The Crumbs3-Pals1 complex participates in the establishment of polarity in mammalian epithelial cells

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Accepted 24 March 2003
Journal of Cell Science 116, 2895-2906 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00500

Summary

In *Drosophila*, the Crumbs–Stardust–Discs-lost complex is required during the establishment of polarized epithelia. Embryos that lack a component of this complex or overexpress Crumbs exhibit defects in epithelial morphogenesis. We recently cloned a novel mammalian epithelial Crumbs isoform, Crumbs3 (CRB3). CRB3 exists in a complex at tight junctions (TJs) with Pals1 and PATJ, the mammalian homologues of Stardust and Discs lost, respectively. Here, we observe that overexpression of CRB3 leads to delayed TJ formation in MDCK epithelial cell monolayers and disruption of polarity in MDCK cysts cultured in collagen. Both phenomena require the last four residues of CRB3. Next, we expressed, in MDCK cells, a dominant-negative Myc-Lin-2–Pals1 chimeric protein, where the PDZ domain of Lin-2 was replaced with that of Pals1. TJ and apical polarity defects were also observed in these cells. Collectively, this suggests that the CRB-Pals1 interaction is important for formation of TJs and polarized epithelia. These results provide insight into the function of the mammalian Crumbs complex during TJ formation and epithelial polarization.

Key words: Crumbs3, Pals1, PDZ domain, Tight junction, Polarity

Introduction

Epithelial cell monolayers play a significant role in the regulation of body homeostasis in vertebrates and invertebrates. Specifically, they function to separate different physiological compartments and, simultaneously, regulate the exchange of solutes across the monolayer. A central feature of epithelia is their polarized nature manifested by asymmetrically localized organelles and protein networks as well as the division of the plasma membrane into apical and basolateral surfaces. Recent work has emphasized the importance of several factors during the establishment and maintenance of epithelial cell polarity. These include cues arising from the extracellular matrix and cell-cell contacts leading ultimately to the polarized sorting of proteins and vesicles from intracellular compartments and the selective retention of proteins at distinct membrane domains (Yeaman et al., 1999b). Many of the proteins involved in these processes contain domains that mediate protein-protein interactions. Recently, studies have highlighted the importance of proteins containing the PSD95/Discs large/ZO-1 (PDZ) domain during epithelial polarization (Fanning and Anderson, 1999; Yeaman et al., 1999a). PDZ domains often function by binding to the extreme C-termini of their ligands (Songyang et al., 1997). There are several cases where PDZ domains dimerize with other PDZ domains thereby facilitating the formation of multimeric protein complexes (Sheng and Sala, 2001; Tochio et al., 2000; Xu et al., 1998).

The apical and basolateral membranes are demarcated by the tight junction (TJ), a specialized site of cell contact at the apical aspect of the lateral membrane. TJs are composed of a complex network of integral and peripheral membrane proteins. Claudins, occludin, and junctional adhesion molecule mediate cell adhesions at this site. In turn, these proteins associate with a diverse collection of cytoplasmic proteins that serve a variety of functions (Zahraoui et al., 2000). For instance, Zona occludens-1 (ZO-1), Zona occludens-2, and Zona occludens-3 link the above proteins to the underlying actin cytoskeleton (Fanning et al., 1998; Fanning et al., 2002). A subset of TJ-associated proteins (Rab3b, Rab8, Rab13, Sec6, Sec8, etc.) are involved in trafficking and docking of vesicles (Zahraoui et al., 2000). Recently, an increasing number of studies have elucidated an intimate functional relationship between the TJ and the establishment of apico-basal polarity. The majority of these reports have focused on an evolutionarily conserved signaling complex composed of aPKC and the PDZ domain containing proteins, Par6 and Par3/ASIP (Joberty et al., 2000; Lin et al., 2000). DaPKC, D-Par6, and Bazooka represent the orthologues of these proteins in *Drosophila*, respectively. In *Drosophila*, this complex has been shown to play important roles in establishing asymmetry in epithelia and delaminating neuroblasts during embryogenesis (Petronczki and Knoblich, 2001; Wodarz et al., 2000). In mammalian epithelia, the Par6-ASIP-aPKC complex, in association with the monomeric GTPase CDC42, has been shown to regulate the assembly of TJs (Gao et al., 2002; Hirose et al., 2002; Izumi et al., 1998; Joberty et al., 2000; Suzuki et al., 2001; Yamanaka et al., 2001).

We and others have recently characterized another evolutionarily conserved TJ complex composed of the orthologues of *Drosophila* Crumbs (CRB), Stardust (Sdt), and Discs lost (Dlt): Crumbs 3 (CRB3), Pals1, and Pals1 associated

TJ protein (PATJ), respectively (Lemmers et al., 2002; Makarova et al., 2003; Roh et al., 2002b). CRB and CRB3 are transmembrane proteins whereas the other proteins are cytoplasmic scaffolding proteins. Sdt and Pals1 are membrane associated guanylate kinase (Maguk) proteins each containing a single PDZ domain (Bachmann et al., 2001; Hong et al., 2001; Kamberov et al., 2000). In contrast, Dlt and PATJ bear multiple PDZ domains (Tepass, 2002). In mammalian epithelia, Pals1 acts as an adaptor mediating the indirect interaction between Crumbs and PATJ (Roh et al., 2002b). In *Drosophila*, Sdt is predicted to be involved in similar types of interactions with CRB and Dlt (Tepass, 2002).

Fly embryos lacking CRB and Sdt expression exhibit similar phenotypes – failure to form a zonula adherens and disruption of apico-basal polarity (Knust et al., 1993; Tepass and Knust, 1993). The CRB null phenotype can be rescued by exogenously expressing full-length or only the transmembrane and intracellular portion of CRB (Klebes and Knust, 2000). Furthermore, CRB overexpression in a wild-type background also leads to polarity defects (Wodarz et al., 1995). These two phenomena specifically require the extreme C-terminus of CRB, which binds to the PDZ domain of Sdt (Klebes and Knust, 2000; Roh, 2002b). These results demonstrate the intimate relationship between CRB and Sdt during the regulation of apico-basal polarization in *Drosophila* epithelial cells. Although it has been shown that Sdt contains multiple protein-protein interaction domains, the identities of Sdt binding partners that are important for epithelial polarity are less understood.

Although *Drosophila* epithelia lack TJs, the CRB-Sdt-Dlt and CRB3-Pals1-PATJ complexes both target to the apical aspect of cell contacts in *Drosophila* and mammalian epithelia, respectively (Tepass, 2002). To date, the physiological roles of the CRB3-Pals1-PATJ complex in mammalian epithelia are poorly understood. However, the similarity in the subcellular localization of these complexes suggests that they perform analogous functions. In the present study, we investigate the functional relationship between CRB3 and mammalian epithelial cell polarity. We demonstrate the importance of the CRB3-Pals1 interaction in the regulation of TJ biogenesis and apico-basal polarization.

Materials and methods

Antibodies

The mouse monoclonal anti-Myc antibody (9E10) was used for immunoprecipitation and immunoblotting. The following antibodies were utilized only in immunostaining experiments: rabbit polyclonal anti-Myc (clone A-14; Santa Cruz Biotechnology Inc., Santa Cruz, CA); mouse monoclonal anti-ZO-1 (Zymed Laboratories, San Francisco, CA); mouse monoclonal anti-gp135 (gift from George Ojakian, SUNY Health Science Center, Brooklyn, NY); mouse anti-β-catenin (BD Transduction Laboratories, San Jose, CA); and rat monoclonal anti-uvomorulin/E-cadherin (Sigma-Aldrich). The mouse monoclonal anti-ezrin ascites fluid (3C12) was obtained from Sigma and utilized for immunoblots and immunostaining. Rabbit polyclonal antibodies directed against Pals1, PATJ, and CRB3 have been described previously (Makarova et al., 2003; Roh et al., 2002b). Secondary antibodies conjugated to Alexa Fluor 488, 594, and 647 (Molecular Probes, Eugene, OR) were used in immunostaining experiments.

DNA constructs

The DNA encoding CRB3, excluding the signal peptide sequence, was PCR amplified and cloned into the pSecTag2B vector (Invitrogen, Carlsbad, CA). The pSecTag2B-MycCRB3 construct has been described previously (Makarova et al., 2003). This construct was used as a template during PCR amplification of the sequence encoding the cytoplasmic 37 residues of CRB3. This DNA was subsequently cloned into the pGSTag vector and the resulting construct used to express the GST-CRB3 fusion protein in bacterial cells. Point and deletion mutagenesis in these constructs was carried out as previously described (Makarova et al., 2000).

The pRK5-Myc-Lin-2 construct has also been described elsewhere (Lee et al., 2002). The PDZ domain was removed via deletion mutagenesis to yield the pRK5-Myc-Lin-2ΔPDZ construct. To design the Myc-Lin-2–Pals1 PDZ chimeric construct, the encoding sequence of the Lin-2 PDZ domain was replaced with the DNA encoding the Pals1 PDZ domain. First, the DNA encoding the Pals1 PDZ domain was PCR amplified using the following primers: 5′-CCAGAGTT-CGGCTGGTACAGTTT**GAAAAGGCTCGGGATATT**-3′ (forward) and 5′-GGACGAAGACTGAGTGCGGTA**CTGTTGACTAGGAA-TCAGAAC**-3′ (reverse). The underlined flanking bases correspond to the sequences flanking the DNA encoding the PDZ domain of Lin-2. The bases shown in bold represent the start and end of the DNA sequence encoding the Pals1 PDZ domain. Next, the sense and antisense strands of the resulting PCR product were utilized as primers in a mutagenesis reaction using the pRK5-Myc-Lin-2 template and Pfu turbo polymerase (Stratagene, Cedar Creek, TX). All constructs were verified by automated sequencing at the University of Michigan DNA Sequencing Core.

Cell culture and transfection

MDCK cells and HEK293 cells were transfected and cultured as described previously (Roh et al., 2002b). MDCK cell cysts were grown in three-dimensional collagen gels as previously described (O'Brien et al., 2001; Pollack et al., 1998). Essentially, MDCK cells were trypsinized, triturated into a single cell suspension, and then mixed into an ice-cold solution containing 2 mg/ml calf skin type I collagen (Sigma), 1× DMEM, 20 mM Hepes pH 7.4, and 5% FBS. The resulting suspensions were added to 10 mm diameter Transwell membrane filters (0.4 μm pore size; Corning Costar) and allowed to form a solidified gel at 37°C prior to addition of culture media. The media was replaced every two days and the cysts were allowed to develop over 5-6 days.

Immunoprecipitation and immunoblotting

Lysates were prepared from MDCK and HEK293 cell lines as previously described (Roh et al., 2002b). Immunoprecipitation, GST pulldown, and immunoblotting experiments were performed according to previously published protocols (Kamberov et al., 2000).

Calcium switch experiments

Approximately 5×10^5 MDCK cells were seeded onto 10 mm diameter Transwell membrane filters and cultured overnight in normal calcium media (DMEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine) containing 1.8 mM Ca^{2+} allowing for the formation of a fully confluent monolayer. Subsequently, the monolayers were washed five times with PBS and grown in low calcium media containing 5% dialyzed FBS and 5 μM Ca^{2+} overnight to dissociate cell-cell contacts. The next day, the low calcium media was replaced with pre-warmed normal calcium media and this was designated as the t=0 timepoint.

Immunostaining and confocal microscopy

MDCK monolayers and cysts were processed and immunostained as described previously (O'Brien et al., 2001; Roh et al., 2002b). For

staining of cysts, collagen gels were detached from the filter supports, washed with PBS, and incubated in PBS supplemented with 100 U/ml collagenase VII (Sigma) for 15 minutes at room temperature. Subsequently, gels were fixed in 4% paraformaldehyde/PBS for 30 minutes, permeabilized in 0.25% Triton X-100/PBS for 30 minutes, and then incubated in 2% goat serum/PBS for 1 hour. Next, gels were incubated with primary antibodies in 2% goat serum/PBS for two days at 4°C under constant agitation. Gels were then washed extensively with 2% goat serum/PBS and then soaked in the 2% goat serum/PBS supplemented with the appropriate fluorochrome-conjugated secondary antibodies overnight at 4°C. Staining of actin microfilaments was achieved by adding fluorochrome-conjugated phalloidin (Molecular Probes, Eugene, OR) to the secondary antibody solution. Finally, gels were washed extensively in PBS and mounted onto glass coverslips using ProLong antifade reagent (Molecular Probes). Cysts were visualized using a ZEISS LSM510 Axiovert 100M inverted confocal laser-scanning microscope. Images were analyzed using the LSM Image Examiner and Adobe Photoshop software.

Results

Overexpression of CRB3 results in apical surface expansion in MDCK cells

In *Drosophila*, the Crumbs–Stardust–Discs-lost complex contributes to the establishment of apico-basal polarity in epithelial cells. Insight into the function of this complex stems from the characterization of mutant flies where components of this complex are absent. Furthermore, Crumbs has been shown to function as an apical polarity determinant because its overexpression results in the expansion of the apical surface (Wodarz et al., 1995). We recently showed that the orthologous CRB3-Pals1-PATJ complex localizes to TJs in MDCK renal epithelial cells. Interestingly, the subcellular distribution of endogenous CRB3 also extends to the apical plasma membrane (Makarova et al., 2003). This led us to hypothesize that CRB3 also could function as an apical polarity determinant in mammalian epithelia.

We first investigated this possibility by isolating MDCK stable cell lines that vastly overexpressed human CRB3, Myc-CRB3, or moderately overexpressed Myc-CRB3 (Fig. 1A). In monolayers overexpressing CRB3 cultured on filter supports in normal calcium media (1.8 mM Ca^{2+}) for 12 hours, a fraction of CRB3 displayed an apical distribution (Fig. 1B,C). However, significant amounts of CRB3 localized to the lateral membrane. The amount of CRB3 present at the lateral surface seemed to correlate with the degree of CRB3 overexpression. Specifically, in MDCK cells vastly overexpressing Myc-CRB3, a significant fraction of Myc-CRB3 targeted laterally (Fig. 1D). In the moderately overexpressing Myc-CRB3 cell line, lateral Myc-CRB3 was also observed but to a slightly lesser extent. It should be noted that CRB3 expression was above endogenous levels in all of these cell lines (Fig. 1A).

To assess the degree of apical expansion respective to apical marker proteins, we immunostained these cell lines with anti-gp135 and anti-ezrin at 12 hours post-calcium switch. In monolayers overexpressing CRB3 and Myc-CRB3, a fraction of ezrin and gp135 was observed to target laterally (Fig. 1C, data not shown). These effects were not observed in the parental MDCK cells cultured in a similar manner (Fig. 1E). Collectively, these observations suggest that CRB3 overexpression results in the expansion of the apical membrane in agreement with *Drosophila* studies on Crumbs overexpression.

CRB3 overexpression negatively regulates tight junction biogenesis in a Pals1-dependent manner

In MDCK cells vastly overexpressing CRB3, CRB3 and apical surface markers were localized to both the apical and lateral surface suggesting that CRB3 overexpression can result in apical membrane expansion. Because a CRB3-Pals1-PATJ complex probably exists at TJs (Makarova et al., 2003), we wondered if CRB3 could regulate TJ assembly. Therefore, we analyzed TJ formation in MDCK cells overexpressing Myc-CRB3. We also generated specific mutations in Myc-CRB3 (summarized in Fig. 2A) and transfected the mutant Myc-CRB3 constructs into MDCK cells. The cytoplasmic tail of 37 amino acids contains two conserved regions: a putative juxtamembrane protein 4.1/ezrin/radixin/moesin (FERM)-binding domain and the extreme C-terminal PDZ-binding motif (Izaddoost et al., 2002; Medina et al., 2002b). Three highly conserved residues in the FERM-binding region were all replaced with alanine to yield the Myc-CRB3 FERMmut construct. The PDZ-binding sequence, ERLI, was also deleted (Myc-CRB3ΔERLI). The Myc-CRB3N→D mutation has been described previously (Makarova et al., 2003).

MDCK stable cell lines expressing each of these mutant constructs were isolated. Each cell line expressed the various Myc-CRB3 proteins above endogenous CRB3 levels (data not shown). Immunostaining these cells with anti-Myc antibody revealed that Myc-CRB3 and each of the Myc-CRB3 mutant proteins predominantly localize to the apical surface (Fig. 2B). Next, we studied the ability of these four Myc-CRB3 proteins to bind Pals1. We prepared lysates from the MDCK cell lines expressing the various Myc-CRB3 proteins, and anti-Myc immunoprecipitations were performed. The presence of Pals1 in the immunoprecipitates was examined by anti-Pals1 immunoblot. We found that the extreme C-terminal ERLI sequence of CRB3 is necessary to bind Pals1 since Myc-CRB3ΔERLI was the only protein that failed to co-immunoprecipitate Pals1 (Fig. 2C). These results are consistent with previous studies on the interactions between Crumbs proteins and Pals1 (Makarova et al., 2003; Roh et al., 2002b). It should be noted in Fig. 2C that the amount of Myc-CRB3ΔERLI expression is underestimated since the anti-CRB3 antibody was raised against the last 20 residues of CRB3 encompassing the ERLI sequence. As predicted, this antibody still detects CRB3 tail harboring the ERLI deletion but with lower affinity with respect to full length CRB3 tail (Fig. 2D). Unfortunately, our attempts to more accurately quantify relative expression of the various Myc-CRB3 proteins via anti-Myc immunoblot were unsuccessful.

To understand the effects of overexpressing Crumbs3 on TJ formation, we performed calcium switch experiments using the MDCK stable cell line expressing Myc-CRB3. MDCK cells grown in low calcium media usually exhibit a rounded morphology and lack TJs as well as adherens junctions. Upon re-addition of 1.8 mM calcium, parental MDCK cells form intact adherens junctions as well as TJs within six hours (Fig. 3A). In MDCK cells expressing Myc-CRB3, the ZO-1 staining pattern was fragmented within the first six hours post-calcium switch (Fig. 3B). In contrast, adherens junction formation was not significantly affected. The ZO-1 distribution still did not circumscribe the entire circumference of every cell in the monolayer even 12-24 hours after calcium switch; however, TJs seemed to be completely assembled by

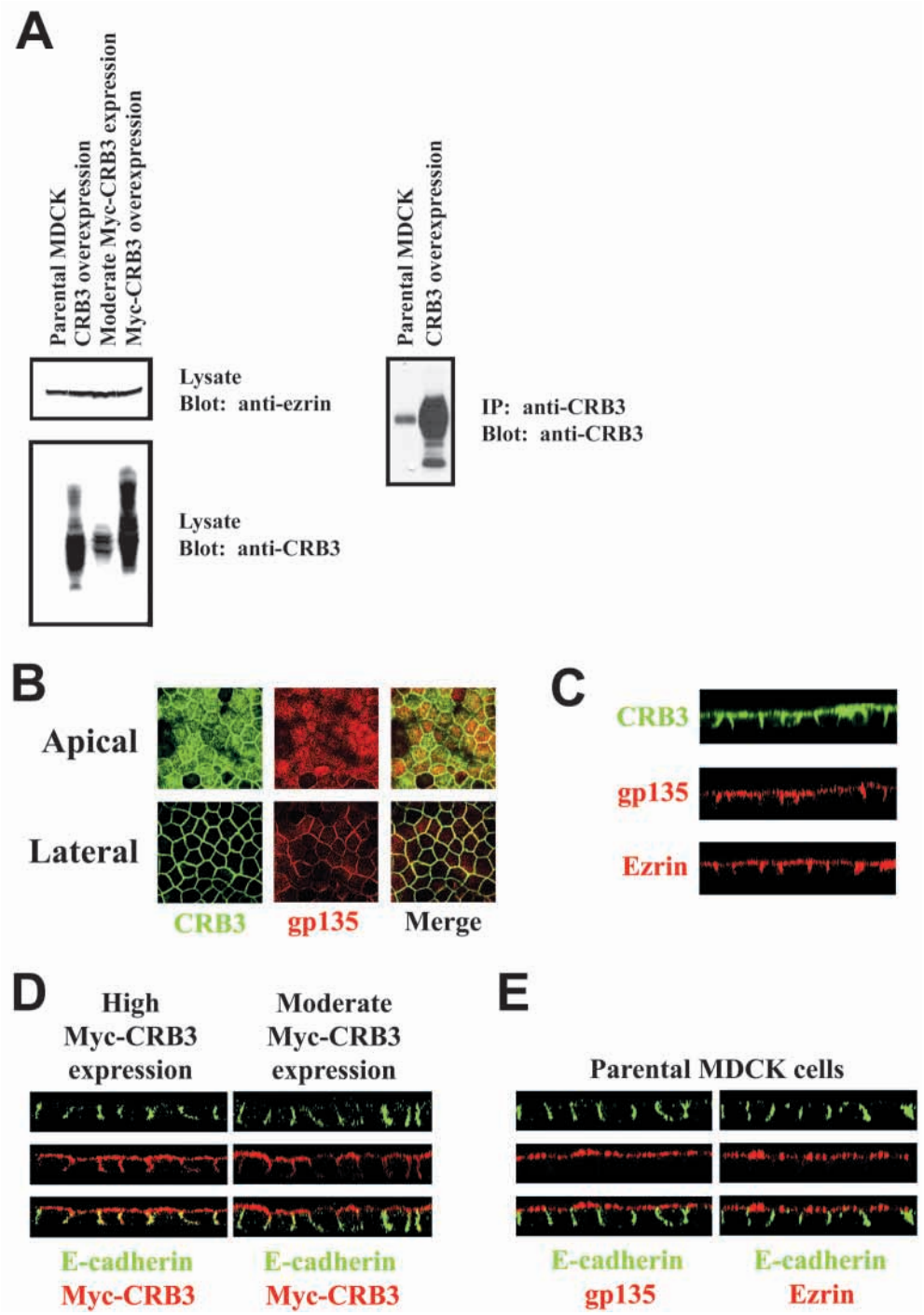

Fig. 1. Overexpression of human Crumbs homologue 3 in MDCK renal epithelial cells. (A) Lysates were prepared from the parental MDCK cells, MDCK stable cell line that overexpresses Crumbs 3 (CRB3) or Myc-CRB3, and cells moderately expressing Myc-CRB3. Lysates proteins (left panels) and anti-CRB3 immunoprecipitates (right panel) were resolved via SDS-PAGE and the indicated immunoblots were performed. (B-E) The indicated cell lines were initially cultured on Transwell filters in low calcium media (5 μM Ca^{2+}). Subsequently, the media was replaced with normal calcium media (1.8 mM Ca^{2+}). Cells were grown in this media for 12 hours and immunostained as indicated according to Materials and Methods. Ezrin and gp135 are markers of the apical surface, whereas E-cadherin marks the lateral surface. Square and rectangular panels represent X-Y photomicrographs and X-Z series (Z-sections), respectively.

48 hours (data not shown). Defects in TJ biogenesis were observed in stable cell lines expressing non-tagged CRB3 or moderately expressing Myc-CRB3 above endogenous CRB3 levels (data not shown).

We next sought to determine the moiety of CRB3 that was important for inhibiting TJ formation. Therefore, we performed calcium switch experiments with the MDCK stable cell lines that expressed the three mutant Myc-CRB3 proteins described above. We found that the ability of overexpressed Myc-CRB3 to delay TJ formation required the Pals1 PDZ-binding motif. Specifically, ZO-1 staining was observed to fully circumscribe the cell periphery of MDCK cells overexpressing Myc-CRB3ΔERLI by 6 hours after calcium re-addition as observed in parental MDCK cells (Fig. 3A,B). In contrast, moderate overexpression of Myc-CRB3N→D also inhibited TJ biogenesis (Fig. 3B). Cells overexpressing Myc-CRB3 FERMmut also exhibited delayed TJ assembly (not shown).

The inhibition of TJ formation by overexpressed Myc-CRB3 correlates with its ability to bind Pals1. In light of these results, we next investigated the localization of endogenous Pals1 in cell lines expressing Myc-CRB3 and Myc-CRB3ΔERLI at six

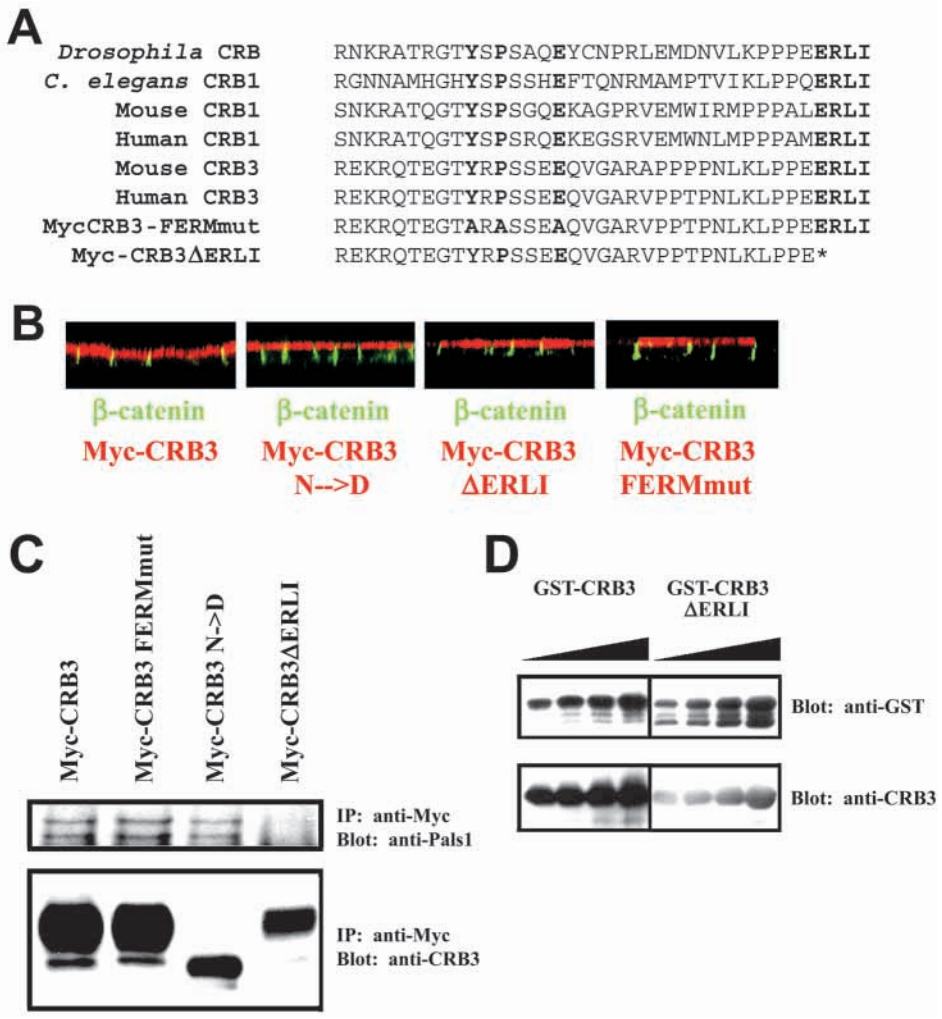

Fig. 2. Wildtype and mutant Myc-CRB3 constructs expressed in MDCK cells. (A) Sequence alignment of the intracellular portion of Crumbs homologues from indicated organisms. There are two conserved motifs: the juxtamembrane region predicted to bind a protein of the FERM superfamily and the extreme C-terminal PDZ-binding motif. Conserved residues in these two regions are shown in bold. The three most conserved residues in the FERM-binding region were all mutated to alanines (also shown in bold) in the Myc-CRB3 FERMmut construct. The Myc-CRB3ΔERLI protein lacks the PDZ-binding motif. Not shown is the Myc-CRB3N→D sequence where the intracellular portion of CRB3 is unchanged; instead, the extracellular N-glycosylation site is mutated as described previously (Makarova et al., 2002). (B) MDCK stable cell lines expressing wild-type Myc-CRB3 and the indicated Myc-CRB3 mutant proteins were grown for 24 hours in normal calcium media and then co-immunostained with anti-Myc and anti-β-catenin antibodies. The β-catenin is used as a marker of the lateral membrane. (C) Lysates were prepared from the stable cell lines used in (B). Anti-Myc immunoprecipitates were resolved by SDS-PAGE. The various Myc-CRB3 proteins and co-precipitated endogenous Pals1 were visualized by blotting with anti-CRB3 and anti-Pals1 antibodies, respectively. (D) Increasing amounts of GST-CRB3 and GST-CRB3ΔERLI fusion proteins were resolved by SDS-PAGE and immunoblotted as indicated.

hours post-calcium switch. In the former, we found that Pals1 was enriched at the fragmented TJs along with ZO-1 (Fig. 3C). In the Myc-CRB3ΔERLI cell line, Pals1 and ZO-1 staining pattern was observed to co-localize to TJs.

CRB3 overexpression disrupts apico-basal polarity in MDCK cells

Overexpression of CRB3 in MDCK cell monolayers results in delayed TJ formation. This effect is dependent on the last four residues of CRB3, the binding site for Pals1. Studies using *Drosophila*, an organism that lacks TJs, have shown that Crumbs overexpression leads to a disruption of epithelial morphogenesis. Thus, we wanted to investigate the global effect of CRB3 overexpression on apico-basal polarization of mammalian epithelia. In MDCK cells overexpressing CRB3, the majority of gp135 and ezrin distributed to the apical membrane. In the cell lines examined in Figs 2 and 3, the wild-type and mutant Myc-CRB3 proteins mainly localized apically. These results suggest that overexpression of CRB3 and Myc-CRB3 do not result in a significant disruption of apico-basal polarity in MDCK cell monolayers grown on synthetic tissue culture supports.

Recently, it has been demonstrated that MDCK cysts, grown in three-dimensional collagen gels, represent a sensitive model system to detect perturbations in apico-basal polarity (O'Brien et al., 2001). Consequently, we cultured each of the MDCK stable cell lines expressing the various Myc-CRB3 constructs in collagen. In parallel, we also cultured the parental MDCK cell line in the same manner. When grown in collagen for six days wild-type MDCK cells develop into cysts in which the epithelial cells surround a central lumen (Fig. 4A). The apical membranes of the cells face the lumen and stains positive for the apical marker gp135. PATJ and ZO-1 were localized to TJs, which demarcate the apical surface from the basolateral membrane (visualized by E-cadherin). The cortical actin cytoskeleton was localized along the entire plasma membrane of each epithelial cell; however, it was clearly concentrated at the apical membrane.

In contrast, when MDCK cells overexpressing Myc-CRB3, Myc-CRB3N→D, or Myc-CRB3 FERMmut were cultured in collagen, a multicellular aggregate of cells formed (Fig. 4B, data not shown). These aggregates did not contain any recognizable lumina. In addition, the subcellular distribution of ZO-1 and gp135 staining exhibited a disorganized pattern suggestive of TJ and apical surface defects (Fig. 4C). Furthermore, E-cadherin still remained localized to the plasma membrane at sites of cell contacts. MDCK cells expressing

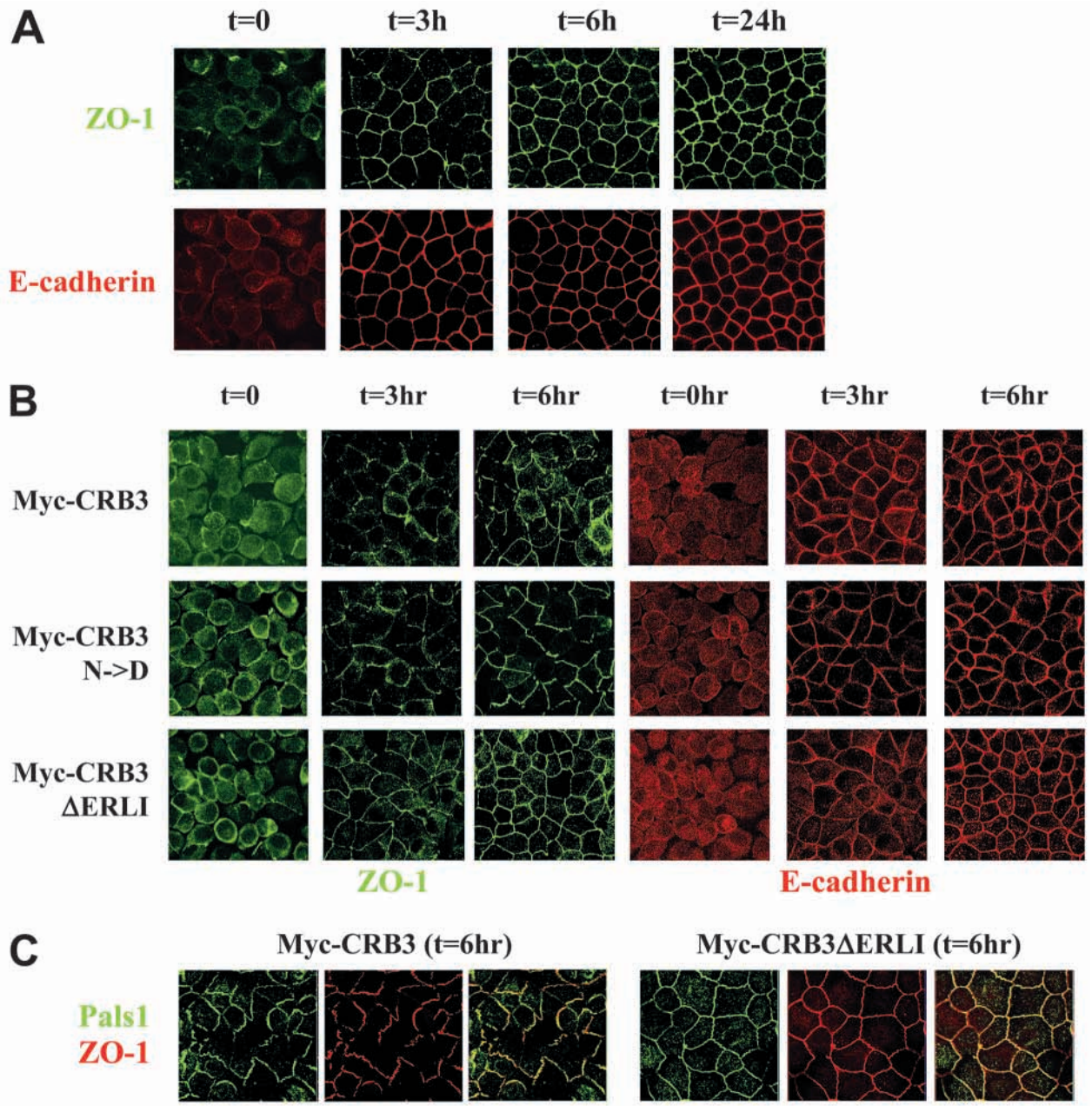

Fig. 3. The effect of CRB3 overexpression on the biogenesis of tight junctions and adherens junctions. (A) Parental MDCK cells were initially cultured in low calcium media. At t=0, the low calcium media was replaced with normal calcium media. At the indicated timepoints, cells were fixed, permeabilized, and co-immunostained with anti-ZO-1 and anti-E-cadherin. (B) The same calcium switch experiment was performed on MDCK cells expressing the indicated Myc-CRB3 proteins. At the indicated times after re-addition of calcium, cells were stained with anti-ZO-1 (green) and anti-E-cadherin (red). ZO-1 and E-cadherin are markers of the TJ and adherens junction, respectively. (C) MDCK cells expressing either Myc-CRB3 or Myc-CRB3ΔERLI were immunostained with anti-Pals1 (green) and anti-ZO-1 (red) antibodies at 6 hours post-calcium switch.

Myc-CRB3ΔERLI formed relatively normal cysts as the apical marker gp135 clearly outlined a central lumen. The distribution of ZO-1 was similar to that in wild-type MDCK cysts as well suggesting normal TJ formation (data not shown). Staining these cysts with anti-Myc antibodies revealed that Myc-CRB3ΔERLI was localized all along the plasma membrane.

The ability of overexpressed Myc-CRB3 to disrupt apico-basal polarity in three-dimensional MDCK cysts again correlated with its ability to bind Pals1. We therefore sought to determine the distribution of Pals1 in these cysts; however, our attempts to immunostain endogenous Pals1 in wild-type MDCK cysts or multicellular aggregates overexpressing Myc-CRB3 were unsuccessful because of relatively high background staining. Alternatively, we were able to immunostain an endogenous Pals1-binding partner, PATJ, in these cysts and aggregates. PATJ co-localized with ZO-1 to TJs in wild-type MDCK cysts (Fig. 4A). In Myc-CRB3 overexpressing cell aggregates, however, PATJ exhibited a

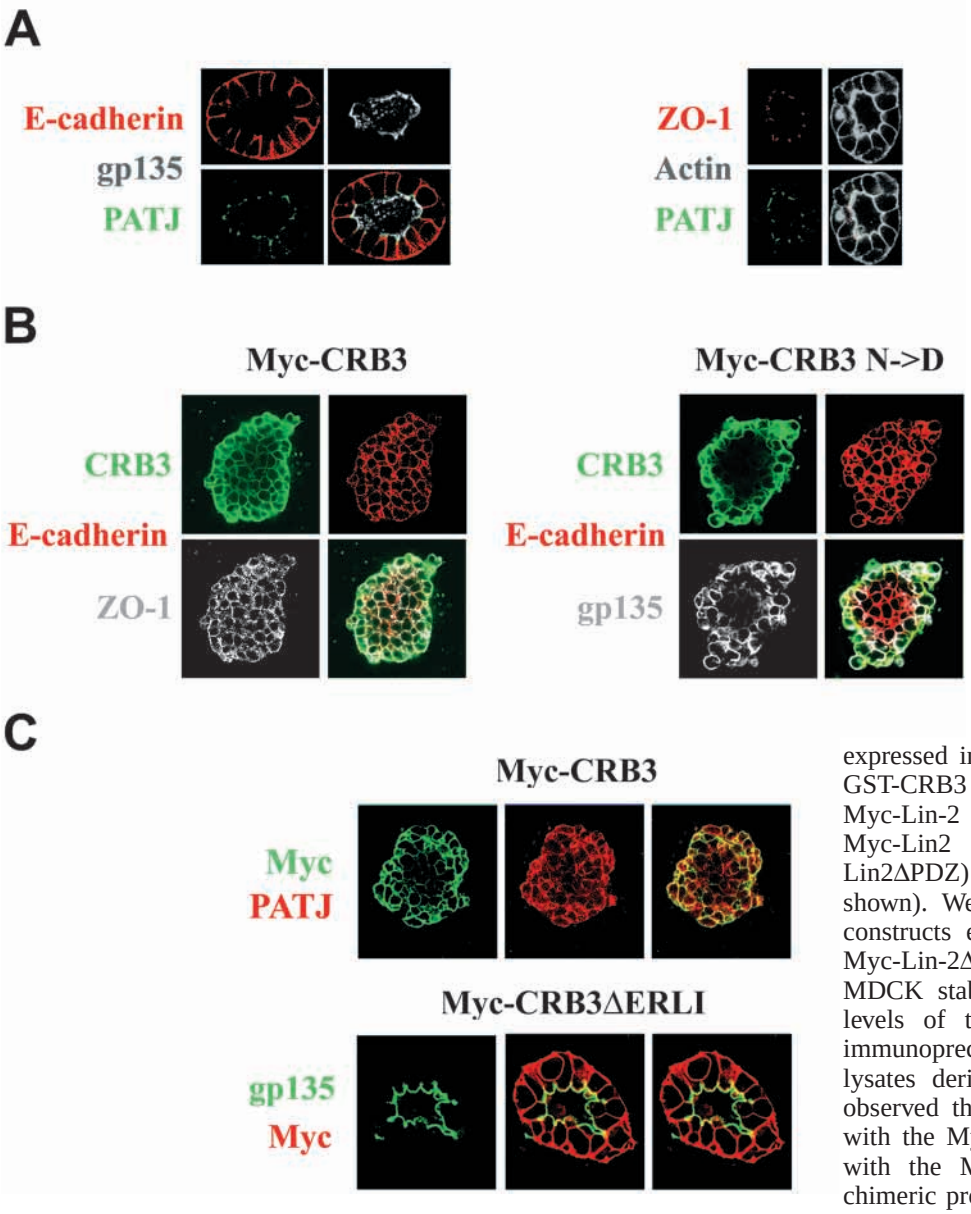

Fig. 4. Crumbs 3 overexpression leads to a disruption of apico-basal polarity in MDCK cysts cultured in collagen matrix. (A) Wild-type MDCK cells were cultured in collagen gel according to Materials and Methods to induce three-dimensional cyst formation. Cysts were co-stained with the indicated antibodies. (B,C) MDCK cells expressing the various Myc-CRB3 proteins were cultured in collagen and subsequently immunostained using the indicated antibodies.

disorganized localization pattern that partially, but not completely, overlapped with the overexpressed Myc-CRB3 (Fig. 4C).

Disruption of the association between endogenous CRB3 and Pals1 perturbs tight junction formation and apical polarity

Overexpression of CRB3 leads to disruption of TJs and apico-basal polarity in mammalian epithelial cells. These events require the extreme C-terminus of CRB3 suggesting that Pals1 is an important element of CRB3 function. We previously demonstrated that the PDZ domain of Pals1 directly interacts with the extreme C-terminal ERLI motif of mammalian Crumbs proteins (Makarova et al., 2003; Roh et al., 2002b). We next wanted to examine the role of the CRB3-Pals1 association during the establishment of TJs and polarity. We sought to disrupt this endogenous interaction using a dominant-negative approach. Therefore, a construct encoding a Myc-Lin-2–Pals1 PDZ chimeric protein in which the mLin-2/CASK PDZ domain was replaced with the Pals1 PDZ domain (Fig. 5A) was designed.

Recently, we demonstrated that Myc-Pals1 associates with a GST fusion protein containing the last 20 amino acids of CRB3 (Makarova et al., 2003). Here, we observed that the Myc-Lin-2–Pals1 PDZ chimera, expressed in HEK293 cells, also associates with GST-CRB3 tail (Fig. 5B). In contrast, wild-type Myc-Lin-2 failed to associate with GST-CRB3. Myc-Lin2 missing the PDZ domain (Myc-Lin2ΔPDZ) also did not bind GST-CRB3 (not shown). We next transfected MDCK cells with constructs encoding Myc-Lin-2–Pals1 PDZ and Myc-Lin-2ΔPDZ (negative control) and isolated MDCK stable cell lines expressing comparable levels of these proteins (Fig. 5C). Anti-Myc immunoprecipitations were performed using lysates derived from these two cell lines. We observed that endogenous CRB3 co-precipitated with the Myc-Lin-2–Pals1 PDZ chimera but not with the Myc-Lin-2ΔPDZ suggesting that the chimeric protein could compete with endogenous Pals1 for binding CRB3 (Fig. 5D).

To investigate the effects of expressing the Myc-Lin-2–Pals1 PDZ chimera on TJ formation in MDCK cells, we performed a calcium switch experiment similar to that shown in Fig. 3. We compared the assembly of cell-cell contacts in MDCK cells expressing the chimera and those expressing Myc-Lin-2ΔPDZ. Junctional assembly was monitored 0, 3, 6, and 96 hours after calcium switch (Fig. 6). The formation of TJs proceeded normally in the Myc-Lin-2ΔPDZ expressing cell line as ZO-1 staining labeled the entire circumference of each cell at cell contacts by six hours after re-addition of calcium (Fig. 6A). In contrast, TJ assembly in the chimera expressing cell line was significantly delayed and was not complete even at 96 hours post-calcium switch. This effect was also observed but to a lesser extent in another stable cell line expressing lower amounts of the chimeric protein. For the Myc-Lin-2–Pals1 PDZ cell line, adherens junctions were visualized at the same timepoints as in Fig. 6A by staining with anti-E-cadherin antibody (Fig. 6B). Within the first three hours after the calcium switch, E-cadherin was observed to target to the lateral

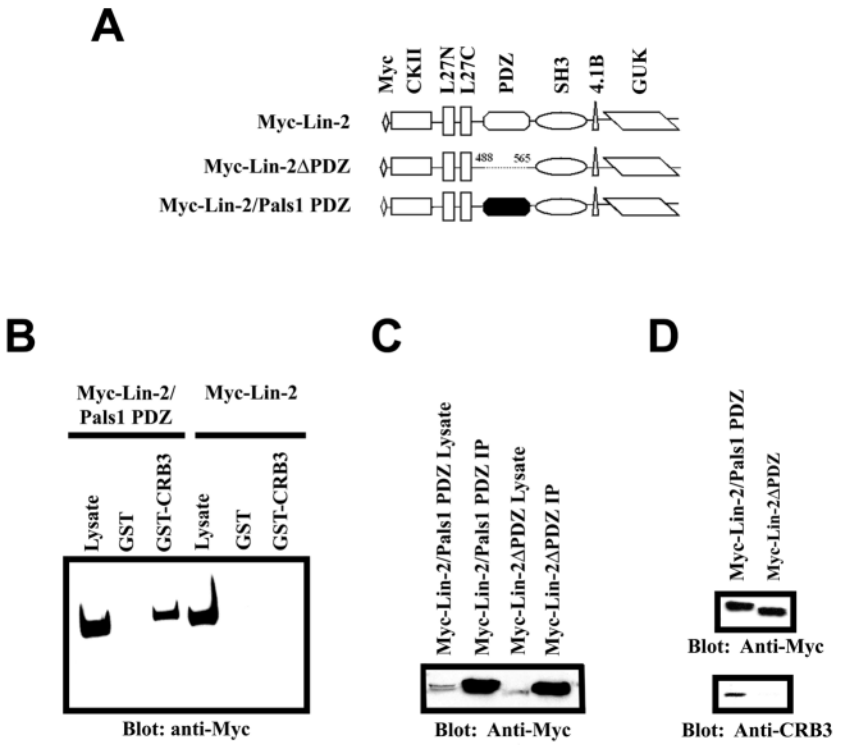

Fig. 5. Biochemical analysis of the Myc-Lin-2/Pals1 PDZ dominant-negative chimeric protein. (A) Schematic diagram illustrating the domain organization of wild-type Myc-Lin-2, Myc-Lin-2 missing the PDZ domain (Myc-Lin-2ΔPDZ), and the Myc-Lin-2 in which the PDZ domain was replaced with the Pals1 PDZ domain (shown in black). Recognized domains are denoted above the protein schematics: CKII, calmodulin serine/threonine kinase II like domain; L27, Lin-2/Lin-7 domain; PDZ, PSD-95/Discs Large/ZO-1 domain; SH3, Src homology 3 domain; 4.1B, protein 4.1 binding domain; and GUK, guanylate kinase domain. (B) Full length Myc-Lin-2 and the Myc-Lin-2/Pals1 PDZ chimera constructs were individually expressed in HEK293 cells. Pulldown experiments with GST (negative control) and GST fused to the last 20 residues of CRB3 were performed on the lysates prepared from the transiently transfected 293 cells. (C) Lysates were prepared from MDCK stable cell lines expressing the indicated proteins. Lysate proteins and anti-Myc immunoprecipitates were resolved by SDS-PAGE and the proteins visualized via anti-Myc immunoblot. (D) Myc-Lin-2/Pals1 PDZ chimeric protein and Myc-Lin-2 missing the PDZ domain were immunoprecipitated from MDCK cells. Co-precipitated endogenous CRB3 was revealed by immunoblotting with anti-CRB3 antisera.

surface in both the Myc-Lin-2–Pals1 PDZ and the Myc-Lin-2ΔPDZ cell lines. Strikingly, however, some E-cadherin was seen to localize to the apical membrane in a fraction of the MDCK cells expressing the Myc-Lin-2/Pals1 PDZ chimera. This was especially evident within the first 24 hours post-calcium switch (Fig. 6B,C). In contrast, this effect was not observed in the Myc-Lin-2ΔPDZ control cell line.

The above observations prompted us to examine the apical membrane of these two cell lines. Therefore, we co-stained the MDCK cell monolayers expressing Myc-Lin-2/Pals1 PDZ and Myc-Lin-2ΔPDZ with antibodies directed against CRB3 and gp135 (Fig. 6C). At 24 hours, all of the Myc-Lin-2ΔPDZ expressing cells exhibited apical CRB3 and gp135 staining. In contrast, these proteins were absent from the apical surface in a fraction of cells expressing the chimera. The absence of these apical proteins seemed to correlate with positive E-cadherin staining at the apical surface.

These results suggest that perturbing the endogenous Pals1/CRB3 interaction leads to apical membrane defects. To further assess the effect of expressing Myc-Lin-2/Pals1 PDZ on apico-basal polarity, we cultured cells expressing the chimera and Myc-Lin-2ΔPDZ (control) in collagen gels. The latter cells developed into normal cysts (Fig. 7A) similar to parental MDCK cell cysts (Fig. 4A). Cells expressing the chimera, however, formed disordered multicellular aggregates that lacked single continuous lumina. Specifically, gp135 appeared in discontinuous patches (Fig. 7B), which is consistent with the previous observation that a fraction of cells in monolayers expressing the chimera lack gp135-positive plasma membranes. Collectively, these results highlight the functional importance of the CRB3/Pals1 association in apical surface determination in mammalian epithelial cells.

Discussion

We recently demonstrated that an evolutionarily conserved complex consisting of Pals1, PATJ and Crumbs exists in mammalian epithelia (Roh et al., 2002b). A similar tripartite complex containing Stardust, Discs lost and Crumbs exists in *Drosophila* epithelial cells (Medina et al., 2002a; Tepass, 2002). In both systems, Pals1/Stardust serves as an adaptor protein that mediates the indirect interaction between PATJ/Discs lost and Crumbs. The Crumbs-Pals1-PATJ and Crumbs–Stardust–Discs-lost complexes both localize to a discrete region of the plasma membrane where the apical and basolateral membranes meet (TJs and subapical zone, respectively).

Fly embryos lacking Crumbs, Stardust, and Discs lost all exhibit defects in epithelial polarity. It has also been shown that Crumbs and Stardust are essential for zonula adherens formation as embryos missing either protein fail to form this structure from spot adherens junctions during early epithelial morphogenesis (Grawe et al., 1996; Klebes and Knust, 2000). Since the zonula adherens represents the most apical cell junction, it could be inferred that Crumbs and Stardust play important roles in demarcating the apical surface and determining the final localization of the zonula adherens. Strikingly, overexpression of Crumbs also leads to a disruption of the zonula adherens, expansion of the apical membrane, induction of multilayered epithelia, and embryonic lethality (Klebes and Knust, 2000; Wodarz et al., 1995). These data suggest that Crumbs is an apical polarity determinant and that the regulation of its expression is crucial for proper establishment of epithelial polarity.

The first reported mammalian homologue of Crumbs was Crumbs1 (CRB1). CRB1 is expressed highest in the retina and neuronal tissues (den Hollander et al., 2002; den Hollander

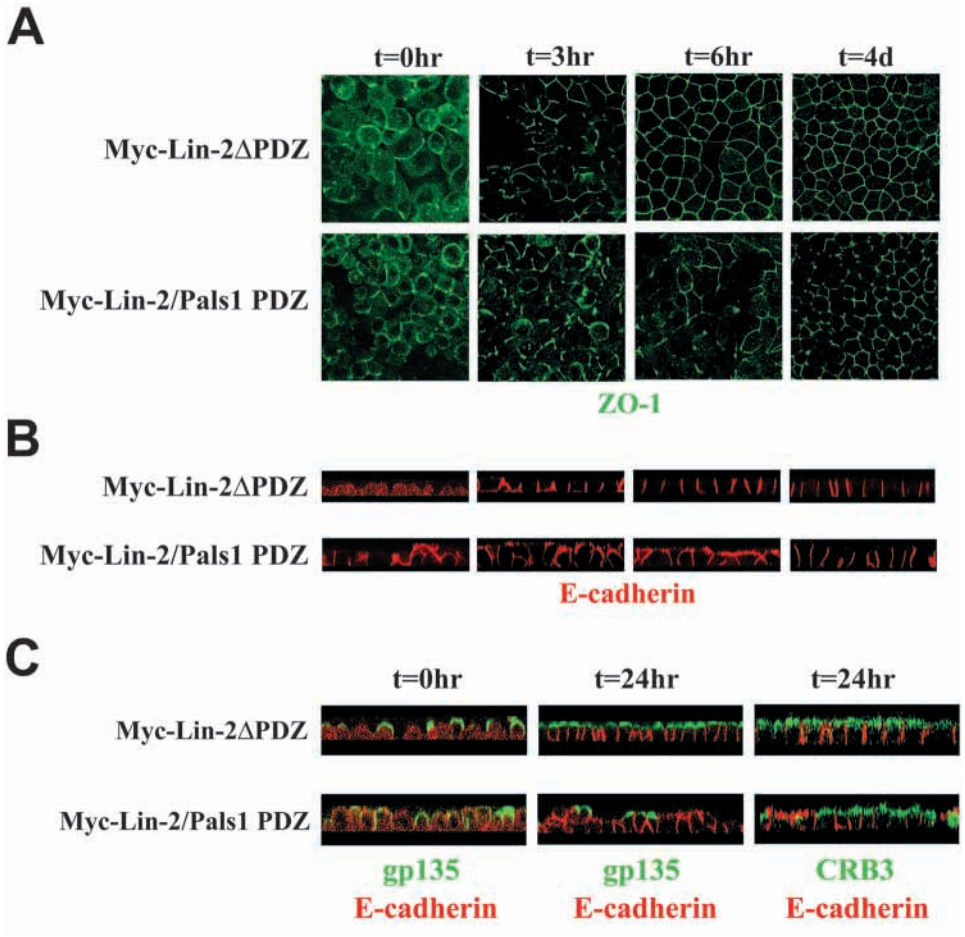

Fig. 6. Effects of Myc-Lin-2/Pals1 PDZ chimera expression on junctional assembly and apico-basal polarity in MDCK monolayers. (A-B) MDCK stable cell lines expressing either Myc-Lin-2/Pals1 PDZ or Myc-Lin-2ΔPDZ (negative control) were subjected to a calcium switch experiment as in Fig. 3. At the indicated timepoints, cells were co-immunostained with anti-ZO-1 and anti-E-cadherin antibodies (green and red, respectively). (C) MDCK cells expressing the indicated proteins were grown in low calcium media (t=0) or in normal calcium media for 24 hours. Subsequently, they were co-stained with antibodies directed against E-cadherin (red) and either gp135 or CRB3 as indicated in green.

et al., 1999). Interestingly, when CRB1 was exogenously expressed in MDCK renal epithelial cells, it co-localized with Pals1 and PATJ at TJs thereby illustrating for the first time that a conserved Crumbs complex could exist in mammalian epithelia (Roh et al., 2002b). With the sequencing of the human genome, two other putative Crumbs genes were recognized and designated as *CRB2* and *CRB3* (Medina et al., 2002a; Tepass et al., 2001). Recently, we reported the cloning of *CRB3* and its native expression in mammalian epithelial cells (Makarova et al., 2003). Unlike the distribution of exogenously expressed CRB1, endogenous CRB3 localizes not only to TJs but also to the apical plasma membrane. On the basis of its subcellular distribution, it seemed likely that CRB3 could serve as an apical polarity determinant and the functional Crumbs homologue in mammalian epithelial cells.

We initially studied CRB3 function by isolating and examining MDCK cells that expressed high levels of CRB3 relative to endogenous levels. In these cells, the localization of two apical markers extended to the basolateral surface domain indicative of apical surface expansion. This is in agreement with previous studies on *Drosophila* Crumbs overexpression. Next, we sought to determine the effects of CRB3

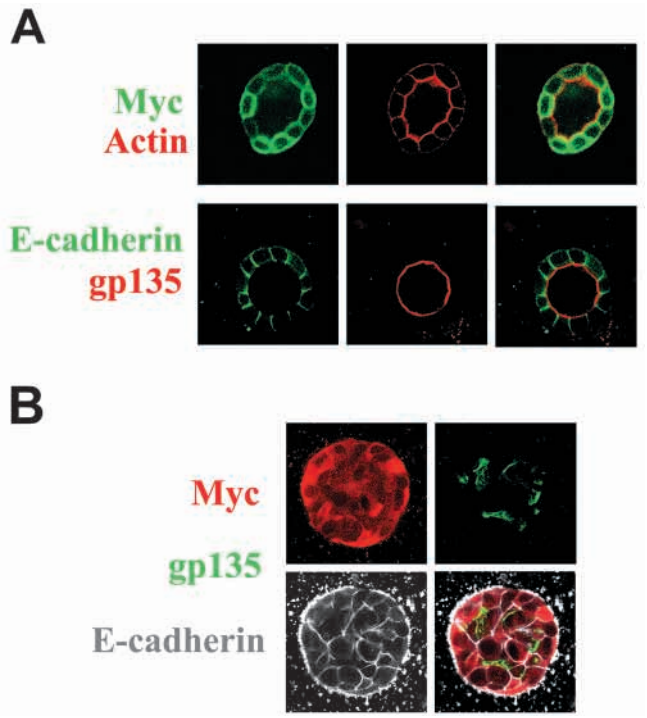

Fig. 7. Effects of Myc-Lin-2/Pals1 PDZ chimera expression on apico-basal polarity in MDCK cysts cultured in collagen gels. (A) Cysts derived from MDCK cells expressing Myc-Lin-2ΔPDZ were grown in collagen matrix and co-stained with either anti-Myc/rhodamine-phalloidin or anti-E-cadherin/anti-gp135 antibodies. (B) In parallel, cysts derived from MDCK cells expressing the Myc-Lin-2/Pals1 PDZ chimeric protein were co-immunostained with the indicated antibodies.

overexpression on the formation of cell junctions. Mammalian epithelial cell lines represent a useful model system to examine the sequential assembly of adherens junctions and TJs using a calcium switch assay (Rajasekaran et al., 1996). We observed that in wild-type and CRB3 overexpressing MDCK cells, adherens junctions were formed at relatively similar rates. In contrast, TJ biogenesis was significantly delayed in the cells overexpressing CRB3. The ability of overexpressed CRB3 to inhibit TJ formation depended only on the last four residues (ERLI). This motif is responsible for binding the PDZ domain of Pals1, and MDCK cells expressing elevated levels of CRB3 missing this sequence formed TJs in a timely manner. Thus, high Crumbs expression in mammalian and fly epithelia seems to negatively regulate the formation of the cell junction positioned closest to the apical surface.

Although TJs were disrupted, the overall polarity of MDCK monolayers overexpressing CRB3 was not dramatically affected. Specifically, the majority of CRB3, ezrin, and gp135 were localized apically, whereas E-cadherin was still present at the lateral membrane. Thus, in spite of apical surface expansion, overall apico-basal asymmetry was maintained. This could be because of the presence of a free surface and cell-substratum/cell-cell contacts providing sufficient cues to establish polarity in the monolayer independent of the Crumbs complex (Yeaman et al., 1999b).

The establishment and maintenance of mammalian epithelial cell polarity has also been addressed from a different experimental angle – the growth of MDCK cysts in a collagen matrix. The utility of three-dimensional culture as a sensitive system to examine molecular polarity signals is becoming increasingly appreciated. For instance, the expression of dominant-negative Rac1 (Rac1-N17) does not affect polarity of MDCK cell monolayers; however, cysts derived from these cells in collagen gels exhibit an inversion of polarity (O'Brien et al., 2001). MDCK cells overexpressing CRB3 do not develop into polarized cysts; instead, they form non-polarized multicellular aggregates. This is in contrast to cysts derived from parental MDCK cells or cells expressing CRB3 missing the Pals1 PDZ-binding motif; these cysts exhibit relatively normal apico-basal polarity. These results suggest that Pals1 is an important downstream mediator of the CRB3 overexpression phenotypes. This is in agreement with previous studies in *Drosophila* that established that *crumbs* functions upstream of *stardust* (the *Drosophila* Pals1 orthologue) in a common genetic pathway (Tepass and Knust, 1993).

Two other mutant versions of CRB3 were exogenously expressed above endogenous levels in this study: CRB3N→D and CRB3FERMmut. MDCK cells overexpressing either of these two proteins exhibited similar degrees of TJ assembly and polarity disruption as those overexpressing wild-type CRB3. This suggests that N-glycosylation of the extracellular domain or presence of an intact intracytoplasmic FERM-binding region do not play significant roles in the CRB3 overexpression phenotype. This is in agreement with studies using Myc-Crumbs-intra, a truncated version of Crumbs in which the large extracellular portion of Crumbs is replaced with a Myc epitope tag, and Myc-Crumbs-intra harboring point mutations in the FERM domain. Flies expressing high levels of either of these proteins in a wild-type background exhibit similar polarity phenotypes as those overexpressing full length Crumbs (Klebes and Knust, 2000).

The exact role of the N-glycosylation of CRB3 is not known; however, it was recently reported that the FERM domain of Crumbs binds D-moesin in flies (Medina et al., 2002b). Crumbs overexpression in flies leads to D-moesin redistribution suggesting that Crumbs could be linked to the apical cortical cytoskeleton. In our study, we observed that high CRB3 expression leads to the redistribution of ezrin raising the possibility that the FERM domain of CRB3 could bind ezrin. However, attempts to demonstrate a physical interaction between these two proteins via co-immunoprecipitation assays have not been successful (data not shown). Consequently, the identity of the binding partner for the CRB3 FERM domain remains to be elucidated. In *Drosophila* lacking Crumbs, exogenous Crumbs expression is able to rescue the embryonic phenotype. Here, both the FERM domain and the extreme C-terminal PDZ-binding motif are required. Hence, it can be inferred that the FERM domain, in concert with the Pals1 PDZ-binding sequence, could play some role during the establishment and/or maintenance of mammalian epithelial polarity.

Another issue that remains unresolved is the moiety of CRB3 that mediates its trafficking to the apical membrane. Myc-CRB3 and the three mutants tested in this study all targeted to the apical surface. It is somewhat surprising that the Myc-CRB3ΔERLI protein, which does not associate with Pals1, still distributes apically suggesting that a Pals1-independent mechanism underlying CRB3 targeting exists. In *Drosophila* expressing Crumbs missing the last 23 residues (*crb*^{8F105} gene product), this mutant protein displays a diffuse cytoplasmic localization pattern in some ectodermally derived epithelia such as the epidermis and pharynx. However, in other epithelia including those found in the salivary glands and Malpighian tubules, this truncated Crumbs is expressed exclusively to the apical membrane (Knust et al., 1993). Coincidentally, in *stardust* mutant flies apical Crumbs expression is maintained in these same tissues (Tepass and Knust, 1993). Therefore, further mutations need to be made in CRB3 to determine the exact residue(s) involved in CRB3 membrane trafficking in mammalian epithelia.

CRB3 overexpression studies have illustrated a functional link between CRB3 and Pals1. To confirm the importance of the CRB3-Pals1 association during epithelial polarization, we next sought to disrupt this interaction in MDCK cells. We initially addressed this task by attempting to express only the PDZ domain of Pals1. However, we were not able to isolate stable clones expressing the Pals1 PDZ domain. Thus, we employed an alternative strategy in which the Pals1 PDZ domain would be expressed as a part of a more stably expressed protein. Thus, we decided to express a Myc-Lin-2/Pals1 PDZ chimera as expression of exogenous Myc-Lin-2 or its various deletion mutants that do not disrupt apico-basal polarity (Lee et al., 2002). This chimera, in contrast to Myc-Lin-2 or Myc-Lin-2 missing the PDZ domain, was able to co-immunoprecipitate CRB3 confirming that CRB3 can associate with the PDZ domain of Pals1. Expression of this chimera resulted in a disruption of TJs in MDCK monolayers as the ZO-1 staining pattern was fragmented. In addition, these cells exhibited apical membrane defects in a cell autonomous fashion, a phenomenon that correlated with the presence of the lateral membrane marker, E-cadherin, to the apical surface. In *stardust* mutant flies, apical surface defects are also detected. Specifically, the

apical marker, Stranded at Second, is absent from cells lacking Stardust expression (Bachmann et al., 2001). Collectively, these results suggest that the CRB3/Pals1 complex and other proteins associated with it could be important for establishing apical membrane identity, influencing the distribution of lateral proteins, and the coordinated assembly of continuous TJ fibrils at apico-lateral membrane thereby demarcating the apical and basolateral surfaces.

What are the exact mechanisms through which the CRB3/Pals1 interaction influences the above processes during epithelial cell polarization? In MDCK cells overexpressing CRB3 or the Lin-2–Pals1 PDZ chimeric protein, endogenous Pals1 localized predominantly to the fragmented TJs (Fig. 3C, data not shown) suggesting that Pals1 mislocalization or sequestration alone cannot account for the phenotypic effects observed throughout this study. Pals1 consists of multiple protein-protein interaction domains and some of their binding partners are known. For instance, Pals1 contains two Lin-2/Lin-7 (L27) heterodimerization domains, L27N and L27C. These domains bind to the L27 domains of PATJ and mLin-7, respectively (Kamberov et al., 2000; Roh et al., 2002b). We recently demonstrated that the N-terminal portion of Pals1, probably the U1 domain, binds to the Par6-Par3-aPKC polarity complex through the direct interaction with Par6 (Hurd et al., 2002). It is possible that the CRB3-Pals1 association influences the binding of Pals1 to its partners (e.g. PATJ and/or Par6) and that these dynamic interactions regulate various stages of polarized membrane trafficking, junction formation, and demarcation of apical and basolateral membrane subdomains. Furthermore, although a CRB3-Pals1-PATJ complex localizes to TJs of fully polarized epithelial cells (Makarova et al., 2003), it is not absolutely certain where the functionally relevant CRB3-Pals1-containing complexes exist.

Polarity proteins acting downstream of Stardust are not well known seeing that Discs lost is the only known binding partner of Stardust besides Crumbs. We have partially addressed this issue in mammalian epithelia by analyzing MDCK cells expressing a Myc-PATJ (1-238) dominant-negative protein. We were able to show in these cells that endogenous Pals1 and aPKCζ were mislocalized away from TJs (Hurd et al., 2002). Interestingly, TJ biogenesis was also disrupted suggesting that the Par6/Par3/aPKC complex could represent important effectors that lie downstream of CRB3/Pals1. This is supported by studies demonstrating the intimate relationship between Par6/Par3/aPKC and TJ formation (Gao et al., 2002; Hirose et al., 2002; Izumi et al., 1998; Joberty et al., 2000; Ohno, 2001; Yamanaka et al., 2001). Further experiments will have to be performed to determine the relative contributions of Par6 and PATJ containing complexes during TJ biogenesis and the establishment of polarity in epithelial cells. Nonetheless, the results of this study provide initial insight into the importance of the CRB3 and its associated proteins in mammalian epithelial cell biology.

We would like to thank Stephanie Laurinec for the affinity purification of antibodies. We are grateful to George Ojakian and Ian Macara for providing reagents used in this study. We also thank the Morphology and Image Analysis Core at the University of Michigan for permitting us to use their confocal microscope. This work was partially supported by NIDDK, National Institutes of Health Grant DK58208. M.H.R. was supported by the Medical Scientist Training Program Grant T32 GM07863 and the Genetics Predoctoral Training Program Grant T32 GM07544024 to the University of Michigan. B.M. is an investigator of the Howard Hughes Medical Institute.

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