Mammalian Lin-7 Stabilizes Polarity Protein Complexes

Received for publication, July 25, 2006, and in revised form, September 20, 2006. Published, JBC Papers in Press, October 1, 2006, DOI 10.1074/jbc.M607059200

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Mammalian Lin-7 forms a complex with several proteins, including PALS1, that have a role in polarity determination in epithelial cells. In this study we have found that loss of Lin-7 protein from the polarized epithelial cell line Madin-Darby canine kidney II by small hairpin RNA results in defects in tight junction formation as indicated by lowered transepithelial electrical resistance and mislocalization of the tight junction protein ZO-1 after calcium switch. The knock down of Lin-7 also resulted in the loss of expression of several Lin-7 binding partners, including PALS1 and the polarity protein PATJ. The effects of Lin-7 knock down were rescued by the exogenous expression of murine Lin-7 constructs that contained the L27 domain, but not the PDZ domain alone. Furthermore, exogenously expressed PALS1, but not other Lin-7 binding partners, also rescued the effects of Lin-7 knock down, including the restoration of PATJ protein in rescued cell lines. Finally, the effects of Lin-7 knock down appeared to be due to instability of PALS1 protein in the absence of Lin-7, as indicated by an increased rate of PALS1 protein degradation. Taken together, these results indicate that Lin-7 functions in tight junction formation by stabilizing its membrane-associated guanylate kinase binding partner PALS1.

Polarization of mammalian epithelia is characterized by the asymmetrical partitioning of proteins to specific membrane domains. The cellular boundary of the apical and basolateral membranes in polarized epithelia is demarcated by the tight junction that forms a fence between the two membrane compartments. Junctions within and between cells are defined by the localization of, and interactions between, specific protein complexes. The interaction of these proteins is mediated by evolutionarily conserved protein-protein interaction domains. Many studies have shown an important role for PDZ-95/Discs large/ZO-1 (PDZ) domain-containing proteins in tight junctions and the polarized localization of proteins(1, 2). One of the earliest studies to show this relationship was the work of Kim and co-workers(3, 4) that demonstrated an important role for the Lin-2, Lin-7, and Lin-10 proteins in the basolateral targeting of the Caenorhabditis elegans epidermal growth factor receptor. Mutation in any one of these genes led to a vulva-less phenotype, presumably due to mistargeting of the epidermal growth factor receptor (3, 4). Lin-2, Lin-7, and Lin-10 all contain PDZ domains and form a conserved heterotrimeric protein complex (4–6). Mammalian Lin-7 (also known as vertebrate Lin-7 (VEL1) or MALS) is a small protein (~197 amino acids) (5–8), and in the worm its PDZ domain directly binds the epidermal growth factor receptor (3). Lin-2 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins (9–11), whereas Lin-10 is a multidomain scaffold protein (4–6). Lin-7 binds to Lin-2 via an L27 domain (12); however, it is important to note that Lin-10 is not associated with Lin-2 and Lin-7 in mammalian epithelial cells (13).

Recent work in mammalian cells as well as Drosophila has identified additional binding partners for the Lin-7 L27 domain. The L27 domain of Lin-7 has been demonstrated to bind a family of MAGUK proteins highly related to Lin-2, collectively referred to as PALS or Proteins Associated with Lin Seven (14). The archetype for these proteins is known as PALS1 in mammalian cells and Stardust in Drosophila. PALS1/Stardust contains several protein interaction motifs, including two adjacent L27 domains, and binds to Lin-7 via the L27 domain nearer its carboxyl terminus (L27C) (15). The amino-terminal L27 domain (L27N) of PALS1/Stardust binds to the polarity protein PATJ (15). Additionally, the PDZ domain of PALS1/Stardust binds to the carboxyl terminus of Crumbs, an apical transmembrane protein (16–18). Studies in mammalian and invertebrate systems have shown an important role for the Crumbs-PALS1-PATJ complex in polarity determination in epithelial cells (19). In turn, these proteins bind to the highly evolutionarily conserved Par3-Par6-atypical protein kinase C polarity effector complex via an interaction between PALS1 and Crumbs with Par6 (20, 21). In mammalian cells, the loss of PATJ or PALS1 via RNA interference knock down leads to loss of polarization of epithelial cells under certain experimental conditions.
In addition, the loss of these proteins results in defects in tight junction formation. Although PALS1 binds to Lin-7, the exact role of Lin-7 in tight junction formation and polarity determination by these proteins remains unclear. As mentioned, Lin-7 also binds to other MAGUK proteins related to Lin-2 and PALS1, including PALS2, DLG2, and DLG3 (14). The role of these other Lin-7 binding partners in protein targeting and epithelial polarization is still unclear, although some clues have been provided by studies of invertebrate systems (24, 25).

It is not clear at this time why Lin-7 binds to several mammalian MAGUK proteins. Recent reports of Lin-7 knock-out mice indicate Lin-7 might not be crucial for epithelial morphogenesis (26). Accordingly, further work on the exact roles of Lin-7 is indicated. In this study, we examined the role of Lin-7 in Madin-Darby canine kidney (MDCK) cells. After stable transfection of the mammalian epithelial cell line MDCKII with Lin-7 small hairpin RNA (shRNA), we found that the resulting loss of Lin-7 correlated with the loss of expression of several Lin-7-binding proteins, including PALS1. The loss of these polarity proteins resulted in defects in tight junction formation in the Lin-7 knockdown cell lines. From these data, we hypothesize that Lin-7 is essential for the stable expression of several MAGUK family members that are in turn required for proper formation of tight junctions in mammalian epithelial cells.

EXPERIMENTAL PROCEDURES

DNA Constructs—To create the shRNA constructs, two 19-base pair sites within canine Lin-7C were chosen, and pairs of complementary oligonucleotides were synthesized by Invitrogen custom primers. These oligonucleotides contained the following target sequences: 5′-GGCCTGGTGAAGCTGTTGG-3′, corresponding to the amino acids NH$_2$GSVKLV-COOH, and 5′-GGCCACAGTGGCAGCTTC-3′, corresponding to the amino acids NH$_2$ATVAAFCOOH. These sequences were checked for significant homology to other genes in the canine genome data base, and none was found. The sense and antisense sequences were separated by a 9-base pair loop region, and each oligonucleotide was terminated with restriction endonuclease half-sites. After annealing the complementary oligonucleotides, the dimers were ligated into the pre-cut pSilencer 2.0-U6/neomycin plasmid (Ambion, Austin, TX) as directed by the manufacturer, followed by amplification of the resulting plasmids. All plasmid sequences were verified by automated sequencing at the University of Michigan DNA Sequencing Core.

The various rescue plasmids used in this study express Myc-tagged constructs from a cytomegalovirus promoter in the plasmid pRK5-myc and contain the human or murine gene indicated in the text and figures. These constructs, mycLin-7FL, mycLin-7N (amino acids 1–97), mycLin-7PDZ (amino acids 89–197), mycPALS1, mycLin-2, mycPALS2, and mycDlg2, have been previously published (5, 13, 15, 16). In the case of the murine Lin-7 constructs, these contained such significant differences in their RNA sequence as to be unaffected by the canine Lin-7-targeted shRNA constructs.

Cell Culture and Transfection—MDCKII cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum supplemented with penicillin, streptomycin, and L-glutamine. All cell culture media and supplements were purchased from Invitrogen. To create the cell lines stably expressing shRNA constructs, MDCKII cells were transfected with 5 µg of plasmid DNA using FuGENE 6 reagent (Roche Applied Science). Cells were transfected with either construct individually or with a mixture of the two constructs to create cell lines. After selection with 500 µg of active G418/ml (Invitrogen) for 14 days, surviving clones were isolated for the generation of cell lines. These lines were screened for loss of Lin-7 expression by both immunostaining and Western blot analysis using affinity purified anti-Lin-7 sera.

Rescue clones were created by co-transfection of the C19 and C20 shRNA Lin-7 lines with 5 µg of the Myc-tagged rescue plasmid and 0.5 µg of zeocin resistance plasmid, followed by selection with 200 µg of zeocin/ml (Invitrogen) for 14 days and the isolation of cell lines. Clones were screened for the expression of the Myc-tagged protein and the maintained loss of endogenous Lin-7 expression using both immunostaining and Western blot analysis.

For immunostaining or calcium switch assays, MDCKII cell lines were seeded at confluence onto 12- or 24-mm Transwell clear polyester filters (Corning Inc.; Corning, NY) in low calcium medium (with 5% dialyzed fetal bovine serum, 5 µM Ca$^{2+}$). After allowing the cells to adhere to the substrate overnight, the non-adherent cells were removed by gently washing with PBS and the medium was replaced with normal growth medium. The cells were then grown at least 72 h so that a tightly packed columnar epithelial monolayer was formed.

Calcium Switch Assay and Transepithelial Electrical Resistance Measurement—MDCKII cell lines were grown to confluence on 24-mm Transwell filters and were then washed extensively with PBS and grown in low calcium medium overnight to dissociate cell-cell contacts. The low calcium medium was replaced the next day with normal growth medium (containing 1.8 µM Ca$^{2+}$), and the cells were prepared for immunostaining at various times afterward (typically 0, 3, 6, or 29 h).

To measure the transepithelial electrical resistance (TER) a similar experiment was performed using 12-mm Transwell filters. The TER was determined with a Millicell-ERS volt-ohm meter (Millipore, Billerica, MA) immediately after the addition of normal growth medium (time = 0) and at 30–60-min intervals for up to 48 h. Prior to each measurement, the Millicell was “zeroed” according to the manufacturer’s directions and the background resistance was determined using cell-free filters. Each cell line was measured in triplicate, background was subtracted, and the means and the S.D. from the means (n = 3) for each time point were plotted using Microsoft Excel.

Antibodies—Lin-7 (UM199)-, Lin-2 (UM195 and UM196)-, PALS1 (UM349)-, PATJ (UM356)-, and CRB3 (UM369)-specific antisera were generated in rabbits and affinity purified as previously described (5, 13, 15, 16). Mouse anti-Myc antibody was purified from hybridoma clone 9E10 ascites fluid obtained from the University of Michigan Hybridoma Core. Antibodies directed against ZO-1, occludin, claudin-1, and claudin-4 were purchased from Zymed Laboratories Inc. (San Francisco, CA). Antibodies to E-cadherin and actin were purchased from Sigma. Antibodies to Par3 and protein kinase Cζ were obtained from Upstate (Lake Placid, NY). Fluorochrome-conjugated antibodies used for immu-
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nol fluorescence were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated antibodies used in immunoblotting were obtained from Amersham Biosciences.

**Immunoprecipitation and Immunoblotting**—MDCKII cell lysates were prepared from confluent 15-cm dishes with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and phosphatase inhibitor mixture) (Sigma) and cleared by centrifugation at 14,000 × g for 20 min at 4 °C. A portion of the lysate was reserved, mixed with LDS loading buffer (Invitrogen), and used as input.

For immunoprecipitation, 1–5 ml of antibody was mixed with 200 μl of lysate and 50 μl of 50% slurry of protein A-Sepharose beads (Zymed Laboratories Inc.; San Francisco, CA) and incubated overnight at 4 °C. The beads were washed three times with ice-cold HNTG and resuspended in LDS loading buffer.

Samples were separated on 4–12% NuPAGE NOVEX gels (Invitrogen) in MOPS-SDS running buffer and transferred to nitrocellulose membranes in Bicine-MeOH. The transfer efficiency was assessed by staining with 0.5% Ponceau S red in 10% acetic acid, and then the membranes were blocked by incubation in 5% bovine serum albumin (Calbiochem; San Diego, CA) in Tris-buffered saline (TBS). The membranes were incubated with primary antibody in 5% bovine serum albumin/TBS for 2 h at room temperature and then washed with 0.1% Triton X-100/TBS. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody in 5% skimmed milk/TBS for 1 h at room temperature and then washed with 0.1% Triton X-100/TBS. Protein bands were visualized using ECL reagent (PerkinElmer Life Sciences).

**CRB3 Peptide Beads**—CRB3 peptide-coupled agarose beads were created using the SulfoLink Coupling gel kit (Pierce Biotechnology, Rockford, IL) and were linked via a terminal cysteine residue added to a peptide corresponding to the carboxyl-terminal 18 amino acids of CRB3 (wild type, NH$_2$-CARVPPTNLKLPPEERLI-COOH) or the same sequence cysteine residue added to a peptide corresponding to the carboxyl-terminal 18 amino acids of CRB3 with a canine kidney-derived epithelial cell line, express only one of

were separated by SDS-PAGE, and the gel was fixed in 30% methanol/10% acetic acid, soaked for 30 min in Amplify (Amersham Biosciences), and dried. Densitometry was performed using a Molecular Dynamics laser scanner, model STORM860, and ImageQuant software (GE Healthcare). Each sample was prepared in triplicate (n = 3) so that the standard deviation from the means could be determined.

**Immunostaining and Confocal Microscopy**—Cells grown on Transwell filters were cut from the support with a scalpel, washed with PBS, and fixed with 4% paraformaldehyde/PBS for 30 min, permeabilized with either 0.1% Triton X-100/PBS or 1% SDS/PBS for 15 min, and then blocked with 2% goat serum/PBS for 1 h. The filters were then incubated with primary antibodies in goat serum/PBS for overnight at 30 °C in a humidified chamber. After washing extensively with goat serum/PBS, fluorochrome-conjugated secondary antibodies in goat serum/PBS were added overnight at 4 °C. Finally, filters were washed with PBS and mounted onto glass slides using ProLong antifade reagent (Molecular Probes, Eugene, OR).

All images were obtained using an Olympus Fluoview 500 confocal laser-scanning confocal microscope at the Morphology and Image Analysis Core of the Michigan Diabetes Research and Training Center. Samples were scanned with appropriate lasers and filter sets, and images were collected at 0.5-μm intervals on an Olympus IX-71 inverted microscope using a ×100 oil objective. Fluoview v4.3 software was used to collect images, and subsequent preparation was performed using Adobe Creative Suite software.

**Quantitative Real-time PCR**—Messenger RNA from cell lines was isolated following the manufacturer’s protocol found in the RNeasy kit (Qiagen). cDNA was reverse transcribed from isolated mRNA by Superscript III First-Strand Synthesis Supermix (Invitrogen) according to the published protocol. Real-time PCR was performed using Platinum SYBR Green qPCR Supermix UDG with Rox (Invitrogen) in an Applied Biosystem 7300 real-time PCR system with the following parameters: 50 °C (2 min), 95 °C (10 min), 40 cycles of 95 °C (15 s), 54 °C (30 s), 72 °C (1 min), followed by a dissociation cycle to verify one product by melting curve analysis. Primers to desired amplicons were designed using PerlPrimer software (27) and are as follows: *Canis familiaris* Lin-7C (5′-GCC TTA CAA AGA GTC CTT CA-3′ (forward)), (5′-TGA TGT CCA CAG TCT CAT AG-3′ (reverse)); *C. familiaris* PALS1 (5′-TAG AAC CCT TTA CAG ATG AGA G-3′ (forward)), (5′-ACT ATC CGA CTA ATG ATG ACA G-3′ (reverse)); *C. familiaris* glyceraldehyde-3-phosphate dehydrogenase (5′-ATT CTA TCC ACG GCA AAT CC-3′ (forward)), (5′-GGA CTC CAC AAC ATA CTC AG-3′ (reverse)). Primer pairs were validated by subjecting the quantitative PCR reactions to agarose gel electrophoresis to confirm the presence of single products at the expected size. In addition, each amplicon was sequenced to verify its identity. Experimental samples were performed in duplicate. Results were compiled from two independent experiments.

**RESULTS**

Lin-7 shRNA Affects the Formation of Tight Junctions—Previous work from our group had shown that MDCKII cells, a canine kidney-derived epithelial cell line, express only one of
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We next tested the MDCKII cell lines for the formation of tight junctions using a calcium switch assay in which cells were placed in low calcium medium overnight to dissociate the cells by breaking adherens junctions, followed by the readddition of calcium to allow the MDCKII cells to form junctions. Tight junction function is measured by the establishment of TER. Cell lines in which Lin-7 had been knocked down had markedly abnormal TER profiles (Fig. 2A, solid lines) compared with wild-type and control cells (dashed lines). However, Western blotting indicated no change in expression of several tight junction component proteins including claudin-1, claudin-4, and occludin in cells with or without Lin-7 (Fig. 2B). Furthermore, immunostaining of the MDCKII cells that had been grown on filters for 7 days showed that the tight junction marker proteins claudin-1 and ZO-1 were similarly localized in both control and Lin-7 knockdown cell lines (results not shown). However, using the calcium switch assay, abnormal, incomplete localization of ZO-1 was observed in the Lin-7 knockdown cells (Fig. 2C) in contrast to control cells. In conjunction with the TER data, this suggests that Lin-7 plays a role in the formation of tight junctions and the loss of Lin-7 expression results in a delay in tight junction formation during polarization.

Lin-7 shRNA Affects Expression of MAGUK Family Members—We have previously demonstrated that far-Western blotting with a radiolabeled Lin-7 probe could reveal multiple MAGUK proteins that bind to Lin-7 (14). These include Lin-2/CASK, PALS1/MPP5, PALS2/Vam-1, Dig2/MMP2, and Dig3/MPP3. In the Lin-7 knockdown cells the expression of these MAGUKs, except for Lin-2, was significantly reduced (Fig. 3A). Of the MAGUK family members with reduced expression, we chose to focus our study on PALS1 because of its known roles in polarity and tight junction formation that had been previously demonstrated by our laboratory and others (15, 17, 18, 22, 29, 30). The PDZ domain of PALS1 binds to the carboxyl-terminal amino acids of Crumbs proteins, and these two proteins are in a complex with PATJ (15, 17, 18, 31). Using a Sepharose bead coated with a peptide corresponding to the carboxyl terminus of Crumbs3 (CRB3), we were able to precipitate PALS1 and PATJ from MDCKII cell lysates. In accord with Fig. 3A, Crumbs beads precipitated PALS1 from control cells but not from cells where Lin-7 was knocked down (Fig. 3B). PATJ expression was also reduced in the Lin-7 knockdown cells, although CRB3 expression was similar in control and wild-type cells (Fig. 3C). This agrees with our previously published result that PATJ protein levels are reduced when PALS1 expression is knocked out. We predicted that the reduced expression of PALS1 and PATJ in the Lin-7 knockdown cells would also affect their protein complexes and/or downstream signaling pathways.

The three Lin-7 genes, Lin-7C (13). Lin-7B is not expressed in the kidney tubule (28); Lin-7A is larger than Lin-7C, and this size is not seen on Western blotting of MDCKII cell lysates with anti-Lin-7 antibodies (13). We generated shRNA expression plasmids to specifically target the canine Lin-7C sequence (Fig. 1A) based on the genomic information available in the public data base. These shRNA constructs had several mismatches with mouse Lin-7C allowing for eventual rescue of the pheno-
down (22). However, the knock down of Lin-7 did not affect expression of Par3 or atypical protein kinase C (Fig. 3D).

The L27 Domain of Lin-7C Is Essential for Correct Tight Junction Formation—To determine whether the effects were specific to the loss of Lin-7, we rescued the Lin-7 shRNA MDCKII cells using mouse Lin-7C constructs. Murine Lin-7C is resistant to the effect of the shRNA constructs we used to knock down canine Lin-7 due to nucleotide sequence differences between species (Fig. 1A). We transfected Lin-7 knockdown cell lines with Muc-tagged mouse Lin-7C constructs: full-length Lin-7C (mycLin-7FL), the amino terminus containing the L27 domain (amino acids 1–97; mycLin-7N), and the carboxyl terminus containing the PDZ domain (amino acids 89–197; mycLin-7PDZ). Several MDCKII cell clones expressing zeocin only were also selected as controls. Full-length murine Lin-7 and the amino-terminal/L27 domain construct rescued the expression of the MAGUK proteins, including PALS1, as determined by far-Western blotting with radiolabeled GST-Lin-7 (Fig. 4A). In contrast, the carboxyl terminus/PDZ domain construct and zeocin alone did not rescue MAGUK expression (Fig. 4A). PATJ protein expression was also rescued by the mycLin-7FL and mycLin-7N constructs (Fig. 4A). Both the mycLin-7FL and mycLin-7N constructs also rescued the TER after calcium switch (Fig. 4B, dashed lines and open shapes). Additionally, immunostaining for ZO-1 after calcium switch illustrated that mycLin-7FL and mycLin-7N constructs rescued normal tight junction formation (Fig. 4C). Thus, these experiments indicate that the L27 domain, but not the PDZ domain, of Lin-7 is required to rescue the Lin-7 knockdown phenotype.

Reexpression of PALS1 Can Reverse the Tight Junction Defect Seen in Lin-7 Knockdown Cells—Next, we wanted to determine whether loss of the MAGUK proteins could explain the tight
junction defects. In particular, we suspected that loss of PALS1 was responsible for the tight junction defects in that PALS1 is required for tight junction formation (22). To this end, the Lin-7 knockdown cell lines were transfected with a vector expressing mycPALS1 and stable cell lines were selected. Two Lin-7 shRNA cell lines overexpressing mycPALS1 were chosen for further study. In these cell lines, mycPALS1 rescued the expression of endogenous PATJ (Fig. 5A). Endogenous Lin-7 protein was still low to undetectable in these PALS1 rescue clones (Fig. 5B). mycPALS1 targeted correctly to the tight junc-

FIGURE 4. Rescue of the Lin-7 shRNA phenotype by Myc-tagged murine Lin-7 constructs. A, Lin-7 knockdown cells rescued with full-length Lin-7 (Lin-7FL), N terminus of Lin-7 (Lin-7N) comprising the L27 domain, and Lin-7 PDZ domain (Lin-7PDZ) were far-Western blotted with GST-Lin-7 and immunoblotted with PATJ, CRB3, Lin-2, and actin antibodies. B, the transepithelial electrical resistance of MDCKII wild-type or Lin-7 knockdown cells rescued with mycLin-7FL, mycLin-7N, mycLin-7PDZ, or control vector (zeo). C, immunostaining with antibodies to ZO-1 (green) and Myc (red) in Lin-7 knockdown cells rescued with mycLin-7FL, mycLin-7N, or mycLin-7PDZ 6 h after calcium switch. Scale bars represent 10 μm.
In the absence of endogenous Lin-7 (Fig. 5B) and was also able to partially rescue the TER after calcium switch (Fig. 5C, dashed lines). In contrast, expression of Myc-tagged PALS2,Dlg2, or overexpressing Lin-2 could not rescue either the TER or tight junction immunostaining defects seen in Lin-7 shRNA cells (Fig. 6, A–D). These results indicate that the defects we observed in Lin-7 knockdown cells were primarily due to the loss of PALS1.

Lin-7 Expression Affects Stability of PALS1 in Polarized MDCKII Cells—Finally, we sought to determine the manner by which PALS1 expression was reduced in Lin-7 shRNA cells: The loss of Lin-7 might be affecting the PALS1 mRNA or the expression and stability of the PALS1 protein. We performed quantitative reverse transcription PCR to measure PALS1 transcripts in both Lin-7 shRNA and a previously published PALS1 shRNA cell line (22). We found no difference in the mRNA levels between control and Lin-7 knockdown cells (Fig. 7A), suggesting PALS1 mRNA levels are not altered in Lin-7 shRNA cells. Control PALS1 knockdown cells illustrate our primer pairs were specific for the PALS1 transcript. Subsequently, we undertook pulse-chase radiolabeling to examine the degradation rate of the PALS1 protein. Our antibodies do not immunoprecipitate PALS1, so instead we examined wild-type cells and Lin-7 shRNA cells transfected with mycPALS1. Thus we were able to immunoprecipitate tagged PALS1 and follow its half-life in the presence and absence of Lin-7. The pulse-chase assay found that the half-life of PALS1 was much shorter in the Lin-7 knockdown cells in comparison with controls, with only 1.7–3.9% of radiolabeled mycPALS1 protein remaining in Lin-7 knockdown cell lines after 12 h compared with 18.7% in control.
FIGURE 6. Other exogenously expressed Myc-tagged proteins that bind to the Lin-7 L27 domain cannot rescue the tight junction phenotype of Lin-7 knockdown. A, far-Western blotting with $^{32}$P-GST-Lin-7 showed the expression of the indicated Myc-tagged proteins and endogenous Lin-2 in Lin-7 knockdown cells. Western blotting for Lin-7 confirmed the knock down, and actin is shown as a loading control. Relative molecular mass is indicated to the left in kilodaltons. B, immunofluorescence with anti-Myc antibodies of indicated cell lines with anti-Myc and anti-Lin-7. Scale bars are 10 $\mu$m. C, transepithelial electrical resistance analysis after calcium switch of indicated cell lines. D, immunostaining of cells grown on Transwell filters for ZO-1 (green) and Myc (red) 6 h after calcium switch in indicated cell lines. Scale bars are 10 $\mu$m.
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Lin-7 knockdown cell lines showed a specific reduction in endogenous Lin-7C, and under static conditions cell-cell junction integrity appeared normal. However, when subjected to calcium switch, where we could monitor phenotypes under polarizing conditions, transepithelial electrical resistance and immunostaining data clearly demonstrated that Lin-7 shRNA cells were unable to efficiently form tight junctions, suggesting that Lin-7 affects the formation of tight junctions in polarizing epithelial cells by delaying their formation.

Lin-7 knockdown reduced the expression of MAGUK family proteins that are known binding partners of the L27 domain of Lin-7. The cause for the reduction in expression of the MAGUK PALS1 was due to a reduced half-life as seen by pulse-chase experiments with no difference seen in mRNA levels. The reduced expression of PALS1 and other MAGUKs was rescued by expression of the L27 domain of Lin-7 but not by expression of the Lin-7 PDZ domain. It is likely that the binding of the Lin-7 L27 domain to the PALS1 L27 domain stabilizes the fold of the PALS1 protein, making it more stable. As L27 domains interact via large hydrophobic surfaces, it is not hard to understand how L27 domain interactions would stabilize folding of proteins containing this domain (32–34).

Knock down of PALS1 leads to a loss of PATJ expression (22), and loss of PATJ was also seen in cells missing Lin-7. This was due to the lack of PALS1 as re-expression of PALS1, even in the absence of Lin-7, restored PATJ expression. PALS1 also binds to PATJ via an L27 domain interaction involving a single L27 domain in the amino terminus of PATJ. Again, it is possible that an unliganded L27 domain in PATJ leads to protein misfolding, instability, and reduced expression in cells missing PALS1. It is interesting to note, however, that PALS1 expression in PATJ knockdown cells is only moderately affected (23). This suggests the relationship between L27 domain binding and protein stability is complex. Similarly, the expression of the MAGUK protein Lin-2/CASK appeared unaffected by the Lin-7 knock down although it binds Lin-7 like PALS1. It is interesting to note, however, that Lin-2/CASK expression was reduced in the brains of Lin-7 knock-out mice (26). The differential effects of L27 binding on MAGUK stability may point to simple structural differences among the proteins or might suggest that the L27 domains of these MAGUKs have alternate binding partners.

The effects we saw with Lin-7 were due to loss of the L27 domain and not the PDZ domain. The role of the PDZ domain of Lin-7 was best described in worms where it targets the epidermal growth factor receptor to the lateral surface (3). Several binding partners for the PDZ domain of mammalian Lin-7 have been described. One binding partner is β catenin (35), although we saw no defects in cadherin staining in cells missing Lin-7. Defects in other Lin-7 binding partners such as potassium channels or other transporters (36, 37) would not be detected in our studies. It has been found that mammalian epidermal growth factor receptor family members can bind to the first 13 amino acids of Lin-7C in a region amino-terminal to the PDZ domain (38); however, deleting this region in mouse Lin-7C had
no effect on the ability of this Lin-7 to rescue knockdown MDCK cells.

We demonstrated that the tight junction defect of Lin-7 shRNA was due to the reduction in endogenous PALS1. Even without Lin-7, exogenously expressed PALS1 still trafficked properly to the tight junction and partially rescued tight junction formation. Its inability to completely rescue TER measurements (Fig. 5C) could have been due to the presence of the Myc tag or the expression level of the mycPALS1 protein. Another possibility could be the loss of other Lin-7-binding MAGUK proteins such as DLG2, DLG3, or PALS2 whose function in epithelia is unclear. Again, further studies in the Lin-7 knock-out mice may be instructive. These mice with all Lin-7 genes removed die shortly after birth due to respiratory failure (26). These studies indicate no gross defect in tissue morphogenesis, suggesting epithelial polarity is not overtly perturbed. Defects in tight junctions may not appear until later in knock-out animals where they manifest as deficiencies in the skin barrier (39).

Although it is likely that PALS1 is reduced in these Lin-7 knock-out animals, they may have sufficient PALS1 to mediate epithelial polarity in summary, our results show an expanding role for Lin-7 proteins such as DLG2, DLG3, or PALS2 whose function in tight junctions may not appear until later in knock-out animals, they may have sufficient PALS1 to mediate epithelial polarity. In addition, our results show an expanding role for Lin-7 proteins such as DLG2, DLG3, or PALS2 whose function in epithelia is unclear. Again, further studies in the Lin-7 knock-out mice may be instructive. These mice with all Lin-7 genes removed die shortly after birth due to respiratory failure (26). These studies indicate no gross defect in tissue morphogenesis, suggesting epithelial polarity is not overtly perturbed. Defects in tight junctions may not appear until later in knock-out animals where they manifest as deficiencies in the skin barrier (39).

Acknowledgments—We thank Toby Hurd for critical discussion concerning this manuscript, the Eric Fearon laboratory for use of the quantitative PCR machine, and Ron Koenig for technical advice on quantitative PCR experiments.

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S. Straight and B. Margolis, unpublished observations.