PATJ regulates tight junction formation and polarity in mammalian epithelial cells

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Recent studies have revealed an important role for tight junction protein complexes in epithelial cell polarity. One of these complexes contains the apical transmembrane protein, Crumbs, and two PSD95/discs large/zonula occludens domain proteins, protein associated with Lin seven 1 (PALS1)/Stardust and PALS1-associated tight junction protein (PATJ). Although Crumbs and PALS1/Stardust are known to be important for cell polarization, recent studies have suggested that Drosophila PATJ is not essential and its function is unclear. Here, we find that PATJ is targeted to the apical region and tight junctions once cell polarization is initiated. We show using RNAi techniques that reduction in PATJ expression leads to delayed tight junction formation as well as defects in cell polarization. These effects are reversed by reintroduction of PATJ into these RNAi cells. This study provides new functional information on PATJ as a polarity protein and increases our understanding of the Crumbs–PALS1–PATJ complex function in epithelial polarity.

Introduction

Polarization is important for the function of epithelial cells that exhibit distinct apico–basal polarity (Ohno, 2001; Nelson, 2003; Macara, 2004). The apical and basolateral membranes are characterized by differential lipid and protein contents, and are separated by tight junctions which function as a physical barrier between these membrane domains (Tsukita et al., 2001; Matter and Balda, 2003). When epithelial cells initially polarize, they receive directional cues from the basal surface via adhesion to the ECM and from the lateral surface via cell–cell interactions. To fully polarize, the mammalian epithelial cell must then separate the apical from basolateral surfaces via tight junction formation. However, the exact mechanisms for epithelial polarization such as the movement of polarity proteins during polarization are largely unknown. One theory for initial epithelial polarization and apical membrane formation came with the identification of the vacuolar apical compartment (VAC) that can be detected when epithelial cells lose polarization (Vega-Salas et al., 1988; Low et al., 2000). In models of reversible polarization, VACs are exocytosed to the apical surface as epithelia repolarize, targeting apical proteins such as GP135 and syntaxin3 to the apical membrane domain. This has lead to the theory that before surface polarization, apical membranes are first generated internally then exocytosed to the newly forming apical membrane. However, VACs are seen in only a limited number of polarization models and thus their role in epithelial polarity in vivo is still uncertain (O’Brien et al., 2002).

Despite our incomplete understanding of the events leading to polarization, recent studies in Drosophila and mammalian cells have begun to identify a large number of proteins as polarity determinants (Knust and Bossinger, 2002). Among these proteins, Crumbs3 (CRB3) and protein associated with Lin seven 1 (PALS1) in mammalian cells and their orthologues Crumbs and Stardust in Drosophila are important for proper epithelial polarity determination (Knust and Bossinger, 2002; Roh and Margolis, 2003b). Crumbs are transmembrane proteins that interact with PALS1/Stardust via an interaction between their COOH-terminal ERLI motif and the PSD95/discs large/zonula occludens (PDZ) domain of PALS1/Stardust (Bachmann et al., 2001; Hong et al., 2001; Roh et al., 2002b). Drosophila CRB has a large extracellular domain and is required for the correct localization of Stardust and is essential for photoreceptor morphogenesis as well as functioning in epithelial polarity (Tepass et al., 1990; Izaddoost et al., 2002; Pellikka et al., 2002; Nam and Choi, 2003). CRB3 is predominantly expressed in mammalian epithelial cells and localized to the tight junction and apical surface (Makarova et al., 2003). Overexpression of CRB3 in MDCK cells leads to polarity defects in cysts grown in collagen gels (Roh et al., 2003a).

PALS1 is a membrane-associated Guanylate kinase family protein consisting of two Lin-2 and Lin-7 (L27) domains.
(L27N and L27C), PDZ domain, SH3 (Src Homology3) domain, 4.1 binding domain, and Guk (Guanylate kinase) domain (Anderson, 1996; Kamberov et al., 2000). Both PALS1 and its Drosophila orthologue, Stardust, have been found to be important in the generation of apical polarity (Bachmann et al., 2001; Hong et al., 2001; Straight et al., 2004). Via one of its L27 domains, PALS1 binds to PALS1-associated tight junction protein (PATJ). PATJ is a multiple PDZ containing protein that also contains an L27 domain that interacts with the L27 domain of PALS1 (Lemmers et al., 2002; Roh et al., 2002b; Roh and Margolis, 2003b). It has 10 PDZ domains and the sixth and eighth PDZ domains of PATJ bind to zonula occludens (ZO)-3 and claudin-1, respectively (Roh et al., 2002a). The Drosophila orthologue of PATJ (Dm-PATJ) has one L27 domain and only four PDZ domains. In Drosophila, it had been reported that mutations in Dm-PATJ leads to polarity defects and the discs lost phenotype (Bhat et al., 1999). However, recent studies showed that the Disc Lost phenotype is caused by disruption in the Drosophila codanin-1 homologue, not Dm-PATJ (Bhat et al., 2003; Pielage et al., 2003). This was surprising to us because we found the interaction between mammalian PATJ and PALS1 is evolutionarily conserved and our studies demonstrated that PATJ targeted PALS1 to tight junctions (Roh et al., 2002b). Accordingly, we undertook an in depth analysis of PATJ function in mammalian epithelial cells. In contrast to the results described in Drosophila, we find that PATJ is essential for proper epithelial polarization and promotes tight junction formation. In addition, we find that PATJ localizes to the VAC in nonpolarized cells and targets to the apical region and tight junction during polarization.

Results and discussion

PATJ targets to the apical region then tight junction during polarization

To understand the role of PATJ in epithelial cells, we first examined the movement of PATJ in wild-type MDCKII cells during polarization. In low calcium medium, MDCK cells lost polarization and PATJ appeared to concentrate in the VAC where it colocalized with GP135 but not ZO1 (Fig. 1, A and B; Low et al., 2000; Ivanov et al., 2004). We examined the movement of PATJ to the tight junction during polarization. We fixed cellular monolayers that were in calcium-free media (Fig. 1 B), then 30 min (Fig. 1 C), 90 min (Fig. 1 D), 3 h (Fig. 1 E), and 6 h (Fig. 1 F) after readdition of calcium to reinitiate polarization. We stained the monolayers for PATJ, E-cadherin, and ZO1. We confirmed as previously reported that ZO1 is initially recruited to sites of cell–cell adhesion with E-cadherin (Yone-

![Figure 1. Localization of PATJ during cell polarization.](image-url)
Western blot for PATJ and PALS1. Precipitate proteins from control and PATJ RNAi lysates, followed by control. (B) Beads containing the COOH terminus of CRB3 were used to analyze for PATJ, PALS1, and CRB3. Blotting for actin was used as a loading control.

**Abnormal tight junction formation in PATJ knockdown cells**

To test if PATJ has a role in the formation of tight junctions, calcium switch experiments were performed with control and PATJ RNAi MDCKII cells. Cells were incubated overnight in low calcium media to disrupt the existing junctions. Subsequently, low calcium media was replaced by normal media to initiate junctional formation and the formation of tight junctions was assessed by immunostaining for the tight junction marker protein, ZO1. As shown in Fig. 3 A, tight junctions rapidly redeveloped after calcium switch and were complete within 6 h in control MDCKII cells. ZO1 and PALS1 correctly localized to the tight junctions (Fig. 3 A, left panels). In contrast, there was a significant delay in the formation of tight junctions in PATJ RNAi MDCKII cells (Fig. 3 A, right). At 24 h, a small amount of PATJ can be seen to localize at tight junctions in the PATJ RNAi cell lines. This is consistent with the residual PATJ expression we observe in the CRB3 bead pulldown from these RNAi cell lines (Fig. 2 B). It is not clear if this small amount of PATJ is necessary for the tight junctions to eventually form. The recruitment of ZO1 to the tight junctions did not take place until 6 h after calcium switch. Interestingly, the localization of PALS1 was also affected by the suppression of PATJ (Fig. 3 A), confirming previous studies from our group that PATJ is responsible for the targeting of PALS1 to the tight junctions (Roh et al., 2002b). The disruption of tight junctions was also confirmed by immunostaining for Par3, another tight junction protein. In control MDCKII cells, Par3 and ZO1 colocalized to the tight junctions within 6 h after calcium switch. However, localization of Par3 to the tight junctions in PATJ RNAi MDCKII cells was significantly disrupted (unpublished data).

To confirm that the disruption of tight junctions described above was due to the loss of PATJ expression, we expressed exogenous PATJ in PATJ RNAi MDCKII cells. Using a retroviral system for efficient introduction of exogenous PATJ to MDCKII cells, EGFP-PATJ was expressed in PATJ RNAi MDCKII cells using EGFP alone as a negative control. As shown in Fig. 3 B, EGFP-PATJ rescued cells displayed expression of EGFP-PATJ but not endogenous PATJ (Fig. 3 B, second and fourth lanes). The formation of tight junctions in rescued MDCKII cells was assessed by immunostaining experiments for ZO1 (Fig. 3 C). In control experiments, EGFP alone failed to rescue tight junction formation in PATJ RNAi MDCKII cells 6 h after calcium switch (Fig. 3 C, top). In contrast, the expression of EGFP-PATJ restored the formation of tight junctions at this time point (Fig. 3 C, bottom). In these rescue experiments, EGFP-PATJ localized to the tight junctions with ZO1.

To further confirm that the formation of tight junctions was disrupted in PATJ RNAi MDCKII cells, we measured transepithelial electrical resistance (TER) during the calcium switch experiments with control, PATJ RNAi MDCKII cells and EGFP-PATJ rescued MDCKII cells (Fig. 3 D). Control MDCKII cells showed the normal profile of TER in which TER rapidly reached the maximum level and gradually decreased through the time course (Fig. 3 D, blue long dashed line). However, PATJ RNAi MDCKII cells showed an abnormal profile of TER in which TER failed to reach the maximum

**Figure 2.** PATJ RNAi reduces PATJ but not PALS1 or CRB3 expression. (A) Lysates derived from control and two different PATJ RNAi expressing MDCKII cell lines were resolved by SDS-PAGE, followed by Western blot analysis for PATJ, PALS1, and CRB3. Blotting for actin was used as a loading control. (B) Beads containing the COOH terminus of CRB3 were used to precipitate proteins from control and PATJ RNAi lysates, followed by Western blot for PATJ and PALS1.
value as shown in control MDCKII cells (Fig. 3 D, gray dash-dot line), suggesting that correct barrier functions of tight junctions is significantly disrupted in PATJ RNAi MDCKII cells. In control EGFP rescue PATJ RNAi MDCKII cells, TER failed to reach the maximum value as observed in wild-type MDCKII cells, showing a similar profile as observed in PATJ RNAi MDCKII cells (Fig. 3 D, red short dashed line). In contrast, the TER in EGFP-PATJ rescued MDCKII cells was partially restored (Fig. 3 D, black solid line).

Together, with the immunostaining experiments, these results strongly suggested that PATJ is required for the correct formation of tight junctions in MDCKII cells and that the disruption of tight junctions in PATJ RNAi MDCKII cells is specifically due to the loss of PATJ.

**PATJ is required for proper polarization in three dimensional culture**

To investigate if PATJ controls the correct development of apico–basal polarity in epithelial cells, we used three dimensional cell culture assays in which single cells from control and PATJ RNAi MDCKII cells were grown in collagen gels for up to 10 d until they developed into cysts. The structure of cysts...
was closely examined by immunostaining experiments to assess if the correct polarity was established (Fig. 4). Control MDCKII cells developed into the perfect cysts with a single lumen, demonstrating well defined apico–basal polarity. In these cysts, epithelial cells surround a lumen and the apical membranes face the cyst lumen as demonstrated by staining for the apical membrane protein, GP135 (Fig. 4 A, green). In Fig. 4, lateral membranes are marked by E-cadherin (gray), whereas cortical actin staining is red. In contrast, PATJ RNAi MDCKII cells failed to develop into the normal cysts (Fig. 4 B). These cysts showed severe defects in development of a lumen displaying aberrant GP135 staining (Fig. 4 B, green). These data demonstrated that PATJ plays a critical role in the establishment of apico–basal polarity during the formation of cysts.

To further confirm that PATJ is required for the correct polarization of MDCK cells grown in three dimensional culture, cyst formation assays were performed with EGFP-PATJ rescued MDCKII cells. As shown in Fig. 4, EGFP rescued MDCKII cells have the same defects in apico–basal polarity as PATJ RNAi cells (Fig. 4 C). However, these polarity defects were reversed in a significant number of EGFP-PATJ rescued MDCKII cells, leading to a polarized cyst with a single lumen (Fig. 4, D and E).

In this study using RNAi, we found that PATJ is important for epithelial polarity and the formation of tight junctions. We also found significant rescue of tight junction and polarity defects after reintroducing EGFP-PATJ back to PATJ RNAi cells. However, the rescue was not complete as demonstrated in the TER profiles (Fig. 3 D) or the extent of apical lumen formation in three dimensional culture (Fig. 4 E). The cause for lack of complete rescue is uncertain but may be due to the heterogeneous expression of EGFP-PATJ in the cells or the presence of the EGFP tag. Nonetheless, it was easy to detect the difference in phenotype between cells rescued with EGFP-PATJ versus those rescued with EGFP alone.

We studied the trafficking of PATJ during calcium switch experiments and found that PATJ initially localized to the VAC then proceeded to the apical region. Subsequently, PATJ concentrated at the tight junction as polarization was completed. Based on these findings, we hypothesize the sequential targeting of PATJ from the VAC to the apical region and then to the tight junction. Previous studies from our group have demonstrated an important role for the sixth PDZ domain of PATJ in this targeting and proposed that this PDZ domain binds to members of the ZO family, in particular ZO3 (Roh et al., 2002b). It is interesting to note that ZO proteins appear to traffic differently to the tight junction. In this study, ZO1 first goes to sites of cell–cell adhesion and then presumably migrates apically to form the tight junction. In light of the results in this paper, one can hypothesize that the convergence of PATJ from the apical region with ZO proteins in the lateral membrane leads to efficient tight junction formation. As the ZO and other proteins concentrate at the apical part of the lateral membrane, one can hypothesize that PATJ is bound to these proteins as it diffuses in the apical region. The interaction of PATJ with these junctional proteins then forms a nidus al-
ollowing the formation of the tight junction. Indeed, tight junctions did not form when MDCK cells were grown in three-dimensional culture. Although the tight junction will form in monolayer culture of cells with a marked reduction in PATJ expression, the efficiency of formation is severely reduced. However, the sequential targeting of PATJ from the VAC to the tight junction via the apical region proposed in our studies is still a hypothesis. For example, PATJ might target to the apical region and tight junctions independently rather than sequentially. This hypothesis will require further proof via live cell imaging and immuno-electron microscopy.

In our studies, the reduction of PALS1 had the same effect as reduction of PATJ (Straight et al., 2004). However, because reduction in PALS1 expression also leads to a loss of PATJ, it is difficult to separate the effects of PATJ from those of PALS1. In PATJ RNAi MDCKII cells, there is a reduction of PALS1 expression. Nevertheless, the amount of reduction of PALS1 expression in PATJ RNAi MDCKII cells is not as large as that in PALS1 RNAi MDCKII cells (Straight et al., 2004).

Our studies suggest that the extent of reduction of PALS1 expression seen in PATJ RNAi cells is not sufficient to result in the junctional or polarity defects seen in these cells (unpublished data). Furthermore, the expression of PALS1 in PATJ RNAi MDCKII cells was not able to rescue the junctional defects, suggesting the phenotypes shown in PATJ RNAi MDCKII cells are due to the loss of PATJ (unpublished data). It is not clear at this point why the expression of PALS1 is reduced in PATJ RNAi MDCKII cells. However, RT-PCR data indicate that mRNA level of PALS1 are not reduced in PATJ RNAi MDCKII cells, suggesting that the protein stability of PALS1 might be decreased with the loss of PATJ.

In conclusion, our results clearly point to PATJ as a polarity protein that regulates tight junction formation. This helps to clarify some of the confusion that has resulted from genetic studies of the discs lost phenotype in Drosophila (Bhat et al., 2003; Pielage et al., 2003). The results are consistent with the strong binding observed between PATJ and PALS1/Stardust in mammalian epithelia. Our ability to measurably reverse the phenotype of PATJ RNAi cell lines with wild-type PATJ also provides a unique tool to better understand PATJ function. Although we understand the function of some of the protein–protein interaction domains in PATJ, there are several PDZ domains whose role is unclear. Structure function analysis of PATJ domains using rescue systems should help clarify the molecular mechanisms underlying PATJ action.

### Materials and methods

#### DNA constructs

Two different 19-bp siRNA target sequences for PATJ were chosen by the siRNA Target Finder program (Ambion). A pair of complimentary oligonucleotides for each target sequence was synthesized by custom primers (Invitrogen). The sense and antisense sequences are separated by a 9-bp loop region [TCTAAGAGA], and each oligonucleotide terminates with restriction endonuclease half sites (BamHI and HindIII) for further cloning into the expression vector. Complimentary oligonucleotides were annealed, and cloned into pSilencer 2.1-U6 hygro (Ambion). The chosen sequences for ATJ siRNA follow: PATJ RNAi #1: sense strand, 5’-GGAAAACGGTCAAACAGCCA-3’, antisense strand, 5’-TGGCTCTGTTG-ACTGTTCG-3’; PATJ RNAi #2: sense strand, 5’-GATCGTTAGTTG-GATCG-3’, antisense strand, 5’-GGATCACAACTCAGCTAC-3’.

To generate the rescue construct, six nucleotides of siRNA target sequence #1 region in wild-type PATJ cDNA were mutated by PCR. The resulting PATJ cDNA was subcloned into retroviral EGFP fusion vector, pLE-GFP-C1 (BD Science) for retroviral infection. The rescue construct encodes the same amino acid as wild-type PATJ, but contains a different nucleotide sequence in PATJ siRNA #1 target region: Wild-type PATJ, 5’-GGAAAACGGTCAAACAGCCA-3’; rescue PATJ, 5’-GGATAATGCGACAGGGTA-3’. Mutated nucleotides are shown in bold.

#### Antibodies

PATJ and PALS1 antisera were generated in rabbits and affinity purified as described previously (Borg et al., 1998; Makarova et al., 2003). Mouse anti-ZO1 and mouse anti–E-cadherin antibodies were purchased from Zymed Laboratories and Sigma-Aldrich, respectively. Mouse monoclonal anti-GPI antibody was purchased from Upstate Biotechnology. Secondary antibodies conjugated to Alexa Fluor 488, 594, and 647 were used for immunostaining experiments (Molecular Probes). Mouse monoclonal anti EGFP antibody was used for immunoblotting (BD Biosciences). Immunoblotting and immunostaining were performed as described previously (Straight et al., 2004).

#### CRBs peptide beads pulldown

CRB3 peptide beads were generated using Sulfolink Coupling Gel Kit (Pierce Chemical Co.). The COOH-terminal 18 aa of CRB3 (NH3-CARRYPFFNKLPKPERRU-COOH) were linked to beads via a terminal cysteine residue. For the CRB3 peptide bead pulldown assays, 20 µl of 50% slurry of CRB3 peptide beads were added to 500 µl lysate and incubated overnight at 4°C. Then, beads were washed three times with HNTG buffer (50 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, and 100 mM glycerol).

#### Cell culture, transfection, and retroviral infection

MDCKII cells were grown in DME containing 10% FBS, penicillin, streptomycin, and glutamine (Life Technologies, Inc.). MDCKII cells were transfected with 5 µg of PATJ siRNA construct, pSilencer 2.1-U6 hygro-PATJ, using Fugene6 reagent (Roche) according to manufacturer’s directions to generate PATJ knockdown stable cell lines. After selection with 200 µg/ml of HygromycinB (Invitrogen) for 2 wk, clones were isolated to generate stable cell lines.

MDCKII cells were grown into three-dimensional cysts in collagen gels as described previously (Pollack et al., 1998; O’Brien et al., 2001; Roh et al., 2003a). For retroviral infection, amphotrophic packaging cell line, Phoenix A cells, were transfected with retroviral vector using FuGene6 reagent as described above. After 48 h, virus containing supernatants were collected and filtered through a 0.45-µm membrane to remove cell debris. MDCKII cells were infected with virus by 1 µg/ml polybrene. At 48 h after infection, virus was removed and the medium was replaced by selection media with 600 µg/ml of G418 (Invitrogen). Infected MDCKII cells were allowed to grow for 4 d before they were prepared for further examination.

#### Calcium switch experiment

Confluent MDCKII cells were grown on 24-mm Transwell filters (Corning Costar) in low calcium medium (5 mM Ca2++) overnight to dissociate cell–cell contacts. Then, low calcium medium was replaced by normal media containing 1.8 mM Ca2++. At different time points (0 h, 3 h, and 6 h), cells were prepared for immunostaining. TER measurements were performed as described previously (Straight et al., 2004).

Confocal microscopy was supported in part by the Morphology and Image Analysis Laboratory at the University of Michigan and by National Institute of Diabetes and Digestive and Kidney Diseases grant P50DK20572. This work was also supported by National Institute of Diabetes and Digestive and Kidney Diseases grant DK-58208 and by the National Institutes of Health through the University of Michigan’s Cancer Center Support grant [5 P30 CA46592]. Ben Margolis is an investigator of the Howard Hughes Medical Institute.

Submitted: 10 August 2004
Accepted: 28 December 2004

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