PALS1 Regulates E-Cadherin Trafficking in Mammalian Epithelial Cells[□]

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Submitted July 31, 2006; Revised November 14, 2006; Accepted December 7, 2006
Monitoring Editor: Keith Mostov

Protein Associated with Lin Seven 1 (PALS1) is an evolutionarily conserved scaffold protein that targets to the tight junction in mammalian epithelia. Prior work in our laboratory demonstrated that the knockdown of PALS1 in Madin Darby canine kidney cells leads to tight junction and polarity defects. We have created new PALS1 stable knockdown cell lines with more profound reduction of PALS1 expression, and a more severe defect in tight junction formation was observed. Unexpectedly, we also observed a severe adherens junction defect, and both defects were corrected when PALS1 wild type and certain PALS1 mutants were expressed in the knockdown cells. We found that the adherens junction structural component E-cadherin was not effectively delivered to the cell surface in the PALS1 knockdown cells, and E-cadherin puncta accumulated in the cell periphery. The exocyst complex was also found to be mislocalized in PALS1 knockdown cells, potentially explaining why E-cadherin trafficking is disrupted. Our results suggest a broad and evolutionarily conserved role for the tight junction protein PALS1 in the biogenesis of adherens junction.

INTRODUCTION

Polarity is an intrinsic feature of epithelial cells reflected by the differential distribution of proteins and lipids in the apical and basolateral surfaces (Roh and Margolis, 2003). The apical and basolateral membranes are physically separated by the tight junction seal at the superior aspect of the lateral surface (Tsukita *et al.*, 2001; Matter and Balda, 2003). The adherens junctions lay basal to the tight junctions in mammalian epithelial cells, and they mediate the adhesion between neighboring cells. Studies in *Drosophila* and mammalian cells have identified a large number of proteins as polarity determinants, and these polarity proteins form evolutionarily conserved macromolecular complexes. The complicated interplay among these complexes and their orderly functioning regulates the establishment of cell polarity and the cell–cell junctions.

Included in these polarity proteins are mammalian Protein Associated with Lin Seven 1 (PALS1) and its orthologue *Drosophila* Stardust (Sdt) (Knust and Bossinger, 2002). Genetic and biochemical studies in *Drosophila* have shown that Sdt interacts with the transmembrane protein Crumbs (CRB) through its PDZ domain (Bachmann *et al.*, 2001; Hong *et al.*, 2001), and mutations in either CRB or Sdt cause polarity defects in *Drosophila* epithelia (Tepass and Knust, 1993). Like Sdt, the PDZ domain of PALS1 binds the C-terminal tail of mammalian CRB isoforms, and PALS1 also interacts with a multi-PDZ domain protein, PALS1-Associated Tight Junction protein (PATJ), through binding to the N-terminal L27 (L27N) domain of PALS1 (Roh *et al.*, 2002). The PALS1–PATJ–CRB complex localizes to the tight junction of mammalian epithelial cells, and

the disruption of the complex leads to defects in cell polarity (Straight *et al.*, 2004). The C-terminal L27 (L27C) domain of PALS1 interacts with an L27 domain within Lin-7 (Kamberov *et al.*, 2000), and prior work in our laboratory has shown that an evolutionarily conserved region in the N terminus of PALS1 mediates its interaction with Par6. This interaction links the polarity complexes PALS1–PATJ–CRB and Par3–Par6–aPKC together (Hurd *et al.*, 2003; Wang *et al.*, 2004). The function of the C-terminal SH3 domain and GUK domain of PALS1 is unknown.

Epithelial cells have an adhesive belt that encircles the cell just below the apical surface called the zonula adherens (Knust and Bossinger, 2002). The zonula adherens, which is also called the adherens junction in vertebrates, is based on calcium-dependent engagement of cadherin molecules on adjacent cells. In *Drosophila*, the Sdt–dPATJ–CRB complex is localized to a specialized zone apical to the zonula adherens called the subapical region or marginal zone. The subapical region, like the tight junction, is located apically to the zonula adherens (Knust and Bossinger, 2002). However, the subapical region is not the site of the intercellular seal in *Drosophila* cells, which instead reside below the zonula adherens in the septate junction. In *Drosophila*, the Sdt–dPATJ–CRB complex regulates the formation of the zonula adherens and E-cadherin localization (Grawe *et al.*, 1996; Tepass, 1996; Klebes and Knust, 2000), although how this protein complex interacts with components of the zonula adherens remains unclear (Knust and Bossinger, 2002).

Prior work in our laboratory has demonstrated that the knockdown of PALS1 in Madin Darby canine kidney (MDCK) cells leads to tight junction and polarity defects (Straight *et al.*, 2004). In this study, we created new PALS1 stable knockdown (KD) cell lines with more profound reduction of PALS1 expression. Besides a more severe defect in tight junction formation, we also observed abnormal adherens junction and E-cadherin localization. We think the depletion of PALS1 disrupted the trafficking of E-cadherin to the cell periphery. This is the first report that PALS1 is

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-07-0651>) on December 20, 2006.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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involved in the regulation of adherens junction biogenesis in mammalian epithelial cells, and it may represent a conserved mechanism from *Drosophila*.

MATERIALS AND METHODS

DNA Constructs

The following sequences of nucleotides with a 19-base target site (in bold) and a 9-base loop (underline) were used to generate small hairpin RNA (siRNA)-expressing plasmid against canine PALS1 mRNA: PALS1 KD#1 primer (5'-GATCCGGAGATGAGGTTCTGGAATTCAGAGATTTC-CAGAACCCTCATCTCTTTTGGAAA-3') and PALS1 KD#2 primer (5'-GATCCGGGGATATACTTCATATCATTCAAGAGATGATATGAA-GTATATCCCTTTTGGAAA-3'). After annealing the complementary oligonucleotides, the dimers were ligated into the pre-cut pSilencer 2.1-U6 hygro plasmid (Ambion, Austin, TX), as directed by the manufacturer, followed by amplification of the resulting plasmids. All plasmids were verified by automated sequencing at the University of Michigan DNA Sequencing Core (Ann Arbor, MI). The PALS1 full-length and various PALS1 mutant cDNA sequences were subcloned into a modified pKH3 vector with a tandem FLAG and Myc tag fused to the N terminus of the insert.

Cell Culture and Calcium Switch Experiment

MDCKII cells were cultured in DMEM plus 10% fetal bovine serum supplemented with penicillin, streptomycin, and L-glutamine. All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). To create the cell lines stably expressing siRNA constructs, MDCKII cells were transfected with 5 μ g of the pSilencer 2.1 plasmid DNA by using FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). After selection with 500 μ g/ml hygromycin (Invitrogen) for 14 d, surviving clones were isolated for the generation of cell lines. The PALS1 KD#1 cell line was cotransfected with 5 μ g of the FLAG-myc-PALS1 plasmid and 0.5 μ g of pSV2NEO and selected with 500 μ g/ml Geneticin (G-418) (Invitrogen) for 14 d to generate the rescue cell lines. All established stable cell lines were cultured in medium containing half of the drug concentration for selection.

For the calcium switch experiments, MDCKII cell lines were grown to confluence on 12-mm Transwell filters and were then washed extensively with phosphate-buffered saline (PBS) and grown in low calcium medium (5 μ M Ca^{2+}) overnight to dissociate cell-cell contacts. The low calcium medium was replaced the next day with normal growth medium (1.8 mM Ca^{2+}), and the cells were prepared for immunostaining at various time points afterward (0, 3, 6, or 29 h).

Antibodies

PALS1, PATJ, Lin-7, and Nectin-like 2 (Ncl2)-specific antisera were generated in rabbits and affinity purified as described previously (Borg *et al.*, 1998; Roh *et al.*, 2002). Rabbit anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-Myc (9E10) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-zonula occludens (ZO)-1, mouse anti-transferrin receptor (TfR), and rat anti-E-cadherin (for immunofluorescence) antibodies were purchased from Zymed Laboratories (South San Francisco, CA). Mouse anti-early endosomal antigen (EEA)1, mouse anti- β -catenin, mouse anti-Rab11, mouse anti-GM130, mouse anti-RalA, and mouse anti-E-cad (for Western blot) antibodies were purchased from BD Biosciences (San Jose, CA). Mouse anti- γ -adaptin was purchased from Sigma-Aldrich, rabbit anti-Akt was purchased from Cell Signaling Technology (Danvers, MA), and mouse anti-Sec8 was purchased from Stressgen Bioreagents (Ann Arbor, MI). The hybridoma generating mouse monoclonal anti-E-cadherin antibody (rr1) was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA (Gumbiner and Simons, 1986).

Immunoblotting

Cells cultured on one 10-cm dish were collected in 0.5 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM EGTA, 10 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM Na_3VO_5 , 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 20 μ g/ml aprotinin). Lysates were cleared by centrifugation (12,000 $\times$ g for 15 min at 4°C). Western blotting was performed as described previously (Straight *et al.*, 2004).

Immunostaining and Microscopy

Cells grown on Transwell filters were cut from the support with a scalpel, washed with PBS, fixed with 4% paraformaldehyde/PBS for 15 min, and permeabilized with either 0.1% Triton X-100/PBS or 1% SDS/PBS for 5 min (or without permeabilization as indicated). Alternatively, cells were fixed and permeabilized at room temperature with 1:1 acetone/methanol for 15 min. Then, the cells were blocked with 2% goat serum (GS)/PBS for 1 h. The filters were then incubated with primary antibodies in GS/PBS overnight at 30°C in a humidified chamber. After washing extensively with GS/PBS, fluorochrome-conjugated secondary antibodies in GS/PBS were added overnight at 4°C. Finally, filters were washed with PBS and mounted onto glass slides by

using ProLong antifade reagent (Invitrogen). All confocal images were obtained using an Olympus Fluoview 500 inverted confocal microscope at the University of Michigan Diabetes Center. Epifluorescent images were taken using a Nikon Eclipse TE2000-U microscope.

Cell Surface Biotinylation

Cells were grown on filters and incubated with 1.5 mg/ml sulfo-succinimidyl 2-(biotinamido) ethyldithiopropionate (sulfo-NHS-SS-biotin; Pierce Chemical, Rockford, IL) at different time points after calcium switch. Biotin was applied to the basal side of the Transwell filter. After the rocking at 4°C for 30 min, the filters were washed with PBS containing 100 mM glycine to quench free sulfo-NHS-SS-biotin followed by several further washes in PBS. The cells were then scraped off the filters and suspended in a radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 10 μ g/ml leupeptin, 100 μ g/ml phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin). The cell lysates were centrifuged, and the supernatants were incubated with streptavidin beads (Pierce Chemical) to collect bound biotinylated proteins. The samples were then subjected to SDS-PAGE followed by Western blotting with the anti-E-cadherin monoclonal antibody. The Western blotting result was scanned by the Typhoon scanner and quantified with ImageQuant 5.8.

Metabolic Labeling and Pulse Chase

Cell cultures were preincubated with methionine/cysteine-free medium for 1 h. Then, 300 μ Ci of [^{35}S]methionine/cysteine (NEG EXPRE ^{35}S ; PerkinElmer Life and Analytical Sciences, Boston, MA) was added to both sides of the Transwell filter. The cells were pulse labeled for 2 h, rinsed two times in PBS, and chased in regular medium for indicated time. Then, a surface biotinylation experiment was performed. Immobilized rat anti-E-cadherin antibody was added to the collected cell lysates. After rocking overnight at 4°C, the beads were washed and eluted with 400 μ l of acid elution buffer (0.2 M glycine and 1% Triton, pH 2.6) at room temperature for 1 h. Biotinylated proteins in the supernatant were precipitated by adding 5 μ l of 1 N NaOH, 25 μ l of 1 M Tris, pH 7.4, 40 μ l of 10% bovine serum albumin, and 60 μ l of streptavidin beads. The beads were washed and resuspended in 30 μ l of 1X SDS sample buffer, and after gel running and electrotransfer, the nitrocellulose membrane was exposed to a phosphorimager screen overnight and scanned and quantified.

E-Cadherin Endocytosis Assay

MDCK or the PALS1 KD cells were seeded into the Lab-Tek II chamber slide system (Nalge Nunc International, Rochester, NY). After culturing cells in serum-free medium for 1 h, mouse rr1 ascites was added to make the final concentration 10%. Cells were fixed and stained as indicated after being incubated at 37°C for 3 h. For the combined endocytosis assay, cells were incubated in the low calcium medium overnight and then switched to serum-free medium containing 1/10 rr1 ascites for another 3 h of incubation at 37°C. The serum-free medium contains normal amount of calcium.

10–15–20–30% Opti-Prep Gradient

The Opti-Prep gradient was carried out following a published procedure (Yeaman, 2003) with minor modifications. Briefly, MDCK cells grown on tissue culture plates were washed with PBS twice before being scraped off in HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, and 1 mM EDTA, supplemented with complete protease inhibitors). The cell suspension was passed five times through a 21-gauge syringe and homogenized 20 times with a 10-ml Wheaton homogenator. Cell homogenates were then spun at 3000 $\times$ g for 5 min, and supernatants were collected as postnuclear supernatant (PNS). PNS was mixed with 60% iodixanol (Opti-Prep) to generate a 30% solution, which was overlaid with 20, 15, and 10% iodixanol, respectively. The gradients were spun in a NVT90 rotor at 350,000 $\times$ g for 4 h at 4°C. Once completed, 13 fractions were collected from each gradient for subsequent Western blot analysis.

RESULTS

Depletion of PALS1 Disrupts Both Tight Junctions and Adherens Junctions in MDCK Cells

Prior work in our laboratory showed that when the expression of the tight junction-associated protein PALS1 is markedly reduced in MDCKII cells by siRNA expression, the expression level of PATJ is lowered accordingly, and the formation of tight junctions is significantly delayed (Straight *et al.*, 2004). To further investigate the role of PALS1 in junction formation, we designed two canine-specific siRNA sequences and created PALS1 KD cell lines by stably expressing the siRNA-encoding plasmid in MDCKII cells. The depletion of PALS1 was shown by Western blot, and as reported previously, PATJ but not Crumbs3 expression was also markedly reduced (Figure 1A).

Figure 1. Knockdown of PALS1 in MDCK cells results in defects in the formation of tight junctions and adherens junctions. (A) Lysates of the MDCKII wild-type cells, two independent SiRNA-expressing cell lines (PALS1 KD#1 and PALS1 KD#2), and the PALS1 KD cell line published previously (Old SiRNA#1) were blotted with the antibodies directed against the proteins indicated to the left. Actin level was used as a loading control. (B) Cells grown to confluence on polyester filters were transferred to low calcium medium overnight to dissociate cell–cell contacts and then returned to normal calcium medium. At 6 and 29 h after calcium readdition, cells were fixed, permeabilized with 1% SDS, and immunostained with the antibodies indicated. (C) The pSilencer 2.1 plasmid encoding the two SiRNA sequences in PALS1 KD#1 and PALS1 KD#2 were transfected into MDCKII cells to generate pools of cells with varying degrees of PALS1 expression. Cells were immunostained 3 h after calcium switch with the antibodies indicated. Images of PALS1 staining and E-cadherin staining were merged with DAPI staining to show the loss of E-cadherin staining in PALS1 KD cells. Cells with PALS1 knocked down are indicated with arrowheads, and cells still expressing PALS1 are indicated with arrows.

Compared with the best PALS1 KD cell line reported in the previous work (Old SiRNA#1; Straight *et al.*, 2004), the two new PALS1 KD cell lines (PALS1 KD#1 and PALS1 KD#2) have lower expression of PATJ (Figure 1A), and we have found that the level of PATJ reflects the residual expression of PALS1. The difference in the level of PALS1 remaining is more clearly shown by immunofluorescence, where residual amounts of PALS1 can be detected in the Old SiRNA#1 cell line, whereas it is almost completely depleted from the two new PALS1 KD cell lines (Figure 1B).

To examine the effect that the loss of PALS1 has on the formation of cell–cell contacts, calcium switch experiments were performed. Cells grown to confluence on polyester filters were transferred to low calcium medium overnight to dissociate cell–cell contacts and then switched to normal calcium medium. MDCKII cells reformed tight junction and adherens junction by 6 h after the switch, revealed by the junctional staining of the tight junction marker ZO-1 and the adherens junction marker E-cadherin (Figure 1B, a). As reported in Straight *et al.* (2004), the Old SiRNA#1 cells showed delayed tight junction formation (Figure 1B, d). In contrast, the PALS1 KD#1 and PALS1 KD#2 cells did not reform tight junctions 29 h after the switch, and unexpectedly, the adherens junction structural protein E-cadherin was also missing from the cell–cell contact sites (Figure 1B, b and c). Moreover, tight junctions and adherens junctions remained partially formed in the two new KD cell lines even after 7 d of culturing on filters (data not shown). These results showed that the formation of both tight junctions and adherens junctions is disrupted in the two new PALS1 KD clonal cell lines. The apical protein marker Gp135 is localized to the apical side of the PALS1 KD#1 cells, although more diffusely than the control cells. F-actin also seemed to be more diffuse in the PALS1 KD cells (Supplemental Figure 1).

We then performed the calcium switch experiment in the PALS1 KD pool cells to eliminate clonal effects of stable cell lines. The pSilencer 2.1 plasmids encoding the two SiRNA sequences in PALS1 KD#1 and PALS1 KD#2 were transiently transfected into MDCKII cells. After 72 h, the KD pool cells were transferred to low calcium medium overnight, and 3 h after being switched to normal calcium medium they were fixed and immunostained. In both pools, ZO-1 and E-cadherin staining were missing from the cell–cell contact sites in cells whose PALS1 was depleted (the presence of cells were revealed by 4,6-diamidino-2-phenylindole [DAPI] staining), and they were correctly localized in areas where PALS1 was intact (Figure 1C). These results agreed with the clonal knockdown cells showing that the depletion of PALS1 disrupted adherens junctions.

Wild-Type PALS1 and Two PALS1 Mutants Rescue the Defects in Junction Formation

The PALS1 KD#1 and PALS1 KD#2 SiRNA sequences were designed against the canine-specific regions of canine PALS1, and they do not recognize murine PALS1 due to the difference of nucleotides within the 19mers. We then reintroduced murine PALS1 wild-type and various PALS1 mutants to see whether they can rescue the defects in junction formation. Figure 2A depicts the domain structure of murine PALS1. The U1 region mutant V37G shows reduced binding with the polarity protein Par6 (Wang *et al.*, 2004), the L27N domain mutant disrupts the interaction between PALS1 and PATJ (Roh *et al.*, 2002), and the PALS1 L27C domain mutant does not bind Lin-7 (Kamberov *et al.*, 2000). It has been shown that extensive intramolecular interactions exist between the C-terminal SH3 domain and the GUK domain of MAGUK proteins (McGee *et al.*, 2001; Tavares *et al.*, 2001). To

explore the function of the C terminus of PALS1, which has been largely unknown, we generated a point mutation L379P in the SH3 domain that disrupts the intramolecular interaction between the SH3 domain and the GUK domain (Woods *et al.*, 1996; Wu *et al.*, 2000), and a truncation mutant delC (PALS1 1-345) that lacks both domains. The wild-type PALS1 and the various mutants were fused to tandem FLAG and Myc tags at the N terminus and stably expressed in the PALS1 KD#1 cells. The expression of the exogenous PALS1 is shown in Figure 2B, and it is comparable with endogenous PALS1. Interestingly, PATJ was rescued to close to its original level in the cells with reexpressed PALS1 except in the cells expressing the PALS1 L27N mutant, which is the PATJ binding-defective mutant (Figure 2B). This result reconfirms that PATJ exists in a complex with PALS1 in the cells, and the interaction with the PALS1 L27N domain stabilizes PATJ.

We studied junction formation of the rescued cell lines at 6 h after calcium switch. The cells expressing the PALS1 wild type as well as two mutants, PALS1 V37G and PALS1 L27C, showed full recovery of tight and adherens junctions, as shown by ZO-1 and E-cadherin staining (Figure 2C, c, d, and f), with the exogenously expressed PALS1 at the tight junctions; PATJ is also correctly localized to the junctions (Supplemental Figure 2) in these cells. In cells expressing the PALS1 L27N, L379P, and delC mutant, the exogenous PALS1 is localized diffusely in the cells, and the reformation of tight and adherens junctions is similar to the PALS1 KD cells transfected with empty vector (Figure 2C, b, e, g, and h). Although PATJ expression is rescued in the L379P and delC mutants, it is also mislocalized in these cell lines (Supplemental Figure 2). The inability of the L27N domain deletion to rescue junction formation confirms the important role of PATJ in cell–cell contacts and cell polarity. These results also indicate the importance of the C terminus of PALS1 in these processes.

There Is Less E-Cadherin on the Surface of the PALS1 KD Cells

We were very interested in the possible link between PALS1 and E-cadherin, so we continued to study E-cadherin in the PALS1 KD#1 cells. We performed a reverse transcription-polymerase chain reaction experiment and found that the mRNA level of E-cadherin was not changed in the PALS1 KD cells (data not shown), and Western blot results showed that the PALS1 KD cells had similar E-cadherin protein levels compared with wild-type MDCK cells (data not shown; Figure 6A). In Figures 1 and 2, E-cadherin was shown to be partially missing from the cell–cell contact sites; therefore, we used an antibody that recognized the E-cadherin extracellular domain to examine whether it is on the surface of the PALS1 KD cells. MDCKII cells and the PALS1 KD#1 cells were grown to confluence on Transwell filters for 3 d and fixed. Then, the E-cadherin antibody was applied to either the top well or the bottom well without permeabilizing the cells. The MDCKII cells revealed strong E-cadherin staining when the antibody was added to the bottom well, whereas the E-cadherin signal was weak when the antibody was added to the top well due to the intact tight junctions (Figure 3A). In contrast, the PALS1 KD cells showed similar staining when antibody was added to the top or bottom well due to defective tight junctions, and overall the E-cadherin signal was much weaker than control cells (Figure 3A).

We then combined this assay with the calcium switch experiment. The MDCKII cells and the PALS1 KD#1 cells were fixed at 0, 1, and 3 h after calcium readdition, and the E-cadherin extracellular antibody was applied to the cut filters without permeabilization. The MDCKII cells had a significant portion of E-cadherin remaining on the cell sur-

Figure 2. Reexpression of wild-type PALS1 and two PALS1 mutants rescue defects in junction formation. (A) The domain structure of PALS1. The two point mutations are marked in the diagram, and various interactions and the intramolecular SH3–GUK interactions are depicted with arrows. The delC truncation mutant lacks the C-terminal residues aa 346–675. (B) The MDCKII wild-type cells, the PALS1 KD#1 cells, and the PALS1 KD#1 cells expressing the empty vector and various PALS1 constructs were lysed, and the lysates were blotted with the antibodies indicated to the left. (C) Cells grown to confluence on polyester filters were incubated in low calcium medium overnight and then transferred to normal calcium medium. Six hours later, cells were fixed, permeabilized with 1% SDS, and stained with the antibodies indicated.

face in the low calcium medium, and E-cadherin quickly translocated to the plasma membranes after the cells were transferred to the normal calcium medium (Figure 3B). In contrast, the PALS1 KD cells revealed very weak E-cadherin staining signals at all three time points, indicating that E-cadherin is not delivered to the cell surface as effectively as in control cells (Figure 3B).

We also performed surface biotinylation experiment to quantify the level of E-cadherin on the plasma membrane. The MDCKII cells and the PALS1 KD#1 cells were incubated with biotin at 0, 3, and 6 h after being transferred from low calcium medium to normal calcium medium. Biotinylated proteins were collected by streptavidin bead pull-down, and subsequent E-cadherin blotting showed that there was significantly less surface E-cadherin in the KD cells at all three time points compared with the wild-type cells (Figure 3C). The results of the E-cadherin biotinylation in Figure 3C as well as that of two other independent experiments are quantified in Figure 3D. It is interesting to note that E-cadherin

surface expression is reduced but not eliminated in the PALS1 KD cells. This may explain our finding that adherens junctions can be seen between wild-type cells and PALS1 KD cells but are not seen between PALS1 KD cells (Figure 1C). We think that normal cadherin surface expression in wild-type cells may be able to stabilize the reduced surface E-cadherin on an adjacent PALS1 KD cell, but there is insufficient surface E-cadherin between adjacent KD cells to form detectable adherens junctions detected by immunostaining.

E-Cadherin Is Retained in Intracellular Puncta in the PALS1 KD Cells

Because the total protein level of E-cadherin is not changed and there is less E-cadherin on the surface of the PALS1 KD cells, we speculated that E-cadherin is accumulated intracellularly. However, initial immunofluorescence results did not reveal that (Figure 1B). We thought it could be due to the

Figure 3. Less E-cadherin is on the surface of PALS1 KD cells. (A) MDCKII wild-type cells and the PALS1 KD cells were grown to confluence on polyester filters and fixed. An E-cadherin antibody that recognizes the extracellular domain of E-cadherin was applied to either the top or bottom well without permeabilization. (B) The MDCKII cells and the PALS1 KD cells were grown to confluence on polyester filters, and the calcium switch experiment was performed with the cells fixed at different times after the switch ($t = 0, 1, \text{ or } 3 \text{ h}$). The E-cadherin antibody that recognizes the extracellular domain of E-cadherin was applied to the cut filters without permeabilization. (C) Cells grown to confluence on polyester filters were incubated in low calcium medium overnight and were biotinylated before or 3 or 6 h after being transferred to normal calcium medium. Cells were lysed in radioimmunoprecipitation assay buffer, and biotinylated proteins were precipitated by streptavidin beads. Five percent of lysate and the biotinylated portion were blotted for E-cadherin and

permeabilization method we used, because the PALS1 antibody only recognizes PALS1 after the cells are permeabilized with 1% SDS and SDS could have removed staining of some of the intracellular structures. Accordingly we changed the permeabilization conditions and also increased the concentration of the E-cadherin antibody. In Figure 4A, a, we grew the PALS1 KD pool cells on filters and fixed and permeabilized the cells with 1:1 acetone/methanol 3 h after calcium switch. The cells expressing the PALS1 KD SiRNA plasmid were identified by a lack of PALS1 staining, and in these cells, E-cadherin was seen to be accumulated in puncta-like structures. In Figure 4A, b, the same cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton, and similar result was seen as in panel a with the costaining of E-cadherin and PATJ. All the rest of the immunofluorescence experiments in Figures 4 and 5 were performed as in Figure 4A, b.

Next, we checked the E-cadherin localization in the PALS1 KD#1 cells. MDCKII cells and the PALS1 KD#1 cells were fixed 3 h after calcium switch and costained for E-cadherin and other adherens junction proteins, including actin, Lin-7, and Ncl2 (Figure 4B, a–c). Compared with the junctional localization in wild-type cells, the adherens junction proteins were diffuse in the PALS1 KD cells, and the actin staining showed clearly that the cells were not making cell-cell contacts; E-cadherin was in puncta rather than at the junctions as well (Figure 4B).

We were interested in the formation of these E-cadherin-positive puncta during cell polarization, so we stained for E-cadherin in the MDCKII cells and the PALS1 KD#1 cells at different times after calcium switch by using the alternate staining conditions. The staining revealed an intracellular pool of E-cadherin that quickly translocated to the cell-cell contact sites in the wild-type cells upon the transition to normal calcium medium (Figure 4C, a). On the contrary, the intracellular E-cadherin puncta were seen at the 0-h time point in the PALS1 KD cells, and no obvious translocation was seen after 3 h (Figure 4C, a). The confocal image in Figure 4D, b provides a magnified view of the E-cadherin localization (Figure 4C, b). It is worth noting that the E-cadherin puncta in the PALS1 KD cells are localized in the cell periphery, where cell-cell contacts and junctions are waiting to form.

E-Cadherin Is Not Effectively Exocytosed to the Cell Surface

E-cadherin is a transmembrane protein, and it is exocytosed to the cell surface in vesicles. After delivery to the plasma membranes, E-cadherin is trafficked to and from the cell surface by exocytic and multiple endocytic pathways (Bryant and Stow, 2004). It was important to determine whether the intracellular E-cadherin is the result of disrupted exocytosis or disrupted recycling, and to this end, we performed a pulse-chase experiment with surface biotinylation. The MDCKII cells and the PALS1 KD#1 cells were pulse labeled with [^{35}S]methionine for 2 h and after being chased for 0, 1, 2, or 3 h, the cells were incubated with biotin. The cells were then lysed and sequentially precipitated with immobilized anti-E-cadherin antibody and streptavidin beads. The bands in the autoradiogram in Figure 5A reflect the amount of E-cadherin that reached cell surface in the indicated length of time, and it can be seen that there was less E-cadherin exocytosed to the plasma membrane in the PALS1 KD cells at any time point.

CRB3. Two duplicated wells of cells were immunoprecipitated by the anti-E-cadherin antibody to show the total of E-cadherin. (D) Quantification of C. Error bars represent SD/sqrt(n). t test: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$; $n = 3$ independent experiments.

Figure 4. E-cadherin is retained in the intracellular puncta in the PALS1 KD cells. (A) MDCKII cells transfected with the PALS1 SiRNA plasmid were fixed 3 h after calcium switch with acetone-methanol (a) or paraformaldehyde and permeabilized with 0.1% Triton (b). They were immunostained with the antibodies indicated subsequently. The E-cadherin staining image and the PALS1 staining image (a) or PATJ staining image (b) were merged with the DAPI staining image to show the E-cadherin-positive puncta in the PALS1 KD cells. Cells with PALS1 knocked down are indicated with arrowheads, and cells still expressing PALS1 are indicated with arrows. (B) The MDCKII cells and the PALS1 KD cells were fixed 3 h after calcium switch and stained for E-cadherin and several adherens junction-localized proteins, including actin (a), Lin-7 (b), and Ncl2 (c). (C) Localization of E-cadherin at the times after calcium switch ($t = 0$ h, 15 min, 30 min, 1 h, 2 h, or 3 h) was revealed by immunostaining (a). Magnified view of the 3-h time point by using confocal microscopy (b).

Figure 5. E-cadherin is not effectively exocytosed to the plasma membrane. (A) MDCKII wild-type cells and the PALS1 KD cells were pulse labeled with [35 S]methionine for 2 h and chased for 0, 1, 2, or 3 h. Cells were biotinylated, and the E-cadherin reaching the surface in the period of the chase was revealed by autoradiation after sequential precipitation by the anti-E-cadherin antibody and the streptavidin beads. One duplicate of each cell line was lysed right after the pulse label and immunoprecipitated by the anti-E-cadherin antibody to show the total level; that panel (right) was from a shorter exposure to avoid saturated images. The results are representative of two experiments. Quantification of the intensity of bands is shown below. (B) Cells were incubated with normal calcium medium containing 10% rr1 anti-E-cadherin mouse ascites for 3 h directly (a) or cultured in low calcium medium overnight before the incubation (b). Total E-cadherin was shown by the immunostaining with a rat E-cadherin antibody, and the coupled rr1 mouse antibody was revealed by a fluorochrome-bound secondary antibody. Note the overlap between the total E-cadherin signal and the rr1 signal in the merge picture (arrows), and green vesicle-like structures in the PALS1 KD cells (arrowheads). (C) The PALS1 KD cells were fixed 3 h after calcium switch and costained for E-cadherin (arrowheads) and Rab11 (a), Transferrin receptor (b), EEA1 (c), GM130 (d), and γ -adaptin (e) (arrows).

To further confirm that E-cadherin exocytosis was misregulated, we conducted an E-cadherin endocytosis assay. We added E-cadherin antibody to the medium at a final concentration of 10%, and incubated it with the MDCKII cells or the PALS1 KD#1 cells for 3 h at 37°C. The mouse antibody rr1 recognizes the extracellular domains of E-cadherin, and an anti-mouse secondary antibody was added after the cells were fixed and permeabilized to reveal the rr1 signal. At the same time, total E-cadherin was detected by staining with a rat E-cadherin antibody. As is shown in Figure 5B, a, the rr1 antibody was coupled to the E-cadherin on the cell surface, and a portion of it was endocytosed into the cells highlighting intracellular puncta (Figure 5B, a, arrows). No significant difference was seen in rr1 endocytosis between the MDCKII cells and the PALS1 KD cells, and although there were more prominent intracellular puncta in the PALS1 KD cells revealed by the total E-cadherin immunofluorescence, they only partially overlap with the rr1 signals (Figure 5B, a, arrowheads).

We then combined the rr1 endocytosis and calcium switch assays with the rr1 mouse antibody added at the time when the cells were transferred from low to normal calcium medium. After 3 h of incubation at 37°C, the MDCKII cells showed similar rr1 endocytosis as in the previous experiment (Figure 5B, b, arrows). In contrast, the PALS1 KD cells had very weak rr1 signals due to the lack of E-cadherin on the cell surface, whereas the intracellular puncta were prominently shown by the total E-cadherin staining (Figure 5B, b, arrowheads). These results support our previous conclusion that these E-cadherin-positive puncta in the PALS1 KD cells cannot exocytose to the surface and bind the antibody present in the extracellular media.

To further examine the identity of the E-cadherin-positive puncta in the PALS1 KD cells, we costained E-cadherin and several intracellular organelle markers, including the recycling endosome marker Rab11 and TfR, the early endosome marker EEA1, the *cis*-Golgi marker GM130 and the *trans*-Golgi network (TGN) marker γ -adaptin (Figure 5C, a–e). We did not see colocalization of E-cadherin with any of these markers.

The Exocyst Is Mislocalized in the PALS1 Knockdown Cells

Because there seemed to be a defect in E-cadherin exocytosis, we decided to study the exocyst complex because there is evidence that this complex is involved in the regulation of E-cadherin exocytosis (Grindstaff *et al.*, 1998; Shipitsin and Feig, 2004; Yeaman *et al.*, 2004; Langevin *et al.*, 2005). We used two independent assays to study the localization of the exocyst complex in the PALS1 KD cells. First, we performed an Opti-Prep fractionation experiment. In Figure 6A, a, the top panel shows the distribution of proteins of the MDCKII cells, whereas the lower panel shows that of the PALS1 KD#1 cells. TfR, Rab11, RalA, and Akt were blotted in both panels, and no significant difference was seen in the distribution of these proteins. When we examined E-cadherin and the exocyst component Sec8, we found a shift of peaks of these two proteins in the PALS1 KD cells. Moreover, E-cadherin and Sec8 cofractionate in fraction 6 and 7 in the MDCKII cells, consistent with previous report (Yeaman, 2003), whereas in the PALS1 KD cells, Sec8 is missing from peak 6 and 7 and the peak of E-cadherin is shifted to fraction 8 (Figure 6A, b). These changes in the distribution of E-cadherin and Sec8 were rescued in the PALS1 KD cells expressing wild-type PALS1 (Figure 6A, b). We also examined Sec8 localization by immunofluorescence. Sec8 is localized to the junctions in the MDCKII cells, whereas in the PALS1 KD cells, only fragmented spots can be seen at the cell–cell contact sites (Figure 6B, a). The junctional localiza-

tion is rescued in PALS1 KD cells expressing PALS1 wild type, the V37G mutant, or the L27C mutant; and consistent with the result of junction rescue, Sec8 is mislocalized in PALS1 KD cells transfected with the empty vector, the PALS1 L27N mutant, the L379P mutant, or the delC mutant (Figure 6B, b). These results indicate that the depletion of PALS1 disrupts the localization of the exocyst complex, whereas the rescue cell lines reveal correct Sec8 localization and rescue of junction formation.

DISCUSSION

We report here a further characterization of the tight junction associated polarity protein PALS1, by using a stable canine-specific SiRNA that depleted PALS1 more thoroughly than our previous work (Straight *et al.*, 2004). This canine-directed SiRNA lead to a more severe cellular deficit than we observed previously, including a defect in adherens junction formation. We were able to rescue this adherens junction defect with wild-type PALS1 and to complete a structure–function analysis by using mutant PALS1 constructs to rescue the defect. Consistent with the previous report, the loss of PALS1 resulted in a corresponding loss of expression of PATJ but not CRB3 (Straight *et al.*, 2004). The reintroduction of exogenous PALS1 recovered the expression of PATJ, except for the PALS1 L27N mutant (Figure 2B). We have tested the interacting ability of all the PALS1 mutants used in the rescue study, and the L27N mutant is the only mutant that is defective in binding PATJ. We hypothesize that when PALS1 is reduced the unbound PATJ is destabilized and degraded. The results obtained from the rescue cell lines support this hypothesis, because PATJ expression was rescued in accordance with the PATJ-binding ability of the PALS1 mutants used.

It is important to distinguish the role of PALS1 and PATJ in the process of junction formation. We suppose the defect in junction formation we saw is a direct effect of PALS1 depletion rather than the subsequent result of PATJ loss, because the L379P cell line and the delC cell line had PATJ expressed but did not rescue the defects (Figure 2C). However, the recovered PATJ is mislocalized in the L379P cell line and the delC cell line (Supplemental Figure 2). Our laboratory has done a PATJ knockdown and has not observed adherens junction disruption (Shin *et al.*, 2005). A separate PATJ knockdown study in Caco2 cells reported that PATJ knockdown leads to the mislocalization of PALS1, but no adherens junction defect followed the PATJ depletion and PALS1 mislocalization (Michel *et al.*, 2005). Thus, loss of PATJ alone does not seem to lead to adherens junction defects. However, that PALS1 that cannot bind PATJ (PALS1 L27N) cannot rescue the PALS1 KD cells suggests that loss of PATJ may contribute to the adherens junction defect we observe in the PALS1 KD cells. It is also possible that the L27N of PALS1 has other functions in addition to binding to PATJ.

New insight from these studies reveals that PALS1 is involved in the regulation of E-cadherin trafficking. With the new PALS1 KD cell lines and a modified immunostaining protocol, we were able to observe the retention of E-cadherin puncta inside the PALS1 KD cells. This defect, together with the defect in tight junction formation was corrected in some of the rescue cell lines. PALS1 wild-type, V37G, and L27C mutants rescued both defects 6 h after calcium switch (Figure 2C). In contrast, the PALS1 L27N, L379P, and delC mutants failed to rescue either of the defects. These results have several implications. First, the function of the PALS1 C terminus has been largely unknown. Here, we show for the first time that the C-terminal SH3 and GUK domains are

Figure 6. The exocyst complex is mislocalized in the PALS1 KD cells. (A) MDCKII wild type and PALS1 KD cells were lysed, and the postnuclear lysates were fractionated in a 10–15–20–30% Opti-Prep. The fractions were loaded on the gel in the order of increased density and blotted for various proteins indicated to the left (a). Top, MDCKII cells. Bottom, PALS1 KD cells. PALS1 KD cells expressing the empty vector or mouse rescue wild-type PALS1 also were fractionated and together blotted for E-cadherin and Sec8 (b). (B) Sec8 is localized at the cell–cell contacts, whereas in the PALS1 KD cells Sec8 staining is disrupted (a). Sec8 was also stained in the PALS1 KD cells expressing the empty vector, the rescue wild-type mouse PALS1, and various PALS1 mutants (b).

important for PALS1 function. It will require further study to elucidate how the PALS1 C terminus is functioning and its interacting proteins. Second, the results of the V37G mutation were of interest to us. Although this mutant does rescue the phenotype at 6 h, we did note that junctional

rescue was delayed with this mutant if we looked at 3 h after calcium addition (data not shown). Nonetheless, this mutation that reduces the binding of Par6 to PALS1 in multiple studies (Wang *et al.*, 2004) has at best minor effects on PALS1 function. We think that the interaction of the PALS1 complex

with the Par complex is of importance but that there are redundant ways for the complexes to interact. These redundant interactions, including the direct interaction of CRB3 to Par6 (Lemmers *et al.*, 2004), can compensate for the lack of direct interactions between PALS1 and Par6.

PALS1 is localized to the tight junction in mammalian epithelial cells, and its importance in the formation of tight junctions has been established previously (Roh *et al.*, 2002; Straight *et al.*, 2004). E-cadherin is localized to a distinct membrane domain basal to tight junctions, and it is the major structural component of the adherens junctions. Our report is the first report to indicate that PALS1 has a role in adherens junction formation in mammalian epithelial cells, but results in *Drosophila* are instructive. *Drosophila* epithelial cells have an adhesive belt that encircles the cell just below the apical surface called the zonula adherens, where DE-cadherin and its interacting proteins Armadillo (*Drosophila* β -catenin) and $\Delta\alpha$ -catenin reside. The Sdt-dPATJ-CRB complex is localized to a distinct domain apical to the zonula adherens in the *Drosophila* epithelial cells called the subapical region. It is widely accepted that the Sdt-dPATJ-CRB complex regulates the formation of the *Drosophila* zonula adherens and E-cadherin localization (Grawe *et al.*, 1996; Tepass, 1996; Klebes and Knust, 2000), although how the protein complex interacts with components of the zonula adherens remains unclear (Knust and Bossinger, 2002). The observations in our study suggest that this mechanism is conserved, and PALS1 regulates the formation of adherens junctions in mammalian epithelial cells in addition to its role in tight junction formation.

The intracellular trafficking of E-cadherin is regulated by a variety of exocytic and endocytic machineries that can modulate adhesion (Bryant and Stow, 2004). After exiting the TGN, newly synthesized E-cadherin goes to the Rab11-positive recycling endosome (Lock and Stow, 2005). It is sorted to the basolateral membranes there, and a portion of E-cadherin undergoes constant recycling between the plasma membrane and the recycling endosome (Bryant and Stow, 2004). Using the E-cadherin extracellular antibody and the surface biotinylation assay, we demonstrated that there is lower proportion of total E-cadherin on the surface of the PALS1 KD cells, and with the pulse-chase experiment and the rr1 endocytosis assay, we showed that the newly synthesized E-cadherin is not effectively exocytosed to the plasma membrane (Figures 3 and 5, A and B). We were not able to identify the exact identity of the E-cadherin puncta in the PALS1 KD cells, because they do not colocalize with any of the organelle markers examined (Figure 5C). We found that those E-cadherin puncta are distributed in the cell periphery and at the site of cell-cell contacts, and this distribution is especially obvious when cells are confluent grown on the filters and tightly packed together (Figure 4C). We hypothesize that in the PALS1 KD cells, the E-cadherin exocytic vesicles are correctly sorted to the cell surface from the recycling endosome, but the depletion of PALS1 either abolishes the final cue of targeting or disrupts their fusion with the plasma membrane, which leads to the accumulation of E-cadherin puncta in the cell periphery.

We also found that the exocyst complex was mislocalized in the PALS1 KD cells by using two independent methods (Figure 6). The exocyst is localized to the tight junctions in polarized MDCK cells (Grindstaff *et al.*, 1998; Yeaman *et al.*, 2004), and we have found that the exocyst component Sec8 colocalized with PALS1 during calcium switch experiments (data not shown). It has been reported that the exocyst complex is involved in the regulation of E-cadherin exocytosis in both mammalian cells and in *Drosophila* (Shipitsin

and Feig, 2004; Langevin *et al.*, 2005). Alternatively, exocyst mediates the targeting of intracellular vesicles to the specific sites of plasma membranes (Hsu *et al.*, 1999), which is consistent with our hypothesis that the docking or fusion step of E-cadherin trafficking is disrupted in the PALS1 KD cells. In polarizing MDCK cells, Sec8 and Sec6 are recruited from the cytosol to sites of cell-cell contact upon initiation of E-cadherin-dependent cell-cell adhesion, and Sec8 can be immunoprecipitated with E-cadherin (Grindstaff *et al.*, 1998; Yeaman *et al.*, 2004). Thus, we hypothesize that defects seen in the trafficking of E-cadherin in PALS1 KD cells are related to concomitant defects in exocyst function. However, despite repeated attempts we were not able to detect a physical interaction between PALS1 and components of the exocyst. We also think that the relationship between E-cadherin and exocyst is nonlinear, and there are complicated interactions and feedback loops between the two. When PALS1 is depleted, the interactions and feedbacks are disrupted, and both E-cadherin and exocyst are mislocalized. However, further investigations are needed to study the interplay among polarity proteins, the exocyst, and E-cadherin.

ACKNOWLEDGMENTS

We thank Dr. Alan Saltiel for reagents and support. This work was supported by National Institutes of Health Grants DK-58208 and DK-39255. This work used the Morphology and Image Analysis Core of the Michigan Diabetes Research and Training Center funded by NIH5P60 DK20572 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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