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A Rich1/Amot Complex Regulates the Cdc42 GTPase and Apical-Polarity Proteins in Epithelial Cells

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SUMMARY

Using functional and proteomic screens of proteins that regulate the Cdc42 GTPase, we have identified a network of protein interactions that center around the Cdc42 RhoGAP Rich1 and organize apical polarity in MDCK epithelial cells. Rich1 binds the scaffolding protein angiomotin (Amot) and is thereby targeted to a protein complex at tight junctions (TJs) containing the PDZ-domain proteins Pals1, Patj, and Par-3. Regulation of Cdc42 by Rich1 is necessary for maintenance of TJs, and Rich1 is therefore an important mediator of this polarity complex. Furthermore, the coiled-coil domain of Amot, with which it binds Rich1, is necessary for localization to apical membranes and is required for Amot to relocalize Pals1 and Par-3 to internal puncta. We propose that Rich1 and Amot maintain TJ integrity by the coordinate regulation of Cdc42 and by linking specific components of the TJ to intracellular protein trafficking.

INTRODUCTION

Dynamic cellular organization depends on the selective interactions of signaling proteins. These interactions are commonly mediated by specific protein domains and are frequently controlled by protein phosphorylation and GTPases that toggle between active and inactive conformations. These simple devices can be exploited to build multiprotein networks that yield complex properties, such as cell polarity. Here, we explore a novel protein complex that is integrated with the mammalian epithelial polarity network and influences the activity of the Cdc42 GTPase, the formation of tight junctions (TJs), and cell morphology.

The plasma membrane of epithelial cells is asymmetrically organized into apical and basolateral regions, which have distinct protein and lipid compositions and are separated in vertebrates by a dense network of protein strands mainly composed of claudins and occludins. These apical strands encircle the cell and make lateral contact with neighboring cells, thus forming the TJ, which provides a paracellular barrier to the movement of ions (Yeaman et al., 1999).

Recent data have identified a conserved series of protein complexes that control cell polarity in metazoans. In epithelial cells, these complexes are spatially segregated along the apical-basolateral axis and impart discrete properties to separate regions of the cell. Distinct complexes also interact with one another, either to promote an aspect of polarity or to restrict the actions of polarity proteins to particular domains of the cell. The protein-protein interactions in this polarity network are typically mediated by PDZ domains and are regulated by serine/threonine phosphorylation and Rho-family GTPases. This is exemplified by two dynamic protein assemblies, the Crumbs and Par-3 complexes, which are important for establishing and maintaining apical junctions and defining apical identity (Macara, 2004; Roh and Margolis, 2003).

Mammalian Crumbs3 is an apically localized transmembrane protein that binds through a C-terminal motif to the PDZ domain of Pals1 (a MAGUK protein) or Patj (a protein with ten PDZ domains) (Roh and Margolis, 2003). Patj and Pals1 also interact directly through heterodimerization of their N-terminal L27 domains (Roh et al., 2002). Mammalian Patj also binds the TJ components ZO-3 and claudin through its PDZ domains and can thereby be recruited into the TJ complex. In mammalian epithelia, Pals1 (Straight et al., 2004), Patj (Shin et al., 2005), and Crumbs3 (Roh et al., 2003) are all necessary for proper TJ integrity.

The Par-3 complex is located subapically. Par-3 itself contains three PDZ domains, through which it associates with the PDZ domain of Par-6 (Joberty et al., 2000; Lin et al., 2000) and the C termini of the adhesion proteins Jam-1 (Ebnet et al., 2001) or Nectin (Takekuni et al., 2003). Par-6 recruits both the atypical protein kinase C (aPKC) λ and the GTP bound form of Cdc42 (Joberty et al., 2000;

Lin et al., 2000), which stabilizes a functional conformation of the Par-6 PDZ domain (Garrard et al., 2003). In mammalian cells, Pals1 provides a direct link between the Crumbs and Par-6/Par-3 complexes (Wang et al., 2004).

Cdc42 is a member of the Rho GTPase family, whose members interact with effectors when bound to GTP and terminate signaling upon GTP hydrolysis. Cdc42 signaling is therefore enhanced by guanine exchange factors (GEFs) that stimulate nucleotide release and consequent GTP binding and is inhibited by GTPase-activating proteins (GAPs) that promote nucleotide hydrolysis (Nobes and Hall, 1994). Cdc42 and proteins that regulate its GTPase cycle potentially act early in establishing cell polarity. Cdc42 was first identified in yeast as a mutant allele that disrupts bud formation and polarized distribution of the actin cytoskeleton (Johnson and Pringle, 1990). In *Drosophila*, expression of constitutively active (CA) or dominant-negative (DN) Cdc42 mutants after cellularization causes defects in epithelialization and a loss of polarity (Hutterer et al., 2004). Similarly, overexpression of either CA or DN Cdc42 in mammalian MDCK epithelial cells disrupts polarity by inducing increased paracellular permeability and mixing of apical and basolateral components, consistent with a disruption of TJs (Rojas et al., 2001).

Because Cdc42 participates in a wide variety of cellular processes, including cell division, microtubule orientation, actin reorganization, and protein trafficking (Johnson, 1999), it is difficult to delineate the precise role of Cdc42 in epithelial polarity using approaches that impact global Cdc42 signaling. Furthermore, Cdc42 belongs to a subset of Rho GTPases that potentially have overlapping functions (Czuchra et al., 2005). One approach to this conundrum involves analysis of the GEFs and GAPs that regulate Cdc42 activity. Rho-family GEFs and GAPs are more numerous than the GTPases themselves and typically contain interaction domains through which they are directed to specific subcellular locations and may recruit upstream regulators, downstream targets, and cytoplasmic scaffolds (Peck et al., 2002; Rossman et al., 2005). As a consequence, specific Rho GEFs and GAPs may each target a discrete subpopulation of Cdc42 that undertakes a specialized function. We therefore reasoned that we could probe Cdc42 function at TJs by studying its local regulators.

In a systematic screen of RhoGEFs and RhoGAPs, we identified a Cdc42-selective GAP, Rich1, that localizes to TJs and adherens junctions (AJs), associates with components of the apical-polarity network, and maintains the integrity of TJs in epithelial cells through its regulation of Cdc42. By examining the binding partners of proteins that associate with Rich1, we define a series of overlapping polarity complexes that regulate the maintenance of TJs.

RESULTS

Rich1 Is a GAP for Cdc42 that Associates with Tight Junctions in Epithelial Cells

To identify modulators of Cdc42, we transiently expressed >50 putative mammalian RhoGEFs and 50 RhoGAPS in HEK293T and/or NIH 3T3 cells and measured their intracellular specificity for RhoA, Rac1, and Cdc42 using fluorescence resonance energy transfer (FRET) based probes (data not shown). Proteins that altered the GTPase cycle of Cdc42 were analyzed in parallel for binding to known polarity proteins by stably expressing each RhoGEF or RhoGAP in a HEK293T-derived (Phoenix) cell line and analyzing their coprecipitating proteins by tandem mass spectrometry (MS/MS). Together, these assays identified Rich1 as a Cdc42 GAP that associates with apical polarity components (Figure 1).

We analyzed Rich1 GAP activity in epithelial cells by two approaches. Using Raichu FRET probes (Itoh et al., 2002; Yoshizaki et al., 2003), full-length (FL) Rich1 reduced the fraction of Cdc42 bound to GTP in 293T cells but had no measurable effect on Rac1 or RhoA (Figure 1A). We also analyzed the effects of Rich1 on endogenous levels of GTP bound Cdc42 and Rac1 in MDCK cells by precipitation with the GTPase binding domain (GBD) of the Pak kinase. For this purpose, we used clonal MDCK cells that stably overexpressed wild-type (wt) Rich1, a GAP-deficient mutant of Rich1 (R288A), or a control line. wt Rich1 reduced the levels of Cdc42 GTP by over 70% with no effect on Rac1. The GAP-deficient mutant of Rich1 modestly elevated Cdc42-GTP with no significant effect on the level of Rac1-GTP (Figures 1B and 1C). Taken together, Rich1 has selective GAP activity toward Cdc42 in epithelial cells.

Rich1 (also termed Nadrin) contains an N-terminal BAR domain (Richnau et al., 2004), a RhoGAP domain, and an ~300 amino acid C-terminal tail with multiple proline-rich motifs capable of binding SH3 domains (Richnau and Aspenstrom, 2001) (see below; see also Figure S6A in the Supplemental Data available with this article online). To identify endogenous proteins that associate with Rich1 in epithelial cells, we constructed three clonal cell lines in which Rich1, containing an N-terminal triple-Flag epitope (Flag-Rich1), was stably expressed at low, medium, and high levels (data not shown). Lysates from these three lines were combined and then immunoprecipitated to capture Rich1 and associated proteins, which were identified through peptide sequencing by MS/MS (Figure 1D).

This analysis showed that Rich1 associates with polarity proteins. The most abundant Rich1-associated protein migrated at 85 kDa and yielded 31 peptides matching 46% of the sequence for Amot. This isoform of Amot has an N-terminal coiled-coil structure and a predicted C-terminal PDZ-domain binding motif. In addition, the polarity protein Pals1 and its binding partner Patj were identified (Figure 1D and Table S1). These interactions appear specific, as we did not see these proteins associate with other RhoGAPs such as chimerin (Table S1). Because Pals1 and Patj are interconnected with the Par-3/Par-6/aPKC complex, we tested whether these latter proteins also coprecipitate with Flag-Rich1. Immunoblotting revealed aPKC and the 100 kDa isoform of Par-3, but not Par-6, in Rich1 immunoprecipitates (Figure 1E). Par-3 and aPKC likely associate with Rich1 at lower levels than Pals1 and Patj since they were not identified by MS.

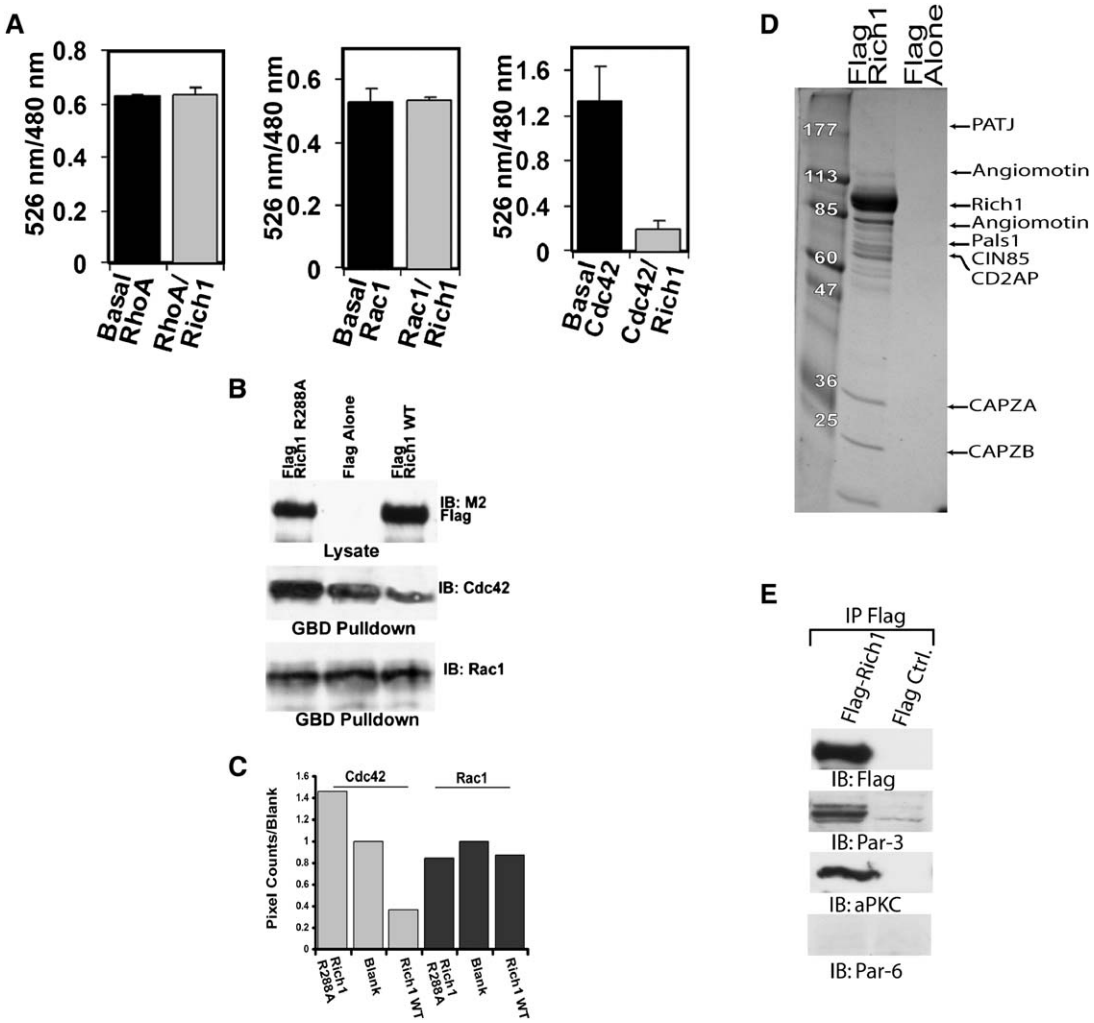

Figure 1. Rich1 Is a Cdc42 GAP that Associates with Signaling Components of the Tight Junction

(A) The effects of transient expression of FL Rich1 on the intracellular levels of GTP bound RhoA, Rac1, and Cdc42 Raichu probes were measured. The normalized FRET peak (526 nm) over the normalized non-FRET peak (480 nm) was then plotted for each condition. Values are representative of three independent experiments performed in triplicate. Error bars correspond to the standard deviation between individual measurements.

(B) Endogenous levels of GTP bound Rac1 and Cdc42 in MDCK cells stably expressing the indicated constructs were assessed with the GBD domain of hPak1. Expression of each protein was determined by immunoblot (IB) analysis with anti-Flag (M2) antibody (top panel). Relative amounts of endogenous GTP bound Cdc42 (middle panel) and GTP bound Rac1 (bottom panel) precipitated from 1 mg of lysates by the GST-GBD beads were determined using the indicated antibodies.

(C) Pixel intensities of bands in the middle (Cdc42, gray bars) or lower panels (Rac1, black bars) in (B) were plotted over the pixel intensities of the Flag alone (Blank) controls.

(D) Colloidal Coomassie-stained proteins coimmunoprecipitated with Flag-Rich1 and their identities as determined by MS/MS.

(E) Immunoblots were probed with the indicated antibodies following Flag immunoprecipitations from cells expressing Flag-Rich1 or a Flag control.

We also found endocytic proteins associated with Rich1. These included the adaptors CIN85 and CD2AP, which directly bind each other and target the EGF (Soubeyran et al., 2002) and HGF (Petrelli et al., 2002) receptors for endocytosis, as well as the actin capping proteins CAPZα and CAPZβ, known binding partners of CD2AP (Hutchings et al., 2003) (Figure 1D and Table S1).

Since Rich1 interacts with polarity proteins that associate with TJs, we examined Rich1 localization in polarized MDCK cells using an antibody raised against Rich1. This revealed that endogenous Rich1 associates with membranes (Figure S1A) and concentrates at sites of cell-cell contact, as well as showing a diffuse intracellular punctate stain (Figure 2A, top panel). The antibody is specific since Rich1 immunoblotting and immunofluorescence were reduced in Rich1-silenced MDCK cells (Figures S2A and S2B). Analysis of endogenous Rich1 staining along the apical-basolateral axis of wt MDCK cells revealed a concentration of Rich1 that overlapped with the basal parts of ZO-1 and with E-cadherin and β-Catenin (Figures 2A

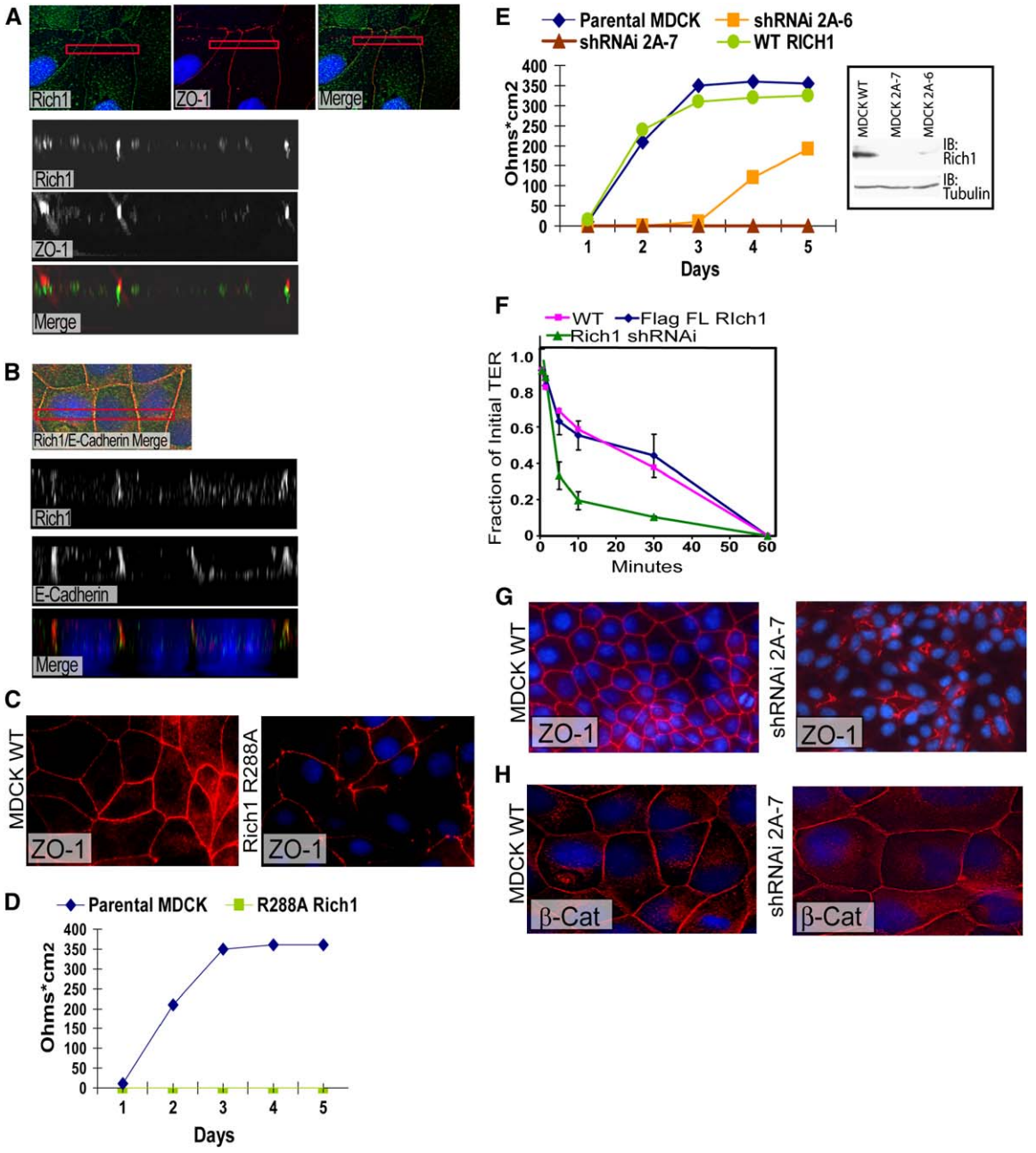

Figure 2. Rich1 Is Necessary for TJ Integrity

(A) Immunofluorescence of endogenous Rich1 (top left panel) and ZO-1 (top middle panel) in polarized MDCK cells. The boxed region was deconvolved to show Rich1 (second panel) and ZO-1 (third panel) along the apical-basolateral axis. The merge of Rich1 (green) and ZO-1 (red) staining is visualized in the bottom panel.

(B) MDCK cells were stained with Rich1 (green) and E-cadherin (red) (top panel) antibodies. Deconvolved Z stack images of boxed regions show Rich1 (second panel), E-cadherin (third panel), and a merge (bottom panel).

(C) MDCK cells stably expressing Flag alone or Flag-Rich1 (R228A) stained with antibodies against ZO-1 (red).

(D) TER measurements of MDCK cells stably expressing Rich1 (R288A) (green boxes) and the parental MDCK cells (blue diamonds) 24 hr after plating on transwell filters.

(E) TER measurements as in (D) for MDCK cells expressing wt Flag-Rich1 (green circles), shRNAi Rich1 clone 2A-7 (brown triangles) or clone 2A-6 (orange boxes), or control cells (blue diamonds). Inset box: immunoblots of Rich1 (top panel) and tubulin (bottom panel) in cellular lysates (50 μg of protein) from the indicated cell lines.

(F) TER measurements from MDCK cells stably expressing Rich1 shRNAi (clone 2A-6) (green triangles), FL Flag-Rich1 (pink boxes), or control cells (blue diamonds) following addition of 250 μM EDTA. Results were plotted as the fraction of TER over the TER before EDTA addition. Values in are representative of three independent experiments performed in triplicate. Error bars correspond to the standard deviation between individual measurements.

and 2B and Figures S1B and S1C). Thus, Rich1 codistributes with markers for both the TJ and AJ.

The GAP Activity of Rich1 Is Required for Proper TJ Maintenance

Since Rich1 is a Cdc42 GAP that localizes to the TJ and AJ, we addressed the effects of reducing or increasing its expression on TJ integrity in MDCK cells. Clonal cell lines stably expressing wt Flag-Rich1, or a mutant of Rich1 lacking the N-terminal 240 amino acids comprising the BAR domain (ΔBAR), displayed a morphology indistinguishable from the parental MDCK cells (data not shown). However, MDCK cells stably expressing a GAP-deficient mutant of Rich1 (R288A) grew slowly and had a de-epithelialized morphology, interspersed with patches of cells with identifiable TJs, as revealed by ZO-1 staining (Figure 2C). To quantitatively assess the integrity of TJs, we measured the transepithelial electrical resistance (TER) of wt MDCK cells, MDCK cells stably overexpressing wt Flag-Rich1, Flag-Rich1 ΔBAR (data not shown), or Flag-Rich1 (R288A). All had TER in excess of 300 ohms × cm^2 2 days after plating (Figure 2D), except monolayers of cells expressing the R288A GAP-deficient mutant, which had no appreciable resistance even after culturing for 5 days (Figure 2D). The slower growth rate of the MDCK R288A cells does not account for their inability to form TER since they still failed to develop any resistance when plated at a higher density (data not shown). To assess the specificity of the defects on TJs induced by Rich1 R288A, we also constructed stable MDCK lines that expressed a distinct Cdc42-specific RhoGAP, chimerin, or a mutant of chimerin (R304A) predicted to lack GAP activity. Both of these lines developed TER similar to wt MDCK cells (data not shown), and their TJs appeared normal by ZO-1 localization (Figure S2G).

To directly assess the involvement of Rich1 in TJ development and maintenance, we isolated two clonal MDCK cell lines (2A-6 and 2A-7) that stably express short hairpin (sh) RNAi to Rich1 and have a reduction in Rich1 expression of at least 60% and 90%, respectively (Figure 2E inset box; Figure S2A). Significantly, the 2A-7 line failed to form monolayers with any measurable TER (Figure 2E). The loss of TJ integrity correlated with the degree of Rich1 silencing since the 2A-6 line formed monolayers that developed TER, albeit more slowly than wt MDCK cells (Figure 2E). Since the defects in TJ structures in Rich1-deficient MDCK cells could reflect alterations in the formation, stability, and/or turnover of TJs, we examined the effects of calcium removal in monolayers of 2A-6 cells. The TER of 2A-6 MDCK cells declined more rapidly than for wt cells following chelation of calcium (Figure 2F), suggesting that Rich1 is important for maintaining the stability of TJs.

To gauge whether the defects observed in MDCK 2A-7 cells silenced for Rich1 correlated with improper localization of TJ proteins, these cells were fixed and stained 48 hr after plating with antibodies to the TJ proteins ZO-1, Par-3, and Pals1 and the AJ protein E-cadherin. All the TJ markers were mislocalized in 2A-7 cells. En face images showed ZO-1, Par-3, and Pals1 at fragmented spots and in circular structures in the 2A-7 (Figure 2G; Figures S2C and S2D) and R288A (data not shown) cell lines as opposed to their localization at cell-cell contacts in wt MDCK cells. Further analysis of ZO-1 staining along the apical-basolateral axis revealed that these circular structures extended into the basal regions of the cell (Figure S2F). Consistent with Rich1's functioning in the maintenance of TJs, we found that Par-3 was partially localized to cell-cell contacts in the MDCK 2A-7 cell line 12 hr after plating but was increasingly mislocalized by 24 and 36 hr (Figure S2H). Furthermore, the overall morphology of these cells became somewhat fibroblastic at these later times (Figure S2I). In contrast, E-cadherin localized normally in both the R288A (data not shown) and 2A-7 cell lines (Figure 2H), indicating a selective loss of TJs. Since overexpressing the R288A mutant of Rich1 gave a phenotype similar to silencing Rich1 expression, we surmise that this construct behaves in a dominant-negative manner and that proper regulation of Cdc42 or closely related GTPases by Rich1 is important for TJ integrity.

An Iterative Approach to Analyzing Rich1-Containing Signaling Complexes

To pursue how Rich1 is integrated into the network of TJ proteins, we used MS/MS to identify further binding partners for Rich1-associated proteins, including Amot and Par-3. To this end, we stably expressed human Amot (KIAA1071) (with an N-terminal triple Flag epitope) in 293T cells, followed by immunoprecipitation with anti-Flag antibodies. Endogenous Par-3 and associated proteins were immunoprecipitated from rat brain lysates. Immunoprecipitates of Amot (Figure 3A; Table S1) contained peptides for Rich1, Pals1, and Patj, providing additional evidence that these four proteins interact in a single complex. Amot also coprecipitated with several proteins that were not detected in Rich1 complexes, including a MAGUK protein (membrane protein, palmitoylated 7; MPP7), the multiple PDZ-domain protein (MUPP1), Amot-like 1 (AMOTL1, also termed junctionally enriched protein, or JEAP)(Nishimura et al., 2002), and Amot-like 2 (AMOTL2, also termed MASCOT) (Patrie, 2005) (Figure 3A; Table S1). These data suggest that Amot is a component of at least two complexes, only one of which contains Rich1.

To verify association of endogenous Rich1, Amot, and Par-3, we examined immunoprecipitations from lysates prepared from tissue culture or rat brains by immunoblot or MS analysis. Endogenous Rich1 coprecipitated with Amot from both 293T (Figure 3B) and rat brain (data not

(G) ZO-1 staining in wt MDCK cells (left panel) and Rich1 shRNAi (clone 2A-7) MDCK cells (right panel).
(H) β-catenin staining in wt MDCK cells (left panel) and Rich1 shRNAi (clone 2A-7) MDCK cells (right panel). Nuclei in (A), (B), (C), (G), and (H) are stained blue with Hoechst dye.

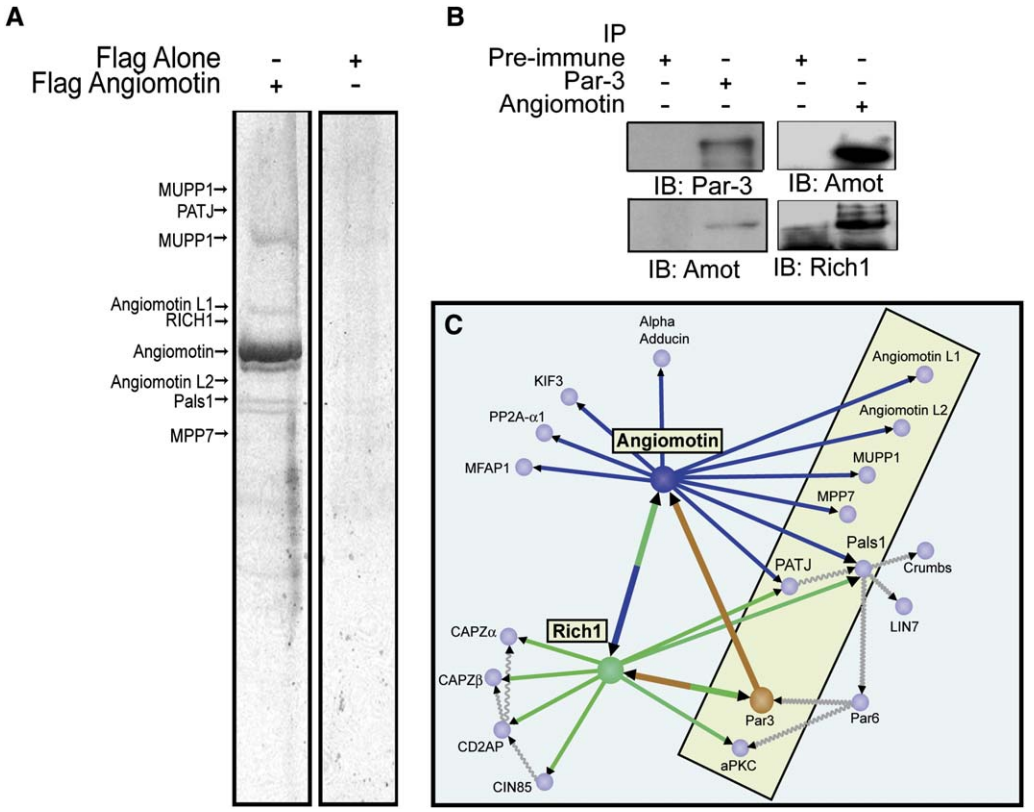

Figure 3. Expanding the Rich1 Interaction Network

(A) Representative image of a colloidal Coomassie-stained gel in which proteins precipitated with Flag-Amot from HEK293T were separated and identified by MS/MS.

(B) Lysates from rat brain (left panels) or HEK293T cells (right panels) were precipitated with the indicated antibodies and immunoblotted as labeled.

(C) Interaction map of proteins that coprecipitated with Rich1, Amot, and Par-3. Wavy lines indicate interactions previously reported in the literature.

shown) lysates. Furthermore, Amot was detected in an immunoprecipitation of Par-3 from rat brain lysate (Figure 3B), and analysis of a Par-3 immunoprecipitate from rat brain lysates by MS/MS detected two peptides matching sequences in Rich1, confirming that Rich1, Amot, and Par-3 associate in vivo (Table S1). These data indicate that Rich1, Amot, Pals1, Patj, and Par-3 form a specific complex in epithelial cells but can also contribute to other complexes with distinct components (Figure 3C).

The PDZ-Domain Binding Motif of Amot Interacts with Patj and Targets Amot to Tight Junctions

Sequence analysis has predicted two Amot isoforms that differ in the extent of their N termini (Moreau et al., 2005). Indeed, rabbit polyclonal antibodies to angiomotin detected two bands of ~130 kDa and 85 kDa in 293T cells (Figure 4A) and rodent brain (data not shown), consistent with the expression of both Amot isoforms. In MDCK cells, however, the 85 kDa isoform was more abundant (Figure 4A).

Amot has a potential PDZ binding motif (EYLI) at its C terminus (Figure S6A), which might recruit proteins such as Pals1 and Patj. Indeed, YFP-Amot separately precipitated Flag-Pals1 and Myc-Patj in 293T cells, whereas a mutant of Amot lacking the C-terminal 5 residues (ΔC-term) did not precipitate either protein (Figure 4B). While these data suggest that Amot interacts with the Pals1-Patj complex via its PDZ binding motif, they do not identify the primary binding partner for Amot because the L27 domains of Pals1 and Patj can heterodimerize (Roh et al., 2002). We therefore examined whether Amot could bind mutants of Patj or Pals1 that lack L27 domains (Patj 3-10PDZ or Pals1 PDZ, respectively) and consequently do not associate with each other. Because Amot bound Patj 3-10PDZ (Figure 4C) but not Pals1 PDZ (Figure S5), it is likely that the C-terminal motif of Amot interacts with one of the eight C-terminal PDZ domains of Patj and that Patj recruits Pals1 to Amot.

These results indicate that Amot might colocalize with the Pals1/Patj complex in polarized epithelial cells through its C terminus. We therefore examined the distribution of stably expressed YFP-Amot 85 kDa or YFP-Amot 85 kDa ΔC-term in MDCK cells. In addition to a diffuse distribution, YFP-Amot 85 kDa was enriched at regions of cell-cell contact where it colocalized with endogenous Par-3, ZO-1, and Pals1 (Figures 4D and 4F; Figure S4A); in

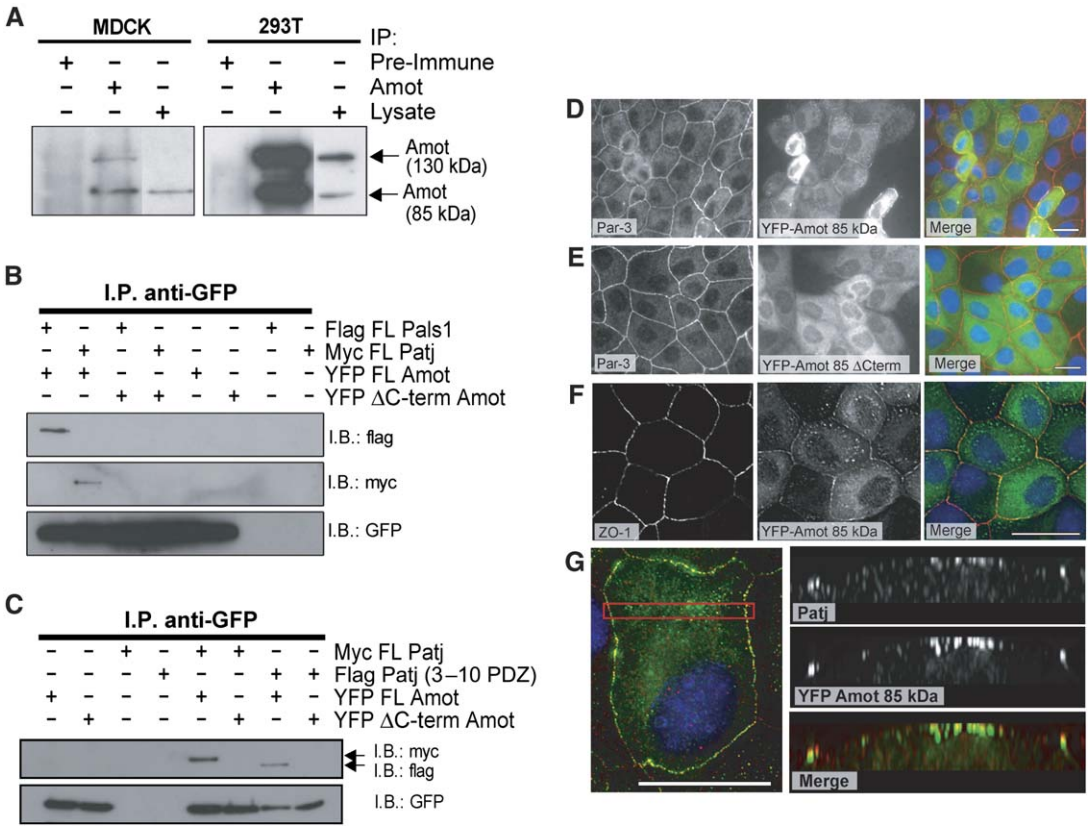

Figure 4. Amot Requires an Intact C Terminus to Associate with Patj and Cell-Cell Contacts

(A) Immunoprecipitations from MDCK (left panel) or HEK293T (right panel) cell lysates were blotted with Amot antibody.

(B) HEK293T cells transfected with the indicated constructs were lysed and immunoprecipitated with GFP antibody and visualized by immunoblot analysis with Flag (top panel), then reprobed with Myc (middle panel) and GFP (bottom panel) antibodies.

(C) Anti-GFP immunoprecipitates of lysates from HEK293T cells transfected as indicated were probed with Myc and Flag antibodies (upper panel) and reprobed with GFP antibodies (bottom panel).

(D–F) MDCK cells stably expressing YFP-Amot 85 kDa (D and F) or YFP-Amot 85 kDa ΔC-term (E) were stained with Par-3 (left panel, [D] and [E]), ZO-1 (left panel, [F]) and GFP (middle panel, [D]–[F]) antibodies. Left (red) and middle (green) images are merged in the right panels (D–F).

(G) MDCK cells stably expressing YFP-Amot 85 kDa were stained with GFP (enface image, left panel and middle right panel) and Patj (enface image, left panel and upper right panel) antibodies. Deconvolved images from the boxed region (left panel) were rotated 90° along the x axis to show the coincident staining of Patj (upper right panel) and YFP-Amot 85 kDa (middle right panel). Merged image, lower right panel. Scale bars = 20 μm.

contrast, YFP-Amot 85 kDa ΔC-term did not colocalize with Par-3 (Figure 4E), indicating that the Amot PDZ binding motif is necessary for its recruitment to TJs. Further, deconvoluted Z stack images showed YFP-Amot 85 kDa codistributed to apical surfaces of MDCK cells with endogenous Patj (Figure 4G). We therefore propose that the PDZ binding motif at the C terminus of Amot binds Patj and that this is necessary for Amot to localize to TJs.

To determine the localization of endogenous Amot, we stained polarized MDCK cells with Amot antibody. Amot was seen at cell-cell contacts and partially colocalized with ZO-1 (Figure 5A), as has been recently reported in endothelial cells (Bratt et al., 2005). In addition, Amot was seen in regions below the TJ, coincident with the AJ protein E-cadherin (Figure 5B). This extended basal staining of endogenous Amot, which is not seen with the exogenous 85 kDa isoform (Figure 4G; Figures S4G and S4H), may represent the 130 kDa Amot isoform, which contains an N-terminal extension that can bind the AJ protein Magi-1b (Bratt et al., 2005; Dobrosotskaya and James, 2000).

Amot Relocalizes to Cell-Cell Contacts following TJ Formation

To determine whether Amot might function in the formation or maintenance of TJs, we imaged its localization in MDCK cells following an overnight Ca^{2+} switch. Unlike ZO-1, which was localized to the TJ within 2–4 hr, Amot did not completely regain TJ staining until 4 to 24 hr following the readdition of Ca^{2+}. Since Amot relocalizes to the TJ later than ZO-1 or Patj, which has been reported to appear at the TJ within 3 hr following a Ca^{2+} switch (Shin et al., 2005), Amot is not likely to play a role in the initial formation of TJs (Figure 5C).

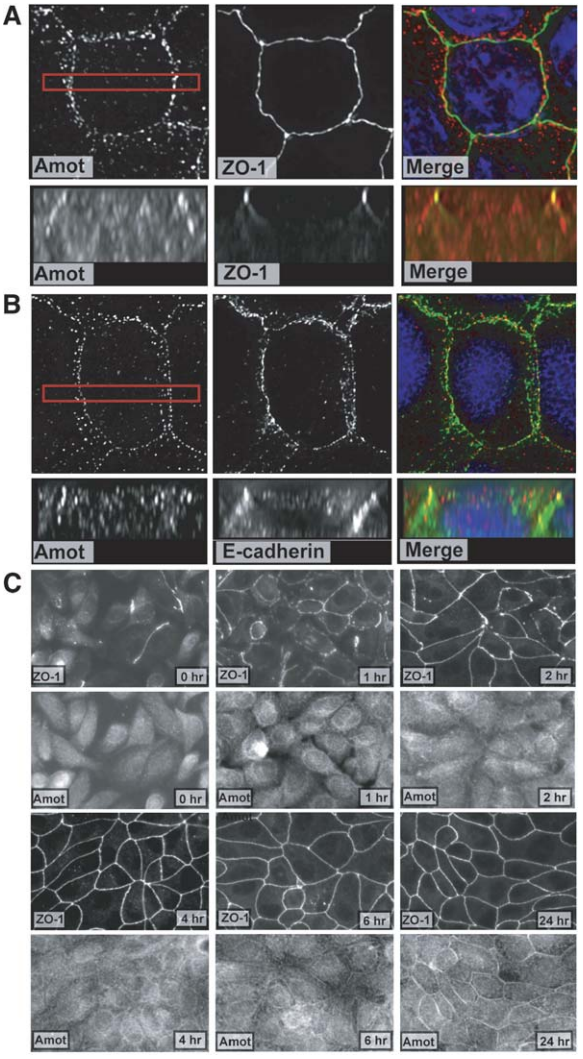

Figure 5. Amot Localizes to Regions of Cell-Cell Contact after TJ Formation

(A and B). Enface images of wt MDCK cells immunostained for Amot (left panels) and either ZO-1 (middle panels, [A]) or E-cadherin (middle panels, [B]). Left (red) and middle (green) images are merged in the right panels. The boxed regions were rotated 90° in the x plane to project Z stack images of Amot (lower left panels, [A] and [B]) and either ZO-1 (lower middle panel, [A]) or E-cadherin (lower middle panel, [B]) and were merged (right lower panels, [A] and [B]).

(C) Wild-type MDCK cells were cultured in low-Ca^{2+} medium, switched to normal medium, fixed at the times indicated, and stained with antibodies against ZO-1 and Amot.

Amot and Rich1 Associate through BAR/Coiled-Coil Domains

The 85 kDa isoform of Amot is predicted to encode a ~240 residue coiled-coil (CC) domain at its N terminus. Because BAR domains are also composed of CC regions of similar length, we modeled this region onto the BAR domain of amphiphysin; this revealed a striking conservation of positively charged residues that in amphiphysin reside on the concave lipid binding surface (Figure S3A). We therefore speculated that the N-terminal CC region of Amot functions as a BAR domain (and will subsequently refer to it as the BAR/CC domain).

Based on reports that BAR domains can homo- or heterodimerize (Navarro et al., 1997), we considered that Amot might directly bind Rich1 and thereby target it to TJ. Coexpression of FL Rich1 and FL Amot in 293T cells resulted in the formation of a complex, as detected by immunoprecipitation of either protein. However, removal of the BAR/CC domain from either Rich1 or Amot greatly reduced this interaction (Figure 6A). Furthermore, the BAR domain of Rich1 alone efficiently bound FL Amot but only weakly recognized a mutant of Amot that lacks the BAR/CC domain (ΔBAR/CC) (Figure 6B). Similarly, the BAR/CC domain of Amot coprecipitated wt Rich1, and this interaction was compromised by deletion of the Rich1 BAR domain (Figure 6B). This indicates that the BAR/CC domains of Rich1 and Amot are necessary and sufficient for these proteins to efficiently associate in cells.

To test whether the BAR/CC domains of Rich1 and/or Amot are required for their localization to membranes and/or TJs, we compared the intracellular localization of FL Rich1 and FL Amot to that of ΔBAR Rich1 and ΔBAR/CC Amot in MDCK cells. As noted above, stably expressed FL Flag-Rich1 was concentrated in small punctate structures and at cell-cell contacts with Par-3 (Figure 6C); Amot 85 kDa expressed stably at low levels or transiently (Figures 4D and 4F; Figure S4E) also localized to TJ and to apical membranes. In contrast, mutants of Rich1 (Figure 6D) or Amot (Figures 6E and 6F) lacking a BAR/CC domain had a diffuse staining pattern. For Rich1, this represents a shift from the membrane to the cytosol (Figure S3B), whereas for Amot, which has been reported to encode a second membrane targeting region (Bratt et al., 2005), there was a loss of staining at cell-cell contacts and apical membranes (Figures 6E and 6F) but no apparent increase in cytosolic localization (Figure S3B). Interestingly, the isolated BAR/CC domain of Amot was highly concentrated in a band at the same apical position as Par-3 (Figure 6G). These data show that the BAR/CC regions of Rich1 and Amot are necessary for heterotypic binding and for targeting within the cell.

Overexpression of Amot Induces a Relocalization of Polarity Proteins and Loss of Transepithelial Electrical Resistance in MDCK Cells

The association of Amot with Rich1 and the Pals1/Patj complex suggests a role in epithelial polarity; we therefore investigated the effects of overexpressing or reducing Amot on polarity in MDCK cells. Cells stably overexpressing YFP Amot 85 kDa failed to develop any detectable TER 30 hr following a calcium switch, unlike wt MDCK cells or cells expressing YFP-Amot 85 kDa ΔC-term, which regained all or 60% of their original TER, respectively (Figure 7A).

The inability of MDCK cells stably expressing wt Amot to form TER is likely explained by the selective relocalization of endogenous TJ components such as Par-3 (Figure 7B)

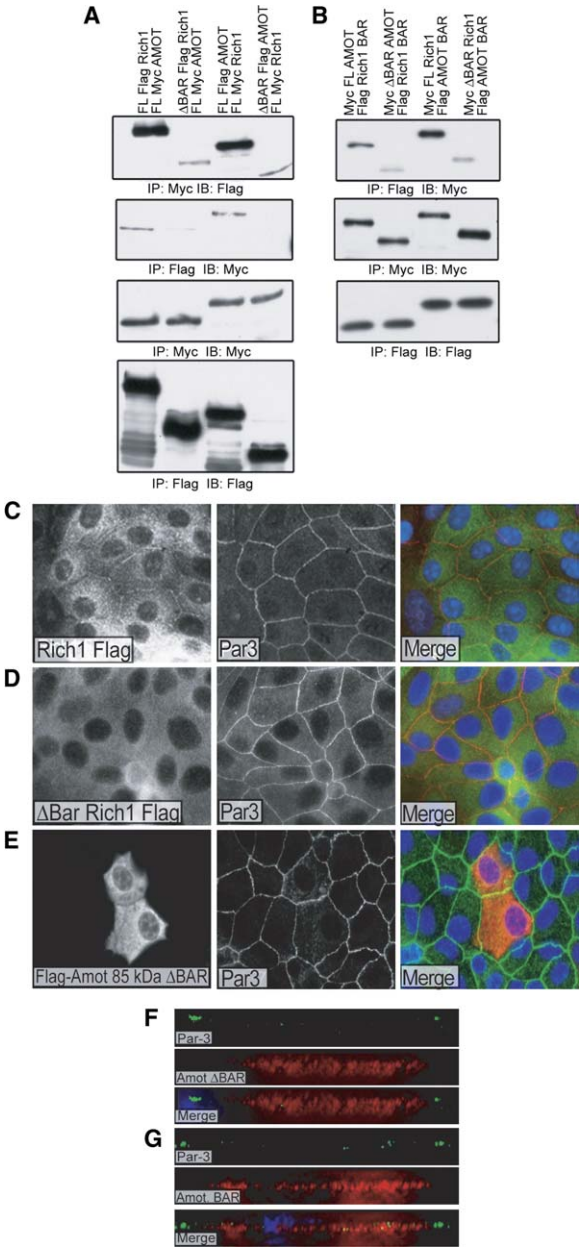

Figure 6. Rich1 and Amot Require Their BAR/CC Domains to Interact for Proper Intracellular Localization

(A) Flag- or Myc-tagged FL Rich1, ΔBAR Rich1, FL Amot, and ΔBAR Amot constructs were transfected as indicated. Lysates from each condition were split and immunoprecipitated with either Myc (panels 1 and 3) or Flag (panels 2 and 4) antibodies and then immunoblotted with Flag (panel 1) or Myc (panel 2) antibodies. Blots were reprobed with Myc (panel 3) or Flag (panel 4) antibodies.

(B) Lysates from HEK293T cells transfected with the indicated constructs were immunoprecipitated and immunoblotted as labeled.

(C) Polarized MDCK cells stably expressing Flag-Rich1 were costained with Flag and Par-3 antibodies.

(D) MDCK cells stably expressing Flag ΔBAR Rich1 were similarly immunostained as in (C).

(E) Polarized MDCK cells were transiently transfected with Flag-Amot ΔBAR and immunostained as in (C).

and Pals1 (Figure S4A), together with Amot, from TJs into punctate structures that partially colocalized with EEA1 (Figures 7D and 7E). Such relocalization only occurs in cells in which Amot is highly expressed (Figure 7B), suggesting that Amot levels must cross a certain threshold to induce internalization of specific TJ components. The localization of ZO-1 to cell-cell contacts was moderately disrupted in such cells, but, since it was not redistributed into puncta with Amot (Figure S4B), this may be a secondary defect due to the loss of Pals1 and Par-3 from TJs. Recent data suggest that endocytosis of proteins at the TJ and subsequent trafficking through differential endosomal populations is important for maintaining cellular polarity (Ivanov et al., 2005). Interestingly, overexpressed YFP-Amot ΔC-term did not recruit TJ components into such structures or disrupt their localization to cell-cell contacts, consistent with the inability of this mutant to disrupt TER (Figures 7A and 7C; Figure S4C). Taken together, Amot connects to polarity proteins through its PDZ binding site and also requires this motif to promote the internalization of proteins at TJs and induce a loss of TJ integrity.

Since a high level of overexpressed Amot recruits components of TJs into internal puncta, reminiscent of the effects of Ca^{2+} depletion on Patj redistribution (Shin et al., 2005), we addressed whether Amot was involved in this latter process by generating an MDCK cell line (Ang A-4) in which Amot expression is partially silenced (Figure 7F inset box). Upon calcium depletion, there was a delay in the loss of TJ integrity as monitored by TER in the MDCK AngA-4 cells compared with wt MDCK cells.

Amot Modulates the Activity of Rich1

Because depletion of Rich1 and overexpression of Amot have the same effects on TJs, we hypothesized that overexpression of Amot may prevent Rich1 from appropriately regulating the Cdc42 GTPase. We explored this possibility using the Cdc42-Raichu FRET reporter in 293T cells. Indeed, Amot overexpression suppressed the ability of Rich1 to reduce the fraction of Cdc42 bound to GTP, whereas the related Amot L1 had no effect on Rich1 GAP activity (Figure 7G). These data suggest that the phenotypes observed upon overexpression of Amot may in part be explained by an inhibition of Rich1 GAP activity.

DISCUSSION

The GTPase-Accelerating Activity of Rich1 for Cdc42 Underlies Its Role in TJ Integrity

We have identified a Cdc42-selective GAP, Rich1, as being important for the integrity of TJs in epithelial cells. Rich1 localizes to TJs, and silencing of Rich1 expression

(F) MDCK cells transfected with Flag-tagged 85 kDa Amot ΔBAR were immunostained for Par-3 (green, top panel) and Flag (red, middle panel). The merge of the top and middle images is shown in the bottom panel.

(G) Polarized MDCK cells transiently expressing the BAR/CC domain of Amot (residues 1–245) were immunostained and visualized as in (F).

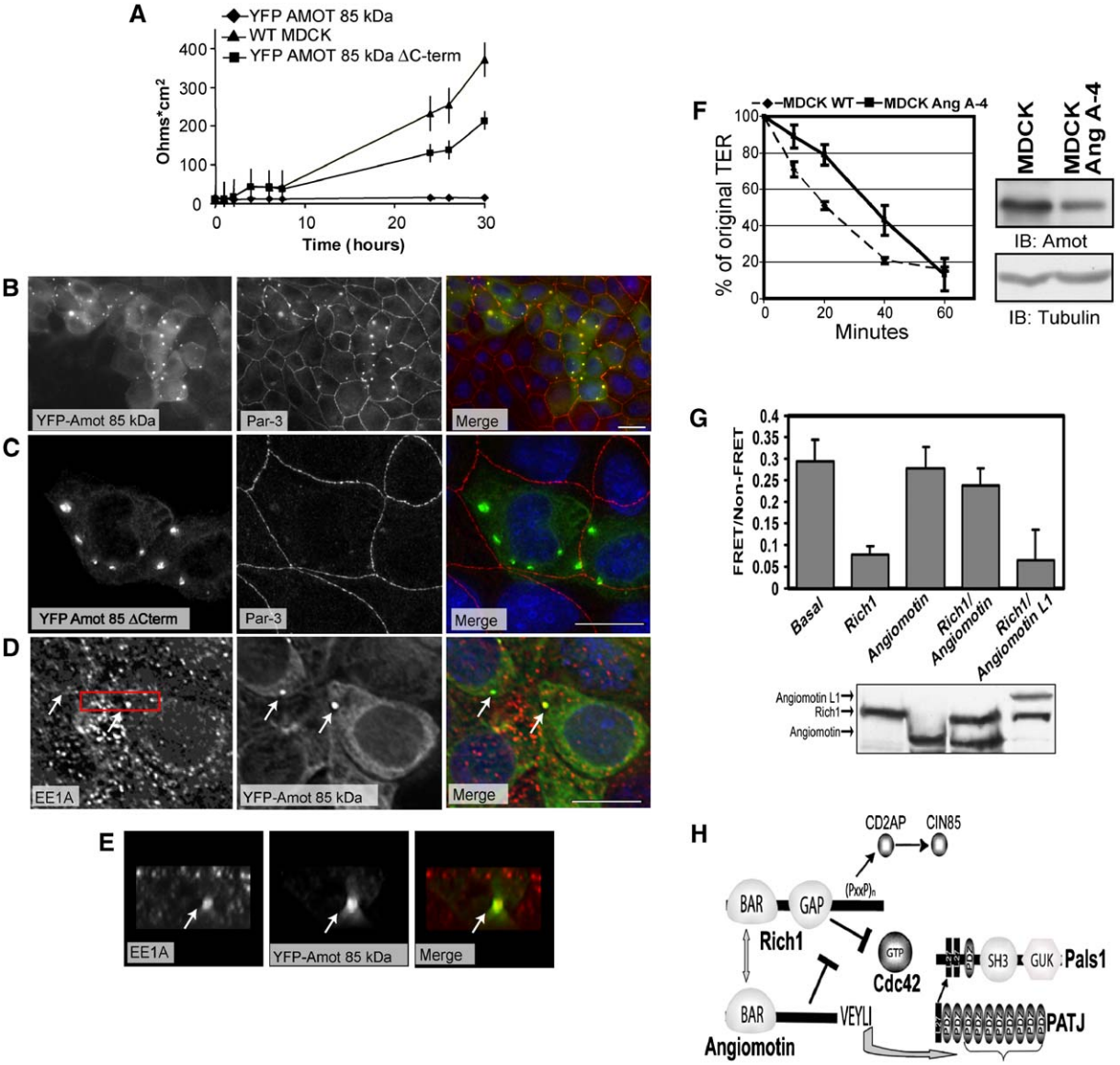

Figure 7. Amot Regulates TJ Integrity and Inhibits Rich1 GAP Activity

(A) TER measurements of wt MDCK cells (triangles) or MDCK cells expressing YFP-Amot 85 kDa (diamonds) or YFP-Amot ΔC-term (boxes) were measured at the indicated times following replacement of normal conditioned media after overnight incubation in low-calcium media.

(B and C) MDCK cells that stably express high levels of YFP-Amot 85 kDa (B) or YFP-Amot ΔC-term (C) immunostained with antibodies against GFP (left panel, [B] and [C]) and Par-3 (middle panel, [B] and [C]). Merged images in right panels show Amot (green) and Par-3 (red) stain. Scale bars = 15 μm.

(D) Deconvoluted images of MDCK cells stably expressing YFP-Amot 85 kDa stained for EEA1 (left panel) and GFP (middle panel). Right panel shows merge (red, EEA1; green, Amot). Punctate structures are arrowed.

(E) Deconvoluted Z stacks of the red boxed region in (D) showing EEA1 and YFP-Amot (arrows).

(F) MDCK cells stably expressing shRNA for Amot (Ang A-4) immunoblotted with Amot antibody (inset box). Percent of original TER of wt (diamonds) and Ang A-4 (squares) MDCK cells following a Ca^{2+} switch is shown.

(G) Ratio of GTP bound over GDP bound Cdc42 Raichu probe in lysates from HEK293T cells expressing the indicated constructs. Lysate controls were immunoblotted with anti-Flag (bottom panel). Values in (A), (F), and (G) represent the mean of data taken in triplicate and are representative of three independent experiments. Error bars show the standard deviation.

(H) A depiction of the domain architectures and proposed functional interactions of Rich1, Amot, Pals1, Patj, and Cdc42.

disrupts their structure and function. A similar phenotype is induced by overexpression of a GAP-deficient mutant of Rich1, arguing that the ability of Rich1 to regulate Cdc42 is important to its role in polarity.

Cdc42 has a conserved role in maintaining the apical-basolateral polarity of epithelial cells, which has been principally explored using CA or DN Cdc42 mutants. Of the two, CA Cdc42 has a greater impact on apical polarity in

cultured MDCK cells (Kroschewski et al., 1999) and specifically inhibits polarity in chick somites (Nakaya et al., 2004). Overexpression of CA Cdc42 and inactivation of Rich1 produce comparable phenotypes, including a redistribution of similar TJ components but the sparing of AJs (Bruewer et al., 2004), arguing that both manipulations disrupt polarity through the aberrant production of GTP bound Cdc42.

Ectopic Cdc42-GTP could simply activate targets that are toxic to TJs; alternatively, it could interfere with cycling of Cdc42 between GDP and GTP bound states and thereby block the ability of Cdc42 to regulate TJ components. In favor of the latter possibility, cycling of Cdc42 has been established as necessary for polarity in *Saccharomyces cerevisiae* (Irazoqui et al., 2003) and is suggested by experiments in flies (Hutterer et al., 2004), MDCK cells (Bruewer et al., 2004), and chick somites (Nakaya et al., 2004). Rich1 may therefore prevent a stable pool of Cdc42-GTP from forming at TJs and/or AJs of mammalian epithelial cells and thereby promote Cdc42 cycling.

The substrate selectivity of FL Rich1 for Cdc42 observed in MDCK and HEK293T epithelial cells is consistent with studies in MDA-MB-231 cells (Parsons et al., 2005). The GAP domain of Rich1 alone, however, exhibits activity for both Cdc42 and Rac1 in a pig aortic endothelial (PAE) cell line (Richnau and Aspenstrom, 2001). Although our assays may have lacked the sensitivity to detect activity toward a less optimal substrate, it is also possible that FL Rich1 is specific for Cdc42 or has context-dependent specificity, as documented for other RhoGAPs (Minoshima et al., 2003). Inactivation of Rich1 signaling in MDCK cells caused a severe mislocalization of ZO-1, similar to CA Cdc42 but distinct from CA Rac1, which produces modest (Jou et al., 1998) or undetectable effects on ZO-1 localization at TJs (Bruewer et al., 2004), consistent with a primary effect on Cdc42 or related GTPases (Czuchra et al., 2005).

Rich1 and Amot Are Novel and Functional Components of Pals1- and Patj-Containing Polarity Complexes

A proteomic screen for Rich1 binding partners converged with a similar analysis of apical-polarity proteins, revealing a series of reciprocal interactions involving Rich1 and Amot, the Patj/Pals1 polarity complex, and Par-3, all of which colocalize to the same region of the TJ in polarized MDCK cells. It appears that these proteins are components of an extensive and potentially dynamic network of interacting complexes (Figure 7H). Our data indicate that Amot is a scaffold that recognizes Patj through its C-terminal PDZ binding motif and also binds Rich1 through a mutual BAR-domain/CC interaction. Thus, Amot links Rich1 to Patj and may thereby target Rich1 to a subpopulation of Cdc42 involved in maintaining TJ structures.

In this regard, mutants of Rich1 or Amot lacking their N-terminal BAR/CC domains are diffusely dispersed. In contrast, variants of Amot containing the BAR/CC domain but lacking the PDZ binding motif are retained at the apical membrane but are not targeted to TJs. These data suggest a model in which the BAR/CC domains of Rich1 and Amot mediate their joint interaction and recruitment to apical membranes. Amot is then further localized to TJs through association of its C-terminal PDZ binding motif with Patj.

Rich1 and Amot Function Primarily by Maintaining Tight Junctions

Recent data indicate that Patj and Pals1 are necessary for apical polarity and that Crumbs3 signaling in MCF10A cells is sufficient to recruit Pals1 and Patj to the apical domain and induce competent TJs (Fogg et al., 2005). Pals1/Patj may function in this context to localize Par-6 or to recruit aPKC to the TJ. However, the loss of Pals1, Patj, or Crumbs3 may also uncouple the Amot/Rich1 complex from TJs, which in turn could deregulate Cdc42 signaling necessary for epithelial polarity.

Amot localizes to TJs following a Ca^{2+} switch, later than the retargeting of ZO-1 or Patj (Shin et al., 2005). This suggests that Amot is not essential for TJ formation. Rich1 also appears to contribute to TJ stability, as MDCK cells partially silenced for Rich1 are abnormally sensitive to a loss of TJ integrity upon Ca^{2+} depletion, while cells highly silenced for Rich1 (2A-7 cells) form islands of cells with intact TJs, interspersed among depolarized cells, although these TJs degrade over time (Figure S2H). These results suggest that Amot and Rich1 are not absolutely necessary for the formation of TJs but are required for their long-term stability.

A Role for Amot and Rich1 in Regulating the Uptake of Polarity Proteins at Tight Junctions

How do Amot and Rich1 function, in a mechanistic sense, to maintain TJs? A possible answer is suggested by the selective internalization of Pals1 and Par-3 as well as the concomitant loss of the TJ permeability barrier upon Amot overexpression. The idea that polarity is maintained by selective endocytosis of polarity proteins is supported by recent work showing that defects in the uptake of *Drosophila* Crumbs, which associates with Pals1 and Patj (Roh et al., 2002; Tepass and Knust, 1993), leads to an expanded apical domain and tumor formation (Lu and Bilder, 2005). The uptake of Crumbs may therefore maintain polarity by preventing excess activity from this apical complex. Two elements within Amot appear important for the regulation of polarity components. The Amot BAR/CC domain is necessary for the formation of Amot-containing puncta, and the C-terminal PDZ binding motif, which recruits the polarity protein Patj, is required for targeting of TJ components into such internal structures.

The requirement for the Amot BAR/CC domain may reflect its involvement in localizing Amot to apical membranes and junctions. Since the BAR domains of proteins such as amphiphysin and endophilin directly regulate vesicle formation by binding and/or bending curved membranes (Peter et al., 2004), it will be of interest to know whether the interacting BAR/CC domains from Amot and Rich1 can modify membranes in a similar fashion. In addition, the ability of the Amot BAR domain to recruit Rich1

may impact on trafficking of TJ components through the association of Rich1 with the endocytic proteins CD2AP/CIN85. This observation supports the notion that Rich1 acts with Amot to mediate the uptake of selected TJ polypeptides.

Interestingly, loss of Rich1 activity and high levels of Amot both induce defective TJs. The finding that Amot suppresses Rich1 GAP activity may explain this inverse relationship and indicates that overexpression of Amot results in an increase in the fraction of Cdc42 bound to GTP. This is consistent with previous findings that DN and CA mutants of Cdc42 disrupt apical endocytosis (Rojas et al., 2001) and that CA Cdc42 induces the basal translocation of ZO-1 (Kroschewski et al., 1999), similar to Rich1 silencing in MDCK cells. Therefore, Amot binding to Rich1 may target Rich1-associated endocytic components to TJs and also regulate the GAP activity of Rich1 to modulate Cdc42 dependent effects on endocytosis.

Amot and Rich1 in the Dynamic Regulation of Polarity

The preceding data raise the possibility that Amot might be regulated by physiological stimuli that modify TJs. For example, Amot overexpression and Ca^{2+} depletion promote a similar uptake of selective TJ components (Shin et al., 2005), and partial silencing of Amot delays the loss of TER induced by Ca^{2+} depletion in MDCK cells. Taken with the localization of Amot and Rich1 to AJs and TJs, it is attractive to speculate that these proteins may participate in the signals leading to breakdown of the TJ in response to loss of cadherin cohesion.

Amot has been described as an angiostatin binding protein that promotes cellular invasion and migration as well as the breakdown of cellular junctions in endothelial cells (Bratt et al., 2005; Levchenko et al., 2004; Troyanovsky et al., 2001). That Amot is required for proper migration of the visceral endoderm in day 7 murine embryos (Shimono and Behringer, 2003) and its specific expression in this structure (Figure S4F) strongly suggest an important role for Amot in migratory processes. However, the molecular mechanisms through which Amot controls migratory and invasive phenotypes are unclear. The finding that FL Amot, but not ΔC-term Amot, leads to a loss of MDCK cell polarity is consistent with data that the C-terminal motif is required for Amot to increase the migratory or invasive index of endothelial cells (Levchenko et al., 2004). Furthermore, the observation that CA Cdc42 specifically induces an epithelial-to-mesenchymal transition in chick somites is consistent with the notion that increased expression of Amot may degrade apical polarity by influencing Rich1 activity and thus the levels of Cdc42 GTP. This loss of polarity may in turn underlie the ability of Amot to promote migration.

In summary, we define a novel network of protein interactions involved in epithelial polarity that links the Cdc42 GAP Rich1 and the scaffold Amot with polarity components such as Pals1/Patj and Par-3. Rich1 and Amot appear to maintain epithelial polarity through the integration of Cdc42 activity and the trafficking of specific polarity proteins at the TJ. The balance of Amot activity therefore appears to control an equilibrium between epithelial and mesenchymal phenotypes. The requirement for the BAR/CC domain of Amot to localize to apical membranes, and to relocalize signaling components, suggests that it may play a direct role in these dynamic aspects of polarity.

EXPERIMENTAL PROCEDURES

Vectors and Antibodies

All vectors are described in Table S2 and the accompanying legend. Antibodies are described in Supplemental Experimental Procedures. The experiments in Figures 7A–7E and Figures S4A–S4C were performed with a variant (E57G, L263P) of human Amot (accession number BAA83023). Equivalent results were obtained with the wild-type sequence (Figure S4D and data not shown).

Tissue Culture

MDCK II, HEK293T, and Phoenix cells were purchased from ATCC and cultured in DME media supplemented with 10% fetal calf serum (HyClone). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Sample Preparation and Protein Identification by Mass Spectrometry

Sample preparation and subsequent peptide identification by MS/MS are described in Table S1 and the accompanying legend.

Immunofluorescence

Cells were fixed and stained as described in Plant et al. (2003) and Figure S2. Rich1, Pals1, Patj, Amot, and Par-3 antibodies were all used at a 1:200 dilution.

Intracellular GTPase Assays

The Raichu probes were kindly provided by Dr. M. Matsuda. Assays were performed as described at http://www-tv.biken.osaka-u.ac.jp/e-phogemon/phomane.htm. Cells were serum starved for 15 hr and treated with 0.5 μM bradykinin and 100 ng/ml of PDGF for 10 min. Clarified lysates containing GBD buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$) were incubated with 5 μg of immobilized GBD protein (from pak1B) for 30 min at 4°C and then washed three times. Cdc42 was detected by immunoblot analysis, and the blot was then stripped and reprobed for Rac1.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, and six figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/3/535/DC1/.

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