Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis

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The establishment of apical–basal polarity within a single cell and throughout a growing tissue is a key feature of epithelial morphogenesis. To examine the underlying mechanisms, the human intestinal epithelial cell line Caco-2 was grown in a three-dimensional matrix to generate a cystlike structure, where the apical surface of each epithelial cell faces a fluid-filled central lumen. A discrete apical domain is established as early as the first cell division and between the two daughter cells. During subsequent cell divisions, the apical domain of each daughter cell is maintained at the center of the growing structure through a combination of mitotic spindle orientation and asymmetric abscission. Depletion of Cdc42 does not prevent the establishment of apical–basal polarity in individual cells but rather disrupts spindle orientation, leading to inappropriate positioning of apical surfaces within the cyst. We conclude that Cdc42 regulates epithelial tissue morphogenesis by controlling spindle orientation during cell division.

Introduction

Cdc42 regulates the generation of cell polarity from yeast to man and in a wide range of biological contexts, and an important breakthrough in understanding the biochemical mechanisms involved came with the identification of the downstream target Par6 (Etienne-Manneville, 2004; Welchman et al., 2007). Par6 was originally identified in Caenorhabditis elegans as being involved in asymmetric positioning of the mitotic spindle during zygotic cell division, and subsequent work showed that Cdc42 is also required (Hung and Kemphues, 1999; Gotta et al., 2001). Other work in C. elegans, Drosophila melanogaster, and mammals has revealed a critical role for Cdc42 and Par6 in generating polarity during directed cell migration and morphogenesis (Etienne-Manneville and Hall, 2001; Hutterer et al., 2004; Na and Zernicka-Goetz, 2006; Welchman et al., 2007; Wu et al., 2007). The exact contribution of Cdc42 in these contexts is less clear. Work on directed cell migration has shown that Cdc42 acts through Par6 to control the orientation of microtubules, but Cdc42 also controls nuclear positioning and polarized actin polymerization through two other targets, MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase) and p65PAK, respectively (Etienne-Manneville and Hall, 2001; Cau and Hall, 2005; Gomes et al., 2005). Cdc42 is not required for random migration, leading to the suggestion that it is not required for the establishment of polarity as such but rather to orient polarity with respect to an external cue (directional sensing; Allen et al., 1998; Srinivasan et al., 2003). A similar conclusion has emerged from experiments on Cdc42 during pheromone mating in yeast (Irazoqui et al., 2004).

Epithelial morphogenesis involves the establishment of an apical surface in an individual cell and the formation of cadherin-based adherens junctions and claudin-based tight junctions between adjacent cells. Accompanying reorganization of the actin and microtubule cytoskeletons and polarized vesicle trafficking reinforces these interactions, leading to a stable tissue of polarized cells. Expression of dominant-negative or constitutively active versions of Cdc42 in the dog kidney epithelial cell line MDCK grown on a two-dimensional surface leads to defective tight junction formation (typically a delay) as well as mislocalized delivery of basolateral proteins (Kroschewski et al., 1999; Rojas et al., 2001; Wells et al., 2006). Similar experiments performed with MDCK grown in three dimensions to provide a more physiological growth context have concluded that Cdc42 is required to form an apical surface through regulated trafficking of a vacuolar apical compartment.

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Figure 1. **Caco-2 intestinal epithelial cells form polarized cysts in three dimensions.** Caco-2 cells plated as a single-cell suspension in three dimensions were fixed and stained after 12 d. (A–C) Single confocal sections through the middle of cysts stained for DNA (blue) and actin (red; A); DNA (blue) and aPKC (red; B); and DNA (blue), E-cadherin (Ecad; green), and ZO-1 (red; C). Phase-contrast images (differential interference contrast [DIC]) are also shown.

(F) DNA, Na+/K+ ATPase, CFTR, overlay.
(Vega-Salas et al., 1987; Martin-Belmonte et al., 2007). In this study, we describe the analysis of Caco-2, a human intestinal epithelial cell line, which, when grown in a three-dimensional matrix, generates polarized cysts with a single central lumen. We show that Cdc42 is not required for the formation of an apical surface, but instead is required to position the apical surface with respect to the growing three-dimensional structure. Our results indicate that Cdc42 regulates apical surface positioning by controlling spindle orientation during cell division.

Results and discussion

Caco-2 form fully polarized cysts in three-dimensional cultures

Caco-2 cells have been used extensively as polarized epithelial monolayers grown on two-dimensional surfaces, although few studies have been performed in three dimensions (Zhang et al., 2003; Guruswamy et al., 2008; Ivanov et al., 2008). 12 d after suspending single cells in Matrigel, Caco-2 form cysts with enhanced actin accumulation at internal surfaces facing a central lumen (Fig. 1 A). Caco-2 cysts have distinct adherens junctions (visualized by epithelial cadherin [E-cadherin] staining) and tight junctions (visualized by ZO-1 staining; Fig. 1 C), and atypical PKC (aPKC), an apical membrane marker, localizes close to the tight junctions facing the luminal space (Fig. 1 B). A three-dimensional reconstruction of a mature cyst is shown in Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200807121/DC1).

cAMP stimulates lumen formation through polarized expansion

Although Caco-2 grown in three dimensions form cysts, the development of a central lumen is relatively inefficient (<50%) in normal culture conditions after 12 d. However, stimulation of cAMP signaling with cholera toxin (CTX) induces dramatic and rapid lumen formation (Fig. 1 D and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200807121/DC1). 6 d after plating, CTX treatment results in a well-defined lumen in >90% of all Caco-2 structures in <12 h (Fig. 1 E). Time-lapse microscopy reveals that lumen formation occurs through a process of internal expansion without cell proliferation or death (Video 3). cAMP can activate PKA and Epac, an exchange factor for the small GTPase Rap1 (de Rooij et al., 1998). PKA is known to activate apical chloride channels such as the cystic fibrosis transmembrane receptor (CFTR), resulting in fluid secretion (Riordan, 2008), whereas Rap regulates epithelial morphogenesis through unknown mechanisms (Boettner and Van Aelst, 2007). To determine which pathway is involved, cAMP analogues that specifically activate PKA (6-Bnz) or Epac (8-Cpt) were used. Activation of PKA but not Epac induces lumen formation (Fig. 1 E). Recent work on intestinal development in zebrafish concluded that lumen formation is induced through fluid accumulation driven by polarized ion transport (Bagnat et al., 2007).

To examine whether Caco-2 use a similar mechanism, three-dimensional cultures were treated with CTX either alone or with ouabain, an inhibitor of the Na+/K+-ATPase pump. Ouabain completely inhibits lumen expansion (Fig. 1 E). Similar to what has previously been reported for Caco-2 cells grown in two dimensions, the CFTR chloride channel and the Na+/K+-ATPase are localized apically and laterally, respectively, in three dimensions (Fig. 1 F). These data indicate that lumen expansion occurs by ion transport–driven fluid flow. Polarized secretion (fluid or protein) to drive the separation of two opposing apical surfaces is emerging as an evolutionarily conserved mechanism for lumen formation in a wide range of epithelial tissues such as the Drosophila retina and trachea (Husain et al., 2006; Tsarouhas et al., 2007), the zebrafish gut (Bagnat et al., 2007), and the zebrafish neural tube (Lowery and Sive, 2005).

Cdc42 depletion inhibits Caco-2 morphogenesis

To determine the role of Cdc42 in cyst development, Caco-2 cells were transfected with nonspecific or Cdc42-specific siRNAs 1 d before plating in Matrigel (Fig. 2 B). Treatment with CTX 6 d later results in the swelling of a single central lumen in control structures, whereas 50% of Cdc42 siRNA–transfected structures appear abnormal (Fig. 2 A and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200807121/DC1). Immunofluorescence reveals that depletion of Cdc42 results in cysts containing multiple lumens (Fig. 2, C–E). Interestingly, the surface of each lumen within the three-dimensional structure is positive for the apical marker aPKC (Fig. 2 D) and the tight junction protein ZO-1 (Fig. 2 E). These data suggest that Cdc42 is not required for the formation of apical–basal polarity and tight junctions during Caco-2 morphogenesis but rather for the correct positioning of the apical surface with respect to the growing structure.

An apical surface is specified at the first cell division and is maintained at the center of the developing cyst by mitotic spindle positioning

To understand the mechanism by which Cdc42 depletion results in multiple lumens, the development of control Caco-2 cysts was examined. Surprisingly, an apical surface is clearly visible at the two-cell stage, as revealed by ZO-1 and aPKC localization (Fig. 3, A and B [second row]). E-cadherin is basolateral and does not overlap with ZO-1 (Fig. 3, A and Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200807121/DC1) such that the membrane interface between two daughter cells is already demarcated into a lateral surface surrounding an
dimensions. 3 d later (4 d after transfection), structures were examined for orientation of mitotic spindles (calculated as described in Fig. 4 A). Depletion of Cdc42 has a dramatic effect on spindle orientation. In control cysts, the majority of spindles are oriented perpendicular to the centroid of the cyst (mean angle = 72.3 ± 3.9°; Fig. 4, B and C [top]). However, after Cdc42 depletion, spindle orientation is randomized (mean angle = 41.2 ± 5.2° and 41.8 ± 5.7° for duplex 2 and duplex 4, respectively; Fig. 4, B and C [bottom]), with some spindles oriented so as to produce a daughter cell in the middle of the structure (Fig. 4 D).

If the apical surface is generated between daughter cells (Fig. 3 A), the consequence of spindle misalignment will be the formation of apical patches at aberrant sites. To examine this, the localization of the apical marker aPKC was determined early in the development of Cdc42-depleted cysts. Figure 4 E (arrows) shows that some cells have multiple distinct apical patches on their surface. These results suggest that loss of Cdc42 causes misoriented division, giving rise to an inappropriately placed apical membrane, which after expansion leads to multiple lumen.

**Cdc42** is required for mitotic spindle orientation

Based on this analysis, a consequence of Cdc42 depletion could be disruption of spindle orientation. To examine this, Caco-2 cells were transfected with Cdc42 siRNA and grown in three
Asymmetric abscission ensures correct positioning of apical surfaces at the center of the cyst.

The analysis of MDCK in two-dimensional and mouse intestinal epithelial cells in vivo has revealed that invagination of the cleavage furrow is asymmetric, proceeding basal to apical such that abscission occurs at the most apical point between two daughter cells (Reinsch and Karsenti, 1994; Fleming et al., 2007). This, in combination with mitotic spindle orientation, would explain the persistence of apical surfaces at the center of growing Caco-2 structures (Fig. 3 B). To examine the abscission site in Caco-2 cells, the cytokinesis midbody was visualized with a tubulin antibody. As seen in Fig. 5 A (top), at the end of the first cell division, the apical marker aPKC is associated with the midbody at the center of the interface between the two emerging daughter cells. This strongly supports the idea that the apical surface is laid down during cytokinesis, although in the case of the first cell division, abscission appears to be symmetric. In multicellular structures (Fig. 5, A [middle] and B), 91% of midbodies are located in the center of the developing cyst, suggesting that after the first cell division, all subsequent abscissions occur asymmetrically and close to the existing apical membrane (i.e., in the center of the growing structure). In Cdc42-depleted Caco-2, <40% of midbodies are located centrally, reflecting the numerous noncentrally located apical surfaces (Fig. 5, A [bottom] and B). We conclude that the loss of Cdc42 results in spindle misorientation, leading to inappropriately placed abscission sites. Because the abscission site establishes the apical surface (Fig. 5 A, midbody position), loss of Cdc42 results in the formation of multiple lumens.

Three-dimensional culture models recapitulate many of the features of in vivo epithelial architecture. Two cell types have been used extensively in this context: the dog kidney cell line MDCK and the human mammary epithelial cell line MCF10A. Both generate cystlike structures, but MCF10A achieve this through clearing internal cells by apoptosis and autophagy, whereas MDCK use a combination of exocytosis of intracellular vacuoles (the vacuolar apical compartment) and apoptosis of interior cells (Martin-Belmonte et al., 2007). In this study, we show that Caco-2 cysts form by polarized fluid filling in a manner similar to gut and neural tube development in zebrafish (Bagnat et al., 2007). We believe that the similarities between Caco-2 cyst formation in vitro and intestinal epithelia in vivo make this a relevant system for studying morphogenesis.

A striking observation is that Caco-2 cells display a distinct apical membrane patch as early as the two-cell stage. This appears to be linked to cytokinesis because the apical marker aPKC associates with the midbody before abscission occurs. The analysis of MDCK in two-dimensional and mouse intestinal epithelial cells in vivo has revealed that invagination of the cleavage furrow is asymmetric, proceeding basal to apical such that abscission occurs at the most apical point between two daughter cells (Reinsch and Karsenti, 1994; Fleming et al., 2007). This, in combination with mitotic spindle orientation, would explain the persistence of apical surfaces at the center of growing Caco-2 structures (Fig. 3 B). To examine the abscission site in Caco-2 cells, the cytokinesis midbody was visualized with a tubulin antibody. As seen in Fig. 5 A (top), at the end of the first cell division, the apical marker aPKC is associated with the midbody at the center of the interface between the two emerging daughter cells. This strongly supports the idea that the apical surface is laid down during cytokinesis, although in the case of the first cell division, abscission appears to be symmetric. In multicellular structures (Fig. 5, A [middle] and B), 91% of midbodies are located in the center of the developing cyst, suggesting that after the first cell division, all subsequent abscissions occur asymmetrically and close to the existing apical membrane (i.e., in the center of the growing structure). In Cdc42-depleted Caco-2, <40% of midbodies are located centrally, reflecting the numerous noncentrally located apical surfaces (Fig. 5, A [bottom] and B). We conclude that the loss of Cdc42 results in spindle misorientation, leading to inappropriately placed abscission sites. Because the abscission site establishes the apical surface (Fig. 5 A, midbody position), loss of Cdc42 results in the formation of multiple lumens.

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Figure 4.  

**Cdc42 depletion disrupts mitotic spindle orientation.** (A) Diagram depicting spindle angle measurement. The centroid of the cyst (dark blue circle) and the center of the spindle axis (pink circles) of a metaphase cell were drawn using ImageJ. The angle (red) between the spindle axis (black lines) and the line connecting the centroid of the cyst to the center of the spindle (dashed lines) was determined. To analyze spindle poles in different z sections, three z sections were taken so as to include both spindle poles and were merged as shown. Three schematic spindles are shown. The right and middle spindle examples represent correctly oriented spindles whose poles are positioned in one section (z2; middle spindle) or in different sections (z1 and z3; right spindle). The left spindle represents a misoriented spindle whose poles are in different z sections. Spindle microtubules, green; centrosomes, yellow; DNA, light blue. (B) Scatter diagram of metaphase spindle angles in cysts that were transfected with control or two Cdc42 siRNA duplexes from three independent experiments. Pink circles indicate mean values, green circles indicate individual data points, and error bars represent the SEM of the total number of spindles analyzed (N). (C) Caco-2 was transfected with control or Cdc42 siRNA and was fixed and stained for DNA (blue), tubulin (green), and F-actin (red).
Apical surfaces are maintained at the center of the growing Caco-2 structure during subsequent cell divisions through a combination of (a) orientation of the mitotic spindle to generate radial cleavage and (b) asymmetric positioning of the midbody to generate apical abscission (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200807121/DC1). Oriented cell division coupled to apical abscission provides a mechanism for maintaining the structural integrity of an epithelial barrier in tissues undergoing continuous proliferation such as the intestine (Reinsch and Karsenti, 1994; Fleming et al., 2007).

Cdc42 depletion in MDCK cells leads to an accumulation of intracellular vesicles containing apical proteins, resulting in intracellular lumens, as well as cells in the middle of the cyst that are eventually cleared by apoptosis, resulting in intracellular lumens (Martin-Belmonte et al., 2007). We find no evidence for cell death after depleting Cdc42 in Caco-2; instead, multiple intercellular lumens are formed, each of which appears to be correctly polarized as judged by aPKC and tight junction localization. Because these ectopic lumens expand through polarized fluid secretion, this provides further evidence for appropriate apical–basal polarity. Further analysis reveals that Cdc42 depletion causes spindle misorientation, leading to disruption of cleavage furrow orientation and mislocalization of the midbody during cytokinesis. Because the apical surface is established at the site of abscission, Cdc42 depletion results in noncentrally located apical surfaces to generate ectopic lumens (Fig. S1 B). Cdc42 has been reported to control spindle orientation in the early C. elegans embryo and in mouse oocytes, and this is linked to its association with Par6 (Gotta et al., 2001; Na and Zernicka-Goetz, 2006), but other potential Cdc42 targets have also been linked to spindle orientation, including LIM kinase and phosphatidylinositol 3-kinase (Toyoshima et al., 2007; Kaji et al., 2008). Finally, it is interesting to note that the loss of asymmetric abscission (Fig. S1 C) could in itself lead to an inappropriately positioned apical surface. The mechanisms controlling apical midbody positioning are not well understood. Recent work on zygotic cell division in C. elegans has implicated septins and anillin (Maddox et al., 2007), and interestingly, the former has been linked to Cdc42 through the Borg family, and the latter is a target of Rho (Pieknny and Glotzer, 2008). We are currently using live cell imaging to characterize in more detail the specific roles of Cd42 in this morphogenetic process.
Materials and methods

Reagents
Cell culture medium was obtained from Invitrogen or an in-house facility. Primary antibodies used in this study were Cdc42 (mouse monoclonal clone 44; BD), α-actin (clone AC-74; Sigma-Aldrich), Flag (clone M2; Sigma-Aldrich), α-tubulin (clone DM1A; Sigma-Aldrich), rat anti–E-cadherin (clone ECD2-2; Invitrogen), rabbit polyclonal PKCζ (C-20; Santa Cruz Biotechnol- ogy, Inc.), ZO-1 (Invitrogen), and CFPTR (NBDR; provided by A.P. Naren, University of Tennessee, Knoxville, TN). The α5 monoclonal antibody to Na+∕K+ -ATPase developed by D.M. Fambrough was obtained from the Devel- opmental Studies Hybridoma Bank and maintained by the University of Iowa. Alexa Fluor 488 and 568 secondary antibodies, rhodamine-conjugated phalloidin, and Prolong gold antifade with DAPI were obtained from Invit- ragen. HRP-conjugated secondary antibodies were obtained from Dako, and DRAGS and 8-Carboxy-1OMe-CAMP sodium salt were obtained from Axoara LLC. N6-benzoyl-cAMP sodium salt was obtained from EMD, and other chemicals were obtained from Sigma-Aldrich.

Cell culture
Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium supple- mented with 10% fetal calf serum and penicillin-streptomycin (100 IU/ml and 100 mg/ml, respectively) at 37°C in 5% CO2. To produce cysts, Caco-2 cells were plated either on top of matrix (for time lapse) or embed- ded in matrix for immunofluorescence. For on-top cultures, cells were trypsin- and resuspended (104 cells/ml) in media plus 2% Matrigel (BD). 400 μl of suspension was plated in each well of an 8-well chamber slide (BD) precoated with 30 μl of Matrigel. For embedded cultures, cells were trypsinized and mixed with Matrigel (final concentration of 0.02% Matrigel) (colla- gen I (final concentration of 1 mg/ml); Trevigen), and Matrigel (final concentra- tion of 40%) to 6 × 104 cells/ml. Approximately 100 μl was plated in each well of an 8-well chamber slide, allowed to solidify for 30 min, and overlaid with 400 μl of media.

Microscopy
Immunofluorescence of embedded Caco-2 cysts was performed as de- scribed previously for MCF10A cysts (Debnath et al., 2003) with the fol- lowing modifications. Before blocking, Caco-2 chambers were rinsed with PBS and treated with 100 μl of 50 U/ml collagenase-I (Sigma-Aldrich) in PBS for 15 min at room temperature. After incubation with fluorescence-conjugated secondary antibodies, DNA was stained with a 1:300 dilution of DRAQ5 and mounted in Prolong gold antifade. Confocal microscopy was performed at room temperature on a microscope (TCS SP2 AOBS; Leica) using a plan Apo 20x 0.7 NA dry differential interference contrast objective (HC; Leica) and 2.29x zoom or on a microscope (TCS SP2; Leica) using a plan Apo 63x 1.32-0.6 oil CS objective (HCX; Leica) and 2 or 4x zoom. Images were collected with confocal software (Leica). Scale bars were added, and images were processed using Velocity (PerkinElmer). Video 1 was generated with Amira 4 (Visage Imaging). For time-lapse video microscopy, Caco-2 cysts were grown on top, treated as indicated in the text, and imaged at 37°C with a plan Neofluar 10 × 0.3 NA Ph1 ob- jective (JC, Carl Zeiss, Inc.) on a microscope (Axiovert 200; Carl Zeiss, Inc.) equipped with a motorized stage and a camera (Orca-ER-1394; Hama- tone). Images were processed using Volocity (PerkinElmer). To measure spindle angle

Video 1 was similar to Video 2, except it shows prolonged treatment with CTX at a lower magnification. Video 4 shows siRNA Cdc42-trans- fected Caco-2 cysts treated with CTX. Video 5 shows a three-dimensional re- construction of a two-cell stage Caco-2 structure stained for DNA, E-cadherin, and ZO-1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200807121/DC1.

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