Par6B and Atypical PKC Regulate Mitotic Spindle Orientation during Epithelial Morphogenesis*

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Cdc42 plays an evolutionarily conserved role in promoting cell polarity and is indispensable during epithelial morphogenesis. To further investigate the role of Cdc42, we have used a three-dimensional matrigel model, in which single Caco-2 cells develop to form polarized cysts. Using this system, we previously reported that Cdc42 controls mitotic spindle orientation during cell division to correctly position the apical surface in a growing epithelial structure. In the present study, we have investigated the specific downstream effectors through which Cdc42 controls this process. Here, we report that Par6B and its binding partner, atypical protein kinase C (aPKC), are required to regulate Caco-2 morphogenesis. Depletion or inhibition of Par6B or aPKC phenocopies the loss of Cdc42, inducing misorientation of the mitotic spindle, mispositioning of the nascent apical surface, and ultimately, the formation of aberrant cysts with multiple lumens. Mechanistically, Par6B and aPKC function interdependently in this context. Par6B localizes to the apical surface of Caco-2 cysts and is required to recruit aPKC to this compartment. Conversely, aPKC protects Par6B from proteasomal degradation, in a kinase-independent manner. In addition, we report that depletion or inhibition of aPKC induces robust apoptotic cell death in Caco-2 cells, significantly reducing both cyst size and number. Cell survival and apical positioning depend upon different thresholds of aPKC expression, suggesting that they are controlled by distinct downstream pathways. We conclude that Par6B and aPKC control mitotic spindle orientation in polarized epithelia and, furthermore, that aPKC coordinates mitotic spindle orientation through the regulated trafficking of vacuolar apical components. To further investigate the role of Cdc42, we have exploited a three-dimensional model of epithelial morphogenesis, in which single Caco-2 cells are cultured in matrigel to form polarized cysts. Using this system, we previously reported that Cdc42 controls mitotic spindle orientation to correctly position the nascent apical surface in a growing cyst. These findings highlight an intriguing relationship between epithelial mitosis and morphogenesis, which couples cell proliferation to tissue architecture.

A major challenge now is to determine the specific upstream regulators and downstream effectors through which Cdc42 controls epithelial morphogenesis. Recent work has focused on the identification of specific guanine nucleotide exchange factors that activate Cdc42 in this context. RNAi-based screens undertaken in Madin-Darby canine kidney (MDCK) cysts have identified two distinct guanine nucleotide exchange factors, Tuba (11) and Intersectin 2 (12), which control the recruitment and activation of Cdc42, at a subapical domain, and the centrosome, respectively, to facilitate mitotic spindle orientation and proper morphogenesis. The downstream effectors through which Cdc42 regulates this process have yet to be systematically investigated.

In non-polarized, adherent cells, Cdc42 orients the mitotic spindle, parallel to the substratum, through two distinct pathways (13). Cdc42 regulates the actin cytoskeleton, through PAK2 and βPix, and activates PI3K during mitosis. Both pathways are required for the localized accumulation of dynein-dynactin complexes at the midcortex and thus for proper spindle alignment (13). However, in polarized epithelial cells, spindle orientation is PI3K-independent (14), indicating that a different mechanism is at play. Furthermore, an additional requirement in these cells is that the spindle is aligned perpendicular to the axis of apical-basal polarity (10, 15, 16). The Cdc42 effector(s) that mediates spindle orientation in polarized epithelia is currently unknown.

Par6 (partitioning-defective 6) is a known Cdc42 effector (17–20) with a critical role in establishing cell polarity during asymmetric division (21, 22), directed migration (4), and the apical-basal polarization of epithelial cells (7, 17, 22). Par6 interacts specifically with Cdc42 in its GTP-bound, active form. Cdc42 binding induces a conformational change in Par6 (23) and promotes its polarized recruitment to specific subcellular

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* The abbreviations used are: MDCK, Madin-Darby canine kidney; aPKC, atypical protein kinase C; CTX, cholera toxin; H2B, histone 2B; nPKC, novel protein kinase C; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.
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compartments (24–27). In the present study, we reasoned that Par6 represents a strong candidate as the Cdc42 effector that controls polarized spindle orientation during mammalian epithelial morphogenesis, and we sought to test this using the Caco-2 cystogenesis model.

Par6 typically functions together with its binding partner, αPKC, downstream of Cdc42 (17–20). In vitro studies suggest that Par6 binding inhibits αPKC, in a manner that is relieved by active Cdc42 (27, 28); we note, however, that these effects may be somewhat context-dependent because other reports indicate that Par6 has either no significant effect on (18) or even enhances αPKC activity (4, 19). αPKC plays a prominent role in promoting cell polarity, during asymmetric division (29, 30), directed migration (4), and axon specification (31). αPKC is also indispensable for epithelial polarity (32). Inhibition of αPKC disrupts tight junction formation in cultured cells (7, 33–36) and induces severe epithelial defects during early embryogenesis (37–42). During epithelial morphogenesis, αPKC is required for apical surface formation (9, 43) and for the exclusion of basolateral proteins (44). Intriguingly, recent work has implicated αPKC in the apical exclusion of LGN, a critical spindle regulator, from mitotic MDCK cells (45). Furthermore, a myristoylated peptide, based on the inhibitory pseudosubstrate sequence of αPKC, randomizes spindle orientation (11). Together, these data suggest that αPKC may control spindle orientation in polarized epithelia. However, although the pseudosubstrate peptide is an effective αPKC inhibitor (46), its specificity is undefined, and in fact, it may inhibit other PKC isoforms (47). As such, it is important to confirm this prediction by other means.

In the present study, we investigated the hypothesis that Par6 and αPKC act downstream of Cdc42 to promote epithelial morphogenesis. We report that Par6B and αPKC function independently to control mitotic spindle orientation and proper positioning of the apical surface. In addition, we find that αPKC activity is indispensable for epithelial cell survival, suggesting that this kinase coordinately regulates multiple processes during epithelial morphogenesis.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning—All primers are listed in the supplemental material. All point mutations were introduced by QuickChange site-directed mutagenesis, using Pfu Turbo (Stratagene). Mouse Par6B was rendered resistant to siRNA duplex siPar6B.3 with two silent mutations and subcloned into pQCXIP (BamHI/EcoRI), with a C-terminal HA tag, by PCR. A ΔP136 deletion mutant was engineered, which is Cdc42 binding-deficient. Rat αPKCζ cDNA was kindly provided by Professor Peter Parker (Cancer Research UK, London). Two silent mutations were introduced to render αPKCζ resistant to siRNA duplex αPKC.1. Full-length or ΔP81 RNAi-resistant αPKCζ was then subcloned into pQCXIP (Agel/EcoRI). Additional point mutations were introduced as follows: D62A, A118E, and D375A. Cdc42 L61 in pRK5myc has been described previously (48). All constructs were fully sequenced.

Cell Culture—293FT cells were seeded as recommended by Invitrogen. Caco-2 (10) and 16HBE1- (7) cells were cultured as described previously; stable lines were selected with 6 μg/ml puromycin (InVivoGen). Three-dimensional cysts were grown on top of or embedded in Matrigel (BD Biosciences, catalog no. 354230) as described previously (10), or in 2% matrigel on glass-bottomed, 4-well Lab-Tek chamber slides (Nunc). Cysts were routinely stimulated with 0.1 μg/ml cholera toxin for 16 h prior to fixation to induce synchronous lumen expansion.

RNAi—All siRNA duplexes were purchased from Dharmacon (see supplemental material). Transfections were performed as described previously (10), except that 10⁵ cells were seeded/6-well dish. 1 day post-transfection, cells were reseeded as indicated, or the medium was changed. For titrations, siAPKC.1 was mixed with siLamin A/C in different proportions to maintain a final siRNA concentration of 50 nM.

Retrovirus Production and Infection—Retroviral particles were prepared for the delivery of pQCXIP-based plasmids (see supplemental material). mCherry-H2B retrovirus was kindly provided by Dr. Oliver Florey (Memorial Sloan-Kettering Cancer Center). 10⁵ Caco-2 cells were seeded/6-well dish and infected with 1.5 ml of viral suspension plus 8 μg/ml Polybrene by centrifugation at 2250 rpm for 30 min. The viral suspension was removed and replaced with fresh medium; where indicated, puromycin selection was initiated 2 days later. To avoid clonal effects, all experiments were performed with short term cultures of selected, stable pools.

FACS Analysis—4 days post-transfection, cells were fixed and stained with propidium iodide as described in the supplemental material. DNA content was measured using a FACSCalibur machine (BD Biosciences) to capture 20,000 events/sample/experiment; analysis was performed using FlowJo software. The data presented are representative of three separate experiments.

Gel Filtration—Gel filtration analysis was performed as described in the supplemental material. Briefly, Caco-2 cells were lysed in the following buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.6% octyl glucopyranoside, 1 mM NaVO₄, 10 mM NaF, 20 mM β-glycerophosphate, 1× protease inhibitors. The soluble fraction was isolated by ultracentrifugation and subjected to gel filtration chromatography using an AKTApurifier, equipped with a calibrated Superose 6 10/300 GL column (Amersham Biosciences).

Immunoprecipitation—293FT cells were seeded on 6-well dishes and co-transfected as indicated, using Lipofectamine 2000 (Invitrogen). 24 h later, cells were lysed and subjected to immunoprecipitation as described previously (49) but using a mouse anti-HA antibody (Covance, catalog no. 16B12; 1:100) and Protein G-Sepharose.

Western Blotting—Total cell extracts or radioimmune precipitation assay lysates were prepared as described in the supplemental material. PAGE and Western blotting were performed as described previously (49); Par6B and Par6B-HA were resolved using 10% BisTris gels. Antibody sources and dilutions are provided in the supplemental material. DNA content was measured using a FACSCalibur machine (BD Biosciences) to capture 20,000 events/sample/experiment; analysis was performed using FlowJo software. The data presented are representative of three separate experiments.

Immunofluorescence and Imaging—For analysis of morphological defects, “on top” cysts were first imaged live using an
inverted microscope and a ×10 objective (Zeiss). For quantification, these cysts were then fixed in 10% formalin and stained for actin (rhodamine phalloidin) and DNA (Hoechst 33342). Structures were visualized using an inverted microscope equipped with a ×40 objective (Zeiss) and scored for “single lumen,” “multilumen,” or “other” phenotypes. For fluorescent imaging, cysts embedded in matrigel or cultured in 2% matrigel on glass were fixed and stained as described previously (10), or with anti-Par6B (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) catalog no. H-64; 1:1000), anti-HA (Roche Applied Science, catalog no. 3F10; 1:100), anti-phosphoezrin (Cell Signaling, catalog no. 3149; 1:100), anti-occludin (Zymed Laboratories Inc., catalog no. 71-1500; 1:200), or cleaved caspase 3 (Cell Signaling, catalog no. 9661). Epifluorescent images were captured using an upright microscope, with a ×40 objective (Zeiss). Confocal images were acquired using a ×63 oil objective and 2× zoom (Leica); individual channels were scanned sequentially, line by line, at 200 Hz, with averaging set at 8. Each confocal image (1024 × 1024) represents a single “Z” optical section. Detailed fixation and staining protocols, microscope, objective, and camera models are provided in the supplemental material. All images are representative of data obtained from at least three separate experiments.

**Time Lapse Microscopy**—For video microscopy, cells were seeded on glass-bottomed, 6-well dishes (MatTek). Time lapse movies were acquired using a Nikon Eclipse Ti-U microscope, equipped with an S Plan Fluor ×20 ELWD objective (Nikon) and a Cool Snap HQ2 CCD camera (Photometrics), controlled by NIS-Elements imaging software (Nikon). Movies were processed and annotated using ImageJ software (National Institutes of Health).

**Line Intensity Scans**—The distribution of aPKC staining in three-dimensional cysts was analyzed using ImageJ (National Institutes of Health); a straight line was drawn along the apical-basal axis of one cell per cyst, along which signal intensities were measured. To determine the ratio between apical and cytosolic signals, intensities at the apical surface (defined by the cross-product of these vectors) was calculated to give the perpendicular line. Using Matlab software (MathWorks), the angle (θ) between the spindle axis (x) and the apical-basal axis (y) was calculated using the formula, cosine θ = dot(x,y)/(norm(x)∗norm(y)); angle θ = acos(cosθ)∗180/π.

**Statistics**—Statistical significance was evaluated using Prism software. Mann-Whitney or unpaired Student’s t tests were performed, with two-tailed p values and 95% confidence intervals.

**RESULTS**

**Par6B Regulates Caco-2 Three-dimensional Morphogenesis**—We recently reported that Cdc42 controls Caco-2 morphogenesis by orienting the mitotic spindle, perpendicular to the apical-basal axis, during cell division (Fig. 1A) (10). Misorientation of the spindle has profound implications for epithelial architecture, promoting cell division in the wrong plane, mispositioning the newly formed apical surface between daughter cells, and ultimately forming defective cysts with multiple lumens. In the present study, we sought to identify the downstream effector through which Cdc42 regulates spindle orientation and epithelial morphogenesis, using the formation of Caco-2 cysts with multiple lumens as a read-out. Taking a candidate approach, we focused on the polarity protein Par6, a known target of Cdc42 (17–20).

Mammalian Par6 comprises a family of four related isofoms, A, B, C, and D (17). Preliminary studies using isofrom-specific pools of siRNA indicated that depletion of Par6B was sufficient to disrupt Caco-2 morphogenesis (supplemental Fig. 1). To validate this observation, four individual siPar6B duplexes were tested, which depleted protein levels to varying extents (Fig. 1B); aPKC provided a loading control. Cells treated with either control or Par6B-specific siRNA were cultured in matrigel, and the resulting cysts were visualized by phase microscopy (Fig. 1C). Depletion of Par6B induced a gross defect in morphogenesis, comparable with the loss of Cdc42. Although >80% of control structures possessed a single, central, fluid-filled lumen, only around half of siPar6B cysts exhibited this normal morphology (arrowheads indicate defective structures).

To examine this morphological defect in more detail, fixed cysts were analyzed by confocal microscopy, staining for DNA to mark individual cells and for actin to visualize the apical surface (Fig. 1, D and E). Depletion of Par6B phenocopied the loss of Cdc42, inducing the formation of aberrant cysts with multiple lumens. Importantly, this phenotype was observed with all four duplexes tested, indicating that this is a specific effect. Moreover, the level of Par6B knockdown correlated using AutoQuant X2.2.0 software (Media Cybernetics). To obtain measurements, three-dimensional reconstructions were analyzed in Imaris software (Bitplane Scientific Software). Each spindle pole was marked as a point in three-dimensional space to give two sets of x,y,z co-ordinates (p1, p2), which define the spindle axis (p2-p1). The axis of apical-basal polarity was defined as a straight line through the center of the mitotic cell, running perpendicular to its apical surface. To determine this axis, three points were marked along the apical surface (p3, p4, p5) to construct two vectors (v1 = p4-p3; v2 = p5-p3), and the cross-product of these vectors was calculated to give the perpendicular line. Using Matlab software (MathWorks), the angle (θ) between the spindle axis (x) and the apical-basal axis (y) was calculated using the formula, cosine θ = dot(x,y)/(norm(x)∗norm(y)); angle θ = acos(cosθ)∗180/π.
closely with the severity of the phenotype (Fig. 1, compare B and D).

Although partial depletion of Par6B promoted the formation of aberrant lumens in Caco-2 cysts, it did not disrupt apical-basal polarity per se. Each individual lumen was surrounded by apical markers, such as actin (Fig. 1E) and ezrin (supplemental Fig. 2A), and both tight junctions and adherens junctions appeared intact, as judged by occludin and E-cadherin staining, respectively (supplemental Fig. 2, B and C). Together, these data reveal that Par6B controls the proper positioning of the apical surface during Caco-2 morphogenesis.

**Par6B Functions Downstream of Cdc42 to Promote Caco-2 Morphogenesis**—To confirm that Par6B functions downstream of Cdc42 during cyst development, an RNAi rescue experiment was performed. Stable pools of Caco-2 cells were derived, expressing either an empty vector (pQCXIP) or a mouse Par6B construct, C-terminally HA-tagged, and rendered resistant to duplex siPar6B.3 (Fig. 2A). The function of wild type Par6B was compared with a ΔP136 mutant, which is Cdc42 binding-deficient (Fig. 2B) but still able to interact with other key partners, such as Par3, aPKC, and Lgl1 (50). As shown in Fig. 2, C and D, expression of wild type Par6B-HA was able to rescue proper morphogenesis following depletion of the endogenous protein. In contrast, expression of the ΔP136 mutant was unable to rescue the multilumen defect, indicating that Cdc42 binding is indispensable for Par6B function in this context. These data are consistent with a model in which Par6B acts as a downstream effector for Cdc42 during Caco-2 morphogenesis.

**aPKC Regulates Caco-2 Three-dimensional Morphogenesis**—Par6 typically functions with its binding partner, aPKC, to mediate signaling downstream of Cdc42 (32, 51, 52). To test whether aPKC is also required during Caco-2 morphogenesis, two non-overlapping duplexes were designed, each of which efficiently co-depletes the two mammalian isoforms of aPKC.
Antibody specificity was established using recombinant proteins (supplemental Fig. 3). Depletion of aPKC with either duplex induced major defects in morphogenesis; only around one-third of siaPKC cysts possessed a single, central, fluid-filled lumen (Fig. 3B; arrowheads indicate defective structures). Notably, aPKC-depleted cysts were also significantly smaller and fewer than control structures, suggesting an additional role for this protein in either cell proliferation or survival (see below). Confocal imaging confirmed that depletion of aPKC increases the formation of Caco-2 cysts with multiple lumens (Fig. 3, C and D). We conclude that aPKC functions with Cdc42 and Par6B to control apical surface positioning.

Surprisingly, no obvious defects in apical-basal polarity or junction formation were detected following aPKC depletion under these conditions; each individual lumen in an aberrant cyst was surrounded by apical markers (e.g. actin (Fig. 3D) or ezrin (data not shown)) and filled with fluid, suggesting intact barrier function. We believe that a stronger knockdown is likely to be required to disrupt polarity and junctions, but we did not explore this further in the present study (see “Discussion”).

**Par6B and aPKC Control Spindle Orientation during Morphogenesis**

(Fig. 3A; antibody specificity was established using recombinant proteins (supplemental Fig. 3)). Depletion of aPKC with either duplex induced major defects in morphogenesis; only around one-third of siaPKC cysts possessed a single, central, fluid-filled lumen (Fig. 3B; arrowheads indicate defective structures). Notably, aPKC-depleted cysts were also significantly smaller and fewer than control structures, suggesting an additional role for this protein in either cell proliferation or survival (see below). Confocal imaging confirmed that depletion of aPKC increases the formation of Caco-2 cysts with multiple lumens (Fig. 3, C and D). We conclude that aPKC functions with Cdc42 and Par6B to control apical surface positioning.

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**Par6B and aPKC Control Mitotic Spindle Orientation**—Cdc42 promotes proper epithelial morphogenesis by orienting the mitotic spindle during cell division (Fig. 1A) (10). To test whether Par6B and aPKC act through the same mechanism, these proteins were depleted, and spindle orientation was measured during the early stages of cyst development (Fig. 4A). Under control conditions, the majority of spindles were oriented perpendicular to the axis of apical-basal polarity (Fig. 4, B and C; median angle = 82.2°), consistent with previous reports (10). However, upon depletion of either Par6B or aPKC, spindle orientation was essentially randomized (Fig. 4, B and C). We conclude that, like Cdc42, the Par6B-aPKC complex controls mitotic spindle orientation during cell division to correctly position the nascent apical surface in a growing cyst.

**Par6B Is Apically Localized**—In many contexts, the ability of Par6 to promote polarization depends upon its own asymmet-
Par6B and aPKC Control Spindle Orientation during Morphogenesis

To determine whether the apical localization of Par6B is induced specifically in a three-dimensional environment, we cultured Caco-2 cells on glass coverslips for immunofluorescence. Mature monolayers were stained for either the endogenous (Fig. 5C) or exogenous protein (Fig. 5D). In both cases, a clear signal was detected at epithelial junctions, indicating that Par6B is polarized in two-dimensional as well as three-dimensional cultures.

To address whether the apical enrichment of Par6B is a cell type-specific phenomenon, we stained monolayers of 16HBE, a human bronchial epithelial cell line. In this cell line, a cytosolic signal could be detected, but both endogenous (Fig. 5C) and exogenous ParB (data not shown) were also observed at apical junctions, consistent with our observations in Caco-2. Together, these data reveal that Par6B is an apical protein and suggest that its asymmetric distribution may be important during epithelial morphogenesis.

Par6B Localizes aPKC to the Apical Surface—During the directed migration of astrocytes, Par6 recruits aPKC to the leading edge of the cell (4). To determine whether Par6B is similarly required to localize aPKC in Caco-2 cysts, siRNA-treated structures were analyzed by immunofluorescence (Fig. 6, A–C). Under control conditions, aPKC was polarized to the apical surface, consistent with previous reports (10). Strikingly, depletion of Par6B attenuated the apical enrichment of aPKC, whereas actin remained concentrated at the apical surface of each individual lumen. The reduction in apical aPKC clearly resulted from a change in its localization because total protein levels did not change following Par6B depletion (Fig. 1B). These data suggest that Par6B localizes aPKC to the apical surface to control morphogenesis. Notably, depletion of Cdc42 did not mislocalize aPKC (Fig. 6, A–C). Because Cdc42 can activate the Par6aPKC complex in vitro (19, 28), we surmise that Par6B acts to localize aPKC for subsequent activation by Cdc42.

To determine whether aPKC kinase activity is required for morphogenesis, pharmacological inhibitors were employed. A myristoylated peptide, based on the pseudosubstrate sequence of aPKCζ, is an effective aPKC inhibitor (46, 47). However, this peptide appeared not to enter matrigel efficiently and was not amenable to these experiments. As an alternative, two small molecule inhibitors with different, but overlapping, specificities for PKC family members were exploited (54). G66983 inhibits all PKC isoforms, whereas BIM1 inhibits classical PKC and novel PKC (nPKC) subtypes but not aPKCs; the comparative use of these compounds is an established means of testing the requirement for aPKC activity (55). As shown in Fig. 6D, G66983, but not BIM1, induced a significant increase in the formation of cysts with multiple lumens, consistent with a specific role for aPKC kinase activity during morphogenesis. We conclude that aPKC promotes proper spindle orientation through the phosphorylation of downstream substrates.

aPKC Stabilizes Par6B through a Kinase-independent Mechanism—During the course of these studies, we also uncovered an unexpected role for aPKC in the regulation of Par6B. Depletion of aPKC, with either of two distinct duplexes, induced a corresponding down-regulation of Par6B protein expression (Fig. 7A). Under these conditions, Par6B levels could be partially

![Image](66x637 to 141x655)

**FIGURE 3.** aPKC regulates Caco-2 three-dimensional epithelial morphogenesis. Caco-2 cells were transfected with siRNA and reseeded as follows. A, on 6-well dishes. Total cell extracts were prepared 6 days post-transfection for Western blotting. B and C, “on top” of matrigel. Seven days post-transfection, cysts were imaged using phase-contrast microscopy to visualize gross morphology; arrowheads indicate abnormal structures. The mean percentage of cysts with a normal morphology (single, central lumen) is indicated ± S.D. Scale bars, 100 μm. C, cysts were fixed and stained for actin/DNA to score for the presence of multiple lumens. At least 100 cysts were counted per condition. Bars, mean ± S.D. (error bars) from three separate experiments. Student’s t tests were performed to determine statistical significance. *, p < 0.02; ***, p < 0.001. D, embedded in matrigel. Seven days post-transfection, cysts were stimulated with CTX and then fixed and stained for actin (red) and DNA (blue). Single confocal sections are presented. Scale bars, 25 μm.

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restored by treatment with the proteasome inhibitor MG132, suggesting that in the absence of aPKC, Par6B is subject to proteasome-mediated degradation. To investigate the molecular basis for Par6B stabilization, we performed RNAi rescue experiments, using a panel of aPKCζ domain mutants (Fig. 7B); these constructs were resistant to duplex siaPKC.1 but sensitive
to siaPKC.2 (Fig. 7C, top). As expected, expression of RNAi-resistant wild-type aPKCΔC rescued the degradation of Par6B following treatment with duplex 1 but not duplex 2 (Fig. 7C). The D375A mutant was equally efficient in restoring Par6B levels, indicating that aPKC kinase activity is dispensable for this function. In contrast, neither Par6 binding mutant (ΔPB1 or D62A) was able to rescue, suggesting that physical association with aPKC is essential. From these data, we predicted that Par6B should be bound stoichiometrically to aPKC in Caco-2 cells. In agreement with this, gel filtration chromatography revealed that Par6B co-eluted with aPKC in fractions containing high molecular weight complexes (Fig. 7D), whereas monomeric Par6B was undetectable. We conclude that aPKC stabilizes Par6B in a kinase-independent manner and that the physical interaction of the two proteins protects Par6B from proteasomal degradation. Together, these data reveal that Par6B and aPKC function interdependently during epithelial morphogenesis.

**aPKC Controls Cell Survival**—In addition to inducing multiple lumens, aPKC depletion also led to the formation of consistently smaller and fewer cysts (Fig. 3, B and D). This observation suggests that aPKC plays an additional role during morphogenesis in the regulation of cell number. To confirm this, Caco-2 cells were transfected with siaPKC.1/2 and counted over 8 days. Growth curves show that depletion of aPKC with either duplex led to a significant decrease in cell number (Fig. 8A). To validate this observation, a rescue experiment was performed using aPKCΔC (resistant to duplex 1 but not duplex 2). Stable pools of cells, expressing either an empty vector or aPKCΔC, were transfected with siRNA and counted 6 days later (Fig. 8B). Expression of aPKCΔC partially rescues the decrease associated with siaPKC.1, but not siaPKC.2, confirming that depletion of aPKC specifically reduces Caco-2 cell number.

To examine this phenotype in more detail, the DNA content of siRNA-treated cells was analyzed by FACS. There was no detectable effect of aPKC depletion on cell cycle progression (Fig. 8C). However, a significant increase in the sub-G1 fraction was observed, indicative of DNA fragmentation and cell death (Fig. 8D). Using time lapse microscopy, Caco-2 morphology was analyzed following treatment with siaPKC; these experiments were performed using cells stably expressing mCherry-histone 2B, so nuclear changes could also be monitored (Fig. 8E and supplemental Movie 1). Depletion of aPKC induces substantial DNA fragmentation and membrane blebbing, characteristic of apoptotic cell death (56). Wide field movies (phase only), captured over a longer time course, reveal the penetrance of this phenotype and highlight the dramatic effect on endpoint cell number (supplemental Movie 2). Importantly, comparable morphological changes were observed in aPKC-depleted cysts (Fig. 8F), suggesting that cell death is similarly induced in both two- and three-dimensional cultures.
Finally, we sought to determine whether aPKC kinase activity is required for cell survival. RNAi rescue experiments were precluded because cell lines expressing kinase-dead aPKCř/H9256 (D375A) could not be stably maintained. This observation suggested that the inactive construct may behave as a dominant negative with respect to cell survival. To explore this further, cells were incubated with the myristoylated, aPKCř/H9256 pseudosubstrate peptide inhibitor; as a control, the myristoylated, nPKCř/H9258 pseudosubstrate peptide was employed (Fig. 8G). Whereas cells treated with the nPKCř inhibitor appear similar to controls, the aPKCř inhibitor induces synchronous cell death. Cells fixed 4 h after treatment show a significant increase in nuclear condensation and cleaved caspase 3 staining, indicative of apoptosis. We conclude that aPKC promotes cell survival in a kinase-dependent manner.

Apical Positioning and Cell Survival Depend upon Different Thresholds of aPKC Expression—The data presented indicate that aPKC controls both apical surface positioning and cell survival during morphogenesis. Because cysts with multiple lumens are readily detectable upon aPKC depletion (Fig. 3), despite the significant increase in apoptotic cell death (Fig. 8), we reasoned that these may represent distinct functions that depend upon different levels of aPKC expression. To test this, different amounts of siPKC.1 were transfected into Caco-2 to titrate aPKC protein levels; cells were then seeded in parallel to analyze either cell number or three-dimensional morphogenesis (Fig. 8H). Cells expressing intermediate levels of aPKC (after treatment with 10 nM siRNA) were significantly impaired in apical surface positioning, with 25% of cysts possessing multiple lumens. However, cell number was unchanged under these conditions. Upon a more robust depletion of aPKC (using 50 nM siRNA), the frequency of morphological defects was further increased to around 40%; however, cell number was now also significantly reduced. We conclude that apical positioning and cell survival depend upon different thresholds of aPKC expression. Positioning of the apical surface is sensitive to even a modest decrease in aPKC, whereas a significantly stronger reduction is required to induce apoptosis.

DISCUSSION

We have used a three-dimensional tissue culture model to explore the downstream effectors though which Cdc42 con-
controls mitotic spindle orientation during epithelial morphogenesis. Here, we report that Par6B and aPKC function interdependently to control this process and, furthermore, that aPKC activity is also indispensable for epithelial cell survival under basal conditions.

During Caco-2 cyst development, the position of the apical surface is determined by the orientation of the mitotic spindle during cell division (10), highlighting an intimate relationship between epithelial mitosis and morphogenesis. Cdc42 is essential for orienting the spindle perpendicular to the apical-basal axis, and accordingly, depletion of Cdc42 promotes mispositioning of the apical surface and the formation of aberrant cysts with multiple lumens. In the present study, we explored the hypothesis that the Par6/H18528aPKC polarity complex may be required to mediate Cdc42-dependent spindle orientation. Using an RNAi-based approach, we found that depletion of Par6 phenocopies the loss of Cdc42, inducing misorientation of the mitotic spindle and the development of defective cysts with multiple lumens. Through rescue experiments, we confirmed that Cdc42 binding is indispensable for Par6 function, consistent with it acting as a direct Cdc42 effector. Specifically, we find that the Par6B species is required for cystogenesis, providing insight into isoform specificity within the mammalian Par6 family. However, our data do not exclude possible roles for other Par6 family members; the knockdown levels of Par6A and/or Par6G achieved here may simply be insufficient to induce a robust phenotype. Related to this, we note that mutation of zebrafish Pard6yb leads to misorientation of the mitotic spindle and formation of multiple lumens in the developing neural tube (57).

Consistent with work in other systems, we find that Par6 functions together with its binding partner aPKC during epithelial morphogenesis. Depletion of aPKC leads to misorientation of the mitotic spindle, mispositioning of the apical surface, and ultimately, to aberrant cystogenesis. Surprisingly, we did not observe an obvious defect in apical-basal polarity or junction formation arising from depletion of either Par6B or aPKC under these conditions. It is likely that a partial depletion of these proteins is insufficient to completely disrupt polarity. With regard to Par6B, either a stronger knockdown may be required or the co-depletion of other Par6 isoforms, which may act redundantly. With respect to aPKC, we show that a more robust knockdown induces apoptotic cell death, precluding the analysis of cell polarity/junctions under these conditions.

Cdc42, Par6, and aPKC are well known to control spindle orientation during asymmetric cell division, for instance during the first cleavage of the C. elegans embryo (30) and in Drosophila neuroblasts (29). In this context, spindle orientation controls cell fate. It is intriguing now to discover that conserved machinery regulates the asymmetrical divisions of epithelia, where spindle orientation couples cell proliferation to tissue architecture. This also adds to the wide range of biological contexts in which Cdc42 signals through the Par6-aPKC complex (51).

With respect to molecular mechanisms, we identify a mutual dependence between Par6B and aPKC (Fig. 9A). First, aPKC
protects Par6B from proteasomal degradation. This mechanism ensures that Par6B is constitutively bound to and able to signal through aPKC. Interestingly, aPKC kinase activity is not necessary for Par6B stabilization. Importantly, this finding excludes the possibility that aPKC functions solely to protect Par6B because its kinase activity is also indispensable for morphogenesis. In addition, these data add to the emerging view that PKC family members can mediate a collection of kinase-
Par6B and aPKC Control Spindle Orientation during Morphogenesis

A

Par6B

proteasome

aPKC

mislocalized

active

substrate phosphorylation

spindle orientation/
apical positioning

basal

B

aPKC

spindle orientation/
apical positioning

cell survival

apical-basal polarity/
junction formation

(threshold of expression required)

epithelial morphogenesis

FIGURE 9. Par6B and aPKC regulate spindle orientation and cell survival during epithelial morphogenesis. A, Par6B and aPKC function interdependently to control spindle orientation downstream of Cdc42. aPKC protects Par6B from proteasome-mediated degradation in a kinase-independent manner. Reciprocally, Par6B localizes aPKC to the apical surface, restraining the kinase in an inactive conformation. Active Cdc42 binds to Par6, provoking a conformational change, which relieves the inhibition of aPKC. This two-step mechanism ensures that aPKC activity is precisely regulated, both spatially, by Par6, and temporally, by Cdc42. B, aPKC performs multiple, distinct functions during mammalian epithelial morphogenesis. Shown is a schematic summarizing the multiple roles of aPKC in mammalian epithelial cells; the threshold of expression required to support apical positioning and cell survival is indicated.

... independent functions (58). To our knowledge, this is the first report to show that aPKC controls Par6 stability, although we predict that this effect may be relevant in other biological contexts that require the Par6-aPKC complex. In particular, it would be pertinent to investigate whether Par6 stabilization is important in processes that require aPKC protein but not kinase activity, such as the control of follicle cell polarity in Drosophila (59).

Conversely, Par6B plays a reciprocal role in localizing aPKC to the apical surface. This observation was initially surprising to us because Par6B itself is reported to distribute throughout the cytosol when exogenously expressed in MDCK cells (17, 53). However, we find that endogenous Par6B is resident at the apical surface in Caco-2 cysts, suggesting that it directly recruits its interacting partner to this compartment. In addition to controlling aPKC localization, Par6 can also regulate its catalytic activity (32). As such, we envisage a two-step mechanism for aPKC activation in epithelial cells (Fig. 9A). Par6B recruits aPKC to the apical surface but is expected to hold the kinase there, poised in an inactive conformation. Binding of active Cdc42 to Par6B induces a conformational change, which relieves this inhibition and permits substrate phosphorylation (23). This mechanism would provide a precise means of regulating aPKC activity both spatially, through Par6B, and temporally, through Cdc42 (52).

The exogenous expression of aPKC was found to perturb Caco-2 cyst morphology, confounding further analysis of molecular mechanisms through RNAi rescue experiments. However, small molecule kinase inhibitors support a specific requirement for aPKC kinase activity during morphogenesis. Consistent with this, an inhibitory aPKC pseudosubstrate peptide has also been shown to misorient the spindle in MDCK cells (11). Furthermore, the zebrafish heart and soul mutation, which renders aPKC kinase deficient, randomizes spindle orientation in retinal progenitors, which can divide either symmetrically or asymmetrically (40). A major goal of our ongoing work is thus to identify substrates through which aPKC controls spindle orientation and positioning of the apical surface.

We have identified an additional function for aPKC during morphogenesis, in the regulation of Caco-2 cell number. Although aPKC is well known as a mitogen (60), we find no evidence for a change in cell cycle progression under the conditions tested. Instead, loss of aPKC activity promotes robust apoptotic cell death. Consistent with this, loss of aPKC function increases TUNEL staining at certain developmental time points in Drosophila (37) and Xenopus (39), and apoptotic markers have been observed in MDCK cells following aPKC depletion or inhibition (61). In the latter case, there was no corresponding change in cell number, possibly due to compensatory increases in proliferation (61, 62). Here, we demonstrate that loss of aPKC has profound consequences for cyst size and number, indicating that support of cell survival is a further, vital function for aPKC during morphogenesis.

Atypical PKC is well known to protect cells from stimulus-induced death. aPKC suppresses apoptosis in leukemic cells following treatment with TNFα, Fas ligand, or chemotherapeutic drugs, generally through activation of the NFκB pathway (63, 64), and in fibroblasts, inhibition of aPKC is induced by UV treatment (65) or Par-4 expression (66) prior to cell death. In the present study, we show that depletion or inhibition of aPKC is sufficient to trigger apoptosis in Caco-2, even under basal conditions. We conclude that aPKC must constitutively transduce critical survival signals. Because epithelial cells are the source of the majority of human tumors, their sensitivity to aPKC inhibition may present a significant therapeutic opportunity. Our data support the emerging view that aPKC represents a potential drug target in certain cancers (67).

Together, our current findings reveal that aPKC performs multiple functions during epithelial morphogenesis (Fig. 9B). Proper spindle orientation and cell survival depend upon different thresholds of aPKC expression, suggesting that they are likely to be regulated by distinct downstream pathways. The multifunctional nature of aPKC could have important implications for its therapeutic targeting. Although robust inhibition induces apoptosis, a desirable outcome in treating a tumor, cells would be expected to survive a partial inhibition but to be impaired in spindle orientation and apical surface positioning. These defects could disrupt epithelial architecture in proliferating tissues, potentially inducing unwanted side effects or even contributing to transformation (68). These may be important...
considerations for designing a therapeutic strategy based on inhibition of aPKC.

In summary, we describe two distinct functions for aPKC during mammalian epithelial morphogenesis. A major goal of our ongoing work is to identify those substrates through which aPKC controls spindle orientation/apical surface positioning and cell survival.

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