Epithelial cell polarity alters Rho-GTPase responses to

\textit{Pseudomonas aeruginosa}

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Abbreviations: LatA, Latrunculin A; MOI, multiplicity of infection; LDH, lactate dehydrogenase.
ABSTRACT

*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 utilized confluent monolayers of Manin Darby Canine Kidney (MDCK) cells cultured on porous filter supports for 1 to 3 days 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 10-100-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 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.

Pseudomonas aeruginosa is an opportunistic pathogen that exploits pre-existing epithelial cell injury. This is apparent clinically, as 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 α5β1 (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 \textit{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 \textit{P. aeruginosa}, including the cystic fibrosis transmembrane receptor (CFTR) (Pier et al., 1997) and the asialoganglioside aGM1 (Hazlett et al., 1993), though their relative importance may be cell type specific. Several studies suggest that \textit{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 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 \textit{P. aeruginosa} receptor(s) to the basolateral surface of polarized cells, no such receptor has been identified to date.

The pathway of \textit{P. aeruginosa} internalization is sensitive to cytochalasin D, an actin depolymerizing agent, is inhibited by the tyrosine kinase inhibitors herbimicin 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,
though recent evidence suggests that MEK and ERK 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 PA, 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 employed 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). Since 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 downregulated 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 PA103pscJ::Tn5 (type III secretion deficient) and PA103ΔUΔT (type III secretion competent, ExoU-, ExoT-) have been described previously (Hauser et al., 1998; Garrity-Ryan et al., 2000). Both strains are internalized by non-professional 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. Plasmids expressing GST-TRBD and GST-CRIB were generously provided by Xiang-Dong Ren and Martin Schwartz (Scripps Institute) and Rick Cerione (Cornell University), respectively.

**Cell Culture:** HeLa cells (ATCC CCL-2) and MDCK cells (Type II) were cultured as described previously (Kazmierczak et al., 2001b). Prior to binding and internalization assays, cells were washed twice with MEM etc. [MEM salts (Sigma)/20 mM Hepes pH 7.4/10 mM sodium bicarbonate].

**Drugs and reagents:** Latrunculin A (LatA, Molecular Probes) was made up as a 2 mg/mL stock in DMSO. Cells were pre-treated for 30 minutes prior to 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 PBS. Cells were pre-treated for 4 hours prior to bacterial infection. We confirmed that neither LatA nor Toxin B inhibited *P. aeruginosa* viability at the concentrations used (data not shown). EDTA (Sigma) was made up in Hanks Ca^{++} Mg^{++} Free BSS (UCSF Tissue Culture Facility) pH 7.6. Cells were routinely pre-treated 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, NY and Barry Gumbiner (Memorial Sloan-Kettering, NY), respectively. Anti-ZO-1 (Chemicon), anti-RhoA (Santa Cruz), anti-Cdc42 (Signal Transduction Laboratories), anti-Rac1 (Upstate Biotechnology), 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$_{600}$=0.1 ($1.5 \times 10^8$/ml) prior to infection unless otherwise indicated. HeLa cell assays
were performed as described (Kazmierczak et al., 2001b). MDCK cells were plated on 12
mm Transwell™ filters (Corning) (0.4 µm pore size) at $1 \times 10^6$ cells/cm$^2$ 16-18h (“1 d”)
or 3 d prior to assaying. For binding assays, 150 µl of bacteria were incubated with
MDCK monolayers for 1 h at 37°C. Non-adherent bacteria were removed by four 5-min
washes with PBS. Adherent and internalized bacteria were released by incubating filters
with 0.25% Triton X-100 in Hanks Ca++Mg ++ free BSS for 30 min at RT. Sterile glass
beads were added to the samples, which were vortexed 2 x 10 sec 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 (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
prior to washing. MEM etc. plus amikacin (400 µg/ml) (Sigma) was added for a further
2 h to kill extracellular bacteria. Internalized bacteria were released by lysing MDCK
cells as described above, then enumerated by plating serial dilutions of the lysate to LB
plates. For basolateral infections, cells were plated to 3 µm pore size 12 mm
Transwell™ filters (Corning) at $1 \times 10^6$ cells/cm$^2$ 1-3 d prior to assaying. Bacteria were
diluted in MEM etc. to an OD$_{600}$ of ca. 0.6. Filters were infected by placing them directly
on top of 40 µl drops of bacterial suspension (MOI ca. 25-50); 200 µl of media were
added to the apical surface of the cells during the infection. After 2 hours incubation
with bacteria, the filters were washed, treated with antibiotic containing medium, and
lysed as for apical infections.
**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 x 10^6 cells/10cm dish two d prior to infection and assay; MDCK cells were plated at 2 x 10^7 cells/7 cm Transwell filter either 16-18 hr or 3 d prior to infection (MOI ≈ 50) and assay. Cells were washed twice with ice-cold TBS pH 7.2 plus 1 mM orthovanadate, then lysed in 600 µl of lysis buffer [20 mM Tris-HCl pH 8.0, 1% Triton X-100, 0.5 M NaCl, 15% glycerol, 10 mM MgCl2, 0.5 mM DTT, 1 mM orthovanadate, Complete™ Protease Inhibitor Cocktail, EDTA-free (Boehringer Mannheim)]. Lysates were cleared by centrifugation for 5 min at 14,000 rpm at 4°C, then divided for (1) affinity precipitation with 75 µg TRBD-GST beads (500 µl of lysate), (2) immunoblotting to determine total RhoA (20 µl of lysate), and (3) total protein concentration determination using the Pierce BCA assay kit (10 µl of lysate). Affinity precipitated samples and aliquots for total RhoA determination were electrophoresed on 13% SDS-PAGE gels, blotted to Immobilon P (Millipore), and incubated with anti-RhoA at 1:200 in 5% milk/TBST for two hours after blocking. After incubation with HRP-conjugated goat anti-mouse secondary Ab (1:2000 in 5% milk/TBST) for 1 hour, blots were washed and developed with the ECL+Plus kit (Amersham) 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 3-5 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. 90% of the cleared lysate was precipitated with 30 µg 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 3-5 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); Texas Red-phalloidin (1:200). Samples were imaged using a Biorad MRC 1024 confocal microscope with a KrAr laser. Z-series were routinely obtained at 1.0 um steps 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 µg/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 (data not shown).

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 *Clostridium 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 (Fig 1B). Bacterial viability was not affected by Toxin B; moreover, these concentrations of Toxin B resulted in stress fiber loss and cell rounding without causing direct HeLa cell injury as measured by LDH release (data not shown).

Similar observations can be made for other internalized *P. aeruginosa* strains. Thus, internalization of PA103ΔUΔT, a mutant capable of type III secretion but carrying large deletions in the genes encoding the cytotoxin ExoU and the anti-internalization factor ExoT, is also sensitive to LatA and Toxin B at 10 µg/ml and 10 ng/ml, respectively (data not shown). Likewise, *P. aeruginosa* strains PAK and PA01 are internalized by HeLa cells via a mechanism that is inhibited by LatA and Toxin B (data not shown), 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.* As 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 ug/ml) that caused a loss of filamentous actin staining, failed to inhibit internalization of PA103pscJ::Tn5 or PA103ΔUΔT (Fig 1C and data not shown). MDCK cell monolayers were nonetheless sensitive to LatA, as the actin-dependent internalization of E. coli MC4100 expressing the Yersinia enterocolitica protein Invasin was inhibited 100-fold under these conditions (Fig 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 (Fig 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 utilized 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 vs. polarized epithelia. MDCK cells plated at high density on permeable filters formed instant monolayers that, at 18-20 hours post plating, 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 FITC-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 (data not shown). 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 (data not shown).

Though d 1 and d 3 monolayers differed in subtle ways morphologically, their responses to *P. aeruginosa* were dramatically different. d 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). d 3 cells, as seen previously, showed no inhibition of internalization following treatment with these agents (Figure 2), while their effects on d 2 monolayers were intermediate to those observed on d 1 and d 3 (data not shown). Internalization was not inhibited as a result of cell injury following exposure to these drugs, as LDH release was not increased by these agents (data not shown). Furthermore, cell loss from the filters following inhibitor treatment was less than 10% and could not account for the decrease in internalized bacteria, although cell retraction and condensation of the actin cytoskeleton were observed (data not shown).

In addition to finding that d 1 and d 3 cells showed different responses to LatA and Toxin B, we also observed that *P. aeruginosa* internalization decreased some 50-fold between d 1 and d 3 MDCK cells (Figure 2A & 2B, compare untreated cells at d 1 and d 3). This difference reflected both a decrease in bacterial adherence to the apical surfaces of d 3 cells, as well as a much greater relative decrease in the frequency with which bound bacteria were internalized (Figure 5A & 5B). Microscopic examination of infected d 1 vs. d 3 MDCK cells stained for internalized versus adherent bacteria also suggested that the efficiency of bacterial internalization by d 1 cells was higher than that of d 3 cells: 2 hr after infection, about 5-10% of d 1 cells contained several internalized
bacteria. In contrast, d 3 cells containing internalized bacteria were extremely rare, with the majority of cell-associated bacteria being extracellular (data not shown). Increasing the inoculum size three-fold or ten-fold resulted in commensurate increases in bacterial binding to the apical surfaces of both d 1 and d 3 cells (Figure 5A). However, increasing the inoculum size did not overcome the relative inability of d 3 cells to internalize *P. aeruginosa*, as d 3 cells infected with a 10-fold greater inoculum internalized only 1.8% as many bacteria as d 1 cells infected with the usual (“1X”) 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 d 1 and d 3 MDCK monolayers, a change that cannot be ascribed to decreased bacterial binding to d 3 cells. These findings could represent either the relocalization 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 um 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 makers of polarity, to ensure that they formed a normal appearing
monolayer. Proteins appeared to be localized correctly: gp135 and E-cadherin were restricted appropriately to the apical membrane and zona adherens, respectively (data not shown). Basolateral infection was established by placing filters on drops of bacterial suspension and resulted in less efficient internalization than apical inoculation. Nonetheless, polarity appeared to affect the number of bacteria internalized following 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 downregulating the pathway utilized by *P. aeruginosa* for internalization. This may result from downregulation or disappearance of either the receptor used by *P. aeruginosa* or of more distal elements in this pathway.

**Basal RhoGTPase activity changes minimally during MDCK cell polarization.** Inhibition of bacterial internalization by Toxin B in non-polarized cells suggests that RhoGTPases participate in bacterial internalization in this setting. If these small GTPases are less active in polarized cells compared to their non-polarized 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 d 1 vs. d 3 MDCK cells 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 d 1 and d 3 cells were compared, the percentage of RhoA and Rac1 in the GTP-bound form did not vary. RhoA-GTP levels were 2.32±0.84% (SD) of total RhoA in d 1 cells and 2.03±1.12% (SD) in d 3 cells. Rac1-GTP made up 1.55±0.30% (SD) of total Rac1 in d 1 cells and 1.23±0.72% (SD) in d 3 cells. However, large changes were observed in Cdc42-GTP levels, which declined approximately 9-fold from d 1 to d 3 (10.1±0.2% (SD) to 1.18±0.04% (SD)).

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 ca. 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 d 1 cells, resulting in increased RhoA-GTP levels at 60 to 90 min post-infection (Figure 4A and data not shown). Increases in RhoA-GTP levels following bacterial infection were not seen in d 3 cells at any time point between 30 min and 180 min post-infection (Fig 4B and data not shown). Instead, polarized MDCK cells responded to *P. aeruginosa* with increases in Cdc42-GTP levels 20 min after infection (Fig 4D). Infection of incompletely polarized (d 1) cells caused small decreases in Cdc42-GTP levels at 20 min post-infection, to 60-70% of uninfected controls (Fig 4C). The changes in RhoA-GTP and Cdc42-GTP levels observed in incompletely polarized MDCK cells are qualitatively similar to the responses observed in non-polarized HeLa cells following *P. aeruginosa*. Thus, Cdc42-GTP levels measured in HeLa cells decrease to 24.8±6.5%
of uninfected control levels at 10 minutes post infection and recover to 82.5±17.8% by 40 minutes post infection (data not shown); RhoA-GTP levels, on the other hand, increase to 277±72% at 90 minutes post infection and 476±110% at 3 hours post infection (data not shown).

*P. aeruginosa* adheres less well to d 3 monolayers as compared to d 1 monolayers (Figure 5A); thus, it is possible that RhoA activation might be observed in d 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 d 3 MDCK monolayers. Although this did result in numbers of adherent bacteria which exceeded those observed for d1 monolayers infected at the usual dose (1X), we still failed to observe activation of RhoA (Figure 5C). This finding, along with the pronounced activation of Cdc42 observed in infected d 3 monolayers, argues that polarized MDCK cells continue to respond to *P. aeruginosa* infection albeit in a qualitatively different way than incompletely polarized (d 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 downregulate 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 (see 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 downregulation 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 prior to bacterial infection showed increased internalization, with 10- to 15-fold more bacteria internalized than untreated cells (data not shown); 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 PA103ΔUΔT 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 downregulated.

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. As a
polarized epithelium physically sequesters basolateral determinants from ingested, inhaled or aspirated bacteria, it limits the ability of pathogens to employ 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, though 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; Plotkowski *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 as compared to 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, while 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 or not, as 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 vs. 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 downregulation 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 hours 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 \textit{P. aeruginosa} binding and internalization may account for the failure of bacteria to activate RhoA in polarized cells. Two results argue against this hypothesis, however. 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 downregulated, 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 \textit{P. aeruginosa} becomes downregulated 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 vs. nonpolarized cell types often differ. For example, Hobert \textit{et al.} recently reported that polarized MDCK cells respond differently to apical \textit{Salmonella typhimurium} infection than nonpolarized cells by showing that Rac1 activity, though still necessary for actin pedestal formation in both cell types, was not required for early pro-inflammatory signaling leading to IL-8
production in MDCK cells (Hobert et al., 2002). A similar discordance between the requirements for RhoGTPase activation in S. typhimurium invasion of nonpolarized cells and polarized MDCK cells was demonstrated earlier by Criss et al., who found that only Rac1 was activated during and required for apical invasion of MDCK cells; in contrast, Cdc42 activation appears to be required for Salmonella invasion of non-polarized cell types. These investigators also found that apical invasion of polarized MDCK cells shows different requirements for RhoGTPase activation than does basolateral invasion (Criss et al., 2001). Hobert et al. propose that different localization patterns of small GTPases in polarized vs. nonpolarized cells may contribute to the different activation patterns observed following bacterial infection. The molecular mechanism underlying the different requirements for RhoGTPase 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, since this virulent pathogen rarely causes clinical disease in the absence of pre-existing 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 pro-inflammatory cytokines, such as IL-8, by epithelial cells in response to pathogenic bacteria (McCormick *et al.*, 1995; Chen *et al.*, 1996; Hobbie *et al.*, 1997; Chen *et al.*, 1999). 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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FIGURE LEGENDS

Fig 1. LatA and Toxin B have different effects on *P. aeruginosa* entry into non-polarized versus polarized epithelial cells. Panels A & B: HeLa cells were infected with PA103::*pscJ*\textsubscript{Tn5} (MOI = 10-20) for 2 hrs. The number of internalized bacteria per well was determined as described in Materials and Methods. Bars represent the mean ± S.D. of triplicate samples from a representative experiment. Panels C & D: MDCK cells were plated on 12 mm Transwell™ filters at 1.5 x 10\(^6\) cells/well 3 d prior to infection with PA103::*pscJ*Tn5, Invasin-expressing *E. coli* (MC4100 pRI203), or *S. typhimurium* (SL1344) for 2 hr. Bars show the mean ± S.D. of triplicate samples from a representative experiment. In some instances, error bars are too small to appear on the log scale used in these graphs. Panels A & C: Latrunculin A or DMSO was added to cells 30 min prior to addition of bacteria and was present for the 2 hr of cocultivation with bacteria. Panels B & D. Cells were pretreated with Toxin B for 4 hr prior to the addition of bacteria.

Fig 2. Sensitivity to inhibitors of actin polymerization is affected by cell polarity. MDCK cells were plated on 12 mm Transwell filters at 1.5 x 10\(^6\) cells/well and allowed to polarize for 1 or 3 d. Cells were pretreated with (A) LatA for 30 min or (B) Toxin B for 4 hr prior to infection with PA103::*pscJ*Tn5 at an MOI of 10-20 for 2 hr. Bars show mean ± S.D. of triplicate samples from a representative experiment. In some instances, error bars are too small to appear on the log scale used in these graphs.
Fig 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 µm pore size) at confluent density (1.5 x 10^6 cells/well). PA103::pscJ Tn5 was grown overnight in LB with shaking, diluted in MEM to A600 0.6. The filter was placed on a 40 ul drop (MOI =50) for 2 hr in a humid chamber to allow infection to occur. Invasion assays were performed as described in Fig 1. The treated MDCK cells were exposed to Toxin B for 4 hours prior to infection. Bars show mean ± S.D. of triplicate samples from a representative experiment. In some instances, error bars are too small to appear on the log scale used in these graphs.

Fig 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 x 10^7 cells/dish) and allowed to polarize for 1 or 3 d prior to infection with PA103ΔUΔT at an MOI of 20. Cells were lysed at indicated times and aliquots of cell lysates incubated with GST-TRBD bound to Glutathione-Sepharose 4B beads (4A & B) or GST-hPAK3 (4C & 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 (4A & B) or anti-Cdc42 (4C & D) antibodies. The gels shown are representative of 2-4 independent experiments carried out in duplicate.
Fig 5. Increasing the number of bacteria bound to polarized MDCK monolayers does not lead to increased bacterial internalization nor to RhoA activation. A & B. MDCK cells were plated to 12 mm Transwell™ filters at 1.5 x 10^6 cells/well and allowed to polarize for 1 or 3 d prior to infection with PA103ΔUΔT at MOIs of 10-20 (1X), 40-60 (3X) or 100-150 (10X). All determinations were carried out in quadruplicate; bars indicate the average number of adherent or internalized organisms (±S.D.). In some instances, error bars are too small to appear on the log scale used in these graphs. C. MDCK cells were plated to 7 cm Transwell™ filters at confluent density (2.0 x 10^7 cells/dish) and allowed to polarize for 3 d before infection with PA103ΔUΔT at an MOI of ca. 200 (10X). RhoA-GTP and total RhoA were determined as described previously at various times post-infection. The gel shown is representative of 4 independent determinations.

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 x 10^7 cells/dish) and allowed to polarize for 3 d. Immediately prior to infection, cells were washed with Hanks’ Ca⁺⁺Mg⁺⁺ free BSS; indicated samples were treated with EDTA (2.5 mM) for 15 min. All samples were then returned to calcium & magnesium-replete tissue culture media prior to infection with PA103ΔUΔT for 1.5 hr. Samples were lysed and total RhoA vs. GTP-bound RhoA levels determined as described previously. The gel illustrates a representative experiment. Bars indicate the mean percentage of GTP-bound vs. total RhoA, normalized to the uninfected/untreated control (100%); error bars show the S.D. for 5 separate determinations.


