Molecular Mechanisms Regulating the Differential Association of Kainate Receptor Subunits with SAP90/PSD-95 and SAP97*

Received for publication, January 23, 2001, and in revised form, February 8, 2001
Published, JBC Papers in Press, February 20, 2001, DOI 10.1074/jbc.M100643200

Sunil Mehta‡, Hongju Wu§, Craig C. Garner§, and John Marshall¶¶

From the ‡Department Of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence, Rhode Island 02912 and the §Department of Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Recent studies have demonstrated that kainate receptors are associated with members of the SAP90/PSD-95 family (synapse-associated proteins (SAPs)) in neurons and that SAP90 can cluster and modify the electrophysiological properties of GluR6/KA2 kainate receptors when co-expressed in transfected cells. In vivo, SAP90 tightly binds kainate receptor subunits, while SAP97 is only weakly associated, suggesting that this glutamate receptor differentially associates with SAP90/PSD-95 family members. Here, green fluorescent protein (GFP)-tagged chimeras and deletion mutants of SAP97 and SAP90 were employed to define the molecular mechanism underlying their differential association with kainate receptors. Our results show that a weak interaction between GluR6 and the PDZ1 domain of SAP97 can account for the weak association of GluR6 with the full-length SAP97 observed in vivo. Expression studies in HEK293 cells and in vitro binding studies further show that although the individual Src homology 3 and guanylate kinase domains in SAP97 can interact with the C-terminal tail of KA2 subunit, specific intramolecular interactions in SAP97 (e.g. the SAP97 N terminus (S97N) binding to the Src homology 3 domain) interfere with KA2 binding to the full-length molecule. Because receptor subunits are known to segregate to different parts of the neuron, our results imply that differential association of kainate receptors with SAP family proteins may be one mechanism of subcellular localization.

An increase in receptor or ion channel density at synapses is important for efficient signaling between neurons. Clustering of ionotropic glutamate receptors as well as the formation of macromolecular signaling complexes with synaptic receptors is believed to involve a class of synapse-associated proteins (SAPs)1 that are members of a superfamily of membrane-associated guanylate kinases (1, 2). Four members of this family have been described: SAP90/PSD-95, SAP97, SAP102, and chapsyn-110/PSD-93. All four are composed of three PDZ domains (PDZ1, PDZ2, and PDZ3), an Src homology 3 domain (SH3), and a catalytically inactive guanylate kinase (GUK) domain (3–5). Each domain has been shown to be a site of protein-protein interaction, allowing SAP90/PSD-95 family members to interact with ligand- and voltage-gated ion channels, cell adhesion molecules, and cytoskeletal proteins as well as proteins involved in intracellular signaling pathways (2, 6). In isolation, analogous domains from each family member have been shown to exhibit similar binding specificities, yet in vivo the full-length molecules are differentially distributed and selectively associate with specific subclasses of ionotropic glutamate receptors. For example, SAP90/PSD-95, SAP102, and chapsyn-110/PSD-93 are all found highly concentrated at and tightly bound to the postsynaptic density (PSD) of type 1 glutamatergic synapses, where they are known to associate with subunits of the N-methyl-D-aspartate receptors and kainate receptors (7–10). In contrast, SAP97, which is less tightly associated with these synapses, interacts preferentially with α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors (11). Differences between SAP97 and the other SAPs are also observed in transfected HEK293 (9, 12). While SAP90/PSD-95 is readily able to cluster Shaker K+ channels at the plasma membrane of HEK293 cells (9), SAP97 traps these channels intracellularly in an endoplasmic reticulum-like compartment (12, 13). Given their striking structural similarities, these observations raise the important issue of how SAP family members differentially select their binding partners.

Recent studies point to at least two types of mechanisms governing the selection process. The first appears to involve small differences in the sequences flanking the conserved PDZ, SH3, and GUK domains. For example, sequences N-terminal to the first PDZ domain in SAP90/PSD-95 and SAP97 (S90N and S97N, respectively) have been found responsible for the differential localization of these two proteins in neurons (14, 15) and nonneuronal cells (16) as well as the ability of SAP90/PSD-95 to cluster ion channels in HEK293 cells (13). The second mechanism appears to involve a series of intramolecular interactions between the individual domains (17), which in turn regulate both GUKAP binding to the GUK domain of SAP97 (18) and Kv1.4 clustering by SAP90/PSD-95 (19).

Typically, kainate receptors are heteromeric receptors composed of various combinations of GluR5, GluR6, GluR7, KA1, or KA2 subunits (20, 21). In a recent study, we found that SAP90 binds the kainate receptor subunits, GluR6 and KA2 (10). In contrast, the SAP97 fails to interact with KA2 and only weakly binds GluR6 (10). Studies on heteromeric GluR6/KA2 receptors also revealed that SAP90/PSD-95 can associate via its PDZ1 domain with the C-terminal ETMA sequence of the GluR6 subunit and the C terminus of KA2 subunits via its SH3 and GUK domains (10). Importantly, these interactions cause reduced desensitization of kainate receptors (10).

To understand better the molecular bases of SAP binding partner specificity, we have evaluated what structural features...
In SAP97 are functionally important for interfering with its association with kainate receptor subunits in vivo. Our results show that GluR6 association with SAP97 is feeble due to a weak interaction between the PDZ1 and GluR6 subunit. In contrast, we also find that although both the SH3 and the GUK domain in SAP97 can interact with KA2, a combination of intrinsic features in SAP97 interfere with its ability to interact with KA2. Specifically, intramolecular interactions between the N terminus of SAP97 and its SH3 domain play a dominant role in preventing its binding to the kainate receptor KA2 subunit.

**Experimental Procedures**

**Mammalian DNA Expression Vectors**—The subcloning of cDNAs encoding SAP97 and SAP90 into eukaryotic expression vectors with a N-terminal green fluorescent protein (GFP) tag has been described previously (16). SAP90 with a C-terminal GFP tag was created by cloning the coding region of SAP90 into the pEGFP-N1 vector (CLONTECH). The SAP97 molecules in which specific subdomains such as the β3 (β339–797), PDZ1–3 (ΔPDZ1–3), and SH3ΔSH3 domains are deleted have been described (16). GFP-PDZ1 ΔS90 and GFP-PDZ1 ΔS97 were constructed by subcloning polymerase chain reaction-amplified DNA fragments directionally into the EcoRI–SalI sites of pEGFP-C2. Single amino acid changes in the SH3 domain of SAP90 were generated by the QuickChange protocol (Stratagene). The cDNAs encoding KA2 and GluR6 (kindly provided by Dr. S. Heinemann) were subcloned into the pcDNA3 expression vector (Invitrogen).

**Cell Culture and Transfection**—Human embryonic kidney cells (HEK293 cells) were purchased from ATCC (Manassas, VA), grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (37 °C, 5% CO₂) and transfected as described (10). In brief, HEK293 cells grown in 75-mm² flasks were transfected at 30–40% confluence using LipofectAMINE according to manufacturers' recommendations (Life Technologies, Inc.). When two plasmids were co-transfected into HEK293 cells (e.g. KA2 and SAP97), a 1:1 ratio was used.

**Protein Preparation, Immunoblotting, and Immunoprecipitation**—For immunoprecipitation experiments, fresh adult rat brains were homogenized and centrifuged at 4 °C for 1 h at 165,000 ×g. Insoluble pellets were dissolved in radioummunoprecipitation buffer with 2% SDS. Solubilized extracts were passed several times through a 25-gauge needle, diluted to a final SDS concentration of 0.33%, and centrifuged at 10,000 ×g for 5 min. Transfected cell extracts were prepared in a similar fashion, except 0.1% SDS was used in the solubilization buffer. Immunoprecipitations were performed by incubating extracts with 5 μg of anti-KA2 (Upstate Biotechnology, Inc., Lake Placid, NY), 5 μg of anti-GluR1 (Upstate Biotechnology), or 5 μg of anti-GFP (Quantum Biotechnology) antibodies for 2 h at 4 °C, followed by incubation with 100 μl of protein G-Sepharose (Amersham Pharmacia Biotech) for 12–16 h and four washes in radioummunoprecipitation buffer. Western blot analysis of the depleted cell extracts showed no detectable anti-KA2 or anti-GFP immunoreactivity, suggesting that immunoprecipitation was complete. Bound proteins were eluted from the beads by boiling and separated by SDS-PAGE. Gels were then blotted and immunostained as previously described (10). Primary antibodies used for Western blotting included mouse monoclonal anti-SAP90 (1:250; Transduction Laboratories), rabbit polyclonal anti-GST (1:1000; Sigma), mouse monoclonal anti-SAP97 (1:2000; Stressgen), mouse monoclonal anti-T7 (1:10,000; Novagen), rabbit polyclonal anti-GluR1 (1 μg/ml; Upstate Biotechnology), rabbit polyclonal anti-KA2 (1 μg/ml; Upstate Biotechnology), and rabbit polyclonal anti-GluR6 (1 μg/ml; Upstate Biotechnology).

**Construction and Purification of Fusion Proteins**—H6KA2(c-term) expressed in pTcHisB (Invitrogen) was prepared as described by Garcia et al. (10). H6S97N was prepared by subcloning the polymerase chain reaction fragment containing amino acids 1–104 of SAP97 in pSETC vector (Invitrogen). Synthesis of recombinant proteins in TOP10 cells (Invitrogen) was induced by 1 mM isopropyl-β-D-thiogalactopyranoside for 12–14 h at 37 °C. Cells were then harvested, resuspended in 50 mM phosphate buffer (pH 8.0) with 300 mM NaCl and 8 μl urea, lysed by French press as described by the manufacturer (SLM Instruments, Inc.), and centrifuged at 70,000 ×g for 25 min at 20 °C. Supernatant containing H6KA2(c-term) was bound to Ni²⁺-nitrilotriacetic acid beads (Qiagen) for 1 h at room temperature with mixing and then loaded into a column. After washing, bound H6KA2(c-term) was eluted using an imidazole step gradient from 50 to 500 mM and then dialyzed in a series of buffers with decreasing urea concentrations (from 6 to 1 M) to renature the protein and remove the imidazole.

**GST fusion proteins of specific regions of SAP90 and SAP97 were constructed by subcloning polymerase chain reaction-amplified DNA fragments directionally into the EcoRI–SalI sites of pGEX-4T (Amersham Pharmacia Biotech). Vectors expressing GST-SAP90 fusion proteins contained the SAP90 cDNA sequence encoding the following amino acids: GST-SH3ΔN (residues 402–500), GST-GUKΔN (residues 521–724), or GST-S90 (residues 402–724).** The GST-SAP97 fusion proteins contained the following amino acids: GST-SH3ΔN (residues 554–653), GST-GUKΔN (residues 727–911), or GST-S97 (residues 554–911). Recombinant proteins were prepared and purified according to the manufacturer's instructions. Protein concentrations were determined with a Protein Assay (BioRad).

**In Vitro Binding Assay**—For each sample, 800 pmol of H6KA2(c-term) or H6S97N were incubated with 200 pmol of GST fusion proteins in radioummunoprecipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A in phosphate-buffered saline) containing 10 μM EDTA and 40 mM imidazole. After 6–8 h of continuous rotation at 4 °C, 25 μl of Ni²⁺-nitrilotriacetic acid-Sepharose beads were added. Samples were then incubated for 1 h and washed four times, and bound proteins were eluted with 50 μl of 500 mM imidazole. The eluted proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose, probed for GST, and visualized by chemiluminescence as directed by the manufacturer (Amersham Pharmacia Biotech). For immunoblotting, 200 pmol of H6KA2(c-term) or H6S97N were incubated with 50 pmol of GST fusion proteins and 0–1600 pmol of H6S97N in radioummunoprecipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A in phosphate-buffered saline) containing 10 mM EDTA and 40 mM imidazole. The samples were processed as above, blotted, and probed using anti-KA2 antibodies (0.05 μg/ml; Upstate Biotechnology). To compare the bindings of SH3ΔN and SH3ΔN(W-A) to H6KA2 and H6S97N, 400 pmol of H6KA2(c-term) or H6S97N were incubated with 200 pmol of GST-SH3 fusion proteins in phosphate-buffered saline with 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 40 mM imidazole. After 6–8 h of continuous rotation at 4 °C, 25 μl of glutathione-Sepharose beads were added, and samples were incubated and washed as above, with elution in 50 μl of 10 mM glutathione.

**Results**

**Differential Interaction of SAP90 and SAP97 with the GluR6 and KA2 Subunits of Kainate Receptors**—In our previous study, co-immunoprecipitations from rat brain extracts showed that the GluR6 and KA2 subunits of kainate receptors associated with SAP90 (10). These experiments also showed a weak association between GluR6 and SAP97 and no association between KA2 and SAP97. Given the striking sequence homology between SAP90 and SAP97, this result was unexpected and raised the possibility that either SAP97 and kainate receptors do not coexist in the same subcellular compartments or that the structure of SAP97 is subtly different from SAP90, preventing its association with kainate receptors. To evaluate the first possibility, GluR6 or KA2 subunits were co-expressed with GFP-tagged SAP90 or SAP97 in HEK293 cells. After solubilization, immunoprecipitation was performed with anti-KA2 or anti-GFP antibodies, and the immunoprecipitated products, separated by SDS-PAGE, were probed with antibodies against SAP90, SAP97, or GluR6 (Fig. 1). Similar to our previous results using rat brain extracts, both the GluR6 and KA2 subunits interacted with SAP90, while GluR6 interacted weakly with SAP97 (Fig. 1, left) and no detectable interaction was seen between KA2 and SAP97 (Fig. 1, right). These results indicate that even when SAP97 and kainate receptors are co-expressed in HEK293 cells, they do not interact, suggesting that structural differences between SAP90 and SAP97 may be responsible for their selective interaction with kainate receptors.

**Binding of PDZ1 Domain of SAPs to GluR6 Determines Their Interaction**—Previously, we showed that SAP90 binding to GluR6 is mediated via its PDZ1 domain (10). Although sequence alignment of the PDZ1 domain of SAP90 and SAP97

Downloaded from http://www.jbc.org/ by guest on July 27, 2018
Fig. 1. Differential interaction of SAP90 and SAP97 with KA2 and GluR6 subunits. A, in comparison with SAP90, GluR6 binds weakly to SAP97. Immunoprecipitation with anti-GFP antibodies was performed from extracts of HEK293 cells expressing GluR6 alone, GluR6 plus GFP-SAP90, or GluR6 plus GFP-SAP97. The immunoprecipitated complexes were resolved on 8% SDS-PAGE and immunoblotted for GluR6 or GFP. GluR6 interacts strongly with SAP90 but not with SAP97 (upper panel). The middle panel shows the immunoprecipitated amounts of GFP-SAPs, and the bottom panel shows the expression levels of GluR6 in extracts. B, SAP90 interacts with KA2, while SAP97 does not. KA2 was immunoprecipitated from HEK293 cell extracts expressing KA2 and SAP90 or SAP97, using anti-KA2 rabbit polyclonal antibodies. The immunoprecipitated complexes were resolved on 8% SDS-PAGE and immunoblotted for SAPs. WCE, whole cell extract; IP, immunoprecipitation.

indicates about 90% identity, subtle change(s) in the amino acid sequence(s) of PDZ1 could be responsible for the selective interaction of SAPs with GluR6. To evaluate this possibility, we co-expressed GFP-tagged PDZ1 domains from SAP90 or SAP97 (GFP-PDZ1SAP90 and GFP-PDZ1SAP97) with the GluR6 subunit in HEK293 cells. Co-immunoprecipitations reveal that the GluR6 subunit binds well to GFP-PDZ1SAP90 and weakly to GFP-PDZ1SAP97 (Fig. 2A, left). This demonstrates that the amino acid sequence of the PDZ1 domain of SAPs is critical for its selective binding to GluR6.

SAP97 has also been shown to interact with the GluR1 subunit of AMPA receptors (11), and it has been speculated that the interaction is mediated via PDZ domains (11, 22). To show that GFP-PDZ1SAP97 forms a functional domain, we co-expressed it with the GluR1 subunit. Immunoprecipitation with the anti-GluR1 antibody reveals that GluR1 binds well to the GFP-PDZ1SAP97 (Fig. 2B, right). Taken together, these data suggest that the PDZ1 domain of SAPs is an important determinant for their differential interaction with GluR6.

The C Terminus of KA2 Can Interact with the SH3 and GUK Domains of SAP97—SAP90 binds to the cytoplasmic C-terminal tail of KA2 via its SH3 and GUK domains (10). Although the SH3 domains are not well conserved between SAP90 and SAP97, GUK domains share a high degree of sequence identity (23). We were therefore curious whether the SH3 and GUK domains of SAP97 could interact with C terminus of KA2. This was addressed by utilizing a “pull down” assay with several segments from the C-terminal half of SAP97 and SAP90, including the SH3 (GST-SH3SAP97 and GST-SH3SAP90), GUK (GST-GUKSAP97 and GST-GUKSAP90), or contiguous SH3 and GUK domains (GST-SG97 and GST-SG90) fused to GST and the C terminus of KA2 expressed with a histidine (His6) tag (His6KA2). The purity of each recombinant purified protein used in this assay was assessed by SDS-PAGE. Each exhibited a single band after staining with Coomassie Brilliant Blue except for the GST-SH3SAP97 and GST-SH3SAP90 proteins (Fig. 3, lower gel), which are subject to some degradation in Escherichia coli.

In our pull down assay, GST fusion proteins were incubated in solution with His6KA2 and then Ni2+ beads to bind out His6KA2 and any interacting GST fusion proteins. Bound proteins were eluted, immunoblotted, and probed with an anti-GST antibody.

Based on the relative amounts of the GST fusion protein detected after immunoblotting (Fig. 3, upper panel), the SH3 domain of SAP97 (SH3SAP97) appears to have a higher affinity for KA2 than the SH3 domain of SAP90 (SH3SAP90). The GUK domains of both SAP97 (GUKSAP97) and SAP90 (GUKSAP90) bind equally well to KA2. Given that the SH3 and GUK domains of SAPs bind to KA2 in a similar manner, we would conclude that the inability of KA2 to bind full-length SAP97 is not due to deficiency in SH3 and/or GUK binding. Previous studies (17, 18) raise the possibility that the inability to bind KA2 may involve sequences that either directly flank the SH3 or GUK domains, sequences upstream of the SH3 domain, or intramolecular interactions either between the SH3 and GUK domains or with other regions in SAP97. To address whether an SH3-GUK intramolecular interaction affected KA2 binding, we assessed KA2 binding to the GST-fused SH3-GUK proteins from both SAP90 and SAP97 (GST-SG90 and GST-SG97). KA2 was able to bind SG90 and SG97, albeit at a slightly lower level than SH3 alone, indicating that this intramolecular interaction is not important for the differential binding of KA2 to SAP90 versus SAP97. These data suggest that some other sequence elements unique to SAP97 must interfere with KA2 binding.

N Terminus of SAP97 Prevents Its Association with KA2—A comparison of the deduced amino acid sequences of SAP97 and SAP90 reveals that the N-terminal region preceding the first PDZ domain (S97N and S90N, respectively) and the sequence situated between the SH3 and GUK domains (U5 region) are the most divergent (18, 23). For example, the amino terminus of SAP97 is 187 amino acids in length and, based on molecular modeling, can be folded into two fibronectin-like domains, while the region containing 63 amino acids in the N terminus of SAP90 has an unordered structure (18). Similarly, the U5 region of SAP97 is longer than that found in SAP90 and can have short insert sequences of 1–33 amino acids that arise from alternative splicing. The most predominantly expressed form of SAP97 used in these studies contains the I3 insert. We therefore hypothesized that differences in the primary structure of SAP97 might govern its unusual behavior. To test this possibility, we assessed the ability of several GFP-tagged SAP97 deletion constructs (Fig. 4A) to interact with KA2 subunits expressed in HEK293 cells. GFP-tagged SAP97 deletion mutants included constructs in which the N terminus (SAP97ΔS97N), PDZ1–3 (SAP97ΔPDZ1–3), SH3 (SAP97ΔSH3), I3 (SAP97ΔI3),
Our N Terminus Can Compete with KA2 for SH3 Binding—The finding that the N terminus of SAP97 prevents its association with KA2 suggests that an intramolecular interaction between the N terminus and SH3 and/or GUK domains of SAP97 may prevent KA2 interaction. Given that the C terminus of KA2 interacts with SH3 via PXXP motifs (10), SAP97, which also has several proline-rich sequences containing PXXP motifs, could potentially bind to its own SH3 domain. We tested this by employing an in vitro binding assay using the histidine-tagged recombinant N terminus of SAP97 (H$_{6}$S97N) and GST-fusion proteins. As described above, binding to H$_{6}$S97N was done in solution, and the ability of GST-fusion proteins to interact with H$_{6}$S97N was identified using Western blots. We find that H$_{6}$S97N binds to the SH3$_{S97N}$ and SG97 domains but not to the GUK$_{S97}$ domain (Fig. 5).

Since the N terminus of SAP97 appears to block its binding to KA2 by intramolecular interaction with its SH3 domain, we speculated that replacement of the N terminus of SAP90 with S97N would result in a chimera (S97N/S90PDZ1-GUK) that is structurally similar to SAP97 and would not associate with KA2. Surprisingly, our results show that the S97N/S90PDZ1-GUK chimera can interact with KA2 (Fig. 4B). To investigate this unexpected finding, we compared the binding of S97N to SH3 and SG proteins from SAP90 and SAP97. As shown in Fig. 5, H$_{6}$S97N binds much better to SH3 and SG proteins from SAP97 than SAP90, indicating that a weaker intramolecular interaction between the S97N and SH3 may allow KA2 binding to the S97N/S90PDZ1-GUK chimera.
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. (B) Differential binding of kainate receptors to SAPs. A, a schematic representation of different recombinant SAP-GST fusion proteins shows the regions of SAPs that were expressed. Amino acid residues of SAPs are indicated as follows: H, His-tagged C terminus of KA2 (H6KA2) overnight. Nickel beads were added for 2 h, and proteins were eluted with imidazole, run on SDS-PAGE, and immunoblotted for GST (upper blot). Individual and contiguous SH3 and GUK domains from both of the SAPs interact with the C terminus of KA2. Neither beads alone nor the GST lane shows any binding to H6KA2, indicating the specificity of interactions. The purity of the recombinant GST-fusion proteins used in this study is shown in the Coomassie-stained lower gel. Lower bands in the GST-SH3 lanes are due to their degradation in bacteria. WB, Western blot.

this type of competition exists in vivo and that intramolecular interactions (between S97N and SH3sg7) interfere with intermolecular interactions (between KA2 and SH3sg7). However, these results do not indicate whether H6S97N and H6KA2 bind to the same site or distinct but overlapping sites on SH3.

We reported earlier (10) that H6KA2 binding to SH3S90 could be blocked by a tryptophan to alanine mutation (at position 470) in SH3, which has also been reported to inhibit other SH3-mediated protein-protein interactions (24). The potential importance of this site was tested by in vitro binding of GST-SH3 or GST-SH3(W-A) to H6KA2 or H6S97N. The bound complexes were pulled down by Ni²⁺ beads and probed for GST. We found that H6S97N binds to the mutated SH3 as well as wild type (Fig. 6B, compare lanes 5 and 6). In contrast, there was minimal binding of H6KA2 to mutated SH3 (Fig. 6B, compare lanes 2 and 3), indicating that tryptophan is a critical residue for SH3 interaction with KA2 but not with S97N. Because the tryptophan is required for most SH3-ligand interactions (24), this suggests that the S97N-SH3 interaction is atypical and that S97N may block the KA2 interaction via steric hindrance.

The sequence of the GUK domain is well conserved between SAP90 and SAP97, as compared with the SH3 domain. Our results indicate that neither the GUK domain nor the SH3 domain of SAP97 is functionally impaired in their capacity to interact with KA2 yet reveal that KA2 does not have access to these sites in full-length SAP97.

Intramolecular Binding between the S97N and the SH3 Domain of SAP97 Prevents Intermolecular Binding of KA2 and SAP97—Although SAP90 and SAP97 are homologous, SAP97 contains an extended N terminus and an insertion in the U5 region between the SH3 and GUK domains. Based on our data indicating that SAP97 lacking S97N binds well to KA2 and that in vitro the S97N can bind its own SH3 domain, we have identified S97N as the negative element preventing the interaction between KA2 and SAP97. Recent biochemical and structural studies have revealed that S97N association with its own SH3 and U5 regions modulates GKAP binding (18). Our findings indicate that an interaction between the SAP97 N terminus and SH3 domain occludes binding to KA2. However, since KA2 interacts with both the SH3 and GUK domains, it should be able to interact with SAP97 via the GUK domain. Nonetheless, SAP97 does not interact with KA2 in vivo or in vitro. One possible reason could be that the affinity of KA2 for the GUK domain is about 5-fold less than for the SH3 domain. Alternatively, an SH3-GUK intramolecular interaction may block the GUK binding site for KA2. Based on our results, a SH3-GUK interaction appears to be an important factor, since the SAP97 with deleted SH3 can bind to KA2 with lower affinity.

The sequence of the SH3 domain is not well conserved between SAP97 and SAP90. Interestingly, the N terminus of SAP97 binds SH3sg7 and SG97 better than SH3sg7 and SG90. These in vitro binding data are consistent with the co-immunoprecipitation results from HEK cells transfected with KA2 and a SAP90/SAP97 chimera with an N-terminal replacement of SAP90 with SAP97. The chimeric protein is similar in structure to SAP97 but binds to KA2. Since S97N binds much better to SG97 than SG90, the inhibition by S97N may not be strong enough to ward off KA2. Competition studies show that in vivo S97N can more easily compete KA2 binding to SG97 than SH3sg7. From these results, it appears that the presence of a
GUK domain contiguous with its SH3 domain has some inhibitory effects on KA2 interaction, possibly due to intramolecular SH3-GUK interactions.

**Interaction between the SH3 Domain and the N Terminal Is Atypical**—SH3 domains are known to interact with proline-rich sequences containing the consensus sequence PXXP (25, 26). However, binding of a SAP SH3 domain to its GUK domain is an exception (17–19), since the GUK does not have any such motif. Our results show that the N terminus of SAP97 to its SH3 may also be atypical. Although the N terminus contains proline-rich sequences and has PXXP motifs, it interacts well with the SH3 containing a W-A mutation, which normally blocks any PXXP-mediated binding to SH3 domains. It is conceivable that the N terminus may still bind to SH3 via its PXXP motifs, but the tryptophan residue is not important. Alternatively, some other sequences at the N terminus may be required for its interaction with SH3. Nevertheless, our results show that intramolecular interactions between the N terminus of SAP97 and SH3 can deny access of KA2 to its potential binding site (Fig. 7).

These effects appear to be similar to the ones described for Src and Tec family kinases (27, 28), where intramolecular interactions...
interactions between the SH3 and catalytic domains cause autoinhibition. However, once an extrinsic ligand binds the SH3 domain, the intermolecular interaction interferes with the intramolecular interaction, freeing the catalytic domain and making it active. It is conceivable that a high affinity ligand for the N terminus of SAP97 may exist in vivo to shift SAP97 into a more open state, exposing its SH3 domain and making it available to bind KA2. Potentially, any resultant binding might be too transient to be detected by standard co-immunoprecipitation or immunocytochemical assays. Alternatively, SAP97 intramolecular interactions could be part of a basic molecular mechanism used by cells to allow the differential association of SAPs with binding partners.

Acknowledgments—We acknowledge Dr. Morgan Sheng for a PSD-95 construct. We also thank Drs. Leslie Blair, Dale Mierke, and Elizabeth Garcia and Zhao Ren for useful suggestions and for carefully reading the manuscript.

REFERENCES
1. Kennedy, M. B. (1997) Trends Neurosci. 20, 264–268
2. Garner, C. C., Nash, J., and Huganir, R. L. (2000) Trends Cell Biol. 10, 274–280
3. Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1992) Neuron 9, 929–942
4. Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993) J. Biol. Chem. 268, 4580–4583
5. Kuhlendahl, S., Spangenberg, O., Konrad, M., Kim, E., and Garner, C. C. (1998) Eur. J. Biochem. 252, 305–313
6. Sheng, M., and Pak, D. T. (1999) Ann. N. Y. Acad. Sci. 866, 483–493
7. Muller, B. M., Kistner, U., Kindler, S., Chung, W. J., Kuhlendahl, S., Fenster,
8. Lau, L. F., Mammen, A., Ehlers, M. D., Kindler, S., Chung, W. J., Garner, C. C., and Huganir, R. L. (1996) *J. Biol. Chem.* **271**, 21622–21628
9. Leonard, A. S., Davare, M. A., Horne, M. C., Garner, C. C., and Hell, J. W. (1998) *J. Biol. Chem.* **273**, 19518–19524
10. Garcia, E. P., Mehta, S., Blair, L. A., Wells, D. G., Sheng, M., and Fallon, J. R. (1998) *Neuron* **21**, 727–739
11. Craven, S. E., and Bredt, D. S. (2000) *J. Biol. Chem.* **275**, 20045–20051
12. Hsueh, Y. P., Kim, E., and Sheng, M. (1997) *Neuron* **18**, 803–814
13. Erpel, T., Superti-Furga, G., and Courtneidge, S. A. (1995) *EMBO J.* **14**, 963–975
14. Wu, H., Reissner, C., Kuhlendahl, S., Coblentz, B., Reuver, S., Kindler, S., Gundelfinger, E. D., and Garner, C. C. (2000) *EMBO J.* **19**, 5740–5751
15. Andreotti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997) *Nature* **385**, 93–97
16. El-Husseini, A. E., Craven, S. E., Chetkovich, D. M., Firestein, B. L., Schnell, E., Aoki, C., and Bredt, D. S. (2000) *J. Biol. Chem.* **275**, 20045–20051
17. Kuhlendahl, S., Chung, W. J., and Garner, C. C. (1998) *J. Cell Sci.* **111**, 2365–2376
18. Wu, H., Reissner, C., Kuhlendahl, S., Coblentz, B., Reuver, S., Kindler, S., Gundelfinger, E. D., and Garner, C. C. (2000) *EMBO J.* **19**, 5740–5751
19. Sheng, M., and C. E. (1996) *Neuron* **17**, 255–265
20. Seeburg, P. H. (1993) *Trends Neurosci.* **16**, 359–365
21. Hollmann, M., and Heinemann, S. (1994) *Annu. Rev. Neurosci.* **17**, 31–108
22. Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., and Malinow, R. (2000) *Science* **287**, 2262–2267
23. Muller, B. M., Kistner, U., Veh, R. W., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., and Garner, C. C. (1995) *J. Neurosci.* **15**, 2354–2366
24. Erpel, T., Superti-Furga, G., and Courtneidge, S. A. (1995) *EMBO J.* **14**, 963–975
25. Weng, Z., Rickles, R. J., Feng, S., Richard, S., Shaw, A. S., Schreiber, S. L., and Brugge, J. S. (1995) *Mol. Cell. Biol.* **15**, 5627–5634
26. Mayer, B. J., and Eck, M. J. (1995) *Curr. Biol.* **5**, 364–367
27. Maccioni, B. R., Lefevre-Bernt, M., Sichesi, F., Huse, M., Lee, C. H., Kuriyan, J., and Miller, W. T. (1997) *Nature* **385**, 650–653
28. Andretti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997) *Nature* **385**, 93–97
