Loss of PALS1 Expression Leads to Tight Junction and Polarity Defects

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Prior work in our laboratory established a connection between the PALS1/PATJ/CRB3 and Par6/Par3/aPKC protein complexes at the tight junction of mammalian epithelial cells. Utilizing a stable small interfering RNA expression system, we have markedly reduced expression of the tight junction-associated protein PALS1 in MDCKII cells. The loss of PALS1 resulted in a corresponding loss of expression of PATJ, a known binding partner of PALS1, but had no effect on the expression of CRB3. However, the absence of PALS1 and PATJ expression did result in the decreased association of CRB3 with members of the Par6/Par3/aPKC protein complex. The consequences of the loss of PALS1 and PATJ were exhibited by a delay in the polarization of MDCKII monolayers after calcium switch, a decrease in the transepithelial electrical resistance, and by the inability of these cells to form luminal cysts when grown in a collagen gel matrix. These defects in polarity determination may be the result of the lack of recruitment of aPKC to the tight junction in PALS1-deficient cells, as observed by confocal microscopy, and subsequent alterations in downstream signaling events.

INTRODUCTION

The establishment of apical-basal polarity in epithelial cells is dependent on the complex interplay of a number of molecules and macromolecular complexes. There has been recent insight into the mechanisms behind these processes and the related function of tight junction determination, by several laboratories. Among the proteins involved are PALS1 (Proteins Associated with Lin Seven 1), PATJ (PALS1-Associated Tight Junction protein), CRB3 (Crumbs 3), aPKC (atypical Protein Kinase C), and Par6 and Par3 (Partition defective proteins). These proteins form two macromolecular complexes: PALS1/PATJ/CRB3 and Par6/Par3/aPKC. The interrelationships between these molecules are evolutionarily conserved in organisms as diverse as nematodes, fruit flies, and vertebrates, and a paradigm for the establishment of polarity in epithelia has emerged based on experimentation with these organisms.

Prior work in our laboratory and others determined that PALS1, PATJ, and CRB3 were bound in a macromolecular complex that localized to the tight junction of mammalian epithelial cells (Roh *et al.*, 2002b; Makarova *et al.*, 2003). PALS1, a membrane-associated guanylate kinase protein, contains a single PDZ domain that binds to the carboxyl-terminal tail of the transmembrane protein CRB3 (Makarova *et al.*, 2003; Roh *et al.*, 2003). PALS1 also binds to the amino-terminus of PATJ through L27 domain interactions, thereby acting as an adaptor between CRB3 and PATJ (Lemmers *et al.*, 2002; Roh *et al.*, 2002b). In turn, the sixth and eighth PDZ domains of PATJ bind to the tight junction-associated proteins ZO-3 and claudin-1, respectively (Roh *et al.*, 2002a). In

Drosophila, the orthologues of these proteins, Crumbs (CRB3), Stardust (PALS1), and Discs Lost (PATJ), also form a complex that localizes to the subapical region (also called the marginal zone), a site that is positionally analogous to the tight junction in mammalian epithelia (Klebes and Knust, 2000; Bachmann *et al.*, 2001; Hong *et al.*, 2001; Medina *et al.*, 2002; Tepass, 2002). The relationships between these molecules have also been reinforced by genetic studies, which have shown these proteins to be indispensable for the proper formation of cell junctions and the establishment of apical-basal polarity in *Drosophila* epithelia (Tepass and Knust, 1993; Grawe *et al.*, 1996; Klebes and Knust, 2000; Bachmann *et al.*, 2001; Hong *et al.*, 2001).

Our laboratory recently established a link between the PALS1/PATJ/CRB3 complex and the Par6/Par3/aPKC complex in mammalian epithelia, in that the amino terminus of PALS1 binds directly to the PDZ domain of Par6 (Hurd *et al.*, 2003). Furthermore, disruptions of either complex interfered with recruitment of the other to the tight junction. Again, the *Drosophila* orthologues of these proteins, D-Par6 (Par6), Bazooka (Par3), and DaPKC (aPKC), have proven important in establishing asymmetry in epithelia as well as neuroblasts during embryogenesis (Muller and Wieschaus, 1996; Wodarz *et al.*, 2000; Petronczik and Knoblich, 2001; Knust and Bossinger, 2002). In addition, the Par6/Par3/aPKC complex has been shown to regulate the assembly of cellular junctions, particularly tight junctions, and the kinase activity of aPKC was required for this process (Yamanaka *et al.*, 2001; Suzuki *et al.*, 2002). Finally, recent studies on *Drosophila* embryonic epithelia and photoreceptor morphogenesis have provided genetic evidence for interaction between the Crumbs and D-Par6 complexes (Bilder *et al.*, 2003; Nam and Choi, 2003; Tanentzapf and Tepass, 2003).

However, many questions remain regarding the specific interactions between these proteins and their relative importance to the process of polarity determination. Recent dis-

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coveries regarding the mechanism and application of small interfering RNA (siRNA) have now made it possible to specifically target mammalian genes for silencing (for review see McManus and Sharp, 2002). Using the expression of PALS1-specific siRNA to suppress the expression of PALS1 in MDCKII cells, we have furthered our studies on the role of PALS1 in determining epithelial cell polarity.

MATERIALS AND METHODS

DNA Constructs

To create the siRNA constructs, seven 19-base pair sites within murine PALS1 were chosen, and pairs of complimentary oligonucleotides were synthesized by Invitrogen Custom Primers (Carlsbad, CA). The sequences chosen were checked for significant homology to other genes in the murine genome database and none was found. The sense and antisense sequences were separated by a nine-base pair loop region, and each oligonucleotide was terminated with restriction endonuclease half-sites. The sequences of the oligonucleotides follow: site 1: 5'-GATCCCGAAGGACAAGAAC TAAC TTTCAAGAGAAGTTAGTTCTTGCTCTCCTTTT TTGGA A A A 3'; 5'-AGCTTTTCCAAAAAAGGAAGAACAAAG AACTA ACTTCTCTTGAAGTTAGTTCTTGCTCTCCTCG G 3'; site 2: 5'-GATCCCGGAGACGAAGTTCTGGAGATTCAA GAGATCTCCAGAACTTCGTCTCTTTTGGAA A 3'; 5'-AGCTTTTCCAAAAAAGGAGACGAAGTTCTGGGAGTC TCTTGAATCTCCAGAACTTCGTCTCCGG 3'; site 3: 5'-GATC CCGGAGATATACTTCATGTGATTCAAGAGATCACAT GAAGTATATCTCTTTTGGAA A 3'; 5'-AGCTTTTCCAA AAAAGGAGATATACTTCATGTGATCTCTTGAATCA CATGAAGTATATCTCCGG 3'; site 4: 5'-GATCCCGGGAAGC CATGAAGCAAACTTGAAGAGATTGCTTCATGGCT TCCCTTTTGGAA A 3'; 5'-AGCTTTTCCAAAAAAGGGA AGCCATGAAGCAAACTCTTGAAGTTTGCTTCATG GCTTCCCGG 3'; site 5: 5'-GATCCCGGAGCCAGAGAAATCA GGATTCAAGAGATCCTGATTTCTCTGGCTCCTTTTTT GGAA A 3'; 5'-AGCTTTTCCAAAAAAGGAGCCAGAGAA A TCAGATCTCTTGAATCTGATTTCTCTGGCTCCCGG 3'; site 6: 5'-GATCCCGGTGAAGGAAAGGACTGTTTTCAAGA GAAACAGTCTTTCTTCACTTTTGGAA A 3'; 5'-AG CTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC TTGAAACAGTCTTTCTTCACTTCCCGG 3'; site 7: 5'-GATCC CGGTCCATTAGGCAATTTGTTTCAAGAGAAACAAATT GCCTAATGACCTTTTTTGGAA A 3'; 5'-AGCTTTTCCAA AAAAGGTCCATTAGGCAATTTGTTTCTTGAACAA A ATTGCCTAATGACCGG 3'. After annealing the complimentary oligonucleotides, the dimers were ligated into the pre-cut pSilencer 2.0-U6 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.

The identity of siRNA constructs expressed in resulting cell lines was determined by PCR using a 5' primer from within the pSilencer vector (either the SP6 or the T7) and 3' primers specific to each oligonucleotide.

Cell Culture and Transfection

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. To create the cell lines stably expressing siRNA constructs, MDCKII cells were transfected with 5 μ g of plasmid DNA and 0.5 mg pSV2NEO using FuGENE 6 reagent (Roche, Indianapolis, IN). Initially, all seven plasmids were used in combination for transfection (~0.7 μ g each), and later only plasmids 1 and 3 were used for transfection to create a second group of cell lines. After selection with 500 μ g active G418/ml (Invitrogen) for 14 days, surviving clones were isolated for the generation of cell lines.

For immunostaining, MDCKII cell lines were seeded at confluence onto 24-mm Transwell clear polyester filters (Corning Inc., Corning, NY) in low-calcium medium (5% dialyzed fetal bovine serum, 5 μ M Ca^{2+}). After allowing the cells to adhere to the substrate overnight, the nonadherent 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.

MDCKII cell cysts were created in collagen gels as described (Pollack *et al.*, 1998; O'Brien *et al.*, 2001; Roh *et al.*, 2003). Briefly, a single-cell suspension was mixed with ice-cold rat's tail collagen type I (Sigma, St. Louis, MO), layered onto 12-mm Transwell clear filters, and allowed to solidify at 37°C. Pre-warmed growth medium was added above and below the gel, and the cysts were allowed to grow for up to 10 days before they were prepared for immunostaining.

Calcium Switch 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 (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 times afterward (0, 3, 6, or 29 h).

A similar experiment was performed using 12-mm Transwell filters to measure transepithelial electrical resistance (TER). 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. Before 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 SD from the means ($n = 3$) for each time point were plotted using Microsoft Excel (Redmond, WA). After the final TER measurement, the cells were prepared as described below for immunostaining to confirm the expression level of PALS1.

Antibodies

LIN7, PALS1, PATJ, and CRB3-specific antisera were generated in rabbits and affinity-purified as previously described (Borg *et al.*, 1998; Roh *et al.*, 2002b; Makarova *et al.*, 2003). Mouse anti-ZO-1, rabbit antioccludin, and rabbit anti-claudin-1 antibodies were purchased from Zymed (San Francisco, CA). GP135 antibody was a gift from George Ojakian at the SUNY Health Science Center (Brooklyn, NY). Antibodies to Par3 and PKC ζ were obtained from Upstate (Lake Placid, NY). Fluorochrome-conjugated antibodies used for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated antibodies used in immunoblotting were obtained from Amersham Biosciences (Buckinghamshire, UK).

CRB3 Peptide Beads

CRB3 peptide-coupled agarose beads were created using the SulfoLink Coupling Gel kit (Pierce Biochemistry, Rockford, IL) and were linked via a terminal cysteine residue added to a peptide corresponding to the carboxyl-terminal 18 amino acids of CRB3 (WT: NH₃-CARVPPTPNLKLPEERLI-COOH) or the same sequence missing the terminal ERLI motif (Δ ERLI: NH₃-CARVPPTPNLKLPE-COOH). The CRB3 peptides were synthesized at the University of Michigan Protein Structure Facility.

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 MgCl₂, 1 mM EGTA, 1 mM phenyl-methylsulfonyl fluoride, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin and phosphatase inhibitor cocktail [Sigma]) and cleared by centrifugation at 14,000 $\times$ 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 the CRB3 peptide bead pull-down assays, 20 μ L of 50% slurry of CRB3 peptide beads were added to 200 μ L cell lysate and incubated overnight at 4°C. The beads were washed three times with ice-cold HNTG (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) and resuspended in LDS loading buffer.

For immunoprecipitation, 1–5 ml of antibody was mixed with 200 ml lysate and 50 ml of 50% slurry of protein A-Sepharose beads (Zymed), 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 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, followed by incubation 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, Boston, MA).

Immunostaining and Confocal Microscopy

Cells grown on Transwell filters were cut from the support with a scalpel, washed with PBS, 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 (GS/PBS) for 1 h. The filters were then incubated with primary antibodies in GS/PBS for 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.

Figure 1. siRNA oligonucleotide sites in PALS1. The top line in A represents the 2835 base pair mRNA of murine PALS1. The open triangle represents the start codon and the solid triangle represents the stop. The numbered lines above the mRNA indicate the approximate location of the sequence chosen for the siRNA constructs 1–7. The diagram below represents the domain structure of the murine PALS1 protein (675 amino acids), showing the two L27 domains, L27N and L27C, a PDZ domain, a Src-homology (SH3) domain and an inactive guanylate kinase (GuK) domain. The mRNA and the protein are drawn approximately to scale, so that the relative location of the siRNA oligonucleotides corresponds to the region of the PALS1 protein below. Note that the site 6 oligonucleotide spans the stop codon, and site 7 is located in the 3' untranslated region of the mRNA. (B) The sequences of the complementary oligonucleotides generated for site 1, showing the 19-base pair sense and antisense sequences corresponding to murine PALS1, the 9-base pair linker sequence used in all the oligonucleotides, the RNA polymerase III termination sequence, and the restriction enzyme half-sites used for ligation into the pSilencer plasmid.

Finally, filters were washed with PBS and mounted onto glass slides using ProLong antifade reagent (Molecular Probes).

For the staining of cysts, collagen gels were removed from the filter supports, washed with PBS, and incubated with 100 U/ml collagenase VII (Sigma) for 10–30 min at 37°C to partially dissolve the collagen. Subsequently, the gel-embedded cysts were fixed in 4% paraformaldehyde/PBS for 1 h, permeabilized in 0.1% Triton X-100/PBS for 30 min, and then blocked with GS/PBS for 1 h. The cysts were then incubated with primary antibodies in GS/PBS for 2 days at 4°C under constant agitation. After washing extensively with GS/PBS, fluorochrome-conjugated secondary antibodies in GS/PBS were added overnight at 4°C. Phalloidin-rhodamine (Sigma) was added to label actin filaments, as necessary. Finally, cysts were washed extensively in PBS and mounted onto glass slides using ProLong.

All images were obtained using a Zeiss LSM 510 Axiovert 100M inverted confocal microscope (Thornwood, NY) at the University of Michigan Morphology and Image Analysis Laboratory and prepared for publication with Zeiss LSM 5 Image Browser software and Adobe Photoshop v5.5 (San Jose, CA).

RESULTS

Figure 1A shows a diagram of the murine PALS1 mRNA and protein that indicates the location of the oligonucleotides chosen to create seven PALS1-specific siRNA expression constructs, and Figure 1B depicts the site 1-specific complementary oligonucleotides that were ligated into the plasmid to create the hairpin siRNA. Initially, all seven siRNA constructs we created were transfected simultaneously into MDCKII cells with a selectable marker, and individual clones were isolated. These initial cell lines were examined by Western blot to determine the level of PALS1 suppression and by PCR to determine which of the siRNA constructs were being expressed. The greatest suppression of PALS1 expression was observed in cells expressing siRNA derived from oligonucleotides 1 and 3 (unpublished data), which targeted the PALS1 mRNA within the L27C and SH3 domains respectively (Figure 1A), and more cell

lines were obtained after transfection with only these two constructs. The apparent failure of some of the siRNA constructs to suppress PALS1 expression in MDCKII cells may have been due to differences between the murine and canine PALS1 mRNA sequences. The data presented in the following figures was collected utilizing the three MDCKII cell lines showing the greatest suppression of PALS1 expression and were obtained from both the first (siRNA 2) and second (siRNA 1 and siRNA 3) rounds of transfection. The expression of siRNA was determined to be stable in these cell lines, and persistent suppression of PALS1 was observed out to 20 or more passages (unpublished data).

To more carefully compare the expression of PALS1 and its binding partners, and the subsequent complexes formed in these cell lines, several pull-down and immunoblotting experiments were performed (Figure 2). Agarose beads coupled to CRB3-specific peptides corresponding to the carboxyl-terminal tail of CRB3 (WT), which binds the PDZ domain of PALS1 (Makarova *et al.*, 2003; Roh *et al.*, 2003) or to a peptide lacking the final four amino acids (Δ ERLI), which comprise the PALS1 binding motif (Makarova *et al.*, 2003; Roh *et al.*, 2003), were used to pull-down protein complexes. WT, but not Δ ERLI, peptide beads were able to pull-down PALS1 and several PALS1-associated proteins, including PATJ, LIN7, and PKC ζ from MDCKII cell lysates (Figure 2A). The WT beads also specifically pulled down Par6 and Par3 (unpublished data). Results similar to untransfected MDCKII cells were obtained with several transfected, G418-resistant cell lines that exhibited no PALS1 suppression (unpublished data). As expected, the CRB3 WT peptide-coupled beads pulled down significantly less PALS1 from the siRNA-expressing cell lines than from control MDCKII cells. Similarly PATJ and LIN7 were also reduced in binding to

Figure 2. PALS1 suppression results in loss of PATJ expression but not CRB3. (A) The results of CRB3 peptide-coupled bead pull-downs using wild-type (WT) and Δ ERLI beads. Lysates from untransfected MDCKII cells and three independent siRNA-expressing cell lines (siRNA 1, siRNA 2, and siRNA 3) were incubated with CRB3-peptide beads and washed with HNTG, and bound proteins were then separated on a 4–12% NuPAGE gel, transferred to nitrocellulose, and blotted with the antibodies directed against the proteins indicated to the right. Input lane shows 10% of the lysate used in the pull-downs. The bottom three panels show the same membrane blotted for PALS1, stripped, and subsequently blotted for PKC ζ . The arrows point to the specific bands recognized by the blotting antibodies. (B) The result of immunoprecipitation with antibodies to PATJ using the same lysates as in A. (C) The immunoblotting of lysates equivalent to those used as input in A. Antibodies were used to assess the expression of CRB3 and the tight junction proteins, occludin, and claudin-1. The numbers to the left of the figures indicate relative molecular weight.

the WT CRB3 peptide beads. Furthermore PKC ζ , which binds to PALS1 through interactions with Par6 (Hurd *et al.*, 2003), was also lost from the complex. Similar results were obtained when blotting for PKC λ (unpublished data). Interestingly, the absolute expression level of PATJ as well as PALS1 was significantly reduced in the siRNA-expressing cell lines, and the relative loss of expression correlated with the decrease in PALS1 expression (Figure 2A, INPUT lanes). In addition, the magnitude of suppression of PALS1 differed between the siRNA-expressing cell lines, with the greatest suppression of PALS1 in the siRNA 1 cell line and the least suppression in the siRNA 3 cell line. Unlike PALS1 and PATJ, however, the expression of LIN7 and PKC ζ was unchanged. The expression of Par6 and Par3 was also unchanged by suppression of PALS1 (unpublished data). Figure 2B shows the results of immunoprecipitation with antibodies directed against PATJ, which confirms the reduction in expression of both PATJ and PALS1 in the siRNA cell lines. Unlike PALS1 and PATJ, however, the expression of CRB3 in the siRNA cell lines was not significantly affected by PALS1 suppression, nor was there any change in the expression of the tight junction proteins occludin or claudin-1 (Figure 2C). Furthermore, neither occludin nor claudin-1 was found to be a significant constituent of the CRB3 complex using the CRB3 peptide bead pull-down assay (unpublished data).

The localization of PALS1 and PATJ in the siRNA-expressing cell lines was determined by immunostaining cell monolayers grown for several days at confluence on polyester filters (Figure 3). In MDCKII cells, PALS1, PATJ, and ZO-1 were localized to the tight junction and GP135 was found at the apical surface (Figure 3, A–C and M–O). In the siRNA-expressing cell lines the localization of these proteins was unchanged, but the relative amount of PALS1 and PATJ proteins was significantly reduced (Figure 3, D–L and P–X). Furthermore, the relative intensity of the immunostaining for PALS1 and PATJ supported the biochemical data in Figure 2A regarding the level of suppression of PALS1 and

loss of expression of PATJ in the siRNA-expressing cell lines: the qualitative differences in staining between the clones showed that siRNA 1 had the least amount of PALS1 and PATJ expression remaining, whereas siRNA 3 had the greatest amount. These cell lines were also immunostained for CRB3, which localized to the apical surface in both control MDCKII cells and the siRNA-expressing cell lines, and we observed no significant decrease in relative intensity between control cells and the siRNA-expressing cell lines (unpublished data).

Next we wished to examine the effect that the loss of PALS1 and PATJ had on the formation of cell-cell contacts in our cell lines. Figure 4 shows the results of a calcium switch experiment using cells grown to confluence on polyester filters, transferred overnight to low-calcium medium to disrupt cell-cell contacts and then placed back in normal growth medium. MDCKII cells rapidly reformed tight junctions, substantiated by the recruitment of the tight junction protein ZO-1 within 3 h after return to normal growth medium (Figure 4D). In contrast, there was a significant delay in the formation of tight junctions in the siRNA-expressing cell line siRNA 1 (Figure 4S), which had the greatest amount of suppression of PALS1 (Figure 2A). Spot-like nascent tight junctions did form in these cells within 3 h (Figure 4P), but did not develop into complete junctions until after 6 h and was complete within 29 h (Figure 4V). Despite the differences in ZO-1 recruitment and tight junction formation between MDCKII and siRNA1 cells, there was no significant difference in the rate of formation of adherens junctions, identified by the localization of E-cadherin to the lateral surface (unpublished data). In MDCKII cells, PALS1 was also recruited to the tight junction within 3 h and colocalized with ZO-1 (Figure 4, E and F). However, in siRNA 1, there was no observable recruitment of PALS1 to the spot-like junctions at 3–6 h (Figure 4, R and U), although PALS1 did colocalize with ZO-1 by 29 h (Figure 4, W and X). Only a small delay in the completion of tight junction formation was observed with the siRNA 2 cell line

Figure 3. PALS1 siRNA expression leads to the loss of PALS1 and PATJ from tight junctions. (A–X) Confocal microscopic X–Y sections near the tight junctions of MDCKII cell lines grown at confluence for 3 days on polyester filters, fixed, permeabilized, and immunostained with the primary antibodies indicated and appropriate secondary antibodies coupled to fluorochromes. Below each panel is the corresponding Z-section showing the apical-basolateral localization of the proteins. Immunostaining for the tight junction protein ZO-1 (A, D, G, and J), PALS1 (C, F, I, and L), the apical protein GP135 (M, P, S, and V) and PATJ (O, R, U, and X) are shown. Merged images (B, E, H, K, N, Q, T, and W) are shown between the individual channels. The cell lines shown here are untransfected MDCKII cells (A–C and M–O), and three siRNA-expressing lines, siRNA 1 (D–F and P–R), siRNA 2 (G–I and S–U), and siRNA 3 (J–L and V–X). All images were acquired using similar settings on the laser-scanning microscope. The scale bars in A–C are 20 μ m long, and the same scale is used throughout this figure.

(Figure 4B'), and no significant delay was observed in the siRNA 3 cell line (unpublished data), correlating with the increasing amount of PALS1 expressed in these cell lines. Furthermore, the PALS1 present in the siRNA 2 cells was recruited to the tight junctions as they were formed (Figure 4, C' and D'). These results indicated that only a small fraction of the PALS1 present in control cells is necessary for properly timed tight junction assembly as assessed with this assay.

In a similar calcium switch experiment, the TER was measured as a means of further assessing tight junction formation (Figure 5). In agreement with the immunofluorescence data in Figure 4, the TER of MDCKII cells rapidly increased to a maximum value of $\sim 310 \text{ } \Omega/\text{cm}^2$ within 6 h (Figure 5, $\circ$). The cell lines expressing PALS1 siRNA all showed significant quantitative and temporal retardation of their TER values, and the defect correlated with the degree of PALS1 suppression. In the case of the two most severely suppressed cell lines, siRNA 1 and siRNA 2, the TER remained significantly the value of control cells over the course of the experiment. At the conclusion of the experiment, the cells were immunostained for ZO-1 and claudin-1: ZO-1 localized to the tight junction, similar to the results shown in Figure 4, V and H', as did claudin-1, although the localization of claudin-1 in the siRNA1 cell line was somewhat less well defined than controls (unpublished data).

Finally, more calcium-switch assays were performed to assess the temporal localization of PKC ζ and Par3 (Figure 6). At $T = 0$ h, the localization of PKC ζ in siRNA 1 was often concentrated in a distinct perinuclear compartment which persisted beyond 6 h (Figure 6A, panels O and R), in contrast to the diffuse cytoplasmic staining in control cells (Figure 6A, panel C). In MDCKII cells, both PKC ζ and Par3 were recruited to tight junctions and colocalized with ZO-1 within 6 h after the return to normal growth medium (Figure 6A, panels D–F, and 6B, panels D–F). In contrast, PKC ζ localization to the tight junction in the siRNA 1 cell line was inhibited at 29 h (Figure 6A, panels S–U) and only weakly colocalized with ZO-1 even after 72 h, remaining somewhat diffusely localized throughout the cytoplasm or within strongly labeled punctuate structures (Figure 6A, panels

V–X). The decrease in PKC ζ recruitment was not due to a decrease in overall PKC ζ expression, which remained unchanged in the PALS1 siRNA-expressing cell lines compared with control cells (Figure 2A, INPUT lanes). In MDCKII cells, Par3 was rapidly recruited to the tight junction and colocalized with ZO-1 (Figure 6B, panels D–F). The localization of Par3 to the tight junction in the siRNA 1 cell line was complete and colocalized with ZO-1 only after 29 h (Figure 6B, panels P–R), corresponding to the apparent delay in tight junction formation in these cells. However, Par3 was also observed colocalizing with ZO-1 at spot-like nascent tight junctions present in siRNA-expressing cells at earlier time points (arrows in Figure 6B, panels M–O). Similar results for both PKC ζ and Par3 were obtained with the siRNA 2 and siRNA 3 cell lines (unpublished data).

The ability of these cell lines to properly determine polarity was examined by growing single cells within a collagen gel matrix for up to 10 days until luminal cysts developed in untransfected MDCKII cells (Figure 7). The development of single cells into multicellular cysts was monitored at several points by immunostaining for E-cadherin and ZO-1 to observe the formation of adherens junctions and tight junctions respectively. As expected, MDCKII cells grew rapidly within the collagen matrix, showing signs of polarization as early as 3–5 days (Figure 7, A and C): immunostaining of the lateral aspects of cells with E-cadherin and inner junctions with ZO-1. These cysts also quickly developed a single large luminal space (Figure 7, D, F, and H). In contrast, the siRNA 1 cell line displayed an aberrant morphology: the majority of cysts possessed no lumen (Figure 7, J, L, N, and P), although some did develop multiple mini-lumens (asterisks in Figure 7, Q and R). These morphologies were also observed in the siRNA 2 and siRNA 3 cell lines (unpublished data). Although tight junctions did not seem to form at any location and staining for ZO-1 remained diffuse (Figure 7, I, K, M, O, and Q), these cysts did not show any apparent defect in the localization of the cortical actin cytoskeleton (Figure 7, J, L, N, P, and R), nor in the formation of adherens junctions (E-cadherin staining in Figure 7, I, K, M, O, and Q). These results corroborate the observed delay in tight junction formation in the calcium switch assays (Figure 4).

Figure 4. Tight junction formation is delayed in PALS1-deficient cells during a calcium switch experiment. Cells grown to confluence on polyester filters were transferred to low-calcium medium overnight to dissociate cell-cell contacts. Normal growth medium was added, and at different times after the addition ($T = 0, 3, 6$, or 29 h) the cells were fixed, permeabilized, and immunostained with the primary antibodies indicated and appropriate secondary antibodies coupled to fluorochromes. (A–J') Confocal microscopic X-Y sections through the tight junctions of MDCKII cell lines. Immunostaining for the tight junction protein ZO-1 (A, D, G, J, M, P, S, V, Y, B', E', and H') and PALS1 (C, F, I, L, O, R, U, X, A', D', G', and J') are shown. Merged images (B, E, H, K, N, Q, T, W, Z, C', F' and I') are shown between the individual channels. The cell lines shown here are untransfected MDCKII cells (A–L) and two siRNA-expressing lines, siRNA 1 (M–X) and siRNA 2 (Y–J'). All images were acquired using similar settings on the laser-scanning microscope. The scale bars in A–C are $20\ \mu\text{m}$ long, and the same scale is used throughout this figure.

Ten-day-old cysts from each cell line were further characterized by immunostaining (Figure 8). Untransfected MDCKII cells primarily grew to form multicellular cysts containing a single lumen, which expressed GP135 on the luminal side only (Figure 8, A–C). Similar normal cysts were observed with several cell lines isolated together with the siRNA-expressing cell lines, but the cells comprising these cysts did not exhibit any significant PALS1 suppression (unpublished data). In contrast, the cell masses formed by the siRNA-expressing cell lines contained either no lumen (Figure 8, D–F and M–O) or several smaller lumens (Figure 8, G–I and J–L). There was no clear localization of GP135 in cysts without obvious lumens, indicating a complete loss of apical-basal polarity, whereas GP135 localization in the multiluminal cysts was partially restored. Occasionally a larger, but incomplete lumen was observed in the siRNA-expressing cells (arrows in Figure 8, M–O). Despite the different levels of PALS1 suppression in the siRNA cell lines, similar polarity defects were found in all the siRNA-expressing cell lines examined with this assay with no apparent correlation between severity of the defects and the level of PALS1 suppression.

DISCUSSION

Utilizing a stable siRNA expression system, we have disrupted expression of the tight junction-associated protein PALS1 in MDCKII cells. The loss of PALS1 resulted in a corresponding loss of expression of PATJ, but had no ap-

parent effect on CRB3 expression or localization. Recently, it was shown that during *Drosophila* embryonic epithelia and photoreceptor development, Discs Lost, the homologue of

Figure 5. Transepithelial electrical resistance is decreased by the expression of PALS1 siRNA. MDCKII cell lines were seeded onto Transwell filters and grown at confluence for several days. After incubation in low-calcium medium to disrupt cell-cell contacts, the cells were incubated in normal growth medium, and the restoration of cell junctions was monitored by measuring transepithelial electrical resistance (TER), expressed in ohms/cm². Wild-type MDCKII cells (○) are shown compared with the PALS1-suppressed cell lines siRNA1 (▲), siRNA2 (◆) and siRNA3 (■). Mean values have been corrected for background, and the error bars show the SD from the mean ($n = 3$).

Figure 6. The localization of PKC ζ , but not Par3, to the tight junction is disrupted by PALS1 siRNA. A calcium switch experiment was performed as described in the legend to Figure 4. (A) Untransfected MDCKII cells (A–L) and a siRNA-expressing cell line (M–X) are shown. Cells were immunostained for the tight junction marker ZO-1 (A, D, G, J, M, P, S, and V) and PKC ζ (C, F, I, L, O, R, U, and X), with a merged image shown between them (B, E, H, K, N, Q, T, and W). (B) Untransfected MDCKII cells (A–I) and an siRNA-expressing cell line (J–R) are shown, and cells were immunostained for ZO-1 (A, D, G, J, M, and P) and Par3 (C, F, I, L, O, and R), with a merged image shown between them (B, E, H, K, N, and Q). Arrows in B (M–O) indicate colocalization of ZO-1 and Par3 at nascent spot-like tight junctions in the siRNA-expressing cells. The scale bars in A–C are 20 μ m long, and the same scale is used throughout this figure.

PATJ, was reduced to an undetectable level in Stardust (PALS1) mutant cells, indicating that the expression or stability of these proteins was in some way linked (Hong *et al.*, 2001; Nam and Choi, 2003). However, in contrast to our results, the mutation of Stardust in *Drosophila* strongly reduced the expression of Crumbs as well (Hong *et al.*, 2001; Nam and Choi, 2003). It is unknown whether the effect of PALS1 suppression on PATJ expression lies at the level of transcription, translation, or protein stability. Because the localization of PALS1 in mammalian epithelia is dependent on interaction with PATJ (Roh *et al.*, 2002b), it seems possible that the interaction between these two proteins is required for their stability. The absence of PALS1 and therefore its ability to act as an adaptor between PATJ and CRB3 may also be significant. In accordance with our findings, in *Drosophila* embryonic epithelia the loss of either Crumbs or Stardust resulted in the disruption of cell polarity and the mislocalization of Discs Lost away from the subapical region/marginal zone (Bachmann *et al.*, 2001). Similarly, the loss of Crumbs in the *Drosophila* photoreceptor resulted in the mislocalization of both Stardust and Discs Lost (Nam and Choi, 2003).

The significant reduction in PALS1 and PATJ expression in the siRNA-expressing cell lines resulted in specific defects in MDCKII cells, including a delay in tight junction formation after calcium switch, reduced TER and the inability of these cells to form luminal cysts when grown in a collagen gel matrix. Interestingly, the cell lines resulting from expressing PALS1-specific siRNA displayed varying levels of suppression that had a corresponding effect on the characteristics of the different cell lines, not unlike a hypomorphic

allele found in genetic studies. For example, the severity of the delay in tight junction formation in the calcium switch assay correlated with the level of PALS1 suppression: only the most severe suppression of PALS1 expression elicited a phenotype. The effect on TER was likewise variable. However, the significant loss of PALS1 in all the cell lines studied here resulted in defects in polarity determination that was most readily observed in the cyst formation assay: the ordered progression toward polarization, as observed in the control cells, was severely altered by the reduction in levels of PALS1 and PATJ, made manifest by the failure of the cysts to properly form a lumen or to localize the tight junction marker ZO-1. The formation of luminal cysts in collagen may be considered a more stringent assay for polarity determination, because the cells lack any initial polarity cues and cyst formation also requires the processes of apoptosis and expansion of the apical membrane (O'Brien *et al.*, 2002), whereas cells grown on filters begin with a pre-determined free apical surface, and thus the results of the filter assay may be less representative of the *in vivo* situation.

The loss of PALS1 and PATJ resulted in an expected decrease in the interaction between the CRB3 peptide beads and members of the Par6/Par3/aPKC complex, notably PKC ζ . This was not unexpected, because previous work in our laboratory demonstrated a direct interaction between PALS1 and Par6, and hence to the other members of the complex including aPKC (Hurd *et al.*, 2003). Furthermore, the localization of PKC ζ to the tight junction was also inhibited in the PALS1 siRNA-expressing cells. Previous work in our laboratory showed that PALS1 and PKC ζ were also

Figure 7. Loss of PALS1 expression results in the impaired development of luminal cysts. Single cells were seeded in collagen gels and grown for 3 (A, B, I, and J), 5 (C, D, K, and L), 7 (E, F, M, and N), or 10 (G, H, O, P, Q, and R) days. The collagen was partially digested, and the cysts were fixed, permeabilized, and stained for confocal microscopy. Polarization of the cysts was assessed by the extent of lumen formation, made visible by staining the cortical actin cytoskeleton (black and white; B, D, F, H, J, L, N, P, and R), and the localization of the cell polarity markers E-cadherin, an adherens junction protein, and ZO-1, a tight junction protein (red and green, respectively; A, C, E, G, I, K, M, O, and Q). Asterisks in Q and R mark small, incomplete lumens. The scale bars in each panel are 10 μ m long.

mislocalized from the tight junction in cells expressing a dominant negative construct of PATJ, and, although PALS1 expression was seemingly unaffected in these cells, the genesis of the tight junction was disrupted (Roh *et al.*, 2002b; Hurd *et al.*, 2003). Many studies support the relationship between the Par6/Par3/aPKC complex and the formation of tight junctions (Izumi *et al.*, 1998; Joberty *et al.*, 2000; Ohno, 2001; Yamanaka *et al.*, 2001; D'Atri and Citi, 2002; Gao *et al.*, 2002; Hirose *et al.*, 2002). E-cadherin-mediated cell-cell contact activates Cdc42 (Kim *et al.*, 2000), which binds Par6 and in turn activates aPKC (Yamanaka *et al.*, 2001), and truncated or mutant Par6 that can no longer interact with aPKC slows down tight junction formation (Gao *et al.*, 2002).

Furthermore, the kinase activity of aPKC is required for the establishment, but not maintenance, of cell polarity (Yamanaka *et al.*, 2001; Suzuki *et al.*, 2002). Because the expres-

sion level of PKC ζ remained unchanged in MDCKII cells that have reduced PALS1 expression, it seems likely that the effect on tight junction formation was due to the change in the localization of PKC ζ activity in those cells. This also suggests that the Par6/Par3/aPKC complex, and particularly aPKC, represent downstream effectors of the PALS1/PATJ/CRB3 complex in tight junction assembly. Mechanistically, the failure of aPKC to be recruited to the tight junction may result in the perturbation of downstream polarity signals, notably the basolateral determinant Lgl, which binds Par6 and is phosphorylated by aPKC during polarization (Betschinger *et al.*, 2003; Plant *et al.*, 2003; Yamanaka *et al.*, 2003), to significantly alter the establishment of apical-basal polarity.

Our results also support the hypothesis that aPKC is recruited to the tight junction through Par6 and its interac-

Figure 8. Polarity in collagen gels is grossly disturbed by suppression of PALS1. Single cells were seeded in a collagen gel matrix and grown 10 days until cysts developed. The collagen was partially digested and the cysts were fixed, permeabilized, and immunostained for examination by confocal microscopy. Cysts were immunostained for the cytoskeletal protein actin (A, D, G, J, and M) and the apical protein GP135 (C, F, I, L, and O). Normal lumen formation was observed with untransfected MDCKII cells (A–C), whereas siRNA cell line-derived cysts lacked any lumen at all (D–F), had multiple small lumens (G–I and J–L), or occasionally developed a larger, but incomplete, lumen (arrows in M–O). All images shown were taken through the center of the cyst. The scale bars in each image are 20 μ m long.

tion with PALS1 and not through Par3, which seems to localize normally to the tight junction and is also found at the spot-like junctions at early time points in the calcium switch assay. This differs from the genetic analysis of *Drosophila* embryonic epithelial development, which suggests that the Bazooka (Par3)/D-Par6/DaPKC complex recruits the Crumbs/Stardust (PALS1)/Discs Lost (PATJ) complex apically (Bilder *et al.*, 2003). However, in *Drosophila* photoreceptor morphogenesis, Bazooka (Par3) does not colocalize with the proteins of the Crumbs complex nor with D-Par6/DaPKC in the rhabdomere stalk, although Bazooka is essential for the proper apical targeting of those proteins (Nam and Choi, 2003). Clearly the interactions between these proteins are complex, and the delay in tight junction formation in MDCKII cells lacking PALS1 indicates that these multimeric complexes have several modes

of localization. Indeed, PATJ can be recruited to the tight junction by interactions with claudin-1 and ZO-3 (Roh *et al.*, 2002a), and Par3 can bind to the tight junction protein JAM (Ebnet *et al.*, 2001). In the PALS1 siRNA-expressing cell lines the latter interaction may recruit Par6/aPKC to the tight junction, thereby resulting in the observed delay in junction formation but not an absolute abrogation.

Taken together, our results show the important role of PALS1 in epithelial cell polarity determination. They agree with previous studies we have published using dominant negative systems as well as results obtained in *Drosophila*. PALS1 is positioned as a crucial adaptor at the tight junction that forms the core of the epithelial polarity complex. What remains unclear is how the dynamic interplay between members of this complex functions to ensure the proper

segregation of apical and basolateral proteins in mature epithelial cells.

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