Molecular Cloning and Characterization of Pals, Proteins Associated with mLin-7*

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In Caenorhabditis elegans, three PDZ domain proteins, Lin-2, Lin-7, and Lin-10, are necessary for the proper targeting of the Let-23 growth factor receptor to the basolateral surface of epithelial cells. It has been demonstrated that homologues of Lin-2, Lin-7, and Lin-10 form a heterotrimeric complex in mammalian brain. Using Far Western overlay assay, we have identified additional proteins that can bind to the amino terminus of mLin-7 and cloned the genes encoding these proteins using bacterial expression cloning. We call these proteins Pals, for proteins associated with Lin-7. These proteins, which include mammalian Lin-2, contain a conserved mLin-7 binding domain in addition to guanylate kinase, PDZ (postsynaptic density 95/discs large/zona occludens-1), and Src homology 3 domains. Using site-directed mutagenesis, we have identified the conserved residues among these proteins crucial for mLin-7 binding. Two of these proteins, Pals1 and Pals2, are newly described. Pals1 consists of 675 amino acids and maps to mouse chromosome 12. Pals2 was found to exist in two splice forms of 539 and 553 amino acids and maps to mouse chromosome 6. Like mLin-2, Pals1 and Pals2 localize to the lateral membrane in Madin-Darby canine kidney cells. Pals proteins represent a new subfamily of membrane-associated guanylate kinases that allow for multiple targeting complexes containing mLin-7.

The proper targeting of proteins at the apical or basolateral surface of epithelial cells is crucial for cellular transport. Similarly, proper targeting of proteins to either axons or dendrites plays an important role in neurotransmission. Many studies have been directed at the molecular basis that controls these targeting decisions. In Caenorhabditis elegans, three proteins (Lin-2, Lin-7, and Lin-10) are crucial for the correct localization of the worm epidermal growth factor receptor, Let-23, to the basolateral surface of the body wall epithelium (1–3). The Lin-10 gene has also been shown to have a role in localization of glutamate receptors in the C. elegans brain (4). A similar complex of mammalian homologues of Lin-2, Lin-7, and Lin-10 have been identified in mammalian brain (5, 6). In mammalian brain, the homologue of Lin-10 is known as X11a or Mint1 (7–9). X11a/Mint1 contains one PTB1 domain and two PDZ domains. PTB domains act as a phosphotyrosine binding domain in proteins such as Shc and insulin receptor substrate-1, but in X11 and many other proteins, the PTB domain binding targets bind independent of phosphotyrosine (10). PDZ domain proteins are named for their first identification in PSD-95, Drosophila Discs Large, and zona occludens-1 proteins (11). They commonly bind to sequences at the extreme carboxyl terminus of proteins. In addition to PTB and PDZ domains, X11a/Mint1 contains an extended amino terminus that can bind mLin-2/Cask and Munc-18 (9, 12).

mLin-2/CASK is a membrane-associated guanylate kinase (Maguk) protein containing a guanylate kinase-like domain, an SH3 domain, a PDZ domain, and a calmodulin kinase-like domain (13). Like many Maguk proteins, mLin-2/Cask also contains a band 4.1 binding site that can potentially interact with members of the family of proteins including ezrin, radixin, and moesin (14). The calmodulin kinase-like domain of mLin-2/Cask binds X11a/Mint1, while a region between the calmodulin kinase domain and the PDZ domain of mLin-2/Cask binds to the amino terminus of mLin-7 (3, 5, 6). mLin-7 is a small protein, which, in addition to the mLin-2/Cask binding amino terminus, contains a PDZ domain. There are several mLin-7 genes also known as vertebrate Lin-7 (Velia) or mammalian Lin-7 (Mals) (5, 15, 16). In worms, the PDZ of Lin-7 domain binds to the carboxyl terminus of the Let-23 receptor (1).

The X11a/Mint, mLin-2/Cask, and mLin-7 system has been studied in mammalian epithelial cells. In epithelia, the mLin-10 homologue is X11/Mint3 (6, 17). This protein is localized to a perinuclear localization and is not bound to mLin-2/Cask or mLin-7 (18). In contrast, both mLin-2/Cask and mLin-7 target to the basolateral surface of epithelial cells (14, 15, 18). One target for the PDZ domain of mLin-2/Cask in epithelia and neurons is the cell surface protein syndecan (14). A target for the PDZ domain in renal epithelia has been identified as BGT-1 (18, 19). BGT-1 localizes to the basolateral side of renal epithelia and permits the uptake of organic osmolytes.
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in hypertonic stress (20, 21). Mutation of the mLin-7 binding site on BGT-1 does not appear to affect its delivery to the basolateral surface but does affect its stability at the surface (19).

Our studies have examined the mechanism that targets mLin-2/Cask and mLin-7 to the basolateral surface of epithelia cells. We have found that the amino terminus of mLin-7 alone is sufficient for lateral targeting (18). However, neither the amino-terminal nor carboxyl-terminal half of mLin-2/Cask targets properly in MDCK cells. The amino-terminal half of mLin-2/Cask not only mislocalizes in cells but also partially mislocalizes endogenous mLin-7. Recent work has suggested that the amino terminus of mLin-7 may have binding partners in addition to Lin-2 (5). In this study we performed Far Western blotting and bacterial expression cloning to identify additional mLin-7-binding proteins in the mouse. We call these mLin-7-binding proteins PALS (proteins associated with Lin-7) and present a characterization of two novel family members, Pals1 and Pals2.

**EXPERIMENTAL PROCEDURES**

**Preparation of 32P-labeled GST-Lin-7 Probe—** Recombinant mouse Lin-7 C (Veli-3) protein (6) was cloned into pGStag vector that encodes glutathione S-transferase (GST) and protein kinase A phosphorylation sequence located amino-terminally of the multiple cloning site (22). The protein was expressed in DH-5α Escherichia coli cells and approximatively 20 μg bound to 100 μl of glutathione-agarose beads (Sigma). After three washes with phosphate-buffered saline buffer containing 1 mM DTT, the beads were equilibrated and resuspended in DK buffer (50 mM potassium phosphate, pH 7.2, 10 mM MgCl2, 5 mM NaF, and 5 mM DTT). Phosphorylation of the protein was performed in DK buffer using 10 units of protein kinase A (Sigma) and 1 μCi of [γ-32P]ATP (NE-N-Life Science Products) as described (22). After washing the beads with 50 ml of cold phosphate-buffered saline buffer containing, 5 mM sodium pyrophosphate, 1 mM EDTA, and 1 mM DTT, the phosphorylated protein was eluted with 500 μl of 100 mM Tris-HCl, pH 8.0, containing 1 mM each of EDTA, DTT, phenylmethylsulfonyl fluoride, 0.005% Nonidet P-40 detergent, and 20 mM reduced glutathione. The protein was re-suspended in FWB buffer (20 mM HEPES, pH 7.5, 1 mM KCl, 5 mM MgCl2, 5 mM DTT, 0.02% sodium azide, and 5% nonfat dry milk) and stored aliquoted at −70 °C. The specific radioactivity of the probe obtained by this method was in the range 4 × 105 to 9 × 105 cpm/μg protein.

**Electrophoretic Procedures and Far Western Overlay Assay—** Proteins were separated on 10% or gradient 4–12% BisTris gels and transferred to nitrocellulose membranes using the NuPAGE electrophoresis system (Novex). After blocking in FWB buffer (see above) for 3 h, membranes were transferred to fresh FWB buffer containing 2–3 × 105 cpm/ml of radiolabeled probe and incubated overnight. Membranes were washed three times with TBS containing 0.1% Triton X-100 and TBS without detergent, air-dried, and exposed to x-ray films with intensifying screen. Immunoprecipitations and pull-downs were performed as described before (8).

**Bacterial Expression Cloning—** Sixteen-day mouse embryo αEloxol library (Novagen) was screened for expression of mLin-7 binding partners essentially as described by Margolis and Young (23). Briefly, αEloxol phage were plated with the recipient E. coli BL21(DE3)pLysE strain at 5 × 108 plaque-forming units/ml on LB soft agarose. After 8–12 h at 37 °C, plates containing plaques were overlaid with nitrocellulose filter discs (Schleicher & Schuell) that have been impregnated with 1 μl isopropyl-1-thio-β-D-galactopyranoside and air-dried. Filters were incubated with the plaques overnight at 37 °C, washed five times with TBS containing 0.1% Triton X-100, and blocked for 3 h in FWB buffer. Filters were then transferred to fresh FWB buffer containing 2 × 105 cpm/ml radiolabeled probe and incubated overnight at 4 °C with shaking. After three washes each of TBS containing 0.1% Triton X-100 and TBS without detergent, filters were air-dried and exposed to x-ray films with intensifying screen. Phages were isolated from positive plaques and grown in 50 ml TBS containing 0.1% Triton X-100 at 37 °C, and then used for in vitro transcriptions using 32P-labeled oligo G+T. Positive clones were verified by DNA sequencing. Recombinant proteins were expressed in bacteria or in mammalian cells as described below and analyzed for expression and size by immunoblotting with polyclonal anti-GFP antibody (Living Color, CLONTECH), polyclonal anti-Pals1, and monoclonal anti-Myc antibody (Santa Cruz).

**Chromosome Mapping—** Pals1 and Pals2 genes were mapped using the mouse T31 Radiation Hybrid (Research Genetics, Huntsville, AL). Pairs of specific primers of the genes were used to amplify mouse-specific fragments. All reactions were performed at least in duplicate, and anomalous results were further checked with extended cycles. The patterns of mouse band positive cell lines were submitted to the Jackson Laboratory Mouse Radiation Hybrid Data Base for analysis against all previously mapped framework data for this T31 panel.

**Sequence Alignments—** Sequence alignments were done using the Lasergene (Megalign) software by the Clustal method.

**Northern Blot Hybridization and PCR-based Tissue Distribution Analysis—** Human multiple tissue Northern blot (CLONTECH) was probed with the small HindIII fragment (base pairs 1568–2383) of Pals1 cDNA following the protocol of the manufacturer. Actin probe (CLONTECH) was used as a normalization control. The probes were radiolabeled with [γ-32P]ATP (NEN Life Science Products) as described above for Lin-7 probe. The patterns of mouse band positive cell lines were submitted to the Jackson Laboratory Mouse Radiation Hybrid Data Base for analysis against all previously mapped framework data for this T31 panel.

**Northern Blot Hybridization—** Expression analysis was performed with appropriate Pals2 and Pals2β sequence-specific primers using mouse multiple tissue cDNA panel 1 normalized for glyceraldehyde-3-phosphate dehydrogenase (CLONTECH) according to the manufacturer’s protocol.

**Production of Antibodies—** Recombinant mouse GST-Pals1 (1–151), GST-Pals1 (163–272), and GST-Pals2 (1–436) were raised in rabbits using the respective fusion proteins coupled to glutathione-agarose beads as immunogens (24). Affinity purification of anti-Pals1 (1–163) was carried out with His-Pals1 (163–272) protein coupled to Affi-Gel support (Bio-Rad) as described before (24). Antibodies against Lin-7 were described elsewhere (6).

**Transfections—** MDCK cells were transfected with Fugene 6 (Roche) according to the manufacturer’s instructions, using 5 μg of NH2- or COOH-terminal EYFP-Pals1 or Pals2 alone or RK5-MycPals1 in combination with 1 μg of PSV-neo on 50% confluent 60-mm dishes. To generate stable MDCK clones, 2 days after transfection cells were split into three 100-mm dishes and were selected with 600 μg/ml G418 (Life Technologies, Inc.). Medium was changed every other day. After 10 days in selection, colonies were picked up and plated on 24-well plates for expansion. The EYFP-Pals and Myc-Pals clones were analyzed by fluorescent microscope directly or following immunofluorescence staining (18), respectively.
Results

Radiolabeled mLin-7 Probe Binds to Multiple Proteins in Far Western Overlay Assay—When electrophoretically separated proteins from Triton X-100 lysates of different cell lines were transferred to nitrocellulose membranes and probed with radiolabeled GST-mLin-7, at least five discrete protein bands were identified, ranging from 55 to 100 kDa (Fig. 1A). The same bands were also present in lysates from whole brain or kidney (data not shown). Both mLin-7 and mLin-2/Cask have been described as ubiquitously expressed proteins (5, 13, 15), but differential patterns of mLin-7-binding proteins were seen in various cells. The band at 100 kDa is contributed in whole or in part by mLin-2/Cask. No bands were detectable with an amino-terminally truncated form of Lin-7 (mLin-7 1–30), which was previously shown to be defective in mLin2/CASK binding in pull-down assay (results not shown). To test the specificity of this interaction, we performed an immunoprecipitation experiment using anti-mLin-7 polyclonal antibody. Fig. 1B shows that the same proteins that are present in lysates specifically co-immunoprecipitated with mLin-7 in both A172 glioblastoma cells or MDCK canine kidney cells. mLin-2/Cask, Dlg2, and Dlg3 belong to the MAGUK superfamily of proteins and have been shown to bind to mLin-7 through an interaction that involves the amino-terminal region of mLin-7 (3, 5, 6). Our results suggest that additional binding partners for mLin-7 exist. This motivated us to screen for such potential target proteins by using bacterial expression cloning (23).

Expression Cloning of mLin-7 Binding Partners—We screened a 16 day mouse embry aEXLox library with radiolabeled GST-mLin-7. We obtained 49 positive clones on primary screen and subjected them to successive rounds of purification until 16 positive clones were purified to homogeneity. Purified phages were converted to pEXLox plasmids by passing them through Cre recombinase-positive E. coli strain. We called these clones, PALS, for proteins associated with Lin-7. Among the six cloned genes, four belong to the MAGUK superfamily. Two did not show any sequence homology to Maguk proteins, and it is not clear if they represent true binding partners. Pals1 and Pals2 were novel MAGUK genes, while Pals3 was mLin-2/CASK (13) and Pals4 was Dlg2/MPP2 (25).

Pals1 (accession no. AF199008) is a gene possessing 56% homology to the mouse Dlg3/MPP3 (26, 27) at the amino acid level. This clone was incomplete in 5’ direction, and the full-length cDNA was subsequently obtained by PCR from a 17-day mouse embryo library using the Rapid Screen Kit (OriGene). It contains an open reading frame encoding 675 amino acids of which the amino-terminal 130 amino acids are unique and do not show any homology to known sequences in the database. The predicted molecular mass of this protein is 77.2 kDa.

Pals2 was represented by two forms differing by an insertion of 14 amino acids. Pals2a cDNA (GenBank™ accession no. AF199009) contained an open reading frame of 539 amino acids and that of Pals2b (GenBank™ accession no. AF199010), 553 amino acids, respectively. The 14-amino acid insertion is located between the PDZ and the SH3 domains of the protein. The predicted protein shows 82% homology to mouse Dlg2/MPP2. During the course of this study, the sequences of mouse and human p55T gene were deposited in GenBank™ (accession nos. AF161181.1 and AF162130, respectively). The mouse P55T gene is similar to Pals2a with only two substitutions. These two amino acids in Pals2a match the human p55T sequence and are conservative substitutions. Searching for gene bank similarities, we have found that the human Pals2 gene matches fragments of a BAC clone sequences on chromosome 7 p15-p21 (BAC clone RG295G08, accession no. AC005084), which spans approximately 115 kb. The 42 bases that confer the longer form of Pals2 are present within this BAC sequence, which suggests that these two forms are splice variants. The significance of this splice variation is unclear at present.

Definition of the mLin-7 Binding (L7B) Site in Pals Proteins—Sequence alignment of five Maguk proteins that bind mLin-7 showed a high degree of conservation at the already described PDZ, SH3, and guanylate kinase (Guk) domains (Fig. 2). All sequences except Dlg3 also contain a conserved 4.1 protein binding site (28). Most differences are related to the amino-terminal portions of these proteins. It has been found that 220 amino acids located between the calmodulin kinase-like domain and the PDZ domain of mLin-2/CASK contain the binding site for mLin-7 (3, 5, 6). Alignment between that region in mLin2/CASK and the sequences located amino-terminally from the PDZ domains in Dlg2, Dlg3, Pals1, and Pals2 reveal a high degree of homology between all five proteins in a region spanning approximately 90 amino acids (amino acids 139–229 in Pals1; Fig. 2). Our original clone of Pals1, obtained by bacterial expression cloning, started at amino acid 163 and lacked part of this conserved region. We cloned and expressed GST-Pals1 (163–590) and GST-Pals1 (163–272) proteins and tested them in a pull-down experiment using lysates containing full-length Myc-mLin-7. Both proteins bound Myc-Lin-7, indicating that amino acids 163–272 of Pals1 are sufficient for binding mLin-7 (Fig. 3 and results not shown). We then produced a series of amino-terminal and carboxyl-terminal truncations of GST-Pals1 (163–272). Fig. 3A shows a pull-down binding assay with six truncated Pals1 GST fusion proteins. The results of these experiments indicated the minimum mLin-7 binding domain is represented by amino acids 181–243 in Pals1. Within
In this region there is a 21-amino acid fragment (amino acids 207–228 in Pals1) with a high degree of conservation (Fig. 2A, L7B domain). However, a GST-Pals1 (201–243) construct containing just this fragment did not bind Myc-Lin-7, suggesting that the highly conserved region is not sufficient for binding and additional upstream residues are required for docking and/or proper folding.

To better characterize the interaction between Pals1 and mLin-7, we further constructed and expressed eight point mutations in GST-Pals1 (163–272), substituting amino acids that are absolutely conserved in all five MAGUK proteins (Fig. 2B, L7B domain). We tested these mutant proteins in a pull-down experiment with Myc-Lin-7 (1–92) (Fig. 3B). Three categories of mutations were obtained based on their effects on binding in vitro: mutations that were without effect, such as E207Q and H216N; mutations that only partially inhibited binding, like L221G and H224N; and mutations that were almost completely inhibiting, such as D225N, V227G, L208G, and L212G. Collectively, these results indicated that the mLin-7 binding domain is a relatively short sequence consisting of 60 amino acids or less with a highly conserved core of 24 amino acids, some of which are critically important for binding.

### Tissue Distribution of Pals1 and Pals2

- **Northern blot** of a human multiple tissue panel (CLONTECH) hybridized with Pals1 DNA probe revealed an approximately 6-kb band (Fig. 4). High level of expression of Pals1 mRNA was found in placenta and kidney, whereas moderate amounts were found in brain, heart, and skeletal muscle. Among the tissues tested, liver expressed the lowest amount of Pals1 mRNA.

- Pals2 expression was analyzed by hybridization with a mouse multiple tissue Northern blot (CLONTECH) and by PCR of mouse multiple tissue cDNA panel normalized for glyceraldehyde-3-phosphate dehydrogenase (CLONTECH). A message of approximately 4.5 kb was detected by Northern blot along with a quantitatively proportional smaller band at about 2.3 kb, the significance of which is unclear. All tested tissues expressed Pals2a and Pals2b. Higher levels were detected in heart, skeletal muscle, liver, testis, kidney, and brain, whereas low expression was found in spleen and lung (results not shown). Preliminary PCR data suggested that the more abundant isoform in all tissues tested was Pals2a.

### Chromosome Mapping of Pals1 and Pals2

Pals1 and Pals2 genes were mapped using the mouse T31 radiation hybrid (Research Genetics, Huntsville, AL). The data for Pals1 most closely matched that of framework markers on chromosome 12, with the highest LOD of 20.8 to D12Mit156, and a LOD of 18.3 to D12Mit143. The best fit location for the data was between D12Mit4 proximal and D12Mit156 distal. These loci are very close together, so the exact local order cannot be reliably determined by this RH panel. The linkage among these loci is...
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A binding partners (Fig. 6).

We can demonstrate that mLin-7 is coimmunoprecipitated with Pals2 antibodies and that the 55-kDa protein is also specifically immunoprecipitated by mLin-7 using Far Western blotting of cell lysates (Figs. 1 and 6A). This represents the smallest protein that is detected in mLin-7 immunoprecipitation of endogenous Pals1 (Fig. 5D), they did not work well in immunoblotting. To determine which of the mLin-7 binding partners in lysates was Pals1, we overexpressed Pals1 in 293 cells and detected the protein using mLin-7 Far Western overlay assay. These results indicate that Pals1 comigrates with a mLin-7-binding protein of approximately 75 kDa (Fig. 6B). Based on these results and additional immunodetection experiments (results not shown), we have tentatively identified some of the mLin-7-binding proteins detected in lysates by Far Western blotting (Fig. 6B).

Pals1 Interaction with Lin-7 and Identification of Pals1 and Pals2 in Cells—We cloned Pals1 (163–675), full-length Pals1, and full-length Pals1 mutation L208G into pRK5 vector encoding amino-terminal Myc tag. The constructs were transfected into MDCK or 293 cells, and the expressed proteins were recognized by anti-Myc as well as by a polyclonal antibody against Pals1 (163–272) (Fig. 5, A and B, and results not shown). We also transfected and expressed the full-length Pals1 clone from pCMV6-XL4 vector (OriGene), which contains the native Kozak sequence of the protein (Fig. 5B). Pull-down experiments with GST-Lin-7 showed that wild type Myc-Pals1 bound to mLin-7, whereas Myc-Pals1[L208G] did not (Fig. 5C). Using antibody against Pals1 (1–181), we were able to coimmunoprecipitate mLin-7 from mouse brain (Fig. 5D).

We also produced a polyclonal anti-Pals2 and used it to coimmunoprecipitate proteins from A172 cell lysates and then performed Far Western overlay assay with mLin-7 as in Fig. 1. A 55-kDa protein band was specifically immunoprecipitated that was detected by mLin-7 in Far Western blotting (Fig. 6A). This represents the smallest protein that is detected in mLin-7 Far Western blotting of cell lysates (Figs. 1A and 6B). Similarly, we can demonstrate that mLin-7 is coimmunoprecipitated with Pals2 antibodies and that the 55-kDa protein is also coimmunoprecipitated by anti-Myc-Pals1 binding partners (Fig. 5D).

Although our Pals1 antibodies were useful for immunoprecipitation of endogenous Pals1 (Fig. 5D), they did not work well in immunoblotting. To determine which of the mLin-7 binding partners in lysates was Pals1, we overexpressed Pals1 in 293 cells and detected the protein using mLin-7 Far Western overlay assay. These results indicate that Pals1 comigrates with a mLin-7-binding protein of approximately 75 kDa (Fig. 6B). Based on these results and additional immunodetection experiments (results not shown), we have tentatively identified some of the mLin-7-binding proteins detected in lysates by Far Western blotting (Fig. 6B).

Localization of Pals1 and Pals2 Proteins in MDCK Cells—Pals1 was cloned into pEYFP-N1 or pEYFP-C1 vector (CLONTECH), resulting in carboxyl-terminally or amino-terminally tagged yellow fluorescent proteins, respectively. Full-length EYFP-N-Pals1 localized to the lateral membranes at the cell-cell contact sites (Fig. 7). Similar results were obtained with EYFP-C-Pals1 (not shown). Immunostaining of MDCK cells stably transfected with full-length Myc-Pals1 showed that the protein was localized to the lateral membranes at the cell-cell contact sites (results not shown). Similarly, a EYFP NH2-terminally tagged Pals2 targeted to the lateral membrane of MDCK cells (Fig. 7).

DISCUSSION

We have identified several proteins that can bind to the amino terminus of mLin-7, and using expression cloning we have begun to identify these proteins. Collectively, we refer to these proteins as Pals. Pals proteins represent a subtype of Maguk protein that contain the mLin-7 binding domain. Pals1 and Pals2 are unique Maguk proteins, whereas Pals3 and Pals4 are previously identified mLin-7 binding partners, Pals2/Cask and Dlg2. All the mLin-7-binding proteins contain the mLin-7 binding domain, the PDZ domain, SH3 domain, and the guanylate kinase domain. Pals2 is primarily composed of just these domains, while Pals1 has a unique extended amino terminus. Pals2 appears to have two splice versions varying in a 14-amino acid insert. During the course of this study, the sequences of mouse and human p55T gene were deposited in GenBank™ (accession nos. AF161181.1 and AF162130, respectively). The mouse p55T gene matches the short splice variant of Pals2.

One question that arises is why there are several proteins that bind mLin-7. One possibility is that these proteins exist in a single complex. Our data to date suggest that this is not the case. Pals2 immunoprecipitates did not contain other Pals proteins (Fig. 6A). Similarly, Pals1 could not be immunoprecipitated with anti-mLin-2/Cask antibodies in 293 cells coexpressing Pals1, mLin-2/Cask, and mLin-7.3 We have failed to detect larger complexes by glycerol gradient centrifugation containing multiple mLin-7 binding partners.3 Our current hypothesis is that the Pals proteins compete for binding to mLin-7.

The presence of multiple binding mLin-7 partners has important implications for the targeting and function of mLin-7. For example, there are multiple mLin-7 binding partners including mLin-2/Cask in brain. mLin-2/Cask binds X11α/Mint1, forming a heterotrimeric complex of X11α/Mint1 with mLin-2/Cask and mLin-7 (5, 6). Our data suggest that binding of

3 E. Kamberov, unpublished observations.

D. Karnak, unpublished observations.
mLin-2/Cask can localize this complex to a perinuclear region where the proteins might play a role in targeting proteins in intracellular compartments, as has been suggested for other PDZ domain proteins (12, 29). In contrast, none of the other Pals proteins should bind to X11a/Mint1 because they lack the calmodulin kinase-like domain. Thus, it is possible that mLin-7 bound to mLin-2/Cask will be targeted differently.

**Fig. 5.** Expression of full-length Pals1 in cultured cells. A and B, Myc-tagged Pals1 (A) or nontagged Pals1 (B) were expressed in MDCK or 293 cells, respectively. Lysates from these cells were then prepared, separated by SDS-PAGE, and transferred to nitrocellulose. Blots were probed with anti-Myc antibody (A) or rabbit polyclonal anti-Pals1 (163–272) antibody (B). Shown as a control in both figures is Myc-tagged Pals1 (163–675) representing the clone first isolated by bacterial expression cloning. C, full-length Myc-Pals1 with or without the mLin-7 binding domain mutation, L208G, were expressed in 293 cells and incubated with immobilized GST or GST-mLin-7 in pull-down assays. After washing the beads, proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Myc antibody. D, Triton X-100 lysates from whole mouse brain were immunoprecipitated with preimmune serum or antibody directed against Pals1 (1–181). After washing the immunoprecipitate, proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti mLin-7 antibody. Input represents 10% of the volume of lysate immunoprecipitated.

**Fig. 6.** Identification of Pals proteins in cells. A, lysates from A172 cells were immunoprecipitated with antibodies to Pals2α or Lin-7. Input represents 5% of the volume of lysate immunoprecipitated. B, Triton X-100 lysate of 293 cells expressing recombinant full-length Pals1 protein (lane 3) was separated by SDS-PAGE along with a panel of non-transfected cell line lysates. Both A and B were transferred to nitrocellulose membrane, and probed in a Far Western overlay assay with radiolabeled GST-Lin-7.

**Fig. 7.** Targeting of Pals1 in MDCK. Full-length Pals1 and Pals2 were fused to yellow fluorescent protein as described under “Experimental Procedures.” MDCK cells were then transfected with the Pals1-EYFP-C1 or Pals2α-EYFP-C1 plasmid using Fugene 6 (Roche). Cells were selected for 5 days under G418 and then examined by fluorescent microscopy. Top panel, EYFP-N-Pals1, amino-terminally tagged Pals1. Bottom panel, EYFP-N-Pals2, amino-terminally tagged Pals2α.
than mLin-7 bound to other Pals proteins. In mammalian epithelia, this difference is not as apparent, as there is no X11a/Mint1 to bind to mLin-2/Cask. However, different Pals proteins may target to different regions of the cells or form microdomains at the basolateral surface. Alternatively, different Pals proteins may enter into different protein complexes. For example, a conserved valine within mLin-2/Cask and Pals1 marks these proteins PDZ domain as class II PDZ domains (30), butDlg2, Pals2, andDlg3 do not contain this conserved valine in their PDZ domains. Similarly,Dlg3, unlike other Pals proteins, does not contain a conserved band 4.1 binding domain.

Another possibility is that different Pals proteins bind to different isoforms of mLin-7. Multiple isoforms of mLin-7 have been detected and isolated (5, 15). We detected multiple isoforms of mLin-7 in our coimmunoprecipitation experiments with anti-Pals2 antibody (Fig. 6A, lower panel). In contrast, we detected only lower molecular weight forms of mLin-7 in similar coimmunoprecipitation experiments with anti-Pals1 antibody (Fig. 5D). While this might suggest differential interactions, these results should be viewed with caution as our anti-Pals1 antibody is of relatively low affinity and does not quantitatively immunoprecipitate Pals1 from cells. Further work will be necessary to examine this issue.

Pals1 and Pals2 appear to localize to the lateral membrane of MDCK cells like mLin-2/Cask and mLin-7 (14, 15, 18). Pals2 is the shortest of all these proteins, yet is also able to target properly. This suggests that the core domains of the Pals Maguk proteins are sufficient to mediate targeting. Studies of another Maguk protein, Sap97/hDlg, have indicated that the amino terminus of this protein might be sufficient for targeting (31). However, additional data suggest that the PDZ and hook domains of Sap97/hDlg also contribute to targeting (31, 32). Our data with mLin-2/Cask indicate that multiple domains are necessary for proper targeting. In the case of mLin-2/Cask, we have generated an amino-terminal fragment, containing the calmodulin kinase-like domain, Lin-7 binding domain, and the PDZ domain; and a carboxyl-terminal fragment of the SH3 domain, the 4.1 binding domain, and the Guk domain. Neither of these fragments targets properly, suggesting that a combination of amino-terminal and carboxyl-terminal domains is required for targeting of the Pals proteins (18).

It is important to note that mLin-7 and its binding partners are not only present in polarized cells like epithelia and neurons but also detected in a variety of cell lines including lymphocytes and fibroblasts. Thus, their role must not be limited to the correct targeting of proteins in polarized cells. One possible universal role for these complexes is to stabilize proteins at cell surfaces, as has been suggested for the binding of mLin-7 to BGT-1. Another possibility is the scaffolding of specific signaling complexes, as has been demonstrated for the INAD protein of Drosophila (33–35), or the modulation of transporter activity, as has been demonstrated for the NHE-RF protein (36). Regardless of its functions in particular context, the ability of mLin-7 to bind to multiple partners in multiple cell types suggests a basic role for this protein in membrane protein function and targeting.

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Identification of mLin-7-binding Proteins

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