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Structure of the SH3-Guanylate Kinase Module from PSD-95 Suggests a Mechanism for Regulated Assembly of MAGUK Scaffolding Proteins

Aaron W. McGee,[1] Srikanth R. Dakoji,[1] Olav Olsen,[1] David S. Bredt,[1,3] Wendell A. Lim,[2,3] and Kenneth E. Prehoda[2,4]

[1] Department of Physiology
[2] Department of Cellular and Molecular Pharmacology
Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143

Summary

Membrane-associated guanylate kinases (MAGUKs), such as PSD-95, are modular scaffolds that organize signaling complexes at synapses and other cell junctions. MAGUKs contain PDZ domains, which recruit signaling proteins, as well as a Src homology 3 (SH3) and a guanylate kinase-like (GK) domain, implicated in scaffold oligomerization. The crystal structure of the SH3-GK module from PSD-95 reveals that these domains form an integrated unit: the SH3 fold comprises noncontiguous sequence elements divided by a hinge region and the GK domain. These elements compose two subdomains that can assemble in either an intra- or intermolecular fashion to complete the SH3 fold. We propose a model for MAGUK oligomerization in which complementary SH3 subdomains associate by 3D domain swapping. This model provides a possible mechanism for ligand regulation of oligomerization.

Introduction

Efficient and specific communication between neighboring cells is often mediated by signaling complexes at cell junctions. Such protein assemblies are observed at excitatory synapses in the brain, where glutamate receptors and associated downstream signaling proteins are concentrated at the postsynaptic density (PSD), an actin-rich cytoskeletal web beneath the postsynaptic membrane (Sheng and Pak, 2000; Ziff, 1997). Similar specialized and highly polarized membrane-associated protein complexes are also observed at diverse sites of cell-cell contact, including neuromuscular and tight junctions. Within these protein complexes, scaffolding proteins may perform two related functions: they recruit and organize receptors and downstream signaling proteins, and they anchor signaling complexes at specific subcellular locations.

Membrane-associated guanylate kinases (MAGUKs) are modular scaffold proteins that are present in many cell-junction complexes (Lahey et al., 1994; Mitic and Anderson, 1998; Rafael et al., 1998). MAGUKs comprise one or three PDZ domains, a Src-Homology 3 (SH3) domain, and a domain homologous to guanylate kinase (GK). The prototypical neuronal MAGUK is PSD-95, which, together with other homologs, such as SAP97, contributes to the organization of the PSD (Craven and Bredt, 1998; Garner et al., 2000; Kennedy, 1998; Kornau et al., 1997; Sheng and Pak, 2000; Sheng and Sala, 2001). In nonneuronal cells, MAGUK proteins such as SAP97, ZO-1, and CASK appear to function as molecular scaffolds to assemble signaling elements at cell junctions (Fanning and Anderson, 1999).

PSD-95 utilizes protein-protein interaction domains to recruit signaling proteins and to mediate assembly with other components of the PSD. PSD-95 binds ion channels (Kim et al., 1995; Kornau et al., 1995) and components of second messenger cascades at synaptic sites through PDZ domain interactions (Brenman et al., 1996; Kim et al., 1998; Harris and Lim, 2001). The SH3 domain is reported to interact with the kainate receptor subunit 2 (Garcia et al., 1998). The PDZ and GK domains associate with cytoskeletal (Passafaro et al., 1999) and cell adhesion proteins (Irie et al., 1997), as well as components of similar multivalent protein complexes (Tu et al., 1999). The GK domain binds neuronal proteins, including AKAP79/150 (Colledge et al., 2000), microtubule-associated protein 1A (MAP1A) (Brenman et al., 1998), guanylate kinase-associated kinesin (GAKIN) (Hanada et al., 2000), and guanylate kinase-associated protein (GKAP) (Kim et al., 1997; Takeuchi et al., 1997), but is catalytically inactive (Kuhlendahl et al., 1998) despite its homology to yeast guanylate kinase.

Oligomerization is another mechanism by which MAGUK-protein assemblies may anchor at cell junctions. PSD-95 and hDLG multimerize through distinct NH_2-terminal sequences (Hsueh et al., 1997; Marfatia et al., 2000), but recent evidence also has focused on the SH3 and GK domains. In addition to interacting with exogenous ligands, fragments encompassing the SH3 and GK domains interact with one another in vitro (Masuko et al., 1999; McGee and Bredt, 1999; Nix et al., 2000; Shin et al., 2000). Studies with the intact tandem SH3-GK module suggest that the intramolecular form of the interaction is preferred over the intermolecular form. Together, these findings have led to the proposal that intermolecular association of SH3-GK modules contributes to the formation of a MAGUK network, but that the intramolecular form of the interaction regulates this assembly (Masuko et al., 1999; Mayer, 2001; Nix et al., 2000). Such a model parallels other signaling proteins in which intramolecular domain interactions play an autoinhibitory role (Anafi et al., 1996; Andreotti et al., 1997; Kim et al., 2000; Prehoda et al., 2000; Sicheri et al., 1997; Xu et al., 1997). A functional role for SH3-GK interactions is supported by genetic studies of MAGUKs in invertebrates (Hoskins et al., 1996; Woods et al., 1996) that have identified numerous loss-of-function mutations in the SH3 and GK domains—all of which disrupt SH3-GK binding (McGee and Bredt, 1999).

Models for how the SH3 and GK domains might interact have assumed that the interaction involves docking of two independently folded domains. However, two

[3] Correspondence: bredt@phy.ucsf.edu (D.S.B.); wlim@itsa.ucsf.edu (W.A.L.)
[4] Present address: Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, OR, 97403

Table 1. Data Collection and Refinement Statistics

MAD Phasing				
Energies	Number of Reflections (Total/Unique)	Completeness (%)[a]	Overall I/σ	R_{sym}[a,b]
λ_1 12,657	164,138/17,911	98.5 (88.8)	10.5	10.2 (45.0)
λ_2 12,655	165,419/18,051	98.8 (90.2)	11.1	8.4 (34.6)
λ_3 12,800	168,966/18,187	99.0 (92.2)	9.3	15.7 (42.0)
Mean overall figure of merit (30.0–2.3 Å) (centric/acentric) = 0.52/0.65.				
Native (30.0–1.8 Å)	294,814/37,032	99.4 (94.4)	11.1	7.7 (35.1)

Refinement and Stereochemical Statistics (All Data 30.0–1.8 Å)	
R value	23%
Free R value	25%
Solvent Molecules	108
Average B Factors (Å^2)	
PSD95 430-724	34
RMS Deviations	
Bonds (Å)	0.005
Angles (°)	1.08

[a] Statistics for highest resolution shell (1.9–1.8 Å) in parentheses.
[b] $R_{sym} = \Sigma|I - <I>|/\Sigma I$, where I is the integrated intensity of a given reflection.

findings suggest that this interaction is unusual. First, SH3 domains typically utilize a conserved aromatic binding surface to recognize ligands bearing a characteristic PxxP motif. The GK domain fragment that is sufficient for interaction lacks PxxP motifs. Moreover, mutations in the canonical peptide binding surface of the SH3 domain do not disrupt the SH3-GK interaction. Second, the SH3-GK interaction requires a GK fragment that includes segments of sequence extending beyond the regions required for binding exogenous GK ligands.

To define the structural basis for SH3-GK interactions in MAGUKs, and to investigate how this interaction may contribute to MAGUK assembly and function, we determined the crystal structure of the SH3-GK region of PSD-95 to 1.8 Å resolution. The structure reveals that the two domains form an integrated structural unit. Unlike typical SH3 domains, the SH3 domain of PSD-95 is composed of noncontiguous elements of sequence, divided by a large hinge region and the GK domain. We find that the SH3-GK interaction does not involve binding between two independently folded domains, but instead represents the reassembly of the SH3 fold from subdomains that are separate in sequence. Based on this structure and complementary biochemical studies, we propose a model in which MAGUK oligomerization by the SH3-GK module occurs through three-dimensional (3D) domain swapping, a mechanism in which complementary substructures from distinct polypeptide chains cross-associate to form dimers or higher order oligomers. This model includes a mechanism by which ligand binding could regulate the formation of an interlocked MAGUK network.

Results and Discussion

Crystal Structure of the SH3-GK Region of PSD-95

The C-terminal region of PSD-95 (residues 417–724) spanning the SH3 and GK domains was crystallized by vapor diffusion against 2 M ammonium sulfate, 2% PEG400, and the structure determined by multiple wavelength anomalous dispersion (MAD) with selenomethionyl-substituted protein (Table 1). The solvent flattened electron density map was readily interpretable and allowed a model of most of the SH3 and the entire GK domain of PSD-95 to be built. The final refined model contains all residues except for 12 NH_2-terminal residues and residues 504–509, for which only weak electron density was observed (Figure 1A). The refined SH3-GK structure has an R value of 23% and a free R value of 25% at 1.8 Å resolution against a native synchrotron data set (Table 1).

A pair of anti-parallel β strands (E and F) links the SH3 and GK domains. A Cα contact map indicates that these linker strands make contact with both globular folds, but that they are primarily part of the SH3 fold (Figure 1B). Thus, the SH3 fold comprises several noncontiguous sequence elements. Aside from the contacts mediated by the E and F β strands, there are remarkably few contacts between the two domains. Below we present the structural properties of each domain, then discuss how this unanticipated domain arrangement explains the previously reported SH3-GK interactions.

The GK Domain Resembles Catalytic Homologs but Lacks Guanine Binding Residues

The GK domain of PSD-95 shares 40% sequence identity with yeast guanylate kinase (Figure 2), the enzyme that catalyzes the phosphorylation of GMP to GDP. Yeast guanylate kinase, like many kinases, has a clamshell-like structure surrounding the active site (Blaszczyk et al., 2001; Stehle and Schulz, 1992). The yeast enzyme can adopt at least two states: an open conformation, observed in the absence of ligand, and a closed conformation, observed in the presence of guanosine monophosphate (GMP), in which the clamshell fold closes down on the substrate (Blaszczyk et al., 2001). The structure of the PSD-95 GK domain superimposes better (RMSD = 1.49 Å) with the open (apo) form of the yeast guanylate kinase structure than the closed conformation (RMSD = 1.94 Å) (Figure 3A). Presumably, the PSD-95 GK fold may also form a similar closed state upon binding of the appropriate ligand. It is also noteworthy that the homology between the yeast guanylate

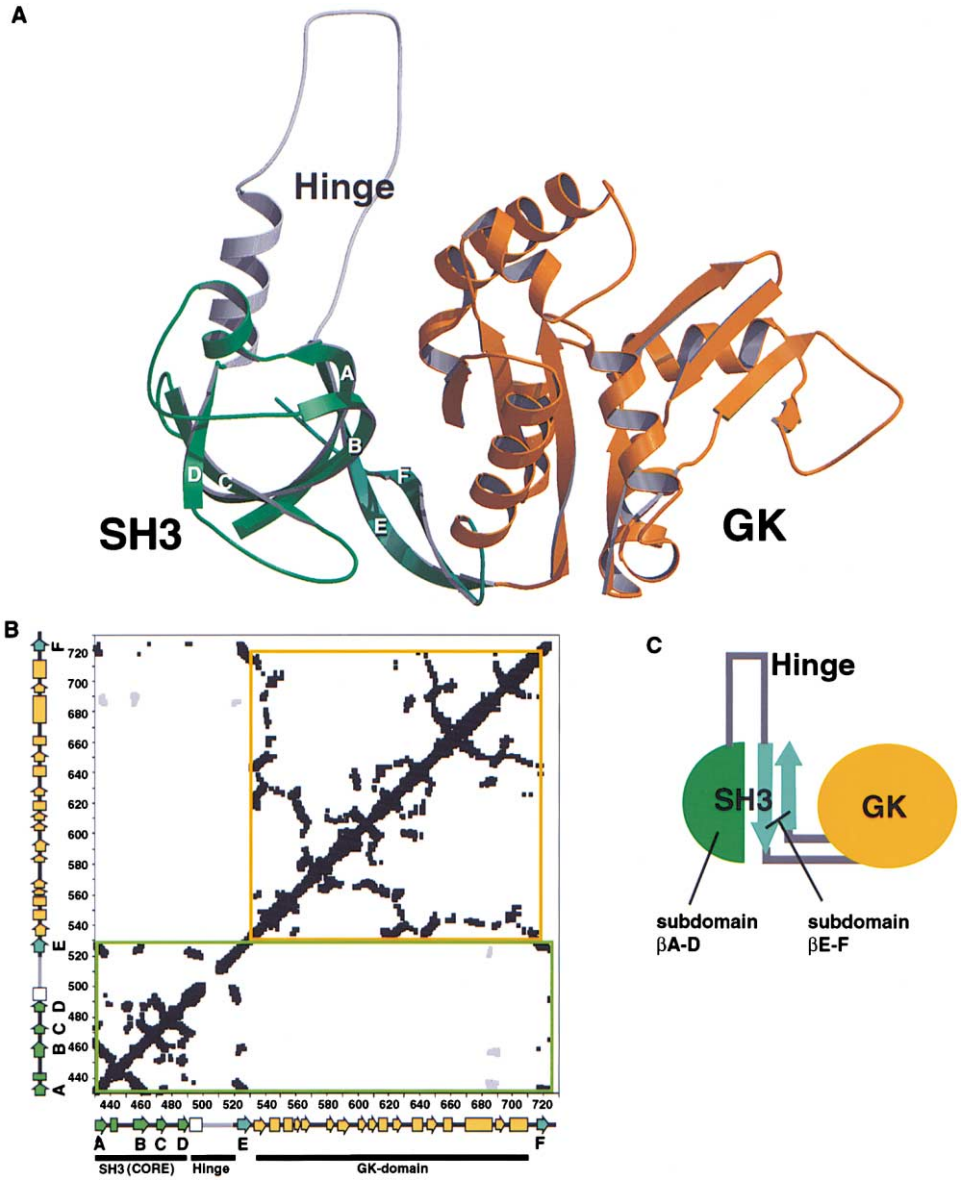

Figure 1. Structure of the SH3-GK Module of PSD-95

(A) Ribbon diagram of the SH3 (green) and guanylate kinase-like (orange) domains of PSD-95. The HINGE (gray) is a large insert (residues 491–522) containing an α-helix that separates structural components of the SH3 fold.

(B) A Cα contact map of the SH3-GK module of PSD-95 reveals that β strand E and β strand F make more numerous and closer contacts with structural components of the SH3. Map indicates pairs of Cα atoms that are <10 Å apart. The rectangles outlined in orange and green outline the GK and SH3 folds, respectively. The structure shows remarkably few interdomain contacts, which are colored in gray.

(C) A schematic representation of the SH3-GK module. The hinge divides the SH3 domain into two subdomains: a core of four β strands A–D (green half-circle), and a pair of β strands (E and F, light green arrows) flanking the GK domain (orange circle). Note that residues 504–509 in the hinge region are not included in the refined model, as only weak electron density was observed for these residues. For clarity, the ends of the protein flanking this gap are shown connected in this figure and in Figure 4. This figure was generated using MOLSCRIPT, RASTER3D, and CNS.

kinase structure and the PSD-95 GK structure does not extend to the flanking E and F β strands, as these strands are not present in the yeast enzyme.

Although PSD-95 lacks guanylate kinase catalytic activity (Kistner et al., 1995; Kuhlendahl, et al., 1998), we wondered whether it binds GMP. The reported binding affinity of yeast guanylate kinase for GMP is 29 μM (Li et al., 1996). Whereas we observed that yeast GK bound GMP with an affinity of 20–50 μM, we were unable to detect binding of GMP to the SH3-GK module using identical equilibrium dialysis conditions (data not shown). These data do not preclude the possibility that SH3-GK binds with a significantly reduced affinity compared to yeast GK. Comparison of the structures suggests why the GK domain from PSD95 may not bind GMP with high affinity. Only two of the four residues that coordinate the guanine

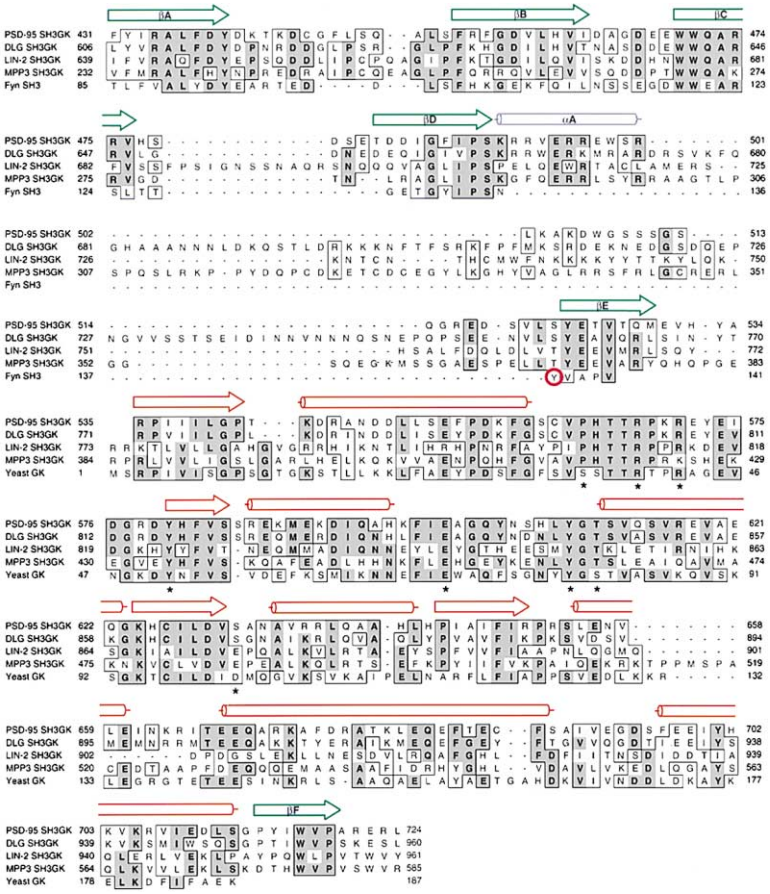

Figure 2. Sequence Alignment of SH3-GK Modules from MAGUKs

Sequences corresponding to the SH3-GK regions of Rat PSD-95, *Drosophila* Discs Large (DLG), *C.elegans* LIN-2, and human palmitoylated membrane protein 3 (MPP3), a p55 subfamily member, were aligned using the program CLUSTALW. For comparison, the sequences of the canonical SH3 domain from Fyn and yeast guanylate kinase were also aligned, using the structure as a guide. Amino acid identities are shaded, and similarities are boxed. Secondary structure elements for the SH3 and GK domains of PSD-95 are shown above the sequences, with β strands as arrows and α helices as cylinders. The βE strand of Fyn SH3 has been displaced to align with the structurally analogous sequence in PSD-95. The tyrosine in this β strand that is critical for binding poly-proline ligands is circled in red. Although this residue is conserved in canonical SH3 domains, it is absent in the MAGUK sequences. The residues of yeast guanylate kinase (yeast GK) that bind GMP are marked with asterisks.

base in yeast guanylate kinase are present in PSD-95. By comparison, the five residues that coordinate the GMP phosphate group are all conserved in sequence and structural arrangement. This observation suggests that the GK domain from PSD-95 may bind a phosphorylated molecule other than GMP.

The SH3 Domain Has a Split Fold Incompatible with Canonical Peptide Binding

The SH3 fold in the SH3-GK module differs radically from typical SH3 domains (Figure 4). First, this domain has an extra strand in its fold. Canonical SH3 domains consist of an anti-parallel β-barrel with orthogonal packing of the β sheets (Kuriyan and Cowburn, 1997; Musacchio et al., 1994b). While the canonical SH3 fold has five β strands (A–E), the PSD-95 SH3 fold has six β strands (A–F). Second, the PSD-95 SH3 domain is composed of discontinuous segments of sequence; a large insert, which we refer to as the hinge region, separates β strands D and E. Additionally, the GK domain is inserted between β strands E and F. Structure-based sequence alignments suggest that this unusual split configuration is conserved within the extended MAGUK family (Figure 2). Third, in canonical SH3 domains, a segment of 3_{10} helix separates β strands D and E (Larson and Davidson, 2000). This conformation is conserved in known SH3 structures and forms part of a conserved hydrophobic binding surface that interacts with proline-containing peptides presented as polyproline type II helices (Figures 4B and 4D) (Feng et al., 1994; Kohda et al., 1994; Lim et al., 1994b; Musacchio et al., 1994a; Wittekind et al., 1994; Yu et al., 1994). A conserved tyrosine in the 3_{10} helix is a crucial element in the peptide recognition interface (Lim and Richards, 1994; Nguyen et al., 1998). In MAGUK SH3 domains, the large hinge insert replaces this short segment. Not only does the PSD-95 SH3 fold lack the conserved tyrosine residue, but the hinge region also sterically occludes the peptide binding site (Figure 4A).

As a consequence of this unusual structure, it seems unlikely that MAGUK SH3 domains bind proline-rich peptide ligands in a canonical manner. Nevertheless, some studies have reported proline-containing ligands for MAGUK SH3 domains (Garcia et al., 1998; Maximov et al., 1999). However, because of the unexpected insertion of the hinge region in the SH3-GK structure, the constructs used in these previous binding studies lack β strands E and F. Thus, ligands identified in previous studies most likely interact in an atypical fashion. The unique features of the PSD-95 SH3 fold suggest that regulated association with the GK domain, and not peptide binding, may be its primary role.

The SH3-GK Interaction Reflects Reassembly of the Split SH3 Domain

While previous models have presumed that the interaction between the SH3 and GK fragments involves docking between two prefolded domains, the structure of the SH3-GK module reveals that these domains are intertwined in sequence (Figure 2). We used the structure to define the boundaries for the interacting fragments.

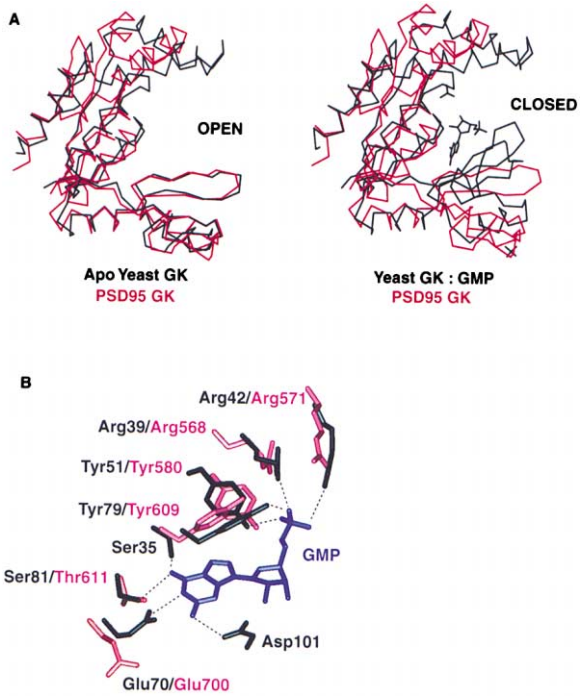

Figure 3. The PSD-95 GK Domain Structurally Resembles Apo-Yeast Guanylate Kinase

(A) Comparison of the PSD-95 GK domain structure (red) with the apo-yeast guanylate kinase structure (black) (top; RMSD = 1.49 Å) and with the yeast guanylate kinase structure in complex with guanosine monophosphate (GMP) (black) (bottom; RMSD = 1.94 Å).

(B) The five residues of yeast guanylate kinase that coordinate the phosphate in GMP are conserved in the PSD-95 GK domain. However, two of the four residues of YGK that bind the guanine ring (Ser35 and Asp101) are not present in PSD-95.

Specifically, we wanted to determine the role of β strands E and F in the SH3-GK interaction, as these β strands appear to be structurally part of the SH3 fold, but are adjacent to the GK domain. We therefore tested a series of deletion constructs for their ability to bind one another by GST-pulldown assays (Figure 5A). In this series, the β strands E and F were either fused to the core SH3 fragment (β strands A–D) or to the GK fragment. This interaction matrix reveals that a GK fragment lacking either the E or F β strands cannot interact with any of the SH3 fragments tested. In contrast, adding the E and F β strands to the core SH3 fragment does not influence binding; even an SH3 fragment only consisting of β strands A through D can bind the GK fragment including β strands E and F. Thus, for binding to occur, the E and F β strands must be part of the GK fragment.

However, the E and F β strands appear to be part of the SH3 fold. To determine if β strands E and F contribute structurally to the SH3 fold, we used guanidine hydrochloride denaturation to measure the stability of four SH3 constructs: β strands A–D, β strands A–E, β strands A–F* (where F is appended to β strand E by a $(Gly\text{-}Ser)_3$ linker), and the full SH3-GK module (Figure 5B). The fragment containing only β strands A–D is unstable. Most likely, this fragment is only partially structured at the experimental temperature of 25°C. In contrast, a fragment including β strand E displays a cooperative unfolding transition, but the protein is still quite unstable ($\Delta G_{fold} = \sim 4$ kcal/mol). Adding β strand F by artificially appending it to β strand E increases domain stability ($\Delta G_{fold} = \sim 5$ kcal/mol). Finally, in the SH3-GK module, SH3 domain is stability is further increased ($\Delta G_{fold} = \sim 10$ kcal/mol). These findings suggest that β strands E and F are part of the SH3 fold. In addition, they show that the GK domain plays a role in stabilizing the SH3 fold. Because the GK domain makes only limited contacts with the core SH3 fold (Figure 1B), its role in stabilizing the SH3 domain appears to be indirect, and most likely occurs by properly orienting β strands E and F.

Thus, the previously described SH3-GK interaction is unusual, because it does not involve docking of two prefolded domains, but rather reassembly of an SH3 fold from two separable subdomains, β strands A–D of the SH3 fold and β strands E and F flanking the GK fold. At least one of the subdomains, the β strand A–D fragment, is unstable in the absence of the complementary subdomain (β strands E and F). This interaction is distinct from canonical domain-domain interactions, as the boundaries of the interacting fragments are different from the boundaries of folded domains (Figure 5C).

Intermolecular Interaction Between SH3-GK Modules May Involve 3D Domain Swapping

The PSD-95 SH3 fold can assemble intramolecularly or intermolecularly with individual subdomains (Figure 5A). The structure solved here represents the intramolecular configuration. Intramolecular assembly is favored, as the intact SH3-GK module does not interact with either isolated subdomain in vitro (Figure 5A). Intermolecular assembly in vitro is only observed between SH3-GK modules if the complementary subdomains are disrupted within separate protein molecules (McGee and Bredt, 1999; Shin et al., 2000). Nonetheless, prior studies indicate that intermolecular SH3 assembly does occur in vivo. First, the MAGUK proteins hCASK and hDLG associate in intestinal epithelia, and this interaction requires the SH3-GK modules (Nix et al., 2000). Second, genetic studies of *discs large*, a MAGUK that clusters receptors and ion channels at the fly neuromuscular junction (Zito et al., 1997) and is also present at septate junctions in epithelia (Woods et al., 1996) demonstrate partial interallelic complementation between mutations that disrupt separate subdomains in the SH3-GK module. Specifically, two different recessive lethal alleles, one containing a missense mutation in the β strand A–D subdomain, dlg^{m30}, the other truncating the protein before the E–F subdomain, $dlg^{XI\text{-}2}$, are viable as transheterozygotes (Woods et al., 1996). Intermolecular assembly of a "functional" SH3 by the two proteins with mutations in separate SH3 subdomains most simply explains this finding.

The SH3-GK structure, along with in vitro binding studies, suggests that the intermolecular SH3 assembly may occur via three-dimensional (3D) domain swapping, a mechanism in which proteins exchange complementary substructures to generate dimers or higher order oligomers (Bennett et al., 1995; Newcomer, 2001; Schlunegger et al., 1997). In SH3-GK 3D domain swapping, the β strand A–D subdomain from one polypeptide would interact with the β strand E and F subdomain from a separate polypeptide. This type of interaction could occur either as a closed dimer or a higher order oligomeric chain (Figure 6A).

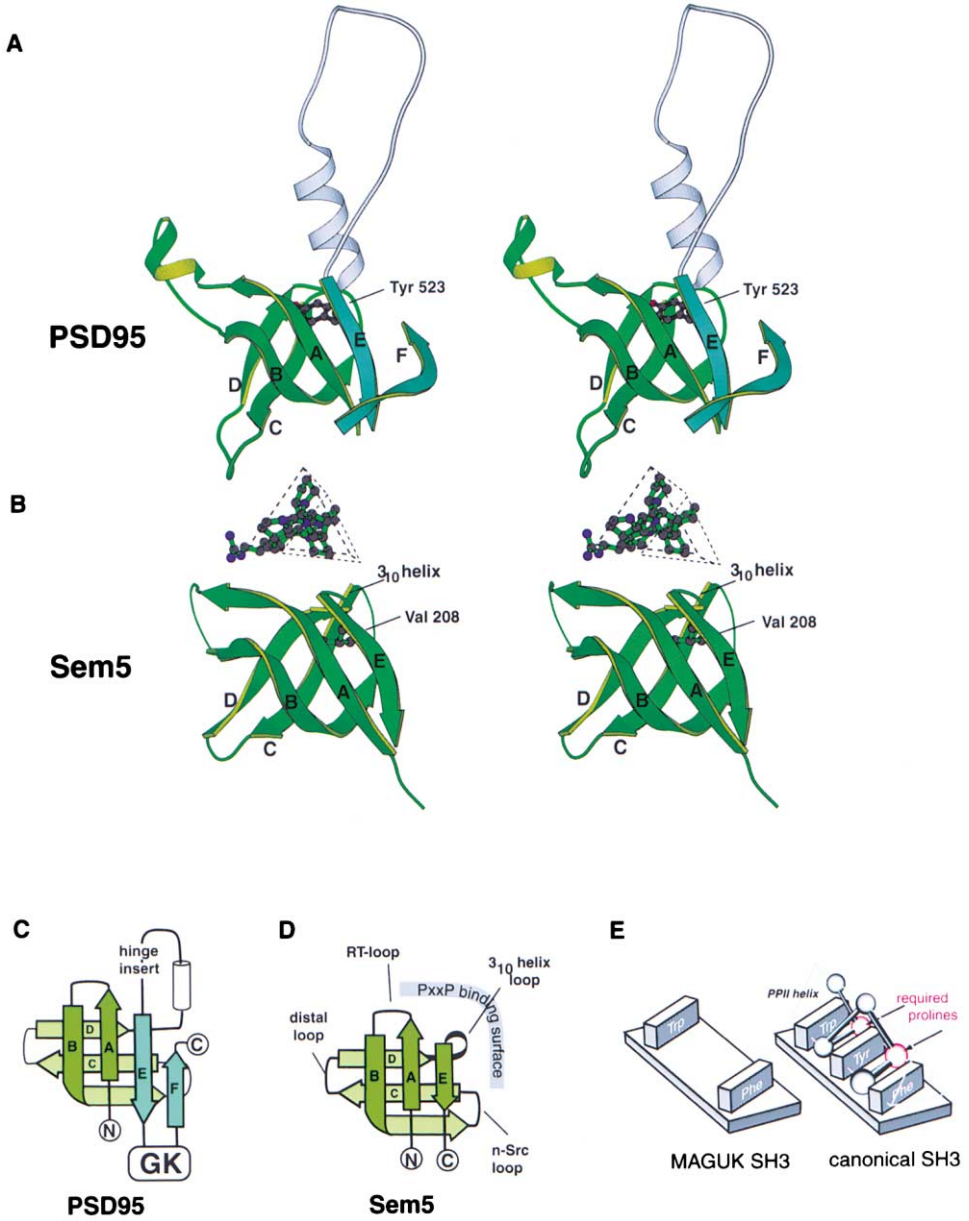

Figure 4. Comparison of the PSD-95 Split SH3 Structure with Canonical SH3 Domain Structure

(A) Stereo ribbon diagrams of the PSD-95 SH3 fold (upper), including the intervening hinge region and the additional β strand F, and the C-terminal SH3 domain of SEM-5 (lower) complexed with a peptide ligand (Lim et al., 1994b). In PSD-95, a tyrosine (523) from the separated E β strand packs into the hydrophobic core of the SH3 fold, in place of a valine normally observed in canonical SH3 domains (Val 208 in SEM-5). The position of the 3_{10} helix in SEM-5 is indicated. The β strands for PSD-95 and SEM-5 are labeled A–F and A–E, respectively.

(B) Secondary structure diagrams of PSD-95 and SEM-5 reveal the conserved overall topology. The hinge region in PSD-95, which separates β strands D and E, occludes the canonical peptide binding surface. The GK domain is inserted between β strands E and F. The positions of the various loops and the conserved segment of 3_{10} helix are shown in SEM-5.

(C) Schematic comparison of the peptide ligand binding surfaces for PSD-95 and SEM-5. Aromatic side chains critical for ligand recognition are represented as planar projections. PSD-95 lacks the central conserved tyrosine residue. A docked polyproline II (PPII) helical ligand is shown for the canonical SH3 domain.

The Hinge Element May Regulate Domain Swap Assembly

In a 3D domain swap model of SH3-GK oligomerization, how could assembly be regulated? Studies of protein systems that undergo domain swapping indicate that the hinge region linking the two interacting subdomains plays an important role in determining the preference for inter- versus intramolecular assembly (Newcomer, 2001; Schlunegger et al., 1997). Experimental manipulation of the hinge loop region in p13suc1 has revealed that imposing a conformational constraint on this region of the protein can alter the kinetics as well as the equilibrium distribution of monomer versus domain swapped dimer (Rousseau et al., 2001; Schymkowitz et al., 2000).

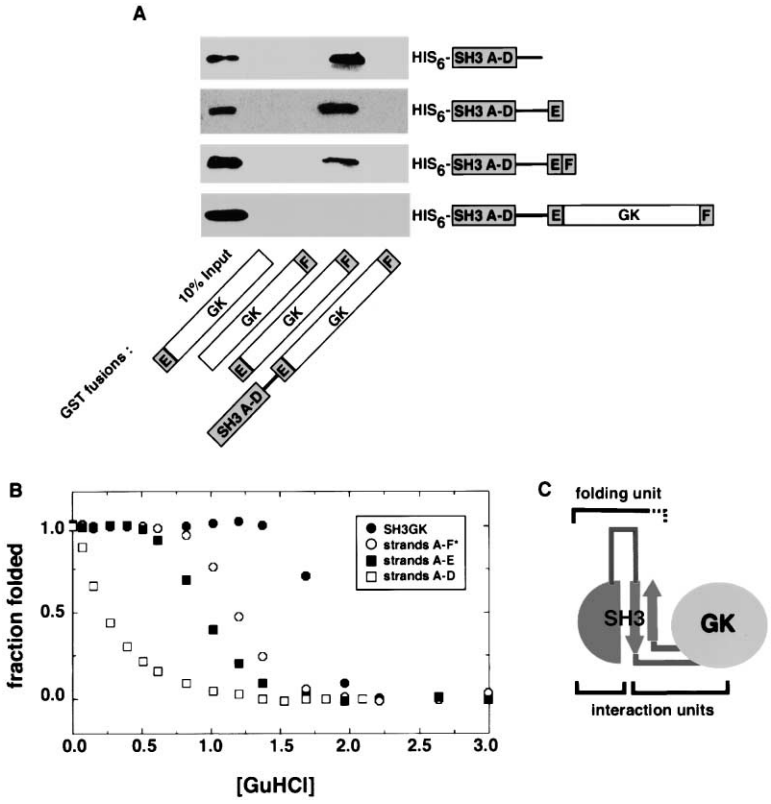

Figure 5. The SH3-GK Interaction Involves Reassembly of the Split SH3 Fold

(A) GST pulldowns demonstrate that the SH3-GK interaction requires that β strands E and F are contiguous with the GK fold. GST-fusions of the GK domain containing one or both of the flanking β strands E and F or GST-SH3-GK were incubated with His-tagged SH3 proteins that contained β strands A–D, A–E, A–F* (with β strand F attached by a $(Gly\text{-}Ser)_3$ linker), or the complete SH3-GK module. Only GST-GK fusion proteins containing β strands E and F bound the His_6-SH3 proteins that did not contain GK.

(B) Stability of corresponding SH3 fragments to guanidine hydrochloride denaturation was monitored by tryptophan fluorescence. Fragments lacking the E and F β strands are not stably folded. Addition of β strands E and F sequentially stabilizes the fold. Stability is most significantly increased by the addition of β strands E, F, and the GK fold.

(C) A schematic comparison of the differences between the folding and interacting units within the SH3-GK module. The SH3-GK interaction requires that β strands E and F be attached to the GK fold. However, these two strands are structural components of the SH3 fold.

Interestingly, in MAGUK SH3 domains, the hinge region replaces a structurally conserved 3_{10} helix (Figure 6B). Moreover, in several MAGUKs, the hinge region has been identified as the binding site for several potential regulatory proteins such as protein 4.1 and calmodulin (Figure 6C) (Cohen et al., 1998; Lue et al., 1994; Marfatia et al., 1995; Masuko et al., 1999). We therefore hypothesize that ligand binding to the hinge region could alter its conformational properties, either by destabilizing the monomeric form or reducing the kinetic barrier, to promote oligomerization. However, no proteins have yet been identified that bind to the hinge region of PSD-95.

To test our hypothesis that the hinge region is a potential point for regulation of inter- versus intramolecular SH3 assembly, we perturbed the conformational properties of this region by mutagenesis. To mimic rigidity that might be imposed by protein binding, we replaced residues 490–523 with a linker containing five prolines (Pro_5Δhinge) (Figure 6D). We predicted that this substitution would stiffen the peptide backbone and extend the distance between the ends of the hinge that separate the SH3 subdomains (MacArthur and Thornton, 1991). In similar constructs, we replaced the hinge with a flexible linker consisting of five glycines (Gly_5Δhinge), or inserted no linker (Δhinge). We analyzed each of these proteins by gel filtration. All of these mutant proteins, like the wild-type SH3-GK module, eluted as monomers, except for Pro_5Δhinge, for which a significant fraction eluted as a dimer (Figure 6E). Rechromatography of the Pro_5Δhinge dimer species revealed that exchange between the monomer and dimer states is relatively slow ($t_{1/2}$ = ~2 hr). Therefore, we conclude that replacement of the hinge with a conformationally constrained segment promotes oligomerization.

To confirm that the dimer observed for this mutant SH3-GK module is formed by 3D domain swapping and not by novel interactions introduced by the inserted Pro_5 element, we performed control experiments to eliminate the possibility that the Pro_5 element served as a docking site for the SH3 domain. First, we mutated the putative peptide binding surface of the SH3 domain within the Pro_5Δhinge construct. Specifically, we changed tryptophan 470 to phenylalanine, as this amino acid is critical for binding of proline-rich peptides to canonical SH3 domains (Lim and Richards, 1994), but does not affect intermolecular assembly of the SH3 fold (McGee and Bredt, 1999). By gel filtration, this mutant protein yielded a dimer species with the same elution profile as the Pro_5Δhinge SH3-GK protein (data not shown). Second, we made an analogous Pro_5 insertion mutant without completely deleting the hinge region (Pro_5+hinge). This variant failed to dimerize, presumably because the remaining hinge sequence retains sufficient flexibility to allow for favorable intramolecular assembly. We conclude that the overall rigidity of the hinge region, and not the specific Pro_5 sequence element, is critical for inducing oligomerization.

Conclusion: A Model of Regulated Assembly by MAGUKs

Assembly of ordered structures such as the PSD requires interaction between many proteins. A fundamental question is how these interactions are coordinated and regulated to achieve spatial and temporal specific-

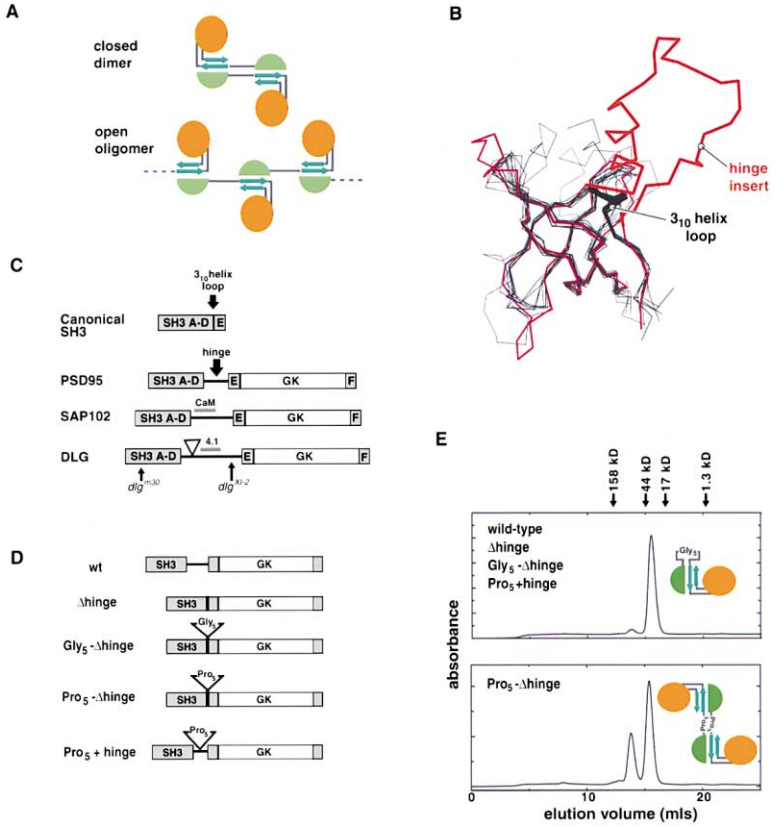

Figure 6. The Hinge Region Is Variable and Can Modulate Inter- Versus Intramolecular SH3 Assembly

(A) Schematic representations of intramolecular SH3 assembly (upper), a 3D domain swapped open oligomeric chain (left), and a closed dimer (right).

(B) Alignment of the PSD-95 SH3 fold (red) with the structures of eight canonical SH3 domains (black) shows that the hinge insert replaces a conserved segment of 3_{10} helix (bold).

(C) The hinge region is variable in length and is the site of protein binding and alternative splicing. Calmodulin and protein 4.1 bind some MAGUKs within the hinge region. Two mutations in *Drosophila* DLG that are capable of interallelic complementation are indicated with arrows.

(D) Schematic representations of the SH3-GK mutant proteins used to probe the role of the hinge region in SH3 3D-domain swapped assembly.

(E) Elution profiles for the proteins diagrammed in (D). Unlike the wt and other mutants, the Pro_5-Δhinge protein elutes with a significant peak corresponding to the dimer species.

ity. Why do these scaffolds only assemble at the correct membrane sites? Previous studies have suggested that the intermolecular SH3-GK interactions may contribute to MAGUK scaffolding (Nix et al., 2000), and that this process may be regulated by extrinsic factors (Masuko et al., 1999). Using the structure of the PSD-95 SH3-GK module, we refine this model by proposing that the intermolecular interactions observed for MAGUKs are mediated by 3D domain swapping of structural components of the split SH3 fold. We hypothesize that ligand binding may constrain the flexibility of the hinge region, thereby promoting the switch from intra- to intermolecular assembly (Figure 7).

This model offers potential advantages as a scaffolding mechanism. First, because 3D domain swapping regenerates complete folds, this mechanism facilitates oligomerization without occluding sites on the SH3 and GK folds that may bind associated signaling proteins. Second, regulatory proteins with the appropriate subcellular localization could direct the correct temporal and spatial assembly of interlocked MAGUK networks. Third, heteromeric 3D domain swapping of MAGUKs, perhaps directed by sets of regulatory proteins, could provide combinatorial scaffold diversity, which could specify differential protein recruitment. This model of regulated assembly is consistent with the function of MAGUK proteins and is one of several mechanisms that may participate in the proper assembly of supramolecular signaling complexes at cell junctions.

The structure of the SH3-GK module reveals how a

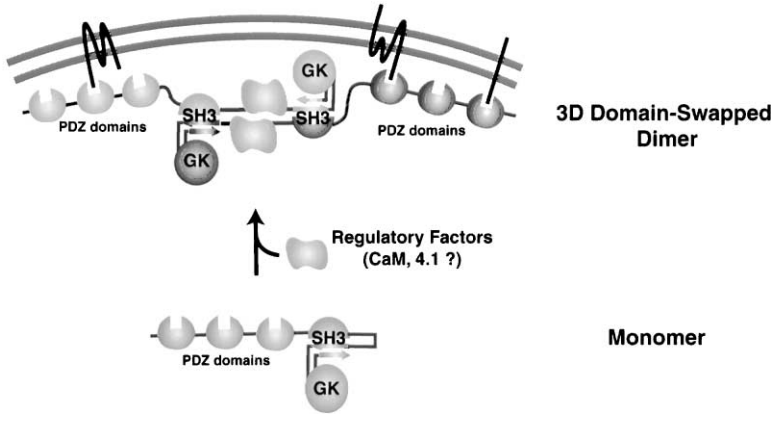

Figure 7. Model for Role of SH3-GK Module in Regulated Assembly—Ligand Binding to the Hinge Region May Promote Oligomerization by 3D-Domain Swapping

Proteins binding to the hinge region, such as calmodulin, protein 4.1, or unknown factors, may constrain this region to promote intermolecular SH3 assembly. Localization of these regulatory components may thereby help to restrict supramolecular assembly to specified subcellular sites.

simple, modular protein fold, such as the SH3 fold, can be used in diverse modes to mediate protein complex assembly. This module provides one of the first structural examples in which normally discrete protein domains have evolved into an integrated functional unit.

Experimental Procedures

Protein Expression and Purification

The DNA sequence encoding residues 417 to 724 of rat PSD-95 (SH3-GK) was amplified by PCR and cloned into a His-tagged expression vector containing a TEV site that permits proteolytic removal of the tag (Hillier et al., 1999). Twelve liters of *E. coli* strain BL21 (DE3; pLysS) expressing SH3-GK were grown to $OD_{600} = 0.8$, induced with 1 mM IPTG for 3 hr, harvested by centrifugation, freeze-thawed with liquid nitrogen, and lysed by sonication in 240 ml of 50 mM Na-PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol. Protein was purified on 12 ml Ni-NTA resin (Qiagen) by elution with 200 mM imidazole following extensive washing with 50 mM Na-PO_4 (pH 6.5), 300 mM NaCl, 10% glycerol, and 20 mM imidazole, 50 mM Na-PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol. The protein was dialyzed against 100 mM Tris-HCl, 400 mM NaCl, 10% glycerol, 2 mM DTT, and the His-tag was removed by cleavage with TEV protease. Following dialysis into 25 mM Tris-HCl (pH 8.0), final purification was achieved by chromatography on a 10 ml Resource Q column (Pharmacia) that was eluted with a gradient of 0–150 mM NaCl in 25 mM Tris-HCl (pH 8.0), 1 mM DTT. Protein was dialyzed against 10 mM HEPES (pH 8.0), 100 mM NaCl, 2 mM DTT, and concentrated to 10 mg/ml by ultrafiltration (Amicon). Purity of >99% was established by SDS-PAGE, mass spectroscopy, and isoelectric focusing gel electrophoresis. For the MAD data set, two additional methionines were inserted into the SH3-GK expression construct used for protein production. Residues T543 and A547 were both mutated to methionine. Both these mutations were conservative, as T543 is a methionine and A547 is a valine or isoleucine in closely related MAGUKs. Selenomethionine (SeMet)-labeled SH3-GK was produced by growth in minimal media supplemented with selenomethionine (Van Duyne et al., 1993) and purified in buffers that were purged with N_2 to prevent selenium oxidation. Labeling was confirmed by mass spectroscopy.

Crystallographic Methods

Small bipyramidal crystals of SH3-GK were grown by vapor diffusion in hanging drops (6 mg/ml) in 2 M ammonium sulfate, 2% PEG400, 10 mM HEPES (pH 7.0) at 15°C (buffer 39; Hampton Research); larger crystals were grown by microseeding in sitting drops. For microseeding, 6 μl of SH3-GK (10 mg/ml) was mixed with 4 μl of buffer 39 and were seeded in serial dilution with microcrystals obtained by crushing a previously obtained crystal. Following 2 weeks of growth, average crystal dimensions were 150 × 150 × 450 μm. Prior to data collection, crystals were transferred to a cryoprotectant solution containing mother liquor and 20% glycerol, then flash frozen in liquid nitrogen.

Crystals of both the native and SeMet protein grew in space group space group $P4_12_12$, with cell constants of a = 205 Å, b = 60.5 Å, c = 60.5 Å, and one molecule per asymmetric unit. Native (1.8 Å diffraction limit) and SeMet protein (2.3 Å diffraction limit) data sets were collected the Advanced Light Source (beamline 5.0.2, Lawrence Berkeley Laboratories). A fluorescence scan of the SeMet crystals was taken to optimize wavelengths for MAD data collection and to calculate anomalous scattering coefficients. Data were collected at three wavelengths (Table 1) and processed with the program HKL2000 (Otwinowski and Minor, 1997). Using the MAD data, the four selenium sites were identified by the Patterson search methods in the program SOLVE (Terwilliger and Berendzen, 1996), and these positions were refined with the program SHARP (De La Fortelle and Bricogne, 1997). The resulting electron density map was subjected to solvent flattening with SOLOMON.

Model Building and Refinement

An initial model was built into the experimental electron density using the program O (Jones et al., 1991). The experimental map was readily traceable and allowed for the construction of a model of residues 430–501 and 518–724 of SH3-GK. Density corresponding to residues 502–517 is visible, but the residues are less well ordered. The model was refined against the native data set using the program CNS (version 1.1) (Brünger et al., 1998) with alternate cycles of rebuilding, positional refinement, and restrained B factor refinement. All diffraction data was used throughout, except a 10% test set for calculation of the free R factor.

In Vitro Binding Assays with Affinity-Tagged Fusion Proteins

Sequences encoding various regions of PSD-95 were amplified by PCR and cloned into either pGEX 4T-1 (Pharmacia) or a His-tagged expression vector (Hillier et al., 1999). The amino acid sequences corresponding to following constructs are in parentheses: His_6-SH3 A–D (417–490), His_6-SH3 A–E (417–532), His_6-SH3 A–F* (417–532-$(GS)_3$-712–724), His_6-SH3-GK (417–724), GST-E-GK (523–711), GST-GK-F (532–724), GST-E-GK-F (523–724), GST-SH3-GK (417–724), GST-Δhinge (417–490-(Δ)-521–724), GST-Gly_5Δhinge (417–490-$(G)_5$-521–724), GST-Pro_5Δhinge (417–490-$(P)_5$-521–724), GST-Pro_5+hinge (417–502-$(P)_5$-514–724). GST fusion proteins and His-tagged proteins were expressed and purified as described (Brenman et al., 1996). The His-tagged proteins were diluted to 0.1 mg/ml with elution buffer (200 mM imidazole, 50 mM Na-PO_4 [pH 8.0], 300 mM NaCl, 10% glycerol) containing 1 mM DTT. Approximately 10 μg of GST-fusion protein was coupled to glutathione-sepharose (Pharmacia) beads and incubated with 250 μl (25 μg) His-tagged proteins at 4°C for 30 min. The beads were washed extensively with a phosphate buffered saline containing 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 1 mM DTT. Retained proteins were eluted with SDS protein loading buffer, separated by PAGE, and analyzed by immunoblotting.

Guanidine Hydrochloride Denaturation

Stability of SH3 constructs to guanidine hydrochloride (GuHCl) denaturation was measured as previously described (Lim et al., 1994a). The folded SH3 structure has a buried tryptophan residue (W471) within its hydrophobic core, but the GK domain has no tryptophan residues. Unfolding was monitored by tryptophan fluorescence (excitation 295 nM; emission 340 nM) at 25°C. Assays were performed using a PTI fluorimeter with His_6-tagged protein at a concentration 1 μM in 50 mM Tris HCl (pH 7.5), 50 mM NaCl. ΔG of unfolding was calculated as described, assuming a two-state model for unfolding. The construct His_6-SH3 A–D showed no folded state baseline, indicating that at 25°C, the protein was already partially unfolded. The fluorescence at 0 M GuHCL was therefore used as an estimate for the upper limit corresponding to folded protein.

Gel filtration

GST fusion proteins were purified on glutathione-sepharose, cleaved from the beads with thrombin (Sigma), concentrated to 5 mg/ml by ultrafiltration in Centricon units, and ultracentrifuged to remove aggregates. Samples (100 μl) were chromatographed on a Superdex 200 column by isocratic elution with 50 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM DTT at a flow rate of 0.5 ml/min.

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Accession Numbers

The coordinates have been deposited with the PDB under accession code 1KJW.