

# Epithelial Cell Polarity Alters Rho-GTPase Responses to *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* is an opportunistic human pathogen that preferentially infects damaged epithelial tissues. Previous studies have failed to distinguish whether the increased susceptibility of injured epithelium results from the loss of cell polarity or increased access to the basolateral surface. We have used confluent monolayers of Madin-Darby canine kidney (MDCK) cells cultured on porous filter supports for 1–3 d as a model system to investigate whether the differentiation state of a polarized model epithelium affected the response of epithelial cells to this pathogen. Confluent incompletely polarized MDCK cell monolayers (day 1) efficiently internalized apically applied *P. aeruginosa* via a pathway that required actin polymerization and activation of Rho-family GTPases and was accompanied by an increase in the amount of activated RhoA. In contrast, *P. aeruginosa* entry into highly polarized MDCK monolayers (day 3) was 10- to 100-fold less efficient and was insensitive to inhibitors of actin polymerization or of Rho-family GTPase activation. There was no activation of RhoA; instead, Cdc42-GTP levels increased significantly. Basolateral infection of highly polarized MDCK monolayers was less efficient and insensitive to *Clostridium difficile* Toxin B, whereas basolateral infection of incompletely polarized MDCK monolayers was more efficient and required activation of Rho-family GTPases. Together, our findings suggest that as epithelial barrier differentiates and becomes highly polarized, it becomes resistant to *P. aeruginosa* infection. Nevertheless, polarized epithelial cells still sense the presence of apically infecting *P. aeruginosa*, but they may do so through a different group of surface proteins and/or downstream signaling pathways than do incompletely polarized cells.

## INTRODUCTION

The portions of the human body that contact the outside world are composed of epithelial cells and specialized cells of the immune system. Such epithelial tissues form a barrier against microbial pathogens, and the breakdown of this barrier often results in clinically apparent infection. Many pathogens have developed strategies to circumvent the barrier function of an intact epithelium. These include crossing an intact epithelial monolayer by invasion or transcytosis of epithelial cells, disrupting cell-cell contacts within the monolayer, or killing cells of the monolayer (reviewed in Kazmierczak *et al.*, 2001a). Other pathogens usually exploit, rather than cause, disruptions in epithelial monolayers. Such disruptions may follow tissue injury secondary to inflammation or trauma, or may result from cell death or division within a monolayer.

*P. aeruginosa* is an opportunistic pathogen that exploits preexisting epithelial cell injury. This is apparent clinically, because *P. aeruginosa* infection follows burns, corneal trauma, catheter-related bladder injury, or local damage to

the upper respiratory tract in mechanically ventilated patients (Salyers and Whitt, 2002). Experimentally, *P. aeruginosa* infection occurs preferentially at sites of epithelial injury (Yamaguchi and Yamada, 1991; Zahm *et al.*, 1991; Tsang *et al.*, 1994; de Bentzmann *et al.*, 1996b). This predilection for injured epithelium has been attributed to increased levels of putative *P. aeruginosa* receptors on repairing cells, such as asialoGM1 (de Bentzmann *et al.*, 1996a), or fibronectin and the integrin  $\alpha 5 \beta 1$  (Roger *et al.*, 1999). The loss of polarity in repairing cells may independently facilitate *P. aeruginosa* infection, as bacterial adhesion, internalization, and cytotoxicity increase in epithelial cells whose polarity has been pharmacologically disrupted (Fleiszig *et al.*, 1998). Epithelial cell activation of the small GTPases Rho and Rac during wound repair (Santos *et al.*, 1997) might also promote *P. aeruginosa* internalization, as we have recently shown that expression of a constitutively active RhoA allele (RhoAV14) is sufficient to increase bacterial internalization (Kazmierczak *et al.*, 2001b).

Several host cell molecules have been identified as internalization receptors for *P. aeruginosa*, including the cystic fibrosis transmembrane receptor (Pier *et al.*, 1997) and the asialoganglioside aGM1 (Hazlett *et al.*, 1993), although their relative importance may be cell type specific. Several studies suggest that *P. aeruginosa* preferentially adheres to and invades the basolateral surface of polarized epithelial cells. Treatment of polarized epithelial monolayers with EGTA, which disrupts intercellular junctions, results in increased binding, cytotoxicity, or invasion (Fleiszig *et al.*, 1997). Manipulations that disrupt polarity, such as plating cells at low

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Abbreviations used: LatA, latrunculin A; LDH, lactate dehydrogenase; MOI, multiplicity of infection.

density or exposure to hepatocyte growth factor, increase cytotoxicity and invasion (Fleiszig *et al.*, 1998; Lee *et al.*, 1999). Finally, susceptibility to invasion correlates with polarity and suggests that polarity is a protective mechanism for the host cell (Fleiszig *et al.*, 1997; Plotkowski *et al.*, 1999). Although these results may be explained by the restriction of *P. aeruginosa* receptor(s) to the basolateral surface of polarized cells, no such receptor has been identified to date.

The pathway of *P. aeruginosa* internalization is sensitive to cytochalasin D, an actin-depolymerizing agent, is inhibited by the tyrosine kinase inhibitors herbimycin and genistein, and may involve the tyrosine kinase src, suggesting that protein phosphorylation events accompany internalization (Fleiszig *et al.*, 1995; Evans *et al.*, 2002a). The components of this signal transduction cascade have not been identified, although recent evidence suggests that mitogen-activated protein kinase kinase and extracellular signal-regulated kinase kinases may be involved (Evans *et al.*, 2002b). Some strains of *P. aeruginosa* trigger the activation of the acid sphingomyelinase and the release of ceramide in sphingolipid-rich rafts. Ceramide reorganizes these rafts into larger signaling platforms that are required to internalize *Pseudomonas aeruginosa*, induce apoptosis, and regulate cytokine response in infected cells (Grassme *et al.*, 2003). We have recently shown that internalization is accompanied by activation of RhoA, a member of the small Rho-family GTPases involved in actin cytoskeleton and membrane rearrangements and that activation is sufficient to promote *P. aeruginosa* internalization by epithelial cells (Kazmierczak *et al.*, 2001b).

Direct modulation of Rho-family GTPase activity is a strategy used by many bacterial pathogens to promote or block their internalization by host cells. *P. aeruginosa* strains synthesize several proteins that are injected into host cells via the bacterial type III secretion system. Two of these, ExoS and ExoT, exhibit GTPase activating protein (GAP) activity toward Rho-family GTPases in vitro (Goehring *et al.*, 1999; Krall *et al.*, 2000; Kazmierczak and Engel, 2002) and block bacterial internalization by both macrophage-like cell lines and epithelial cells (Frithz-Lindsten *et al.*, 1997; Cowell *et al.*, 2000; Garrity-Ryan *et al.*, 2000). Point mutations that abolish the GAP activity of ExoT significantly reduce the ability of this protein to inhibit bacterial internalization, further supporting the hypothesis that RhoA activation is required for efficient bacterial internalization (Garrity-Ryan *et al.*, 2000).

Rho-family GTPase activity is required to establish many of the features of a polarized epithelium, e.g., tight junction formation, polarized protein targeting, focal contact and adhesion formation (Braga *et al.*, 1997; Jou *et al.*, 1998; Kroschewski *et al.*, 1999; Leung *et al.*, 1999; Nobes and Hall, 1999). Because Rho-family GTPase activity is central to *P. aeruginosa* internalization, we investigated whether the limited ability of polarized epithelia to internalize *P. aeruginosa* was regulated at the level of Rho-family GTPase activity. We developed a system for examining confluent model epithelial monolayers polarized to varying extents and demonstrated that decreased internalization of *P. aeruginosa* by polarized cells was accompanied by the loss of a Rho-GTPase dependent uptake pathway. Polarized cells continued to respond strongly to apically infecting bacteria; however, their response shifted from RhoA activation to Cdc42 activation. Basolateral infection of polarized cells was likewise less efficient than basolateral infection of incompletely polarized cells, suggesting that the RhoA-dependent internalization pathway is down-regulated during the development of epithelial cell polarity. These findings support the idea that epithelial cells alter their responses to pathogen

bacteria as a function of polarization and suggest a novel way in which epithelial cell responses to pathogens may be altered by epithelial tissue injury.

## METHODS

### Bacterial Strains

*P. aeruginosa* strains PA103 $\Delta$ pscJ::Tn5 (type III secretion deficient) and PA103 $\Delta$ UAT (type III secretion competent, ExoU-, ExoT-) have been described previously (Hauser *et al.*, 1998; Garrity-Ryan *et al.*, 2000). Both strains are internalized by nonprofessional phagocytes to a similar extent, a phenotype that has been ascribed to the inability of these isogenic mutants to type III secrete the effector protein ExoT (Garrity-Ryan *et al.*, 2000). *Salmonella typhimurium* SL1344 and *Escherichia coli* MC4100 pRI203 (Invasin+) were kindly provided by Stanley Falkow (Stanford University, Stanford, CA). Plasmids expressing GST-Rhotekin binding domain (GST-TRBD) and GST-Cdc42/Rac interacting binding domain (GST-CRIB) were generously provided by Xiang-Dong Ren and Martin Schwartz (The Scripps Institute, La Jolla, CA) and Rick Cerione (Cornell University, Ithaca, NY), respectively.

### Cell Culture

HeLa cells (ATCC CCL-2) and MDCK cells (type II) were cultured as described previously (Kazmierczak *et al.*, 2001b). Before binding and internalization assays, cells were washed twice with minimal essential medium (MEM), etc. [MEM salts (Sigma-Aldrich, St. Louis, MO)/20 mM HEPES, pH 7.4/10 mM sodium bicarbonate].

### Drugs and Reagents

Latrunculin A (LatA; Molecular Probes, Eugene, OR) was made up as a 2 mg/ml stock in dimethyl sulfoxide. Cells were pretreated for 30 min before bacterial infection, and LatA was present throughout the invasion assay. *Clostridium difficile* Toxin B (TechLab, Blacksburg, VA) was supplied at 0.38 mg/ml in phosphate-buffered saline. Cells were pretreated for 4 h before bacterial infection. We confirmed that neither LatA nor Toxin B inhibited *P. aeruginosa* viability at the concentrations used (our unpublished data). EDTA (Sigma-Aldrich) was made up in Hanks' Ca<sup>2+</sup> Mg<sup>2+</sup>-free balanced salt solution (BSS) (UCSF Tissue Culture Facility, San Francisco, CA), pH 7.6. Cells were routinely pretreated for 15 min with 2.5 mM EDTA, washed twice with MEM, etc., and then infected. Anti-gp135 and anti-E-cadherin (RR1) were kindly provided by George Ojakian (SUNY Downstate, Brooklyn, NY) and Barry Gumbiner (Memorial Sloan-Kettering, New York, NY), respectively. Anti-ZO-1 (Chemicon International, Temecula, CA), anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cdc42 (BD Transduction Laboratories, Lexington, KY), anti-Rac1 (Upstate Biotechnology, Lake Placid, NY), Alexa 488-coupled secondary antibodies (Molecular Probes), and Texas-Red phalloidin (Molecular Probes) were purchased as indicated.

### Binding and Internalization Assays

For all assays, single colonies of freshly plated bacteria were used to inoculate 3-ml cultures of Luria Broth (LB), which were grown overnight (14–16 h) at 37°C with agitation. Bacteria were diluted in MEM, etc., to an OD<sub>600</sub> = 0.1 (1.5 × 10<sup>8</sup>/ml) before infection unless otherwise indicated. HeLa cell assays were performed as described previously (Kazmierczak *et al.*, 2001b). MDCK cells were plated on 12-mm Transwell filters (Corning Glassworks, Corning, NY) (0.4- $\mu$ m pore size) at 1 × 10<sup>6</sup> cells/cm<sup>2</sup>, 16–18 h ("1 d") or 3 d before assaying. For binding assays, 150  $\mu$ l of bacteria was incubated with MDCK monolayers for 1 h at 37°C. Nonadherent bacteria were removed by four 5-min washes with phosphate-buffered saline. Adherent and internalized bacteria were released by incubating filters with 0.25% Triton X-100 in Hanks' Ca<sup>2+</sup>Mg<sup>2+</sup>-free BSS for 30 min at room temperature. Sterile glass beads were added to the samples, which were vortexed 2 × 10 s to ensure efficient host cell lysis and bacterial release. Adherent and internalized bacteria were enumerated by plating serial dilutions of the lysates to LB plates. Apical internalization assays were performed as described previously (Kazmierczak *et al.*, 2001b). Briefly, infections were initiated as described for binding assays, except that bacteria were allowed to infect host cells for 2 h before washing. MEM, etc., plus amikacin (400  $\mu$ g/ml) (Sigma-Aldrich) was added for a further 2 h to kill extracellular bacteria. Internalized bacteria were released by lysing MDCK cells as described above, and then enumerated by plating serial dilutions of the lysate to LB plates. For basolateral infections, cells were plated to 3- $\mu$ m pore size 12-mm Transwell filters (Corning Glassworks) at 1 × 10<sup>6</sup> cells/cm<sup>2</sup> 1–3 d before assaying. Bacteria were diluted in MEM, etc., to an OD<sub>600</sub> of ~0.6. Filters were infected by placing them directly on top of 40- $\mu$ l drops of bacterial suspension (MOI ~25–50); 200  $\mu$ l of media was added to the apical surface of the cells during the infection. After 2-h incubation with bacteria, the filters were washed, treated with antibiotic-containing medium, and lysed as for apical infections.

### Lactate Dehydrogenase (LDH) Assays

LDH release was measured as described previously (Kazmierczak *et al.*, 2001b).

### Rho-GTP Determination

The procedure was adapted from Ren *et al.* (1999). Briefly, HeLa cells were plated at  $1 \times 10^6$  cells/10-cm dish 2 d before infection and assay; MDCK cells were plated at  $2 \times 10^7$  cells/7-cm Transwell filter either 16–18 h or 3 d before infection (MOI  $\approx 50$ ) and assay. Cells were washed twice with ice-cold Tris-buffered saline, pH 7.2, plus 1 mM orthovanadate, and then lysed in 600  $\mu$ l of lysis buffer (20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.5 M NaCl, 15% glycerol, 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 1 mM orthovanadate, Complete Protease Inhibitor Cocktail, EDTA-free; Roche Diagnostics, Indianapolis, IN). Lysates were cleared by centrifugation for 5 min at 14,000 rpm at 4°C and then divided for 1) affinity precipitation with 75  $\mu$ g of TRBD-GST beads (500  $\mu$ l of lysate), 2) immunoblotting to determine total RhoA (20  $\mu$ l of lysate), and 3) total protein concentration determination using the BCA assay kit (10  $\mu$ l of lysate; Pierce Chemical, Rockford, IL). Affinity-precipitated samples and aliquots for total RhoA determination were electrophoresed on 13% SDS-PAGE gels, blotted to Immobilon P (Millipore, Bedford, MA), and incubated with anti-RhoA at 1:200 in 5% milk/Tris-buffered saline/Tween 20 for 2 h after blocking. After incubation with HRP-conjugated goat anti-mouse secondary antibody (1:2000 in 5% milk/Tris-buffered saline/Tween 20) for 1 h, blots were washed and developed with the ECL+Plus kit (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions. Film exposed to blots was developed, scanned, and quantified using IPLab Gel H software. All samples were assayed in duplicate or triplicate; data presented are representative of three to five repetitions of each experiment.

### Rac-GTP and Cdc42-GTP Determinations

CRIB-GST was prepared according to Ren *et al.* (1999). HeLa and MDCK cells were plated, infected, and lysed as for Rho-GTP assays. Ninety percent of the cleared lysate was precipitated with 30  $\mu$ g of CRIB beads per sample; 3% of the lysate was reserved to determine total Rac1 or Cdc42 present within the lysate. Electrophoresis and Western blotting were carried out as per Rho-GTP assays, except that blots were incubated with anti-Cdc42 (1:500) or anti-Rac1 (1:1000). Samples were assayed in duplicate or triplicate; data presented are representative of three to five repetitions of each experiment.

### Indirect Immunofluorescence and Confocal Microscopy

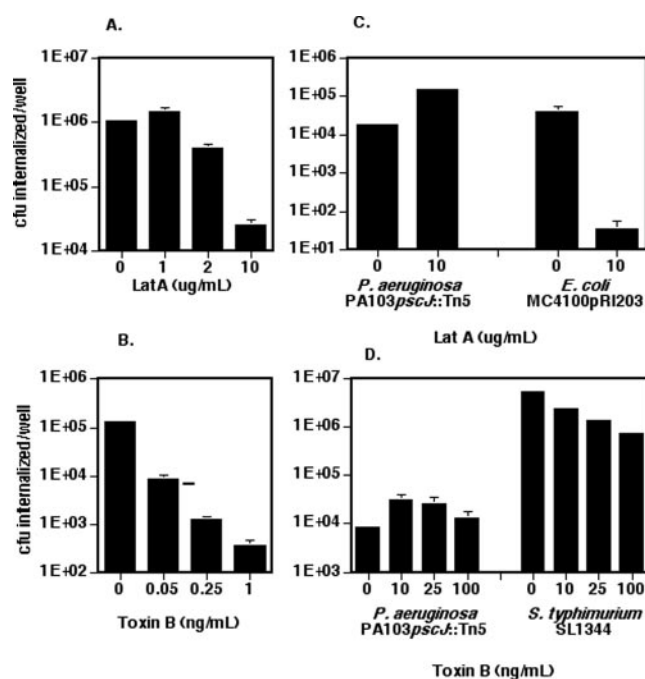
Filter grown MDCK cells were processed for staining and microscopy as described previously (Kazmierczak *et al.*, 2001b). Primary and secondary antibodies were used as follows: anti-gp135 (1:10,000), anti-E-cadherin (RR-1, 1:1), anti-ZO-1 (1:200), Alexa 488 anti-mouse (1:500), Alexa 488 anti-rat (1:200), and Texas Red-phalloidin (1:200). Samples were imaged using a Bio-Rad MRC 1024 confocal microscope with a KrAr laser. Z-series were routinely obtained at 1.0- $\mu$ m steps by using a sequential scanning program. Images were imported using NIH Image 1.67b and pseudocolored with Adobe Photoshop 5.0.

## RESULTS

### Entry of *P. aeruginosa* into HeLa Cells Is Sensitive to Inhibitors of the Actin Cytoskeleton

The entry of several strains of *P. aeruginosa* into HeLa cells is inhibited by cytochalasin D (Fleiszig *et al.*, 1995). We confirmed that the internalization of a type III secretion mutant of PA103 (PA103pscJ::Tn5) is actin dependent by using an alternative inhibitor of actin polymerization, LatA (Lamaze *et al.*, 1997). LatA inhibited invasion in a dose-dependent manner, up to 60-fold at 10  $\mu$ g/ml (Figure 1A). The decrease in internalized bacteria was not due to either direct toxicity of LatA for *P. aeruginosa* or to HeLa cell damage as assayed by LDH release (our unpublished data).

The actin cytoskeleton rearrangements that accompany the internalization of the intracellular pathogens *S. typhimurium* and *S. flexneri* are mediated by small GTPases of the Rho family (Chen *et al.*, 1996; Tran Van Nhieu and Sansonetti, 1999; Criss *et al.*, 2001). We tested the sensitivity of *P. aeruginosa* internalization to *C. difficile* Toxin B, which glucosylates and thereby inactivates Rho, Cdc42, and Rac GTPases (Just *et al.*, 1995). Toxin B inhibited PA103pscJ::Tn5 internalization into HeLa cells in a dose-dependent manner (Figure 1B). Bacterial viability was not affected by Toxin B; moreover, these concentrations of Toxin B resulted in stress



**Figure 1.** LatA and Toxin B have different effects on *P. aeruginosa* entry into nonpolarized versus polarized epithelial cells. (A and B) HeLa cells were infected with PA103::pscJ/Tn5 (MOI 10–20) for 2 h. The number of internalized bacteria per well was determined as described in MATERIALS AND METHODS. Bars represent the mean  $\pm$  SD of triplicate samples from a representative experiment. (C and D) MDCK cells were plated on 12-mm Transwell filters at  $1.5 \times 10^6$  cells/well 3 d before infection with PA103::pscJ/Tn5, Invasion-expressing *E. coli* (MC4100 pRI203), or *S. typhimurium* (SL1344) for 2 h. Bars show the mean  $\pm$  SD of triplicate samples from a representative experiment. In some instances, error bars are too small to see on the log scale used in these graphs. (A and C) Latrunculin A or DMSO was added to cells 30 min before addition of bacteria and was present for the 2 h of cocultivation with bacteria. (B and D) Cells were pretreated with Toxin B for 4 h before the addition of bacteria.

fiber loss and cell rounding without causing direct HeLa cell injury as measured by LDH release (our unpublished data).

Similar observations can be made for other internalized *P. aeruginosa* strains. Thus, internalization of PA103 $\Delta$ UAT, a mutant capable of type III secretion but carrying large deletions in the genes encoding the cytotoxin ExoU and the antiinternalization factor ExoT, is also sensitive to LatA and Toxin B at 10  $\mu$ g/ml and 10 ng/ml, respectively (our unpublished data). Likewise, *P. aeruginosa* strains PAK and PA01 are internalized by HeLa cells via a mechanism that is inhibited by LatA and Toxin B (our unpublished data), suggesting that actin polymerization and Rho-family GTPase activity are required for *P. aeruginosa* internalization.

### Entry of *P. aeruginosa* into Polarized MDCK Epithelial Cells Is Not Sensitive to Inhibitors of the Actin Cytoskeleton

Because polarized epithelial cells are the natural cell type that *P. aeruginosa* encounters, we tested whether LatA and Toxin B inhibit *P. aeruginosa* entry into polarized MDCK type II cells. Surprisingly, LatA, at doses (10  $\mu$ g/ml) that caused a loss of filamentous actin staining, failed to inhibit internalization of PA103pscJ::Tn5 or PA103 $\Delta$ UAT (Figure 1C; our unpublished data). MDCK cell monolayers were none-

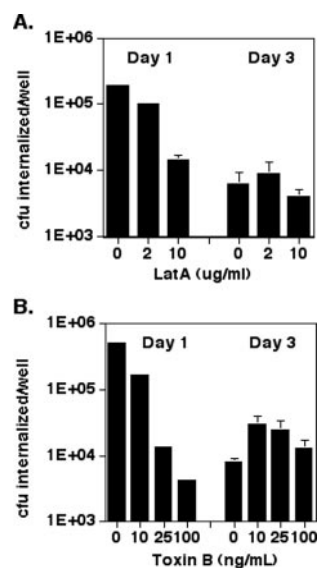
theless sensitive to LatA, because the actin-dependent internalization of *E. coli* MC4100 expressing the *Yersinia enterocolitica* protein Invasin was inhibited 100-fold under these conditions (Figure 1C). Likewise, *P. aeruginosa* invasion of polarized MDCK monolayers was not inhibited by Toxin B at concentrations up to 100 ng/ml, even though *S. typhimurium* invasion of these cells was inhibited 90% by Toxin B (Figure 1D). In fact, both agents slightly stimulated *P. aeruginosa* internalization by polarized MDCK cell monolayers. The amount of increased invasion correlated with and is likely secondary to the disruption of intercellular junctions caused by these agents (see below).

#### *The Pathway Used by P. aeruginosa for Entry into Epithelial Cells Depends upon Their Degree of Polarization*

The differential sensitivity of *P. aeruginosa* internalization to LatA and Toxin B seen in HeLa and in MDCK cells could be a function of cell type or of cell polarity. To distinguish between these two possibilities, we developed a system to model incompletely polarized versus polarized epithelia. MDCK cells plated at high density on permeable filters formed instant monolayers that, at 18–20 h postplating, had acquired many markers of a polarized epithelium. These included a transepithelial resistance statistically indistinguishable from that measured in monolayers 3 d after plating; restricted paracellular diffusion of tracers such as fluorescein isothiocyanate-inulin similar to that seen in 3-d-old monolayers; intact tight junctions, as seen by continuous ZO-1 staining; and correct distribution of apical and basolateral protein markers, such as gp135 and E-cadherin, respectively (our unpublished data). Between 1 and 3 d after plating, the cells continued to differentiate, increasing cell height and restricting even further the membrane distribution of E-cadherin to only the lateral membrane (our unpublished data).

Although day 1 and day 3 monolayers differed in subtle ways morphologically, their responses to *P. aeruginosa* were dramatically different. Day 1 confluent MDCK cell monolayers behaved much like HeLa cells, with *P. aeruginosa* invasion inhibited >10-fold by LatA and >50-fold by Toxin B (Figure 2). Day 3 cells, as seen previously, showed no inhibition of internalization after treatment with these agents (Figure 2), whereas their effects on day 2 monolayers were intermediate to those observed on day 1 and day 3 (our unpublished data). Internalization was not inhibited as a result of cell injury after exposure to these drugs, because LDH release was not increased by these agents (our unpublished data). Furthermore, cell loss from the filters after inhibitor treatment was <10% and could not account for the decrease in internalized bacteria, although cell retraction and condensation of the actin cytoskeleton were observed (our unpublished data).

In addition to finding that day 1 and day 3 cells showed different responses to LatA and Toxin B, we also observed that *P. aeruginosa* internalization decreased some 50-fold between day 1 and day 3 MDCK cells (Figure 2, A and B, compare untreated cells at day 1 and day 3). This difference reflected both a decrease in bacterial adherence to the apical surfaces of day 3 cells, as well as a much greater relative decrease in the frequency with which bound bacteria were internalized (Figure 5, A and B). Microscopic examination of infected day 1 versus day 3 MDCK cells stained for internalized versus adherent bacteria also suggested that the efficiency of bacterial internalization by day 1 cells was higher than that of day 3 cells: 2 h after infection, ~5–10% of day 1 cells contained several internalized bacteria. In con-



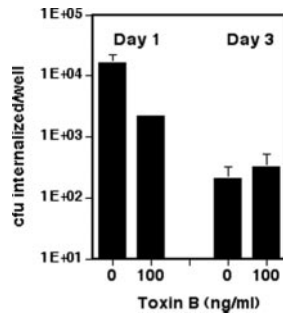
**Figure 2.** Sensitivity to inhibitors of actin polymerization is affected by cell polarity. MDCK cells were plated on 12-mm Transwell filters at  $1.5 \times 10^6$  cells/well and allowed to polarize for 1 or 3 d. Cells were pretreated with LatA for 30 min (A) or Toxin B for 4 h (B) before infection with PA103::psc/Tn5 at an MOI of 10–20 for 2 h. Bars show mean  $\pm$  SD of triplicate samples from a representative experiment. In some instances, error bars are too small to see on the log scale used in these graphs.

trast, day 3 cells containing internalized bacteria were extremely rare, with the majority of cell-associated bacteria being extracellular (our unpublished data). Increasing the inoculum size 3-fold or 10-fold resulted in commensurate increases in bacterial binding to the apical surfaces of both day 1 and day 3 cells (Figure 5A). However, increasing the inoculum size did not overcome the relative inability of day 3 cells to internalize *P. aeruginosa*, because day 3 cells infected with a 10-fold greater inoculum internalized only 1.8% as many bacteria as day 1 cells infected with the usual (“1 $\times$ ”) inoculum (Figure 5B).

Two major conclusions emerge from these experiments. First, bacterial internalization changes from a Toxin B-sensitive to a Toxin B-insensitive process in epithelial cells as they polarize. Second, the absolute amount of apical internalization markedly decreases between day 1 and day 3 MDCK monolayers, a change that cannot be ascribed to decreased bacterial binding to day 3 cells. These findings could represent either the relocation of a Toxin B-sensitive internalization pathway to the basolateral surface and/or the loss of this pathway in polarizing cells.

#### *Basolateral Infection of MDCK Monolayers by P. aeruginosa Decreases as Cells Become More Polarized*

If *P. aeruginosa* internalization decreases in polarized cells because the internalization pathway is increasingly restricted to the basolateral domain of these cells, we would predict that cells infected basolaterally would retain their ability to internalize *P. aeruginosa* even when polarized. MDCK cells were thus plated at “confluent” density on Transwell filters with a 3- $\mu$ m pore size, permitting the physical passage of *P. aeruginosa* through the filter to the basolateral face of the monolayer. The cells were assayed for several markers of polarity, to ensure that they formed a normal-looking monolayer. Proteins seemed to be localized

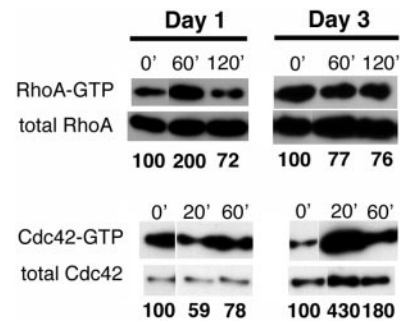


**Figure 3.** Basolateral internalization decreases with increasing MDCK polarity and becomes insensitive to Toxin B inhibition. MDCK cells were plated on 12-mm Transwell filters (3- $\mu$ m pore size) at confluent density ( $1.5 \times 10^6$  cells/well). PA103:*psc/Tn5* was grown overnight in LB with shaking, diluted in MEM to  $A_{600}$  0.6. The filter was placed on a 40- $\mu$ l drop (MOI 50) for 2 h in a humid chamber to allow infection to occur. Invasion assays were performed as described in Figure 1. The treated MDCK cells were exposed to Toxin B for 4 h before infection. Bars show mean  $\pm$  SD of triplicate samples from a representative experiment. In some instances, error bars are too small to see on the log scale used in these graphs.

correctly: gp135 and E-cadherin were restricted appropriately to the apical membrane and zona adherens, respectively (our unpublished data). Basolateral infection was established by placing filters on drops of bacterial suspension and resulted in less efficient internalization than apical inoculation. Nonetheless, polarity seemed to affect the number of bacteria internalized after basolateral infection as well, with more internalized bacteria recovered from day 1 than day 3 cells (Figure 3). Internalization was insensitive to Toxin B in day 3 cells, but inhibited 10-fold by this agent in day 1 cells. These results strongly suggest that the decrease in bacterial internalization seen with increasing polarity is not the result of restricting internalization to an inaccessible basolateral domain, but rather the consequence of down-regulating the pathway used by *P. aeruginosa* for internalization. This may result from down-regulation or disappearance of either the receptor used by *P. aeruginosa* or of more distal elements in this pathway.

#### Basal Rho-GTPase Activity Changes Minimally during MDCK Cell Polarization

Inhibition of bacterial internalization by Toxin B in nonpolarized cells suggests that Rho-GTPases participate in bacterial internalization in this setting. If these small GTPases are less active in polarized cells compared with their nonpolarized counterparts, this might account for the relative resistance of polarized cells to bacterial internalization. We therefore assayed Rho-GTP, Rac-GTP, and Cdc42-GTP levels in day 1 versus day 3 MDCK cells by using portions of downstream effectors specific for the GTP-bound forms of these proteins as "affinity-precipitation" reagents for the activated forms of Rho (GST-TRBD) (Ren *et al.*, 1999), or Rac and Cdc42 (GST-CRIB hPAK3) (Bagrodia *et al.*, 1998; Benard *et al.*, 1999). When day 1 and day 3 cells were compared, the percentage of RhoA and Rac1 in the GTP-bound form did not vary. RhoA-GTP levels were  $2.32 \pm 0.84\%$  (SD) of total RhoA in day 1 cells and  $2.03 \pm 1.12\%$  (SD) in day 3 cells. Rac1-GTP made up  $1.55 \pm 0.30\%$  (SD) of total Rac1 in d 1 cells and  $1.23 \pm 0.72\%$  (SD) in day 3 cells. However, large changes were observed in Cdc42-GTP levels, which declined approximately ninefold from day 1 to day 3 ( $10.1 \pm 0.2\%$  (SD) to  $1.18 \pm 0.04\%$  (SD)).

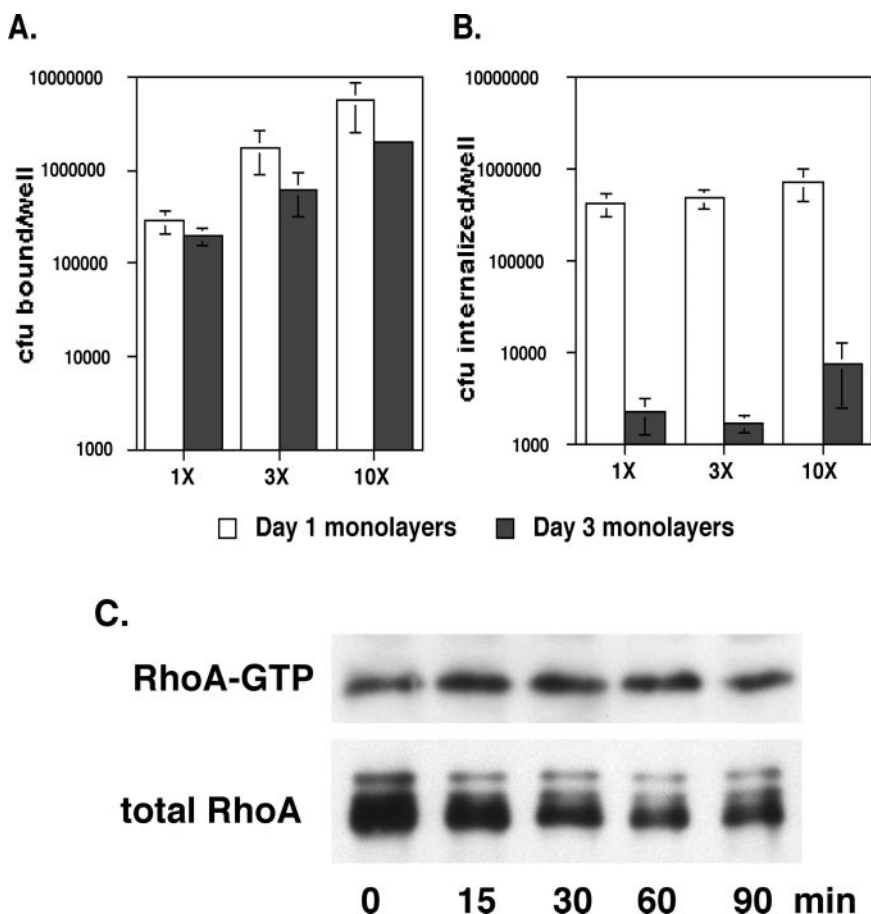


**Figure 4.** MDCK cell polarization is accompanied by a switch from RhoA activation to Cdc42 activation in response to infection. MDCK cells were plated to 7-cm Transwell filters at confluent density ( $2.0 \times 10^7$  cells/dish) and allowed to polarize for 1 or 3 d before infection with PA103 $\Delta$ UAT at an MOI of 20. Cells were lysed at indicated times, and aliquots of cell lysates were incubated with GST-TRBD bound to glutathione-Sepharose 4B beads (A and B) or GST-hPAK3 (C and D) as described in MATERIALS AND METHODS, allowing selective precipitation of GTP-bound RhoA or Cdc42, respectively. Both affinity-precipitated samples (GTP-bound) and aliquots of unprecipitated lysates were Western blotted with anti-RhoA (A and B) or anti-Cdc42 (C and D) antibodies. The gels shown are representative of two to four independent experiments carried out in duplicate.

#### The Ability of PA103 to Activate Rho-Family GTPases during Internalization Is Profoundly Altered by Polarization

Basal levels of RhoA-GTP showed no change as cells became more polarized, suggesting that changes in endogenous RhoA activity, as detected by our assay, could not account for the  $\sim$ 50-fold decrease in internalization we observed. However, the response of MDCK cells to bacterial challenge was altered dramatically by MDCK cell polarization. First, infection with *P. aeruginosa* led to RhoA activation only in day 1 cells, resulting in increased RhoA-GTP levels at 60–90 min postinfection (Figure 4A; our unpublished data). Increases in RhoA-GTP levels after bacterial infection were not seen in day 3 cells at any time point between 30 and 180 min postinfection (Figure 4B; our unpublished data). Instead, polarized MDCK cells responded to *P. aeruginosa* with increases in Cdc42-GTP levels 20 min after infection (Figure 4D). Infection of incompletely polarized (day 1) cells caused small decreases in Cdc42-GTP levels at 20 min postinfection, to 60–70% of uninfected controls (Figure 4C). The changes in RhoA-GTP and Cdc42-GTP levels observed in incompletely polarized MDCK cells are qualitatively similar to the responses observed in nonpolarized HeLa cells after *P. aeruginosa* infection. Thus, Cdc42-GTP levels measured in HeLa cells decrease to  $24.8 \pm 6.5\%$  of uninfected control levels at 10 min postinfection and recover to  $82.5 \pm 17.8\%$  by 40 min postinfection (our unpublished data); RhoA-GTP levels, on the other hand, increase to  $277 \pm 72\%$  at 90 min postinfection and  $476 \pm 110\%$  at 3 h postinfection (our unpublished data).

*P. aeruginosa* adheres less well to day 3 monolayers compared with day 1 monolayers (Figure 5A); thus, it is possible that RhoA activation might be observed in day 3 monolayers if a sufficient number of bacteria could be bound to these monolayers. We thus repeated our assays using a 10-fold higher number of bacteria to infect day 3 MDCK monolayers. Although this did result in numbers of adherent bacteria that exceeded those observed for d1 monolayers infected at the usual dose (1 $\times$ ), we still failed to observe activation of RhoA (Figure 5C). This finding, along with the pronounced



**Figure 5.** Increasing the number of bacteria bound to polarized MDCK monolayers does not lead to increased bacterial internalization nor to RhoA activation. (A and B) MDCK cells were plated to 12-mm Transwell filters at  $1.5 \times 10^6$  cells/well and allowed to polarize for 1 or 3 d before infection with PA103 $\Delta$ U $\Delta$ T at MOIs of 10–20 (1 $\times$ ), 40–60 (3 $\times$ ), or 100–150 (10 $\times$ ). All determinations were carried out in quadruplicate; bars indicate the average number of adherent or internalized organisms ( $\pm$ SD). In some instances, error bars are too small to see on the log scale used in these graphs. (C) MDCK cells were plated to 7-cm Transwell filters at confluent density ( $2.0 \times 10^7$  cells/dish) and allowed to polarize for 3 d before infection with PA103 $\Delta$ U $\Delta$ T at an MOI of  $\sim$ 200 (10 $\times$ ). RhoA-GTP and total RhoA were determined as described previously at various times postinfection. The gel shown is representative of four independent determinations.

activation of Cdc42 observed in infected day 3 monolayers, argues that polarized MDCK cells continue to respond to *P. aeruginosa* infection albeit in a qualitatively different way than incompletely polarized (day 1) monolayers.

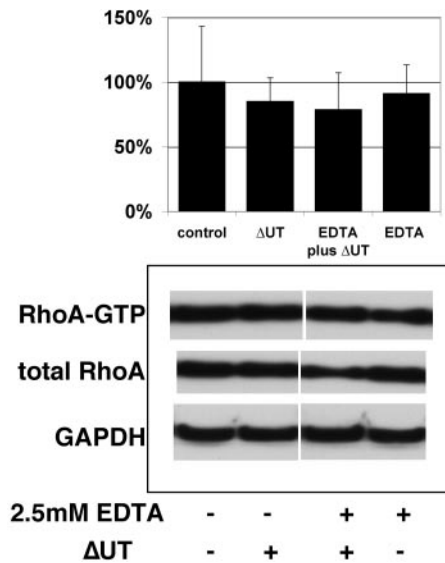
#### Disruption of Tight Junctions Increase *P. aeruginosa* Internalization via a Rho-independent Pathway

The data presented above are consistent with a model in which polarized epithelial cells down-regulate a Rho-dependent internalization pathway for apically infecting *P. aeruginosa*. It is possible that this pathway remains accessible at the basolateral surface of these polarized cells. Indeed, the observation that treatment of polarized epithelial cell monolayers with calcium chelators markedly increases *P. aeruginosa* internalization has led several investigators to conclude that *P. aeruginosa* preferentially interacts with polarized epithelial cells via a basolateral receptor (Fleiszig *et al.*, 1997). Although EDTA-treated cells avidly internalize bacteria and are no longer “polarized” by virtue of having disrupted tight junctions, we hypothesized that internalization would not occur via a Rho-dependent pathway based on several observations. First, Toxin B treatment of a polarized MDCK monolayer, which efficiently disrupts tight junctions, results in increased *P. aeruginosa* internalization even though Rho-family GTPases are inhibited (Figure 1). Second, polarized cells treated with specific RhoA inhibitors such as C3 ADP-ribosyltransferase show disrupted tight junctions and internalize *P. aeruginosa* more avidly than untreated controls (Kazmierczak *et al.*, 2001b). Last, recent studies have shown that tight junction disruption leads to RhoA down-regula-

tion by increasing cytoplasmic pools of p120 catenin (Anastasiadis *et al.*, 2000; Noren *et al.*, 2000). MDCK cells treated with EDTA to disrupt tight junctions immediately before bacterial infection showed increased internalization, with 10- to 15-fold more bacteria internalized than untreated cells (our unpublished data); however, there was no detectable increase in RhoA-GTP levels in the infected EDTA-exposed cells over levels seen in uninfected cells or cells challenged with PA103 $\Delta$ U $\Delta$ T in the absence of EDTA (Figure 6). This observation argues that the Rho-dependent pathway present in incompletely polarized cells is not spatially restricted to the basolateral surface in polarized cells, but rather is down-regulated.

#### DISCUSSION

It has long been appreciated that an intact epithelial layer serves as a physical barrier to bacterial infection and penetration. Recent studies reveal that certain pathogens invade or damage cells only through interaction with basolateral receptors or targets. Because a polarized epithelium physically sequesters basolateral determinants from ingested, inhaled or aspirated bacteria, it limits the ability of pathogens to use some of their virulence factors. Our work, however, reveals a novel “defense mechanism” linked to polarity: the acquisition of a polarized phenotype alters the response of epithelial cells to an apically presented pathogen. Moreover, although it has been previously noted that different cell lines internalize *P. aeruginosa* to an extent that inversely correlates with their degree of polarization (Fleiszig *et al.*, 1998; Plot-



**Figure 6.** Exposure of polarized MDCK cells to EDTA increases internalization without activating RhoA. MDCK cells were plated to 7-cm Transwell filters at confluent density ( $2.0 \times 10^7$  cells/dish) and allowed to polarize for 3 d. Immediately before infection, cells were washed with Hanks'  $\text{Ca}^{2+}\text{Mg}^{2+}$ -free BSS; indicated samples were treated with EDTA (2.5 mM) for 15 min. All samples were then returned to calcium and magnesium-replete tissue culture media before infection with PA103 $\Delta\text{U}\Delta\text{T}$  for 1.5 h. Samples were lysed and total RhoA versus GTP-bound RhoA levels determined as described previously. The gel illustrates a representative experiment. Bars indicate the mean percentage of GTP-bound versus total RhoA, normalized to the uninfected/untreated control (100%); error bars show the SD for five separate determinations.

kowski *et al.*, 1999), this is the first demonstration that activation patterns of Rho-family GTPases that accompany internalization are also altered by the acquisition of polarity.

Activation of Rho-family GTPases is required for several events that lead to the establishment of polarity, including tight junction formation and membrane protein targeting. It is therefore possible that Rho-family GTPase activity may be measurably greater in incompletely polarized cells compared with fully polarized cells. When we explicitly measured GTP-bound RhoA, Rac1, and Cdc42 levels in cells during the acquisition of polarity, we found that the percentage of GTP-bound Cdc42 declined markedly over time, but that the percentage of GTP-bound RhoA and Rac1 remained constant. Thus, it is difficult to invoke decreases in basal RhoA activity as the reason for decreased *P. aeruginosa* internalization by polarized cells. However, epithelial cell responses to infection with *P. aeruginosa* change markedly with the acquisition of polarity. RhoA is strongly activated in incompletely polarized cells, which show high levels of actin-dependent Toxin B-inhibited bacterial internalization, whereas Cdc42 is inactivated. In contrast, polarized cells show minimal RhoA activation, brisk Cdc42 activation, and low levels of bacterial internalization. These patterns of Rho-GTPase activation and inactivation likely play a role in governing whether *P. aeruginosa* is internalized by epithelial cells, because the expression of either constitutively active RhoA (RhoAV14) or dominant negative Cdc42 (Cdc42N17) in MDCK cells strongly promotes *Pseudomonas* internalization (Kazmierczak *et al.*, 2001b). The observation that polarized cells respond to invasive *P. aeruginosa* strains by rapidly activating Cdc42 demonstrates that the cells still sense the

presence of bacteria; thus, the absence of RhoA activation in polarized cells cannot be the result of the failure of the cells to detect the presence of *P. aeruginosa*. Activation of Cdc42 is not accompanied by bacterial internalization, however, which is consistent with our previous observation that the expression of constitutively active Cdc42 (Cdc42V12) in MDCK cells does not promote *P. aeruginosa* internalization (Kazmierczak *et al.*, 2001b). We currently cannot distinguish whether the bacteria interact with different receptors on incompletely versus completely polarized cells, or whether the same receptor engages a different downstream signaling pathway as cells become polarized. An analogous developmental change has been described in dendritic cells, which mature from highly endocytic immature cells into poorly endocytic antigen-presenting cells via a process involving down-regulation of Cdc42 activation (Garrett *et al.*, 2000). The mechanism by which Cdc42 activation is regulated in maturing dendritic cells remains unknown.

The events occurring during MDCK II polarization are well studied. As cell-cell contacts form, transepithelial resistance rapidly increases, lipid diffusion between the apical and basolateral membranes is prohibited, and ZO-1 staining indicates the presence of a complete tight junction ring (Bacallao *et al.*, 1989). We observe similar markers of tight junction formation in our system 18 h after plating cells at "instant monolayer" density to porous filters. Nonetheless, these cells continue to refine their segregation of apical and basolateral components, removing (by transcytosis) or degrading components present on the wrong side of the tight junction (Bacallao *et al.*, 1989). Thus, restriction of the receptor(s) for *P. aeruginosa* binding and internalization may account for the failure of bacteria to activate RhoA in polarized cells. Two results, however, argue against this hypothesis. Bacterial infection established at the basolateral surface of MDCK cells remains sensitive to the degree of cell polarization, arguing that the receptor or pathway is more likely down-regulated, rather than spatially restricted in polarized cells. Second, disrupting MDCK cell tight junctions by transient exposure of polarized cells to EDTA allows bacteria access to basolateral and apical membranes and results in large increases in internalization; however, internalization occurs without detectable activation of RhoA. These findings are consistent with a model in which a RhoA-dependent uptake pathway for *P. aeruginosa* becomes down-regulated in polarized cells. We have not yet defined the apparently RhoA-independent mechanism by which bacterial internalization occurs in the setting of monolayer disruption.

The study of pathogen interactions with epithelial cells in vitro has been instrumental in revealing multiple mechanisms by which bacteria manipulate host cell biology. The recent use of polarized epithelial cell models to study such interactions has made it clear, however, that the signaling pathways evoked by pathogens in polarized versus nonpolarized cell types often differ. For example, Hobert *et al.* (2002) recently reported that polarized MDCK cells respond differently to apical *S. typhimurium* infection than nonpolarized cells by showing that Rac1 activity, although still necessary for actin pedestal formation in both cell types, was not required for early proinflammatory signaling leading to interleukin (IL)-8 production in MDCK cells. A similar discordance between the requirements for Rho-GTPase activation in *S. typhimurium* invasion of nonpolarized cells and polarized MDCK cells was demonstrated earlier by Criss *et al.* (2001), who found that only Rac1 was activated during, and was required for, apical invasion of MDCK cells; in contrast, Cdc42 activation seems to be required for *Salmo-*

*nella* invasion of nonpolarized cell types. These investigators also found that apical invasion of polarized MDCK cells shows different requirements for Rho-GTPase activation than does basolateral invasion (Criss *et al.*, 2001). Hobert *et al.* (2002) propose that different localization patterns of small GTPases in polarized versus nonpolarized cells may contribute to the different activation patterns observed after bacterial infection. The molecular mechanism underlying the different requirements for Rho-GTPase activity in host cell signaling pathways, however, remains obscure.

The question of how an intact epithelial tissue successfully resists infection by an opportunistic pathogen such as *P. aeruginosa* is of particular interest, because this virulent pathogen rarely causes clinical disease in the absence of preexisting epithelial trauma. Our approach of examining how an epithelial monolayer responds to infection as it becomes more polarized has allowed us to make the novel observation that the response of an epithelial cell to *P. aeruginosa* shifts from RhoA activation and Cdc42 inactivation to rapid Cdc42 activation as the cell becomes polarized. Incompletely polarized MDCK cells avidly internalize *P. aeruginosa*, a result that fits well with our previous observation that expression of either constitutively active RhoA (RhoAV14) or dominant negative Cdc42 (Cdc42N17) is sufficient to promote *Pseudomonas* internalization in polarized MDCK cells (Kazmierczak *et al.*, 2001b).

It is particularly noteworthy that the response of polarized cells to *P. aeruginosa* involves Cdc42 activation, an event linked to the production of proinflammatory cytokines, such as IL-8, by epithelial cells in response to pathogenic bacteria (McCormick *et al.*, 1995; Chen *et al.*, 1996, 1999; Hobbie *et al.*, 1997). Thus, part of the protection afforded by a polarized epithelium may be derived from its ability to trigger a rapid and effective immune response against pathogens. *P. aeruginosa* has been shown to elicit IL-8 production by respiratory epithelial cells (DiMango *et al.*, 1995); whether disruption of this signaling pathway in the setting of epithelial trauma and loss of polarity facilitates infection by this opportunistic pathogen is currently under investigation.

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