Identification of Multiple Binding Partners for the Amino-Terminal Domain of SAP97

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Running Title: Multiple MRE domain partners of SAP97
SUMMARY

Multiprotein complexes mediate static and dynamic functions to establish and maintain cell polarity in both epithelial cells and neurons. Membrane Associated Guanylate Kinase (MAGUK) proteins are thought to be scaffolding molecules in these processes and bind multiple proteins via their obligate PSD-95/Disc Large/Zona Occludens-1 (PDZ), Src Homology 3 (SH3), and Guanylate Kinase-like (GUK) domains. Subsets of MAGUK proteins have additional protein-protein interaction domains. An additional domain we identified in SAP97 called the MAGUK Recruitment (MRE) domain, binds the LIN-2,7 amino-terminal (L27N) domain of mLIN-2/CASK, a MAGUK known to bind mLIN-7. Here we show that SAP97 binds two other mLIN-7 binding MAGUK proteins. One of these MAGUK proteins, DLG3, coimmunoprecipitates with SAP97 in lysates from rat brain and transfected MDCK cells. This interaction requires the MRE domain of SAP97 and surprisingly, both the L27N and L27 carboxy terminal (L27C) domains of DLG3. We also demonstrate that SAP97 can interact with the MAGUK protein, DLG2, but not the highly related protein, PALS2. The ability of SAP97 to interact with multiple MAGUK proteins is likely to be important for the targeting of specific protein complexes in polarized cells.
INTRODUCTION

Polarized cells such as neurons and epithelia establish and maintain a non-homogenous plasma membrane distribution of both integral and peripheral membrane proteins (1-3). Apical and basolateral membranes of epithelial cells are subdivided into even smaller sub-domains, e.g. lipid rafts, adherens junctions and tight junctions, each with unique sets of proteins and distinct functions. Neurons are also highly polarized cells segregating different protein complexes in the presynaptic axon and postsynaptic dendrite. Polarity can be a dynamic event where proteins are shuttled from one place to another or a more static process where proteins are retained at a particular cell surface (4,5). Large complexes of proteins are involved in each of these cellular processes.

Several members of the Membrane Associated Guanylate Kinase, or MAGUK, family of proteins have been implicated in polarity processes. MAGUKs are a subclass of PDZ domain containing proteins. Several members of the PDZ domain containing family have been reported to form large multi-protein complexes to accomplish specific cellular tasks. One example is the mLIN-10/mLIN-2/mLIN-7 complex of proteins (6-8) where each member contains one or more PDZ domains. In neurons, the scaffolding MAGUK mLIN-2 binds mLIN-10 which in turn binds KIF17, a motor protein (9). Movement of protein cargo along microtubules was shown to be dependent on the interaction of KIF17 with mLIN-10. Intriguingly, mLIN-2 binds mLIN-7 in epithelial cells where mLIN-7 acts to retain BGT-1 at the basolateral surface (10). Hence, protein complexes that include mLIN-2 seem to serve both dynamic and static functions.

Recently, a subset of MAGUKs was defined by the presence of a novel protein-protein interaction domain found to bind mLIN-7 (11). This group has several members including mLIN-2/CASK, DLG2 (MPP2), DLG3 (MPP3), MPP4, PALS1 (MPP5) and, PALS2 (MPP6).
Each protein contains a PDZ domain, SH3 domain and catalytically inactive guanylate kinase (GUK) domain in addition to two novel domains that were named L27N (located closer to the amino terminus) and L27C (positioned closer to the carboxy terminus) (12). The L27C domain of mLIN-2 and PALS1 has been shown to bind mLIN-7 (11,13). The L27N domain of mLIN-2/CASK binds SAP97 (13,14) while the L27N domain of PALS1 binds PATJ (15). Subtle differences were noted between the mLIN-7 binding proteins. For example, a protein 4.1 binding domain (or Hook domain) is present in all except DLG3 and MPP4. Protein 4.1 binding domains provide a link to the cortical actin cytoskeleton by binding to members of the Protein 4.1/Ezrin/Radixin/Moeisin superfamily (16-18). Also, mLIN-2 and PALS1 have an extra domain at their extreme amino termini, the CAMKII and U1 respectively.

Since large complexes of PDZ domain containing proteins seem to be important mediators of polarity establishment and maintenance, defining these complexes could elucidate novel cellular functions. SAP97, a mammalian homolog of the Drosophila tumor suppressor Dlg (19), is a MAGUK protein that was reported to bind mLIN-2/CASK via a newly described MAGUK Recruitment (MRE) domain (13,15). Here we show that SAP97 binds two other mLIN-7 associated MAGUKs via this MRE domain.

**EXPERIMENTAL PROCEDURES**

*DNA constructs, Cloning Methods, and Mutagenesis*- The carboxy terminal amino acid sequence of mouse DLG3 reported in GenBank contained the amino acids EPAASSELS which was highly divergent from other mLIN-7 binding proteins and the reported sequence for human DLG3. We obtained and sequenced a mouse EST (GenBank Accession Number AI1498811) which contained sequence that coded for a carboxy terminus highly homologous to both human
DLG3 and other mLIN-7 binding proteins. We amplified mouse DLG3 from this clone and subcloned it into PCDNA3.1 in frame with an amino-terminal Myc tag and sequenced this construct. The sequence coded for a protein that was almost identical to human DLG3. The coding sequence for DLG3 was also subcloned into the RK5-Myc vector. The pGSTag-DLG3(1-222) construct was created by subcloning a restriction enzyme digested PCR product using standard methods. Construction of pGSTag-SAP97(1-78), pGSTag-mLIN-2/CASK(L27N)(329-403) for expression as glutathione-S-transferase (GST) fusion proteins has been described elsewhere (13). The deletions constructs RK5-Myc-DLG3(ΔL27N), RK5-Myc-DLG3(ΔL27C) and RK5-Myc-DLG3(ΔPDZ) were made by the single primer method as described elsewhere (20). The point mutation constructs RK5-Myc-DLG3(L91G) and RK5-Myc-DLG3(L34G:F38G) were also made by the single primer method. The coding sequence for DLG2 was amplified from a mouse cDNA library and subcloned into PCDNA3.1 in frame with an amino-terminal Myc tag.

Preparation of 32P-labeled GST-fusion proteins- Bacterial expression, purification and labeling of GST-mLIN-7 (mouse), GST-DLG3(1-222) (mouse) and GST-mLIN-2(329-403) (human) for use in Far Western blotting was performed as previously described (11).

Protein Procedures and Far Western Overlay Assay- MDCK cells were lysed as previously described (13). Briefly, cells were washed in PBS and then lysed in buffer containing 0.1% Triton X-100. Immunoprecipitations and GST-pull-downs were performed as previously described (11,21). Far Western blotting was performed as previously described (11) with the following modifications. Blots were done either overnight at 4 °C or for 2 hours at room temperature. Additionally, dried membranes were either exposed to film with intensifying screens or processed for analysis using a phosphorimagery.
Antibodies- Polyclonal antibodies against GST-DLG3(289-365) were raised in rabbits with the fusion protein coupled to glutathione-agarose beads as the immunogen. Affinity purification of anti-DLG3 antibodies was carried out with DLG3(289-365) expressed as a NusA fusion protein coupled to Affi-Gel support as described elsewhere (22). This antibody was only used for Western blotting. Immunoprecipitation of DLG3 was performed using an antibody which was the kind gift of Elior Peles. Antibodies directed against PALS2 and mLIN-7 were reported elsewhere (11,22). A monoclonal antibody to SAP97 was purchased from StressGen Biotechnologies. The mouse monoclonal antibody 9E10 was used wherever an anti-Myc antibody was indicated.

Transfections and Immunostaining- MDCK cells were transfected with Fugene 6 (Roche) as previously described (13). For constructs cloned into the PCDNA3.1/zeocin vector, stably expressing clones were selected in 200 µg/mL zeocin, picked, and maintained in 100 µg/mL zeocin. For constructs cloned into the pRK5-Myc vector, cells were cotransfected with either PCDNA3.1/Hyg or pSV2/Neo. Antibiotic selection and maintenance concentrations were 500 µg/ml and 250 µg/ml for Hygromycin and 600 µg/ml and 300 µg/ml for G418. Cells were processed for immunostaining and imaged as previously described (22). Confocal microscopy was performed at the Microscopy and Image-analysis Laboratory at the University of Michigan using a ZEISS LSM510 Axiovert 100M inverted confocal microscope.

RESULTS

Multiple mLIN-7 binding proteins associate with SAP97

Previous studies have demonstrated that several MAGUK proteins can bind mLIN-7 including mLIN-2/CASK (6,8,11) and that mLIN-2/CASK (Fig. 1A) can directly interact with
SAP97 (13, 23). We have utilized $^{32}$P-GST-mLIN-7 as a probe in Far Western blotting to identify mLIN-7 binding proteins in immunoprecipitates (11). Using this technique, we found that in addition to mLIN-2/CASK other mLIN-7 binding proteins were present in SAP97 immunoprecipitations (11, 13).

To explore this further, rat brain lysates were immunoprecipitated with antibodies directed against known mLIN-7 binding proteins including mLIN-2/CASK, DLG3, and PALS2. Immunoprecipitations were also performed with mLIN-7 and SAP97 antibodies (Fig. 1B). Pre-immune serum was included as a control for non-specific binding to the Protein-A Sepharose beads. Three mLIN-7 binding proteins clearly coimmunoprecipitated with SAP97. Two co-migrated with proteins in the DLG3 and mLIN-2 lanes at approximately 64 kDa and 110 kDa, respectively. The third migrated close to PALS2 at approximately 55-60 kDa. However, PALS2 seemed to migrate slightly below this molecular weight and was seen as a doublet when blotting with a PALS2 antibody (data not shown).

**DLG3 directly binds SAP97**

Based on the immunoprecipitation experiments in rat brain, DLG3 was investigated as a putative partner for SAP97. To see if the interaction was direct, we employed Far Western blotting using $^{32}$P-GST-DLG3(1-222) as a probe. This probe, which includes both L27 domains and the PDZ domain of DLG3, was chosen because shorter probes containing one or both L27 domains were poorly expressed in bacteria. Lysates from MDCK cells transfected with Myc-tagged mouse DLG3 were immunoprecipitated with antibodies against Myc, SAP97, mLIN-7, or pre-immune serum (Figure 2A). It should be noted that the mouse DLG3 we subcloned was obtained from an EST and differs from the sequence reported in GenBank (see “Experimental Procedures”). The probe bound a protein that migrated at approximately 130 kDa in all lanes.
except pre-immune. This membrane was stripped and reprobed with $^{32}$P-GST-mLIN-7 to confirm that Myc-DLG3 was efficiently immunoprecipitated (data not shown). This experiment strongly suggested that DLG3 directly bound SAP97 via either its L27N domain, L27C domain, PDZ domain, or some combination of the three.

Before discriminating between those possibilities we wanted to study the interaction further. Previously published data from our lab showed that the first 78 amino acids of SAP97 containing the MRE domain were necessary and sufficient for mLIN-2/CASK binding (13). By GST-pull down, we tested if SAP97 bound DLG3 via the same domain (Fig. 2B). Bacterially expressed GST-SAP97(1-78) or GST alone were immobilized on glutathione agarose beads and incubated with lysates from MDCK cells expressing Myc-DLG3 or Myc-mLIN-2. Both Myc-DLG3 and Myc-mLIN-2 bound SAP97 via this MRE domain.

We next wanted to determine if SAP97, Myc-DLG3 and mLIN-7 exist in a complex in MDCK cells. Antibodies against Myc, SAP97 or pre-immune serum were incubated with lysates from MDCK cells expressing Myc-DLG3. Resolved immunocomplexes were subjected to western blotting with anti-mLIN-7, anti-Myc, and anti-SAP97 antibodies (Fig. 2C). Both Myc and SAP97 immunocomplexes contained all three proteins which strongly suggested that they form a complex in MDCK cells.

To show that this interaction may have some physiological relevance we repeated the rat brain immunoprecipitation experiment. However, instead of a Far Western blot, we immunoblotted with both anti-SAP97 and an affinity purified DLG3 antibody raised against GST-DLG3(289-365). This epitope was chosen because it was the region of DLG3 that shared the least homology with the other mLIN-7 binding MAGUKs. Both DLG3 and SAP97 can be detected when anti-DLG3 or anti-SAP97 antibodies were used to immunoprecipitate proteins.
from brain lysates (Fig. 2D). These data indicated that endogenous DLG3 and SAP97 interact in rat brain.

Both L27N and L27C domains of DLG3 are required to bind SAP97

In Figure 2A we demonstrated that SAP97 and DLG3(1-222) directly interact. In other reports, the L27C domain of mLIN-7 binding proteins bound mLIN-7 while the L27N domain bound the MRE domain of another partner (13,15). DLG3 constructs lacking the L27N, L27C, or PDZ domain were made to further investigate the exact region of DLG3 necessary for the interaction with SAP97. Lysates from MDCK cells overexpressing Myc-tagged full length DLG3, DLG3 deletion constructs, or Myc-mLIN-2 were immunoprecipitated with anti-Myc antibody. SAP97 was visualized by a previously reported Far Western assay utilizing $^{32}$P-GST-mLIN-2(L27N) (13).

SAP97 coprecipitated with Myc-mLIN-2, Myc-DLG3 and Myc-DLG3(ΔPDZ) (Fig. 3B). The L27N deletion still bound mLIN-7 which suggests that the L27C domain was intact. As expected, deleting the L27C domain of DLG3 resulted in the inability of mLIN-7 to interact with this protein. Surprisingly, deleting either the L27N or L27C of DLG3 resulted in the failure of SAP97 to coprecipitate. This result was unexpected since the L27N domain of mLIN-2 was solely responsible for SAP97 binding (13).

Point mutations were made in DLG3 to further investigate the possibility that both L27 domains of DLG3 are necessary for SAP97 binding. Point mutations of conserved hydrophobic residues, either singly in the L27C domain or in tandem in the L27N domain, have been shown to negate binding of the domains to target proteins (11). For example, mLIN-2(L422G), a mutation in the L27C domain, does not bind mLIN-7 (D.K. unpublished observation) and a double point mutation in the L27N domain of PALS1(L150G;V154G) does not bind PATJ (15).
Hence, analogous mutations were created in the L27C and L27N domains of DLG3, Myc-DLG3(L91G) and Myc-DLG3(L34G:F38G), respectively. The experiment shown in Figure 3B was repeated using these constructs and omitting the Myc-mLIN-2 control. As expected Myc-DLG3(L91G) did not bind mLIN-7, but it also did not bind SAP97 (Fig. 3C). SAP97 only coprecipitated with wild type Myc-DLG3. Taken together with the deletion analysis, these data strongly suggested that both L27 domains of DLG3 were required for SAP97 binding.

*Endogenous mLIN-7 localization is perturbed by expression of Myc-DLG3*

Previous work showed that SAP97 and mLIN-2/CASK colocalized to the basolateral surface in MDCK cells and this localization was dependent on the L27N domain of mLIN-2/CASK (13). We wanted to see where Myc-DLG3 would localize. To this end, we seeded MDCK cells overexpressing Myc-DLG3 or Myc-mLIN-2/CASK, as a control, on polycarbonate filters and processed them for immunostaining (Fig. 4A and 4B). As expected, Myc-mLIN-2 and mLIN-7 colocalized at the basolateral surface (22). Interestingly, Myc-DLG3 displayed a diffuse localization and appeared to mislocalize a portion of endogenous mLIN-7. This effect was most evident when comparing a cell expressing Myc-DLG3 to an adjacent cell that was not. One possible explanation for this diffuse localization of Myc-DLG3 is that DLG3 lacks a Hook domain. Hook domains are believed to bind members of the protein 4.1/ezrin/radixin/moeisin superfamily, that can provide a link between MAGUK proteins and the actin cytoskeleton (16-18). All studied mLIN-7 associated MAGUKs except DLG3 contain a Hook domain and localize to a lateral surface in MDCK cells. In accordance with this hypothesis, a chimeric Myc-DLG3 protein with the Hook domain of mLIN-2/CASK inserted in frame (Myc-DLG3:Hook) localized predominantly to the basolateral surface (data not shown).
In Figure 4B, we showed that a portion of endogenous mLIN-7 was mislocalized in cells expressing Myc-DLG3. To confirm that this effect was attributable to DLG3, we compared mLIN-7 localization in cells expressing Myc-DLG3 versus Myc-DLG3(ΔL27C), which lacks the ability to bind mLIN-7 (Figure 4C). Endogenous mLIN-7 was partially mislocalized only if DLG3 had an intact L27C domain. This result strongly suggested that mLIN-7 interacts with Myc-DLG3 and the two exist in a complex in the cytosol. Deleting the L27C domain of Myc-DLG3 ablates binding and hence mLIN-7 remains at the basolateral surface while Myc-DLG3(ΔL27C) localizes diffusely throughout the cell.

*SAP97 and Myc-DLG2 coimmunoprecipitate from MDCK cell lysates*

DLG3 and mLIN-2/CASK have been established as two of the three mLIN-7 associated partners for SAP97. We sought to identify the third partner. Although the third partner migrated close to PALS2 in Figure 1B, DLG2 was also a possibility. Since we had an antibody to PALS2 but not DLG2, we overexpressed Myc-DLG2 in MDCK cells. Immunoprecipitations with anti-SAP97, anti-PALS2, anti-Myc or pre-immune serum were performed on lysates containing exogenously expressed Myc-DLG2. Control immunoprecipitations were performed on mock transfected cells or cells expressing Myc-DLG3 or Myc-mLIN-2.

SAP97 was efficiently coprecipitated with exogenously expressed Myc-DLG2, Myc-DLG3 and Myc-mLIN-2 (Fig. 5A). No SAP97 could be detected in complex with endogenous PALS2 in either Myc-DLG2 expressing cells or mock transfected cells. Admittedly, the amount of immunoprecipitated PALS2 was less than Myc-DLG2, Myc-DLG3 or Myc-mLIN-2. Since DLG2 and PALS2 migrate at approximately the same molecular weight, we wanted to confirm that PALS2 did not associate with SAP97 in brain. Previous experiments in rat brain indicated that PALS2 was a very abundant protein and could be efficiently precipitated with anti-PALS2
antibody (Fig. 1B). Hence, we repeated the rat brain immunoprecipitation experiment of Figure 1B but instead of a Far Western blot, we immunoblotted for SAP97 and PALS2 (Fig. 5B). PALS2 and SAP97 did not coprecipitate in this experiment. Additionally, GST-MRE of SAP97 can precipitate exogenously expressed Myc-DLG2 from MDCK cell lysates (data not shown). We concluded that DLG2, and not PALS2, was a third mLIN-7 associated binding partner for SAP97.

**DISCUSSION**

Multiprotein complexes of PDZ domain containing proteins are important mediators of polarity in epithelial cells and neurons. Several reported protein scaffolds include SAP97 as an integral player (23-25). Here we provide data that a total of three mLIN-7 binding proteins interact with SAP97: mLIN-2/CASK (which was previously described; (13,14,23)), DLG2 and DLG3. Promiscuous binding of MAGUKs to other family members as well as themselves has also been widely reported. Discovering why SAP97 binds DLG2, DLG3, and mLIN-2/CASK but not PALS2 and PALS1 will be an important next step in understanding the molecular mechanisms of L27 domain interactions. In particular PALS2 is 66% identical and 84% similar to DLG2 yet does not bind SAP97. It is possible that other binding partners play some role in determining specificity. One clue as to why SAP97 binds multiple mLIN-7 binding MAGUKs may come from the report that KIF1Bα binds SAP97 (25). This report suggests a role for a SAP97 complex in vesicle targeting. It is possible that SAP97 binds different scaffolds to mediate the targeting of subsets of vesicles or receptors to specific membrane locations or organelles along microtubules.
An unpredicted finding of this study is that DLG3 binding to SAP97 requires both the L27N and L27C domains of DLG3. One possibility is that mLIN-7 somehow assists DLG3 binding to SAP97. This seems unlikely since a direct interaction between GST-DLG3(1-222) and SAP97 can be observed in the Far Western overlay assay. Secondary structure algorithms predict that L27 domains are made up of 2 alpha helices (12). In theory two alpha helices from one L27 domain may fold onto the adjacent L27 domain to form a 4 helix bundle. It is possible that in most mLIN-7 binding MAGUKs, the two helices from the L27C domain interact with mLIN-7 while both of the L27N helices interact with another partner. The DLG3:SAP97 interaction may be different in that it requires one helix from each L27 domain of DLG3 to form a stable complex with SAP97. However, in circular dichroism studies of the L27 domains of mLIN-7, mLIN-2 and orthologues, Harris et al. found that L27 domains seem to be largely unfolded until binding to another L27 domain (26). Thus more structural studies of L27 domains will be required before any definitive conclusions can be reached.

Our group previously reported a possible role for mLIN-7 in endosomal trafficking of proteins (27). However, we have not been able to visualize mLIN-7 in an endosomal compartment. Here we show that endogenous mLIN-7 localization is altered in MDCK cells expressing Myc-DLG3. This staining appears to be diffusely cytosolic, but could also represent an association with intracellular membranes including endosomes. Further staining with markers of different intracellular membranes will be necessary to address this prospect. Due to problems with antibodies, we have been unable to determine if SAP97 is mislocalized in cells expressing Myc-DLG3. Further experimentation is necessary to determine if Myc-DLG3 and SAP97 colocalize in the cytosol.
Most mLIN-7 is associated with mLIN-2 in MDCK cells and is thus tethered to the actin cytoskeleton via the protein 4.1 binding domain of mLIN-2/CASK (11,22,28). Since DLG3 lacks a protein 4.1 binding domain it may play a role at a non-plasma-membrane site, such as an endosome. Previous data indicates that basolateral localization of mLIN-2 requires an intact hook domain. Additionally, we found that a DLG3:Hook chimera localizes to the basolateral surface which suggests that this domain has the power to redirect a cytosolic MAGUK to the basolateral surface. It would be interesting to test if this domain alone could direct any cytosolic protein to the plasma membrane.

In summary we have demonstrated that the MRE domain of SAP97 has multiple binding partners including DLG3, DLG2 and mLIN-2/CASK. These multiple interactions allow SAP97 to be present in different protein complexes within cells. These differential interactions could control the targeting and localization of different SAP97 complexes. Additionally, signaling complexes containing SAP97 (29,30) could be altered depending on the MRE binding partners present in individual cells.
ACKNOWLEDGEMENTS

We thank the following people for contributions and materials: Emmanuel Kamberov and Olga Makarova for generation of PCDNA3.1-Myc-DLG2, Elior Peles for his generous gift of anti-DLG3 antibodies, Samuel Straight and Albert Liu for expert assistance with confocal microscopy, and members of the Margolis lab for suggestions and critical reading of this manuscript.

David Karnak was partially supported by NIH Cellular Biotechnology Training Program Grant (GM08353). Seonok Lee was supported by NIH Genetics Training Grant (5-T32-GM07544). Ben Margolis is an investigator of the Howard Hughes Medical Institute. This work was supported in part by NIDDK grant (2-P50-DK39255). We are also grateful to Stephen Lentz, manager of the University of Michigan Diabetes Research Center Morphology and Image Analysis Core (NIH grant 5-P60-DK20572), for his assistance with confocal microscopy.
FIGURE LEGENDS

Figure 1. **Multiple mLIN-7 binding proteins associate with SAP97.** A. Schematic illustration depicting the recognized protein-protein interaction domains of the mLIN-7 associated MAGUKs (DLG3, DLG2, PALS2, PALS1 and mLIN-2/CASK) as well as mLIN-7 and SAP97. The MRE domain of SAP97 binds the L27N domain of mLIN-2. The L27 domain of mLIN-7 binds the L27C domain of mLIN-2 and PALS1. B. Triton X-100 extracted homogenates from rat brain were immunoprecipitated with the indicated antibodies. Input represents 10% of the material precipitated. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. mLIN-7 associated MAGUKs were detected by Far Western overlay assay with $^{32}$P-GST-mLIN-7 prepared as noted in Experimental Procedures. A non-specific band of 120 kDa is seen in all immunoprecipitation (IP) lanes, but a stronger band is seen in mLIN-7 and SAP97 IP lanes where it represents mLIN-2.

Figure 2. **DLG3 directly binds SAP97.** A. Lysates from MDCK cells expressing amino terminal Myc-tagged DLG3 were immunoprecipitated with the indicated antibodies. Input represents 5% of the material precipitated. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with $^{32}$P-GST-DLG3(1-222) which contains the L27N, L27C, and PDZ domains of DLG3. B. Lysates from MDCK cells expressing either Myc-mLIN-2/CASK or Myc-DLG3 (noted under the gel) were incubated with either GST or GST-SAP97(1-78), which is the minimal portion of the MRE domain found to bind mLIN-2, bound to glutathione agarose beads. Beads were washed three times in HNTG. Inputs are 5% of the material used in the pulldowns. Proteins were treated as above. The
membrane was then immunoblotted with mouse anti-Myc(9E10) antibody. C. Lysates from MDCK cells expressing Myc-DLG3 were immunoprecipitated with the noted antibodies. Proteins were treated as above. The nitrocellulose was cut into strips and immunoblotted with either anti-SAP97, anti-Myc, or affinity purified anti-mLIN-7 antibody. D. Triton X-100 homogenates from rat brain were immunoprecipitated with noted antibodies. Input represents 5% of the material used in the precipitation. Proteins were treated as above. The nitrocellulose was cut into strips and immunoblotted with anti-SAP97 or affinity purified anti-DLG3 antibody.

Figure 3. The L27N and L27C domains of DLG3 are both required to bind SAP97. A. Schematic representation of Myc-DLG3 constructs generated by single primer mutagenesis as noted in Experimental Procedures. B. Lysates from MDCK cells expressing the Myc-tagged proteins noted above the gel were immunoprecipitated with anti-Myc antibody. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was cut into strips and immunoblotted with either affinity purified anti-mLIN-7 or anti-Myc antibody. SAP97 was detected by Far Western blotting with $^{32}$P-GST-mLIN-2(L27N) which has been shown to specifically recognize this protein. C. The above mentioned procedure was performed on lysates from MDCK cells expressing Myc-DLG3(L91G), Myc-DLG3(L34G:F38G), or wild type Myc-DLG3. Mock transfected cells were included in this experiment as a negative control.

Figure 4. The L27C domain of DLG3 is responsible for endogenous mLIN-7 mislocalization. MDCK cells expressing either (A) Myc-mLin2/CASK, (B) Myc-DLG3 or (C) Myc-DLG3(ΔL27C) were seeded at high density on polycarbonate filters as described in Experimental Procedures. Filters were then processed for immunostaining with affinity purified
anti-mLIN-7 and anti-Myc primary antibodies. Secondary antibodies coupled to fluorochromes were added and filters were examined by confocal laser scanning microscopy. The square panels represent digital photomicrographs (x-y dimension of the z series), and rectangular panels show the x-z or y-z dimensions (z-sections).

Figure 5. **SAP97 and Myc-DLG2 coimmunoprecipitate from MDCK cell lysates.** A. Lysates from MDCK cells expressing Myc-DLG2, Myc-DLG3, or Myc-mLIN-2 (noted under the gel) were immunoprecipitated with noted antibodies in duplicate. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. One membrane was immunoblotted with anti-SAP97 antibody and the other was Far Western blotted with $^{32}$P-GST-mLIN-7. B. Triton X-100 extracted homogenates from rat brain were immunoprecipitated with the indicated antibodies. Input represents 10% of the material precipitated. Proteins were treated as above. The membrane was cut into two strips. One strip was immunoblotted with anti-SAP97 antibody and the other was immunoblotted with affinity purified anti-PALS2 antibody.
ABBREVIATIONS

The abbreviations used are: MAGUK, membrane-associated guanylate kinase; PDZ, postsynaptic density 95/discs large/zona occludens 1; SH3, Src Homology 3; GUK, guanylate kinase; SAP97, synapse associated protein 97; MRE, MAGUK recruitment; L27, LIN2,7; KIF17, kinesin superfamily member 17; BGT-1, betaine-glutamate transporter 1; MPP, membrane protein, palmitoylated; PALS, protein associated with Lin Seven; PATJ, Pals1-associated tight junction; CAMKII, calmodulin kinase type 2; Dlg, discs large; MDCK, Madin-Darby canine kidney; GST, glutathione S-transferase; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PBS, phosphate-buffered saline; KIF1Bα, kinesin superfamily member 1B alpha; and IP, immunoprecipitation.
REFERENCES:


A  

- L27
- PDZ
- SH3
- HOOK
- GK

- DLG3
- DLG2
- Pals2
- Pals1
- mLin-2/CASK
- mLin-7
- CKII

- MRE
- SAP97

B  

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Blot: [32P]-GST-mLin-7

Figure 1
Figure 2
Figure 3
Figure 5
Identification of multiple binding partners for the amino-terminal domain of SAP97
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*J. Biol. Chem.* published online September 25, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208781200

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