Multidomain scaffolding proteins are central components of many signaling pathways and are commonly found at membrane specializations. Here we have shown that multiple interdomain interactions in the scaffold Discs Large (Dlg) regulate binding to the synaptic protein GukHolder (GukH). GukH binds the Src homology 3 (SH3) and guanylate kinase-like (GK) protein interaction domains of Dlg, whereas an intramolecular interaction between the two domains inhibits association with GukH. Regulation occurs through a PDZ domain adjacent to the SH3 that allows GukH to interact with the composite SH3-GK binding site, but PDZ ligands inhibit GukH binding such that Dlg forms mutually exclusive PDZ ligand and GukH cellular complexes. The PDZ-SH3-GK module is a common feature of membrane associate guanylate kinase scaffolds such as Dlg, and these results indicate that its supramodular architecture leads to regulation of Dlg complexes.

Communication and adhesion between cells is mediated by specialized regions of the plasma membrane. For example, in excitatory synapses in the brain, the postsynaptic membrane contains an actin-rich cytoskeletal region known as the postsynaptic density (1). Analogous structures are present at sites of cell-cell contact, including the junctions between epithelial cells, which are important for signaling and the formation of physical barriers (2, 3). The establishment and function of these important structures is regulated by a large number of proteins that serve to organize receptors and downstream signaling proteins and to anchor signaling complexes at specific membrane locations.

Membrane-associated guanylate kinases (MAGUKs) are scaffolding proteins that regulate the formation and function of membrane specializations, such as synapses and tight junctions (3, 4). MAGUKs have a unique domain architecture that is typified by one or three PDZ domains, an SH3 domain, a variable HOOK sequence, and a region with homology to the enzyme guanylate kinase (GK) that lacks enzymatic activity but instead acts as a protein interaction domain. The SH3 and GK domains form an intramolecular interaction in the MAGUK PSD-95, which is thought to be a common feature of MAGUK proteins (5, 6).

One of the best studied MAGUK proteins is the Drosophila tumor suppressor Discs Large (Dlg). Dlg plays a role in the formation and function of diverse polarized cellular structures, including epithelial junctions (7), stem cell cortical domains (8, 9), and neuronal synapses (10). In the neuromuscular synapse, Dlg is present at high levels at both pre- and postsynaptic sites (11). Dlg is thought to function at these sites by clustering ion channels and organizing signal transduction pathways.

The intramolecular interaction between the SH3 and GK domains is important for MAGUK function. All genetically identified mutations in the SH3 and GK regions of dlg and the related Caenorhabditis elegans lin-2 gene disrupt the intramolecular interaction (5). However, the exact role of the intramolecular interaction in MAGUK function has remained obscure. The crystal structure of the PSD-95 SH3-GK revealed that the two domains interact through a unique mechanism in which a two-stranded $\beta$-sheet is composed of strands that emerge from the SH3-HOOK and GK domains (12, 13). The nature of this interaction is such that movements of the domains relative to one another could create functionally distinct conformations that result from hinge movements about the linking strands. However, how the distribution among these conformations might be modulated and what their functions are has remained unclear.

One function of the SH3-GK intramolecular interaction may be to regulate the assembly of MAGUK complexes. For example, GK-associated protein (GKAP) binds to a fragment of the MAGUK SAP-97 containing only the GK domain but fails to bind to the SH3-GK, indicating competition between the intramolecular interactions (14). A secondary intramolecular event with an NH$_2$-terminal L27 domain rescues the interaction with GKAP in the full-length protein. However, the mechanism by which this interaction may be regulated in the context of the full-length protein is unknown. Not all SH3-GK ligands compete against the intramolecular interaction, indicating that multiple binding surfaces are utilized by ligands of this unique domain.

Here we have analyzed how binding of Dlg to the synaptic protein GukHolder (GukH) is regulated by complex interdomain interactions within Dlg that involve transitions in the SH3-GK intramolecular interaction. GukH was first identified in a yeast two-hybrid screen as a binding partner for the Dlg GK domain (15), and mammalian homologues have been identified.
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(16, 17). GukH colocalizes with Dlg at synaptic borders, and the interaction of the two proteins appears to be required for proper localization of the tumor suppressor Scribble. GukH also colocalizes with Dlg in neuroblasts, precursors of the Drosophila central nervous system (18). The interaction of GukH with Dlg is mediated by an ~300-residue region at the GukH COOH terminus that contains no known domains.

We find that GukH binding to Dlg is actively regulated by the SH3-GK intramolecular interaction. GukH binds to a composite site formed by not only the GK domain but the SH3 domain as well. However, the intramolecular interaction between the two domains competes against GukH binding. Binding is rescued by a PDZ domain directly NH2-terminal to the SH3 domain, a common feature of MAGUK proteins. The complex interdomain interactions in Dlg cause GukH-Dlg- and PDZ-bound complexes to be mutually exclusive in a cellular context.

These results have implications for the types of complexes that are formed by Dlg and therefore its role in regulating the formation and function of membrane specializations, such as epithelial junctions and synapses.

EXPERIMENTAL PROCEDURES

Molecular Cloning, Protein Expression, and Purification—DNA encoding full-length Drosophila, the PE isoform of Dlg (which contains the “13” exon), and the GukH Dlg binding domain fragment from the PA isoform (1494–1788) were cloned from an embryonic cDNA library. For the PDZ, PDZ-SH3, SH3, GK, HOOK-GK, SH3-GK, linker-SH3-GK, and PDZ-SH3-GK fragments of Dlg, amino acids 481–571, 481–691, 581–681, 764–960, 675–960, 598–960, 560–960, and 474–960 were used, respectively. The sequence of all inserts was verified by DNA sequencing.

For expression of glutathione S-transferase (GST) fusions, cDNAs were ligated into pGEX 4T-1, whereas the pET-19b derivative pBH was used for hexahistidine tags. The pBH vector encodes for a tobacco etch virus protease site following the derivative pBH was used for hexahistidine tags. The pBH vector encodes for a tobacco etch virus protease site following the SK site. The complex interdomain interactions in Dlg cause GukH-Dlg- and PDZ-bound complexes to be mutually exclusive in a cellular context.

For qualitative “pulldown” assays, E. coli cell lysates containing the GST fusion protein of interest were incubated with glutathione-agarose beads and washed with binding buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 0.5% Triton X-100). Potential interacting proteins were added to a concentration of 10 μM and incubated with the beads at room temperature for 15 min. The reactions were washed three times with binding buffer to remove unbound proteins. Bound proteins were eluted with SDS loading buffer and analyzed by staining with Coomassie Blue and/or Western blotting using an anti-histidine antibody (Qiagen).

For quantitative binding measurements, a peptide with the sequence from the last ten residues of CRIP and an NH2-terminal rhodamine was synthesized by Fmoc solid phase peptide synthesis. A sulfonyl chloride rhodamine derivative (Molecular Probes L-20) was used after the addition of the final Fmoc amino acid. Following cleavage from the solid support, the peptide was purified by reverse-phase high pressure liquid chromatography and its sequence verified by MALDI-TOF mass spectrometry. A series of solutions were prepared with increasing concentrations of the appropriate Dlg fragment and a concentration of 100 nM rhodamine-labeled peptide. The anisotropy of each solution was measured using an ISS PC1 fluorometer. The $K_d$ of interactions was determined by nonlinear fitting of the data to a bimolecular binding equation.

Cell Culture and Immunoprecipitation—We transiently transfected Drosophila S2 cells grown in Schneider’s insect medium supplemented with 10% fetal bovine serum with expression vectors for full-length Dlg and/or the GFP-CRIPT and hemagglutinin (HA)-GukH Dlg binding domains using 1 μg of total DNA, which resulted in an efficiency of ~30%. We induced protein expression after 24 h using 0.5 mM copper sulfate and collected the cells after an additional 24 h of growth. To collect the cells, we centrifuged them for 5 min at 1000 × g and washed the resulting pellet with ice-cold phosphate-buffered saline twice.

For immunoprecipitation experiments, extracts were prepared by incubation with lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Cell lysate was precleared by gently mixing with protein A-Sepharose beads (Amersham Biosciences) at 4 °C for 1 h. The beads were then removed by centrifugation at 12,000 × g for 20 s. The proteins were immunoprecipitated by incubating anti-HA, anti-GFP, or anti-His with precleared lysate at 4 °C for 1 h. Protein A-Sepharose beads were added to the mixture and incubated at 4 °C for 1 h with rotation. The pellets were collected at 12,000 × g for 20 s and washed three times with lysis buffer and once with phosphate-buffered saline. The final pellets were suspended in protein loading buffer and analyzed by SDS-PAGE followed by western.

For immunostaining, Dlg localization was detected with an anti-Dlg antibody and Cy3-labeled secondary antibody (endogenous Dlg was below the level of detection for immunostaining). After labeling, the cells were imaged by confocal microscopy on a Nikon Eclipse TE2000-U microscope with a Photometrics CoolSNAP fx CCD camera. Images were analyzed with the ImageJ software (NIH), and cells were binned into cortical or cytoplasmic localization based upon the pixel intensity distribution across the cell.
Cells having cortical signal intensity 2× or greater than the cytoplasmic pool were scored as cortically localized. Results are reported from two independent experiments.

RESULTS

The Third Dlg PDZ Domain Modulates the Interaction of Dlg with GukH—The interaction of Dlg with GukH has been shown to occur through the Dlg GK domain (15). While analyzing fragments of Dlg for their ability to bind to GukH, we found that, although the GUK is able to bind GukH, a fragment that also includes the SH3 domain does not bind (Fig. 1A). This result indicates that the intramolecular interaction between the SH3 and GK domains competes with GukH binding. However, elements NH₂-terminal to the SH3-GK control the ability of Dlg to interact with GukH, as larger fragments of Dlg, including the full-length protein, are able to bind GukH.

We analyzed several Dlg fragments to identify the minimal components necessary for regulation of the Dlg-GukH complex assembly using a GST fusion of the GukH Dlg binding domain (Fig. 1B). A fragment containing the third PDZ domain along with the short linker that connects it to the SH3-GK module is necessary and sufficient to allow GukH binding (Fig. 1A and C). As shown in Fig. 1D, the PDZ-SH3-GK architecture is highly conserved among MAGUK proteins with the number of residues linking the PDZ and SH3 domains ranging from 5 to 40. The functional linkage between the Dlg PDZ and SH3-GK domains and the conserved architecture of these domains in MAGUK proteins suggests that the PDZ domain is an integral component of a larger PDZ-SH3-GK module.

GukH Binds to a Composite Site on Dlg Formed by Both the SH3 and GK Domains—In experiments using purified components, we noticed that a proteolytic fragment of the PDZ-SH3-GK also interacts with GukH (Fig. 1C, starred band). This proteolytic fragment corresponds to a COOH-terminal truncation that lacks the GK domain (based on the molecular weight of the fragment and the presence of the NH₂-terminal His tag), indicating that one or more additional GukH binding sites exist outside of the GK domain. We tested both the Dlg PDZ and SH3 domains for the ability to bind GukH. Consistent with a GukH binding site in Dlg outside of the GK domain, purified fragments of Dlg that lack the GK but contain the SH3 domain are able to bind GukH (Fig. 2A). However, the third PDZ domain is not required for GukH binding in this context. This interaction is qualitatively weaker than with the PDZ-SH3-GK (Fig. 2B), consistent with the SH3 domain being only one part of a larger interaction surface that includes the GK domain. As the region of GukH that binds to Dlg contains several proline-rich sequences (Fig. 2C) and SH3 domains bind to a consensus sequence of PXXP (19), we tested these sequences for their ability to bind the Dlg SH3 domain (using an SH3 domain that lacked the “HOOK” segment that links the SH3 and GK domains). Although MAGUK SH3s deviate from canonical SH3 domains (12), we found that each of the GukH proline-rich segments is able to bind to the Dlg SH3 domain (Fig. 2D). Similar to the interaction of GukH with the Dlg GK domain, the binding site on the SH3 domain is obscured when the GK domain is present (Fig. 2E), presumably because of the intramolecular interaction between the two domains. Another similarity between the two binding sites is that GukH binding is rescued by the presence of the third PDZ domain (Fig. 2E). Binding of proline-rich sequences requires the presence of specific proline residues as mutation of the PXXP to AXXA completely disrupts binding (Fig. 2D). We therefore conclude that GukH binds to a composite binding site formed by the SH3 and GK domains and that this binding site is obscured by the intramolecular interaction between the two domains. The presence of three SH3 ligand sequences in GukH indicates that each GukH may bind multiple Dlg proteins.

Modulation of GukH Binding by COOH-terminal PDZ Ligands—The interplay between the Dlg PDZ and SH3-GK modules appears to be a mechanism for communication between the PDZ and SH3-GK binding sites. PDZ domains are common protein interaction domains that bind short, COOH-terminal sequences present in target proteins (20), although binding to internal motifs can also occur (21). The third PDZ domain from Dlg or its mammalian homologues has been shown to bind two COOH-terminal sequences, one from CRIP1 (cysteine-rich interactor of PDZ; sequence DTNKNYK-QTSV-COOH) and Drosophila neuroligin (sequence KRVIQEISV-COOH) (22, 23). CRIP1 binds to microtubules providing a link between MAGUK scaffolding proteins and the
PDZ binding is similar for neuroligin (data not shown). The difference between PDZ-SH3-GK and CRIPT for the isolated PDZ domain is qualitatively weaker than to the full PDZ-SH3-GK. C, proline-rich sequences present in GukH. An alignment of the three putative SH3 ligand sequences in GukH is shown. D, each of the proline-rich sequences in GukH can bind the Dlg PDZ domain. GST fusions of the GukH proline-rich sequences, as labeled in C, bind to Dlg. Mutation of the proline residues to alanine abrogates binding. E, interdomain regulation of SH3 binding. Similar to the larger GukH fragment, binding of the individual proline-rich sequences is inhibited by the SH3-GK intramolecular interaction and rescued by the PDZ domain.

The communication between the Dlg PDZ and SH3-GK domains must have energetic consequences. To explore the coupling between the two, we measured the affinity of the CRIPT or neuroligin peptide for the isolated PDZ domain and the entire PDZ-SH3-GK fragment using the fluorescence anisotropy of NH2-terminal tetramethylrhodamine (Fig. 3A). These results indicate that PDZ ligand binding influences the GukH (SH3-GK) binding site.

The difference between the affinity for the two fragments is consistent with the coupling of PDZ ligands and GukH binding sites. However, as the affinity for PDZ-SH3-GK is higher than for PDZ alone, the data excludes a simple model in which the Dlg PDZ domain no longer recruits Dlg to the cell cortex. Expression of a GFP fusion of the Dlg binding domain shows a large percentage of cells with cortical localization (Fig. 4C), whereas full-length Dlg is consistently found in the cytoplasm, excluded from the nucleus (Fig. 4D). Expression of CRIPT induces localization of Dlg at the cell cortex in a significant fraction of the transfected cells (Fig. 4E), indicating that CRIPT recruits Dlg to the cell cortex. Expression of a GFP fusion of the Dlg binding portion of CRIPT (which localizes to the cytoplasm) leads to a significant decrease in the fraction of cells with cortical Dlg localization (Fig. 4F), which we interpret as arising from competition between the CRIPT and GukH complexes of Dlg. Additionally, a Dlg mutant in which the PDZ domain no longer rescues the ability to bind GukH (DlgΔA; see below) is not significantly localized to the cortex by GukH. These results are also consistent with mutually exclusive PDZ ligand and GukH-Dlg complexes.

Mechanism of PDZ-based Regulation of the SH3-GK Module—How does binding of COOH-terminal ligands to the PDZ domain alter the ability of SH3-GK to bind GukH? The affinity of SH3-GK for GukH is ~15-fold weaker than to the full PDZ-SH3-GK. As CRIPT and GukH binding sites are likely to be fairly distant from one another, competition through a direct steric mechanism is unlikely. One possible mechanism for PDZ regulation of the SH3-GK module is that the PDZ domain disrupts the intramolecular interaction between the SH3 and GUK domains to expose the composite GUK binding site. In this model, the PDZ-SH3 would fail to interact with the GUK. However, in an intermolecular assay, in which the PDZ-SH3 and HOOK-GK are separately expressed, we find that these two domains are able to bind one another and that this binding is not qualitatively altered by the presence of CRIPT peptide (Fig. 5A). This indicates that the SH3-GK intramolecular interaction is not qualitatively affected by the PDZ domain.
The Dlg PDZ-SH3-GK module contains a conserved linker between the PDZ and SH3 domains (Fig. 5B). In the structure of the Dlg PDZ domain (25), a portion of the sequence following the PDZ domain forms a short helix that packs against the PDZ domain. The conservation and structure of this ~40-residue sequence prompted us to examine the role of the linker in functionally coupling the PDZ and SH3-GK modules. To test the contribution of this sequence to the coupling between CRIP and GukH binding, we constructed a series of PDZ-SH3-GK fragments with short deletions in the sequence. When either half of the linker is removed (Δ1 and Δ2), the PDZ domain no longer efficiently rescues binding of GukH to the composite SH3-GK binding site (Fig. 5C). Combining both deletions (ΔΔ) results in an even more severe effect. In addition, we find that the PDZ domain is unable to relieve GukH inhibition in trans.3

To determine whether the sequence of the linker is important or, alternatively, whether the spacing provided by the linker is only required, we replaced the deleted residues with glycine-serine repeats (Fig. 5C, GS). As this flexible linker is unable to restore GukH binding activity, the linker does not function solely to provide proper spacing between the domains. These results indicate that the covalent attachment of the PDZ and SH3 domains through a conserved linker is necessary for the regulation of the composite GukH binding site and that this regulation does not occur by disruption of the SH3-GK intramolecular interaction.

**DISCUSSION**

We have demonstrated a set of interdomain interactions in the Drosophila tumor suppressor Dlg that leads to regulation of the ligand binding activity of these domains. The Dlg SH3-GK

3 Y. Qian and K. E. Prehoda, unpublished observations.
module, a defining feature of MAGUK proteins, forms a composite binding site for the synaptic protein GukH, but the intramolecular interaction between the SH3 and GK domains obscures this binding site. The third PDZ domain from Dlg relieves this inhibition, making use of the short linker that attaches it to the SH3 domain. Binding of CRIPT to the PDZ domain induces a change in the SH3-GK module that again obscures the GukH binding site, effectively leading to competition between CRIPT and GukH binding that influences the organization of Dlg-mediated protein complexes in a cellular context. The active scaffolding of Dlg complexes does not utilize disruption of the SH3-GK intramolecular interaction but requires a conserved, structured linker that attaches the PDZ and SH3 domains.

**PDZ-based Regulation of MAGUK Complex Formation**—How might the third PDZ domain from Dlg expose the composite GukH binding site within the SH3-GK module? Our data exclude a simple model in which the PDZ domain directly competes against the interaction between the SH3 and GK domains as the PDZ-SH3 and GK domains bind to one another in an intermolecular assay (Fig. 5A). We propose that the PDZ domain stabilizes the linker that connects it to the SH3 domain and that this linker interacts with the SH3 domain to induce an SH3-GK conformation that allows for GukH binding. The structure of the PSD-95 SH3-GK indicates that the SH3 ligand binding site is obscured in the closed conformation. The PDZ must therefore alter the position of residues that occupy the PDZ ligand binding site to allow for GukH binding. Although there is no direct structural information on the position of the PDZ domain, based on the N terminus of the SH3 domain, we can infer that the approximate position of the PDZ domain is likely to be in close proximity to the PX domain binding site. Binding of CRIPT would then return the SH3-GK to its basal conformation.

**Active Scaffolding of MAGUK Complexes**—Cellular signaling relies on the formation of specific protein complexes, and scaffolding proteins play a central role in this process (26). Although scaffolds are critical components of many signaling pathways, their exact function has remained unclear (27). In particular, are scaffolds simple tethers that passively bind to their many ligands or is ligand binding actively regulated? The answers to these questions have significant implications for the types of scaffold-mediated complexes that are formed in cells. In the yeast mitogen-activated protein (MAP) kinase scaffold Ste5, heterologous protein interaction domains can functionally replace the native kinase recruitment modules, although at reduced levels (28). As the heterologous domains are unlikely to participate in interactions that would lead to regulated binding of scaffold ligands, this suggests that certain scaffolds may function in a passive manner. However, the fact that domain-swapped Ste5 scaffolds do not function at wild-type levels leaves open the possibility that this scaffold also has characteristics of an active scaffold. Clearly, in the case of Dlg, however, the complexes that it forms are regulated by dynamic interdomain interactions. We are currently assessing how these interactions affect the diversity of Dlg complexes that might be formed in different cellular contexts.

**FIGURE 5. Modulation of GukH binding leaves the Dlg intramolecular interaction intact and requires the PDZ-SH3 linker.** A, the Dlg PDZ domain does not inhibit the SH3-GK intramolecular interaction. A GST fusion of a fragment containing the PDZ and SH3 domain is able to pull down a fragment containing the HOOK (the linker between the SH3 and GK domains) and GK. The presence of saturating concentrations of CRIPT peptide also does not affect the interaction. B, conservation of the linker PDZ-SH3-GK module. An alignment of PDZ SH3 linker sequences from diverse Dlg sequences shows the high degree of conservation of this portion of the protein. C, the conserved PDZ-SH3 linker is required for PDZ rescue of GukH binding. Deletions in the linker between the PDZ and SH3 domains renders the PDZ domain unable to rescue GukH binding to SH3-GK. Δ1, Δ569–574; Δ2, Δ584–593; ΔΔ, both deletions; GS, deleted residues replaced with glycine-serine repeats.
tion, which binds GukH with low affinity. This would explain why the affinity of CRIPT for the PDZ-SH3-GK fragment is higher than that for the PDZ alone. Such a supramolecular interaction induced by a PDZ ligand has been observed in the PDZ-regulated protease DegS (29, 30) (see below).

The PDZ domain of the MAGUK PSD-93 has also been shown to be involved in the regulation of its SH3-GK module, although in this case, the PDZ domain negatively regulates ligand binding to SH3-GK. A fragment of PSD-93 containing the SH3 and GK domains binds to microtubule-associated protein 1A (MAP1A), but MAP1A fails to bind to full-length PSD-93 (31). Binding of MAP1A is restored by the presence of a COOH-terminal ligand for the third PSD-93 PDZ domain, although the other two PDZ domains appear to play a small role. In this system, the PDZ domain appears to repress binding to the SH3-GK and the PDZ ligand somehow restores binding. The distinct behavior of these two systems indicates that ligands can utilize the interdomain interactions in MAGUK proteins to achieve very different regulatory effects.

Regulation of interactions that are modulated by the SH3-GK intramolecular interaction have also been shown to occur by PDZ-independent mechanisms. The GK domain from the Dlg homologue SAP97 binds to GKAP (32). GKAP binding is inhibited by the SH3-GK intramolecular interaction (14). In this case, binding is rescued by an L27 domain present at the very NH2 terminus of the protein. However, how GKAP binding might be modulated in the context of the full-length protein is unknown.

PDZ domains have been utilized in the regulation of diverse functions including the enzymatic activity of proteases. In the DegS protease, which is responsible for initiation of the misfolded protein proteolytic cascade in the periplasm of bacteria, a NH2-terminal PDZ domain regulates the protease domain activity using a mechanism that may be similar to PDZ regulation of the Dlg SH3-GK module. In DegS, the PDZ domain does not directly repress the protease domain. Instead, the protease is normally found in an inactive conformation (30). Activation occurs when a COOH-terminal ligand binds to the PDZ domain, which induces an interaction between a loop within the protease domain and PDZ ligand. This interaction causes a large change in the protease to an active conformation. Such an interaction with the ligand of the Dlg PDZ domain would be consistent with the higher affinity of the PDZ-SH3-GK module for this ligand.

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