Identification of an Intramolecular Interaction between the SH3 and Guanylate Kinase Domains of PSD-95*  

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POSTSYNAPTIC DENSITY-95 (PSD-95/SAP-90) is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins that assemble protein complexes at synapses and other cell junctions. MAGUKs comprise multiple protein-protein interaction motifs including PDZ, SH3 and guanylate kinase (GK) domains, and these binding sites mediate the scaffolding function of MAGUK proteins. Synaptic binding partners for the PDZ and GK domains of PSD-95 have been identified, but the role of the SH3 domain remains elusive. We now report that the SH3 domain of PSD-95 mediates a specific interaction with the GK domain. The SH3 domain lacks a poly-proline motif that typically binds to SH3 motifs; instead, SH3/GK binding is a bi-domain interaction that requires both intact motifs. Although isolated SH3 and GK domains can bind in trans, experiments with intact PSD-95 molecules indicate that intramolecular SH3/GK binding dominates and prevents intermolecular associations. SH3/GK binding is conserved in the related Drosophila MAGUK protein DLG but is not detectable for Caenorhabditis elegans LIN-2. Many previously identified genetic mutations of MAGUKs in invertebrates occur in the SH3 or GK domains, and all of these mutations disrupt intramolecular SH3/GK binding.

Postsynaptic density-95 (PSD-95/SAP-90) is a major protein constituent of the postsynaptic density (PSD) at excitatory synapses and participates in assembly of the PSD (1–3). PSD-95 is a member of a large family of membrane-associated guanylate kinase (MAGUK) proteins, which play critical roles in regulating both structure and signal transduction at sites of cell-cell contact (4–7). These functional roles for MAGUKs have been established by genetic studies of invertebrates. Mutations of discs large (dlg), which encodes a MAGUK in Drosophila, disrupt epithelial cell septate junctions and epithelial cell polarity and cause overgrowth of the imaginal discs (6). A related MAGUK, LIN-2 in Caenorhabditis elegans, is also essential in regulating cell junctions and differentiation (7). LIN-2 is required for polarized sorting of a receptor tyrosine kinase in C. elegans vulval epithelial cell precursor cells (8). In lin-2 mutant worms, vulval precursor cells do not respond to an inductive signal appropriately, preventing vulval formation (7).

MAGUK proteins regulate cell junctions and cell differentiation by functioning as molecular scaffolds that organize intracellular signaling pathways (9). This scaffolding function is explained by the structure of MAGUK proteins, which comprise multiple protein-protein interaction domains. MAGUKs contain one or three PDZ domains, an SH3 domain, and a region homologous to yeast guanylate kinase (GK) (1, 2, 6, 7). PDZ domains are modular protein-protein interaction motifs (3, 5, 9, 10). Concatenated PDZ domains from PSD-95 can help specify and accelerate signal transduction at the synapse by interacting with multiple components of a signaling pathway (9). Although functions for the PDZ domains of MAGUKs are established, physiological roles for the GK and SH3 domains are less clear (11, 12). The GK domains of MAGUKs are highly homologous to the enzyme guanylate kinase, but catalytic activity has not been reported. Instead, the GK domains of certain MAGUKs have been characterized as protein-protein interaction interfaces (13–15). The GK domain of PSD-95 binds with high affinity to both microtubule-associated protein 1A (MAP1A) (15) and to a novel family of guanylate kinase associated proteins (GKAPs or synapse-associated protein associated proteins (SAPAPs)) (13, 14). Although the functional implications of MAP1A and GKAP interaction with the GK domains are uncertain, genetic screens have identified several mutations in the GK domain of MAGUKs in invertebrates (7, 16), indicating that this region is critical for gene function.

Genetic studies have determined that the SH3 domains of MAGUKs also are essential (12, 16); however, the molecular functions for the SH3 domains remain elusive. SH3 domains classically mediate protein-protein interactions by binding to proline-rich sequences (17–19). Although this function would appear to fit well with the scaffolding role for MAGUKs, binding partners for their SH3 domains are unknown. Recent studies have shown that SH3 domains in certain tyrosine kinases not only mediate protein-protein interactions, but can also regulate protein function through intramolecular interactions (20, 21). Accordingly, we now identify a specific interaction between the SH3 and GK domains within PSD-95. Binding studies with protein fragments of PSD-95 demonstrate that the SH3 and GK domains can bind in trans. However, experiments with intact PSD-95 molecules indicate that intramolecular SH3/GK binding dominates, preventing intermolecular associations. SH3/GK interactions are conserved and occur in both PSD-95 and DLG. All previously identified genetic mutations of...
the SH3 or GK domains in dlg disrupt the intramolecular binding, correlating the critical functional role for these domains with the SH3/GK interaction.

### EXPERIMENTAL PROCEDURES

**Antibodies**—Monoclonal antibodies to PSD-95 (clone 7E3-18; Affinity Bioreagents) and green fluorescent protein (GFP) (CLONTECH) and polyclonal antibodies to GKAP (14), actin-associated LIM protein (ALP) (22), and the GAL4 DNA binding domain (Santa Cruz Biotechnology) have been previously characterized. A polyclonal antiserum to the PDZ domains of PSD-95 was raised by immunizing a sheep with a GST fusion protein of amino acids 1–386 of rat (595). Polyclonal antiserum to the GK domain of PSD-95 was raised by injecting rabbits with a GST fusion of GFP. All antiserum were affinity purified on Affi-Gel-10 columns charged with the immunizing antigen.

**Construction of cDNA Plasmids**—For yeast two-hybrid experiments, domains of PSD-95 were amplified by PCR with primers containing endonuclease restriction sites, and PCR products were restricted with the appropriate enzymes and ligated into pGBK9 or pGAD424 (CLONTECH). All constructs were sequenced to confirm inserted nucleotide sequences were correct. The primers for PSD-95 sequences are as follows: PSD-95 SH3 sense, 5′-CGG-GAA-CAG-CTC-ATG-AAT-3′; PSD-95 SH3 antisense, 5′-GGA-GCC-CCA-GTC-CTT-GGC-3′; PSD-95 GK sense, 5′-AAG-GCC-GAG-TAC-GGC-TGC-TCC-3′; and PSD-95 SH3 antisense, 5′-CTA-GAG-TCT-CTC-GCC-TGG-3′; and PSD-95 GK611 sense, 5′-ACC-AGC-TGA-GTC-CTT-GTG-3′. The constructs containing the W470F mutation were assembled by sequential PCR with a codon change in both the sense and antisense primer from W to TTT: sense, 5′-GAGA-TGG-TTT-GGA-GCA-CGC-GCG-GTG-AAC-3′; and antisense, 5′-GGA-GTC-GAC-CGC-CCG-TGC-GTA-GCA-GAA-GAG-TGG-TGG-GAC-3′.

### Table 1

**Structural requirements for the PSD-95 SH3/GK domain interaction**

| Gal4 DNA binding hybrid | Gal4 activation hybrid | β-Galactosidase activity | Growth |
|-------------------------|-----------------------|-------------------------|--------|
| Interactions between PSD-95 SH3 and guanylate kinase-like domain deletions |                           |                         |        |
| GK-(503–724)            | SH3-(408–509)         | ++                      | +      |
| GKN57-(560–724)         | SH3-(408–509)         | –                       | –      |
| GKN109-(611–724)        | SH3-(408–509)         | –                       | –      |
| GKC25-(503–699)         | SH3-(408–509)         | –                       | –      |

| Interactions between PSD-95 SH3 point mutations and the guanylate kinase-like domain |                           |                         |        |
|-----------------------------------------------|-------------------------|-------------------------|--------|
| GK-(503–724)                                 | SH3 L460P-(408–509)     | ++                      | +      |
| SH3 L460F-(408–560)                         | GK-(503–724)            | +                       | +      |
| SH3 W470F-(408–560)                         | GK-(503–724)            | +                       | +      |
| SH3-(408–560)                                |                         |                         |        |
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GAC-CTT-TTT-AAG-GCT-3' and GKAP antisse, 5'-CTG-TAT-CCC-AAT-AGA-TAG-GCA-3'. The nNOS and PSD-95 PDZ1–3 constructs were previously described (4). The C-terminal GFP fusion constructs were subcloned by ligating a PCR-amplified sequence of PSD-95 SH3 or GK (with the primers described above) into EGFP-C2 (CLONTECH).

**Yeast Two-hybrid Analysis—** Yeast co-transformation, colony lift β-galactosidase filter assays, and ONPG liquid culture assays were done as described in the Matchmaker Library protocol (CLONTECH) with the yeast strain HF7c or SFY526. Growth on Hist – was tested by streaking several individual colonies to -LW plates. Positives were scored by the presence of individual colonies after 3 days at 30 °C.

**Cell Transfection—** HEK 293 cells were transiently transfected with EGF-C2 constructs using LipofectAMINE Plus (Life Technologies). Transfections were done in six-well culture plates with cells at 50–80% confluence. Two μg of plasmid DNA were used for each transfection. Six h after transformation, the media was changed to Dulbecco's modified Eagle’s medium–21 plus 10% heat-inactivated fetal bovine serum, and the cells were harvested 48 h later.

**In Vitro Binding Assays with GST Fusion Proteins—** To test for interactions between SH3 and GK domains in vitro, GST fusion proteins were incubated with HEK cell extracts expressing GFP fusion proteins. GST fusion proteins were expressed and purified as described previously (23). Extracts from transfected HEK 293 cells were prepared from a six-well plate. Cells were washed with 1 ml of phosphate-buffered saline and then scraped from the substrate into resuspension buffer: 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA. Cells were sheared with 10 passes through a 25-gauge needle. Phenylmethylsulfonyl fluoride was added to 1 mM, and Triton X-100 was added to 1%. The solution was gently agitated at 4 °C for 30 min and then centrifuged for 20 min at 14,000 rpm in a microcentrifuge. The soluble fraction of the extracts was removed to new tubes. Three μg of GST protein coupled to Sepharose beads was added, and the samples were incubated at 4 °C for 60 min. The protein-coupled GST beads were pelleted by centrifugation and then washed 5 times with 1 ml of resuspension buffer. The beads were resuspended in 5 μl protein loading buffer. Proteins were separated by sodium-polyacrylamide gel electrophoresis with 12% acrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore) and analyzed by Western blotting as described previously (4).

**Immunoprecipitations—** Adult rat brain was homogenized in 20 volumes of resuspension buffer. After centrifugation at 15,000 × g to remove the soluble fraction, membranes were solubilized for 30 min in resuspension buffer + 0.5% Triton (Triton Extracts) or + 0.2% SDS (SDS Extracts). The Triton X-100 was then added to 1% to the Triton extracts to sequester the ionic detergent. Solubilized proteins were recovered by centrifuging the extracts at 100,000 × g for 30 min. Immunoprecipitations were performed by first adding the appropriate antibody, and then, after a 60-min incubation at 4 °C, adding 20 μl of protein G-Sepharose to precipitate the antibodies. The protein G beads were then washed five times with resuspension buffer and immunoprecipitated proteins were recovered in 5× protein loading buffer. Immunoprecipitates were resolved by 10% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

**RESULTS**

Because SH3 domains can regulate protein function through intramolecular interactions (20, 21), we determined whether the SH3 domain of PSD-95 might associate with other domains from the protein. We first evaluated this possibility using the yeast two-hybrid system. Yeast strains HF7c and SFY526 were transformed with appropriate GAL4-fusion expression vectors encoding the SH3 domain of PSD-95 together with either the PDZ, SH3, or GK domains. Using both an assay for β-galactosidase activity and an assay for growth on His(−) plates, we found that the SH3 domain specifically interacts with the GK domain and that the SH3 domain does not interact with the PDZ or SH3 domains (Fig. 1A). A quantitative liquid culture assay indicated that this association is robust and appears similar in magnitude to the PDZ-95 PDZ domain interaction with the PDZ domain of neuronal nitric-oxide synthase (4) and to the PSD-95 GK domain interaction with GKAP (13, 14). To verify that the SH3/GK interaction is authentic, we also evaluated binding of protein fragments in vitro. We found that the SH3 domain of PSD-95 fused to GFP, expressed in HEK 293 cells, binds specifically to a bacterial GST fusion protein of the GK domain. Similarly, the GK domain of PSD-95, expressed in HEK cells, interacts with a GST-SH3 fusion protein (Fig. 1B).

The SH3/GK interaction identified here is atypical because the GK domain lacks a P-X-X-P motif that typically binds to SH3 domains (17–19). We therefore characterized the structural requirements within the GK domain that are essential for interaction. Using the yeast two-hybrid system, we found that an intact GK domain is required to bind the SH3 domain. The GK domain in PSD-95 comprises amino acids 534–712 (1). Yeast transformed with constructs encoding GK domains that were missing 36 amino acids from the N terminus or 13 amino acids from the C terminus do not turn blue in β-galactosidase assays nor do they form colonies on synthetic media plates lacking histidine (Table I).

We also characterized features of the SH3 domain that mediate GK domain binding. Three-dimensional structures of SH3 domains are well characterized and are conserved (18). Trp-470 of PSD-95 corresponds to an amino acid in other SH3 domains that mediates interaction with proline-containing peptides (23). Mutation of this tryptophan to phenylalanine typically disrupts SH3/proline-containing peptide interactions without compromising the SH3 domain structure (23), but we found it does not abolish SH3/GK binding (Table I). One allele of dlg, a Drosophila MAGUK, contains a point mutation that changes a conserved leucine in the SH3 domain to proline (16). We introduced the corresponding mutation, L460P, into the PSD-95 SH3 domain constructs and found it disrupts the SH3/GK domain interaction (Table I).

We next asked whether the SH3/GK domain interaction found in PSD-95 is unique or is conserved in other MAGUK proteins. We tested for SH3/GK binding in both Drosophila DLG (6), which is highly homologous to PSD-95, and in C. elegans LIN-2 (7), a MAGUK that is more distantly related. We found that the SH3 domain from DLG interacts with its GK domain but that the SH3 and GK domains from LIN-2 do not (Fig. 2). Furthermore, SH3/GK domain binding was detected in SH3 and GK combinations between PSD-95 and DLG (Fig. 2). Our inability to detect SH3/GK interactions with the protein domains from LIN-2 either reflects the lower affinity for this interaction within LIN-2 or suggests that this interaction is not a general feature of more distantly related MAGUK proteins.

Intramolecular SH3/GK domain binding could mediate multimerization of MAGUKs, whereas intramolecular SH3/GK binding could mediate a regulatory interaction. To determine which mode of binding predominates, we evaluated binding of isolated SH3 or GK domains to intact PSD-95 in vitro. We found that GST fusions of either the SH3 or GK domain alone did not bind to a full-length PSD-95-GFP fusion expressed in HEK 293 cells (Fig. 3B). Further, in yeast two-hybrid experiments, no binding was detected between isolated SH3 or GK domains and SH3GK (Fig. 3A). This absence of binding may indicate that intramolecular SH3/GK binding in intact PSD-95 molecules is highly favored and prevents intermolecular associations. This interpretation predicts that introducing mutations that disrupt the intramolecular binding would facilitate intramolecular binding of isolated SH3 or GK domains to intact PSD-95 or SH3GK. Indeed, we found that an isolated GK domain interacts only with an SH3GK construct that contains a C-terminal GK domain truncation (Fig. 3A). We also determined, by yeast two-hybrid analysis and with GST-fusion protein binding experiments, that an isolated SH3 domain can bind full-length PSD-95 or SH3GK constructs that contain a disruptive L460P point mutation in the SH3 domain (Fig. 3B). This latter interaction is specific because an SH3GK construct
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**Fig. 2.** The SH3/GK domain interaction is conserved between PSD-95 and DLG, but is not detectable with LIN-2. Yeast strains SFY526 were transformed with plasmids encoding combinations of SH3 and GK domains from PSD-95, DLG, and LIN-2. Constructs encoding SH3 domains were fusions with the GAL4 activation domain, and those encoding GK domains were fusions with the GAL4 DNA binding domain. Interactions were assayed with the β-galactosidase colony filter-lift assay. β-Galactosidase activity was scored as the time required for colonies of transformed yeast to turn blue at 30 °C: ++, < 60 min; +, 60–240 min; −, no significant activity.

As an additional tool to evaluate the SH3/GK domain interaction in PSD-95 protein extracted from brain, we generated an antibody to the last 14 amino acids of the GK domain (amino acids 698–711). We find that this antibody immunoprecipitates PSD-95 from whole brain homogenates solubilized with 0.2% SDS (SDS extracts) but does not immunoprecipitate PSD-95 from brain homogenates solubilized with 1% Triton X-100 alone (Fig. 4A). In contrast, another antibody directed to the PDZ domains immunoprecipitates PSD-95 equally from both the SDS and Triton extracts (Fig. 4A).

The selective immunoprecipitation of PSD-95 by the GK domain antibody in SDS extracts may indicate that the antibody only interacts with a denatured epitope. Alternatively, the antigenic epitope of the GK domain may not be accessible to the antibody under native conditions because of intermolecular protein interactions, such as with the GKAPs or MAP1A, or of intramolecular interactions, such as with the SH3 domain. To help distinguish between these possibilities, we evaluated immunoprecipitation of PSD-95, SH3GK-GFP, or GK-GFP expressed in HEK 293 cells, which lack GKAPs and MAP1A, and those encoding GK domains were fusions with the GAL4 activation domain, and those encoding GK domains were fusions with the GAL4 DNA binding domain. Interactions were assayed with the β-galactosidase colony filter-lift assay. β-Galactosidase activity was scored as the time required for colonies of transformed yeast to turn blue at 30 °C: ++, < 60 min; +, 60–240 min; −, no significant activity.

With both the L460P SH3 mutation and a truncation of the GK domain does not bind to SH3 (Fig. 3B)

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To determine whether the SH3 domain interaction might regulate GK binding to GKAP or MAP1A, we evaluated the association of wild-type and mutant PSD-95 with GKAP. We co-transfected HEK cells with expression constructs encoding GKAP (13, 14) together with either full-length PSD-95, GFP-SH3GK, or with PSD-95 or GFP-SH3GK constructs containing the SH3 L460P mutation, which disrupts SH3/GK binding. In extracts from these cells, we immunoprecipitated PSD-95 and evaluated association of GKAP (Fig. 5). We found that GKAP co-immunoprecipitates equally with all these PSD-95 con-
structs, indicating that SH3/GK binding does not influence GK binding activity.

**DISCUSSION**

This work identifies a specific intramolecular association between the SH3 and GK domains within PSD-95 and shows that this interaction is conserved in other MAGUK proteins. Although isolated SH3 and GK domains of PSD-95 can bind one another in trans, this intermolecular interaction is not detected between intact PSD-95 molecules or fusion proteins that contain both the SH3 and GK domain. Instead, an intramolecular SH3/GK association appears to predominate within full-length PSD-95 molecules, suggesting a regulatory rather than scaffolding role for the interaction.

Several lines of evidence suggest that the intramolecular interaction between the SH3 and GK domains identified here is likely to play a physiological role in MAGUK function. First, SH3/GK binding is conserved even between the distantly related MAGUK proteins PSD-95 and DLG. Second, genetically identified mutations of the SH3 and GK domains in *dlg* disrupt the intramolecular SH3/GK association. A point mutation in the SH3 domain that disrupts SH3/GK binding was identified by its strong phenotype (16). Conversely, C-terminal GK domain truncations that resemble genetic mutations of *dlg* disrupt the SH3 domain interaction. These truncations of DLG in *Drosophila* are lethal in the absence of maternal contribution (16). It is also intriguing that certain genetic mutants of the SH3 and GK domains of *dlg* complement one another in mixed heterozygous flies, negating the lethality of the recessive mutations (16). Our data suggest that a MAGUK protein with a mutant SH3 domain will bind in trans to a MAGUK with a GK

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**FIG. 4.** Immunoprecipitations from whole brain homogenate and transfected HEK 293 cells. PSD-95 was detected with a monoclonal PSD-95 antibody (Affinity Bioreagents, Inc.). GFP-fusion constructs were detected with a monoclonal antibody (CLONTECH). Positions of marker proteins are given on the left (in kDa). A, rat brain extracts were solubilized with 1% Triton (Triton extracts) or with 0.2% SDS (SDS extracts) and were immunoprecipitated with antibodies against either ALP (negative control), the GK domain of PSD-95, or the PDZ domains of PSD-95. Note that the PDZ antibody immunoprecipitates PSD-95 from both Triton and SDS extracts whereas the GK antibody only immunoprecipitates from SDS extracts. Extract lanes represent 10 and 5% of the volume of Triton and SDS extracts used for immunoprecipitations, respectively. B, Triton and SDS extracts were prepared from HEK 293 cells expressing the GK domain of PSD-95 fused to GFP. Immunoprecipitations and analyses were done as in panel A. The extract lanes contain 5% of the extract used for each condition. C, immunoprecipitations were performed with Triton and SDS extracts prepared from transfected HEK 293 cells expressing PSD-95, PSD-95 containing the SH3 mutation L460P (*PSD-95*), GFP-SH3GK, or GFP-SH3GK with the L460P point mutation. The extract lanes contain 5% of the extract used for each condition.

**FIG. 5.** Co-immunoprecipitation of GKAP is unaffected by the SH3 mutation L460P. HEK 293 were co-transfected with expression constructs for GKAP and either PSD-95, PSD-95 containing the SH3 mutation L460P (*PSD-95*), GFP-SH3GK, or GFP-SH3GK with the L460P point mutation. Cell extracts were solubilized with 1.0% Triton. Immunoprecipitations of these extracts were analyzed by immunoblotting for GKAP. The extract lanes represent 5% of the cell extract used for immunoprecipitations. Positions of molecular mass marker proteins (in kDa) are on the left.
domain mutation. If such a mixed bimolecular complex restores dlg function, this could explain the genetic complementation that has been observed.

The SH3 domain interactions described here are atypical. SH3 domains have classically been shown to interact with proline-containing motifs that contain the consensus sequence P-X-X-P (17). This binding model cannot explain the SH3/GK interactions, as GK domains lack a P-X-X-P motif. Moreover, mutation of the SH3 domain of PSD-95 at the conserved Trp-470 that normally mediates interaction with proline-containing peptides (23) does not disrupt SH3/GK binding. Rather than the SH3 domain recognizing a short peptide sequence within the GK domain, our data suggest that SH3/GK binding represents a bi-domain interaction and requires proper folding of both intact motifs.

The intramolecular interactions described here are reminiscent of recent studies showing that intramolecular SH3 domain associations mediate autoinhibition of Src and Tec family tyrosine kinases (20, 21). In the Src family kinase, Hck, the SH3 motif binds to and blocks the catalytic activity of the adjacent tyrosine kinase domain. This intramolecular SH3 domain interaction within Hck is displaced, and tyrosine kinase activity is restored when the Hck SH3 domain binds to an appropriate protein ligand in trans. By analogy, the intramolecular SH3 domain interaction within PSD-95 may regulate the GK domain. While we find that this interaction does not alter GK domain binding to either GKAP (Fig. 5) or MAP1A (data not shown), the SH3 domain may regulate an as yet unidentified catalytic activity of the GK domain. It is also possible that the intramolecular SH3/GK interaction mediates functional interactions between the SH3 domain and other unknown proteins, as studies with DLG in Drosophila have suggested that the SH3 domain may act as a negative regulator of DLG in controlling cell proliferation (12). Recent studies indicate that PDZ domains within PSD-95 can negatively regulate GK binding activity (15), though the PDZ domains themselves do not bind to GK (15). As the SH3 domain is interposed between the PDZ and GK domains, the SH3/GK interaction described here could play a role in autoinhibition of GK binding by PDZ domains. Alternatively, while our data best support a model in which intramolecular associations between the SH3 and GK domains predominate, it does not eliminate the possibility that other factors present in vivo may facilitate intermolecular interactions that could contribute to the scaffolding functions of PSD-95.

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