Loss of PALS1 Expression Leads to Tight Junction and Polarity Defects

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Prior work in our laboratory and others determined that PALS1, PATJ, and CRB3 are essential components of 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 have led to the evolution of conserved proteins and their important roles in epithelial asymmetry. In particular, the PALS1/PATJ/CRB3 complex and the Par6/Par3/aPKC complex have 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., 2003; Nam et al., 2003).

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., 2000). 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 epithlia as well as neuroblasts during embryogenesis (Muller and Wieschaus, 1996; Wodarz et al., 2000; Petronczki 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., 2003; 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-
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 (Carlabad, 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'-GATCCGGAGGCAATTTGTTTCAAGAGAACAAATTCTTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC-3'; site 2: 5'-GAATCCTCCAGAAGAACGTAATGATTAGGAAAGTTAGTCTTGTCCTTCCGCT-3'; site 3: 5'-GATCCGGAGGCAATTTGTTTCAAGAGAACAAATTCTTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC-3'; site 4: 5'-GATCCGGAGGCAATTTGTTTCAAGAGAACAAATTCTTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC-3'; site 5: 5'-GATCCGGAGGCAATTTGTTTCAAGAGAACAAATTCTTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC-3'; site 6: 5'-GATCCGGAGGCAATTTGTTTCAAGAGAACAAATTCTTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC-3'; site 7: 5'-GATCCGGAGGCAATTTGTTTCAAGAGAACAAATTCTTTTCCAAAAAAGGTGAAGGAAAGGACTGTTTCTC-3'.

**Antibodies**

L17, 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 anticolchicine, and rabbit anticaudin-1 antibodies were purchased from Zymed (San Francisco, CA). CRB3 polyclonal antibody was a gift from George Ojakian at the SUNY Health Science Center (Brooklyn, NY). Antibodies to Par3 and PKCɛ were obtained from Upstate (Lake Placid, NY). Fluorochrome-conjugated antibodies used for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase–conjugated secondary antibodies for immunoblotting were obtained from Amersham Biosciences (Buckinghamshire, UK).

**CRB3 Peptide**

CRB3 peptide-coupled agarose beads were created using the SulfoLink Coupling Gel kit (Pierce Biotechnology, Rockford, IL) and were linked via a terminal cysteine residue added to a peptide corresponding to the C-terminal 18 amino acids of CRB3 (WT: NH3-CARPVPPTPNLKLPPEERLI-COOH) or the same sequence missing the terminal ERLI motif (ÆRLI: NH3-CARPVPPTPNLKLPPE-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-mm dishes with 1 mL of ice-cold lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 15 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. 10 μg/mL leupeptin, 20 μg/mL aprotinin and phosphatase inhibitor cocktail [Sigma]) and cleared by centrifugation at 14,000 × g for 20 min at 4°C. A portion of the lysate was reserved, mixed with LDS loading buffer (Invitrogen), and used as input. For the CRB3 peptide bead pull-down assay, 20 μL of 50% sherry of CRB3 peptide beads were added to 200 μL 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 μL of antibody was mixed with 200 μL lysate and 50% of sherry 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. In some cases, the membranes were 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. The membranes were incubated 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. The membranes were 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.

**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 0.5% SDS/PBS for 15 min, and then blocked with 2% goat serum/PBS (GS/PBS) for 1 h. The cells were then washed with 0.1% Triton X-100/PBS, followed by incubation with horseradish peroxidase–conjugated secondary antibody in 5% skimmed milk/TBS for 1 h at room temperature. The membranes were 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 SH3 domains respectively (Figure 1A), and more cell data), which targeted the PALS1 mRNA within the L27C siRNA derived from oligonucleotides 1 and 3 (unpublished of PALS1 expression was observed in cells expressing constructs were being expressed. The greatest suppression suppression and by PCR to determine which of the siRNA examined by Western blot to determine the level of PALS1 individual clones were isolated. These initial cell lines were simultaneously into MDCKII cells with a selectable marker, and siRNA constructs we created were transfected simulta-
obtained when blotting for PKC-ε/H9261 (2003), was also lost from the complex. Similar results were
binds to PALS1 through interactions with Par6 (Hurd et al., 2003). 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-ε 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 confirm the reduction in expression of both PATJ and PALS1 in the siRNA cell lines. Unlike PALS1 and PATJ, however, the expression of LIN7 and PKC-ε was unchanged. The expression of Par6 and Par3 was also unchanged by suppression of PALS1 (unpublished data). Figure 2A shows the results of immunoblotting with antibodies directed against the proteins indicated to the right. 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 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, PALS, 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 PALS 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.
In contrast, PKCζ recruited to tight junctions and colocalized with ZO-1 within strongly labeled punctuate structures (Figure 6A, panels C). In MDCKII cells, both PKCζ 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, PKCζ 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 PKCζ recruitment was not due to a decrease in overall PKCζ 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 PKCζ 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).
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 lumenal 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 multilumenal 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 apparent effect on CRB3 expression or localization. Recently, it was shown that during *Drosophila* embryonic epithelia and photoreceptor development, Discs Lost, the homologue of

**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, I, and J) 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 μm long, and the same scale is used throughout this figure.

**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).
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 lumenal 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 lumenal 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 predetermined 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 PKCζ. 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 PKCζ to the tight junction was also inhibited in the PALS1 siRNA-expressing cells. Previous work in our laboratory showed that PALS1 and PKCζ were also

Figure 6. The localization of PKCζ, 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 PKCζ (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 μm long, and the same scale is used throughout this figure.
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 expression level of PKCζ 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 PKCζ 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 7. Loss of PALS1 expression results in the impaired development of lumenal 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 μm 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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