Functional Analysis of the Nucleotide Binding Domain of Membrane-associated Guanylate Kinases*

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Membrane-associated guanylate kinases (MAGUKs) regulate cellular adhesion and signal transduction at sites of cell-cell contact. MAGUKs are composed of modular protein-protein interaction motifs including L27, PDZ, Src homology (SH) 3, and guanylate kinase domains that aggregate adhesion molecules and receptors. Genetic analyses reveal that lethal mutations of MAGUKs often occur in the guanylate kinase domain, indicating a critical role for this domain. Here, we explored whether GMP binding to the guanylate kinase domain regulates MAGUK function. Surprisingly, and in contrast to previously published studies, we failed to detect GMP binding to the MAGUK postsynaptic density-95 (PSD-95) and CASK. Two amino acid residues in the GMP binding pocket that differ between MAGUK and authentic guanylate kinase explain this lack of binding, as swapping these residues largely prevent GMP binding to yeast guanylate kinase. Conversely, these mutations restore GMP binding but not catalytic activity to PSD-95. Protein ligands for the PSD-95 guanylate kinase domain, guanylate kinase-associated protein (GKAP) and MAPI1A, appear not to interact with the canonical GMP binding pocket, and GMP binding does not influence the intramolecular SH3/guanylate kinase (GK) interaction within PSD-95. These studies indicate that MAGUK proteins have lost affinity for GMP but may have retained the guanylate kinase structure to accommodate a related regulatory ligand.

Tissue development, differentiation, and physiology require specialized cellular adhesion and signal transduction at sites of cell-cell contact. Scaffolding proteins that tether adhesion molecules, receptors, and intracellular signaling enzymes organize macromolecular protein complexes at cellular junctions to integrate these functions. One family of such scaffolding proteins is the large group of membrane associated guanylate kinases (MAGUKs) (1–4). Genetic studies have highlighted the critical roles for MAGUK proteins in the development and physiology of numerous tissues from a variety of metazoan organisms. Mutation of Drosophila discs large (dlg) disrupts epithelial septate junctions and causes overgrowth of the imaginal discs (5). Similarly, mutation of lin-2, a related MAGUK in Caenorhabditis elegans, blocks vulval development (2), and mutation of the postsynaptic density protein PSD-95 impairs synaptic plasticity in mammalian brain (6).

MAGUK proteins are composed of a set of protein-protein interaction domains that explain their important roles in organizing protein complexes at sites of cell-cell contact. MAGUKs contain L27 heterodimerization domains, one or three PDZ domains, an SH3 domain and a C-terminal domain homologous to guanylate kinase (GK) (7, 8). PDZ domains are small modular motifs that contain a single peptide binding groove that associates with specific sequences often found at the extreme C termini of interacting ion channels (9, 10), cell adhesion molecules (11, 12), and other membrane-associated proteins (13–16). Multiple PDZ domains in PSD-95 organize and accelerate signal transduction at synapses by linking receptors to downstream signaling enzymes (17, 18).

Whereas the functional roles for PDZ domains in MAGUKs are well established, functions for the SH3 and GK domains are less certain. SH3 domains classically bind to proline-rich motifs (19–23); however, the structure of the PSD-95 SH3 domain suggests that such interactions are unlikely because a conserved helix in MAGUK SH3 domains occludes the canonical polyproline binding site (24, 25). A variety of high affinity protein ligands have been identified for GK domains from several MAGUKs (26–30), but it is not yet clear how these interactions regulate MAGUK functions. In addition to binding exogenous ligands, protein fragments containing the proposed SH3 and GK regions of MAGUK proteins interact with each other (31–34). The crystal structure of the SH3/GK region of PSD-95 reveals that this interaction is the assembly of the SH3 fold from discontinuous structural components (24, 25). This SH3/GK interaction, may oligomerize MAGUK scaffolds, but factors that regulate intermolecular SH3 assembly remain uncertain. Despite our limited understanding of the biochemical roles for the SH3 and GK domains, these regions are clearly critical as most genetically identified mutations of MAGUKs occur in the SH3 and GK domains (2, 5).

The GK domains of MAGUKs share 40% sequence homology with authentic GK enzymes, which phosphorylate GMP to form GDP. The connection of MAGUK GK domains to guanine nucleotide metabolism is uncertain. Most MAGUKs lack enzymatic activity, although p55, a MAGUK in red blood cells, apparently shows modest GK catalysis (35). Rather than having robust GK activity MAGUKs are suggested to bind potently to GMP (36, 37), which may then regulate GK domain interactions.

To explore for a regulatory role of GMP on MAGUK function,
GMP Binding to MAGUK Proteins

We have now quantitated GMP binding using a straightforward and sensitive assay, equilibrium dialysis. Surprisingly, we found that MAGUK proteins PSD-95 and CASK fail to interact detectably with GMP, whereas yeast GK (YGK) binds with an affinity of 30 μM. This lack of binding by MAGUKs is explained in part by two residues in the GMP binding pocket of YGK that are not conserved in MAGUK proteins. Mutating these residues in YGK to those in PSD-95 drastically reduces GMP binding by YGK. Conversely, replacing these two residues in PSD-95 with those occupying the identical positions in YGK facilitates GMP binding, albeit of low affinity (~1 mM), but does not confer GK activity on the double mutant PSD-95 GK domain. This double mutant PSD-95 construct can still properly assemble the SH3 domain as well as bind the exogenous GK ligands GKAP and MAP1A, and these protein binding activities of the double mutant PSD-95 are not competitive with GMP. These studies demonstrate that MAGUK proteins lack detectable GMP binding activity and suggest that other related ligands may associate with the canonical GMP binding pocket in MAGUK proteins.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—DNA sequences encoding YGK (residues 2-187), rat PSD-95 (SH3GK, residues 417-724; GK, residues 532-711), and rat CASK (SH3GK, residues 591-909) were amplified by PCR and cloned in-frame into a His6-tagged expression vector (16). Mutations were introduced by site-directed PCR-based mutagenesis. Encherichia coli strain BL21 (DE3) (Stratagene) expressing PSD-95 and YGK constructs were grown in LB to an OD600 of 0.8, induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h, harvested by centrifugation, and sonicated in MTPBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 2 mM EDTA). Approximately 20 μg of GST fusion protein was coupled to glutathione-Sepharose (Amersham Biosciences) beads and washed extensively with MT-PBS. Prior to the interaction assays, His6-tagged proteins, COS cell lysate, and coupled GST proteins were incubated in the presence or absence of 5 mM GMP at 100°C. Coupled GST fusion proteins were then incubated with 20 μg of His6-tagged fusion proteins or 80 μL of COS cell lysate for 40 min ≥ 5 mM GMP at 4°C and extensively washed with either Buffer A or TEEN ≥ 5 mM GMP. Retained proteins were eluted with SDS protein loading buffer, separated by PAGE, and analyzed by immunoblotting.

RESULTS

We assessed possible binding of [3H]GMP to the SH3GK domain of PSD-95 by equilibrium dialysis. For a typical experiment, we dialyzed SH3GK or YGK at 100 μM protein concentration in a volume of 100 μL against a 1-liter volume of buffer containing [3H]GMP at varying concentrations. When only tracer concentrations of [3H]GMP were present in the buffer (100 nM), the YGK dialysis compartment accumulated [3H]GMP levels 3-fold those in the dialysis buffer. On the other hand, dialysate containing either the SH3GK region of PSD-95 or of CASK showed no increased accumulation of [3H]GMP above that in the dialysis buffer.

Previous studies have suggested that the SH3GK domain of PSD-95 binds GMP with an affinity of 300 nM (36). Such an affinity would have yielded counts in the dialysis bag ≥250-fold above those in the dialysis buffer in our experiments containing only tracer levels of [3H]GMP. An affinity as low as 1 μM would have yielded counts in the dialysis bag 10% above those in the buffer, and this would have been detectable. Therefore, we conclude that the SH3GK domains of PSD-95 and CASK do not bind GMP with affinities <1 μM. Our protein appeared stable during the dialysis, as it was not degraded when evaluated by SDS-PAGE (Fig. 1A), and dialyzed PSD-95 SH3GK was able to specifically interact with GST-MAP1A (data not shown).

By varying the concentrations of [3H]GMP in the dialysis buffer, we determined the affinity for YGK to be ~30 μM (Fig. 1A and Table I), which is similar to that reported by others (39). We also evaluated GK activity and found that YGK was active with a Km of ~40 μM, but that neither PSD-95-SH3GK nor CASK-SH3GK showed catalytic activity (Fig. 1B and Table I).

The SH3 domain structurally flanks the GK domain in MAGUK proteins potentially constraining the GK domain from binding GMP. We therefore expressed the GK domain from PSD-95 alone. Again, we found that the isolated GK domain failed to bind [3H]GMP (Fig. 2). A previously published crystal structure of the SH3GK domain of PSD-95 showed that GMP co-crystallized with PSD-95 only in the presence of 100 mM added guanidine (25). However, we found that addition of guanidine did not influence [3H]GMP binding to PSD-95 (Fig. 2).

The GK domain of PSD-95 shares 40% sequence identity with YGK (Fig. 3A), and the crystal structures of these domains are similar (24). The crystal structure of YGK bound to GMP showed that nine residues from the enzyme make specific contact with the GMP molecule (40). Seven of these nine residues are conserved in the GK domains of PSD-95 and CASK (Fig. 3, A and B). To test whether these amino acid differences explained the failure of PSD-95 or CASK GK domains to bind GMP, we first mutated one or both of these residues in YGK and evaluated the effects on GMP binding and GK activity. Strikingly, we found that mutation of serine 35 in YGK to
proline, which resembles the residue in PSD-95 and is conserved within all PSD-95 members, reduced the affinity for GMP by 40-fold (Table I). This mutation also drastically reduced the guanylate kinase activity nearly 1000-fold (Fig. 4 and Table I). A double mutation of YGK, changing both serine 35 to proline and aspartic acid 101 to serine to mimic that in PSD-95, yielded an enzyme with compromised binding proper-

ties (Table I) and GK catalytic activity similar to the single mutation (Fig. 4).

These changes suggested that differences at these crucial residues may explain our failure to detect \(^3\text{H}\)GMP binding to PSD-95. Therefore, we mutated these residues in the SH3GK domain of PSD-95 to resemble those in YGK. This double mutant of PSD-95 showed very weak binding to GMP (\(K_d = 1.3 \text{ mM}\); Table I). This mutant, however, lacked guanylate kinase activity (Fig. 4).

Rather than binding to GMP, PSD-95 interacts with several synaptic proteins, including GKAP/SAPAP (synapse-associated protein-associated protein) (29, 30) and MAP1A (26). We therefore asked whether the canonical GMP binding pocket in PSD-95 might be the site for interaction with these proteins. To evaluate this possibility, we examined binding of GKAP to PSD-95 GK constructs containing the mutations in the GMP binding pocket that permit low affinity GMP binding. As previously reported, we found that the isolated GK domain of PSD-95 expressed as a GST fusion protein binds to GKAP protein expressed in heterologous cells in a "pull-down" assay. The double mutation of the GK domain of PSD-95 that permits binding to GMP does not abolish binding to GKAP (Fig. 5A).

Similarly, an SH3GK construct containing the double mutations in the GMP binding site also preferentially assembled in an intramolecular manner as it failed to bind to the partial SH3 domain. Addition of GMP failed to promote an intermolecular SH3/GK interactions (Fig. 5C), suggesting that...

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**TABLE I**

| Specific activity  | \(K_d\) (mM) | \(K_m\) (mM) | Specific activity |
|-------------------|-------------|-------------|-----------------|
| YGK              | 28.5 ± 4.3  | 40.6 ± 0.1  | 1581 ± 39       |
| YGK S35P         | 1.22 ± 0.3  | 2.01 ± 0.5  | 2.5 ± 0.2       |
| YGK S35P/D101S   | 1.1 ± 0.3   | 2.5 ± 0.3   | 2.5 ± 0.2       |
| SH3GK           | ND          | ND          | ND              |
| SH3GK*          | 1.3 ± 0.2   | ND          | ND              |

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**Fig. 1.** PSD-95 and CASK do not bind GMP or have GK activity. A, YGK (closed circles) binds GMP with an affinity of \(29 \text{ mM}\) as determined by equilibrium dialysis. In contrast, GMP binding was not observed for the SH3GK domains of PSD-95 (open circles) and CASK (closed triangles). Little to no degradation of protein occurred during dialysis as shown by the Coomassie-stained gel. B, among the proteins tested, only YGK was catalytically active (\(K_m = 41 \text{ mM}\)).

**Fig. 2.** Removal of the SH3 domain does not facilitate GK binding of GMP. Expression of the GK domain alone (open circles) is not sufficient to promote GMP binding. Additionally, 100 mM guanidine, which may help coordinate GMP in the nucleotide binding pocket, does not facilitate GMP binding by PSD-95 (closed triangles).
Fig. 3. The GK domains of MAGUKs resemble YGK. A, amino acid sequences corresponding to the GK domains of rat PSD-95 and rat CASK aligned to YGK using CLUSTALW. Shaded amino acids show identity, boxes denote similarity, and asterisks mark the amino acids in YGK that bind GMP. Circled asterisks specify GMP binding residues in YGK that are not conserved in PSD-95. Arrows and cylinders above the sequences represent β-strand and α-helix secondary structures, respectively. B, residues that bind GMP in YGK superimposed with the corresponding amino acids of PSD-95. The amino acid residues in YGK that coordinate the phosphate group of GMP are completely conserved in identity and spatial position in PSD-95. In contrast, two of the four residues in YGK (marked by asterisks) that bind the guanine ring are different in PSD-95.

Fig. 4. Mutation of the GMP binding residues of YGK to those in PSD-95 dramatically reduces GK activity. The YGK S35P (open circles) and S35P/D101S (open triangles) mutants have a significantly higher K_m and dramatically reduced velocity compared with the wild type (closed circles) enzyme (note different scales, left axis for closed symbols and right axis for open symbols). Despite conferring GMP binding to PSD-95, the F564S/S631D mutations (95-SH3GK*) do not produce a catalytically active enzyme (open squares).

the GMP binding site is not involved in assembly of SH3GK modules.

**DISCUSSION**

This study shows that the GK domains of MAGUK proteins PSD-95 and CASK do not bind to GMP with detectable affinity. This lack of binding is explained in part by two residues that differ between MAGUK proteins and authentic GK enzymes. However, even a PSD-95 protein with "back mutations" to contain these residues shows only weak binding, which suggests that other differences between MAGUK proteins and authentic GK also contribute to the lack of binding. Our study also suggests that the residues involved in GMP binding likely do not contribute to interactions of MAGUK proteins with certain neuronal protein ligands, including GKAP and MAP1A.

Previous studies suggested that MAGUK proteins bind to GMP with very high affinity (36, 37). One study showed that full-length PSD-95 as a GST fusion protein bound to a Cibacron blue dye column and could be eluted from such a column with 1 mM GMP (36). We repeated those experiments and indeed found that PSD-95 either expressed in bacteria or from brain extracts does bind to a Cibacron blue dye column. However, we were unable to elute PSD-95 with concentrations of GMP up to 1 mM (data not shown). Reasons for this discrepancy are unclear; it is conceivable that differences in the Cibacron blue preparation might explain the disparate results. Another study used equilibrium dialysis and found that the GK region of CASK binds to [3H]GMP with an affinity of 300 nM (37). This high affinity, which is 100-fold greater than that of YGK for GMP, is very difficult to reconcile with our results. Unfortunately, the binding reported in that study (37) was presented as "data not shown," so it is impossible for us to compare our conditions with theirs.

Conceivably, the GK constructs used in our study could have failed to bind GMP, because they were inactivated during pu-
His6-GK fusion proteins of the wild type or the GMP binding mutant. The SH3/GK interaction. Binding of His6-GK fusion constructs to GST MAP1A to either of the GK fusion proteins.

Wild type (95-GK*) fusions of the PSD-95 GK domain were incubated with lysates from CASK crystals. Indeed, a guanidine bound near the GMP and formed guanidine (25), an additive screened to obtain high quality crystallization with GMP was achieved only in the presence of 100 mM. As no crystal structure has yet been solved for a MAGUK molecule occupies this pocket and how this binding controls function has not yet been investigated at the genetic level. Future biochemical and genetic studies of this region are warranted. Also critical will be to determine whether another nucleotide or other phosphorylated molecule occupies this pocket and how this binding controls MAGUK protein function.

Fig. 5. GMP does not influence PSD-95 interactions. A, GST fusions of the PSD-95 GK domain were incubated with lysates from COS cells expressing GFP-GKAP. GKAP bound similarly to both the wild type (GK) and GMP binding mutant (95-GK*), in the presence or absence of 5 mM GMP. B, GST fusions of MAP1A were incubated with His6-GK fusion proteins of the wild type or the GMP binding mutant. Similar to the GKAP interactions, GMP did not influence binding of MAP1A to either of the GK fusion proteins. C, GMP does not influence the SH3/GK interaction. Binding of His6-GK fusion constructs to GST constructs containing strands A–D of the SH3 domain (GST 95-SH3) was evaluated by pull-down assay. Whereas the GK domain flanked by the E and F strands of the SH3 domain (His 95-E-GK-F) binds, the intact SH3GK domain does not. An SH3GK construct containing the two GMP binding mutations (SH3GK*) also fails to bind. In all cases, GMP does not promote SH3/GK binding.

We feel this is unlikely for three reasons. First, our proteins migrated as single bands by SDS-PAGE and were not degraded during the dialysis experiments. Second, we used very mild conditions for purification of these proteins from bacteria. Third, our GK constructs retained binding to other ligands such as GKAP and MAP1A. Another possibility is that GMP may have been tightly bound to our constructs purified from bacteria; therefore, the site would have been unavailable for binding to exogenous GMP. This is also unlikely because the SH3GK protein used here is identical to that which we previously crystallized and found no GMP in the canonical binding site (24).

Crystal structures of GK domains from PSD-95 and CASK have yielded ambiguous results concerning GMP binding (24, 25, 37). For all three published structures, GMP did not copurify with the GK domain from bacteria and was not found in the native structure. Attempts to co-crystallize CASK with added GMP were unsuccessful (37). For PSD-95, co-crystallization with GMP was achieved only in the presence of 100 mM guanidine (25), an additive screened to obtain high quality crystals. Indeed, a guanidine bound near the GMP and formed one of the crucial hydrogen bonds with the phosphate group of GMP and with an Asp residue from a crystallographically related GK molecule (25). Based on these results and our new findings, GMP binding in the crystal likely occurred as a result of the crystallization conditions and likely has no biological relevance.

Rather than binding GMP, the GK domain of MAGUK proteins associate with specific protein ligands such as GKAP (29, 30), MAP1A (26), BEGAIN (41), GAKIN (28), and SPAR (42). As no crystal structure has yet been solved for a MAGUK bound to a protein ligand, the binding site(s) for these protein ligands remain(s) uncertain. One reasonable location would be the crevice into which GMP sits in the structure of YGK. Our experiments suggest that this is not the site for binding to MAP1A or GKAP. That is, mutation of two residues in this binding pocket that produces weak binding to GMP do not abolish binding to MAP1A or GKAP. Furthermore, adding high concentrations of GMP to these mutants, which should occupy the nucleotide binding site, does not prevent additional contemporaneous binding of MAP1A or GKAP. This does not exclude the possibility that MAP1A and GKAP interactions are not disrupted, because the GMP binding affinity is too low to efficiently compete. Future structural studies of MAGUK proteins complexed with ligands would seem essential to determine decisively binding sites for these protein partners.

Why might the GK structure be so tightly conserved in MAGUK proteins? This conservation includes all four residues that coordinate the phosphate of GMP and three of five residues that bind the guanine base. This may suggest that MAGUK proteins interact with a distinct phosphorylated nucleotide or even a phosphorylated residue from a polypeptide. Our results here showing that back mutations of the residues in PSD-95 that differ from GK restore weak binding suggests that MAGUK proteins may have lost their ability to bind GMP in favor of a distinct ligand.

In addition to binding exogenous ligands, the SH3 and GK domains of MAGUK proteins mediate a unique intramolecular association (31–34). This SH3/GK interaction actually reflects assembly of the unique MAGUK SH3 domain, which has a peculiar pair of β-strands that surround the GK domain (24, 25). Interestingly, this assembly of MAGUK SH3 domains can occur in both an intramolecular as well as an intermolecular fashion and can thereby mediate a regulated oligomerization of MAGUK proteins (32, 33, 43). It is unlikely that residues of the NBD are directly involved in intermolecular assembly, as the GK double mutant retains the ability to interact with the SH3 domain (data not shown). Instead, this swapping between monomer and MAGUK oligomer may be regulated by protein interactions or increased rigidity of the HOOK insert within the SH3 domain (24, 32). It is conceivable that conformational changes associated with binding sites in the GK domain of MAGUKs could also induce oligomerization.

Although biochemical functions for the SH3GK region of MAGUK proteins remain uncertain, genetic studies indicate that this region is critical for MAGUK function (2, 5). A single point mutation in the SH3 domain of Drosophila discs large yields a lethal phenotype, as do small truncations in the GK domain (5). Furthermore, truncations of the GK domain of lin-2 in C. elegans cause a vulvaless phenotype (2). These mutations all disrupt the previously identified SH3/GK interaction, suggesting a critical role for that mode of protein assembly (31). Whether the largely conserved GMP binding pocket also is essential for MAGUK protein function has not yet been investigated at the genetic level. Future biochemical and genetic studies of this region are warranted. Also critical will be to determine whether another nucleotide or other phosphorylated molecule occupies this pocket and how this binding controls MAGUK protein function.
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