Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks

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Summary

Postsynaptic density protein 95 (PSD-95/SAP-90) is a palmitoylated membrane-associated guanylate kinase that oligomerizes and clusters ion channels and associated signaling machinery at excitatory synapses in brain. However, the mechanism for PSD-95 oligomerization and its relationship to ion channel clustering remain uncertain. Here, we find that multimerization of PSD-95 is determined by only its first 13 amino acids, which also have a remarkable capacity to oligomerize heterologous proteins. Multimerization does not involve a covalent linkage but rather palmitoylation of two cysteine residues in the 13 amino acid motif. This lipid-mediated oligomerization is a specific property of the PSD-95 motif, because it is not observed with other palmitoylated domains. Clustering K^+ channel Kv1.4 requires interaction of palmitoylated PSD-95 with tetrameric K^+ channel subunits but, surprisingly, does not require multimerization of PSD-95. Finally, disrupting palmitoylation with 2-bromopalmitate disperses PSD-95/K^+-channel clusters. These data suggest new models for K^+ channel clustering by PSD-95 – a reversible process regulated by protein palmitoylation.

Key words: Synapse, Clustering, Receptor, Palmitoylation

Introduction

Efficient synaptic transmission requires aggregation of neurotransmitters and associated signal transduction machinery at synaptic sites. On the postsynaptic side, receptors and downstream signaling enzymes are clustered and thereby poised to respond to released neurotransmitters. Postsynaptic clustering is understood best at the neuromuscular junction, where nicotinic acetylcholine (nAChR) receptors are concentrated 10,000-fold above levels at extrajunctional sites (Hall and Sanes, 1993). Clustering of nAChR requires association with rapsyn, a myristoylated peripheral membrane protein at the motor endplate (Gautam et al., 1995; Marchand et al., 2000). In the brain, mechanisms for receptor clustering are less certain. However, the postsynaptic density protein PSD-95/SAP-90 (Cho et al., 1992; Kistner et al., 1993) and related membrane-associated guanylate kinases have recently emerged as candidate scaffold molecules that can organize transduction machinery at excitatory synapses (Craven and Bredt, 1998; Kennedy, 1998; O'Brien et al., 1998; Sheng and Wyszynski, 1997).

A role for PSD-95 in receptor clustering was first suggested based on the potent interaction of both *N*-methyl-D-aspartate (NMDA) receptors and Shaker-type K^+ channels with the PDZ domains of PSD-95 (Kim et al., 1995; Kornau et al., 1995). Furthermore, mutations of *discs large* (*dlg*), a *Drosophila* homolog of PSD-95, prevent proper post-synaptic clustering of Shaker-type K^+ channels at the larval neuromuscular junction (Tejedor et al., 1997). In addition to clustering ion channels, PDZ domains from PSD-95 organize signaling enzymes (Brenman et al., 1996) and cell adhesion molecules (Irie et al., 1997) at the postsynaptic density. PSD-95 also contains SH3 and guanylate kinase (GK) domains, which both mediate regulatory intramolecular interactions (McGee et al., 2001; Tavares et al., 2001) and recruit additional proteins to the macromolecular complex (Brenman et al., 1998; Kim et al., 1997; Takeuchi et al., 1997). Through this network of interactions, PSD-95 and related MAGUK protein complexes regulate postsynaptic development (El-Husseini et al., 2000b; Sala et al., 2001) and plasticity (Migaud et al., 1998).

Biochemical mechanisms for ion channel clustering by PSD-95 remain uncertain. Some aspects of channel clustering can be reproduced in a heterologous cell transfection assay in which co-expression of PSD-95 and Kv1.4 yields formation of plasma membrane patches containing both PSD-95 and Kv1.4 (Kim et al., 1995). One model for this clustering posits that the clusters of PSD-95 and Kv1.4 reflect a molecular lattice, which forms based on the multivalent nature of both PSD-95 and Kv1.4 (Hsueh et al., 1997). Shaker-type K^+ channels are

multimeric based on their inherent tetrameric structure. Although the oligomeric structure of PSD-95 is unclear, previous studies have proposed that PSD-95 occurs as a tetramer formed through intermolecular disulfide bonds involving cysteine residues 3 and 5 (Hsueh et al., 1997).

Here, we mechanistically analysed the oligomerization and clustering of PSD-95. We find that multimerization of PSD-95 involves only its first 13 amino acids. Remarkably, these 13 amino acids are sufficient for oligomerization of two heterologous proteins. Multimerization of PSD-95 does not involve disulfide bonds but instead requires palmitoylation of two cysteine residues in the 13 amino acid motif. This lipid-dependent multimerization is a unique property of the N-terminus of PSD-95 and is not seen with other palmitoylation domains. Clustering K^+ channel Kv1.4 requires interaction of palmitoylated PSD-95 with tetrameric K^+ channel subunits but, surprisingly, does not require multimerization of PSD-95. Finally, blocking palmitoylation with 2-bromopalmitate disperses PSD-95/ion-channel clusters, indicating that K^+ channel clustering by PSD-95 is a reversible process regulated by protein palmitoylation.

Materials and Methods

Antibodies

The following antibodies were used: monoclonal antibodies to PSD-95 (clone 7E3-18; Affinity Bioreagents) and green fluorescent protein (GFP; Clontech) and polyclonal antibodies to Kv1.4 (Kim et al., 1995), PSD-95 and GFP (El-Husseini et al., 2000a).

Construction of cDNA plasmids

Epitope-tagged constructs of PSD-95 were generated by PCR as described previously (El-Husseini et al., 2000a). To generate the Kv1.4 constructs, nucleotides encoding amino acids 1-350 and 351-655 of Kv1.4 were amplified separately by PCR with a hemagglutinin (HA) epitope encoded in the 5′ primer at amino acid 351. A three-part ligation reintroduced Kv1.4 into GW1 *Hin*dIII/*Eco*RI with a *Kpn*I site joining the two separate PCR products. To produce $(Kv1.4)_2$, the coding region of HA-tagged Kv1.4 was amplified by PCR twice: once with a 5′ primer containing a *Hin*dIII site and a 3′ primer with a *Bgl*II site and a second time with a 5′ primer containing an in-frame *Bgl*II site and a 3′ primer with a stop codon and an *Eco*RI site. A three-part ligation reintroduced both Kv1.4 channel subunits into GW1 *Hin*dIII/*Eco*RI with a *Bgl*II site between the two subunits. To link four Kv1.4 channel subunits together to produce $(Kv1.4)_4$, the entire coding region of HA-tagged Kv1.4 was amplified twice and subcloned back into GW1 as described above for $(Kv1.4)_2$ but without the stop codon in the second subunit. A third subunit was added in-frame at the *Bgl*II site and a fourth in-frame with a stop codon at the *Eco*RI site. The Tac (interleukin-2 receptor α-subunit) cDNA, modified by the introduction of an *Xba*I site at the 5′ end, was kindly provided by R. Edwards (University of California, San Francisco). A binding consensus for PSD-95 from the C-terminal 12 amino acids of the NR2B subunit was subcloned into this cDNA with oligos using the introduced *Xba*I site and an *Apa*I site in the multi-cloning site of the vector. The C-terminal prenyl motif of paralemmin (-DMKKHRSKSCSIM) was added to the extreme C-terminus of PSD-95(C3,5S)-GFP with PCR primers encoding this motif.

Cell transfection and immunoprecipitation

COS7 or HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin and streptomycin. Cells were transfected using Lipofectamine reagent according to the manufacturer's protocol (Gibco). For routine immunoprecipitation studies, transfected cells were incubated in lysis buffer [25 mM Tris HCl, pH 7.4, plus 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF] at 4°C for 30 minutes. In some experiments, as indicated, 0.1% SDS replaced Triton X-100 in the cell lysis buffer; in these experiments, 1% Triton X-100 was added after cell lysis. Lysates were cleared by centrifugation at 17,000 ***g*** for 15 minutes and incubated for 1 hour at 4°C with 2 μg sheep anti-PSD-95 antibody (Brenman et al., 1996) or rabbit anti-GFP antibody (Clontech).

Immunofluorescent labeling

COS7 cells were grown on coverslips in 24-well plates and transfected as described above. Coverslips were removed from culture wells and fixed in 2% paraformaldehyde for 30 minutes. After washing with PBS containing 0.3% Triton X-100 (PBST) three times for 5 minutes each, the cells were incubated in PBST containing 3% normal goat serum for 1 hour at room temperature to block non-specific antibody interactions. Primary antibodies were added in block solution for 1 hour at room temperature, followed by donkey anti-mouse or goat anti-rabbit secondary antibodies conjugated to Cy2 or Cy3 fluorophores (diluted 1:200 in block solution) for 1 hour at room temperature. Images were taken under fluorescence microscopy with a 100× oil-immersion objective (NA=1.4) affixed to a Leica upright microscope.

Results

PSD-95 forms non-covalent homo-multimers

To determine whether PSD-95 forms multimeric protein complexes in intact cells, we constructed expression vectors encoding PSD-95 fusion proteins containing either a FLAG epitope tag or GFP at the C-terminus. These two proteins were co-expressed in HEK293 cells, proteins were solubilized with Triton X-100, lysates were immunoprecipitated with an antibody to GFP and analysed by western blotting for the presence of PSD-95/FLAG. As previously reported (Hsueh et al., 1997), we readily detect formation of PSD-95 multimers in the co-transfected cells (Fig. 1).

To determine whether PSD-95 multimerization involves intermolecular covalent bonds, we denatured the protein lysates with 0.1% SDS in the absence of reducing agents. This procedure disrupts non-covalent protein/protein interactions but does not disrupt disulfide bonds. We found that denaturing cell lysates with SDS disrupted the PSD-95 multimers (Fig. 1), indicating that they are not linked by covalent bonds.

Palmitoylated N-terminal 13 amino acids of PSD-95 mediate oligomerization

To define the interaction interface between the PSD-95 multimers, we constructed and analysed a series of progressively larger deletion constructs encoding PSD-95 fused to GFP. Each of these constructs was transfected into COS cells and assessed for interaction and coimmunoprecipitation with full-length PSD-95 (data not shown). These experiments demonstrated that a construct containing only the first 13 amino acids of PSD-95 associates with full-length PSD-95 (Fig. 2A). This interaction between 1-13 and full length PSD-95 is disrupted by SDS, indicating that it is not covalent (Fig. 2B).

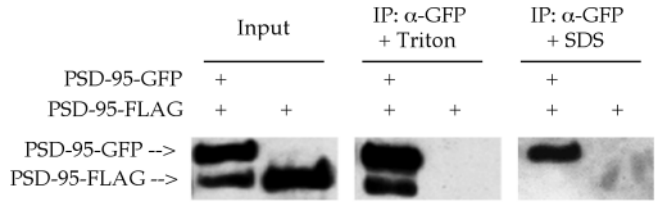

Fig. 1. PSD-95 forms non-covalent multimers. COS cells were transfected with plasmids encoding GFP- and FLAG-tagged PSD-95 proteins as indicated. Cells were lysed with either 1% Triton X-100 or 0.1% SDS and proteins were immunoprecipitated with an antibody to GFP. Western blotting reveals that PSD-95/FLAG co-immunoprecipitated in cells lysed with Triton X-100 but not in SDS-treated lysates.

Having determined that the first 13 amino acids of PSD-95 are sufficient for interaction with the full-length protein, we next asked whether this 13 amino acids motif alone could mediate oligomerization. To assess this, we co-transfected COS cells with constructs encoding the first 13 amino acids of PSD-95 linked both to GFP and to maltose binding protein. Remarkably, these two fusion proteins efficiently co-immunoprecipitated (Fig. 2C).

The first 13 amino acids of PSD-95, which mediate multimerization, are also necessary and sufficient for palmitoylation (El-Husseini et al., 2000a), raising the possibility that multimerization and palmitoylation are interrelated. Consistent with this possibility, mutating palmitoylated cysteines 3 and 5 prevents multimerization of PSD-95 (Fig. 3A) (Hsueh et al., 1997). More importantly, mutating leucine-4 to serine, which disrupts palmitoylation of PSD-95 (El-Husseini et al., 2000a), also prevents multimerization (Fig. 3B).

To assess more directly the role of palmitoylation, we treated transfected cells with 2-bromopalmitate, which blocks the palmitoyl transferase that mediates protein palmitoylation (Webb et al., 2000) and blocks PSD-95 palmitoylation (El-Husseini et al., 2002). Cells co-transfected with PSD-95-GFP and 1-13-GFP were treated with 20 μM 2-bromopalmitate for 4 hours and multimerization was assessed by co-immunoprecipitation. These experiments showed that 2-bromopalmitate blocks multimerization of PSD-95 (Fig. 3C). As a control, we treated cells with palmitate and found that this does not interfere with multimerization (Fig. 3C).

We next asked whether another palmitoylated motif, from the growth associated protein GAP-43, would also mediate multimerization. Previous studies have shown that the N-terminal ten amino acids of GAP-43 determine its palmitoylation (Liu et al., 1993) and we previously reported that fusing these ten amino acids to PSD-95 yields a robustly palmitoylated chimera (El-Husseini et al., 2000a). To test whether the palmitoylation motif of GAP-43 also mediates multimerization, we tagged such GAP-43/PSD-95 chimeras with either GFP or FLAG at their C-termini. In co-transfected COS cells, we find that these differently tagged GAP-43 chimeras do not interact (Fig. 3D), indicating that oligomerization is a specific property of the PSD-95 palmitoylation motif.

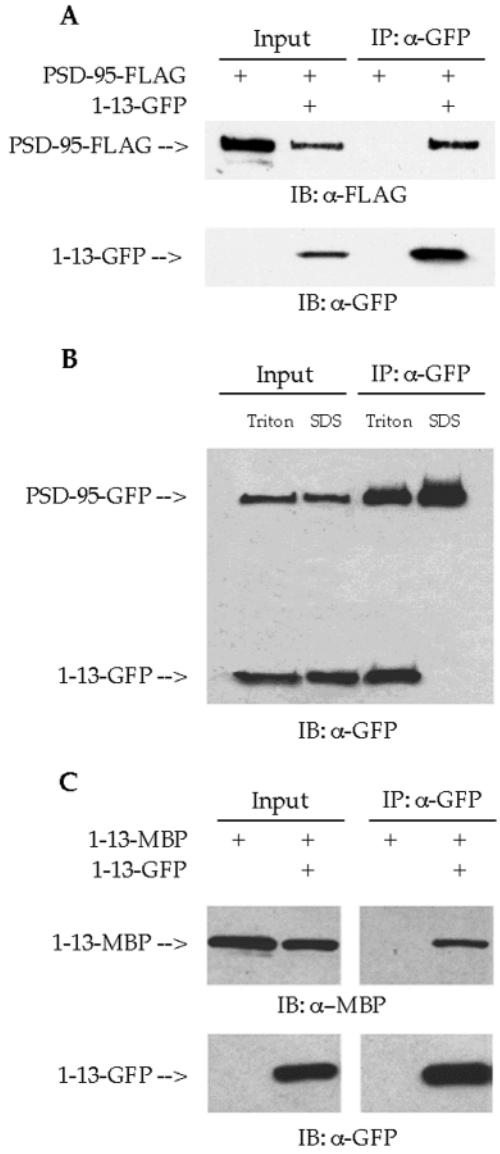

Fig. 2. The N-terminal 13 amino acids of PSD-95 can mediate homomultimerization. COS cells were transfected with plasmids as indicated. Cell lysates were immunoprecipitated with antibodies to GFP or to the PDZ domains of PSD-95 as indicated and western blotting was used to monitor co-immunoprecipitation. (A) FLAG-tagged full-length PSD-95 and amino acids 1-13 of PSD-95 fused to GFP efficiently co-immunoprecipitated. (B) COS cells were co-transfected with constructs encoding PSD-95-GFP and amino acids 1-13 of PSD-95 fused to GFP. Co-immunoprecipitation of these constructs is disrupted when cells are lysed in buffer containing 0.1% SDS. (C) Chimeras of GFP and maltose binding protein (MBP), each containing the first 13 amino acids of PSD-95, efficiently co-immunoprecipitate.

Clustering by PSD-95 requires interaction with a multivalent ion channel

We next asked whether protein multimerization participates in ion channel clustering with PSD-95. Certain aspects of channel clustering by PSD-95 can be reproduced in a co-clustering experiment in heterologous cells (Kim et al., 1995). In this assay, co-transfection of PSD-95 with Shaker K^+ channel Kv1.4 causes a striking redistribution of both proteins to raft-like clusters on the cell surface (Fig. 4A,B). To determine whether the tetrameric structure of Kv1.4 contributes to its clustering with PSD-95, we constructed expression plasmids

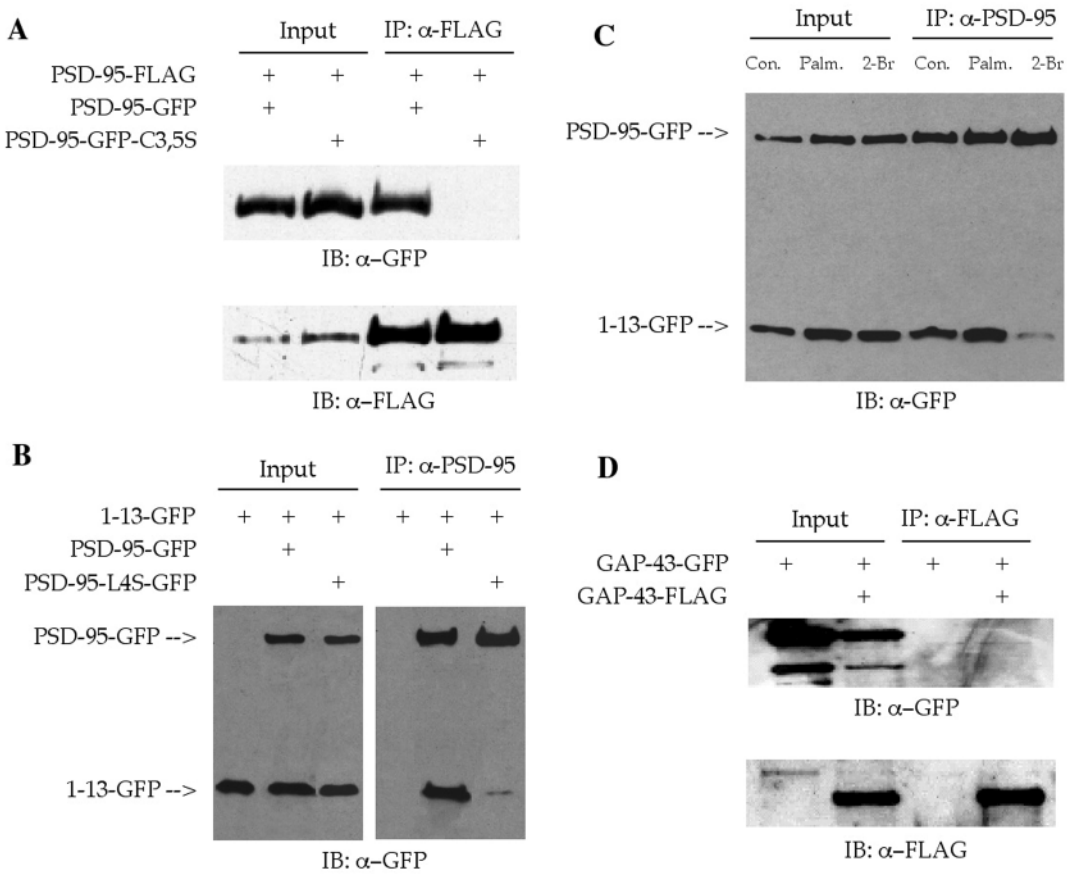

Fig. 3. Multimerization is a specific property of the PSD-95 palmitoylation motif. COS cells were transfected with plasmids encoding plasmids as indicated. Cell lysates were immunoprecipitated with an antibody to GFP, FLAG or the PDZ domains of PSD-95 as indicated and western blotting was used to monitor co-immunoprecipitation. (A) Mutating palmitoylated cysteine residues 3 and 5 of PSD-95 blocks co-immunoprecipitation. (B) Mutating leucine 4 of PSD-95 to serine (which blocks palmitoylation) disrupts co-immunoprecipitation. (C) Treating PSD-95-transfected COS cells for 4 hours with 20 μM 2-bromopalmitate – but not palmitate – disrupts multimerization mediated by the N-terminus of PSD-95. (D) Differently tagged chimeras of PSD-95 (containing just the palmitoylated N-terminal ten amino acids of GAP-43 fused to PSD-95 lacking a palmitoylation motif) do not oligomerize.

containing two or four Kv1.4 subunits linked in tandem. Previous studies have shown that Shaker-type K^+ channel subunits, which contain six transmembrane domains, can be expressed as functional channels when fused together in tandem arrays (Isacoff et al., 1990). As was done in these previous studies, our constructs were designed such that the intracellular C-terminus of one Kv1.4 subunit is linked in-frame to the N-terminus of another subunit to form a single polypeptide with 12 transmembrane domains and two C-terminal cytoplasmic tails per channel to interact with PSD-95. We also linked together two $(Kv1.4)_2$ subunits to form a $(Kv1.4)_4$ channel containing 24 transmembrane domains and a single C-terminal cytoplasmic tail per channel. These Kv1.4 channels contain an HA epitope in the extracellular domain so that surface expression can be monitored by immunofluorescent staining of intact cells.

We find that $(Kv1.4)_2$ and $(Kv1.4)_4$ channels are expressed on the surface of the cells. However, in co-transfections with PSD-95, they do not efficiently form clusters (Fig. 4C,D and data not shown). Co-expression of PSD-95 with wild-type Kv1.4 yields clusters in ~60% of cells, whereas PSD-95 and $(Kv1.4)_2$ form clusters in 3% of cells, and PSD-95/$(Kv1.4)_4$ did not form clusters in any cells (n=100 for each). To determine whether monomeric single-pass transmembrane proteins can be recruited to clusters, we appended the PSD-95 binding consensus (-SDV) from the C-terminus of NMDA receptor 2B (NR2B) to the short cytoplasmic tail of the interleukin-2 receptor α-subunit (IL-2 or Tac), a well-characterized plasma membrane protein. When expressed with PSD-95 in COS7 cells, no clusters of Tac-SDV are formed (Fig. 4E,F). However, when Kv1.4 is co-expressed with PSD-95 and Tac-SDV, clusters of all three proteins are formed (Fig. 4G,H). This co-clustering requires the PDZ binding site on Tac, because the native Tac construct remains diffuse in cells containing PSD-95/Kv1.4 clusters (Fig. 4I,J). These results suggest that the tetrameric nature of the Kv1.4 ion channel is necessary for efficient formation of clusters.

Ion channel clustering by PSD-95 requires palmitoylation but not multimerization

One model for protein clustering by PSD-95 suggests that clusters represent a macromolecular lattice that relies on the multivalent nature of both PSD-95 and Kv1.4. However, this model is inconsistent with experiments showing that co-transfections of Kv1.4 with the monomeric GAP-43/PSD-95 chimera (Fig. 3D) shows clusters that appear identical to those formed with Kv1.4 and wild-type PSD-95 (Fig. 5A-D). Although difficult to address experimentally, it is possible that binding of this chimera to Kv1.4 induces multimerization of the chimera. To assess further the role of palmitoylation in K^+ channel clustering, we acutely blocked palmitoylation with 2-bromopalmitate. Co-transfected COS cells showing patches of Kv.1.4 and PSD-95 were treated with 20 μM 2-bromopalmitate

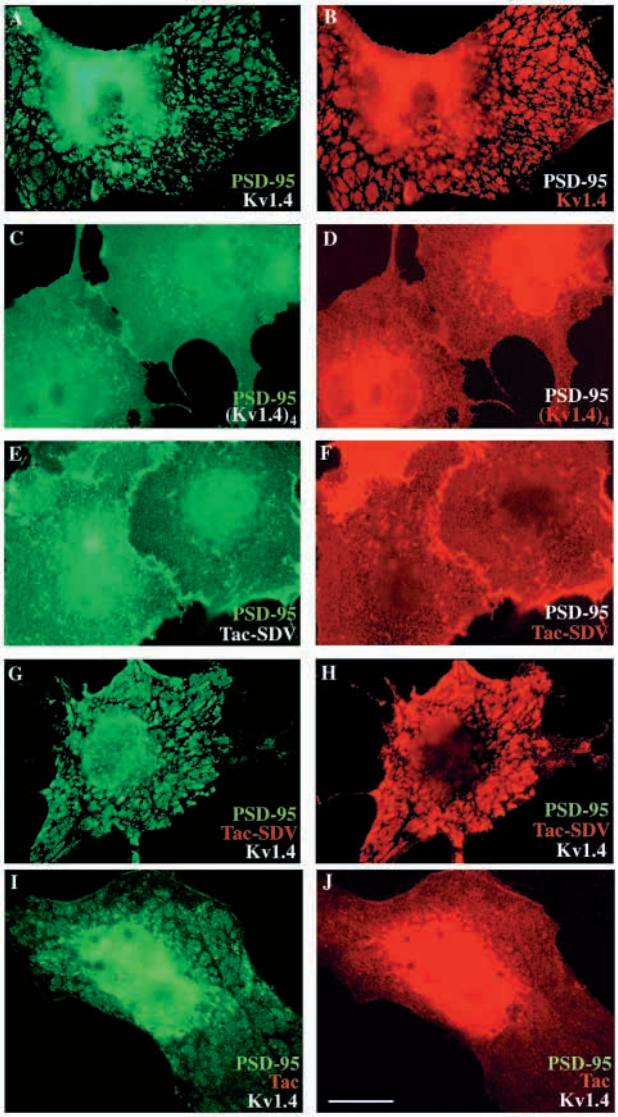

Fig. 4. Ion channel clustering requires interaction of PSD-95 with a tetrameric ion channel. (A,B) PSD-95 forms clusters when co-transfected with K^+ channel Kv1.4. (C,D) A tandem construct containing four covalently linked Kv1.4 subunits does not form clusters with PSD-95. (E,F) A monomeric transmembrane protein containing a C-terminal PDZ binding site (Tac-SDV) does not cluster when simply co-transfected with PSD-95. (G,H) However, this Tac-SDV construct is recruited to clusters when co-transfected with PSD-95 and Kv1.4. (I,J) This co-clustering requires the PDZ binding site on Tac, as the native Tac construct remains diffuse in cells containing PSD-95/Kv1.4 clusters. Bar, 10 μm.

and, within 4-8 hours, the patches disappeared and were replaced by round perinuclear blobs that appear to be intracellular protein aggregates (Fig. 5E,F). These perinuclear blobs resemble those seen when palmitoylation-deficient mutants of PSD-95 are co-transfected with Kv1.4 (Fig. 5G,H). Cells treated with palmitate as a control showed normal patches of PSD-95 and Kv.1.4 (Fig. 5I,J).

To help verify that the effects of 2-bromopalmitate on K^+ channel clustering are specific for palmitoylation of PSD-95, we used a non-palmitoylated construct of PSD-95 containing a C-terminal CAAX domain from paralemmin, which is isoprenylated (El-Husseini et al., 2000a). This PSD-95 construct forms clusters with Kv1.4 that are somewhat smaller but generally resemble those formed with wild-type PSD-95 (Fig. 5K,L). As expected, 2-bromopalmitate does not affect clustering mediated by isoprenylated PSD-95 (Fig. 5M,N). Finally, we asked whether the prenylated PSD-95-CAAX can multimerize and found that PSD-95-prenyl multimerizes with neither itself nor palmitoylated PSD-95 (Fig. 6).

Discussion

A primary finding of this study is that the first 13 amino acids of PSD-95 are necessary and sufficient to mediate homomultimerization. The protein interaction mediated by this domain requires a novel non-covalent lipid-mediated binding. This capacity for multimerization is a unique property of the N-terminus of PSD-95, because it is not seen with other palmitoylation domains. Surprisingly, multimerization of PSD-95 is not needed for receptor clustering, which instead requires palmitoylation of PSD-95 and its interaction with a tetrameric ion channel.

A remarkable aspect of this study is that only 13 amino acids of PSD-95 are needed for oligomerization. Although the mechanism for this multimerization remains uncertain, it is lipid dependent and requires protein palmitoylation. The interaction is not disrupted by solubilization with Triton X-100 but is disrupted by SDS. We sought to determine the stoichiometry of the PSD-95 oligomers by performing gel filtration chromatography studies on PSD-95 extracted from both transfected cells and brain homogenates. In both cases, we found that PSD-95 migrates in the excluded volume, suggesting a molecular mass of greater than 1000 kDa (data not shown). This behavior is consistent with PSD-95 being present in a very high molecular weight complex or perhaps in lipid rafts.

Many palmitoylated proteins partition in specialized non-ionic detergent resistant membrane (DRM) domains (Lisanti et al., 1995; Moffett et al., 2000; Shaul et al., 1996) and a small proportion of PSD-95 occurs in these raft-like domains (Perez and Bredt, 1998). However, this partitioning cannot by itself explain the multimerization of PSD-95, because the palmitoylated protein GAP-43 efficiently sorts to DRMs (Arni et al., 1998), but we find that GAP-43 does not form multimers. Although both PSD-95 and GAP-43 are doubly palmitoylated, the palmitoylation motif of GAP-43 differs from that of PSD-95 in that it contains two adjacent cysteines as well as nearby basic residues. It is possible that the structural differences between these palmitoylation motifs favors, in the case of PSD-95, interactions between palmitoyl groups.

Whatever the mechanism for multimerization, the 13 amino acid motif of PSD-95 is one of the smallest domains found in any protein capable of mediating specific homomultimerization. Appending this short motif to heterologous proteins provides a novel mechanism for artificially forming protein multimers at the cell surface. This facile tool for constructing multimeric protein networks is likely to have general utility for both cell biological and pharmaceutical research.

Another surprise from this work is that multimerization of PSD-95 is not essential for ion channel clustering. This finding

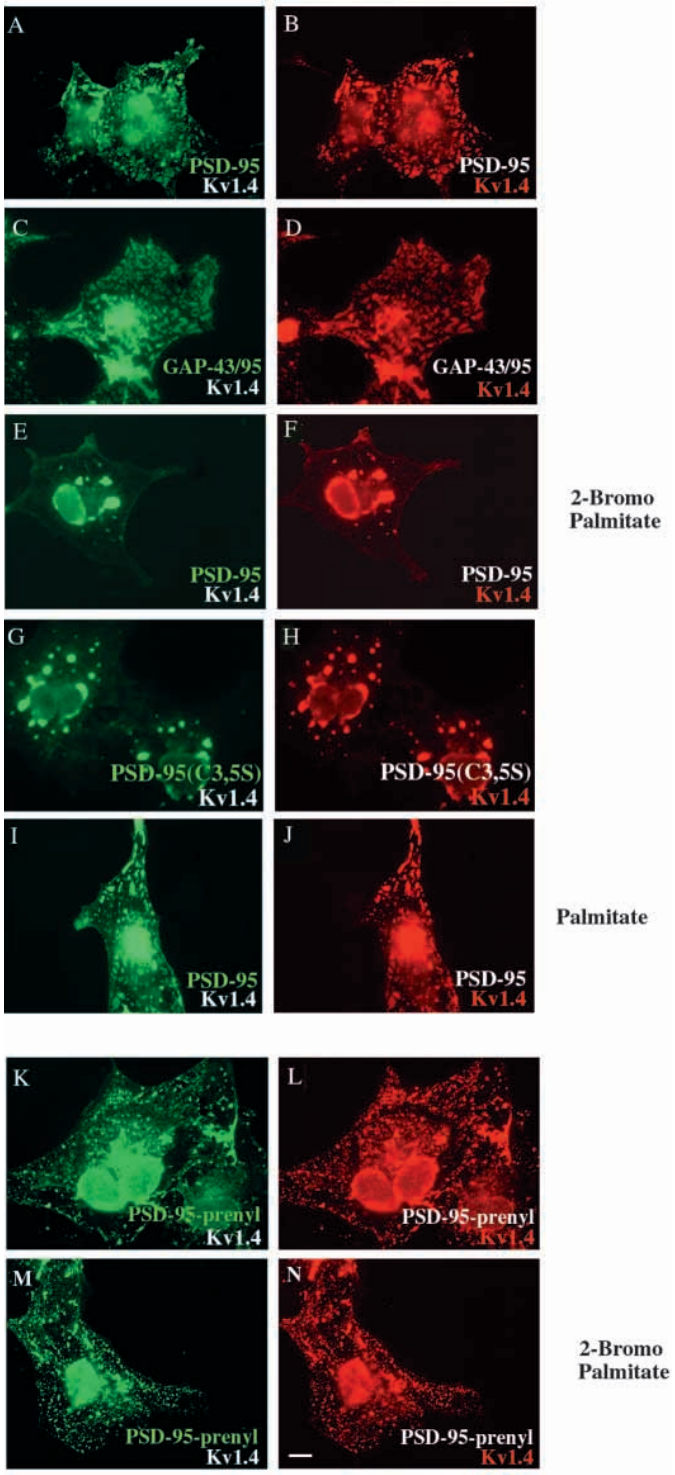

Fig. 5. PSD-95/ion-channel clusters do not require PSD-95 multimerization but are reversibly dispersed by blocking palmitoylation of PSD-95. (A-D) PSD-95 clusters normally with a monomeric chimera containing the palmitoylated N-terminus of GAP-43 fused to PSD-95. (E,F) Incubating PSD-95/Kv1.4 co-transfected COS cells for 4 hours with 2-bromopalmitate disrupts PSD-95 ion channel clusters and causes both proteins to accumulate in perinuclear aggregates that resemble those formed (G,H) in cells co-transfected with Kv1.4 and the palmitoylation-deficient mutant of PSD-95 (C3,5S). (I,J) Treatment with palmitate as a control does not disrupt PSD-95/ion-channel clusters. (K,L) Somewhat smaller clusters are observed when Kv1.4 is co-transfected with a palmitoylation-deficient PSD-95 mutant that contains the paralemmin (PSD-95-prenyl) C-terminal consensus sequence for isoprenylation. (M,N) A 4-hour treatment with 20 μM 2-bromopalmitate does not disrupt ion channel clustering by PSD-95-prenyl. Bar, 10 μm.

questions the model that a multivalent lattice of both PSD-95 and an interacting ion channel mediates ion channel clustering by PSD-95. However, we do find that N-terminal palmitoylation of PSD-95 is essential for channel clustering. Because palmitoylation also targets PSD-95 to cellular endomembranes (El-Husseini et al., 2000a), it has been proposed that PSD-95 actively recruits ion channels from endosomes to the plasma membrane. Indeed, this model has recently been supported by similar studies showing that rapsyn, a myristoylated peripheral membrane protein, actively recruits nAChR from endosomes to mediate synaptic clustering (Marchand et al., 2000).

If multimerization of PSD-95 is not needed for channel clustering, what might be the function for this unique property of the N-terminus? One possibility is that multimerization of PSD-95 might be crucial for its roles in early synaptic development. Studies in cultured cells indicate that PSD-95 clusters at earlier developmental stages than do other postsynaptic proteins (Rao et al., 1998). Oligomerization of PSD-95 through a lipid-dependent mechanism could provide an autonomous mechanism for formation of these antesynaptic complexes. An alternative possibility is that multimerization of PSD-95 is important for its assembly of synaptic protein networks. Previous studies have shown that coupling of NMDA receptors to downstream effectors such as neuronal nitric oxide synthase (nNOS) and to physiological responses such as synaptic plasticity and learning requires PSD-95

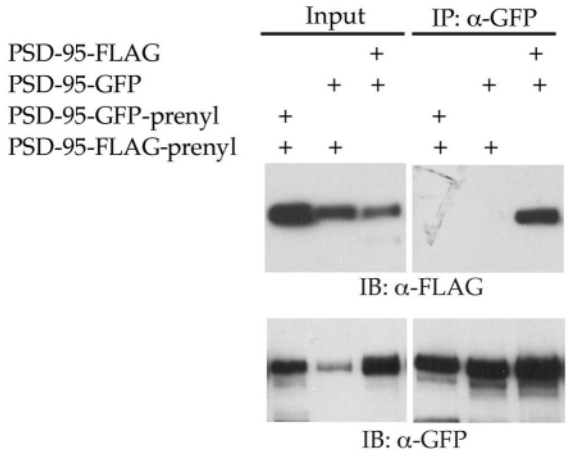

Fig. 6. Prenylated PSD-95 does not multimerize with itself or with palmitoylated PSD-95. COS cells were transfected with plasmids encoding GFP- and FLAG-tagged PSD-95 constructs, some of which contain the paralemmin (PSD-95-prenyl) C-terminal consensus sequence for isoprenylation. Cells were lysed with 1% Triton X-100 and proteins were immunoprecipitated with an antibody to GFP. Western blotting reveals that PSD-95-prenyl multimerizes neither with itself nor with wild-type (palmitoylated) PSD-95.

(Migaud et al., 1998). Through protein multimerization, PDS-95 might help to recruit nNOS and other intracellular proteins to the NMDA receptor complex (Craven and Bredt, 1998; Kennedy, 1998; O'Brien et al., 1998; Sheng and Wyszynski, 1997).

Because protein palmitoylation is reversible (Dunphy and Linder, 1998; Milligan et al., 1995), it provides a mechanism for regulating multimerization and ion channel clustering of PSD-95. Indeed, blocking palmitoylation with 2-bromopalmitate disperses preformed PSD-95/K^+ channel clusters, indicating that maintenance of these clusters requires continued palmitoylation of PSD-95. It will now be important to identify the palmitoyl transferase and palmitoyl thioesterase enzymes that regulate palmitoylation, multimerization and clustering of PSD-95.

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References

Arni, S., Keilbaugh, S. A., Ostermeyer, A. G. and Brown, D. A. (1998). Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. *J. Biol. Chem.* **273**, 28478-28485.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Huang, F., Xia, H., Peters, M. F., Froehner, S. C. et al. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α-1 syntrophin mediated by PDZ motifs. *Cell* **84**, 757-767.

Brenman, J. E., Topinka, J. R., Cooper, E. C., McGee, A. W., Rosen, J., Milroy, T., Ralston, H. J. and Bredt, D. S. (1998). Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. *J. Neurosci.* **18**, 8805-8813.

Cho, K. O., Hunt, C. A. and Kennedy, M. B. (1992). The rat brain postsynaptic density fraction contains a homolog of the *Drosophila* discs-large tumor suppressor protein. *Neuron* **9**, 929-942.

Craven, S. E. and Bredt, D. S. (1998). PDZ proteins organize synaptic signaling pathways. *Cell* **93**, 495-498.

Dunphy, J. T. and Linder, M. E. (1998). Signalling functions of protein palmitoylation. *Biochim. Biophys. Acta* **1436**, 245-261.

El-Husseini, A. E., Craven, S. E., Chetkovich, D. M., Firestein, B. L., Schnell, E., Aoki, C. and Bredt, D. S. (2000a). Dual palmitoylation of PSD-95 mediates its vesiculotubular sorting, postsynaptic targeting, and ion channel clustering. *J. Cell Biol.* **148**, 159-172.

El-Husseini, A. E., Schnell, E., Chetkovich, D. M., Nicoll, R. A. and Bredt, D. S. (2000b). PSD-95 involvement in maturation of excitatory synapses. *Science* **290**, 1364-1368.

El-Husseini, A. E., Schnell, E., Dakoji, S., Sweeney, N., Zhou, Q., Prange, O., Gauthier-Campbell, C., Aguilera-Moreno, A., Nicoll, R. A. and Bredt, D. S. (2002). Synaptic strength regulated by palmitate cycling on PSD-95. *Cell* **108**, 849-863.

Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. and Merlie, J. P. (1995). Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377**, 232-236.

Hall, Z. W. and Sanes, J. R. (1993). Synaptic structure and development: the neuromuscular junction. *Cell* **72 Suppl.**, 99-121.

Hsueh, Y. P., Kim, E. and Sheng, M. (1997). Disulfide-linked head-to-head multimerization in the mechanism of ion channel clustering by PSD-95. *Neuron* **18**, 803-814.

Irie, M., Hata, Y., Takeuchi, M., Ichtchenko, K., Toyoda, A., Hirao, K., Takai, Y., Rosahl, T. W. and Sudhof, T. C. (1997). Binding of neuroligins to PSD-95. *Science* **277**, 1511-1515.

Isacoff, E. Y., Jan, Y. N. and Jan, L. Y. (1990). Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes. *Nature* **345**, 530-534.

Kennedy, M. B. (1998). Signal transduction molecules at the glutamatergic postsynaptic membrane. *Brain Res. Brain Res. Rev.* **26**, 243-257.

Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N. and Sheng, M. (1995). Clustering of Shaker-type K+ channels by direct interaction with the PSD-95/SAP90 family of membrane-associated guanylate kinases. *Nature* **378**, 85-88.

Kim, E., Naisbitt, S., Hsueh, Y. P., Rao, A., Rothschild, A., Craig, A. M. and Sheng, M. (1997). GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. *J. Cell Biol.* **136**, 669-678.

Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D. and Garner, C. C. (1993). SAP90, a rat presynaptic protein related to the product of the *Drosophila* tumor suppressor gene *dlg-A*. *J. Biol. Chem.* **268**, 4580-4583.

Kornau, H.-C., Schenker, L. T., Kennedy, M. B. and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269**, 1737-1740.

Lisanti, M. P., Tang, Z., Scherer, P. E., Kubler, E., Koleske, A. J. and Sargiacomo, M. (1995). Caveolae, transmembrane signalling and cellular transformation. *Mol. Membr. Biol.* **12**, 121-124.

Liu, Y., Fisher, D. A. and Storm, D. R. (1993). Analysis of the palmitoylation and membrane targeting domain of neuromodulin (GAP-43) by site-specific mutagenesis. *Biochemistry* **32**, 10714-10719.

Marchand, S., Bignami, F., Stetzkowski-Marden, F. and Cartaud, J. (2000). The myristoylated protein rapsyn is cotargeted with the nicotinic acetylcholine receptor to the postsynaptic membrane via the exocytic pathway. *J. Neurosci.* **20**, 521-528.

McGee, A. W., Dakoji, S. R., Olsen, O., Bredt, D. S., Lim, W. A. and Prehoda, K. E. (2001). Structure of the SH3-guanylate kinase module from PSD-95 suggests a mechanism for regulated assembly of MAGUK scaffolding proteins. *Mol. Cell* **8**, 1291-1301.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Makhinson, M., He, Y., Ramsay, M. F., Morris, R. G., Morrison, J. H. et al. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* **396**, 433-439.

Milligan, G., Parenti, M. and Magee, A. I. (1995). The dynamic role of palmitoylation in signal transduction. *Trends Biochem. Sci.* **20**, 181-187.

Moffett, S., Brown, D. A. and Linder, M. E. (2000). Lipid-dependent targeting of G proteins into rafts. *J. Biol. Chem.* **275**, 2191-2198.

O'Brien, R. J., Lau, L. F. and Huganir, R. L. (1998). Molecular mechanisms of glutamate receptor clustering at excitatory synapses. *Curr. Opin. Neurobiol.* **8**, 364-369.

Perez, A. S. and Bredt, D. S. (1998). The N-terminal PDZ-containing region of postsynaptic density-95 mediates association with caveolar-like lipid domains. *Neurosci. Lett.* **258**, 121-123.

Rao, A., Kim, E., Sheng, M. and Craig, A. M. (1998). Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *J. Neurosci.* **18**, 1217-1229.

Sala, C., Piech, V., Wilson, N. R., Passafaro, M., Liu, G. and Sheng, M. (2001). Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* **31**, 115-130.

Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. and Michel, T. (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271**, 6518-6522.

Sheng, M. and Wyszynski, M. (1997). Ion channel targeting in neurons. *BioEssays* **19**, 847-853.

Takeuchi, M., Hata, Y., Hirao, K., Toyoda, A., Irie, M. and Takai, Y. (1997). SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. *J. Biol. Chem.* **272**, 11943-11951.

Tavares, G. A., Panepucci, E. H. and Brunger, A. T. (2001). Structural characterization of the intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. *Mol. Cell* **8**, 1313-1325.

Tejedor, F. J., Bokhari, A., Rogero, O., Gorczyca, M., Zhang, J., Kim, E., Sheng, M. and Budnik, V. (1997). Essential role for dlg in synaptic clustering of Shaker K^+ channels in vivo. *J. Neurosci.* **17**, 152-159.

Webb, Y., Hermida-Matsumoto, L. and Resh, M. D. (2000). Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J. Biol. Chem.* **275**, 261-270.