Intramolecular interactions regulate SAP97 binding to GKAP

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Membrane-associated guanylate kinase homologs (MAGUKs) are multidomain proteins found to be central organizers of cellular junctions. In this study, we examined the molecular mechanisms that regulate the interaction of the MAGUK SAP97 with its GUK domain binding partner GKAP (GUK-associated protein). The GKAP–GUK interaction is regulated by a series of intramolecular interactions. Specifically, the association of the Src homology 3 (SH3) domain and sequences situated between the SH3 and GUK domains with the GUK domain was found to interfere with GKAP binding. In contrast, N-terminal sequences that precede the first PDZ domain in SAP97, facilitated GKAP binding via its association with the SH3 domain. Utilizing crystal structure data available for PDZ, SH3 and GUK domains, molecular models of SAP97 were generated. These models revealed that SAP97 can exist in a compact U-shaped conformation in which the N-terminal domain folds back and interacts with the SH3 and GUK domains. These models support the biochemical data and provide new insights into how intramolecular interactions may regulate the association of SAP97 with its binding partners.

Keywords: cell adhesion/GKAP/molecular modeling/SAP97/SH3

Introduction

The rat synapse-associated protein SAP97 and its human homolog hDlg are members of a family of membrane-associated guanylate kinase homologs (MAGUKs). MAGUKs are thought to play central organizational roles in the assembly of distinct membrane specializations such as synaptic and tight junctions (Fanning and Anderson, 1998; O'Brien *et al.*, 1998; Garner *et al.*, 2000) and have been implicated in the suppression of epithelial and brain tumors (Woods *et al.*, 1996; Thomas

et al., 1997; De Lorenzo *et al.*, 1999; Pim *et al.*, 2000). MAGUK family members are composed of one or more PDZ domains, a Src homology 3 (SH3) domain and a guanylate kinase (GUK)-like domain (Fanning and Anderson, 1998; Hata *et al.*, 1998; Garner *et al.*, 2000). SAP97 belongs to a subfamily of MAGUKs that includes SAP97/hDlg, SAP90/PSD-95, SAP102/NhDlg, PSD93/Chapsyn110 and *Drosophila* DLG (Hata *et al.*, 1998; Garner *et al.*, 2000). In neuronal cells, SAP97 subfamily members have been localized to the cytoskeletal matrix assembled at the cytoplasmic face of synaptic junctions (Müller *et al.*, 1995; Sans *et al.*, 2000). In addition to its pre- and postsynaptic localization, SAP97 is found along the epithelial lateral membrane and at the lymphocyte immune synapse (Lue *et al.*, 1994; Müller *et al.*, 1995; Reuver and Garner, 1998). Recruitment to these cellular contact sites is promoted by cell–cell adhesion and requires the assembly of the cortical cytoskeleton (Reuver and Garner, 1998). Protein sequences N-terminal to the first PDZ domain (referred to as the S97N region) and those situated between the SH3 and GUK domains (referred to as the U5 region) have been shown to be essential for efficient localization and cytoskeletal attachment of SAP97 at cell–cell adhesion sites (Wu *et al.*, 1998). The former has recently been found to be a multimerization domain (Marfatia *et al.*, 2000), while the latter a protein 4.1 binding site (Lue *et al.*, 1994).

Each conserved domain in SAP97 subfamily members is also a site of protein–protein interaction (Hata *et al.*, 1998; O'Brien *et al.*, 1998; Garner *et al.*, 2000). For example, PDZ domains bind voltage- and ligand-gated ion channels, cell adhesion molecules as well as cytoskeletal components, while SH3 domains interact with PXXPR-like sequences in several proteins (Garcia *et al.*, 1998; Hata *et al.*, 1998; Garner *et al.*, 2000). Interacting partners of the GUK domain include members of the GKAP/SAPAP1/DAP1 family (Kim *et al.*, 1997; Satoh *et al.*, 1997; Takeuchi *et al.*, 1997), the microtubule-associated protein MAP1A (Brenman *et al.*, 1998) and brain-enriched guanylate kinase-associated protein (BEGAIN) (Deguchi *et al.*, 1998). These observations have led to the hypothesis that SAP97 is an adaptor protein involved in the assembly of macromolecular protein complexes at various membrane specializations. This concept is supported by studies on DLG, the *Drosophila* homolog of SAP97, showing that DLG plays an essential role in the suppression of tumors (Woods *et al.*, 1996; Thomas *et al.*, 1997; De Lorenzo *et al.*, 1999) and the assembly of neuromuscular junctions (Budnik, 1996). Moreover, deletion studies on DLG have revealed that the GUK domain plays an essential role in its localization at neuromuscular junctions, implicating its association with GKAP family members in the recruitment of SAP97 family members to synaptic junction (Thomas *et al.*, 2000).

A fundamental question regarding the assembly of macromolecular protein complexes by MAGUKs at specific membrane specializations is what regulates the availability of individual domains for their binding partners. In SAP90/PSD-95, ligand binding to the PDZ domains has been found to promote MAP1A binding to the GUK domain (Brenman *et al.*, 1998), indicating that intrinsic features within MAGUKs can modulate access of interacting proteins to specific subdomains. Recently, the SH3 domain in SAP90/PSD-95 was found to interact with its own GUK domain (McGee and Bredt, 1999; Shin *et al.*, 2000). A role for this intramolecular interaction in regulating access to the GUK or SH3 domains has not been described.

In this study, we have defined elements in SAP97 that regulate GKAP binding. Our results show that intramolecular interactions of the SH3 domain and U5 region with the GUK domain prevent GKAP binding. In contrast, S97N counteracts the inhibitory effect of the SH3 and U5 regions and facilitates GKAP binding. To gain insights into the 3D structure of SAP97, we developed molecular models based on the crystal structures of PDZ, SH3 and GUK domains from other proteins (Stehle and Schulz, 1992; Maignan *et al.*, 1995; Doyle *et al.*, 1996; Morais Cabral *et al.*, 1996). These independently derived models allow a better geometric understanding of the intramolecular regulation of GKAP binding and suggest a fundamental role for intramolecular interactions within SAP97 in its recruitment and assembly at cellular junctions.

Results

Dual regulation of GKAP binding to SAP97

In a previous study, we observed that full-length SAP97 and SAP90 exhibited different binding affinities to GKAP compared with their individually expressed GUK domains (Kuhlendahl *et al.*, 1998). This suggests that sequences N-terminal to the GUK domains may modulate GKAP binding. To explore this possibility, we assessed the relative binding affinities of several SAP97 deletion constructs for GKAP. Initially, an ELISA-based binding assay was employed to compare the ability of a recombinant GKAP containing a six-histidine (His₆) and thioredoxin (thio) epitope tag (thio-GKAP) to bind a glutathione *S*-transferase (GST)-tagged full-length SAP97 (GST-SAP97), the C-terminal half of SAP97 (GST-SG97) or the GUK domain alone (GST-GUK) (Figure 1A). Whereas full-length SAP97 and GUK bound GKAP with similar affinity, SG97 failed to bind GKAP (Figure 1B). This result was confirmed in a yeast two-hybrid (YTH) binding assay for which the GKAP cDNA was placed in the prey vector (pGAD10) and SAP97 cDNAs were inserted into the bait vector (pBHA5). Again, GKAP bound the GUK domain alone, but not SG97 (Figure 1A). To further explore this finding in living cells, GKAP was co-transfected into CACO-2 cells together with GFP-tagged SAP97, SG97 or GUK. When transfected alone, GKAP exhibited a lateral membrane distribution identical to endogenous SAP97 (Figure 1C; Reuver and Garner, 1998). Co-expression of GKAP with GFP-tagged SAP97 did not alter this pattern. However, when GKAP was co-expressed with GFP-GUK,

a diffuse cytoplasmic pattern was observed for both recombinant proteins (Figure 1C). In the absence of GKAP, GFP-GUK remained cytoplasmic (Wu *et al.*, 1998). These results suggest that the lateral membrane localization of GKAP in these cells is mediated by its binding to the GUK domain of endogenous or recombinant SAP97. GFP-SG97, co-expressed with GKAP, remained cytoplasmic and had no effect on the lateral membrane localization of GKAP (Figure 1C). This finding indicates that SG97 does not interact with GKAP and thus is unable to block the recruitment of GKAP to the lateral membrane. Together, these results suggest that sequences situated between PDZ3 and GUK domains interfere with GKAP binding, while regions situated N-terminal to SH3 facilitate GKAP binding.

SH3 and U5 regions hinder GKAP binding to SAP97

N-terminal of the GUK domain in SG97 are three discernable regions, the SH3 domain and two unique regions called U4 and U5 (Figure 1A). In tyrosine kinases, such as *Src*, the SH3 domain functions as a *cis*-acting negative regulator of kinase activity (Andreotti *et al.*, 1997; Moarefi *et al.*, 1997; Tatosyan and Mizenina, 2000). Recent studies have shown that the U5 region in SAP97 contains a binding site for protein 4.1 (Lue *et al.*, 1994) and most likely also a calmodulin (CaM) binding site (CBS, Figure 1A), originally detected in SAP102/NE-Dlg (Masuko *et al.*, 1999). The 4.1 binding site is present within the I3 insert (Figure 1A). I3 is one of several different inserts present in SAP97/hDlg, including I2, that can be found in the U5 region and arise via alternative splicing (Lue *et al.*, 1994; Müller *et al.*, 1995; Mori *et al.*, 1998). To identify which of these regions interferes with GKAP binding, GKAP binding to a series of SG97 deletion constructs was assessed by ELISA. While deleting the U4 region had no effect on the inhibition of GKAP binding, deleting U4 and the SH3 domain or just the SH3 domain permitted GKAP to bind with an affinity similar to the GUK domain alone (Figure 2). Deleting the middle part of U5 including I3 (I0) allowed GKAP binding to SG97 albeit with a lower affinity than SG97ΔSH3. These results indicate that SH3 and U5 act in concert to prevent GKAP binding.

Intramolecular interactions in SAP97 regulate GKAP binding

To assess whether the inhibitory action of the SH3 and U5 regions might be mediated by physically masking the GKAP binding site on the GUK domain, we examined whether either region could bind the GUK domain directly. By ELISA, the isolated SH3 and U5 regions interacted *in trans* with the GUK domain with a slightly lower affinity than a construct containing both domains (U4-SH3-U5) (Figure 3A). The isolated U4 region exhibited only weak binding to the GUK domain (Figure 3A). To test whether the SH3 and U5 regions also interact *in cis* with the GUK domain, we assessed whether the SH3 domain binds SG97 *in trans*. Utilizing a two-hybrid binding assay, the SH3 domain bound the GUK domain alone, but not SG97 (Figure 3B). This indicates that the intramolecular *cis*-interaction between

SH3 and GUK domains is preferred over the intermolecular *trans*-interaction.

Classically, SH3 domains interact with proline-rich motifs (consensus sequence -P-X-X-P-R) in their binding partners (Pawson and Scott, 1997; Sicheri *et al.*, 1997). Although the GUK domains of SAP97 and SAP90 do not contain classical -P-X-X-P-R sequences, there are several regions that resemble class II polypyrroline sequences. We therefore examined whether mutations in the SH3 domain (SH3P489L or SH3W470A) that disrupted binding of the SH3 domain in SAP90 (S90-SH3) to the PXXPR motifs in KA2 subunits of the kainate receptors (Garcia *et al.*, 1998) affect the SH3-GUK association. Neither mutation affected the ability of the SH3 domain to interact with the GUK domain *in trans* (Figure 3C). These data indicate that the SH3 domain binds the GUK domain via a non-classical mechanism and support similar studies on SAP90/PSD-95 (McGee and Bredt, 1999; Shin *et al.*, 2000).

In DLG, a single amino acid substitution (L556P) in the SH3 domain, called m30, was identified to promote tumor formation in the epithelium of the imaginal disc (Woods *et al.*, 1996). To examine whether this mutation can affect the ability of the SH3 domain to interact with the GUK domain, we created an analogous mutation in the SH3 domain of SAP97 (L579P; SH3m30). SH3m30 did not bind to the GUK domain *in trans* (Figure 3C). This finding was confirmed by placing the m30 mutation into SG97 (SG97m30). SG97m30 bound the SH3 domain supplied *in trans* indicating that the m30 mutation disrupted the intramolecular interaction between the SH3 and GUK domains (Figure 3B). To assess whether a stable SH3-GUK interaction is necessary to suppress GKAP binding, we also tested whether SG97m30 could bind GKAP. Similar to U5-GK, SG97 Δ SH3 and GUK, SG97m30 bound GKAP (Figures 2 and 3B). Taken together, these data show that an intramolecular

Fig. 1. Comparison of GKAP binding to different regions of SAP97. (A) Schematic diagram of SAP97 deletion constructs. Labeled are each of the three PDZ domains, SH3 and GUK domains. Also labeled are the regions unique to SAP97 (U1-U5) situated between each of the core domains. The U1 region is also referred to as the S97N domain. Present in the U5 region is a calmodulin binding site (CBS) and the alternatively spliced I3 insert. Yeast two-hybrid (YTH) and ELISA binding data are summarized for each construct. ND, not done; -, no binding detected; +++ strong binding; ++ moderate binding. (B) An ELISA assay shows that SG97 did not bind GKAP, while full-length SAP97 and GUK domain bound GKAP well. In the experiment, 20 pmol of thio-GKAP (residues 28-79) peptide was bound to a 96-well plate and incubated with increasing concentrations of GST, GST-tagged SG97, GUK or full-length SAP97. To detect the binding, the plate was incubated with anti-GST antibody, followed by AP-conjugated secondary antibody. The binding activity was determined by measuring the absorbance at 405 nm. The values were converted into relative binding by taking the value for maximal 405 nm absorbance as 100%. (C) Double labeling immunofluorescence analysis of the effect of SAP97 deletion constructs on the lateral membrane localization of recombinant GKAP transiently transfected into the epithelial cell line CACO-2. (a and b) Distribution of GKAP (a) versus endogenous SAP97; (c-h) Distribution of GKAP (c, e and g) and GFP-tagged SAP97 (d), SG97 (f) or GUK (h) constructs co-transfected into CACO-2. The lateral membrane localization of GKAP was disrupted when co-expressed with GFP-GUK, but not by GFP-SG97 or GFP-SAP97.

Fig. 2. SH3 and I3 interfere with GKAP binding to SG97.

(A) Schematic diagram of SG97 deletion constructs. ELISA binding data are summarized for each construct. (B) Binding of SG97 deletions to GKAP in an ELISA assay. The assay was performed as described for Figure 1. Deleting the SH3 or U5/I3 regions of SG97 allowed GKAP binding, indicating that these domains work in concert to prevent GKAP binding to the GUK domain.

SH3–GUK interaction regulates the ability of GKAP to bind the GUK domain.

Does the SH3 domain mask the GKAP binding site on the GUK domain? To help resolve this point, we examined whether the SH3–GUK region of SAP90 (SG90) also contains an element that disrupts GKAP binding. This region of SAP90 shares a high degree of sequence homology with SAP97 particularly in the U4, SH3 and GUK domains (Müller *et al.*, 1995). The U5 region in SAP90, in contrast, is smaller and mostly unrelated to the U5 region in SAP97 (Figure 4A). Interestingly, in both ELISA and two-hybrid binding assays, GKAP bound SG90 with an affinity similar to the GUK domain from SAP97 alone (Figures 1B, and 4B and C). These data indicate that SG90 lacks an element that interferes with GKAP binding. The ability of the SH3 domain from SAP90 (S90-SH3) to interact with the SAP97 and SAP90 GUK domains but not with SG90 (Figure 4B) indicates that this is not due to a general inability of S90-SH3 to interact with the GUK domain and that the SH3 domain *per se* does not mask the GKAP binding site in SG97. Instead, these data indicate that the U5 region in SAP97 may mask the GUK binding surface. To test this hypothesis, we compared the binding affinity of several SG97 constructs containing different insert sequences (I0, I2, I3) and SG90 (see Figure 4A) for GKAP by ELISA. SG90 bound GKAP with the highest affinity, similar to the GUK domain alone, followed by I0 > I2 > I3-containing

Fig. 3. Intramolecular interactions between the GUK domain and the SH3 and U5 regions. (A) SH3 and I3/U5 regions interacted with GUK domain in an ELISA assay. In the experiment, 20 pmol of GUK protein (cleaved from GST–GUK fusion protein with thrombin) were bound to a 96-well plate and incubated with increasing concentrations of GST or GST-tagged SG97 deletion proteins. The binding activity was detected as described in Figure 1. (B) Schematic diagram of SG97 construct used in YTH assays. Relative binding for SG97, SG97 containing the m30 mutation in the SH3 domain and the GUK domain are shown. –, no β -galactosidase activity within 6 h; +++, robust β -galactosidase activity appearing within 1 h; ++, moderate β -galactosidase activity appearing after 1–2 h. Data show that the m30 mutation disrupts the intramolecular SH3–GUK interaction and permits GKAP and SH3 to bind *in trans*. (C) Binding of SH3 mutations to SAP97 GUK indicates that SH3 interacts with the GUK domain via an unconventional mechanism. In the ELISA experiment, 20 pmol of SAP97 GUK protein (cleaved from GST–GUK fusion protein with thrombin) were bound to a 96-well plate and incubated with increasing concentrations of GST or GST-tagged SH3 mutations. The binding activity was detected as described for Figure 1. Data show that W470A and P489L mutations that disrupt classical SH3 binding did not affect GUK binding. SH3m30 (L579P) mutation abolished the SH3–GUK interaction.

SG97 isoforms (Figure 4C). These data imply that a larger U5 region has an increased capacity to interfere with binding to the GUK domain. However, since the U5 region in SG97-I0 is similar in size to the U5 region in SAP90 (Figure 4A), masking is in part sequence dependent.

Fig. 4. Insert sequences in the U5 region of SG97 differentially affect GKAP binding. (A) Protein sequence alignment of the U5 regions of SAP90 and SAP97 with alternatively spliced inserts I2 and I3 (bold letters) as well as the extent of the I0 deletion in this region. Identical residues are boxed in gray. (B) Schematic diagrams of C-terminal SAP90 and SAP97 constructs used in YTH binding assays showing that SG90 and SG97 exhibit different GKAP binding activity although neither can bind an SH3 domain supplied *in trans*. These data imply that the SH3 domains in SG90 and SG97 are stably associated with their GUK domains and that the SH3 domain is unlikely to mask the GKAP binding site. (C) SG90 and SG97 proteins with I3, I2 or I0 exhibited differential GKAP binding activity in an ELISA assay. In this experiment, 20 pmol of thio-GKAP (amino acids 28–79) peptide were bound to a 96-well plate and incubated with increasing concentrations of GST or GST-tagged SG90, SG97 (has I3 insert), SG97(I2) or SG97 (I0). The binding activity was detected as described in Figure 1. The data suggest that I3 interferes with GKAP binding to SG97.

S97N facilitates GKAP binding to SAP97

Given the inhibitory effect of the SH3 and U5 regions on GKAP binding to SG97, we next investigated why GKAP binds full-length SAP97 with a similar affinity to the GUK domain alone. To test whether the N-terminal half of SAP97 contains an element that neutralizes the inhibitory effect of SH3 and U5 on GKAP binding, we examined the binding of several additional SAP97 deletion constructs to GKAP. SAP97 lacking the first 186 amino acids comprising the S97N domain (Wu *et al.*, 1998) failed to bind GKAP (Figure 5A). In contrast, deleting PDZ1–3 and placing N_{1–186} next to SG97 (N_{1–186}-SG97) resulted in a

Fig. 5. S97N facilitates GKAP binding to SAP97. (A) Schematic diagram of SAP97 deletion constructs used to identify the region in SAP97 that relieves the inhibition of GKAP binding by the SH3/U5 regions. Relative GKAP binding as assessed by either ELISA or YTH assays are shown. Deleting S97N from SAP97 abolished GKAP binding, while deleting the PDZ domains had no effect on GKAP binding. SG97 constructs containing N_{1–104} or N_{66–186} promoted GKAP binding indicating that the positive regulatory element is situated in this region of the S97N domain. (B) Three-component ELISA assay evaluating whether N_{1–186} facilitates GKAP binding to SG97 when supplied *in trans*. Twenty pmoles of GST–SG97 were bound to a 96-well plate and incubated with increasing concentrations of thio-GKAP in either the absence or presence of 250 nM T7–N_{1–186}. Relative absorbance at 405 nm versus [GKAP] (nM) from ELISA binding data is shown. (C) S97N interacts with SH3 and I3/U5 domains in an ELISA assay. Twenty pmoles of T7–N_{1–104} was bound to a 96-well plate and incubated with increasing concentrations of GST or GST-tagged SG97, SH3, U5 and GUK. The binding activity was detected as described for Figure 1. (D) S97N interferes with the binding of SH3 to GUK. Twenty pmoles GUK were bound to a 96-well plate (in which the GST domain had been cleaved off with thrombin) and then incubated with increasing concentrations of GST-tagged SAP97 SH3 in the absence or presence of 1 μM of T7–N_{1–104}. High concentrations of T7–N_{1–104} reduced SH3 binding to GUK. (E) N_{1–104} interacts with the SH3 domain via a non-classical PXXPR mechanism. ELISA binding assays comparing the ability of N_{1–104} to bind SH3 domains with or without a P489L mutation. While this mutation disrupts SH3 domain binding to PXXPR sequences in KA2 subunits of the kainate receptors (Garcia *et al.*, 1998), it did not affect N_{1–104} binding.

molecule that bound GKAP similarly to the GUK domain (Figure 5A). Significant binding was also observed when the first 65 residues were removed (N_{66–186}-SG97) or

when residues 1–104 were fused to SG97 (N_{1–104}-SG97) (Figure 5A). Fusing residues 1–65 to SG97 (N_{1–65}-SG97) had no effect on GKAP binding. These data suggest that amino acid residues 66–104 (N_{66–104}) compensate the inhibitory effect of the SH3 and U5 regions and allow GKAP to bind.

To further investigate the molecular mechanisms by which this N-terminal element facilitates GKAP binding, we evaluated whether this effect can be mediated *in trans*. For these experiments, 20 pmol of GST-SG97 were bound to each well of a 96-well ELISA plate. The T7-epitope-tagged first 186 amino acid residues from SAP97 (T7-N_{1–186}) and a thio-tagged GKAP were added in increasing amounts to the columns or rows, respectively, of the ELISA plate. At low concentrations of T7-N_{1–186} (<50 nM), thio-GKAP did not bind GST-SG97 (Figure 5B). However, at higher N_{1–186} concentrations (≥200 nM), GKAP was found to interact with SG97 (Figure 5B). Similar results were obtained with N_{1–104} (data not shown). Thus, N_{1–104} can promote binding of GKAP to the GUK domain *in trans*. This suggests that N_{1–104} interacts directly with SG97 and/or GKAP. This possibility was evaluated in an ELISA assay. While N_{1–104} bound SG97 with an affinity of ~60 nM (Figure 5C), no interaction with GKAP was detected (data not shown). To identify the binding element in SG97, we tested the ability of N_{1–104} to bind with different SG97 subregions. N_{1–104} bound strongly to SG97, the SH3 domain and the U5 region, but not the GUK domain alone (Figure 5C). This indicates that N_{1–104} interacts with the SH3 as well as the U5 regions in SG97. Moreover, N_{1–104} bound the SH3 domain from SAP90 with and without a P489L mutation (Figure 5E), indicating that N_{1–104} in SAP97 interacts with the SH3 domain in a non-classical manner.

Taking these data together, N_{1–104} appears to facilitate GKAP binding to the GUK domain by interacting with the SH3/U5 regions. This concept was also tested by assessing whether N_{1–104} modulates SH3 binding to the GUK domain in a competitive ELISA. Addition of N_{1–104} at 1 μM was found to greatly decrease binding of the SH3 domain to the GUK domain (Figure 5D). Thus, GKAP binding to the GUK domain in SAP97 appears to be regulated through a series of intramolecular interactions that act to mask and unmask its binding site.

Molecular modeling of SAP97

Our *in vitro* binding data suggest that SAP97 is not simply a linear molecule comprised of domains tethered together like beads on a string but is composed of a series of domains that interact with each other to regulate the availability of intermolecular binding sites. To better understand this regulation, we used structural information provided by X-ray crystallographic studies on PDZ domains from SAP97 (Morais Cabral *et al.*, 1996), SAP90 (Doyle *et al.*, 1996), SH3 domains in Grb2 (Maignan *et al.*, 1995) and the yeast guanylate kinase (Stehle and Schulz, 1992) to create a molecular model of SAP97. The primary objectives of these studies were (i) to determine the geometric arrangement of the SAP97 domains, and (ii) to assess whether this arrangement can explain the regulatory role of the SH3, U5 and S97N domains on GKAP binding. More specifically, we wanted to know whether the geometry of the domains would

structurally allow S97N to associate *in cis* with the SH3/U5 regions.

The molecular modeling of SAP97 was accomplished by first performing a Blastp (PDB) analysis at the NCBI and EMBL databases to identify sequences in SAP97 that match NMR and crystal structures (Table I). In addition to the PDZ, SH3 and GUK domains, this analysis revealed structural information for the U regions. Template selection and construction of all unknown domains were guided by their ability to form hydrophobic folding units.

The core of the U1 region (residues 77–234) consists mainly of β-strand elements that resemble two fibronectin type III-like domains, referred to as S97N-1 and S97N-2. The U2 sequence (situated between PDZ1 and PDZ2) is part of a β-sheet from PDZ1 and continues into PDZ2. The larger U3 region is predicted to be a closed element composed of a β-turn. U4 is similar to a region adjacent to the SH3 domain in Grb2 (Maignan *et al.*, 1995). U5 most closely resembles linker sequences between the A and the B domains of topoisomerase II, 1BGW (Berger *et al.*, 1996). This structure provides the coordinates of a helix and β-turn for the core region of U5 (see Figures 3B and 4A). The corresponding U5 helix in SAP102/NE-Dlg serves as a calmodulin binding site (Masuko *et al.*, 1999). I2 and I3 inserts resemble the topoisomerase disordered linker between the A and B domains for which coordinates are not available. The required modeling of these inserts is based on the assumption that the inserts exhibit a similar fold (Figure 6B).

Crystal symmetry-related contacts between PDZ domains (Morais Cabral *et al.*, 1996) as well as geometric criteria provided the basis for building the SAP97 structure. Importantly, the surfaces of the SH3, PDZ and GUK domains are well defined due to the rigidity of their geometry (Martinez and Serrano, 1999; Yan and Tsai, 1999; Tochio *et al.*, 2000). As such there is a limited number of possible arrangements for these domains. The packing of domains was guided by the following criteria. First, the contact areas between two domains should be as large as possible and of complementary shape. Secondly, the resulting protein structure should show high order symmetry. The final model was positionally refined and evaluated using standard software Procheck (Laskowski *et al.*, 1996) and Whatif (Vriend, 1990). It is important to note that the models were derived independently of all biochemical data and based solely on structural criteria.

A model of SAP97 in a compact conformation is presented in Figure 6A. This model, with the PDZ1 and PDZ2 domains at the bottom, predicts that SAP97 resembles a planar U-shaped molecule with the N-terminus folded back onto the C-terminus (see 90° rotation around y-axis, Figure 6A). In this model, the peptide binding pockets for PDZ1 and PDZ2 are predicted to face out and down. These pockets are not equally accessible from the solvent as the U3 β-turn forms a lid over the PDZ2 pocket. PDZ3 is stacked above PDZ2 with its binding pocket facing the SH3 domain. Bound peptides at PDZ1 and PDZ2 will be anti-parallel, while that at PDZ3 is orthogonal to the others. The SH3 domain is predicted to engage the GUK domain in a pocket that serves as the ATP binding site in authentic guanylate kinases. The

Table I. Structural templates used for the modeling of SAP97

Residues		Templates	Fold used	Aminoacid Identity Similarity		Quality of the model	
<u>SAP97</u>	<u>Templates</u>					<u>Ramachandran</u>	
<u>N1</u> 49-84 89-109 116-126	<u>1FNF</u> 157-192 198-218 227-237		Fibronectin domain type III	87.2%	27.9%	44.1%	75.2/20.2/4.7
<u>N2</u> 127-133 137-156 162-211	238-244 254-273 276-325			90.6%	10.4%	33.8%	71.0/29.0/0.0
<u>PDZ1</u> 222-242 250-311	<u>1BE9</u> 311-333 333-394		The third PDZ domain of PSD-95	92.2%	44.4%	66.7%	89.4/10.6/0.0
<u>PDZ2</u> 315-336 344-410 415-425	310-331 333-399 402-412			90.1%	37.0%	67.0%	78.6/17.3/4.1
<u>U3</u> 428-450	<u>2VSG</u> 318-340		Variant surface glycoprotein	100.0%	34.8%	43.5%	76.5/17.6/5.9
<u>PDZ3</u> 456-553	<u>1PDR</u> 304-401		The third PDZ domain of the human homologue of disc large protein (hDlg)	100.0%	98.0%	98.0%	89.9/8.9/1.3
<u>SH3</u> 554-594 602-641	<u>1GRI</u> 130-170 173-212		Grows factor bound protein 2, SH3 domain	92.0%	27.6%	46.6%	81.8/18.2/0.0
<u>U5/HOOK</u> 636-674 718-724	<u>1BGW</u> 38-76 108-114		Topoisomerase II	100.0%	26.1%	39.1%	70.3/16.2/13.5
<u>GUK</u> 735-743 746-786 789-841 844-858 861-878 880-913	<u>1GKY</u> 1-9 15-55 57-109 113-127 133-150 153-186		Guanylate kinase	95.0%	33.9%	55.2%	84.0/12.3/3.7

Each of the modeled domains is compared with its original template coordinates from Brookhaven Protein Database (<http://www.rcsb.org/pdb/>). Column 'Fold used' gives the percentage of main chain atoms that have not been altered after the modeling. The Ramachandran values are given as percentage of residues with preferred/allowed/generously allowed ψ and ϕ angles. The structural overall Ramachandran values are 80/17/3, while the G -factor is 0.24. A more detailed description of structural parameters is presented at <http://www.synprot.de>

Fig. 6. Structural model of SAP97. (A) Ribbon structure of SAP97 in a U-shaped compact conformation. Two views rotated by 90° along the imaginary axis from the PDZ1 (light blue) to the GUK (magenta) domains are shown. The first 48 residues of the N-terminus are missing. Identifiable structural domains, shown in different colors, include the FN type III-like domains S97N-1 (green) and S97N-2 (blue-green), PDZ1-3 (light blue, purple, blue, respectively), SH3 (white), GUK (magenta) as well as unique (U) regions. PDZ binding peptides and GMP bound to the GUK domain are shown as space filling models. The core region of U5 (Hook) and CaM binding helix (CaM) are shown in red, while the I3 insert is shown in yellow. U3 (2VSG) (pink) is predicted to form a β -turn that forms a lid over the PDZ2 peptide binding pocket. (B) Detailed view of the U5 regions from SAP90, SAP97-I0, SAP97-I2 and SAP97-I3. The potential binding site for CaM is indicated. The core regions of U5 (helix in red and β -turn in green) are based on the TopoII structure. In SAP97, a conserved loop is seen in all U5 elements into which the inserts are added. In I3, an additional loop is predicted to extend up and over the GUK domain. The structure of I0 closely resembles the U5 from SAP90.

PXXPR binding site of the SH3 domain points downwards, away from the GUK domain, and faces the PDZ3 peptide binding pocket. The calmodulin binding helix, and the remainder of the U5 region, wraps around the back of the GUK domain and up along its left side (Figure 6A). The S97N-1 domain (amino acid residues 65–114) is predicted to interact with the C-terminal half of SAP97 lying in a pocket formed by the SH3, U5 and GUK domains. S97N-1 interacts with the SH3 domain on a face that does not include its PXXPR binding pocket, while the S97N-2 domain has contact to surfaces of S97N-1, PDZ3 and PDZ1. This analysis shows that the individual domains in SAP97 can be arranged in a compact structure. Subsequent dissociation of each domain from this compact conformation would lead to multiple ‘open’ states (see Figure 7 for open states of S97N1, SH3, U5 and GUK domain) and ultimately to a completely extended form of SAP97.

Discussion

Previous studies have shown that MAGUKs are multi-domain proteins possessing the capacity to interact with a variety of integral membrane proteins, cytoskeletal components and cytosolic proteins involved in intracellular signaling. Cellular mechanisms regulating the interaction of MAGUKs with their binding partners and thus their recruitment and assembly into the cytoskeleton at sites of cell–cell adhesion are poorly understood. In this study, we have assessed a possible role of intramolecular interactions between individual domains in SAP97 in regulating the binding of its GUK domain to the postsynaptic density protein GKAP. This interaction may be of physiological significance given the essential role the GUK domain plays in synaptic localization of the MAGUK DlgA in flies (Thomas *et al.*, 2000) and the ability of SAP97 to interact with GKAP/SAPAPs/DAP-1 *in vivo* (Kim *et al.*, 1997; Satoh *et al.*, 1997; Takeuchi *et al.*, 1997).

Fig. 7. Ribbon models of intramolecular interactions of S97N-1, SH3, U5 and GUK domains in partly dissociated SAP97. Individual domains are color coded: GUK, pink; SH3, gray; core U5, red; I3 insert, yellow; S97N-2, blue; S97N-1, light green. Space-filling model of GMF bound to the GUK domain is also shown. The models illustrate the concept of ordered assembly in which the association of one pair of domains can hinder the association of the next depending on the orders of association/dissociation placing SAP97 in a conformation that favors GKAP binding. In the sequence shown, the dissociation of I3 from GUK and S97N-1 (N-1) permits S97N-1 and then GUK to dissociate from the SH3 domain (steps 1–3). Re-association of S97N-1 with the SH3 domain would hinder the rebinding of GUK (step 4). In this configuration, U5 is prevented from interacting with S97N-1 and GUK by displacing it from its favored position. This in turn allows GKAP to bind. Binding of the I3 insert to S97N-1 at this stage (step 5) will sterically hinder the formation of a closed conformation (step 6). The conformational changes are shown as rigid body motions of the domains around the five hinge regions. These hinge regions are located between S97N-1 and S97N-2 (steps 2 and 4), between U5 and GUK (step 3), at the splice site for I3 insert (steps 1 and 5) and at the CaM binding helix (steps 3 and 5). The CaM binding helix is partly unfolded in the closed conformation, while freely accessible in the open conformation. Hence, step 6 would require two hinge motions at the same time.

Our data revealed that SAP97, and thus other MAGUKs, can exist in a compact structure in which intramolecular binding sites are likely to be masked by intramolecular domain interactions. Specifically, we found that interactions between the SH3, U5 and GUK domains in SAP97 interfere with GKAP binding, whereas the S97N domain was found to support GKAP binding through its intramolecular interactions with the SH3 and U5 regions. These intramolecular interactions are predicted to not only modulate GKAP binding, but also the association of SAP97 with other interacting partners and to regulate its recruitment and assembly at cell–cell adhesion sites. This conclusion is supported by recent studies showing that intermolecular interactions between the S97N domain and SH3 domain modulate binding of SAP97 to KA2 subunits

of the kainate receptors at synapses (S.Mehta, H.Wu, C.Garner and J.Marshall, in preparation), SH3–GUK interactions SAP97 binding to CASK in epithelial cells (Z.Walther and J.Anderson, in preparation) and SAP90/PSD-95 binding to potassium channels (Shin *et al.*, 2000).

In the process of defining elements that interfere with the binding of GKAP to the GUK domain in SAP97, we discovered that the SH3 and U5 regions can interact intramolecularly with the GUK domain. SH3 binding to the GUK domain does not involve its PXXPR binding pocket. Recent studies on other MAGUKs indicate that intramolecular SH3–GUK interactions are a shared feature of SAP90, CASK and ZO-1 subfamilies (McGee and Bredt, 1999; Shin *et al.*, 2000; Z.Walther and J.Anderson, in preparation). Cross-talk between the SH3 and GUK

domains within and between subfamilies is possible *in vitro*, although it is currently unclear whether such intermolecular interactions occur *in vivo*. These SH3–GUK interactions do not appear to involve classical PXXPR–SH3 interactions (McGee and Bredt, 1999; Shin *et al.*, 2000). Instead, sequences flanking the GUK domain have been suggested as regions involved in this interaction (McGee and Bredt, 1999; Shin *et al.*, 2000). Our molecular modeling studies indicate that the SH3 domain does not bind at this site but rather interacts with the bases of the GUK domain at a site that in authentic guanylate kinases represents the ATP binding pocket (Stehle and Schulz, 1992). This region of the GUK domain forms a rigid structure in contrast to the flexible GMP binding site (data not shown) and has a face that is complementary in shape to that of the SH3 domain. Regulation of SH3 binding by ATP seems unlikely given that the ATP binding activity of this pocket has apparently been lost during evolution (Kuhlendahl *et al.*, 1998).

Our biochemical data indicate that the SH3–GUK interaction does not directly interfere with GKAP binding but may be an intramolecular regulator of GKAP binding. This conclusion is suggested by experiments showing that while GKAP can bind SG90, SG97-I0 and SG97-m30, SH3 domain supplied *in trans* can only bind SG97-m30. This implies that the SH3 domain binds GUK at a position that is distinct from the GKAP binding site. Nevertheless, the SH3 domain appears to be essential for the inhibition, since deleting the SH3 domain or introducing a single amino acid mutation into the SH3 domain (m30) relieves the inhibition. The best candidate for the sequence element that directly masks the GKAP binding site is the U5 region and in particular the I3 insert. *In vitro*, I0 and I2 forms of U5 of SG97 did not induce a strong suppression of GKAP binding. Moreover, SG90 containing a U5 region that lacks I2 or I3-like inserts bound GKAP. Molecular modeling of the U5 regions in SAP90, SAP97-I2, SAP97-I3 and SAP97-I0 (Figure 6B) discloses a potential structural explanation for these observations. Blastp (PDB) analysis revealed that these U5 regions closely resemble the hinge region between domains A and B of topoisomerase II (TopoII). This element in TopoII is composed of a helix and β -turn flanking a loop (Berger *et al.*, 1996). We predict that the helix and β -turn in the U5 elements form a core structure into which loops of differing sizes can be added. The loops formed by inserts I2 and I3 in splice variants SAP97-I2 and SAP97-I3 are essentially absent from the U5 region of SAP90 and SAP97-I0. Assuming that I2 and I3 loops share a common fold, our models predict that I3 creates a secondary loop that extends from the side of the I2 loop. Modeling the U5 regions within the context of the SH3 and GUK domains indicates that U5 wraps around the back of the GUK domain (Figure 6A) and that the surface of its core region fits favorably that of the GUK domain. U5 binding to GUK is supported by our *in vitro* binding data. As such, these data suggest that the GKAP binding site may lie beneath this region of the GUK domain. However, it should be noted that the U5 region by itself does not interfere with GKAP binding but requires a functional SH3 domain. Taken together these data suggest that the SH3 acts to position the U5 region through its own association with the GUK domain and that factors that can modify the binding

of either U5 or SH3 to the GUK domain may regulate GKAP binding to the GUK domain.

One factor found to relieve the inhibitory effect of SH3 and U5 on GKAP binding to SG97 is the S97N region in SAP97. Biochemically, the S97N-1 region was able to promote GKAP binding when present either *in cis* or *in trans* and has the capacity to bind the SH3/U5 region and disrupt the SH3–GUK interaction. Molecular modeling reveals that the domains in SAP97 can be geometrically arranged in a compact conformation such that the S97N-1 is placed in a pocket formed by the SH3, U5 and GUK domains. In this arrangement S97N-1 is not expected to disrupt SH3–GUK binding. None the less, our biochemical data show that S97N-1 can interfere with SH3 binding to the GUK domain. Thus, we hypothesize that the sequential order in which intramolecular domain interactions are established in SAP97 influences the overall conformation of the molecule. For example, initial SH3–S97N binding would interfere with a subsequent SH3–GUK association. In this model, the GUK domain is fixed in a dissociated state allowing it to interact with GKAP (Figure 7, step 6). Alternatively, the association of S97N with the SH3–GUK domains in the closed conformation may change the position of the flexible I3 loop in U5, thereby unmasking the GKAP binding site on the GUK domain (Figure 7, step 1). This possibility is supported by the ability of S97N to bind to U5. In addition to these intramolecular interactions, intermolecular associations, such as CaM binding to the U5 helix or protein 4.1 binding to I3 may regulate GKAP binding (Figure 7, step 3). Similarly, peptide binding to the PDZ domains may affect the dissociation state of these domains; a scenario supported by studies examining factors that regulate MAP1A binding to the GUK domain in SAP90 (Brenman *et al.*, 1998).

Modeling of the SAP97 structure reveals that individual domains potentially toggle between associated and dissociated states with their neighboring domains. These include interactions between SH3 and GUK, U5 and GUK, S97N and SH3, and S97N and U5 described above, as well as S97N1 and S97N2, S97N2 and PDZ1, PDZ2 and PDZ3, PDZ2 and U3, and PDZ3 and SH3. Only, the PDZ1–PDZ2 interaction is unlikely to exist in a dissociated state given that the potential hinge between these domains is very short and is part of a presumably rigid β -strand that starts in PDZ1 and continues into PDZ2. Some intramolecular domain interactions are predicted to interfere with intermolecular interactions. For example, the S97N1–SH3 interaction may affect the dimerization of SAP97/hDlg via this N-terminal domain (Marfatia *et al.*, 2000) and/or the localization of SAP97/hDlg to the epithelial lateral membrane which requires the S97N region (Wu *et al.*, 1998). Likewise, the SH3–PDZ3 interaction could mask the PXXPR binding site on the SH3 domain.

The ability of each domain in SAP97 to exist in either an associated or dissociated state permits SAP97 to exist in numerous conformational states from completely compact (Figure 6A) to fully extended, as diagrammed for the S97N-1, SH3, U5 and GUK domains in Figure 7. Within cells, these different conformational states may play a role in the recruitment of SAP97 from cytoplasmic pools to adhesion sites and, as discussed above, its association with its cytoskeletal and ion channel binding partners.

Presumably, cytosolic SAP97 is in a mostly closed conformation, while at the plasma membrane where SAP97 associates with cytoskeletal proteins, ion channels and cell adhesion molecules, a mostly open conformation is predicted. Interestingly, the dissociation of each domain–domain interaction is predicted to have a differential effect on the scaffolding function of SAP97, i.e. potential to tether binding partners. For example, the dissociation of the U5–GUK, GUK–SH3, U3–PDZ2 interactions may have minimal effects on the association status of the rest of the domains. In contrast, disruption of the PDZ3–SH3 or S97N1–S97N2 interactions is predicted to dramatically affect the dissociation status of most domains. The ability of multidomain proteins to exist in open and closed states that exhibit distinct biological activities is not unique to SAP97 and has been shown to be present in Src, radixin and kinesins. For example, in the Src family of tyrosine kinases, the intramolecular association of the SH3 and kinase domains blocks its catalytic activity (Andreotti *et al.*, 1997; Moarefi *et al.*, 1997).

Given the established structural homologies between the various MAGUK family members, our studies on SAP97 suggest that intramolecular interactions are likely to play a fundamental role in regulating the assembly of MAGUK-based multiprotein complexes at membrane specializations such as tight and synaptic junctions.

Materials and methods

Antibodies

Goat polyclonal anti-GST antibodies were purchased from Amersham Pharmacia Biotech Inc. Anti-T7 and anti-thio antibodies were purchased from Novagen and Invitrogen, respectively. Rabbit anti-GKAP antibody described by Kim *et al.* (1997) was a generous gift from Dr Morgan Sheng.

Construction and purification of fusion proteins

GST–SAP97 fusion proteins were created by subcloning various regions of SAP97, amplified by PCR, in-frame into the pGEX-2T vector (Amersham/Pharmacia). SAP97 cDNA clones containing the I3 insert were used as templates for all constructs unless stated specifically. Amino acid numbering for each construct was used as described (Wu *et al.*, 1998). SG97, U5–GUK, I3–GUK, GUK, SH3 contain amino acid residues 473–909, 620–909, 635–909, 700–909 and 559–619, respectively. Removal of amino acid residues 473–559, 559–619 and 636–682 from SG97 gave rise to SG97ΔU4, SG97ΔSH3 and SG97(I0), respectively. His₆/thio-tagged GKAP fusion constructs (full-length or amino acid residues 28–79) were created in the pET32a vector (Novagen) (Kim *et al.*, 1997). His₆/T7-tagged N_{1–104} and N_{1–186} constructs were created by subcloning PCR fragments into the pRSETC vector (Invitrogen).

The recombinant proteins were expressed in bacterial strain BL21 GOLD (Stratagene) after induction with 0.1 mM isopropyl thio-β-D-galactopyranoside (Fisher Biotech) as described (Kuhlendahl *et al.*, 1998). Fusion proteins were purified using glutathione–agarose beads (Sigma) for GST proteins, or TALON metal affinity resin (Clontech) for His₆ proteins. Protein concentrations were determined with the Coomassie Plus protein assay reagent (Pierce). To remove GST from GST–GUK, thrombin (Amersham Pharmacia) cleavage was performed before elution with glutathione elution buffer [5 mM reduced glutathione (Sigma) in 50 mM Tris–HCl pH 8.0] according to the manufacturer's protocol (Amersham/Pharmacia).

Yeast two-hybrid binding assay

Yeast two-hybrid analysis was performed using Matchmaker two-hybrid system (Clontech) with yeast strain L40. Interactions between two domains were assessed based on growth on –His plates and the time it took colonies to turn blue in the filter-lifting β-galactosidase assay as described (Clontech). For GKAP–SAP97 interactions, GKAP (clone gk2.18; Kim *et al.*, 1997) was cloned into the pGAD10 vector (Clontech) and different regions of SAP97 were cloned into the pBHA5 vector

(Clontech). Regions in SAP97 cloned into the pBHA5 vector include GUK (amino acid residues 700–909), SH3–GUK (SG97, amino acid residues 473–909), SG97m30, S97N_{1–186}–SG97, S97N_{1–104}–SG97, S97N_{1–65}–SG97 and S97N_{66–186}–SG97. SH3–GUK (SG90, amino acid residues 352–721) and GUK (residues 513–721) from SAP90 were cloned into the pBHA5 vector as well.

ELISA

ELISA-based binding assays were performed as described previously (Kuhlendahl *et al.*, 1998). The absorbance of ELISAs at 405 nm was measured in a microplate reader (Cambridge Technology Incorporated). The values for each experiment were changed into relative binding activity by defining the maximal absorbance at 405 nm as 100%. All assays were repeated three times in duplicate.

Cell culture and transfection

The human colon carcinoma cell line CACO-2 was cultured in MEM (Gibco-BRL) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. Single and double transfections of CACO-2 cells with GKAP in the GW1 vector (Kim *et al.*, 1997) and GFP-tagged SAP97, SG97 or GUK were performed as described in Wu *et al.* (1998). Fixation, antibody staining and fluorescent images were performed as described (Wu *et al.*, 1998).

Structural model building

A Blastp (PDB) search of structural templates for SAP97 protein was performed using databases at NCBI and EMBL. The template selection and mapping of adjacent fragments was supported by hydrophobic cluster analysis program Octopus (Durand, 1998). The template fragments were mutated by the sequence of SAP97 (SAP97_RAT, accession No. Q62696) using the program Swiss PDB Viewer (Guex and Peitsch, 1997). The constructed structural fragments of SAP97 were evaluated and optimized according to their ability to build hydrophobic folding units. Domains PDZ1 and PDZ2 of SAP97 were oriented to fit a symmetry-related dimer found in PDZ crystal structure of SAP97 (Morais Cabral *et al.*, 1996) resulting in a mostly compact dimer of PDZ domains. The remaining structural fragments were assembled to form a compact structure of U-shape type with the program FTDOCK (Gabb *et al.*, 1997) on an SGI Origin 200 multi-processor computer server. A number of structural templates suggested U1 to be divided into two domains (referred to as S97N-1 and S97N-2). Fibronectin III (FNIII) domains were chosen as templates since SAP97 shows a high degree of identical residues at the transition from the S97N-1 to the S97N-2 domain. The structural model is presented at <http://www.synprot.de/>.

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