The Stardust Family Protein MPP7 Forms a Tripartite Complex with LIN7 and DLG1 That Regulates the Stability and Localization of DLG1 to Cell Junctions*

Joanna Bohl, Nicole Brimer, Charles Lyons, and Scott B. Vande Pol

From the Department of Pathology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

MPP7, a previously uncharacterized member of the p55 Stardust family of membrane-associated guanylate kinase (MAGUK) proteins, was found in a tripartite complex with DLG1 and LIN7A or LIN7C. MPP7 dimersize with all three LIN7 family members (LIN7A, -B, and -C) through interaction of the single L27 domain of LIN7 with the carboxy-terminal L27 domain of MPP7, thereby stabilizing both proteins. The dimer of MPP7 with LIN7A or LIN7C associates with DLG1 through an interaction requiring the amino-terminal L27 domain of MPP7. The amino-terminal L27 domain of MPP7 is not sufficient for interaction with DLG1 but interacts efficiently only if MPP7 is in a complex with LIN7A or -C. Thus the specificity of interaction of DLG1 with the LIN7-MPP7 complex is determined by L27 interactions with both MPP7 and LIN7. The tripartite complex forms in a ratio of 1:1:1 and localizes to epithelial adherens junctions in a manner dependent upon MPP7. Expression of MPP7 stabilizes DLG1 in an insoluble compartment. Expression of MPP7 deleted of the PDZ or Src homology 3 domain redistributes MPP7, DLG1, and LIN7 out of adherens junctions and into the soluble cytoplasmic fraction without changing the localization of E-cadherin. Thus, the stability and localization of DLG1 to cell-cell junctions are complex functions determined by the expression and association of particular Stardust family members together with particular LIN7 family members.

MAGUK family proteins are critical adapter proteins for the assembly of multiprotein complexes at sites of cell-cell contact. MAGUK proteins are found at synapses, adherens junctions, and tight junctions and contain at least one PDZ domain (that interacts with short peptide sequences typically at the carboxyl terminus of interacting proteins), an SH3 domain, and a GUK domain (catalytically inactive guanylate kinase domain) that serve as docking sites for cellular proteins. Many MAGUK proteins also contain one or two L27 domains that heterodimerize with L27 domains found on other MAGUK proteins, resulting in multimerization of MAGUK proteins (recently reviewed in Ref. 1).

The prototype MAGUK protein is DLG1, which has served as a model protein in studies of cellular localization and protein association for this family. Drosophila DLG was identified as a tumor suppressor gene in flies with a tumor-like overgrowth and disorganization of the larval imaginal disks (2). The Drosophila DLG protein localizes to septate junctions, and mutants were found to have a loss of septate junctions (the invertebrate analog of mammalian tight junctions). Mammalian DLG1 complements the defects observed in DLG flies (3). In Caenorhabditis elegans DLG is required for proper assembly of the zonula adherens (4). Although DLG1 in mammals has not yet been shown to have a tumor suppressor phenotype, loss of DLG1 or other MAGUK genes results in developmental defects, illustrating the importance of this family in the control tissue morphogenesis (5, 6). It is possible that the large numbers of mammalian MAGUK genes are able to complement defective alleles of individual genes.

A second subgroup of MAGUK proteins is represented by the Drosophila Stardust protein that is required for the establishment of cell polarity in the developing Drosophila embryo (7). The single PDZ domain of Stardust associates with the apical transmembrane protein Crumbs through a PDZ ligand on Crumbs (8, 9); both Crumbs and Stardust are required for the establishment of cell polarity. Mammals have at least seven Stardust homologs termed MPP1–7. Mammalian MPP5, also known as PALS1 (for “partner associated with LIN7”), acts similarly to Stardust, linking the mammalian homolog of Crumbs and the tight junction protein PATJ (10). In C. elegans, LIN7 (also known as Veli or MAL5) is necessary for vulva development and is required for localization of the epidermal growth factor receptor (11). LIN7 contains single L27 and PDZ domains. There are three mammalian LIN7 homologs (LIN7A, -B, and -C) and three mammalian Stardust-like proteins described as associating with LIN7 as follows: MPP3, MPP5 (also known as PALS1), and MPP6 (also known as PALS2) (12, 13).

Because of the large number of MAGUK proteins with overlapping functions, the role of MAGUK proteins in the regulation of mammalian cell growth remains unresolved. One pro...
vocative observation has been the association of MAGUK proteins with viral regulatory proteins and oncoproteins. Human T-cell lymphotrophic virus type I Tax has a PDZ-binding ligand at the carboxyl terminus of the protein that is necessary for full virus-induced T-cell proliferation in vivo and full infection and persistence in vitro (14); the PDZ ligand of Tax interacts with a number of PDZ-containing proteins, including DLG1 (reviewed in Ref. 15). The E4orf1 protein is required to cause rat mammary mesenchymal sarcomas and myoepitheliomas by adenovirus type 9 (16–18); E4orf1 contains a PDZ ligand that interacts with a number of cellular PDZ proteins at adherens and tight junctions (19), and this ligand is required for the transformation of rat mammary cells by E4orf1 (20). E4orf1 associates with the DLG1 protein, resulting in the activation of phosphatidylinositol 3-kinase (21). Papillomavirus E6 oncoproteins also associate with the DLG1 protein (22, 23) as well as other cellular PDZ proteins through a PDZ ligand at the carboxyl terminus of E6 (24–27). Papillomaviruses are causative agents of benign tumors of cutaneous and mucosal squamous epithelia. Subsets of papillomaviruses induce mucosal epithelial tumors that may develop into epithelial malignancies; these types are termed “high risk” HPV types (prototypes are HPV-16, -18, and -31). Transgenic expression of E6 oncoproteins within the mouse skin is sufficient to induce epithelial hyperplasia and focal squamous cell carcinomas; analysis of E6 mutants established that this phenotype requires the presence of a PDZ ligand at the carboxyl terminus of E6. This implicates cellular PDZ proteins as targets for E6 oncogenicity.

To identify cellular PDZ proteins that interact with E6, we have used a proteomics approach to identify complexes of cellular proteins that interact with the HPV-16 E6 (16E6) PDZ ligand, and we have characterized a tripartite complex containing LIN7, a Stardust family protein termed MPP7, and DLG1.

**MATERIALS AND METHODS**

**Cells and Tissue Culture and Transfections**—293T, CV-1, and MDCK cells were obtained from the ATCC and maintained in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum, glutamine, and antibiotics. HaCat squamous keratinocytes were maintained in 7.5% calf serum and 2.5% fetal bovine serum in Dulbecco’s modified Eagle’s media. Cell lines were transfected with FuGENE 6 in accordance with the manufacturer’s instructions.

**Plasmids**—Glutathione S-transferase (GST) fusions to E6 proteins have been described previously (28). The last 10 amino acids of 16E6 (amino acids 142–151, the intact PDZ ligand) or amino acids 142–149 (the mutated PDZ ligand) were cloned as fusions to GST followed by a tobacco etch virus protease cleavage site. For transient expression of proteins in mammalian cells, genes were cloned into pcDNA3 (Invitrogen) with two tandem copies of monoclonal antibody epitope tags for EE, FLAG, HA, or MYC monoclonal antibodies. For retroviral transduction of mammalian cells, cDNA open reading frames or site-directed mutant genes were cloned into pQCXi with monoclonal antibody epitope tags and retrovirus-packaged by transient transfection of Phoenix Ampho cells (provided by Dr. Gary Nolan, Stanford University) together with an expression plasmid for the vesicular stomatitis virus G protein. MPP7 was cloned from human keratinocyte NIKS cells (29) by RT-PCR using 5′-ATGccagctttttgccaccggtcg and 3′-ttatagaaggct-caaccgtactgg oligonucleotides. cDNA expression plasmids were generously provided for LIN7A, -B, and -C (Paul Welling, University of Maryland) and DLG1 (Lawrence Banks, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).

**Affinity Purification of PDZ Domain Proteins**—GST fusions to the PDZ ligand of 16E6 or a mutated PDZ ligand were expressed in bacteria and purified as described previously (30). 25 confluent 15-cm plates of HaCat cells per sample were washed three times with phosphate-buffered saline and lysed on ice with 0.5× Nonidet P-40 lysis buffer (1× Nonidet P-40 lysis buffer is 150 mM NaCl; 50 mM Tris, pH 7.5; 50 mM NaF; 5 mM NaPP; 1% IPEGAL CA-630; 0.01% phenylmethylsulfonyl fluoride; 1 mM sodium vanadate; 1 μg/ml leupeptin/aprotinin). Lysates were centrifuged at 15,000 × g for 10 min. Clarified lysates were pre-cleared on 3.0-mL columns of glutathione-agarose. The flow-through was then incubated with 20 μg of GST fusion protein immobilized on glutathione-agarose beads for 1 h on ice. The beads were washed three times with Nonidet P-40 lysis buffer and twice with Nonidet P-40 buffer without protease inhibitors, and bound proteins were released by cleavage with tobacco etch virus protease in the manufacturer’s supplied buffer supplemented with 0.1% IPEGAL CA-630. Eluted proteins were freeze-dried, resuspended in SDS-PAGE sample buffer, and applied to 4–20% NOVEX polyacrylamide gel (Invitrogen), stained first with Coomassie Blue, and then re-stained with silver to visualize protein bands. The gel bands were subsequently cut from the gel and destained. The proteins were reduced, alkylated, and digested with trypsin in the gel. The peptides formed in the digestion were extracted, concentrated, and characterized by capillary column liquid chromatography-tandem mass spectrometry. Data base searches using the program SEQUEST were used to identify the proteins by matching the collision-induced dissociation spectra. These matching spectra were verified by manual inspection of the collision-induced dissociation spectra.

**In Vitro Translation and in Vitro Binding Assays**—In vitro coupled translations and translations were performed utilizing standard nuclease-treated reticulocyte lysate (Promega), according to the manufacturer’s recommendations, and supplemented with 1.5 mM MgCl₂, 25 mM nucleotide triphosphates, and 25 units of T7 RNA polymerase (Invitrogen) per 50-μl translation reaction. For in vitro binding assays, 25 μl of reticulocyte lysate programmed to express the indicated proteins were incubated on ice for 30 min at 4 °C, and then 175 μl of 0.5× Nonidet P-40 lysis buffer containing precipitating antibody and protein A-Sepharose or bead-immobilized GST fusion were added, and binding was allowed to proceed at 4 °C with rocking for 1 h. Beads were washed three times with 1.5 ml of 0.5× Nonidet P-40 lysis buffer. Retained proteins were eluted with SDS sample buffer, resolved by 15% SDS-PAGE, blotted to PVDF membranes, Ponceau-stained, and subjected to autoradiography or quantitative β-scanning with a Packard Instant Imager.

**Western Blot Analysis**—Cell lysates in Nonidet P-40 lysis buffer or cell lysates from cells lysed in 1% SDS were equalized...
for protein content as determined with a commercial kit (Bio-Rad) before electrophoresis; equalized proteins boiled in complete SDS sample buffer were resolved by SDS-PAGE and transferred to PVDF membranes. Monoclonal antibody sources were as follows: epitope tags were obtained from Sigma (M2 FLAG monoclonal and rabbit polyclonal antibody), the Developmental Studies Hybridoma Bank (MYC clone 9E10), Cell Signaling (anti-MYC epitope tag clone 9B11), and Dr. Gernot Walter (EE, University of California, San Diego, and rabbit polyclonal antibody from Covance), and 12CA5 monoclonal antibody to the influenza hemagglutinin protein from Roche Applied Science. Monoclonal antibodies to E-cadherin and DLG1 were from BD Biosystems; monoclonal antibody to tubulin were from Sigma, and monoclonal antibody to dog ZO-1 was from the Developmental Studies Hybridoma Bank (clone R26.4C).

RESULTS

We performed an in vitro binding assay using just the last 10 amino acids of HPV-16 E6 (16E6) compared with the same peptide minus the very last 2 amino acids, destroying the PDZ ligand of 16E6. Mass spectrometry identified Scribble/Vertau, DLG1/SAP-97, CASK/LIN2, PSD-95/DLG4, MPP7 (a human p55 family member analogous to Drosophila Stardust), and LIN7 family members (primarily LIN7C; data not shown). We have focused this study upon the uncharacterized MPP7 protein and its interaction with DLG1 and LIN7.

*p55 MAGUK Protein MPP7—The MPP7 open reading frame cDNA was isolated using RT-PCR and mRNA from immortalized human keratinocytes. Four full-length open reading frame clones were fully sequenced. Three were identical and corresponded to human EST sequences described previously in a study that had predicted the existence of MPP7 by sequence data base examination (31), and they differed by two silent nucleotide changes from the open reading frame of GenBank™ accession number NM_173416. The domain structures of MPP7 and MPP7 mutants used in this study are shown in Fig. 1.

Preliminary Analysis of the Association of PDZ Proteins with Each Other and with 16E6—Initial experiments were performed to identify potential direct associations with 16E6 by in vitro translation of each of the proteins in rabbit reticulocyte lysate followed by association with immobilized 16E6. Because of the common association of DLG1 with viral oncoproteins and the presumed importance of the 16E6 interaction with DLG1, we have in this study analyzed potential complexes formed with DLG1 first. Initial in vitro binding of in vitro-translated DLG1, LIN7A, and MPP7 each bound 16E6; however, a clear enhancement of the LIN7 and MPP7 interaction with 16E6 was found when both were incubated together, indicating that either a possible complex of MPP7 plus LIN7A bound better to 16E6 or that such a complex efficiently bound to DLG1 and that this complex bound efficiently to the PDZ ligand of E6 (supplemental Fig. S1).

Association of MPP7 with LIN7—To determine whether MPP7 could form a complex with LIN7A, both proteins were translated in vitro, mixed together, and then immune precipitated with an epitope tag on LIN7A, revealing that a complex formed in vitro (Fig. 2A). To determine the stoichiometry, the two proteins were translated in vitro at constant specific activity with separate epitope tags on each protein. The complex was tandem affinity-purified (TAP) first by immune precipitation with the FLAG tag on MPP7, and the peptide was eluted with FLAG peptide and then re-precipitated with antibody to the HA epitope on LIN7A. The LIN7A protein in vitro-translated in two forms, a slower migrating form containing the HA epitope and a faster migrating form that initiated translation internal to the HA epitope. Because only the slower migrating form was present in the TAP product (Fig. 2A, lane 2), it can be inferred that the LIN7A-MPP7 complex was a dimer in which higher order associations were not detected.

To determine which domains of MPP7 associated with LIN7A, amino-terminal deletions of MPP7 (Fig. 1) were tested for in vitro association with LIN7A, revealing that although the L27N domain of MPP7 was dispensable, the L27C domain was required for the association (Fig. 2B). Similarly, carboxyl-terminal deletions of MPP7 revealed that the GUK, hook, SH3, and PDZ domains were dispensable, whereas the L27C domain was required (Fig. 2C). These experiments reveal that the interaction of LIN7A with MPP7 required the L27C domain of MPP7.

Association of DLG1 with the LIN7-MPP7 Complex—To determine whether DLG1 associated with MPP7 with or without LIN7A, the three proteins were separately translated in vitro and then mixed together in various combinations and immune precipitated using amino-terminal monoclonal antibody epitope tags (supplemental Fig. S2). DLG1 failed to associate with either LIN7A or MPP7 alone but efficiently associated with MPP7 together with LIN7A.

In the initial identification of MPP7 by mass spectrometry, we identified peptides that were unique to LIN7C as well as peptides common to all three LIN7 gene products. To determine whether all three LIN7 family members can associate with MPP7 and to determine their potential role in association with DLG1 as well, each protein was translated in vitro and tested for in vitro association with MPP7 and DLG1. All three LIN7 family members (LIN7A, -B, and -C) associated with MPP7 in vitro, but only complexes formed between LIN7A and LIN7C together with MPP7 efficiently associated with DLG1 (Fig. 3A). To determine the ratio of the three proteins in the complex, the three proteins were translated in vitro to the same specific radioactivity, mixed together, then immune precipitated first with FLAG antibody tag on DLG1, washed, eluted with FLAG

FIGURE 1. MPP7 domain structure. The domain structure of MPP7 and the mutants used in this study. The amino acids of the illustrated domains in MPP7 are shown.
peptide, and then re-precipitated using MYC antibody directed against tagged MPP7. The MPP7 translation product, like LIN7A in Fig. 2A, contains both a slower migrating epitope-tagged and a faster migrating untagged product (Fig. 3B, lanes 6–8), which are both present in the FLAG-DLG1 immune precipitate (lane 4). However, only MYC-tagged MPP7 is present in the TAP product (Fig. 3B, lane 2), demonstrating that in vitro complexes between DLG1, MPP7, and LIN7A contain single molecules of MPP7, with multimers of MPP7 not observed. The TAP products were quantitated and normalized for methionine content, and net counts above background were calculated, with the ratio of DLG1:MPP7:LIN7A being 1.0:0.98:1.2 which is close to a theoretical 1:1:1 ratio. Thus, the three proteins form a tripartite complex.

Initial experiments indicated that DLG1 only associated with either MPP7 or HA-LIN7 if the L27N domain was present on MPP7 (supplemental Fig. S3). Thus, LIN7A forms a complex with MPP7 requiring the L27C domain of MPP7, and this dimer associates with DLG1 requiring the L27N domain of MPP7.

It was possible that DLG1 might interact directly with only the L27N domain of MPP7. In this model, interaction of LIN7A with the L27C domain of MPP7 might make the L27N domain of MPP7 available for interaction with DLG1. To test this possibility, carboxyl-terminal deletions of MPP7 were incubated with a GST fusion to the DLG1 L27 domain (DLG1 amino acids 1–228) in the presence or absence of LIN7A (Fig. 3C). A very weak interaction between DLG1 and MPP7 was observed in the absence of LIN7A that was not affected by deletion of the MPP7 L27C (Fig. 3C, middle panel, lanes 4 and 5), indicating that MPP7 L27N interactions are not repressed by the L27C domain. In the presence of LIN7A, there was a 12.4-fold enhancement of MPP7 bound by DLG1. (In the presence of LIN7A, 5.1% of the input counts of MPP7 were retained on the GST-DLG1_1–228 beads, whereas in the absence of LIN7A, only 0.41% of the input counts was retained.) The enhancement of the MPP7-DLG1 interaction required the presence of the L27C domain of MPP7 (Fig. 3C, top panel, compare lanes 4 and 5).

To confirm that the MPP7 interaction with the DLG1 L27 domain was similar to the interaction with the full DLG1 molecule, the same series of MPP7 deletion mutants was incubated together with cold-translated full-length EE-DLG1 with or without cold-translated LIN7A (Fig. 3D). DLG1 was immune precipitated, and associated MPP7 or MPP7 mutants were identified by autoradiography. Similar to the results of Fig. 3C, the efficient association of DLG1 with MPP7 required LIN7A and the presence of the L27C domain of MPP7. Addition of LIN7A caused a 13.3-fold increase in DLG1-associated full-length MPP7 compared with vector-programmed reticulocyte lysate.

**Stabilization of MPP7, LIN7, and DLG1 by Formation of a Tripartite Complex**—To determine whether the LIN7-MPP7-DLG1 tripartite complex defined in vitro also forms in vivo, mammalian cells were transfected with epitope-tagged DLG1, MPP7, and LIN7A and analyzed by immune precipitation for formation of the tripartite complex. Fig. 4A shows that DLG1 does not associate with either MPP7 or LIN7A alone, but it associates only when both LIN7A and MPP7 are co-expressed.
DLG1 Association with MPP7/LIN7

A. MPP7, DLG1, and LIN7A were transiently co-expressed and pull-down assays were performed as described. 5% input samples were precipitated with MPP7 or LIN7A antibodies, washed, and analyzed by Western blotting. B. In vitro translation of the indicated proteins was performed and the GST-DLG1 amino-terminal 228 amino acids were expressed as a fusion protein. The complexes were analyzed by SDS-PAGE and quantitated by liquid scintillation of excised bands. The ratio of normalized protein band intensity was determined for each band. C. The indicated proteins were expressed in MDCK cells and analyzed for association with DLG1. D. The indicated proteins were expressed in MDCK cells and analyzed for association with LIN7A. 

FIGURE 3. Analysis of the MPP7-LIN7-DLG1 tripartite complex. A, in vitro association of LIN7 family members with DLG1 and MPP7. In vitro translations of the indicated proteins were immune precipitated (IP) with HA antibody and analyzed by SDS-PAGE and autoradiography. B, ratio of MPP7, DLG1, and MPP7 in the tripartite complex. The indicated epitope-tagged proteins were translated in vitro at the same specific activity and combined as indicated in vitro. The complex was immune precipitated first with FLAG antibody beads, eluted with FLAG peptide, and then re-precipitated with antibody to the MYC epitope. The TAP-purified products were analyzed by SDS-PAGE and quantitated by liquid scintillation of excised bands. The ratio of normalized decay from DLG1 to MPP7 to LIN7A was 1:1:1.2. C, carboxyl-terminal sequences of MPP7 required to form a complex with LIN7 and the L27 domain of DLG1. MPP7 and the indicated MPP7 deletion mutants were translated in vitro with [35S]methionine and incubated together with nonradioactive (“cold”) in vitro-translated LIN7A (top and bottom panel) or vector-programmed nonradioactive reticulocyte lysate (middle panel). Complexes were isolated on immobilized GST fusion to the DLG1 amino-terminal 228 amino acids (top and middle panels) or onto unfused immobilized GST (bottom panel). Proteins retained on the washed beads were resolved by 15% SDS-PAGE and transferred to PVDF membranes, and proteins were visualized by Ponceau staining of the membrane and then autoradiography. In the presence of LIN7A, 5.1% of the input counts of MPP7 were retained on the GST-DLG1_1–228 beads, whereas in the absence of LIN7A, only 0.4% of the input counts was retained. At the bottom of each panel an image shows Ponceau-stained GST or GST fusions. Protein band distortions in lanes 9 and 10 of input gels are because of co-migrating globin in the reticulocyte lysate. PD refers to pulldown with the indicated GST fusion protein. D, domains of MPP7 required to form a complex with LIN7 and DLG1. In vitro translations were incubated together with cold-translated EE-DLG1 and cold-translated LIN7 (top panel), or cold-translated EE-DLG1 together with vector-programmed cold reticulocyte lysate. Complexes were isolated with rabbit antibody to the EE epitope fused to DLG1, and washed complexes were analyzed by SDS-PAGE. In the presence of LIN7A, 2.4% of the input counts were precipitated, whereas in the absence of LIN7A, 0.18% of the input counts was precipitated.

together with DLG1. Fig. 4B shows the whole cell lysates for Fig. 4A, revealing that MPP7 and LIN7A are both expressed at higher levels when co-expressed together (Fig. 4B, lanes 3 and 4 compared with lane 6). Similarly, DLG1 is expressed at high levels only when co-expressed together with both LIN7A and MPP7, indicating that the complex of MPP7 and LIN7 stabilizes each other and that the dimer of LIN7 and MPP7 stabilizes DLG1.

To determine whether the associations defined in vitro and in transient transfections in vivo were found under stable expression conditions in vivo, MDCK cells were separately and retrovirally transduced with epitope-tagged LIN7A, -B, and -C. Fig. 5A shows that expression levels of DLG1 in these cells were similar to untransduced MDCK cells, and DLG1 was found in a complex with LIN7A and -C but to a much lesser extent than LIN7B, correlating with the in vitro binding assay of Fig. 3A.

In separate retroviral transductions, FLAG-MPP7 and FLAG-MPP7Δ135–576 (expressing only MPP7, L27N, and L27C) were introduced into MDCK cells and selected by G418 drug resistance, followed by LIN7A, -B, or -C selected by hygromycin resistance. Drug-selected cells were passaged for 6 weeks, followed by analysis of DLG1 expression levels. Introduction of MPP7 into MDCK cells resulted in increased expression of DLG1 (Fig. 5B, lane 4 compared with lane 6). Further introduction of LIN7 proteins resulted in enhanced expression of MPP7 compared with expression of MPP7 alone (Fig. 5B, lanes 1–3 compared with lane 4). This correlates with the enhanced expression observed by transient co-expression of MPP7 and LIN7A (Fig. 4B). Introduction of MPP7Δ135–576 resulted in a dramatic elevation of DLG1 expression. Immune precipitation of wild type MPP7 revealed that DLG1 was associated with both full-length and the deleted MPP7 (possibly through associations with endogenous LIN7), and in cells stably expressing MYC-LIN7 proteins, DLG1 associated with MPP7 together with LIN7A and LIN7C but not LIN7B, paralleling the in vitro binding assays of Fig. 3A. Quantitative RT-PCR analysis showed no differences in RNA expression of DLG1 between MDCK cells and MDCK cells expressing MPP7, LIN7C, or both MPP7 and LIN7C (data not shown), whereas the half-life of DLG1 protein was prolonged in MDCK cells expressing MPP7 compared with vector-transduced MDCK cells (supplemental Fig. S4).

Cytoskeleton Association of MPP7 and DLG1—Deletion mutants of MPP7 (illustrated in Fig. 2) were also stably transduced into MDCK cells and analyzed for association with DLG1 and partitioning between the soluble cellular extract and the cytoskeleton-containing pellet. Mutants of MPP7, which contained both L27 motifs and associated with both LIN7 and DLG1 in vitro, formed a complex with DLG1 in vivo and increased expression levels of DLG1 in vivo (Fig. 5C). Mutants of MPP7 that did not associate with either DLG1 or LIN7 in vitro (Fig. 2C and Fig. 3, C and D) did not enhance DLG1 expres-
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**FIGURE 4. In vivo association of LIN7A, MPP7, and DLG1.** A, tripartite complex formation in mammalian cells. Transient transfection of HEK 293 cells with the indicated epitope-tagged plasmids was followed by Nonidet P-40 lysis (with a portion removed for SDS-lysis, used in B) and immune precipitation (IP) with EE antibody directed to the EE tag on DLG1. Immunoblots were analyzed by sequential immunoblots with EE, FLAG, and HA antibodies with the result shown after probing with the three immunoblotting antibodies. B, stabilization of LIN7 and MPP7 by co-expression and stabilization of DLG1 by co-expression with MPP7 and LIN7A. SDS-denatured lysates from A were analyzed by immunoblotting with EE, FLAG, and HA antibodies. The bands marked with asterisks are cellular proteins cross-reactive with the HA antibody and demonstrate equal loading. WB, Western blot.

**FIGURE 5. Tripartite complex formation in stably transduced polarized epithelial cells.** A, association of LIN7A and LIN7C but not LIN7B with DLG1. MDCK cells stably transduced with MYC-tagged LIN7A, B, or C were lysed in Nonidet P-40 lysis buffer and clarified lysates immune precipitated (IP) with antibodies to MYC followed by immunoblotting (IB) with the indicated antibodies. B, stable co-expression of MPP7 and LIN7 stabilizes DLG1. MDCK cells stably and retrovirally transduced to express the indicated cDNAs were lysed in Nonidet P-40 buffer and analyzed by immune precipitation with rabbit anti-FLAG and immunoblotting with mouse monoclonal antibodies as indicated. **Vertical white lines** indicate where irrelevant lanes were excised. C, effect of MPP7 mutants upon DLG1 expression and association of DLG1 with the insoluble cell fraction. MDCK cells stably and retrovirally transduced to express the indicated cDNAs were lysed in Nonidet P-40 buffer. MPP7 or the indicated MPP7 mutants were analyzed by immunoblotting of the soluble (left, top panels) and insoluble cell fraction (pellet, left bottom panels) after lysis and clarification of the cells. The same cell lysates were also immune precipitated with FLAG antibody and analyzed by immunoblotting with the indicated antibodies (right panels).

**Localization of MPP7 and DLG1—MDCK cells are a model system for the localization of DLG1 and other MAGUK proteins because they form well structured tight adherens junctions.** FLAG-MPP7 and mutants were introduced by stable retroviral transduction. In MDCK cells E-cadherin was localized at cell junctions as expected. DLG1 was localized in three cellular compartments as follows: at cell junctions co-localized with E-cadherin, in intranuclear spots, and distributed in small cytoplasmic dots (Fig. 6A) (32). In cells transduced with MPP7, MPP7 was co-localized with DLG1 and E-cadherin at cell junctions (Fig. 6A). In cells expressing MPP7, there was clearly enhanced localization of DLG1 to cell junctions and reduced nuclear and cytoplasmic localization, whereas E-cadherin localization was unaffected. In cells expressing the MPP7 deletion mutant Δ135–576, MPP7 and DLG1 were both redistributed from cell junctions into the cytosol, whereas E-cadherin was unaffected. Compared with wild-type MPP7-transduced cells, there is enhanced nuclear DLG1 expression in the MPP7Δ135–576-expressing cells (Fig. 6A). Although MPP7 co-localized with DLG1, there was no co-localization of MPP7 with the tight junction proteins ZO1 (Fig. 6B). Deletion mutants of MPP7 were examined for localization of the MPP7 molecule and DLG1 (Fig. 7). Deletion of the GUK domain alone from MPP7Δ380–576 did not alter either MPP7 or DLG1 localization, whereas further sequential deletion of the hook, SH3, and PDZ domains progressively redistributed both MPP7 and DLG1 from cell junctions into the cytoplasm (Fig. 7), whereas the final deletion of the L27C domain in MPP7Δ87–576, which ablates the interaction of MPP7 with DLG1, restored the localization of DLG1 to cell junctions (data not shown). Although not shown in Fig. 7, amino-terminal deletions of MPP7 did not mislocalize DLG1, but deletion of or beyond the PDZ domain gave rise to unstable proteins.

Although all three LIN7 proteins associated with MPP7 in vitro (Fig. 3A), the principal LIN7 member associated with MPP7 in MDCK cells was unknown. Accordingly, FLAG-
tagged MPP7 was immune precipitated from MDCK cells using FLAG antibody beads, and the associated proteins were analyzed by mass spectrometry of trypsinized peptides. Only peptides from endogenous LIN7C were found in association with MPP7 (data not shown). To determine whether LIN7C expression independently altered the localization of DLG1, LIN7C protein was stably transduced into MDCK cells that had been previously transduced with MPP7 or MPP7Δ135–576. LIN7C localized with cell junctions in MDCK cells (Fig. 8). Expression of MPP7 enhanced cell junction staining of DLG1 and LIN7C. Expression of MPP7Δ135–576 completely mislocalized both DLG1 (as observed before in Fig. 6A) and LIN7C (Fig. 8).

DISCUSSION

MPP7 was found in a complex with cellular proteins isolated through affinity for the PDZ ligand of HPV-16 E6. Peptides from no additional Stardust family proteins were found in this complex. This study has focused upon complexes containing DLG1. Studies are underway as to how this complex relates to the other proteins associated with the PDZ ligand of 16E6 and the effect of 16E6 upon these complexes.

MPP7 was found to dimerize with all three LIN7 proteins through association of the sole L27 motif LIN7 with the carboxyl-terminal L27 motif of MPP7. The formation of this dimeric complex results in the in vivo stabilization of MPP7 and of LIN7, implying that unpaired MPP7 and LIN7 proteins may be reduced in stability. Similarly, transient expression of DLG1
without both LIN7 and MPP7 was associated with lower expression levels than co-transfections (Fig. 4B); similar results were observed in stable expression in MDCK cells (Fig. 5) where expression of MPP7 together with LIN7 results in substantial increases in DLG1 expression. Thus, DLG1 expression can be potentially modulated by increases in the expression of LIN7, MPP7, or possibly other L27-containing proteins that interact with DLG1 by L27 motifs. This parallels a previous observation where the knockdown of the related MAGUK protein PALS1 by short hairpin RNA resulted in reduced expression of the PALS1-associated protein PATJ (33). Although our results show that LIN7 stabilizes the expression of MPP7 and thereby DLG1, it does not show that either MPP7 or LIN7 is essential for such an effect, as it is possible that other proteins interacting through L27 motifs might serve this function as well. Indeed, this might be predicted as mice with targeted gene disruptions of all three LIN7 isoforms form normal-appearing organs (although such mice die shortly after birth) (34), whereas in worms, LIN7 is essential for normal tissue development (11). However, a recent study of MDCK cells where LIN7C expression was reduced through expression of short hairpin RNA showed reductions in the expression of LIN7C-associated PALS1 expression and stability, which is very analogous to the results presented here with LIN7C and MPP7 (35). These observations demonstrate that the expression of MAGUK proteins is inter-related. Overall expression of DLG1 could be regulated by the abundance of LIN7A, LIN7C, or MPP7 proteins (or similar Stardust family members that interact with DLG1) that are available to interact with each other, or by post-translational modifications of these proteins that modulate their ability to form a tripartite complex. DLG1 protein expression has been found to progressively decrease in colonic mucosa during the transition from benign to malignant and finally invasive cancers (36). Our experiments raise the possibility that changes in the expression of all three components of the tripartite complex could be responsible in some cases.

Although a very weak interaction in vitro between the L27N of MPP7 and an overexpressed GST-L27 of DLG1 was observed (Fig. 3C), the addition of LIN7A strongly enhanced the interaction of DLG1 L27 with the L27N of MPP7 (Fig. 6B, upper panel). In experiments using the whole DLG1 molecule expressed at similar levels to MPP7 and LIN7, little interaction between DLG and MPP7 was observed in the absence of LIN7A or -C (Fig. 3, A and D). From this we conclude that the likely association of MPP7 with DLG1 involves the formation of a tripartite complex containing a tetrameric L27 complex at the amino terminus, and the interaction of the L27N of MPP7 with DLG1 is determined in part by the prior interaction of L27C of MPP7 with the L27 of LIN7A or -C. We saw little evidence for the formation of a DLG1-MPP7 L27N heterodimer in the absence of an LIN7-MPP7 L27C heterodimer (Fig. 3, C and D). Our results suggest the concerted formation of a tetrameric complex rather than the tetramerization of two independently formed heterodimers.

Our results are similar to an earlier study of the interaction of SAP97 with MPP3, a protein related to MPP7 (12), which in far Western blots showed a similar requirement for the L27N and -C domains of MPP3 for interaction with SAP97. Unlike our study, those authors identified a putative direct interaction by far Western blotting between SAP97 and MPP3 that did not require the presence of LIN7. It is possible that non-native folding conditions on membranes might have favored such a finding, that the interaction observed was less than would have been observed in the presence of a LIN7 protein, or that MPP3 and MPP7 differ in the interactions of their L27N domains with the L27 of DLG1.

MPP7 co-localized with E-cadherin and DLG1 at cell junctions. Deletion of the MPP7 GUK domain had little effect, whereas progressive deletion of the hook, SH3, and finally PDZ domains removed all apparent localization of MPP7 to cell junctions (see MPP7Δ135−576, Figs. 6 and 7). In-frame deletion of only the SH3, hook, or PDZ domains each had intermediate phenotypes compared with deletion of the contiguous PDZ-SH3-hook region (Fig. 7B). This indicates that our ability to make confident distinctions in localization is limited or that there may be redundant mechanisms to partially localize MPP7 to cell junctions.

MPP7 co-localized with DLG1 and altered the localization of DLG1. Ectopic stable MPP7 expression localized DLG1 from the nucleus and cytoplasm to cell junctions where the two proteins co-localized (Fig. 6). Mutants of MPP7 that interacted with DLG1 but failed to localize to cell junctions (such as MPP7Δ135−576) completely re-localized DLG1 out of cell junctions (Figs. 7 and 8) and out of association with the insoluble cell fraction and predominantly into a soluble cytoplasmic/nuclear fraction (Fig. 5C). Mutants of MPP7 that no longer associate with DLG1 because of deletion of the L27C of MPP7 did not alter the expression, fractionation, or localization of DLG1 (Figs. 5C and 7, compare MPP7Δ135−576 and Δ86−576). Also, for expression of MPP7Δ1−60, deleting the L27N domain only does not alter the localization of DLG1.4

The complete mislocalization of DLG1 by MPP7Δ135−576 was surprising. Previous studies of DLG1 localization had revealed two mechanisms for the association of DLG1 with cell junctions. First, expression of the amino-terminal L27-containing region upstream of the first PDZ motif as a fusion to green fluorescent protein localized green fluorescent protein to cell junctions (37), indicating that the L27 region alone was sufficient to localize DLG1 to cell junctions. This study indicates that such a result could be due to the association of the DLG1 L27 with MPP7 + LIN7A or -C. Second, a subsequent study demonstrated that the I3 splice variant of DLG1 could localize to cell junctions in the absence of an intact L27 motif, whereas the I2 splice variant required an intact L27 motif (38). Thus, DLG1 can localize to cell junctions either through associations at the L27 domain or through associations at the I3 region. In preliminary experiments, we have purified FLAG-MPP7 from MDCK cells and verified by mass spectrometry that peptides derived from the I3 region of DLG1 are associated with MPP7.3 It is therefore curious that expression of MPP7Δ135−576 would disrupt the localization of DLG1-I3, which should be able to localize independently of L27 interactions. This raises the possibility that interactions at the L27 region of DLG1 can

3 J. Bohl and S. Vande Pol, unpublished observations.
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alter the function of the SH3-GUK region that can target the localization of DLG1. Consistent with the in vitro binding data and the localization of MPP7, expression of MPP7 enhanced the localization of LIN7C to cell junctions, and MPP7Δ135–576 mislocalized both DLG1 and LIN7C out of cell junctions (Fig. 8). Thus all three components of the in vitro tripartite complex mislocalize together.

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