

Revised 3/10/00

Synaptic targeting of the postsynaptic density protein PSD-95 mediated by a tyrosine-based trafficking signal

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Running title: Postsynaptic targeting of PSD-95

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Abstract

Synaptic function requires proper localization of proteins at synaptic sites. Targeting of the postsynaptic protein PSD-95 relies on multiple signals within the protein, including twelve C-terminal amino acids. We now show that this C-terminal targeting domain of PSD-95 mediates postsynaptic localization through a short tyrosine-based motif followed by a pair of hydrophobic amino acids. Consistent with a role in cellular trafficking, the tyrosine motif resembles the canonical motif for interactions with clathrin adaptor proteins. In fact, we find that the C-terminal targeting domain of PSD-95 is sufficient to mediate clathrin-dependent endocytosis when appended to a transmembrane protein. Furthermore, systematic mutagenesis reveals that endocytosis mediated by this domain depends on both the tyrosine motif and the di-hydrophobic amino acid pair. Thus, postsynaptic targeting of PSD-95 requires a tyrosine-based signal that can mediate clathrin-coated vesicle formation.

Introduction

Proper synaptic function requires assembly of protein complexes at specialized pre- and postsynaptic membranes. The postsynaptic density (PSD) is an electron-dense cytoskeletal structure beneath the plasma membrane of excitatory synapses where neurotransmitter receptors and signaling molecules aggregate to respond to synaptic stimuli. Biochemical studies have helped to define the protein composition of the PSD, but the mechanisms for targeting and assembly of these protein complexes are just beginning to be elucidated. Some components of the PSD may be recruited into signaling networks by the PSD-95 family of membrane-associated guanylate kinase (MAGUK) proteins (1,2).

PSD-95 and its homologs contain three PDZ protein-protein interaction motifs that bind to specific synaptic proteins including N-methyl-D-aspartate- (NMDA-) type glutamate receptors, Shaker-type K⁺ channels, and intracellular signaling enzymes (3-7). MAGUK proteins also contain an orphan SH3 domain and a C-terminal guanylate kinase-like domain, which binds proteins of the neuronal cytoskeleton (8-11). Postsynaptic targeting of PSD-95 relies on multiple signals within the protein including N-terminal palmitoylation, the first and second PDZ domains, and a twelve amino acid C-terminal targeting domain (12,13). The mechanisms by which palmitoylation and the PDZ domains contribute to postsynaptic targeting likely involve membrane association and protein-protein interactions, respectively. However, the mechanism by which the C-terminal domain regulates targeting is unknown.

The presence of a C-terminal targeting domain in PSD-95 is reminiscent of cytosolic C-terminal motifs in integral membrane proteins that mediate a diverse set of intracellular sorting events, including endocytosis from the plasma membrane and targeting to endosomes, lysosomes, the trans-Golgi network, and the basolateral and dendritic plasma membrane (14,15). A subset of

these C-terminal sorting signals contain a critical tyrosine residue and conform to the motif YXX Φ , where Y is a tyrosine, X is any amino acid, and Φ is a hydrophobic amino acid. These tyrosine motifs interact directly with the medium chain of the clathrin adaptor proteins, AP-1 and AP-2, to assemble clathrin-coated transport vesicles (14,16). Interestingly, the C-terminal targeting domain of PSD-95 contains a similar tyrosine motif.

Here we define the functional signals within the C-terminal targeting domain of PSD-95. We find that postsynaptic targeting of PSD-95 in cultured hippocampal neurons requires the C-terminal YXX Φ tyrosine motif as well as a nearby pair of hydrophobic amino acids. When appended to an integral membrane protein, the tyrosine-based signal of PSD-95 is sufficient to mediate clathrin-dependent endocytosis. Systematic mutagenesis of the C-terminus indicates a strict correlation between postsynaptic targeting and endocytosis, suggesting that a tyrosine-based signal able to mediate clathrin-coated vesicle formation participates in postsynaptic targeting of PSD-95.

Experimental Procedures

cDNA Constructs—Construction of GW1 PSD-95 Δ 13-GFP was described previously (12). Mutations within the 12 amino acid targeting domain (amino acids 700 to 711) were made with oligos, encoding the targeting domain with the appropriate mutation and restriction sites, that were annealed and subcloned into GW1 PSD-95 Δ 13-GFP at the Bgl II site (amino acid 700) and the Kpn I site between PSD-95 Δ 13 and GFP. Mutations in the targeting domain in full length PSD-95 were made with oligos, encoding the C-terminal 25 amino acids of PSD-95 with the appropriate mutation and restriction sites, that were annealed and then subcloned into GW1 PSD-95-GFP at the Bgl II site (amino acid 700) and Kpn I site between PSD-95 and GFP. The Tac (interleukin-2 receptor α -subunit) cDNA, modified by the introduction of a Xba I site at the 5' end (17), was kindly provided by Dr. Robert Edwards (University of California, San Francisco). The 12 amino acids of the C-terminal targeting domain of PSD-95 or TGN 38 were 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. cDNAs for hemagglutinin (HA)-tagged wild type and dominant negative (K44A) dynamin I were kindly provided by Dr. Sandra Schmid (The Scripps Research Institute, La Jolla, CA).

Preparation and Transfection of Hippocampal Cultures and Immunofluorescence—Neuronal cultures were prepared from the hippocampi of E18/E19 rats. Hippocampi were dissociated by enzyme digestion with papain followed by brief mechanical trituration. Cells were plated on poly-D-lysine (Sigma) treated glass coverslips (Fisher: 12 mm in diameter) in 24 well plates (Falcon). Cultures were plated and maintained in Neurobasal media (Gibco) supplemented with B27, penicillin, streptomycin, and L-glutamine as described (18). Hippocampal cultures were

transfected by lipid-mediated gene transfer as described previously (12). Briefly, cells were transfected prior to plating in a balanced salt-solution at approximately 1-1.5 million cells/0.25 mL. Two micrograms of DNA and 10 μ l of DOTAP (Boehringer-Mannheim) were mixed in 25 μ l of HBS (20 mM Hepes, 150 mM NaCl, pH 7.4) and were added to the cells with gentle mixing. The cells were incubated for 1 hr at 37°C and then plated at a density of 600-1000/mm². Transfection efficiency was low and never exceeded 0.01%. On average, 15-20 transfected cells were obtained for each independent transfection.

To visualize cells transfected with GFP-tagged constructs, coverslips were removed from culture wells and GFP was visualized live under fluorescence microscopy. For co-localization of transfected PSD-95-GFP with endogenous NMDA-type glutamate receptors, cells were fixed in -20°C methanol and stained with primary antibodies against GFP (polyclonal; Clontech) and NR1 (monoclonal; Pharmingen). Secondary antibodies conjugated to Cy2 and Cy3 fluorophores were used, and the cells visualized under fluorescence microscopy using a Zeiss Axiovert inverted microscope equipped with a 63x oil objective and a Hamamatsu 12-bit ORCA interline CCD camera.

Quantitative Measurement of GFP Expression—For quantitation, transfected neurons were chosen at random from 2 to 5 coverslips from 2 to 3 independent transfections of each construct (9-13 cells per construct). Depending on transfection efficiency, 20-50 transfected neuron were qualitatively examined for each construct. Images were acquired with an exposure time adjusted to limit photobleaching and such that maximum pixel intensity was at least half-saturating. The synaptic clustering ratio (SCR) was determined by dividing the average pixel intensity in the dendrites by that in the synaptic clusters from individual cells. The perimeter of the dendrites was traced

(excluding clusters) and the average pixel intensity calculated in NIH Image. Similarly, the clusters were traced and the average pixel intensity obtained. The ratio of the average pixel intensity in dendrites versus clusters was defined as the SCR; a ratio of zero indicates complete synaptic clustering with no cytosolic fluorescence in dendrites, whereas a ratio of 1 indicates diffuse dendritic fluorescence with no clusters. Data were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons with Prism software (Graph Pad, San Diego, CA). To compare the expression levels of neurons transfected with different constructs, the total pixel intensity of individual transfected cells was measured by tracing the perimeter of the entire cell and calculating the total pixel intensity in NIH Image.

Endocytosis Assay—COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin, and streptomycin. Cells were transfected with Lipofectamine reagent according to the manufacture's protocol (Gibco). To assess endocytosis, intact cells were incubated at 4°C for 1 hr with a monoclonal antibody against the extracellular domain of Tac (BAbCO) diluted 1:500 in DMEM to allow surface binding of antibody. Cells were then washed with ice-cold PBS and incubated in pre-warmed media at 37°C for 1 hr to allow endocytosis of bound antibody. After fixation in 4% paraformaldehyde and permeabilization in TBS with 0.1% Triton-X-100, cells were incubated with Cy3-conjugated antibodies to mouse Ig. To quantitate endocytosis of chimeric Tac-95 receptor constructs, the percentage of cells that showed receptor endocytosis versus cells where the receptor remained predominantly on the cell surface was calculated. Approximately one hundred cells were counted from each of two independent assays for each construct. The counter was blinded to the identity of the constructs. Data were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons.

Results

Postsynaptic Targeting of PSD-95 is Mediated by a C-terminal Tyrosine-Based Motif—As described previously, PSD-95 protein missing its C-terminal 25 amino acids (700 to 724) is not fully synaptically targeted. Although there is some accumulation of the PSD-95 Δ 25-GFP protein at synapses, the majority of the protein is found diffusely in the cell body and dendrites (12; Fig. 1A). However, addition of 12 amino acids (700 to 711) fully restores postsynaptic targeting, such that most of the PSD-95 Δ 13-GFP protein is concentrated at synaptic sites with very little visible in the cell body or dendrites (12; Fig. 1A). These PSD-95-GFP clusters are synaptic as they co-localize with synaptophysin (12) and with the NMDA-type glutamate receptor (Fig. 1A). Thus, a twelve amino acid motif between 700 to 711 is necessary for postsynaptic targeting of PSD-95.

Inspection of these twelve amino acids reveals a consensus sequence for tyrosine-based trafficking that conforms to the motif YXX Φ , where Y is a tyrosine, X is any amino acid, and Φ is a hydrophobic amino acid (Fig. 1A). To determine if this tyrosine motif is involved in localizing PSD-95 to synapses, we mutated critical amino acids and assessed the effects on protein targeting in cultured hippocampal neurons. First we mutated the tyrosine residue to either alanine within PSD-95 Δ 13-GFP (Δ 13 Y701A) or to serine within full length PSD-95-GFP (FL Y701S). Mutations of this tyrosine significantly perturb PSD-95 targeting, as the mutant proteins do not localize properly to postsynaptic sites. Rather both Δ 13 Y701A and FL Y701S occur diffusely within the cell body and dendrites and only modestly accumulate at synapses (Fig. 2A), a distribution similar to PSD-95 Δ 25-GFP protein lacking the entire C-terminal targeting domain (Fig. 1A). This mostly diffuse distribution is not explained by differences in expression level, as

the range of total pixel counts is similar for many constructs analyzed regardless of expression pattern (Table 1). To quantitate the extent of synaptic targeting, we calculated a synaptic clustering ratio (SCR) for these and the other mutations described below (Table 1). The SCR compares the average protein expression in dendrites with that at synaptic clusters (see Experimental Procedures). The SCR calculated for synaptically targeted PSD-95 Δ 13-GFP (Δ 13 WT) protein is close to zero, 0.11 ± 0.019 , indicating that the average fluorescence in the cell processes is near background and that the majority of the protein is synaptically localized. In contrast, SCRs of 0.62 ± 0.039 calculated for Δ 13 Y701A and 0.60 ± 0.028 for FL Y701S, indicate that much of these proteins are diffusely distributed (Table 1). The SCRs for Δ 13 Y701A and FL Y701S are statistically different from Δ 13 WT and also from diffusely expressed mutants of PSD-95 or GFP alone that have SCRs closer to 1 (12). We therefore define the expression pattern of Δ 13 Y701A and FL Y701S as semi-clustered to distinguish them from wild type synaptic clustering and from diffuse protein expression.

To implicate further the YXX Φ tyrosine motif in targeting of PSD-95, mutations were made in the hydrophobic amino acid of this motif, valine 704. Mutations of this valine to a charged residue, lysine or glutamate (Δ 13 V704K and Δ 13 V704E), or to alanine (Δ 13 V704A) in PSD-95 Δ 13-GFP, or to serine (FL V704S) in full length PSD-95-GFP disrupt PSD-95 synaptic targeting (Fig. 2B; data not shown). Again, although these mutant proteins accumulate somewhat at synapses, much of the protein occurs diffusely in the cell body and dendrites. And again, the difference in targeting is not explained by differences in expression levels (Table 1). These valine mutations yield SCRs statistically different from Δ 13 WT but not from Δ 13 Y701A or FL Y701S and are therefore semi-clustered (Table 1). In contrast, conserved mutations of the valine to leucine

or to isoleucine ($\Delta 13$ V704L and $\Delta 13$ V704I) do not disrupt targeting of PSD-95 (Fig. 2B; data not shown) and yield SCRs that are not statistically different from $\Delta 13$ WT (Table 1).

Tyrosine-based Targeting Motifs are Present in PSD-95 Homologs—PSD-93 is a homolog of PSD-95 that also localizes to the postsynaptic membrane in hippocampal neurons (12). To determine if proper postsynaptic localization of PSD-93 also relies on a C-terminal targeting motif, we deleted the C-terminal 25 amino acids. Whereas wild type PSD-93-GFP is efficiently targeted to the postsynaptic membrane, PSD-93 $\Delta 25$ -GFP is more diffusely expressed, similar to PSD-95 $\Delta 25$ -GFP (Fig 1B; Table 1).

The tyrosine-based motif is conserved in the C-termini of PSD-93 and other PSD-95 homologs (Fig. 3). However, the hydrophobic amino acid in PSD-93 is a cysteine, as compared to the more common leucine, isoleucine, and valine residue (19). To determine if a cysteine can function in the context of this motif, the valine 704 of PSD-95 was replaced with a cysteine ($\Delta 13$ V704C). Consistent with a conserved function, $\Delta 13$ V704C protein is predominantly synaptically targeted similar to PSD-95 $\Delta 13$ -GFP (Fig. 2B; Table 1). A second discrepancy is the substitution of the tyrosine with a phenylalanine residue in DLG2. This conservative change occurs in the endocytic signal of some transmembrane proteins (20), and a mutation of the tyrosine in PSD-95 to a phenylalanine ($\Delta 13$ Y701F) does not disrupt synaptic targeting of PSD-95 (Fig. 2; Table 1). These results suggest a conserved targeting function for the tyrosine-based signal in homologs of PSD-95.

A Pair of Hydrophobic Amino Acids within the C-terminus of PSD-95 is also Necessary for Postsynaptic Targeting—Additional mutagenesis was performed to further define the functional targeting motif. Mutations in the amino acids between the tyrosine and valine of the YXX Φ motif ($\Delta 13$ H702A and $\Delta 13$ K703A) do not influence postsynaptic targeting (Fig. 4; Table 1; data not

shown). Similarly, mutations of the two amino acids following valine 704 or the last three amino acids of the domain have minimal ($\Delta 13$ K705A) or no effect on targeting ($\Delta 13$ R706A; $\Delta 13$ E709A; Fig. 3; Table 1; data not shown). In contrast, mutating a pair of hydrophobic amino acids C-terminal to the tyrosine motif either separately ($\Delta 13$ V707S and $\Delta 13$ I708S) within PSD-95 $\Delta 13$ -GFP or together (FL V707S, I708S) in full length PSD-95-GFP disrupts postsynaptic targeting (Fig. 4; data not shown). Again these mutants are diffusely expressed with only minimal accumulation at synapses (Table 1). Interestingly, these critical hydrophobic amino acids are conserved among PSD-95 homologs (Fig. 3), suggestive of a functional conservation.

The C-terminal Targeting Domain of PSD-95 Confers Endocytosis on the Transmembrane Protein Tac—To determine if the tyrosine-based targeting signal of PSD-95 can recruit proteins into clathrin-coated vesicles, we assessed the ability of this motif to mediate endocytosis. The C-terminal domain of PSD-95 was appended to the short cytoplasmic tail of the interleukin-2 receptor α -subunit (IL-2 or Tac), a well-characterized plasma membrane protein. Tac itself does not contain signals for active endocytosis, and the protein remains on the plasma membrane of heterologous cells (Fig. 5A). In contrast, addition of the C-terminal targeting domain of PSD-95 to Tac (Tac-95) is sufficient for endocytosis, and the chimeric protein is almost entirely displaced from the plasma membrane to endocytotic vesicles (Fig. 5A). Quantification reveals that $56.3 \pm 2.0\%$ of transfected cells endocytose the Tac-95 chimera similar to the $62 \pm 3.3\%$ and $54 \pm 2.5\%$ of cells that endocytose the Tac receptor fused to known endocytotic signals: the tyrosine-based motif of TGN38 and the di-leucine motif of the neuron-specific vesicular monoamine transporter (VMAT2), respectively (16,17). In contrast, 0% of cells endocytose Tac receptor alone. Consistent with clathrin-coated vesicles mediating the Tac-95 fusion protein endocytosis, co-expression of Tac-95 with dominant-negative (K44A) dynamin, a GTPase required for endocytic clathrin-coated vesicle formation,

disrupts endocytosis (Fig. 6). In contrast, co-expression of wild type dynamin has no effect (Fig. 6).

We next investigated the specificity of endocytosis conferred on the Tac receptor by the C-terminal targeting domain of PSD-95. Inspection of the extreme C-terminus of PSD-95 (amino acids 712-724) reveals a second YXXV consensus sequence. Previously, we reported that amino acids 712-724 neither participate in postsynaptic targeting nor substitute for the C-terminal targeting domain between amino acids 700-711 (12). Consistent with this, addition of this second tyrosine motif to the Tac receptor does not confer endocytosis on the receptor (data not shown), as only a statistically insignificant percentage of cells, $3.6 \pm 1.2\%$, show any endocytosis. Therefore, the capacity of the C-terminal trafficking signal of PSD-95 to mediate endocytosis is a distinct property of this domain and not simply of any tyrosine signal within PSD-95.

Endocytosis Mediated by the C-terminal Targeting Domain of PSD-95 is Dependent on both the Tyrosine Motif and the Pair of Hydrophobic Amino Acid—Mutagenesis was used to determine the signals within the C-terminal targeting domain of PSD-95 required for endocytosis. Mutation of the tyrosine to alanine or non-conserved mutations of the valine disrupt endocytosis of the Tac-95 chimeric protein (Fig. 5A; data not shown). A tyrosine to alanine mutation (Tac-95 Y701A) dramatically reduces the percentage of cells showing endocytosis to $1.2 \pm 0.1\%$. In contrast, a mutation to phenylalanine does not significantly reduce endocytosis ($51 \pm 3.4\%$; data not shown), consistent with the observed tolerance for a phenylalanine residue in postsynaptic targeting (Fig. 2A). In addition to the tyrosine motif, the pair of hydrophobic amino acids involved in postsynaptic targeting is also required for endocytosis. Mutation of these hydrophobic amino acids (V707S, I708S) disrupts endocytosis of the Tac-95 chimeric receptor ($12.8 \pm 1.1\%$; Fig. 5B). In contrast, mutations of the remaining amino acids not involved in postsynaptic targeting (Tac-95

H702A, K703A; Tac-95 K705A, R706A; and Tac-95 E709A, D710A) have no effect on endocytosis ($54.7 \pm 1.8\%$, $47.9 \pm 1.3\%$, and $54.9 \pm 1.2\%$ of cells, respectively, endocytose the Tac-95 receptor; Fig. 5B; data not shown). Thus there is a strict correlation between the capacity of the PSD-95 C-terminal motif to mediate postsynaptic targeting and endocytosis via clathrin-coated vesicles.

Discussion

Proper postsynaptic targeting of PSD-95 requires a C-terminal targeting domain between amino acids 700 to 711 (12; Fig.1A). The important targeting signal within this domain is a tyrosine-based motif, YXX Φ , followed by a pair of hydrophobic amino acids. This motif is functionally conserved in other PSD-95 family members, and is sufficient to confer clathrin-mediated endocytosis on a transmembrane receptor. Mutagenesis of the C-terminus indicates a tight correlation between postsynaptic targeting and endocytosis. These data suggest that cellular trafficking of PSD-95, and likely PSD-95 homologs, involves a tyrosine-based signal that can mediate clathrin-dependent vesicular transport.

Protein sorting signals often consist of either a tyrosine-based motif or a di-leucine motif (14). Though some proteins contain both motifs, either motif can be sufficient to mediate protein internalization from the plasma membrane (21). Thus it is unusual that endocytosis mediated by the C-terminal trafficking signal of PSD-95 requires both a tyrosine motif and a pair of hydrophobic amino acids that superficially resemble a di-leucine motif. Perhaps both these motifs in PSD-95 have weak affinities for the clathrin adaptor proteins so that a combination of both is necessary for internalization to occur. Alternatively, since the presence of both a tyrosine and a di-leucine motif can result in protein sorting that differs from sorting that occurs with either motif alone (21), the combination within PSD-95 may be key for protein trafficking that contributes to postsynaptic targeting.

Attempts to establish a direct link between postsynaptic targeting and clathrin-mediated trafficking were unsuccessful. Overexpression of dominant negative (K44A) dynamin has no effect on postsynaptic targeting of PSD-95 (unpublished results). However, dynamin may participate in clathrin-dependent transport solely from the plasma membrane (22), leaving open the

possibility that the tyrosine-based signal of PSD-95 is involved in clathrin-mediated intracellular trafficking. To evaluate this possibility, we overexpressed the clathrin hub domain to act as a dominant negative inhibitor of all clathrin coat formation (23), yet we found no effect on targeting of PSD-95 (unpublished results). One caveat is that cells overexpressing dominant negative dynamin or the clathrin hub may compensate for the loss of clathrin-dependent endocytosis by inducing an alternative endocytic pathway (24). To circumvent this problem, we co-transfected mature neurons with the dominant negative proteins and PSD-95. However, PSD-95 overexpressed this way in mature neurons is not properly targeted to the synapse, precluding analysis of the dominant negative effects. This latter result may explain why a second group studying the postsynaptic targeting PSD-95 did not identify the C-terminal targeting motif (13). In their study, PSD-95 localization in cultured cortical slices was assessed 40 hrs. post-infection, a time at which PSD-95 expression in our system is not fully synaptic.

We were also unable to detect direct interactions between the C-terminus of PSD-95 and clathrin adaptor proteins that must occur to mediate endocytosis. Yeast two-hybrid did not yield positive results, even when the motif was repeated three times in tandem (16; unpublished results). However, a bait containing the PSD-95 motif did not express as highly in yeast as did that containing the tyrosine motif of TGN38, which served as positive control. Co-immunoprecipitation experiments from heterologous cells or directly from brain also failed to detect a direct interaction (unpublished results). However, because interactions with clathrin adaptors are transient, they may not be easily revealed by these methods.

The strong correlation between the ability of the C-terminal targeting domain of PSD-95 to mediate both postsynaptic targeting and receptor endocytosis suggests that clathrin-dependent trafficking is an important step in localizing PSD-95 to synapses. However, the relevance of

clathrin-mediated transport for peripheral membrane proteins such as PSD-95 is not well established. One example is the cytosolic Nef protein of the simian immunodeficiency virus (SIV), which interacts with clathrin AP-2 adaptors to internalize the transmembrane protein CD4 (25). It is thought that viral Nef may mimic endogenous proteins involved in linking cell surface proteins to components of the endocytic machinery. We failed, however, to uncover evidence that PSD-95 facilitated the endocytosis of interacting membrane proteins such as the NMDA receptor or Kv1.4 potassium channel (unpublished results). Rather, a recent report suggests that palmitoylated PSD-95 suppresses the endocytosis of Kv1.4, whereas non-palmitoylated PSD-95 can enhance endocytosis (26), suggesting that internalization mediated by PSD-95 is regulated by lipidation.

Vesicle transport may play one of several possible roles in synaptic targeting of PSD-95. Membrane association of PSD-95 via palmitoylation may occur in the secretory pathway where sorting of many transmembrane proteins occurs (27). Indeed PSD-95 localizes to an intramembranous compartment that partially overlaps with the trans-Golgi and endosomal vesicles in heterologous cells and in developing neurons in culture (28). PSD-95 may be sorted from these intracellular membranes into clathrin-coated vesicles for transport to the postsynaptic membrane. Alternatively, PSD-95 may target diffusely to the plasma membrane, and from there, clathrin-coated endocytic vesicles may mediate transport to the synapse. Because PSD-95 protein lacking the tyrosine-based targeting motif does accumulate at the synapse to some degree, there may be multiple mechanisms for synaptic localization – one dependent on and one independent of clathrin-dependent vesicular transport. Alternatively, this partial clustering may result from passive association of the PSD-95 C-terminal mutants with postsynaptic membranes rather than active targeting. Future research into the cellular trafficking of PSD-95 will help to differentiate between

these models and to determine mechanisms by which additional postsynaptic proteins are targeted and assembled at the PSD.

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Figure Legends

Figure 1. Postsynaptic Targeting of PSD-95 and PSD-93 Require A C-Terminal Targeting Domain. A.) A domain map shows N-terminal palmitoylation and the relative locations of the protein domains within PSD-95. The twelve amino acid targeting domain of PSD-95 (amino acids 700-711) is expanded. When expressed in hippocampal neurons, PSD-95-GFP lacking the final 25 amino acids (PSD-95 Δ 25-GFP; upper left; div 11) is only partially clustered at synapse. Addition of the twelve amino acid C-terminal targeting domain (PSD-95 Δ 13-GFP; upper right; div 11; scale bar = 4 μ m) yields wild type synaptic clustering. PSD-95-GFP clusters (green; bottom left; div 11) co-localize with NMDA receptors (red; bottom middle): merged image (yellow; bottom right). B.) PSD-93 lacking the final 25 amino acids (PSD-93 Δ 25-GFP; left; div 11) is semi-clustered whereas wild type PSD-93 is synaptically targeted (PSD-93-GFP WT; right; div 11).

Figure 2. Mutations of the Tyrosine Motif Disrupt Postsynaptic Targeting of PSD-95. A.) The amino acid sequence of the C-terminal targeting domain is at the top with the tyrosine and the hydrophobic residues of the YXX Φ motif in red. Mutating the tyrosine to alanine (Y701A) disrupts postsynaptic targeting (top middle; div 11) as compared to wild type PSD-95 Δ 13-GFP (WT; top left; div 11; scale bar = 4 μ m). In contrast, a conserved mutation of the tyrosine to phenylalanine (Y701F; top right; div 11) does not disrupt postsynaptic targeting. Mutating the tyrosine to serine within full length PSD-95-GFP (Y701S; div 11; bottom) also disrupts targeting; the same neuron is shown at two different magnifications (bottom left, scale bar = 10 μ m; bottom right, scale bar = 5 μ m). B.) Conserved mutations of the valine to isoleucine (V704I; top left; div

11) or cysteine as found in PSD-93 (V704C; top right; div 11) does not disrupt the targeting of PSD-95. In contrast, non-conserved mutations to alanine (V704A; bottom left; div 11), glutamate (V704E; bottom middle; div 11), or serine within full length PSD-95-GFP (V704S; bottom right; div 12) disrupt postsynaptic (see Table 1).

Figure 3. Conservation of the Tyrosine-Based Signal Amongst PSD-95 Homologs. Alignment of the final 25 amino acids of PSD-95, PSD-93, SAP-97, SAP-102, *Drosophila* DLG, DLG2, and DLG3. The functionally defined synaptic targeting domain of PSD-95 is overlined. The conserved tyrosine and hydrophobic residues of the putative clathrin interacting YXX Φ motif and the critical pair of hydrophobic amino acids are noted in bold.

Figure 4. A Pair of Hydrophobic Amino Acids C-Terminal to the Tyrosine Motif is also Involved in Postsynaptic Targeting of PSD-95. The amino acid sequence of the C-terminal targeting domain is at the top with the hydrophobic pair of amino acids in red. A representative sample of mutations that do not disrupt targeting is shown: left top, K703A; right top, R706A; and bottom, E709A (all div 11; scale bar = 4 μ m). In contrast, mutation of V707S (left middle; div 11) or I708S (right middle; div 11) disrupt the postsynaptic targeting of PSD-95.

Figure 5. The C-Terminal Motif of PSD-95 is Sufficient to Mediate Receptor Endocytosis. A.) The amino acid sequence of the C-terminal targeting domain is at the top with the critical amino acids for postsynaptic targeting in red. The Tac receptor does not undergo endocytosis and the protein remains on the plasma membrane in heterologous cells (Tac; left). In contrast, addition of the PSD-95 targeting domain to the Tac receptor results in substantial endocytosis (Tac-95;

middle) and the chimeric protein is almost entirely displaced from the plasma membrane to intracellular vesicles. Mutation of the critical tyrosine residue to alanine disrupts endocytosis and this protein is retained on the plasma membrane (Tac-95 Y-A; right). B.) Mutations of the pair of hydrophobic amino acids involved in postsynaptic targeting of PSD-95 disrupt endocytosis (Tac-95 IV-SS; middle). In contrast, mutations of other amino acids within the PSD-95 C-terminus not involved in targeting have no effect on endocytosis (Tac-95 HK-AA; left; Tac-95 KR-AA; right).

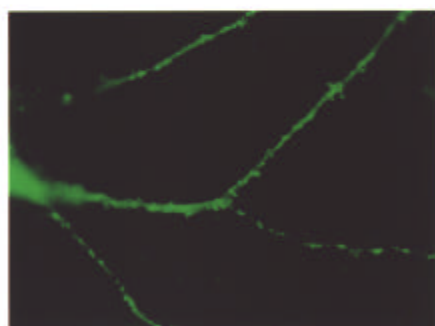
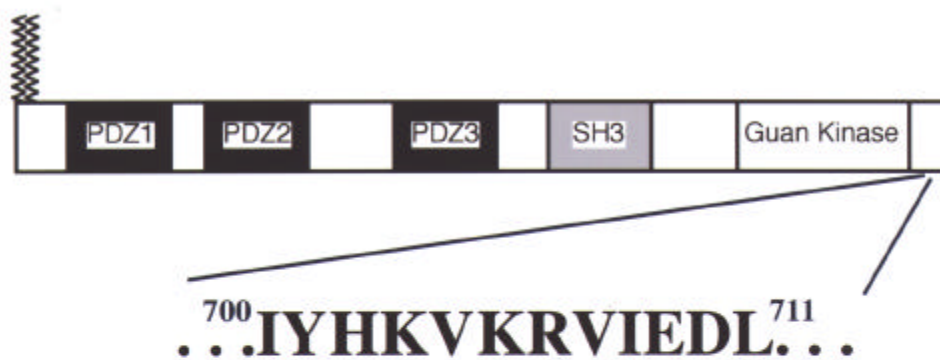
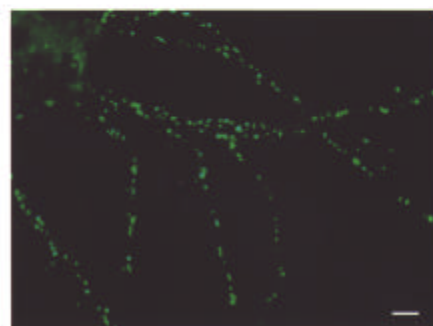
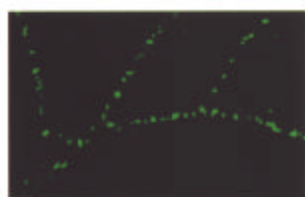
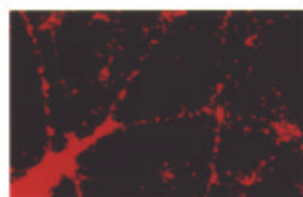
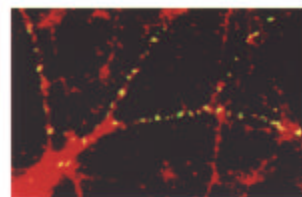
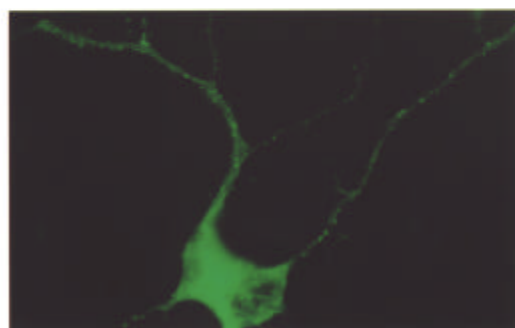
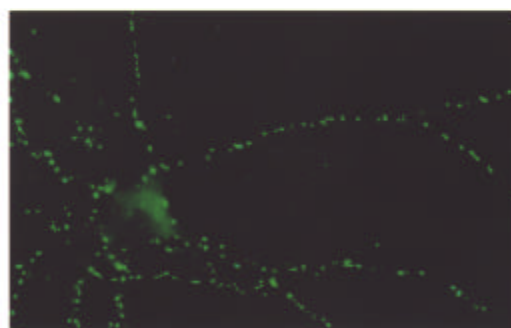
Figure 6. Endocytosis is Dependent on Clathrin-Coated Vesicle Formation. Co-expression of dominant negative (K44A) but not wild type dynamin blocks the ability of the tyrosine-based signal of PSD-95 to mediate receptor endocytosis. The top left panel shows two cells expressing Tac-95, but only the top cell is co-expressing hemagglutinin (HA)-tagged dominant negative dynamin (DN DYN; top right) as revealed by immunocytochemistry for the HA-tag. The Tac-95 protein of the singly transfected cell undergoes endocytosis and localizes to intracellular vesicles. In contrast, the Tac-95 protein in the cell co-expressing DN dynamin remains on the plasma membrane. The bottom panels shows two cells expressing both Tac-95 (Tac-95; bottom left) and wild type dynamin (WT DYN; bottom right). Both cells show the majority of Tac-95 in intracellular vesicles following endocytosis.

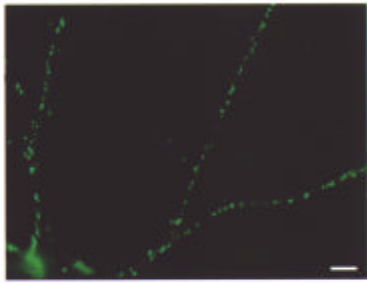
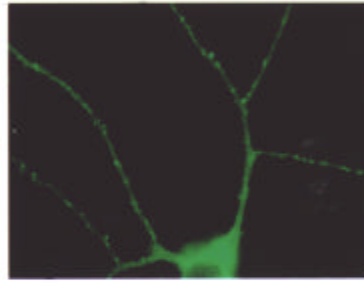
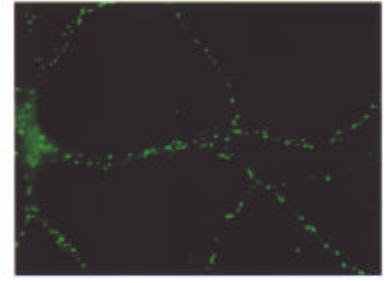
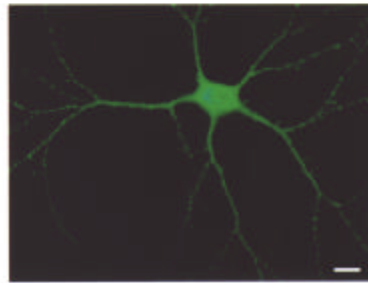
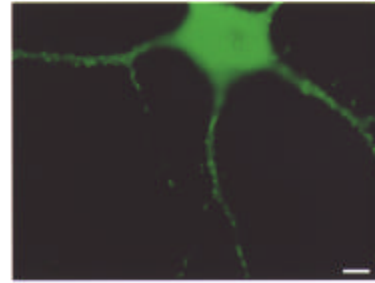
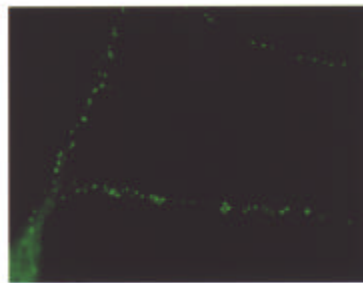
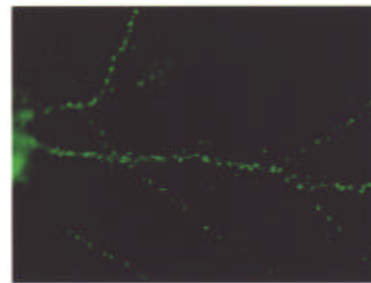
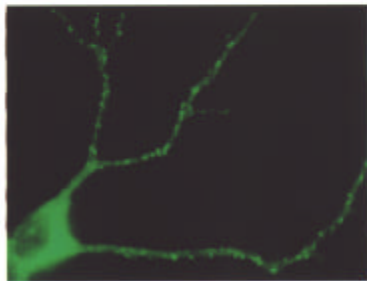
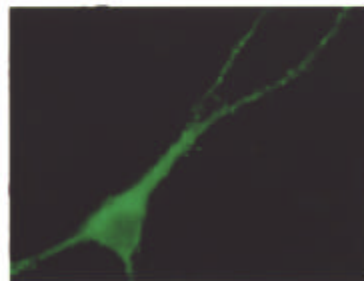
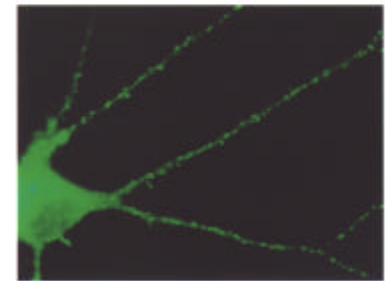
Table 1. Synaptic Clustering Ratio (SCR) of PSD-95 GFP-Fusion Proteins

Construct	n=	SCR\pmSEM	Expression Category	Fluorescence Intensity \pm SEM
Δ 13 WT	9	0.11 \pm 0.019	S	1800 \pm 220
Δ 13 Y701A	13	0.62 \pm 0.036***	SC	1600 \pm 340
FL Y701S	10	0.60 \pm 0.028***	SC	
Δ 13 Y701F	10	0.15 \pm 0.014	S	
Δ 13 V704A	10	0.52 \pm 0.059***	SC	1700 \pm 170
Δ 13 V704E	10	0.57 \pm 0.056***	SC	2000 \pm 290
FL V704S	12	0.58 \pm 0.047***	SC	
Δ 13 V704I or L	11	0.10 \pm 0.017	S	
Δ 13 V704C	8	0.097 \pm 0.024	S	
Δ 13 H702A	10	0.092 \pm 0.013	S	
Δ 13 K703A	10	0.086 \pm 0.035	S	
Δ 13 K705A	10	0.39 \pm 0.047*	S	
Δ 13 R706A	10	0.11 \pm 0.020	S	
Δ 13 V707S	9	0.50 \pm 0.064***	SC	2100 \pm 200
Δ 13 I708S	10	0.53 \pm 0.045***	SC	
FL V707S,I708S	9	0.62 \pm 0.056***	SC	
Δ 13 E709A	10	0.084 \pm 0.0092	S	
PSD-93 WT	9	0.13 \pm 0.020	S	
Δ 25 PSD-93	10	0.67 \pm 0.047***	SC	

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The SCR quantitates the intensity of fluorescent protein in dendrites compared to at synaptic clusters with 0 indicative of complete synaptic clustering and 1 indicative of no clusters (see Experimental Procedures). Values are mean \pm SE for the number of cells (n=) indicated. A one-way ANOVA was performed with Bonferroni correction for multiple comparisons: *** indicates statistical significance from wild-type Δ 13 (p<0.001), * (p<0.05). A letter code indicates the expression pattern: S, synaptic clustering not statistically significant from Δ 13 WT and SC, semi-clustered statistically different from Δ 13 WT. Fluorescence intensity is the total cellular GFP fluorescence in arbitrary units (see Experimental Procedures).

A**PSD-95 $\Delta 25$** **PSD-95 $\Delta 13$** **PSD-95****NR1****MERGE****B****PSD-93 $\Delta 25$** **PSD-93 WT**

A**...⁷⁰⁰IYHKV⁷¹¹KRVIEDL...****WT****Y701A****Y701F****Y701S****B****V704I****V704C****V704A****V701E****V704S**

-25

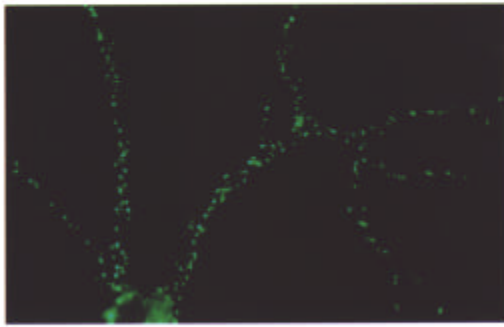
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PSD-95	-I Y H K V K R V I E D L	SGPYIWVPARERL-COOH
PSD-93	-I Y N Q C K L V I E E Q	SGPFIWIPSKEKL-COOH
SAP-97	-I Y N Q V K Q I I E E Q	SGPYIWVPAKEKL-COOH
SAP-102	-I Y N K I K Q I I E D Q	SGHYIWVPSPEKL-COOH
DLG	-I Y S K V K S M I W S Q	SGPTIWVPSKESL-COOH
DLG2	-T F R E L Q T A M E K L	RTEPQWVPVSWVY-COOH
DLG3	-A Y S Q L K V V L E K L	SKDTHWVPVSWVR-COOH

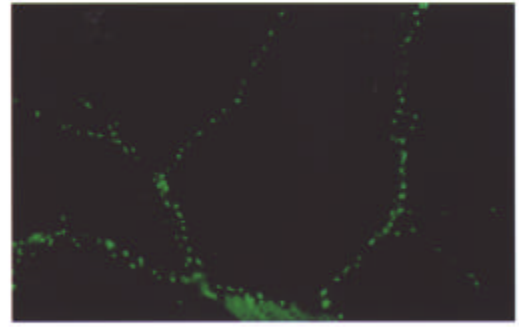
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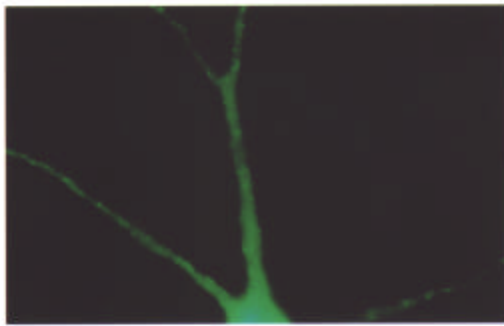
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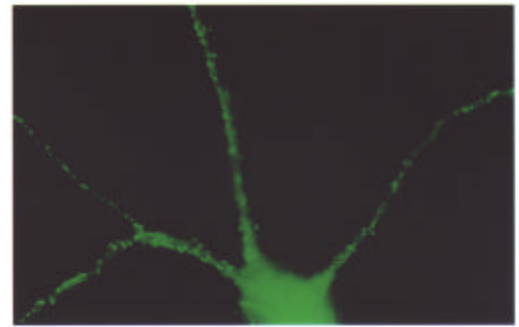
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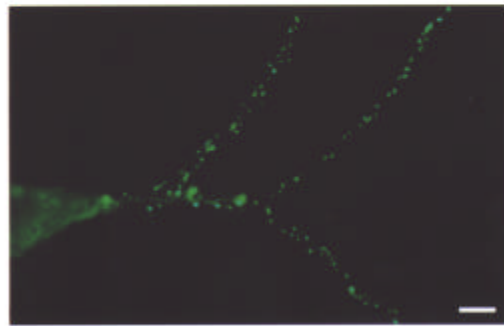
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V707S



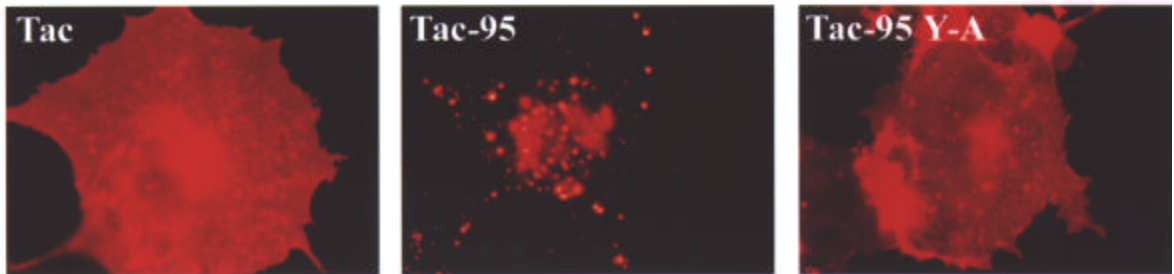
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E709A

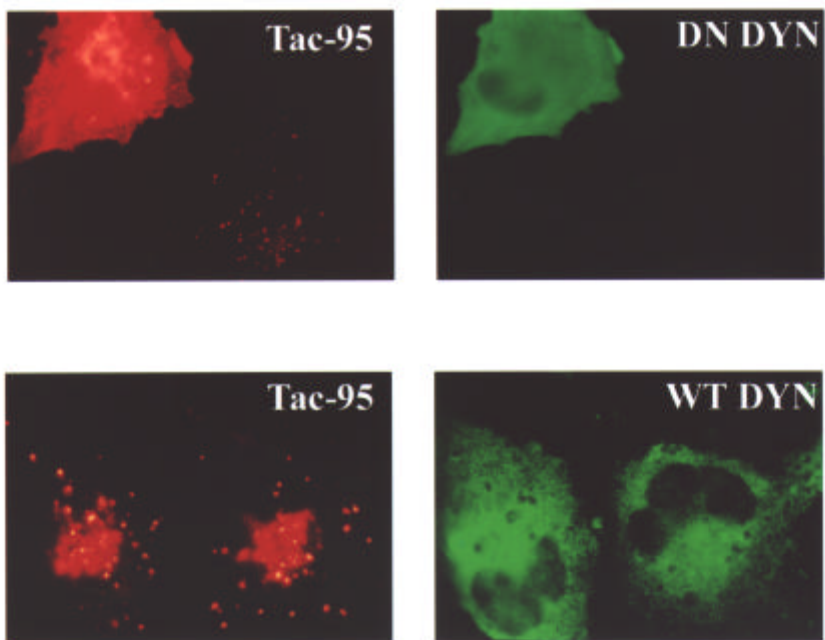
A

...⁷⁰⁰**IYHKVKRVI**⁷¹¹**EDL**...



B





Synaptic targeting of the postsynaptic density protein PSD-95 mediated by a tyrosine-based trafficking signal

Sarah E. Craven and David S. Bredt

J. Biol. Chem. published online March 23, 2000

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