Exocyst complex subunit sec8 binds to postsynaptic density protein-95 (PSD-95): a novel interaction regulated by cypin (cytosolic PSD-95 interactor)

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The PDZ domains of postsynaptic density (PSD) protein-95 play a role in the localization of PSD-95 and binding partners to neuronal synapses. The identification of binding partners to these PDZ domains can help us in understanding how signalling complexes are assembled. We observed that one of the subunits in the sec6/8 or exocyst complex, sec8, contains a C-terminal consensus sequence for PDZ binding. Sec8 binds to PDZ1–2 of PSD-95, and this binding can be competed with a peptide that binds to PDZ1 and PDZ2 in the peptide-binding site. In addition, binding of sec8 is dependent on its C-terminal-binding sequence namely Thr-Thr-Val (TTV). Immunoblotting of rat tissue extracts shows that sec8 and PSD-95 are enriched in the same brain regions, and sec8 and PSD-95 have the same subcellular distribution in pheochromocytoma cells, suggesting that these proteins may interact *in vivo*. Immunoprecipitation studies of sec8 and PSD-95 in brain provide further evidence of a sec8 and PSD-95 interaction. Furthermore, the cytosolic PSD-95 interactor competes with sec8 for interaction with PSD-95. Taken together, our results suggest that the cytosolic PSD-95 interactor may function to regulate the ability of sec8 to bind to PSD-95.

Key words: brain, exocyst, postsynaptic density, protein targeting.

INTRODUCTION

For proper synaptic transmission to occur, proteins must be localized to appropriate sites in the neuron. The postsynaptic density protein-95/synapse-associated protein-90 (PSD-95/SAP-90) and related membrane-associated guanylate kinases (MAGUKs) act to assemble synaptic-signalling complexes at excitatory synapses [1–5]. PSD-95 and related neuronal MAGUKs contain protein–protein interaction motifs including three N-terminal PDZ domains, an src homology 3 domain (SH3 domain) and a C-terminal region homologous with yeast guanylate kinase (GK) [6–9]. PDZ domains from PSD-95 and other MAGUK proteins bind to a number of receptors and channels [5,10–11], which contain a C-terminal PDZ-binding consensus sequence Thr/Ser-Xaa-Val/Ile [5]. Furthermore, we have shown previously [12] that PSD-95 can recruit actively the PDZ-binding Kv1.4 Shaker K^+ channel to synapses when transfected into hippocampal neurons. By aggregating neurotransmitter receptors and downstream enzymes, MAGUK proteins act as molecular scaffolds that mediate subcellular protein compartmentalization, which ensures selective activation of different signal-transduction cascades within a single cell [13–14].

PSD-95 has a crucial role in the development of excitatory synapses. Thus it is an excellent model system for determining the molecular mechanisms that underlie synaptic targeting during development and plasticity. Through its concurrent interaction with neuronal nitric oxide synthase (nNOS), PSD-95 serves as a physical tether to allow nNOS signalling by *N*-methyl-D-aspartate (NMDA) receptor activity [5,7]. By abolishing expression of PSD-95, either by knock-out technology in mice or by antisense technology in tissue culture, it has been shown that the presence of PSD-95 is essential for NO production by glutamate stimulation [7,15–16]. In addition, recent studies suggest that PSD-95 expression enhances maturation of excitatory synapses and postsynaptic clustering and activity of glutamate receptors [17] via its interaction with stargazin [18]. Furthermore, time-lapse fluorescence imaging revealed that PSD-95 expression and clustering correlates with the formation and stabilization of dendritic spines [19]. The mechanisms by which PSD-95 is rapidly localized to and removed from dynamically changing spines remain unclear. Thus proteins that serve to regulate PSD-95 targeting may play an important role in regulating processes such as development and synaptic plasticity.

In the present study, we identified sec8, a member of the exocyst complex, as a binding partner to PSD-95. Using a glutathione S-transferase (GST) fusion protein column containing PSD-95 for affinity chromatography with brain extract, we show that sec8 binds to PSD-95 via its C-terminal consensus sequence. Furthermore, sec8 binds to PDZ1 and PDZ2 of PSD-95, and this interaction is at the peptide-binding site of PSD-95 PDZ domains, since the peptide KLSSIESDV competes with sec8 binding. Both PSD-95 and sec8 are enriched in most brain regions. PSD-95 and sec8 associate *in vivo* as immunoprecipitation of PSD-95 co-immunoprecipitates sec8. The binding of sec8 can be competed with cytosolic PSD-95 interacting protein (cypin) for binding to PSD-95, suggesting that cypin may act to decrease PSD-95 localization by disrupting the sec8/PSD-95 interaction. Taken together, these results suggest that competition at PDZ1 and PDZ2 of PSD-95 may play a role in regulating its localization.

Abbreviations used: PSD, postsynaptic density; cypin, cytosolic PSD-95 interactor; DIV, days *in vitro*; DLG, discs large; GFP, green fluorescent protein; GK, guanylate kinase; GST, glutathione S-transferase; MAGUK, membrane-associated guanylate kinase; MTOC, microtubule organizing centre; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; PC12, pheochromocytoma; SAP, synapse-associated protein; SH3 domain, src homology 3 domain.

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EXPERIMENTAL

Materials

Peptides were from Research Genetics (Birmingham, AL, U.S.A.). Glutathione–Sepharose and pGEX4T-1 vector were from Pharmacia Biotech (Piscataway, NJ, U.S.A.). Horseradish peroxidase-coupled donkey anti-mouse antibody was from Sigma. SuperSignal Chemiluminescent Substrate was from Pierce (Rockford, IL, U.S.A.). Full-range rainbow protein molecular-mass standards were from Amersham Life Science (Piscataway, NJ, U.S.A.). cDNA encoding sec8 wild-type or lacking the last three amino acids, Thr-Thr-Val (sec8-TTV) was constructed using PCR and a 5′-oligo-GCTCTAGACATGGCGGCAGAAG-CAGCTGGTG and a 3′-oligo-GCGTCGACCTACACAGTGG-TTATTTTCTTG (wild-type) or GCGTCGACCTATTTCTTGT-CCTTGGTGGCTTG (-TTV) and subcloning the fragment into pGEX-Kg at *Xba*I and *Sal*I sites. Monoclonal antibodies 2E12 and 15E12 were raised as reported previously [20]. All other reagents were from Sigma.

Expression and purification of GST proteins

GST-fusion proteins encoding PSD-95 regions were constructed by PCR and were expressed and purified from *Escherichia coli* as described previously [12]. cDNAs encoding GST-fusion proteins of sec8 and sec8-TTV were transformed into *E. coli* and purified using standard techniques [21]. For protein affinity-chromatography studies, GST-fusion proteins were not eluted from the beads.

GST-fusion protein binding

One adult rat brain was homogenized in 10 ml of TEEN [25 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/150 mM NaCl/ 1 mM PMSF/1 mM dithiothreitol]. The extract was spun at 1000 ***g*** for 5 min twice to pellet unwanted nuclei. The brain extract (1 ml) was incubated with glutathione–Sepharose bound to 25 μg of the appropriate GST-fusion protein at 4 °C for 1 h. Extract/beads were loaded into the appropriate sized column and were washed three times with 20 ml of TEEN lacking NaCl. Bound proteins were eluted with 100 μl of 0.5 % SDS/100 mM NaCl, resolved on 10 % (w/v) polyacrylamide gels, and transferred to PVDF membrane as described above. Blots were probed using monoclonal antibody 15E12 raised against sec8 and monoclonal antibody 045 (Affinity Bioreagents, Golden, CO, U.S.A.) raised against PSD-95, and visualized using a secondary antibody coupled with horseradish peroxidase and SuperSignal Chemiluminescent Substrate (Pierce).

For affinity chromatography using pheochromocytoma (PC12) extracts, PC-12 cells were scraped into TEEN and passed through a 25 gauge needle five times to break cell membranes and shear DNA. Triton X-100 was added to a final concentration of 1 % (v/v) and proteins were extracted for 1 h at 4 °C on a nutator. The extract was centrifuged at 12 000 ***g*** for 15 min, and the supernatant was incubated with GST-fusion proteins on beads as described above.

For competition studies, GST–cypin was prepared as described previously [12] and eluted from glutathione–Sepharose beads by cleaving with thrombin. Thrombin cleaves the cypin from GST. GST–PSD-95, immobilized on beads, was incubated with brain or PC12 extract in the absence or presence of increasing amounts of cypin. Elutates were analysed for the presence of sec8 as described above.

Immunoprecipitation

Frozen rat brains were homogenized in 30 ml of TEEN2 [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/100 mM NaCl/ 1 mM PMSF]. Soluble proteins were extracted with 1 % (v/v) Triton X-100 by incubation for 1 h at 4 °C. Insoluble proteins were pelleted by centrifugation at 20 000 ***g*** for 20 min and the supernatant was pre-cleared with Protein A–Sepharose for 1 h. Lysates were diluted 10-fold in TEEN2 and PSD-95 was immunoprecipitated with 10 μg/ml polyclonal anti-PSD-95 antibody [12] or preimmune antiserum linked to Protein A–Sepharose (Pharmacia) for overnight at 4 °C. Beads were washed, and bound proteins were eluted in SDS loading buffer and analysed by immunoblotting using a mouse antibody (15E12) raised against sec8 [20].

For competition studies, recombinant cypin protein was added to the lysates before performing the co-immunoprecipitation.

Primary neuronal culture

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days gestation. The hippocampi were dissociated with papain followed by brief mechanical trituration. Cells were plated on poly-D-lysine-treated glass coverslips (12 mm in diameter) at a density of 200–250 cells/mm^2. Cultures were plated and maintained in Neurobasal media, supplemented with B27, penicillin, streptomycin and L-glutamine. For immunohistochemistry, neurons were fixed in ice-cold methanol for 15 min and stained using monoclonal antibody 2E12 raised against sec8 and rabbit polyclonal antibody raised against PSD-95 as described previously [12,21]. Labelled cells were visualized by immunofluorescence (Olympus IX50 microscope with a Cooke Sensicam charge-coupled-device-cooled camera, fluorescence-imaging system and Image Pro software).

PC-12 cell transfection

For biochemical assays, PC-12 cells were plated at 50–70 % confluency. Cells were transfected with cDNAs encoding PSD-95–GFP (where GFP stands for green fluorescent protein) and cypin using LIPOFECTAMINE™ 2000 (Gibco–BRL, Gaithersburg, MD, U.S.A.). Cells were treated with 100 ng/ml nerve growth factor (NGF) for 2 days after transfection to induce differentiation. For immunohistochemical studies, the cells were fixed in ice-cold methanol and stained using monoclonal antibody 2E12 raised against sec8 and rat antibody raised against GFP as described previously [21]. Labelled cells were visualized by immunofluorescence as described above.

RESULTS

Sec8 binds to PSD-95 via its C-terminal amino acids

To gain more insight into the mechanism of PSD-95 targeting to the PSD, we scanned sequences of proteins involved in protein trafficking for PDZ-binding motifs. Remarkably, we found that two of the exocyst subunits, sec8 and exo84 (Figure 1), have sequences that end with a valine residue, as do most consensus sequences for PDZ binding [22]. Both of these sequences contain a serine or threonine residue at the second position from the C-terminus, which fits a consensus for PDZ type I. Since the MAGUK proteins contain type I PDZ domains and interact potentially with sec8 and/or exo84, we performed ‘pull down’ assays in which GST-fusion proteins bearing sec8 or sec8 lacking the last three amino acids (-TTV) were incubated with brain extract. As seen in Figure 2(A), PSD-95 binds to sec8. Furthermore, deletion of the last three amino acids of sec8, which

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Sec5     nkfkssmhlqltcfqaasptvmkt
Sec6     aspnyvpifqeivvpslnvakllk
Sec8     rlkeiiceqaaikqatkdkkiTtV
Sec15    drqklietvvkqlrglvtgmsqhm
Exo70    pekyikyrveqvgdmidrlfdtsa
Exo84    kqlqdlrnasrllrvnpesttSvV
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Figure 1 C-terminal sequences of subunits of the exocyst complex

Sequences from rodent sec5, sec6, sec8, sec15, exo70 and exo84 are shown. Both sec8 and exo84 contain consensus motifs for PDZ binding. Conserved amino acids for binding are shown by boldface letters and underlined.

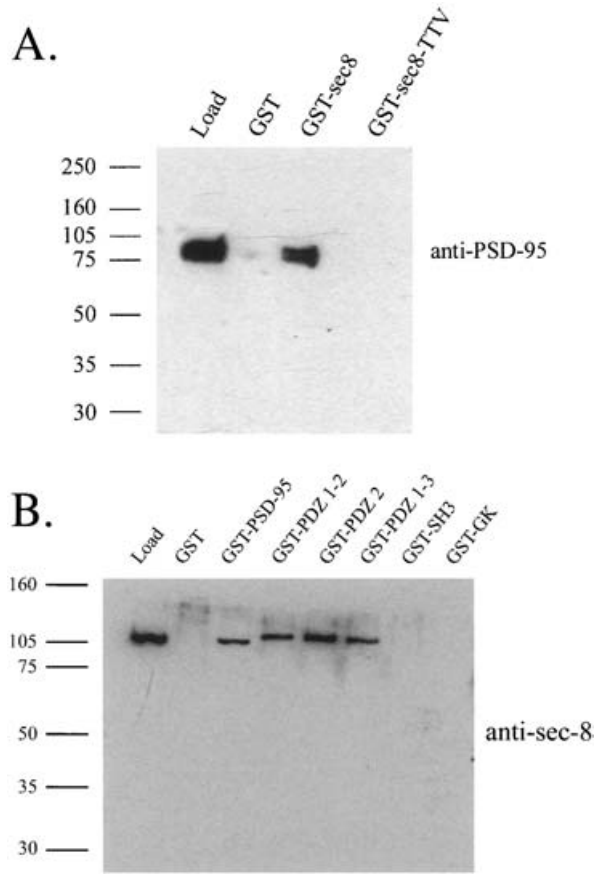

Figure 2 Sec8 binds to PDZ1–2 of PSD-95 via its C-terminus

(**A**) Detergent-soluble extracts of rat brain were incubated with glutathione–Sepharose bound to 25 μg of GST, GST-sec8, or GST-sec8-TTV (sec8 lacking the last three amino acids). The Sepharose was washed and eluted, and proteins were resolved by SDS/PAGE [8 % (w/v) gel] and transferred to Immobilon-P. Western blotting of eluates demonstrated that PSD-95 binds wild-type but not mutant sec8. (**B**) Brain extract was incubated with glutathione–Sepharose bound to 25 μg of GST or GST-fusion proteins of PSD-95 domains as indicated. The Sepharose was washed and eluted, and proteins were resolved by SDS/PAGE (8 % gel) and transferred to Immobilon-P. Western blotting of eluates demonstrated that sec8 binds to PDZ1–2 of PSD-95 but not the SH3 domain or GK domains.

contain the PDZ-binding motif, eliminates PSD-95 binding to sec8 (Figure 2A). PSD-95 also binds to exo84 and this binding depends on the last three amino acids (results not shown).

Sec8 binds to the PDZ domains of PSD-95

Since deletion of the PDZ-binding consensus of sec8 eliminates PSD-95 binding, we predicted that sec8 binds to PSD-95 via PDZ

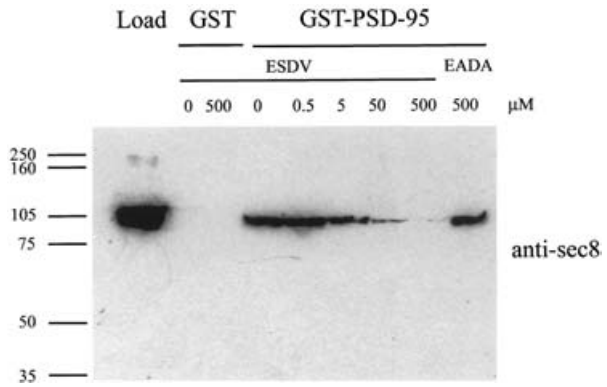

Figure 3 Sec8 binds competitively to the peptide-binding site in the PSD-95 PDZ1 and PDZ2 domains

Detergent-soluble brain extracts were incubated with glutathione–Sepharose bound to 25 μg of GST or GST–PSD-95 in the presence of increasing concentrations (μM) of the peptide KLSSIESDV, which binds to the first and second PDZ domains of PSD-95 or KLSSIEADA, which does not bind to the PDZ domains of PSD-95. The Sepharose was washed, eluted and proteins were resolved by SDS/PAGE (8 % gel) and transferred to Immobilon-P. Western blotting of eluates demonstrates that sec8 binds specifically to the PDZ domains of PSD-95 and that binding is blocked by the peptide KLSSIESDV (ESDV) with an IC_{50} of approx. 10 μM. The peptide KLSSIEADA (EADA) does not block sec8 binding.

domains of PSD-95. To determine if our prediction was correct, GST-fusion protein affinity chromatography was performed using PDZ1, PDZ2, PDZ1 and PDZ2, PDZ1–3 together, SH3 and GK domains, as well as full-length PSD-95. Figure 2(B) shows that sec8 binds to PDZ1 and PDZ2 together and to PDZ2 alone. Binding to PDZ1 and PDZ2 is quantitatively similar to sec8 binding to full-length PSD-95 (Figure 2B). In addition, sec8 does not bind to the SH3 or GK domains (Figure 2B).

PSD-95 binds sec8 at its peptide-binding site

It has been shown previously that the PDZ domains of PSD-95 bind preferentially to peptides ending with the sequence (S/T)XV [23,24]. To determine whether sec8 binds to the peptide-binding site in the PDZ domains of PSD-95, we performed GST affinity chromatography using full-length PSD-95 immobilized on beads incubated with brain extract in the presence of a peptide KLSSIESDV, which has high affinity for PDZ1 and PDZ2. As seen in Figure 3, the peptide competes with sec8 binding to PSD-95 with an IC_{50} of approx. 10 μM, suggesting that sec8 binds the PDZ domains of PSD-95 at the peptide-binding sites. A control peptide, KLSSIEADA, which does not bind to the PDZ domains, has no effect on sec8 binding to PSD-95, suggesting that this interaction is specific (Figure 3).

Sec8 and PSD-95 are expressed in the same brain regions

If the interaction between sec8 and PSD-95 is relevant physiologically, these proteins should be expressed in the same regions of the brain. To address this question, we subjected extracts of different brain regions to SDS/PAGE and performed Western blotting using either an antibody raised against sec8 or PSD-95. As seen in Figure 4 (top and middle panels), both sec8 and PSD-95 are expressed in striatum, olfactory bulb, medulla, hippocampus, cerebellum and cortex. Both sec8 and PSD-95 are expressed in the spinal cord, although PSD-95 appears to be expressed at extremely low levels (Figure 4, top and middle

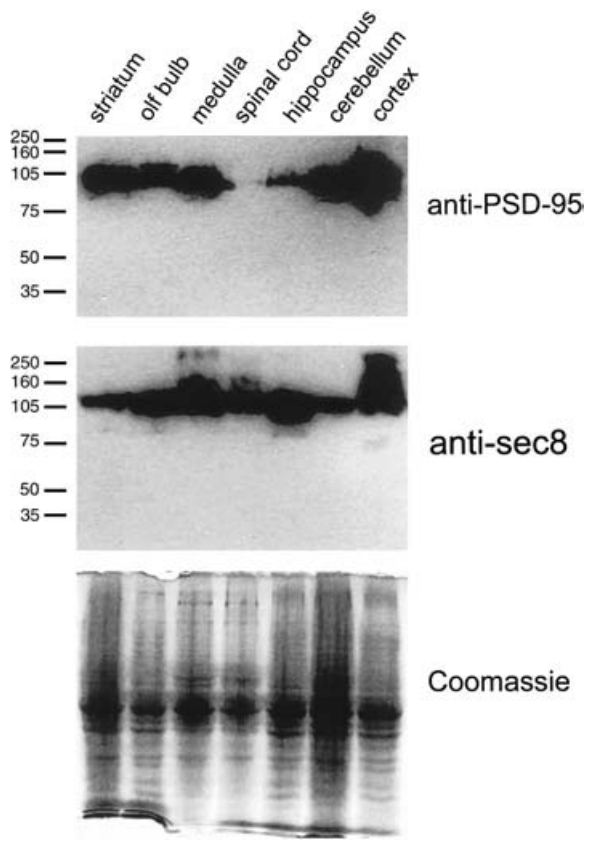

Figure 4 PSD-95 and sec8 proteins are enriched in the brain regions

Top and middle panels: extracts of tissue from different rat brain regions were resolved on a 10 % polyacrylamide gel and transferred to Immobilon-P. Western blotting of eluates demonstrates that both PSD-95 and sec8 are detected readily in all regions with the exception that PSD-95 is detected at low levels in the spinal cord. PSD-95 is detected as a doublet. Bottom panel: Coomassie Blue staining of a 10 % polyacrylamide gel run with 10 μg of each sample, as determined by Bradford assay, shows that there is equal loading of each sample. olf, olfactory.

panels). Coomassie Blue staining confirms that similar levels of protein were used for all regions of brain and spinal cord (Figure 4, bottom panel). Thus the co-expression of PSD-95 and sec8 in brain areas supports the possibility of interaction between these two proteins.

Exogenously expressed PSD-95 co-localizes with endogenous sec8 in PC-12 cells

Vega and Hsu [21] have shown previously that the exocyst complex associates with the microtubule organizing centre (MTOC) in undifferentiated PC-12 cells and that the exocyst complex then extends to the growing neurite when PC-12 cells are differentiated with NGF. To determine whether sec8 and PSD-95 are localized at or near the MTOC in neurons, we stained neurons that had been in culture for 24 h or 1 day *in vitro* (DIV 1) with antibodies to sec8 and PSD-95. We reasoned that DIV 1 neurons may not be differentiated fully and that sec8 and PSD-95 would localize to the MTOC before differentiation. As seen in Figure 5(A), this is not the case. DIV 1 neurons are not undifferentiated. Furthermore, sec8 and PSD-95 co-localize in the neuronal cell body; however, PSD-95 is clustered at the PSD in some dendrites this early in culture (arrows). To study the co-localization of sec8 and PSD-95 in undifferentiated versus differentiated cells, we made use of PC-12 cells. As seen in Figure 5(B), sec8 shows a perinuclear distribution in undifferentiated cells and is localized throughout the cell body and neurites in differentiated cells. We then transfected PC-12 cells with a cDNA encoding PSD-95–GFP and fixed the cells 48 h later (undifferentiated) or added NGF (100 ng/ml) 24 h after transfection and fixed the cells after 48 h of NGF treatment (differentiated). As seen in Figure 5(C), in a majority of undifferentiated cells, PSD-95 localizes to a perinuclear region (white arrows). Endogenous sec8 is also present at this region, as it is in untransfected cells, suggesting that it is near the MTOC and that transfection of PSD-95 does not change sec8 localization. Furthermore, on differentiation, both PSD-95 and sec8 are expressed in a more diffuse pattern and their expression extends into the neurites in many cells (Figure 5D). These results suggest that PSD-95 and sec8 associate near or at the MTOC in undifferentiated cells and are trafficked to the neurite as cells differentiate.

Sec8 co-immunoprecipitates with PSD-95 from brain extract

To determine whether sec8 and PSD-95 interact *in vivo*, we immunoprecipitated PSD-95 from brain extract. As shown in Figure 6, sec8 co-immunoprecipitates with PSD-95. When pre-immune serum is used, neither PSD-95 nor sec8 is immunoprecipitated. These results suggest that sec8 associates with PSD-95 *in vivo*.

Cypin competes with sec8 for binding to PSD-95

Since both cypin and sec8 bind to the PDZ domains of PSD-95, we asked whether they compete for binding of the same pool of PSD-95. First, we performed affinity chromatography of brain or PC-12 cell extracts using GST–PSD-95 bound to glutathione–Sepharose beads in the absence and presence of increasing amounts of recombinant cypin protein. As seen in Figure 7(A), cypin competes with sec8 for binding to PSD-95 with an IC_{50} of approx. 18 nM. This suggests that the binding of sec8 and cypin to PSD-95 is mutually exclusive. Furthermore, as a control, mutation of SXV to IXV (cypin SΔI) in cypin's C-terminal which results in decreased binding to PSD-95 [12], competes sec8 binding with much lower affinity (Figure 7A). To test whether cypin may compete with the binding of other proteins to PSD-95, we probed the eluates with an antibody to nNOS. As seen in Figure 7(A), cypin competes nNOS binding to PSD-95 with an IC_{50} similar to that of sec8 binding. Thus regulation of PDZ binding by cypin may be a general cypin function.

We also transfected PC-12 cells with a construct coding for PSD-95–GFP. We then immunoprecipitated the PSD-95 and immunoblotted to confirm the co-immunoprecipitation of sec8. We also performed the immunoprecipitation in the presence of increasing amounts of recombinant cypin protein. As seen in Figure 7(B), cypin attenuates the co-immunoprecipitation of sec8 with PSD-95 with an IC_{50} of 27 nM, a value similar to the competition in affinity chromatography (Figure 7A). Taken together, these results suggest that cypin competes with sec8 for binding to PSD-95.

DISCUSSION

The main finding of the present study is that sec8, a member of the exocyst complex, associates with PSD-95. Sec8 binds specifically to the peptide-binding site in PDZ1 and PDZ2 of PSD-95 because its binding can be competed with the peptide.

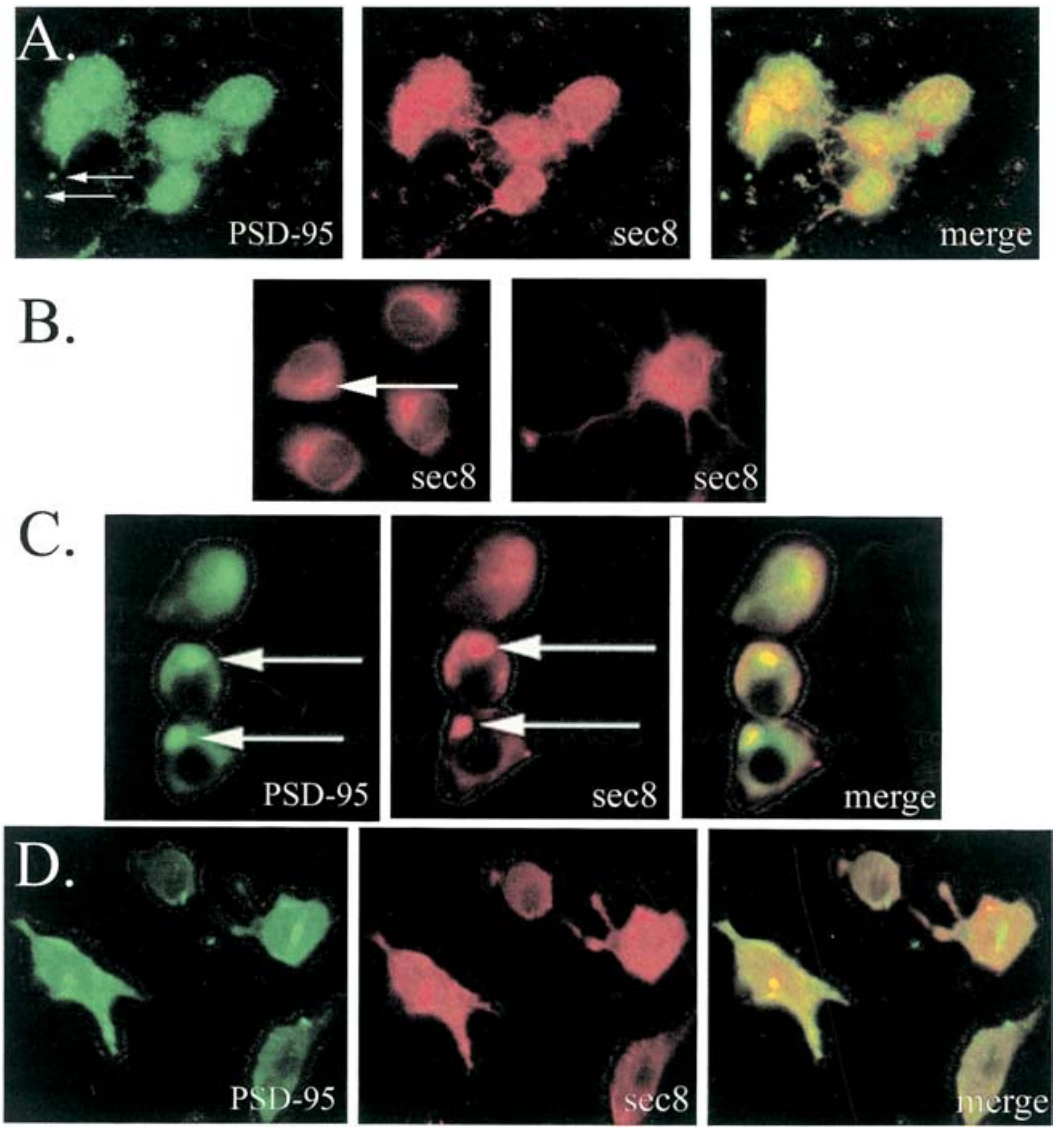

Figure 5 Localization of Sec8 and PSD-95 in hippocampal neurons and PC-12 cells

(**A**) Hippocampal neurons were dissected from E18 rats and were grown in culture for 1 day. The cells were then fixed in methanol and immunostained for endogenous sec8 and PSD-95. Both sec8 and PSD-95 show a diffuse expression pattern within the cell bodies. Some punctate staining of PSD-95 is seen in the dendrites (arrows). (**B**) Untransfected PC12 cells were stained for endogenous sec8. In undifferentiated cells (left panel), sec8 is localized to a perinuclear region (arrow). On differentiation of the cells with NGF (right panel), sec8 localization becomes diffuse throughout the cell body and neurites. (**C**, **D**) PC-12 cells were transfected with a cDNA encoding PSD-95–GFP. The cells were then fixed in methanol and immunostained for endogenous sec8. In a majority of undifferentiated cells (**C**), both PSD-95 and sec8 are concentrated in a perinuclear region (white arrows). In many cells differentiated with NGF (**D**), both PSD-95 and sec8 show a more diffuse pattern of expression.

Sec8 contains a C-terminal consensus site (-TXV) that binds to the peptide-binding site, and mutation of the threonine and valine to alanine residues eliminates binding. Furthermore, sec8 and PSD-95 are co-expressed in various regions of the central nervous system. Importantly, cypin competes binding of sec8 to PSD-95, a mechanism that may explain the role of cypin in the regulation of PSD-95 localization as discussed below.

Postsynaptic localization of PSD-95 is dependent on multiple signals, including an N-terminal palmitoylation motif, PDZ domains and a C-terminal motif [25–27]. Similarly, other MAGUKs rely on N-terminal signals for postsynaptic targeting [28]. Based on these studies, it is possible that partners that bind to motifs are responsible for regulating targeting. In fact, we previously isolated cypin as a regulator of the targeting of PSD-95 family members to the PSD by binding to the PDZ domains [12]. The mechanism by which cypin acts is unknown. Our results in the present study suggest that cypin may compete with other PSD-95-binding partners to regulate PSD-95 localization.

In addition to identifying protein motifs responsible for synaptic targeting, a number of groups have attempted to identify the molecular mechanisms by which proteins are targeted to the synapse. In the case of PSD-95 and the related *Drosophila* protein DLG (discs large), it appears that trafficking occurs via a subcellular membrane pathway. In cultured hippocampal neurons and heterologous cells, membrane localization of PSD-95 is preceded by a transient association with a perinuclear endosomal compartment and the smooth endoplasmic reticulum [29]. Furthermore, PSD-95 trafficking is dependent on microtubules, since nocodazole blocks PSD-95 transport intermediates [29]. Similarly, DLG trafficking is a

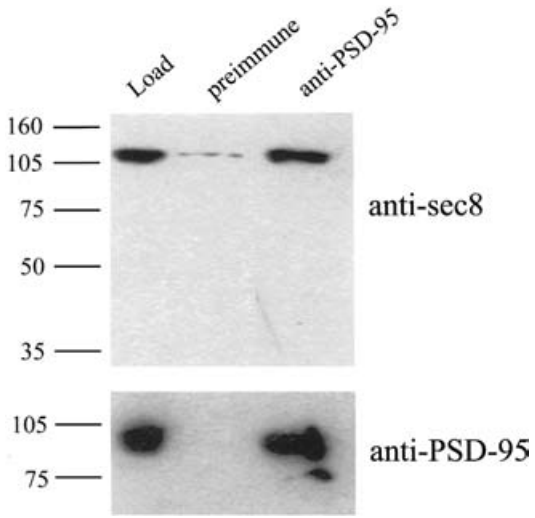

Figure 6 Sec8 co-immunoprecipitates with PSD-95

Detergent soluble brain extract was incubated with rabbit preimmune serum or rabbit anti-PSD-95. Antibody complexes were collected with Protein A–Sepharose, and the immunoprecipitated complexes were separated by SDS/PAGE (8 % gel). Western blotting for sec8 reveals a protein band at approx. 110 kDa that is much more intense in the anti-PSD-95 complexes than in the preimmune complexes. In addition, PSD-95 is precipitated with antibody, but not with preimmune serum. Load represents 10 % (v/v) of extract proteins.

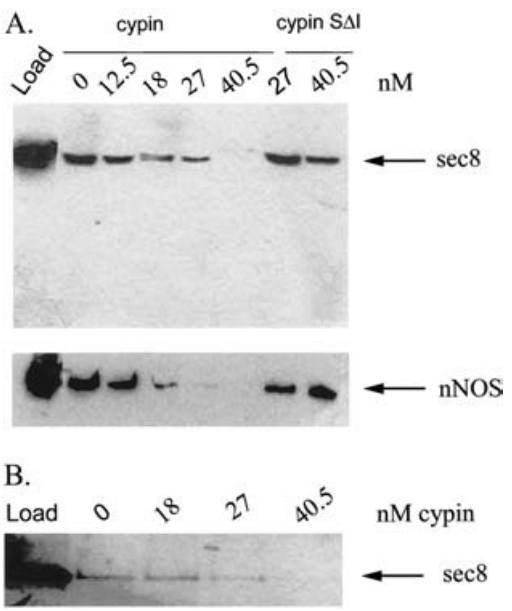

Figure 7 Cypin competes sec8 binding to PSD-95

(**A**) Detergent-soluble extracts of rat brain or PC-12 cells were incubated with glutathione–Sepharose bound to 25 μg of GST or GST–PSD-95 in the presence of increasing amounts of purified recombinant cypin. The Sepharose was washed and eluted, and proteins were resolved by SDS/PAGE (8 % gel) and transferred to Immobilon-P. Western blotting of eluates demonstrates that the presence of cypin results in decreased sec8 binding to PSD-95 with an IC_{50} of approx. 18 nM. As a control, cypin SΔI, a mutant of cypin that does not bind to PSD-95, competes with sec8 for binding to PSD-95 with much lower affinity. (**B**) Detergent-soluble extracts of PC-12 cells transfected with a cDNA encoding PSD-95–GFP were incubated with a polyclonal antibody raised against PSD-95 in the presence of the indicated amounts of cypin. The immunoprecipitates were resolved by SDS/PAGE and Western blotting was performed to assess the presence of sec8. Increasing amounts of purified recombinant cypin were added to the extracts before immunoprecipitation. Western blotting of immunoprecipitates demonstrates that the presence of cypin results in decreased co-immunoprecipitation of sec8 with PSD-95 for an IC_{50} of approx. 27 nM.

three-step process that encompasses localization at an intracellular network, the plasma membrane and the synaptic membrane [30]. In contrast with these results, Bresler et al. [31] have found that PSD-95 is recruited to nascent synaptic junctions from a diffuse dendritic cytoplasmic pool, which is partly cytosolic and partly associated with a light membrane fraction. Thus, by identifying PSD-95-binding partners, we may elucidate the roles of each of these pathways, vesicular and microtubule-associated versus cytosolic and light membrane-associated, in PSD-95 targeting.

What role could sec8 be playing in PSD-95 localization? Sec8 is one of the eight subunits that comprise the exocyst complex. It was identified originally in a screen in the budding yeast *Saccharomyces cervisiae* for mutants that contain secretory and polarized growth defects [32,33]. The exocyst complex is conserved from yeast to mammals [32,34] and has been found to concentrate at sites of membrane addition [35] and in growth cones of hippocampal and PC12 cells [21,36–37]. It is thought that the exocyst may be involved in maintaining cell polarity (as reviewed in [38,39]), neurite outgrowth [21] and exocytosis [40]. Furthermore, the exocyst complex has been found to associate with vesicles [35,37,41] and microtubules [21], where the complex is found distributing from the MTOC to the vicinity of the plasma membrane [21]. These associations of the exocyst complex, along with the binding of sec8 to the PDZ domains of PSD-95, support the idea that PSD-95 associates with vesicles and/or microtubules as it is localized to the synapse. Based on the fact that sec8 and PSD-95 co-localize in undifferentiated cells, we believe that sec8 and PSD-95 may transiently associate as PSD-95 is trafficked to the synapse.

Previously, we identified cypin as a protein that regulates the targeting of PSD-95 family members to the PSD by binding to the PDZ domains [12]. To our knowledge, cypin is the only protein to date that mediates PSD-95 targeting away from the synapse. The mechanism by which cypin acts is unknown. We now show that cypin can act to compete the binding of sec8 to the PDZ domains of PSD-95 (Figure 7), which are essential for synaptic localization of PSD-95 [26]. Since cypin is a highly soluble protein [12], this competitive binding can serve to regulate the association of PSD-95 with vesicles and/or microtubules, which is thought to be involved in PSD-95 trafficking [29]. By performing this function, cypin may act to regulate the amount of PSD-95 localized to the synapse, which in turn regulates the amount of nNOS that can be activated by stimulation of the NMDA receptor [16]. This process is important for maintaining proper neuronal function since NMDA receptor-induced NO production has been implicated in learning and memory (as reviewed in [42]).

The association of PSD-95 with sec8 suggests that synaptic localization of PSD-95 may be coupled with neurite outgrowth during early stages in development. In fact, we see clustering of PSD-95 in the dendrites of cultured hippocampal neurons as early as DIV 1, when dendrites develop (Figure 5; [29,43]). Once localized to synapses, PSD-95 clusters tend not to move within the dendrite [43]. Thus by competing with sec8 for PSD-95 binding, cypin may regulate PSD-95 localization independent of other exocyst functions.

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