-delphilin formed clusters mainly in dendritic shafts (Fig. 6, A and B), whereas S-delphilin was preferentially expressed in spines (Fig. 6, C and D); this result indicates that delphilin isoforms were distinctly localized in neurons. Interestingly, S-del-C3A or C-del, which lacked the palmitoylation signal, did not accumulate in spines (Fig. 6, G–J). However, S-del-C3A and C-del did not form clusters in dendritic shafts (Fig. 6, G–J) or COS cells (Fig. 4) under conditions where L-delphilin formed large clusters. These results suggest that the palmitoylation signal is responsible for the accumulation of S-delphilin within spines and that the unique N-terminal region causes L-delphilin clustering within dendritic shafts.

To examine whether L-delphilin clusters were located on the cell surface, L-delphilin was coexpressed with NT-HA-GluR62, which had an HA tag at the N-terminal extracellular region, and the cells were stained with anti-HA antibodies under nonpermeabilizing conditions. L-delphilin immunostained under nonpermeabilizing conditions did not colocalize with surface GluR62 proteins (Fig. 7, A and B). Thus, L-delphilin formed cytosolic clusters within the dendritic shafts, similar to its activity in heterologous cells (Fig. 5H). Indeed, L-delphilin immunoreactivity was colocalized with a dendritic intracellular protein, MAP2 (Fig. 7C), but not with a synaptic protein, PSD-95 (Fig. 7E). In contrast, S-delphilin immunoreactivity overlapped with PSD-95 (Fig. 7F), but not with MAP2 (Fig. 7D). These results indicate that the two delphilin isoforms are differentially localized, thereby modulating GluR62 signaling in neurons.

DISCUSSION

This report describes the cloning and functional characterization of a novel splicing variant of delphilin, L-delphilin, that contains an additional PDZ domain at its extreme N terminus in addition to the original PDZ domain found in the original S-delphilin. We also demonstrated that S-delphilin, but not L-delphilin, underwent palmitoylation at its third cysteine in its N-terminal region. As a result, S-delphilin was mainly associated with the plasma membrane in COS cells (Fig. 5C) and concentrated at the dendritic spines in hippocampal neurons.
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Differential localization of delphilin isoforms and its mutants in transfected COS cells. A, schematic drawing of the structures of delphilin isoforms and deletion mutants. An HA tag was attached to the C-terminal end of each construct. The number on right represents the mean ± S.E. of cells exhibiting clusters of delphilin in cytosols (n = 4 for each construct). B–G, typical confocal images of delphilin isoforms and mutants. COS cells expressing L-delphilin (L-del; B), S-delphilin (S-del; C), delphilin containing only a common region (C-del; D), L-delphilin lacking a coiled-coil domain (l-del-ΔCC; E), L-delphilin lacking a PDZ1 domain (l-del-ΔPDZ1; F), and L-delphilin lacking a linker domain (l-del-Δlinker; G) were immunostained with anti-HA antibody. H, localization of coexpressed GluR2 and L-delphilin. GluR2/Δ2 and HA-tagged L-delphilin were coexpressed in COS cells and immunostained with anti-GluR2 (red) and anti-HA (green) antibodies. The right panel shows an overlay image. The asterisks correspond to the nucleus. L-delphilin was partially colocalized with GluR2 at perinuclear regions (arrow heads), I, localization of cell surface GluR2 and coexpressed L-delphilin. GluR2 that had an HA tag at the N terminus was immunostained by anti-HA antibody under nonpermeabilizing conditions. Immunopositive GluR2 on the cell surface (surface HA-Δ2; red) was not co-localized with L-delphilin immunoreactivity (green). Each scale bar corresponds to 20 μm.

Interestingly, several PDZ-containing anchoring proteins for ionotropic glutamate receptors have splicing variants that have differential palmitoylation and clustering capabilities (14, 15, 25). For example, an isoform of ABP/GRIP2, pABP-L, is palmitoylated and condensed at the spines, whereas the non-palmitoylated isoform ABP-L is abundant in the cell body and dendritic shafts (14). It is presumed, but not proven, that these two forms of ABP/GRIP2 provide two separate AMPA receptor anchorages: ABP-L for internal AMPA receptor pools and pABP-L for synaptic pools. During some forms of long-term potentiation and LTD, AMPA receptors are shuttled between intracellular and synaptic pools. Thus, palmitoylated and non-palmitoylated isoforms of ABP/GRIP2 could be involved in the trafficking of AMPA receptors during synaptic plasticity. Unlike AMPA receptors, GluR2 is mainly localized at plasma membranes (17); however, it is rapidly endocytosed when intracellular Ca2+ levels are elevated by synaptic activities (26), and the presence of GluR2 at postsynaptic membranes is thought to play an essential role in controlling AMPA receptor endocytosis and LTD induction in Purkinje cells (7). Therefore, although the distribution and functions of endogenous S- and L-delphilin isoforms need to be further clarified in vivo, these isoforms may be involved in certain aspects of synaptic plasticity by providing two separate GluR2 anchorages (Fig. 7), similar to the function of ABP/GRIP2 isoforms for AMPA receptors.

Because S-delphilin mRNA did not exhibit the cerebellum-dominant expression pattern observed with the common probe (Fig. 3A), L-delphilin may be the predominant isoform in the adult cerebellum. It is also possible that other splicing variants at the N terminus that could not be detected with the probe used for S-delphilin may exist. Indeed, during preparation of this report, another isoform of delphilin, designated as delphilin β, was reported to be produced by alternative splicing at the N terminus (27). Delphilin β also lacks the cysteine residue for palmitoylation; however, unlike...
L-delphilin, it does not contain an additional PDZ domain. Interestingly, delphilin/H9252 mRNA was shown to have stable expression throughout the cerebellar development, whereas S-delphilin mRNA gradually decreased following the first postnatal week (27). Although relative expression level of each isoform during development should be determined, these results suggest that L-delphilin and delphilin/H9252 may be the major isoforms in adult cerebellum.

Palmitoylation is thought to stabilize proteins at the plasma membrane by partitioning proteins into special lipid microdomains or rafts (28). S-delphilin, which was mainly associated with the plasma membrane in COS cells (Fig. 5C), was concentrated at the dendritic spines in hippocampal neurons (Fig. 6, C and D). When the third cysteine residue was replaced by alanine (S-del-C3A) or the initial five residues including cysteine were removed (C-del), S-delphilin did not accumulate in the spines (Fig. 6, G–J). These findings support the hypothesis that dendritic spines have a distinct lipid microdomain where palmitoylated proteins like S-delphilin, pABP-L, and PSD-95/H9251 can be stably retained.

The ability of L-delphilin to form large clusters (Fig. 5B) cannot be explained by the lack of palmitoylation in L-delphilin because S-del-C3A and C-del, which were not palmitoylated, did not form large clusters in COS cells (Fig. 5D) or hippocampal neurons (Fig. 6, G–J). Self-association is a common feature of many PDZ-containing proteins and is thought to be involved in the clustering of these proteins and their targets at specific sites (29). Multimerization is achieved by N-terminal interactions in PSD-95 (30, 31); it can also be mediated by direct interactions between PDZ domains in GRIP (32), INAD (33), and Shank (34). In addition, coiled-coil domains are involved in the multimerization of a wide variety of proteins (35), including a PDZ protein, PICK1 (36). The variety of mechanisms involved in self-association may explain the differences in the behavior of PDZ protein clusters. For example, unlike S-del-C3A and C-del, mutants that disrupt the palmitoylation of PSD-95 block both synaptic targeting and the clustering of PSD-95 (22). Because L-del-ΔCC, which lacked the coiled-coil domain, no longer formed clusters in COS cells (Fig. 5E), the coiled-coil domain is thought to play an essential role in the aggregation of L-delphilin. Interestingly, S-delphilin, S-del-C3A, or C-del, which also contained a coiled-coil domain, did not form large clusters in COS cells. In addition to the coiled-coil domain, either the linker domain (Fig. 5G) or the PDZ1 domain (Fig. 5F) was necessary to cause the clustering of L-delphilin in COS cells. These results indicate that the unique N-terminal region of L-delphilin works in concert with the coiled-coil domain to form clusters. Because a coimmunoprecipitation analysis indicated that S-delphilin formed homomers (data not shown), the unique N-terminal region of L-delphilin may be necessary to form larger mac-

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**FIGURE 6.** Different localization of delphilin isoforms in cultured hippocampal neurons. A–D, confocal images of HA-tagged L-delphilin (L-del; A, B) and S-delphilin (S-del; C, D) expressed in hippocampal neurons stained with anti-HA antibodies. EGFP-expressing vector was co-transfected to visualize the morphology of the transfected neurons. E–I, conventional fluorescent microscope images of HA-tagged S-delphilin (S-del; E, F), S-delphilin whose third cysteine residue was replaced with alanine (C3A; G, H), a region common to both S- and L-delphilins (C-del; I, J). B, D, F, H, and J are enlarged views of the boxes in panels A, C, E, G, and I, respectively. Unlike S-del or L-del, the C-del and C3A mutants were distributed diffusely throughout the dendritic shaft and spine structures. Each scale bar corresponds to 50 μm.
romolecular complexes. Alternatively, the unique N-terminal region of L-delphilin may allosterically stabilize the interaction at the coiled-coil domain.

The presence of the second PDZ domain, PDZ1, in L-delphilin could have significant effects on intracellular signaling, in addition to cluster formation of L-delphilin. If the PDZ1 domain can bind to ligands that are not recognized by the common PDZ2 domain, L-delphilin might mediate a new mechanism of intracellular signaling in Purkinje cells. PDZ domains arranged in tandem can also achieve additional functions. For example, the fourth PDZ domain of GRIP does not bind to its ligand but rather facilitates the folding and function of the fifth PDZ domain (37). In addition, the combined PDZ1 and PDZ2 domains of PSD-95 have significantly different binding specificities for ligands (38). However, unlike the tandem PDZ domains of GRIP or PSD-95, the two PDZ domains of L-delphilin likely function in an independent manner because L-delphilin lacking either the first or second PDZ domain assembled with GluRδ2 in similar manners (Fig. 2D). The identification of cognate ligands for each PDZ domain of L-delphilin and S-delphilin should further clarify the nature of the signaling mechanisms mediated by these two isoforms.

Because GluRδ2 does not contribute to normal excitatory postsynaptic currents at PF-Purkinje cell synapses (8) and is involved in postsynaptic AMPA receptor endocytosis (7), GluRδ2 may convey signals by interacting with intracellular signaling molecules, most likely via its long C terminus (39). Therefore, to obtain clues to the function of GluRδ2 in synapse formation and synaptic plasticity, further characterization of the signaling pathways mediated by delphilin and its isoform is warranted.

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