

Host Cell Polarity Proteins Participate in Innate Immunity to *Pseudomonas aeruginosa* Infection

Cindy S. Tran,^{1,10} Yoni Eran,^{2,10} Travis R. Ruch,² David M. Bryant,³ Anirban Datta,³ Paul Brakeman,⁴ Arlinet Kierbel,⁵ Torsten Wittmann,⁶ Ross J. Metzger,³ Keith E. Mostov,^{3,7,8} and Joanne N. Engel^{2,9,*}

¹Division of Neonatology, Department of Pediatrics

²Division of Infectious Disease, Department of Medicine

³Department of Anatomy

⁴Division of Pediatric Nephrology, Department of Pediatrics
University of California, San Francisco, San Francisco, CA 94143, USA

⁵Institut Pasteur de Montevideo, Montevideo 11400, Uruguay

⁶Department of Cell and Tissue Biology

⁷Department of Biochemistry and Biophysics

⁸Cardiovascular Research Institute

⁹Department of Microbiology and Immunology
University of California, San Francisco, San Francisco, CA 94143, USA

¹⁰Co-first authors

*Correspondence: jengel@medicine.ucsf.edu

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SUMMARY

The mucosal epithelium consists of polarized cells with distinct apical and basolateral membranes that serve as functional and physical barriers to external pathogens. The apical surface of the epithelium constitutes the first point of contact between mucosal pathogens, such as *Pseudomonas aeruginosa*, and their host. We observed that binding of *P. aeruginosa* aggregates to the apical surface of polarized cells led to the striking formation of an actin-rich membrane protrusion with inverted polarity, containing basolateral lipids and membrane components. Such protrusions were associated with a spatially localized host immune response to *P. aeruginosa* aggregates that required bacterial flagella and a type III secretion system apparatus. Host protrusions formed de novo underneath bacterial aggregates and involved the apical recruitment of a Par3/Par6 α /aPKC/Rac1 signaling module for a robust, spatially localized host NF- κ B response. Our data reveal a role for spatiotemporal epithelial polarity changes in the activation of innate immune responses.

INTRODUCTION

The mucosal barrier, composed of adherent sheets of polarized epithelial cells with distinct apical and basolateral membranes that are connected by tight junctions (TJs) and adherens junctions (AJs), is one of the most fundamental components of the innate immune system. Initiation and maintenance of the polarized epithelium requires the spatial and temporal orchestration

of a large network of proteins and lipids. Apical-basolateral polarity is initiated by the formation of primordial AJs that lack TJ components, while cadherins extending from adjacent cells interact to create homophilic intercellular adhesions. Subsequent Rho family guanosine triphosphatase (GTPase) activation leads to cytoskeletal rearrangements, resulting in the formation of mature TJs and AJs. In addition, cell polarity and junction integrity are regulated by three different apical- and basolateral-specific polarity complexes, including the apical Par complex composed of Par3, Par6, and aPKC. The asymmetric distribution of phosphatidylinositol phosphates (PIPs) also contributes to cell polarity, with PI-(4,5)-bisphosphate (PIP₂) enriched in the apical surface and PI-(3,4,5)-trisphosphate (PIP₃) localized to the basolateral surface (Rodriguez-Boulan and Macara, 2014).

Epithelial cell polarity plays a critical role in defense against microbial pathogens, including the often lethal opportunistic Gram-negative bacterium *P. aeruginosa*. When the epithelial barrier and the host immune system are intact, *P. aeruginosa* is unable to efficiently colonize the mucosal epithelium and cause disease. However, in the setting of injured or incompletely polarized epithelium, *P. aeruginosa* can initiate colonization and unleash its arsenal of potent virulence factors, which include the type III secretion system (T3SS) and its secreted effectors (Engel and Balachandran, 2009). This simple paradigm explains why *P. aeruginosa* is a leading cause of hospital-acquired infections, including ventilator-associated pneumonia, skin infections in burn patients or at the site of surgical incisions, and catheter-related infections (Mandell et al., 2010). *P. aeruginosa* is also a cause of chronic lung infections and ultimately death in patients with cystic fibrosis (Mandell et al., 2010). The molecular mechanisms and signal transduction pathways that connect pathogen sensing to the innate immune response in epithelial cells, however, remain incompletely understood (Artis, 2008; Ryu et al., 2010).

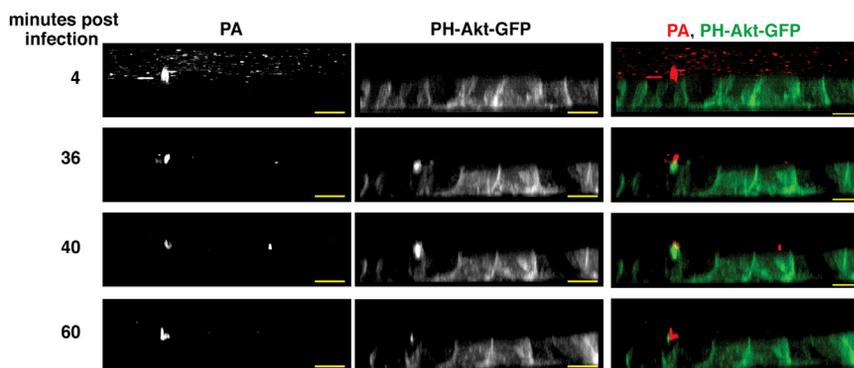


Figure 1. PIP₃-Rich Protrusions Are Formed De Novo at the Site of Bacterial Aggregate Binding on the Apical Surface of Polarized Epithelial Cells

Selected xz frames from time-lapse spinning disk confocal images of MDCK cells expressing PH-Akt-GFP (green, a marker for PIP₃) infected with PAK-mCherry (red) (see also [Movie S1](#)). At 4 min, a bacterial aggregate bound to the apical surface is detectable. Many nonadherent individual bacteria are also observed. At 36 min postinfection, a PIP₃-rich protrusion forms underneath the bacterial aggregate and is less intense at 60 min postinfection. Scale bars, 10 μ m. See also [Movie S1](#).

We have previously used *P. aeruginosa* infection of filter-grown epithelial cells to model host-pathogen interactions at the mucosal barrier (Bucior et al., 2010, 2012; Kazmierczak et al., 2001). When grown for several days on semiporous filters (Transwells), Madin-Darby canine kidney (MDCK) epithelial cells form well-polarized, confluent monolayers with distinct apical and basolateral surfaces (Mostov, 1995). Notably, the degree of cell polarity negatively correlates with the final outcome of *P. aeruginosa* infection (Kazmierczak et al., 2001).

When *P. aeruginosa* is added to the apical surface of polarized epithelial cells, cell-associated bacterial aggregates are formed from free-swimming individual bacteria within minutes, often near cell-cell junctions (Lepanto et al., 2011). The binding of bacterial aggregates, but not individual bacteria, is associated with the transformation of a small patch of apical membrane into one with basolateral characteristics within 30 to 60 min of infection (Kierbel et al., 2007), prior to translocation of the type III secreted effectors and associated cytotoxicity (Balachandran et al., 2007; Soong et al., 2008). This spatial and temporal cortical domain transformation involves the production of a host membrane protrusion that is enriched for phosphatidylinositol 3-kinase (PI3K), its normally basolateral lipid product PIP₃, actin, and several basolateral proteins. Importantly, TJs are not disrupted during the initial stages of protrusion formation, suggesting that protrusions result from localized rearrangement of the apical membrane rather than overt loss of cell polarity (Kierbel et al., 2007). How such remarkable polarity rearrangement can occur in an otherwise stable epithelium remains an unanswered question. Furthermore, the consequences of protrusion formation for both host and pathogen have not yet been examined. Here, we have elucidated the molecular regulation and function of these membrane events, including the critical host and pathogen factors required for their formation.

RESULTS

PIP₃-Rich Protrusions Are Formed De Novo at the Site of Bacterial Aggregate Binding

To investigate whether protrusions are formed in response to bacterial aggregate formation, we analyzed the dynamics of protrusion formation using time-lapse spinning disk confocal microscopy of filter-grown MDCK cells stably expressing PH-Akt-GFP, a probe for the normally basolateral lipid PIP₃ (Watton and Downward, 1999) that robustly localizes to aggregate-associated protrusions (Kierbel et al., 2007). *P. aeruginosa* (strain

PAK) expressing mCherry was added to the apical surface of MDCK monolayers, and confocal optical stacks were acquired. Within 30 min, PIP₃-rich protrusions rapidly formed de novo at the binding site of a bacterial aggregate and subsequently disappeared by 60 min (Figure 1; [Movie S1](#) available online). Thus, protrusions are dynamic structures that can be formed de novo on the apical surface of polarized cells in response to binding of bacterial aggregates.

Bacterial Flagella and the T3SS Are Required for a Localized Host Innate Immune Response

P. aeruginosa binding to epithelia has been shown to activate NF- κ B, a central regulator of the innate immune response (DiMango et al., 1998; Lavoie et al., 2011; Mijares et al., 2011; Sadikot et al., 2006; Schroeder et al., 2002), though the mechanism that connects *P. aeruginosa* binding to NF- κ B activation remains poorly understood (Hybiske et al., 2004). Thus, we examined whether aggregate binding and protrusion formation are involved in a spatially localized host response to bacteria.

We first determined whether the binding of *P. aeruginosa* aggregates was associated with NF- κ B activation, as assessed by nuclear translocation of the NF- κ B subunit p65. Immunofluorescence staining of p65 revealed a strong nuclear signal in epithelial cells underneath or adjacent to bacterial aggregates, in contrast to only a very weak nuclear signal in most nonadjacent cells (Figure 2A, left panel; [Movie S2](#)). We defined NF- κ B activation as a nuclear:cytoplasmic p65 ratio of ≥ 1 (see [Supplemental Experimental Procedures](#)). Notably, the vast majority of bacterial aggregates (67%), but no adherent single bacteria (0/24), were associated with NF- κ B activation in underlying epithelial cells (Figure S1). Uninfected cells also did not exhibit NF- κ B activation (Figure S1). We further determined that actin-rich protrusions and local NF- κ B activation occur at the site of bacterial aggregate binding in 16HBE14o⁻ cells (a human airway epithelial cell line) (Figure S2). These data suggest that localized protrusion formation and NF- κ B activation may occur as a host response to bacterial aggregation on the apical surface.

We next tested whether key virulence factors of *P. aeruginosa*, specifically flagella and the T3SS, were involved in protrusion formation and localized NF- κ B activation. To evaluate protrusion formation and localized NF- κ B activation, bacterial aggregates and the underlying MDCK cells were imaged, and recruitment of cortical actin or PIP₃ to the protrusion was analyzed in 3D reconstructions using a well-defined set of criteria that minimized observer-to-observer variation (see [Supplemental Experimental Procedures](#)). *P. aeruginosa* flagellin

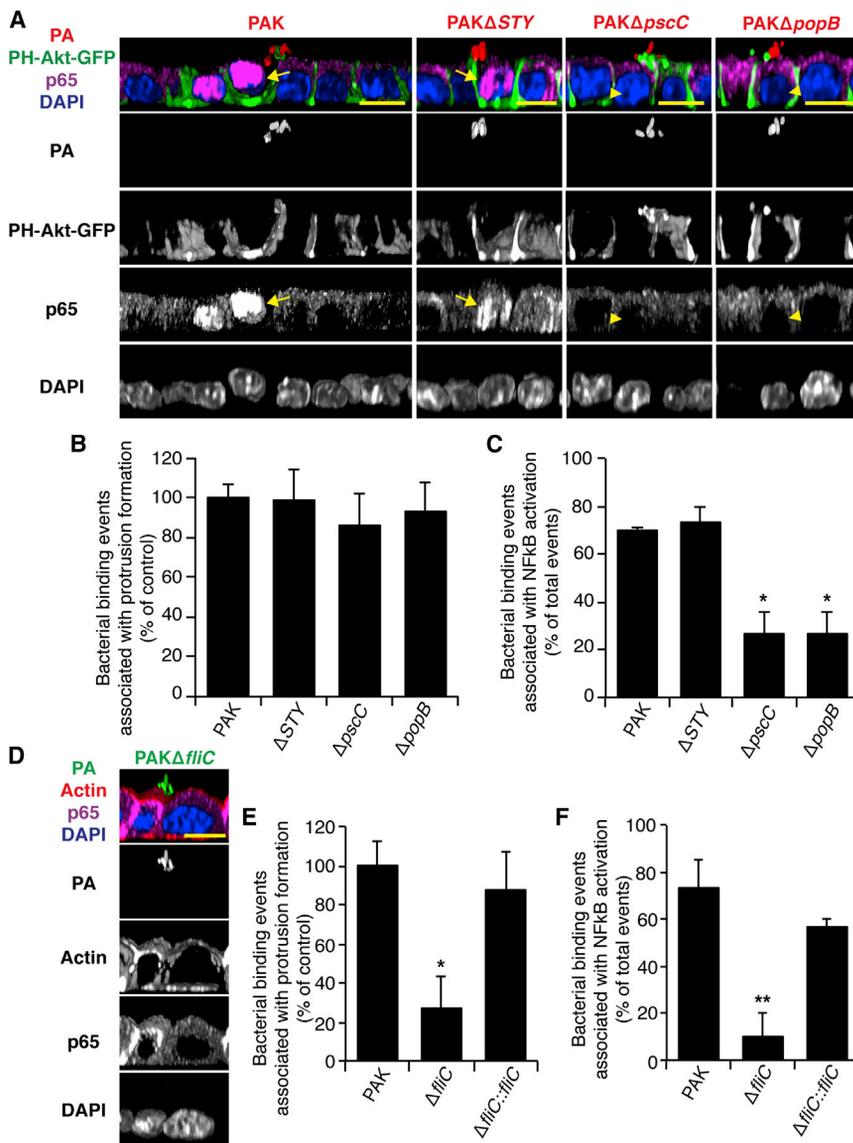


Figure 2. Bacterial Factors Required for Aggregate-Associated Protrusion Formation and NF- κ B Activation

(A) 3D reconstruction and xz view, of MDCK cells expressing PH-Akt-GFP infected with PAK or isogenic T3SS mutants expressing mCherry (red) for 30 min. Cells were fixed and stained with p65 antibody (NF- κ B, purple) and DAPI (nucleus, blue). Arrows indicate increased nuclear p65 staining in cells underneath or adjacent to bacterial aggregates; arrowheads indicate cells that show no change.

(B and C) Bacterial binding events associated with protrusion formation (B) or NF- κ B activation (C) by aggregates (≥ 10 bacteria) of PAK or isogenic T3SS mutants ($n = 3$). Protrusion formation was measured by PIP₃ as judged by PH-Akt-GFP recruitment.

(D) 3D reconstruction and xz view, of MDCK cells infected with PAK Δ fliC expressing GFP (green) for 30 min. Cells were fixed and stained with p65 antibody (NF- κ B, purple), phalloidin (actin, red), and DAPI (nucleus, blue).

(E and F) Bacterial binding events associated with protrusion formation (E) or NF- κ B activation (F) by aggregates (≥ 10 bacteria) of PAK or isogenic mutants lacking flagellin ($n = 3$). Protrusion formation was measured by actin recruitment as judged by fluorescent phalloidin staining. Scale bars, 10 μ m. Data are mean \pm SEM. * $p < 0.05$; ** $p < 0.01$ compared to PAK control. See also [Figures S1 and S2](#), [Movie S2](#), and [Supplemental Experimental Procedures](#).

is known to activate NF- κ B both through Toll-like receptor 5 (TLR5) and through intracellular cytosolic sensors (Lavoie et al., 2011). The mutant PAK Δ fliC, which fails to produce flagellin, shows $\sim 50\%$ reduction in binding to the apical surface of polarized MDCK cells (Bucior et al., 2012). Despite less-efficient binding by PAK Δ fliC, this mutant still formed aggregates on the apical surface of cells, albeit at a reduced frequency (C.S.T. and J.N.E., unpublished data). We found that protrusion formation associated with bacterial aggregates, as assessed by actin recruitment, was decreased by 75% in PAK Δ fliC (Figure 2E). Protrusion formation was restored in the complemented mutant (Figure 2E). Strikingly, NF- κ B activation was also reduced in cells infected with PAK Δ fliC and restored in the complemented mutant (Figures 2D and 2F). Our results suggest that flagella, in addition to their role in adhesion, are necessary for both protrusion formation and localized NF- κ B activation.

We next focused on the T3SS, which is necessary for virulence in both in vitro and in vivo models of infection and is associated

gesting that the T3SS effectors are not involved in these processes. Aggregates and protrusions could be observed in host cells infected with PAK Δ pscC (which lacks the T3SS needle apparatus and pore-forming translocon) or PAK Δ popB (which has an intact T3SS needle apparatus but lacks the translocon), albeit at a slightly reduced level (Figure 2B; C.S.T. and J.N.E., unpublished data). Remarkably, both PAK Δ pscC and PAK Δ popB were significantly deficient in inducing NF- κ B activation (Figures 2A and 2C). Thus, our data suggest that *P. aeruginosa* aggregation and protrusion formation on the epithelium may both be necessary, but are not sufficient, for NF- κ B activation. Furthermore, a functional needle apparatus and translocon, but not the effectors, are required for local activation of the innate immune response.

The Par Complex, Rac1, and AJ Proteins Are Recruited to Protrusions

We next qualitatively and quantitatively assayed potential host factors that are recruited to protrusions, with the hypothesis

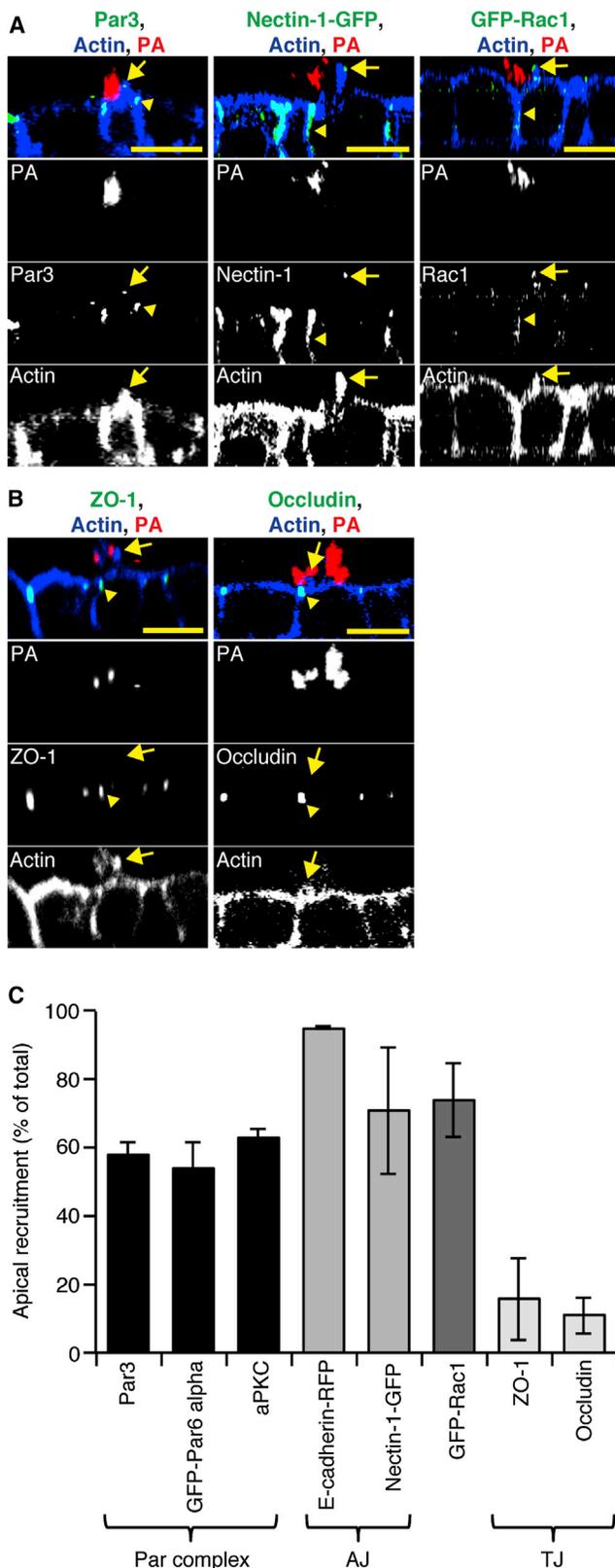


Figure 3. Par3, AJ Proteins, and Rac1 Are Recruited to Protrusions
(A) Confocal xz scans of MDCK monolayers infected with PAK-mCherry for 30 min. Samples were stained with fluorescent phalloidin (actin, blue) and

that components that are recruited to protrusions may also be necessary for activation of the NF- κ B response. The dramatic rearrangement of membrane composition in protrusions could involve polarity-regulating components such as the Par complex. The Par6/Cdc42/aPKC complex, together with Par3, has been implicated in the establishment and maintenance of cell polarity and is normally localized at TJs (Rodriguez-Boulan and Macara, 2014). We observed robust recruitment of Par3 and aPKC coincident with F-actin to protrusions (Figures 3A, arrows, 3C, and S3A). Notably, the Par complexes may exist in different subtypes, with basolateral Rac1-Par6 α potentially substituting for the apical Cdc42-Par6 β complex (Macara, 2004). Accordingly, exogenously expressed GFP-Par6 α and GFP-Rac1 (Figures 3A, 3C, and S3A) and endogenous Rac1 (Figure S3A), which are normally basolaterally located, were all strongly enriched in protrusions. Our efforts to analyze the recruitment of Cdc42 and RhoA were complicated by the largely cytoplasmic localization of these proteins, even when ectopically expressed. Thus, while both of these proteins were present within the protrusion, it was not clear if they were specifically enriched in the protrusion (data not shown). Nonetheless, these data suggest that a Par6 α /Rac1/aPKC/Par3 module is recruited to the protrusion.

Par3 is a multi-PDZ domain-containing protein that is recruited to cell-cell junctions through multiple mechanisms that include interactions with both AJ and TJ proteins (Takekuni et al., 2003). We examined whether AJ or TJ proteins were similarly associated with the aggregate-induced protrusion. Strikingly, both the prototypical AJ protein E-cadherin and the Par3-interacting dual AJ/TJ adhesion protein Nectin-1 were enriched in protrusions (Figures 3A and 3C), whereas no other TJ proteins examined, including ZO-1 and occludin, were present (Figures 3B and 3C). In other systems, Nectin-1 initially localizes to developing cell-cell contacts before redistributing to TJs once contacts mature (Takekuni et al., 2003). Our data indicate that protrusions contain characteristics of rudimentary types of cell-cell contacts, including polarity and AJ proteins, but largely lack TJ proteins. These results suggest that bacterial aggregate detection may mimic the early phases of a nascent cell-cell contact.

PI3K and Rac1 Are Required for Protrusion Formation

We tested the necessity and potential hierarchy of proteins recruited to protrusions, assessing the effect of chemical inhibitors or shRNA-mediated depletion on the recruitment of two robust markers of protrusions: PIP₃ and F-actin (see Supplemental Experimental Procedures). None of the inhibitors or RNAi

immunostained for Par3 to examine recruitment to protrusions. For some experiments, MDCK cells stably expressing fluorescent protein fusions (Nectin-1-GFP, GFP-Rac1) were used. Arrowheads indicate proteins at their expected localization; arrows indicate the location of the protrusion.

(B) Confocal xz scans of MDCK monolayers infected with PAK-mCherry for 30 min. Samples were stained with fluorescent phalloidin (actin, blue) and immunostained for ZO-1 or occludin to examine recruitment to protrusions. Arrowheads indicate proteins at their expected localization; arrows indicate the location of the protrusion.

(C) Quantification of host protein recruitment to protrusions as described in the Supplemental Experimental Procedures ($n \geq 2$ independent experiments). The different classes of protein function are indicated. Scale bars, 10 μ m. Data are mean \pm SEM. See also Figures S3A and S4.

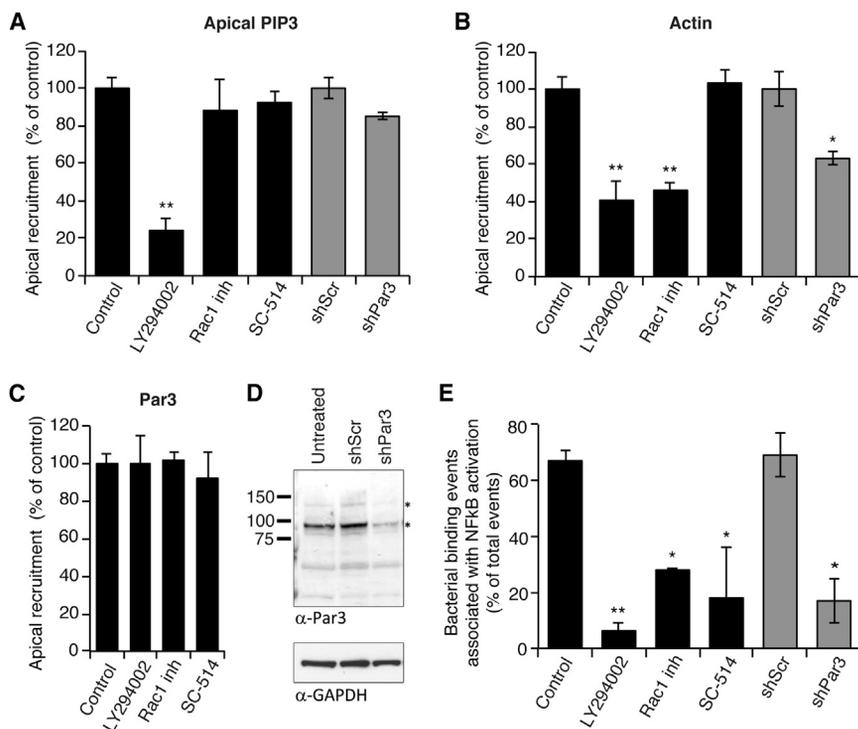


Figure 4. PI3K and Rac1 Are Required for Protrusion Formation, while PI3K, Rac1, and Par3 Are Required for Aggregate-Associated NF- κ B Activation

(A) The effect of chemical inhibition (LY29004 for PI3K, Rac1 inhibitor for Rac1, SC-514 for NF- κ B; black bars) or shRNA-mediated protein depletion (gray bars) on the recruitment of PIP₃ to the protrusion as judged by recruitment of PH-Akt-GFP to the protrusion. In control experiments, PH-Akt-GFP was recruited to 82% (control) and 86% (shScr) of bacterial aggregates analyzed, and all results were normalized to controls ($n \geq 2$ independent experiments).

(B) The effect of chemical inhibition (black bars) or shRNA-mediated protein depletion (gray bars) on the recruitment of actin to the protrusion as judged by fluorescent phalloidin staining. All results were normalized to controls ($n \geq 2$ independent experiments).

(C) Chemical inhibition of PI3K, Rac1, or NF- κ B does not affect Par3 recruitment to the protrusion. (D) Western blot for Par3 in untreated, shScr-expressing, or shPar3-expressing MDCK cells. The two major Par3 isoforms are indicated with an asterisk. GAPDH serves as a loading control.

(E) NF- κ B activation underneath bacterial aggregates following chemical inhibition (black bars) or shRNA-mediated protein depletion (gray bars). NF- κ B activation was scored the same as in Figure 2B ($n \geq 2$ independent experiments). Data are mean \pm SEM. * $p < 0.05$; ** $p < 0.01$ compared to control. See also Figures S3B and S4 and Supplemental Experimental Procedures.

perturbed monolayer integrity (data not shown). Inhibition of PI3K by LY294002 significantly decreased aggregate-induced PIP₃ accumulation on the apical membrane (Figure 4A), confirming previous observations (Kierbel et al., 2007) and validating our quantification methodologies. PI3K inhibition similarly decreased F-actin recruitment to the protrusion (Figure 4B), consistent with a function for PIP₃ in regulation of the actin cytoskeleton (Czech and Corvera, 1999). In accordance with its recruitment to protrusions, chemical inhibition of Rac1 (Rac1 inhibitor) decreased F-actin recruitment underneath protrusions (Figure 4B) while only minimally affecting apical PIP₃ recruitment (Figures 4A and S3B). Our findings indicate that both PIP₃ production by PI3K and actin reorganization mediated by Rac1 are required for protrusion formation. Furthermore, our results suggest that actin reorganization at protrusions occurs downstream of apical PIP₃ generation.

We similarly examined the effect of PI3K and Rac1 inhibition on recruitment of Par3. Par3 is known to bind a number of proteins, including PI3K, the Rac1-specific guanine nucleotide exchange factor Tiam1 (Rodriguez-Boulan and Macara, 2014), and the GTPase-activating protein Bcr (Narayanan et al., 2013). Thus, Par3 could locally control the activity of PI3K and Rac1 at the protrusion by linking these molecules into a complex. Par3 recruitment to protrusions was not affected by inhibiting PI3K or Rac1 (Figure 4C), suggesting that its localization to the protrusion occurs prior to, or independently of, PI3K or Rac1 activity. shRNA-mediated depletion of Par3 decreased F-actin recruitment to protrusion by approximately 40% (Figure 4B) while only minimally affecting PIP₃ recruitment (Figure 4A). These

data suggest that Par3, possibly via Rac1, is involved in robust F-actin polymerization at protrusions, downstream of aggregate-induced apical PIP₃ generation.

Protrusion Formation Is Associated with a Spatially Localized Innate Immune Response

We tested whether host components involved in protrusion formation were required for NF- κ B activation (Figure 4E). Aggregate-induced NF- κ B activation was almost completely abolished by PI3K inhibition (LY294002), confirming that ectopic apical PIP₃ generation is a critical event in the host response. Likewise, Rac1 chemical inhibition or Par3 depletion resulted in a robust and significant reduction in NF- κ B activation similar to treatment with the I κ B kinase-2 inhibitor SC-514. We eliminated the possibility that NF- κ B is required for protrusion formation by demonstrating that SC-514 had no effect on protrusion formation, as assessed by PIP₃ or actin recruitment (Figures 4A and 4B). In summary, our results indicate an intimate association between protrusion formation and NF- κ B activation, with PI3K activity, Rac1, and Par3 required for both processes. Our data suggest that—in response to the binding of bacterial aggregates—protrusion formation may play a critical role in generating a spatially localized innate immune response at the epithelial surface.

DISCUSSION

How epithelial cells specifically recognize microbial pathogens and activate an effective immune response is a central issue in

understanding host-pathogen interactions. In this work, we describe a role for cell polarity proteins in innate immunity to *P. aeruginosa* infection. Our results allow us to construct an integrated model for the cellular signaling events involved in innate immunity to *P. aeruginosa* (Figure S4), which may be applicable to other mucosal pathogens.

The first step in the remarkable membrane remodeling of the apical surface is the recruitment of individual free-swimming bacteria to multicellular aggregates on the host cell surface, often at cell-cell junctions (Lepanto et al., 2011). Time-lapse video microscopy demonstrated that, in intact epithelium, protrusions were formed de novo in response to aggregate binding, as opposed to the aggregates binding to preexisting protrusions. This process is rapid and dynamic, potentially allowing for tightly controlled activation of downstream pathways. Par3 and PI3K are recruited to the site of aggregate binding, leading to ectopic production of PIP₃. Recruitment of Rac1, along with aPKC-Par6 α and additional AJ proteins, completes the transformation of a small patch of apical membrane to a basolateral-like protrusion rich in F-actin. We observed recruitment of AJ proteins, but not TJ proteins, suggesting that *P. aeruginosa* aggregate binding results in the de novo formation of a primordial AJ at the site of aggregate binding. Notably, these events occur early, within 30 min of infection. At later times, after translocation of the T3SS effectors or other virulence factors, cell-cell junctions and epithelial polarity can be disrupted (Kang et al., 1997; Soong et al., 2008).

Protrusion formation is associated with spatially and temporally localized translocation of NF- κ B to the nucleus, indicating activation of an innate immune response to bacterial aggregates. Neither protrusion formation nor NF- κ B activation was observed upon binding of individual bacteria, suggesting that either a minimal number of bacteria is required to incite this response or the bacterial aggregates have distinct functional characteristics. We favor the latter possibility because we have not found a correlation between the number of bound bacteria contained within the aggregate, the size of the protrusion, or the activation of NF- κ B (C.S.T. and J.N.E., unpublished data).

T3SS needle and translocon mutants induced protrusions, but not NF- κ B activation, suggesting that protrusion formation is necessary, but not sufficient, for activation of the innate immune response. We postulate that protrusion formation is part of a crucial first step in a signaling cascade within the epithelial cell that ultimately results in NF- κ B activation. However, additional host components may be necessary for activation of the localized innate immune response.

This process may be triggered by T3SS pore formation and/or flagella. Our work complements and extends previous studies that showed that flagellar-mediated binding of *P. aeruginosa* to the basolateral surface of polarized cystic fibrosis transmembrane regulator (CFTR)-deficient airway cells resulted in robust activation of NF- κ B at 4 hr postinfection (Hybiske et al., 2004). Both cytosolic flagellin and the rod component of the T3SS apparatus are sensed by NLR4, although their detection involves distinct accessory proteins (Kofoid and Vance, 2011). Flagella can also activate NF- κ B through TLR5 (Gewirtz et al., 2001). How detection of the T3SS or flagella is linked to Par3 and PI3K is still unknown but may represent a sensing mechanism in polarized epithelial cells. It will be of interest to determine

whether TLR5 is recruited or is required for protrusion formation and NF- κ B activation.

Our results suggest that changes in host cell polarity may function as a danger signal that alerts the host to the presence of a pathogen. In addition to conserved microbial products such as lipopolysaccharide (LPS) or peptidoglycan, host cell components, including glycans and actin, as well as pathogen-induced alteration of small GTPase signaling pathways have been shown to function as danger signals that are sensed by immune cells (Ahrens et al., 2012; Cerliani et al., 2011; Keestra et al., 2013; Varki, 2011). Thus, perturbations in cell polarity may be sensed by epithelial cells as an early warning system for detecting the presence of mucosal pathogens that target junctional complexes.

This work underscores an emerging theme that host cell polarity determinants are important targets by which specific pathogens subvert epithelial or endothelial barriers, though the consequences can be vastly different. The endothelial pathogen *Neisseria meningitidis* has been shown to recruit the Par3/Par6/aPKC complex away from TJs in endothelial cells, weakening the intercellular barrier and providing an explanation for disruption of the blood brain barrier (Coureuil et al., 2010; Coureuil et al., 2009). Rhesus papillomavirus type 1 (RPV1) targets Par3 for degradation during infection, which facilitates barrier breakdown (Tomaić et al., 2009). In contrast, initial binding of *P. aeruginosa* aggregates is accompanied by augmentation of the barrier, as evidenced by enhanced transepithelial resistance (Kierbel et al., 2007).

Given our data showing that Par3 is involved in the innate immune response, we speculate that RPV1 and *N. meningitidis* may target the Par complex not only to bypass the physical barrier of the epithelium, but also to block activation of the innate immune system. The ability to subvert Par3 function could represent an intermediate step in the evolution of a pathogen; as an opportunistic pathogen, *P. aeruginosa* activates the innate immune response but lacks the virulence capacity to initiate destruction of the epithelial barrier at early time points. In contrast, more potent pathogens may have the ability to subvert or destroy Par3 to circumvent the epithelial and endothelial barrier. These studies illustrate the sophisticated interplay between pathogen-directed damage and the host response and highlight the Par complex as a key target of both pathogen and host defense systems.

EXPERIMENTAL PROCEDURES

Bacterial strains, reagents, antibodies, cell lines, RNAi, microscopy, and statistical analysis are described in the [Supplemental Experimental Procedures](#).

Analysis of Protein Recruitment to Protrusions

Images were analyzed with NIS-Elements software (Nikon Instruments) to assess recruitment of the protein of interest to bacterial binding sites. MDCK cells that were bound to aggregates of ≥ 10 bacteria were included for analysis. Internalized bacterial aggregates and aggregates located near extruded MDCK cells were excluded from analysis. After ensuring that the protein of interest showed appropriate localization in the surrounding epithelial cells, the 3D space within and adjacent to the bacterial aggregate was examined for protein recruitment. Recruitment was scored as positive if the protein of interest was found on or above the apical surface of the cell and if the fluorescent intensity was equal to the intensity at basolateral or junctional locations. In pilot studies to ensure minimal interobserver variability, over 90 images were

independently analyzed by two observers, with less than 10% variation in scoring. Results are reported for $n \geq 2$ and at least 14 bacterial aggregate binding events for each protein of interest or condition. For experiments with chemical inhibitor or shRNA treatments, appropriate negative controls (no treatment or scrambled shRNA) were used for statistical comparisons. Results were normalized to control experiments.

Quantitative Analysis of NF- κ B Localization

Confocal microscopy images were analyzed with NIS-Elements software (Nikon Instruments) to quantify NF- κ B p65 nuclear and cytoplasmic fluorescence intensity. A confocal z slice with the strongest DAPI signal was chosen for analysis from each confocal image stack. For each cell in contact with an aggregate of ≥ 10 bacteria, regions of interest (ROI) were defined for the nucleus and the cytoplasm, and sum fluorescence intensity of NF- κ B p65 was quantified in each ROI. To establish an appropriate negative control value, we determined the nuclear and cytoplasmic sum fluorescence intensity of NF- κ B p65 in 600 uninfected MDCK cells from three independent experiments (Figure S1). All cells showed a ratio of nuclear/cytoplasmic p65 fluorescence intensity of <1 , with an average ratio of 0.47 ± 0.08 SD; we therefore defined NF- κ B nuclear activation as a nuclear/cytoplasmic p65 fluorescence ratio of ≥ 1 . Results are reported for $n \geq 2$ and at least 20 bacterial aggregate binding events for each bacterial strain or treatment condition.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Statistical Analysis, four figures, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2014.04.007>.

AUTHOR CONTRIBUTIONS

C.S.T. and Y.E. contributed equally to the design of the experiments, performance of the experiments, analysis of the data, and writing of this manuscript.

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