Rac1 is required for reorientation of polarity and lumen formation through a PI 3-kinase-dependent pathway

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ONE OF THE HALLMARKS of epithelial cells is the capacity to form highly polarized, differentiated sheets of cells that line the majority of organs, including the gastrointestinal tract, kidney, and lungs (12, 34). These cells have distinct and specialized membrane domains. The apical surface of the cells typically faces the interior surface or lumen of the organ and is characterized by the presence of specific cellular proteins. Cells are attached to one another at their lateral membranes. The lateral membrane is characterized by a series of junctions, including the tight junction (25), which separates the basolateral surface from the apical surface. The basal surface of cells is attached to extracellular matrix (ECM). Epithelial cell polarity requires the sorting of proteins to the appropriate apical or basolateral surface or junctional complex (5, 19).

The requirements for polarization can be further divided into signals required for polarity itself and signals required for the appropriate orientation of polarity. One powerful model system for distinguishing these two types of signals is the Madin-Darby canine kidney (MDCK) three-dimensional cell culture system (22). In this model system, a suspension of MDCK cells is plated in a collagen gel matrix. These single cells proliferate and differentiate to form multicellular, highly polarized, fluid-filled cysts. The architecture of these cysts recapitulates the normal architecture of epithelial cell-lined organs, with the apical cell surface facing the lumen. In contrast to two-dimensional culture systems (29), where epithelial cells receive an overwhelming cue from a culture support (such as a semipermeable filter) to direct the polarization process, this three-dimensional model requires appropriate cell-ECM interactions in order for cells to orient their polarity. Loss of function of either Rac1 or β1-integrin causes the cysts to develop inverted polarity, with the apical surface facing the outside of the cyst (21, 32).

A complexity of this system is that the cells proliferate as they form polarized cysts. Because some signaling events may be required for both polarity and proliferation, we were interested in identifying a model to examine events required for polarization, distinct from a requirement for proliferation. Wang and colleagues (30, 31) have described a culture system in which MDCK cells are grown in suspension. These cells form multicellular cysts with a small amount of ECM in the space between the cells. The apical surface of the cells is oriented toward the culture medium. When these cysts are removed from suspension and plated in collagen, the cells reverse their polarity: a new apical surface is formed that faces a new lumen in the interior of the cyst, and the new basolateral surface contacts the ECM surrounding the cells. These events occur rapidly (within 12–24 h), before significant proliferation.

We have used this system to characterize the signaling requirements for the reorientation of polarity. Because cysts are simultaneously subjected to the cue to reorient their polarity (the addition of cysts to collagen), signaling pathways that are activated by integrin-ECM interactions are synchronized, facilitating biochemical characterization. Thus this is an ideal model system in which to test the signaling requirements for reorientation of polarity in response to cues from the ECM.
Using this system, we have examined the requirements for Rac1, phosphoinositide 3'-kinase (PI 3-kinase) and atypical protein kinase C (aPKC) during reorientation of polarity. Studies from our laboratory have previously demonstrated that Rac1 plays a critical role in the orientation of polarity in three-dimensional culture systems, but the molecular events downstream of Rac1 remain unclear. PI 3-kinase and protein kinase B (Akt) 1/2 are known to be downstream of Rac1 as well as other Rho family GTPases in other signaling contexts, so we hypothesized that these molecules might play an important role in the repolarization events induced by ECM. We also investigated the role of aPKC, given the important role of this molecule in polarization in other systems (11). We demonstrated that Rac1, PI 3-kinase, and aPKC, but not Akt1/2, appear to be critical for the reorientation of cell polarity in response to ECM cues.

MATERIALS AND METHODS

Antibodies and reagents. The mouse anti-pp135 antibody was the generous gift of George Ojakian (SUNY-Downstate, Brooklyn, NY). The β1-integrin blocking rat monoclonal antibody AIIB2 was the generous gift of Caroline Damsky (University of California, San Francisco, CA). The rat anti-ZO-1 (R40.76) was a gift of Bruce Stevenson (University of Alberta). Other primary antibodies used in this study were mouse anti-cis-Golgi enzyme GM130 and mouse-anti-aPKC (BD Biosciences, San Jose, CA). Rabbit anti-β-catenin and rabbit anti-phospho-aPKC were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Akt, rabbit anti-phospho-Akt (T308), rabbit anti-GSKβ, and rabbit anti-phospho-GSKα/β (Ser21/9) were obtained from Cell Signaling (Danvers, MA). LY-294002 and 1,3-diydro-1-[(4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)-phenyl)methyl]-4-piperidinyl)-2H-benzimidazol-2-one (Akt inhibitor VIII) were from Calbiochem (San Diego, CA). The myristoylated aPKC pseudosubstrate was obtained from Biosource (Camarillo, CA). Secondary antibodies used were anti-mouse Alexa fluor 488, anti-rat Alexa fluor 488, and anti-rabbit Alexa fluor 555; actin filaments were stained with Alexa Fluor 555 or 633 phalloidin, and nuclei were stained with Hoescht 33342 (all from Invitrogen, Carlsbad, CA).

Cell culture. MDCK cells were maintained in minimal essential media containing Earle’s balanced salt solution (Cellgro, Washington, DC) supplemented with 5% FBS and penicillin/streptomycin. T23 MDCK cells expressing N17Rac1 under the control of the tetracycline-repressible transactivator (14) were maintained in growth medium supplemented with 20 ng/ml tetracycline. MDCK cell lines expressing constitutively active (CA) Akt and a control cell line transfected with the parental vector were the generous gift of Emma Shitivelman and have been described previously.

For the inside-out cyst preparation, cells were trypsinized in a single-cell suspension. The cell suspension (1.5 ml) was plated in each well of a 24-well plate pretreated with poly(2-hydroxyethyl)methacrylate (P-3932; Sigma, St. Louis, MO) or in a 24-well ultra low attachment plate (3473; Corning, Acton, MA). Cells were resuspended by pipetting 24 h after plating. Inside-out cysts were allowed to form for the next 4–5 days. To induce expression of N17Rac1, inside-out cysts were pelleted on day 4, washed three times with medium to remove doxycycline, and then replated in doxycycline-free growth medium. Cysts were plated in collagen 24–48 h after induction of transgene expression.

Inside-out cysts were plated in collagen as follows. Briefly, cysts were gently pelleted and resuspended in a type 1 collagen solution (66% Vitrogen, 3 mg/ml; Cohesion, Palo Alto, CA). Collagen mixture (160 μl) was plated on a 10-mm, 0.02-μm pore Anopore filter (Nalgene Nunc, Rochester NY) for immunofluorescence studies or on a 12-mm, 0.4-μm pore polyester Transwell insert (Corning 3460) on top of a cell-free gelled collagen layer for biochemical studies. The collagen solution was allowed to gel at 37°C, and then medium was added. To treat inside-out cysts with AIIB2, the antibody was added to the collagen gel as well as to the culture medium to a final concentration of 8 μg/ml.

Immunofluorescence staining. The procedure for the immunofluorescence of MDCK cells embedded in a collagen gel has been described previously in detail (21, 23, 33). Briefly, samples were rinsed twice with PBS containing calcium and magnesium, treated with collagenase type VII (Sigma-Aldrich) at room temperature for 7 min, and fixed with 4% paraformaldehyde for 30 min. Cells were permeabilized with 0.25% Triton X-100 for 30 min and blocked with 0.7% fish skin gelatin in PBS/0.025% saponin (PFS) for an additional 30 min. Samples were then incubated in primary antibodies at 4°C overnight and washed extensively with PFS, followed by an overnight incubation with Alexa fluor-conjugated secondary antibodies (1:400) and phallolidin (1:400). Nuclei were stained with Hoescht 33342 (1:500) for 30 min, and then the samples were washed with PBS and mounted with Prolong antifade (Invitrogen). For the time-course experiments, time 0 cysts were washed, pelleted, and fixed in 4% paraformaldehyde for 30 min. The cysts were then washed and plated in a collagen gel for immunofluorescence staining.

Image analysis. To quantitate cyst polarity, cysts were stained for gp135, actin, and nuclei and analyzed with a Zeiss 510 LSM confocal microscope. Cysts were classified based on the localization of gp135 and scored as gp135 on the outside and interior surfaces, or on the interior surface exclusively. Each experiment was done at least three times, and 25 cysts/condition were analyzed per experiment.

Inhibitor treatment of cysts. For inhibitor experiments, day 5 inside-out cysts were gently pelleted and resuspended in medium containing the appropriate inhibitor for 1–2 h before plating in collagen. Specifically, for LY-294002, cells were pretreated with 20 μM LY-294002 and then either fixed (for the time 0 time point) or plated in collagen for 16 h; our laboratory has previously demonstrated that MDCK cells show good viability under these conditions (33). Alternatively, cells were pretreated with the Akt inhibitor VIII at a concentration of 5 μM for 1 h or the myristoylated aPKC pseudosubstrate at a concentration of 25 μM for 2 h.

In vitro kinase assay/immunoprecipitation/Western blotting. Active Akt1 was detected using a nonradioactive Akt kinase assay kit (Cell Signaling) according to the manufacturer’s instructions. Protein concentrations were quantitated using the DC protein assay (Bio-Rad, Hercules, CA) and normalized before immunoprecipitation. The products of the kinase reaction were separated by SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA). Membranes were probed with the phospho-GSKα/β antibody. To confirm equal loading, blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford IL) and reprobed with the anti-GSKβ antibody.

For other biochemical experiments, cells were pelleted as above and lysed in 1% Nonidet P-40 (NP-40) lysis buffer (140 mM NaCl, 10 mM Tris·HCl, 1% NP-40, 5 mM EDTA, pH 7.5 ± Pls). Protein concentrations were quantitated, and equal amounts of protein were separated by SDS-PAGE, transferred to Immobilon-P, and subjected to Western blotting with the appropriate antibody. To confirm equal protein loading, blots were stripped and reprobed with antibodies directed against the total protein (for example, total aPKC) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody.

RESULTS

Dynamics of inside-out cyst formation and reversion. MDCK cells were grown in suspension by plating in tissue culture wells coated with poly-HEMA (to prevent the cells from attaching to the surface) and allowed to form cysts with inside-out polarity. Cells were grown in suspension for 5–6 days before embedding in collagen (Fig. 1). As shown in Fig. 2A, apical markers such as gp135/podocalyxin were found...
on the outer free surface of the cysts, and basolateral markers such as β-catenin were excluded from the free surface. In polarized epithelial cells, the Golgi lies between the nucleus and the apical surface of the cell; in these inside-out cysts, the Golgi is localized to the exterior surface of the cysts (Fig. 2F). Tight junction markers such as ZO-1 are localized to the exterior surface of the cysts (Fig. 2K).

To induce repolarization, cysts were embedded in type I collagen. At the indicated time points, the cysts were fixed, and the localization of cell polarity markers was examined by confocal microscopy. Of the markers examined, the relocalization of gp135 occurred most rapidly; at 8 h, gp135 had disappeared from significant portions of the free surface of the cells and started to appear at cell-cell junctions at the site of nascent lumens (Fig. 2B). By 16 h, gp135 was significantly localized to the interior surfaces of the cysts, and many small lumens were beginning to take shape (Fig. 2C). The relocalization of the Golgi and tight junctions occurred more slowly and only became a prominent feature at 16 h. By 24 h, a number of distinct lumens were present within the cysts, and by 48 h these multiple lumens appeared to coalesce into larger lumens (Fig. 2 and Liu, unpublished observations). These findings confirm the results of Wang and colleagues (30, 31) and further extend these studies by demonstrating the highly synchronized and dynamic nature of the reorientation process.

Role of Rac1 in inside-out cyst reversion. Integrins are often critical mediators of cellular interactions with ECM. As previously shown by Wang and colleagues (30, 31), we found that blocking β1-integrin function by exposure to the inhibitory antibody AIIB2 blocks polarity reorientation (Fig. 3, A and B). Intracellular integrin signaling is often mediated by Rac1, a small GTPase. Our laboratory has recently reported that cysts grown in the presence of AIIB2 resemble cysts expressing dominant negative (DN) Rac1 in that both have inverted polarity (although these two manipulations have divergent effects on cell proliferation, emphasizing the need for a system where cells do not proliferate; see Refs. 6 and 21). Collagen binding to β1-integrin transiently activates Rac1 (6, 32). To

Fig. 1. Schematic of inside-out cyst reversion. Top: collagen-based Madin-Darby canine kidney (MDCK) cyst system: a single cell suspension is plated in collagen. Cells must proliferate to form polarized, multicellular cysts. Bottom: inside-out MDCK cyst system. Cysts with inverted polarity are grown in suspension culture. Following plating in collagen, these cysts rapidly reverse their polarity.

Fig. 2. Time course of inside-out cyst reversion. Inside-out cysts were grown in suspension, harvested, and plated in collagen. At the indicated times, cysts were fixed with 4% paraformaldehyde for subsequent studies. In A–O, nuclei are stained with Hoescht 33342 (blue). A–E: immunofluorescent staining of cysts for gp135 (green) and β-catenin (red) as markers of the apical and basolateral surfaces, respectively. F–J: immunofluorescent staining of GM-130 (green) as a marker of the subapical Golgi compartment; F-actin staining with phalloidin (red). K–O: immunofluorescent staining of ZO-1 (green) as a marker of tight junctions; F-actin staining with phalloidin (red). Scale bar, 10 μm.
Rac1 is required for inside-out cyst reversion. A and B: treatment of cells with a β1-integrin blocking antibody blocks inside-out cyst reversion. MDCK inside-out cysts were grown in suspension. Cysts were spun down and pretreated with antibody for 2 h before plating in collagen. At the indicated times, cysts were fixed with paraformaldehyde for immunofluorescent staining with gp135 (green), β-catenin (red), and nuclei (blue). C–F: expression of dominant-negative Rac1N17 blocks inside-out cyst reversion. Rac1N17 is expressed under the control of the tetracycline transactivator. Inside-out cysts were grown in suspension in the presence of doxycycline. Before plating in collagen (2 days), doxycycline was washed out as described in MATERIALS AND METHODS. Later (48 h), cysts were spun down and plated in collagen. At the indicated times, cysts were fixed and stained as in A and B. gm135/βcat/nuclei

Fig. 3. Rac1 is required for inside-out cyst reversion. A and B: treatment of cells with a β1-integrin blocking antibody blocks inside-out cyst reversion.

investigate the role of Rac1 in polarity reorientation, we used an MDCK cell line that expresses a DN N17 Rac1 under control of the tetracycline transactivator (14). Inside-out cysts were allowed to form in the presence of doxycycline (to repress Rac1 expression). To induce expression of DN-Rac1 in the inside-out cysts, doxycycline was removed from the culture medium 48 h before the initiation of cyst reversion. In suspension, inside-out cysts expressing DN-Rac1 are indistinguishable from control cysts grown in the presence of doxycycline (Fig. 3, C and D). However, after 48 h in collagen, cysts expressing DN-Rac1 fail to reorient polarity (Fig. 3E) and appear identical to cysts treated with the β1-integrin blocking antibody, whereas control cysts grown in the presence of doxycycline have completely reoriented their polarity (Fig. 3F).

Because expression of the DN-Rac1 blocks inside-out cyst reversion, we tested the ability of cysts expressing a CA-Rac1 to reverse their polarity. The morphology of cysts was examined at 16 h, since this is an intermediate time point in the course of cyst reversion. Representative cysts were scored for the presence of gp135 on interior surfaces only (the de novo apical surface) or on the free surface as well. CA-Rac1 appears to accelerate remodeling of gp135 from the original apical surface so that a smaller proportion of cysts have gp135 remaining on the original apical surface at 16 h (Liu, unpublished observations). gp135 is present on the new interior lumens of cysts expressing CA-Rac1. Because the new lumens appear to form at cell-cell contacts and then expand, gp135 appears continuously on this new surface; whether or not there is acceleration of de novo gp135 appearance is unclear. Thus Rac1 appears to be an important regulator for reorientation of polarity.

PI 3-kinase/Akt1 are activated in inside-out cysts exposed to collagen. Because Rac1 is known to activate the PI 3-kinase/Akt pathway (2), we tested whether Akt1 is activated when inside-out cysts are exposed to collagen. We used Akt1 phosphorylation as a downstream readout of PI 3-kinase activation, since Akt1 activation is mediated by PI 3-kinase and is a widely accepted surrogate measure of PI 3-kinase activation (1, 7). Inside-out cysts were plated in collagen for the indicated time periods. Cells were subsequently harvested and lysed. Total Akt1 was immunoprecipitated, and activated Akt1 was detected using a phosphospecific antibody directed against threonine-308. Indeed, Akt1 is rapidly activated following plating of inside-out cysts in collagen (Fig. 4A). Pretreatment of cells with LY-294002 (an inhibitor of PI 3-kinase) blocked Akt1 activation (Fig. 4A), suggesting that PI 3-kinase is upstream of Akt1 in this pathway.

LY-294002 blocks inside-out cyst reversion in response to ECM cues. To test the functional role of the PI 3-kinase/Akt1 pathway in inside-out cyst reversion, we tested the ability of cysts to revert in the presence of LY-294002. Cysts grown in the presence of LY-294002 for 16 h are viable (33) but exhibited increased staining of gp135 on the cyst periphery, suggesting that reorientation of polarity was inhibited by loss of PI 3-kinase activity (Fig. 4B). To quantitate this result, representative cysts were scored for the presence of gp135 on the interior surfaces only (the de novo apical surface) or remaining on the peripheral surface as well (Fig. 4C). Indeed, a smaller proportion of cysts had gp135 exclusively on the interior surface in the presence of LY-294002, suggesting that PI 3-kinase plays an important role in the remodeling of gp135 from the peripheral surface to the de novo apical surface.

Collagen and Rac1 induce PI 3-kinase/Akt1 but not GSK3β activation during reorientation. To confirm that PI 3-kinase/Akt1 activation requires collagen-integrin interactions, we measured Akt1 activation in the presence of AIIB2. Akt1 activation was completely blocked in the presence of AIIB2 (Fig. 5A), suggesting that PI 3-kinase/Akt1 are downstream of collagen-integrin binding. We next examined Akt1 activation in the presence of dominant-negative Rac1 expressed under the control of the tetracycline transactivator. DN-Rac1 blocked Akt1 activation (Fig. 5B) compared with control cells grown in the presence of doxycycline. We conclude that Akt1 is downstream of collagen-integrin binding, Rac1, and PI 3-kinase in this system. However, these experiments do not prove that Akt1 is the critical downstream effector of PI 3-kinase required for inside-out cyst reversion.

Last, we hypothesized that GSK3β might be phosphorylated during the inside-out signaling response, since GSK3β phosphorylation is mediated by Akt1 in a number of signaling
pathways (18). Furthermore, phosphorylation of GSK3β/α/Akt (PI 3-kinase)/protein kinase B (Akt) is activated when inside-out cysts are plated in collagen and PI 3-kinase is required for Akt activation. Inside-out cysts were pretreated with LY-294002 at a concentration of 20 μM for 1 h before plating to collagen. Cysts were then plated in collagen in the presence or absence of LY-294002 for the indicated times, harvested, and lysed as detailed in MATERIALS AND METHODS. Total cell lysate (20 μg) was subjected to SDS-PAGE electrophoresis and Western blotting with the indicated phospho-antibody. The membrane was subsequently stripped and reprobed to measure levels of total protein.

![Fig. 4](image)

A: PI 3-kinase/Akt is activated when inside-out cysts are plated in collagen and PI 3-kinase is required for Akt activation. Inside-out cysts were pretreated with LY-294002 as in A and plated in collagen. Later (16 h), cysts were fixed and subjected to staining with gp135 (green), actin (red), and nuclei (blue). C: quantitation of inside-out cyst reversion. Cysts were treated as described in B, and the localization of gp135 at the cyst surface was quantitated. Cysts were scored as having gp135 on interior de novo apical surfaces only or with residual gp135 on the original apical surface. The values shown are means ± SD for 5 separate experiments for the wild-type MDCK cysts.

![Fig. 5](image)

A: Akt activation requires collagen-integrin interactions. Where indicated, cells were pretreated with AIB2 for 2 h in suspension and then plated in collagen with AIB2. Cysts were harvested and lysed, and Western blotting was performed as in Fig. 4A. B: expression of a dominant-negative Rac1N17 blocks PI 3-kinase activation. Cysts were plated in collagen for the indicated times, harvested, and lysed as in MATERIALS AND METHODS. Total cell lysate (20 μg) was subjected to SDS-PAGE and Western blotting with the indicated phospho-antibody. The membrane was subsequently stripped and reprobed to measure levels of total protein.

aPKC is required for inside-out cyst reversion. aPKC has been implicated in a signaling pathway with Cdc42 and the Par proteins that is critical for epithelial cell polarization (13, 15). To test the role of this pathway in cyst reversion, we examined the effect of a myristoylated aPKC inhibitor on inside-out cyst reversion. This inhibitor blocked the earliest stages of inside-out cyst reversion, that is, breakdown of the free apical surface and relocation of apical proteins to de novo lumens (Fig. 7A). Inhibition of aPKC activity did not affect the activation of PI 3-kinase through Rac1; in the presence of the aPKC inhibitor, Akt1 was phosphorylated by PI 3-kinase in response to collagen stimulation (Fig. 7B). Thus aPKC is also required for inside-out cyst reversion. PI 3-kinase does not appear to modulate aPKC phosphorylation, which is required for aPKC activity (Liu, unpublished observations). However, pharmacological inhibition of aPKC does not appear to affect signaling pathways (18). Furthermore, phosphorylation of GSK3β at serine-9 inhibits its activity and has been shown to be critical for cell polarization in neurons (8, 26). Western blotting of total cell lysates was performed using an antibody directed against phospho-GSK3β. We did not detect any changes in phosphorylation of GSK3β following the addition of collagen (Fig. 5C). Thus changes in GSK3β activity do not appear to modulate reorientation of polarity in this system, in contrast to other model systems.

CA-Akt1 fails to restore inside-out cyst reversion. To determine if Akt1 is the critical downstream mediator of PI 3-kinase for inside-out cyst reversion, we took advantage of an MDCK cell line that expresses a CA-Akt1 mutant (CA-Akt). This mutant has a deletion of the pleckstrin homology domain as well as point mutations to replace serine-473 and threonine-308 (the regulatory phosphorylation sites) with aspartic acids and has previously been shown to be resistant to treatment with LY-294002 and to have increased levels of Akt activity compared with wild-type cells (28). The activity of CA-Akt was measured using an in vitro kinase assay and was indeed resistant to pretreatment of the cells with LY-294002 (Fig. 6A). However, treatment of the CA-Akt-expressing cells with LY-294002 blocked remodeling of the apical surface, as in wild-type cells (Fig. 6B). Similarly, an Akt-specific inhibitor [that blocks Akt1 and Akt2 (33)] blocked Akt1 activation in response to collagen (Fig. 6C). However, when cells were treated with this inhibitor, there was no significant effect on the repolarization of inside-out cysts in response to ECM (Fig. 6D). Taken together, these results suggest that a molecule other than Akt is the critical downstream effector of Rac1/PI 3-kinase.
events downstream of Rac1, such as PI 3-kinase/Akt1 activation.

We next examined the effects of constitutive Rac1 activity on inside-out cyst reversion in the presence of the aPKC inhibitor using an MDCK cell line that inducibly expressed the activated V12 form of Rac1 under the control of the tet-repressible transactivator (14). We confirmed that these cell lines express appropriate levels of CA-Rac1 (Liu, unpublished observations). As shown in Fig. 7C, addition of the aPKC inhibitor significantly reduces the proportion of cysts that have initiated the process of flipping their polarity at 8 h. This is not overcome by the expression of CA-Rac1, suggesting that both Rac1 and aPKC are required for inside-out cyst reversion.

**DISCUSSION**

Under normal conditions, epithelial cells maintain a highly polarized architecture, characterized by distinct cell surface domains. We have used a model system to further characterize the molecular events required for the reorientation of polarity in response to ECM cues. In this system, MDCK cells are grown in suspension culture and allowed to form cysts with an “inside-out” architecture, that is, with the apical surface facing the culture medium. When these cysts are placed in an appropriate ECM (such as collagen), these cysts rapidly repolarize in response to the ECM cues. Because all of the cysts are exposed to ECM simultaneously, this system has made it possible to analyze the molecular signaling events required for polarity reversal.

First, we have demonstrated that, in this system, Rac1 plays a critical role in the reorientation of polarity. Inside-out cysts expressing a dominant-negative form of Rac1 fail to reorient polarity when embedded in collagen, suggesting that Rac1 plays a critical role in signaling cues from the ECM to orient the cell. Indeed, blocking β1-integrin with AIIB2 results in a similar phenotype, suggesting that integrin-ECM signaling is upstream of Rac1. This result is in agreement with results from other MDCK three-dimensional culture systems in which the phenotype of DN-Rac1-expressing cells resembles that of cells treated with the integrin blocking antibody (21, 32).

The polarity reversion events are highly synchronized, allowing for biochemical analysis of the signaling events required for inside-out cyst reversion. We found that the PI 3-kinase/Akt pathway was activated following exposure of inside-out cysts to collagen and that these signaling intermediates were not activated in cells expressing DN-Rac1. Although we did not directly measure PI 3-kinase activity, it is well established that Akt1 phosphorylation is modulated by PI 3-kinase; furthermore, in our system, Akt1 phosphorylation was blocked by the addition of LY-294002, a known PI 3-kinase inhibitor. Thus PI 3-kinase and Akt1 appear to be downstream of Rac1 in this system. We also found that PI 3-kinase, but not Akt1 or GSKβ, is an important downstream
process either (Liu, unpublished observations), suggesting that mammalian target of rapamycin is not the relevant downstream intermediate. Although the exact downstream effector of PI 3-kinase relevant to the reorientation process is unclear, the PI 3-kinase pathway appears to play an important role in polarity reversion. It may be the phospholipids themselves that play a critical role in the pathway, since, in other systems, the PI 3-kinase lipid products themselves can orient polarity (27). Indeed, we have recently shown that PI 3-kinase and phosphatidylinositol 3-phosphate (PIP3) are key to the formation of the basolateral surface. Exogenous PIP3 ectopically implanted in the apical surface of MDCK cells causes the relocalization of basolateral markers to the apical surface, whereas partial inhibition of PI 3-kinase reduces the size of the lateral surface (10). We were unable to demonstrate that Rac activity in inside-out cysts was significantly affected by pretreatment of cysts with LY-294002 (Liu, unpublished observations). Thus, at present, we have no evidence of a feedback loop between Rac1 and PI 3-kinase in this model system; in other model systems, Rac1 has been implicated as a downstream modulator of PI 3-kinase.

A potential limitation of this work is the use of DN-Rac1-expressing cell lines, since others have reported that other DN-Rho family GTPases (specifically, Cdc42) can modulate Rac1 activity (4). However, in the same report, Cdc42 does not regulate the activation of other Rho family GTPases, notably RhoA. To the best of our knowledge, there are no published reports that DN-Rac1 has nonspecific effects on other Rho family GTPases. Indeed, Garcia-Bernal and colleagues (9) demonstrated that a Rac1 dominant negative and Rac1 small-interfering RNA have similar phenotypes in a T lymphocyte-based signaling system. Furthermore, in both the MDCK three-dimensional cyst culture system and in the collagen overlay system, DN-Rac1 and DN-Cdc42 have slightly different phenotypes (although polarity is inverted in both cases), suggesting that there is some difference in the pattern of Rho family GTPase inactivation (24 and Datta, unpublished observations). Efforts in our laboratory to block Rac1 activity through other biochemical techniques to confirm and extend our current findings are ongoing.

The Cdc42/aPKC pathway also plays key role in the signaling events required for reorientation of polarity, since pharmacological blockade of aPKC results in a failure of cells to initiate the reorientation of polarity. The Cdc42 pathway interacts with the Rac1 pathway via the recently described interaction of Par3 with Tiam1, a Rac1 GTP exchange factor (3, 17, 20). One of the important effects of the Cdc42/aPKC pathway may be the modulation of Rac1 activity through Tiam1. However, CA-Rac1 was unable to overcome the effects of pharmacological aPKC blockade, suggesting that the Cdc42/aPKC pathway has other effects on the orientation of polarity in this system. Indeed, our laboratory has recently shown that formation of the apical surface and lumen requires Cdc42 and aPKC (16).

In summary, we have used a suspension culture system to generate cysts with inverted epithelial cell polarity. When these cysts are exposed to collagen, they are able to reorient their polarity in response to cues from the ECM. This model system has allowed us to examine signaling events that are required for the reorientation of polarity in response to ECM cues. We found that Rac1, PI 3-kinase, and aPKC are required for this process. Rac1 activation in response to collagen-matrix inter-
actions activates multiple pathways, including the PI 3-kinase pathway. PI 3-kinase signaling through Akt does not appear to be required for cyst formation, and thus alternate downstream targets of PI 3-kinase such as the lipids themselves may be critical for polarity reorientation. Future work will focus on elucidating the signaling events that may be downstream of PI 3-kinase during the formation of polarity, as well as understanding the cross talk between the Rac1 and aPKC pathways, which may occur through Tiam1.

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REFERENCES