Role of host cell polarity and leading edge properties in *Pseudomonas* type III secretion

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Type III secretion (T3S) functions in establishing infections in a large number of Gram-negative bacteria, yet little is known about how host cell properties might function in this process. We used the opportunistic pathogen *Pseudomonas aeruginosa* and the ability to alter host cell sensitivity to *Pseudomonas* T3S to explore this problem. HT-29 epithelial cells were used to study cellular changes associated with loss of T3S sensitivity, which could be induced by treatment with methyl-beta-cyclodextrin or perfringolysin O. HL-60 promyelocytic cells are innately resistant to *Pseudomonas* T3S and were used to study cellular changes occurring in response to induction of T3S sensitivity, which occurred following treatment with phorbol esters. Using both cell models, a positive correlation was observed between eukaryotic cell adherence to tissue culture wells and T3S sensitivity. In examining the type of adhesion process linked to T3S sensitivity in HT-29 cells, a hierarchical order of protein involvement was identified that paralleled the architecture of leading edge (LE) focal complexes. Conversely, in HL-60 cells, induction of T3S sensitivity coincided with the onset of LE properties and the development of actin-rich projections associated with polarized cell migration. When LE architecture was examined by immunofluorescent staining for actin, Rac1, IQ-motif-containing GTPase-activating protein 1 (IQGAP1) and phosphatidylinositol 3 kinase (PI3 kinase), intact LE structure was found to closely correlate with host cell sensitivity to *P. aeruginosa* T3S. Our model for host cell involvement in *Pseudomonas* T3S proposes that cortical actin polymerization at the LE alters membrane properties to favour T3S translocon function and the establishment of infections, which is consistent with *Pseudomonas* infections targeting wounded epithelial barriers undergoing cell migration.

Received 31 July 2009

Revised 3 November 2009

Accepted 6 November 2009

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Abbreviations: CTB, cholera toxin B subunit; dsPFO, prepore locked PFO; HA, haemagglutinin; IF, immunofluorescent/immunofluorescence; IQGAP1, IQ-motif-containing GTPase-activating protein 1; LatB, latrunculin B; LE, leading edge; MβCD, methyl-beta-cyclodextrin; MT, microtubule; *Pa*-ExoS-HA, *P. aeruginosa* strain PA103ΔUT expressing plasmid-encoded ExoS-HA; *Pa*-T3S, *P. aeruginosa* type III secretion; PFO, perfringolysin O; ROCK, Rho kinase; siRNA, small interfering RNA; ssPFO, monomer locked PFO; T3S, type III secretion; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Five supplementary figures and a supplementary table are available with the online version of this paper. The supplementary figures show the relationship between eukaryotic cell adherence mechanisms and *Pa*-T3S sensitivity, the role of focal adhesions in host cell *Pa*-T3S sensitivity, the role of LE IQGAP1 in host cell *Pa*-T3S sensitivity, the relationship between HL-60 cell LE architecture and *Pa*-T3S sensitivity, and the relationship between HT-29 cell LE architecture and *Pa*-T3S sensitivity. The supplementary table lists effects of *Pa*-T3S drug treatments.

INTRODUCTION

Originally identified because of its role in *Yersinia* virulence (Cornelis *et al.*, 1989), type III secretion (T3S) is now recognized to contribute to the pathogenesis of a large number of Gram-negative bacteria. T3S allows the direct translocation of ‘effectors’ from the bacterial cytosol into eukaryotic cells, enabling bacteria to manipulate host cells to establish infections while evading immune responses. The T3S system includes a bacterially formed ‘injectisome’ needle-like nanostructure that serves as a conduit for transferring bacterial effectors to eukaryotic cells. A bacterially formed ‘translocon’ channel is then believed to mediate effector translocation across host cell membranes. The mechanism underlying T3S translocon channel formation and host involvement in this process remain the least understood events in T3S. We have used the

opportunistic pathogen *Pseudomonas aeruginosa* and insights into T3S provided by it to study this problem.

P. aeruginosa is a ubiquitous environmental bacterium that is capable of causing infections of high morbidity and mortality in individuals compromised by wounds, immune defects or cystic fibrosis. While the cellular alteration predisposing individuals to *P. aeruginosa* infections is not known, factors that have been implicated include alterations in expression of putative *P. aeruginosa* receptors, such as asialo-GM1, alterations in the cystic fibrosis transmembrane conductance regulator, and cellular changes affecting epithelial cell polarity (de Bentzmann *et al.*, 1996; Fleiszig *et al.*, 1997; Kazmierczak *et al.*, 2004; Plotkowski *et al.*, 1999). Studies have also drawn attention to the role of T3S during initial phases of *P. aeruginosa* infections (Dacheux *et al.*, 2001; Goodman *et al.*, 2004; Jain *et al.*, 2004). Consistent with this notion, cellular properties influencing *P. aeruginosa* infections, such as apical-basolateral polarity (Fleiszig *et al.*, 1997; Kazmierczak *et al.*, 2004; Plotkowski *et al.*, 1999), also influence sensitivity to *P. aeruginosa* T3S (*Pa*-T3S) (McGuffie *et al.*, 1999). The integral role of T3S in the establishment of *P. aeruginosa* infections is evident in the ability of the T3S translocon protein, PcrV, to induce adaptive immune protection against *P. aeruginosa* infections (Sawa *et al.*, 1999). *P. aeruginosa* offers an advantage in studying mechanisms underlying T3S translocon function and host cell involvement in this process for, as an opportunistic pathogen, eukaryotic cells sensitive or resistant to *Pa*-T3S have been identified and are available for mechanistic comparisons (McGuffie *et al.*, 1999).

Current studies support the suggestion that in *Pa*-T3S translocon formation, the needle tip protein PcrV oligomerizes into a ring-like structure in association with the T3S needle complex and serves as an assembly platform for membrane insertion and pore formation by hydrophobic translocator proteins, PopB and PopD (Gebus *et al.*, 2008; Goure *et al.*, 2004; Mueller *et al.*, 2005). Consistent with this model, PcrV is required for T3S pore formation in red blood cells and for translocation of effectors into nucleated cells (Goure *et al.*, 2005). Also consistent with this model, PopB and PopD maintain intrinsic pore-forming properties, and PopB and PopD, but not PcrV, can penetrate into and be detected in eukaryotic cell membrane fractions (Faudry *et al.*, 2006; Schoehn *et al.*, 2003). A precise understanding of the mechanism of T3S translocon formation and function, however, has been hampered by an inability to detect the direct interaction of PcrV with either PopD or PopB (Allmond *et al.*, 2003; Goure *et al.*, 2004; Schoehn *et al.*, 2003). A role of host cell membrane properties in *Pa*-T3S translocon function has been revealed by studies that have found PcrV to be required for T3S pore formation in eukaryotic cells, whereas PcrV is not required for pore formation in lipid vesicles (Goure *et al.*, 2004; Mueller *et al.*, 2005; Schoehn *et al.*, 2003).

The limited understanding of host cell involvement in T3S translocon function attests to experimental difficulties in

identifying bacterial-host cell interactions during T3S. Current studies have implicated host cell lipid rafts and their cholesterol component in T3S translocon formation, but the mechanism underlying their role in T3S is unclear (Hayward *et al.*, 2005; Lafont *et al.*, 2004; Lafont & van der Goot, 2005; Riff *et al.*, 2005; van der Goot *et al.*, 2004). In our analyses of over 40 cell lines (including epithelial, fibroblasts, endothelial, macrophage and T-cells), two cell types have been identified to be resistant to T3S: confluent polarized epithelial cells and promyelocytic HL-60 cells (McGuffie *et al.*, 1999; Rucks & Olson, 2005). We have also found host cell sensitivity to *Pa*-T3S to be an alterable property. Polarized epithelial cells can become T3S-sensitive by disruption of tight junctions and loss of apical-basolateral polarity (McGuffie *et al.*, 1999). HL-60 cells can be converted to *Pa*-T3S-sensitive by treatment with the differentiating phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Rucks & Olson, 2005). In studies of HL-60 cells, resistance to *Pa*-T3S has been found to occur at the level of membrane translocation.

The questions being asked in this study are: (1) how do eukaryotic cells change in conjunction with alterations in *Pa*-T3S sensitivity, and (2) how can this be applied to understanding the role of host cell properties in T3S translocon function? Two basic approaches were used to answer these questions. First, *Pa*-T3S-sensitive HT-29 epithelial cells were used as a model to examine alterations in cellular properties occurring in conjunction with loss of host cell sensitivity to *Pa*-T3S. Second, *Pa*-T3S-resistant HL-60 cells were used to examine alterations in cellular properties that occur upon induction of *Pa*-T3S sensitivity. Both approaches led to the recognition of a relationship between host cell polarity, directional migration and leading edge (LE) properties with *Pa*-T3S sensitivity. These findings have led us to hypothesize that as an opportunistic pathogen *P. aeruginosa* relies on alterations in membrane properties at the LE for *Pa*-T3S translocon insertion and function.

METHODS

Bacterial strains. *P. aeruginosa* strain PA103Δ*exoU*Δ*exoT*::Tc (PA103ΔUT) (Vallis *et al.*, 1999) and PA103ΔPopB, a T3S translocon mutant strain, were provided by Dara Frank (Medical College of Wisconsin, Milwaukee, WI, USA). Haemagglutinin (HA)-tagged ExoS (ExoS-HA) (Pederson *et al.*, 2002) was provided by Joseph Barbieri (Medical College of Wisconsin). PA103ΔUT expressing plasmid-encoded ExoS-HA (*Pa*-ExoS-HA) was the primary strain used in this study to assay for host cell sensitivity to *Pa*-T3S. PA103ΔUT expressing a plasmid-encoded enzymically inactive ExoS-GAP/ADPRT mutant (having R146A-GTPase activating protein and E379A/E381A-ADP-ribosyltransferase mutations) was used as a control to evaluate the role of ExoS enzymic function in our analyses and was constructed as previously described (Fraylick *et al.*, 2001).

Eukaryotic cell culture. HT-29, T24 and HL-60 cells were obtained from the American type Cell Culture Collection (ATCC, Manassas, VA) and were cultured according to ATCC specifications in media supplemented with 10% fetal bovine serum, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Hyclone).

Bacterial–eukaryotic cell co-culture studies and drug treatments. In co-culture studies of T3S-sensitive cells, HT-29 or T24 cells were seeded at 2.5×10^5 or 1.0×10^5 cells ml^{-1} , respectively, unless otherwise specified, and grown to 70–90% confluency. Both HT-29 and T24 cells were examined using the drug treatments listed in Supplementary Table S1 to confirm that drug effects were not cell line-specific. Prior to drug treatment, cells were washed twice with Dulbecco's PBS (DPBS; HyClone), and drugs were added in cell line-specific medium containing 0.6% BSA (Sigma–Aldrich). Cells were treated with drugs as specified and then co-cultured with 10^7 c.f.u. ml^{-1} of the indicated *P. aeruginosa* strain or no bacteria for 4–5 h, as previously described (Olson *et al.*, 1997). In studies using perfringolysin-O (PFO, C459A; Shepard *et al.*, 1998), PFO was activated with 5 mM DTT. Monomer locked PFO (ssPFO, T319C/V334C; Ramachandran *et al.*, 2004) and prepore locked PFO (dsPFO, S190C/G57C; Hotze *et al.*, 2001) were dialysed against HEPES-buffered saline prior to addition to cells. All PFO constructs were provided by Rodney Tweten (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA).

In co-culture studies of T3S-resistant cells, HL-60 cells were seeded at a density of 1×10^5 cells ml^{-1} 12 h prior to TPA differentiation or co-culture with *P. aeruginosa*. Terminal differentiation was induced with 20 nM TPA (Calbiochem) (Collins *et al.*, 1978; Leglise *et al.*, 1988) for the indicated times. Prior to co-culture, non-adherent HL-60 cells were collected, centrifuged and reseeded with the corresponding adherent fractions (when present) in medium containing 0.6% BSA. Cells were co-cultured for 4–5 h with *Pa*-ExoS-HA (m.o.i. 30:1).

Analysis of eukaryotic cell cytotoxicity, adherence and *Pa*-T3S sensitivity. Following co-culture of HT-29 cells with *P. aeruginosa* strains, bacteria were removed, cells detached and extracellular proteins degraded with 0.25% trypsin/1 mM EDTA (Hyclone). Cells were washed two times in medium containing 200 µg ciprofloxacin ml^{-1} , and an aliquot of cells was examined for cell viability, adherent cells and total cell number using trypan blue staining. The percentage of adherent HT-29 cells was calculated relative to non-drug-treated control adherent cells remaining in tissue culture wells following co-culture studies. A second aliquot of cells was examined for total protein (BCA Protein Assay, Pierce). The remainder of the cells was processed for either cell extract or membrane fractionation analysis. For cell extract analysis, cells were resuspended and lysed in $4 \times$ Laemmli sample buffer (Laemmli, 1970). For cell membrane analysis, cells were resuspended in digitonin fractionation buffer and fractionated as previously described (Rocha *et al.*, 2005). RalA localization to the membrane fraction was used to confirm the specificity of the fractionation procedure.

Following studies of co-culture of HL-60 cells with *P. aeruginosa* strains, cells were collected and washed with medium containing ciprofloxacin, extracellular proteins were degraded with trypsin, and cell membrane fractionation was performed as described for HT-29 cells. To quantify HL-60 cell adherence to tissue culture wells relative to *Pa*-T3S sensitivity, HL-60 cells were seeded in duplicate, treated with TPA for increasing times, and then co-cultured with *Pa*-ExoS-HA. One sample was assayed for T3S effector translocation, as described below. The other sample was assayed for the percentage of adherent cells by enumerating cells adherent and non-adherent to tissue culture wells in each sample using trypan blue staining; the percentage of adherent cells was calculated relative to total cells.

Eukaryotic cell sensitivity to *Pa*-T3S was assayed based on the translocation of ExoS-HA, ExoS ADP ribosylation of RalA, or membrane insertion of translocon proteins, PopB and PopD, as determined by SDS-PAGE and immunoblot analysis (Fraylich *et al.*, 2001, 2002; Rucks & Olson, 2005). Comparisons of host cell T3S sensitivity were based on equal protein loading, and detection

antibodies included: monoclonal anti-HA to detect ExoS-HA (Covance Research Products); monoclonal anti-RalA to detect ExoS effector function (BD-Transduction Laboratories); and polyclonal anti-PopB and anti-PopD to detect translocon membrane insertion (provided by Joseph Barbieri and Dara Frank). Monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Chemicon International) was used as a protein expression/loading control. Immunoblots were developed using horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Sigma–Aldrich) and detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences). Percentage RalA ADP ribosylation was quantified relative to total RalA based on densitometry analyses using ImageJ 1.40 g software (<http://rsbweb.nih.gov/ij/>), averaging a minimum of three independent experiments.

Analysis of effects of drug treatments on bacteria. Effects of drug treatments on *P. aeruginosa* growth were determined following co-culture experiments by diluting and plating co-culture supernatants on Luria broth agar plates, using a micro-dilution plating technique (Peltz *et al.*, 2001). *P. aeruginosa* adherence to HT-29 cells was determined following a 3 h co-culture period by scraping cells into DPBS containing 0.6% BSA (DPBS–BSA), washing three times with DPBS–BSA, lysing cells on ice in 0.25% Triton X-100, then diluting and plating the lysate. The number of bacteria per eukaryotic cell was calculated. The efficiency of T3S effector production and secretion in the presence of drugs was determined by harvesting co-culture supernatants and detecting ExoS-HA and PopB by SDS-PAGE and immunoblot analysis.

Immunofluorescence (IF) analyses. HT-29 cells were seeded at 1.5×10^5 cells ml^{-1} in chamber slides (Nalge Nunc International) 48 h prior to drug treatment and co-cultured with *Pa*-ExoS-HA or enzymically inactive ExoS for 3 h. This shorter co-culture period allowed for bacterial–host cell interaction with limited bacterial effects on eukaryotic cell morphology. Cells were then fixed with 3% paraformaldehyde in actin stabilization buffer (ASB; Goeckeler & Wysolmerski, 1995), permeabilized with 0.2% Triton X-100 in ASB, blocked and stained using monoclonal antibodies to: IQ-motif-containing GTPase-activating protein 1 (IQGAP1) (Invitrogen), α -tubulin (Invitrogen), Rac1 (BD Transduction Laboratories) or phosphatidylinositol 3-kinase (PI3K) (BD Transduction Laboratories) coupled with anti-mouse Alexa Fluor 488 conjugate (Invitrogen). Actin was stained using phalloidin conjugated to tetramethylrhodamine B isothiocyanate (Phalloidin–TRITC, Sigma–Aldrich). Lipid rafts were stained with Alexa Fluor 488-labelled cholera toxin B subunit (CTB; Invitrogen). *P. aeruginosa* was stained with a polyclonal guinea pig antibody against *P. aeruginosa* (Biogenesis) or a polyclonal rabbit antibody against *P. aeruginosa* LPS (provided by Joseph Lam, University of Guelph, Canada), followed by an appropriate Alexa Fluor conjugate (Invitrogen). Cells were mounted using Fluoromount-G (Southern Biotechnology Associates) and visualized using a Plan-Neofluar $\times 100/1.3$ oil objective on a Zeiss LSM 510 confocal microscope, or a Plan-Apochromat $\times 63/1.40$ oil objective on a Zeiss Imager.Z1 LSM 510 confocal microscope. Images were captured and exported as 8-bit TIF files into Adobe Photoshop CS2 (Adobe Systems) and cropped.

For IF staining of TPA-differentiated HL-60 (dHL-60) cells, cells were seeded on glass coverslips, differentiated with 20 nM TPA for the indicated time, and then co-cultured for 3 h with *Pa*-ExoS-HA. Samples were prepared for IF analysis and stained for the indicated proteins, as described above. For IF staining of undifferentiated, non-adherent T3S-resistant HL-60 (rHL-60) cells or T3S-sensitive (sHL-60) cells, cells were seeded in tubes, collected by centrifugation and prepared for IF analysis as above, with the exception that cells were centrifuged between washes and fixed to glass slides using a cytospin.

IQGAP1 small interfering RNA (siRNA) knockdown. ON-TARGETplus SMARTpool RNA oligomers against human IQGAP1 (Dharmacon) were transfected into HT-29 cells using TransIT-TKO Transfection Reagent (Mirus Bio), according to the manufacturer's specifications. siRNA sequences targeting multiple IQGAP1 mRNA sequences included: (1) GAACGUGGCUUAUGAGUAC, (2) GCAGGUGGAUUACUUAUAAA, (3) CGAACCAUCUUACUGAAUA and (4) CAUUGAGCAGUUCAGUUA. siCONTROL Non-Targeting siRNA #1 (Dharmacon) was used as a control. Cells were co-cultured with *Pa*-ExoS-HA for 48 or 72 h post-transfection. IQGAP1 expression was examined by Western blot analysis and quantified by densitometry using ImageJ, and percentage IQGAP1 expression was determined relative to quantified levels of IQGAP1 in non-drug-treated cells that were co-cultured with *Pa*-ExoS-HA.

Focal complex, focal adhesion and LE functional analyses. To assess the role of focal complexes and focal adhesions in *Pa*-T3S sensitivity of adherent HT-29 cells, cells were seeded 24–48 h prior to treatment with the following inhibitors: (1) Rac1 inhibitor, NSC23766 (EMD Chemicals), added to cells 16 h prior to co-culture; (2) Rho-kinase (ROCK) inhibitor, Y-27632 (Alexis Biochemicals), added to cells 1 h prior to co-culture; (3) Src kinase inhibitor, PP2 (Calbiochem), added to cells 1 h prior to co-culture; or (4) Cdc42 inhibitor, secramine A ($C_{35}H_{39}BrN_2O_5S$; provided by Thomas Kirchhausen, Harvard Medical School, MA, USA and Gerald B. Hammond, University of Louisville, KY, USA), added to cells in medium containing 0.5% BSA and 1% DMSO 1 h prior to co-culture. In examining the effects of inhibitors on non-adherent HT-29 cells, cells were seeded at 5.0×10^5 cells ml^{-1} , followed by the immediate addition of drugs as described above. To assess the role of LE associated actin, microtubules (MTs) and PI3K in *Pa*-T3S, HT-29 cells were seeded as described above for pre- and post-adherence drug treatment studies and treated with the following inhibitors for 1 h: latrunculin B (LatB; BIOMOL International), nocodazole (Sigma-Aldrich), AS605240 or LY294002 (Cayman Chemicals). To disrupt existing MTs, cells were placed at 4 °C for 3 h prior to drug treatment. Cells were co-cultured with *Pa*-ExoS-HA in the presence of the drug, and drug toxicity and *Pa*-T3S sensitivity were examined as previously described.

Statistical analysis. Statistical significance was calculated based on a Student's *t* test, using JMP, Version 7 software (SAS Institute). The relationship between eukaryotic cell adherence to tissue culture wells and *Pa*-T3S effector translocation was computed using a Pearson's correlation coefficient based on comparisons of percentage adherence with percentage RalA modification for individual samples under the indicated experimental conditions.

RESULTS

Inhibition of host cell *Pa*-T3S sensitivity

The finding that most cultured cells are sensitive to *Pa*-T3S underscores the conserved nature of host cell properties conferring T3S sensitivity. To help characterize these properties, cell lines were treated with agents that alter membrane properties, cytoskeletal structure or cell signalling pathways, and their effects on host cell *Pa*-T3S sensitivity were determined. *Pa*-T3S sensitivity was monitored based on effector internalization following co-culture with *P. aeruginosa* strains expressing the T3S effector ExoS. Of the many agents tested (summarized in Supplementary Table S1), two membrane-modulating

agents, M β CD and PFO, were identified to inhibit host cell *Pa*-T3S sensitivity. Similar results were obtained with HT-29, T24 and other tested *Pa*-T3S-sensitive cell lines. The finding that agents modulating membrane structure, but not those altering cytoskeletal structure or common cell signalling pathways, affected T3S sensitivity provided an initial indication that host cell sensitivity to *Pa*-T3S was linked to membrane properties.

With the identification of M β CD and PFO as *Pa*-T3S inhibitory agents, these agents were then used as tools to characterize alterations in host cell properties associated with loss of *Pa*-T3S sensitivity. M β CD depletes membrane cholesterol, and in dose-response studies, treatment of HT-29 epithelial cells with 2–3 mM M β CD, prior to co-culture with *Pa*-ExoS-HA, caused a decrease in T3S effector ExoS translocation (Fig. 1a, indicated by asterisks). The decrease in ExoS translocation corresponded with a decreased shift in the apparent molecular mass of RalA, reflecting the decreased internalization and functional ADP ribosylation of host cell RalA by ExoS. As shown in Fig. 1(a), RalA exhibits variable shifts in molecular mass upon ADP ribosylation by ExoS. This relates to RalA having primary (Arg52) and secondary (Arg135 and Arg161) sites of ExoS ADP ribosylation, which results in one or two shifts in RalA as assayed by SDS-PAGE and Western blot analysis (Fraylick *et al.*, 2002; Rucks *et al.*, 2003). Quantification of the shift in molecular mass of RalA (determined by densitometry) can in turn be used as a readout of the efficiency of ExoS effector translocation (Fraylick *et al.*, 2002; Rucks *et al.*, 2003). M β CD also caused a dose-dependent decrease in the translocation of enzymically inactive ExoS (a GAP/ADPRT mutant), but inhibition required a slightly higher, 4–5 mM, concentration of M β CD (data not shown). This finding suggests that the mechanism underlying the inhibitory effects of M β CD on *Pa*-T3S is somehow augmented by ExoS.

Inhibition of *Pa*-T3S by M β CD occurred in the absence of major effects on HT-29 cell viability, as determined by trypan blue exclusion assays. However, a decrease in HT-29 cell adherence to tissue culture wells (a 29–34% loss of adherence) occurred in conjunction with a decrease in T3S sensitivity (initial decrease in adherence indicated by an asterisk in Fig. 1a). Membrane fractionation studies confirmed that inhibition of *Pa*-T3S by M β CD occurred at the level of *Pa*-T3S translocon insertion and effector translocation, as apparent in the decrease in PopB and ExoS in membrane fractions of M β CD-treated cells (Fig. 1b). Effects of M β CD were also targeted to host cells, as evidenced by the lack of effect of M β CD on T3S effector/translocator production (Fig. 1c), or on *P. aeruginosa* growth or adherence to host cells (Fig. 1d), at 3 mM inhibitory concentrations. Inhibitory effects of M β CD on *Pa*-T3S were consistent with findings in other Gram-negative bacteria that support the role of cholesterol-rich membrane rafts in T3S (Hayward *et al.*, 2005; Riff *et al.*, 2005; van der Goot *et al.*, 2004). CTB binds GM1 gangliosides that partition to lipid rafts, and when coupled

Fig. 1. Effect of MβCD treatment on *Pa*-T3S sensitivity. HT-29 cells were treated with the indicated concentration of MβCD for 30 min, prior to and during co-culture with strain *Pa*-ExoS-HA. (a) To assay for *Pa*-T3S sensitivity, HT-29 cell extracts were immunoblotted for translocation of T3S effector, ExoS and for internalized effector function based on ExoS ADP ribosylation of RalA (as recognized by a shift in molecular mass of RalA). GAPDH served as a protein expression/loading control. Percentages of viable and adherent cells were enumerated using trypan blue staining. Asterisks mark initial decreases in *Pa*-T3S sensitivity and loss of HT-29 cell adherence. (b) Effects of MβCD on *Pa*-T3S translocon insertion were assessed by immunoblotting HT-29 cell membrane fractions for PopB and ExoS. Inhibition of *Pa*-T3S coincided with interference of PopB translocon and ExoS membrane insertion. PA103 ΔPopB served as a translocon mutant control in these studies. sn, T3S-induced *P. aeruginosa* culture supernatant used as a molecular mass marker for ExoS and PopB. (c) Effects of MβCD on *P. aeruginosa* induction and production of T3S effectors were determined by harvesting and assaying co-culture supernatants for PopB and ExoS by immunoblot analysis. (d) Effects of MβCD on *P. aeruginosa* (*Pa*) growth were determined by quantifying *P. aeruginosa* c.f.u. in co-culture supernatants at the end of the culture period. Results are expressed as the ratio (mean ± SD) of c.f.u. of MβCD-treated versus non-drug-treated cells, co-cultured with *Pa*-ExoS-HA. Effects of MβCD on *P. aeruginosa* adherence to HT-29 cells were determined following the co-culture period by harvesting and plating HT-29 cell lysates to quantify c.f.u. Results are expressed as number (mean ± SEM × 10⁻³) of *P. aeruginosa* adhering per HT-29 cell. (e) The relationship between the effects of MβCD on *Pa*-T3S sensitivity and host cell lipid raft structure was determined following co-culture with *Pa*-ExoS-HA and using Alexa Fluor 488-labelled CTB to stain GM1 gangliosides that partition to lipid rafts. Results of all studies are representative of three to six independent experiments. Bars, 10 μm.

to Alexa Fluor 488 can be used to monitor alterations in lipid rafts relative to increasing concentrations of MβCD (Wolf *et al.*, 2002). Previous studies using CTB to track lipid rafts have found increasing concentrations of MβCD to disrupt lipid raft organization (Larbi *et al.*, 2004). When CTB was used to monitor lipid rafts in HT-29 cells relative to increasing concentrations of MβCD and inhibition of

T3S, CTB staining changed from a diffuse punctate pattern to an aggregated pattern at 0.5 mM MβCD, a concentration of MβCD lower than that required to inhibit T3S. These results indicated that lipid raft reorganization preceded inhibition of *Pa*-T3S. Our conclusions from studies using MβCD to identify host cell properties involved in *Pa*-T3S sensitivity were that: (1) membrane

cholesterol influences T3S translocon insertion, and (2) membrane alterations more severe than lipid raft reorganization were required to inhibit T3S.

PFO affects membrane properties differently from M β CD, causing oligomerization of membrane cholesterol in conjunction with pore formation (Tweten, 2005). PFO

was found to inhibit HT-29 cell sensitivity to *Pa*-T3S in a dose-dependent range of 0.04–0.2 $\mu\text{g ml}^{-1}$ (Fig. 2a) and was used to further examine host cell properties linked to *Pa*-T3S sensitivity. As observed with M β CD, inhibition of *Pa*-T3S by PFO occurred in the absence of major effects on HT-29 cell viability but in conjunction with loss of HT-29 cell adherence to tissue culture wells (a 26–52 % loss of

Fig. 2. Effect of PFO treatment on *Pa*-T3S sensitivity. HT-29 cells were treated with the indicated concentration of PFO 1 h prior to and during co-culture with strain *Pa*-ExoS-HA. Effects of PFO on: (a) inhibition of *Pa*-T3S, (b) T3S translocon insertion, (c) *P. aeruginosa* induction and production of T3S effectors, (d) *P. aeruginosa* (*Pa*) growth and *P. aeruginosa* adherence to HT-29 cells, and (e) lipid raft structure were determined as described in Fig. 1. In membrane fractionation studies, an increase in PopB and ExoS membrane association occurred relative to increasing concentrations of PFO, indicating that PFO inhibited ExoS translocation and translocon function after membrane insertion. (f) To examine the stage in PFO function inhibiting *Pa*-T3S, HT-29 cells were treated with wild-type PFO, ssPFO or dsPFO for 1 h prior to and during co-culture with strain *Pa*-ExoS-HA. *Pa*-T3S was assayed based on ExoS ADP ribosylation of RalA, and asterisks indicate initial decreases in RalA ADP ribosylation and loss of HT-29 cell adherence to tissue culture wells. Results of all studies are representative of three to six independent experiments. Bars, 10 μm .

adherence), which was greater than that observed for M β CD (Fig. 2a). PFO also caused a dose-dependent decrease in the translocation of enzymically inactive ExoS, but inhibition required a slightly higher, 0.3–0.4 $\mu\text{g ml}^{-1}$, concentration of PFO (results not shown). The increased toxic effects of PFO on HT-29 cell adherence limited the co-culture time, which was reflected in decreased detection of ExoS in cell extracts. In membrane fractionation studies, increasing concentrations of PFO caused translocator protein PopB and effector ExoS to accumulate in membranes rather than to decrease, as was observed with M β CD (Fig. 2b). PFO was also confirmed to target host cell properties based on its lack of effect on T3S effector/translocator production (Fig. 2c), or on *P. aeruginosa* growth or adherence to host cells (Fig. 2d). Comparisons of the effects of M β CD and PFO on T3S thus found depletion of membrane cholesterol by M β CD to interfere with T3S translocon insertion, while oligomerization of membrane cholesterol by PFO allowed translocon insertion but not effector translocation.

Cholesterol-dependent cytolysins, such as PFO, have been reported to bind to and serve as probes for cholesterol-rich lipid rafts (Ohno-Iwashita *et al.*, 2004; Shimada *et al.*, 2002; Waheed *et al.*, 2001). When CTB was used to track how PFO altered lipid rafts in relation to inhibition of *Pa*-T3S, lipid raft reorganization occurred at subT3S-inhibitory concentrations (0.02 $\mu\text{g ml}^{-1}$) of PFO (Fig. 2e). More severe aggregation and eventual loss of lipid raft staining occurred at T3S inhibitory concentrations (0.1–0.2 $\mu\text{g ml}^{-1}$) of PFO. These findings are consistent with studies of the cholesterol-dependent cytolysin listeriolysin O, which has been found to be a potent aggregator of lipid rafts (Gekara *et al.*, 2005). The fact that alterations in lipid rafts by both PFO and M β CD preceded inhibition of T3S sensitivity provided evidence that lipid raft organization was indirectly related to host cell *Pa*-T3S sensitivity.

PFO pore formation has been dissected into distinct steps: (1) binding of PFO monomers to the eukaryotic cell receptor, cholesterol; (2) oligomerization of PFO monomers in association with membrane cholesterol to form a pre-pore complex; and (3) pore formation, associated with the extension of amphipathic transmembrane β -hairpins (Tweten, 2005). We were able to clarify the stage in PFO function inhibiting *Pa*-T3S through the use of PFO mutants locked at either the cholesterol monomer-binding step (ssPFO) (Ramachandran *et al.*, 2004) or at the cholesterol oligomerization pre-pore complex step (dsPFO) (Hotze *et al.*, 2001). Treatment of HT-29 cells with ssPFO or dsPFO, prior to exposure to *Pa*-ExoS-HA, caused no inhibition of T3S, even at concentrations 1000-fold higher than that of wild-type PFO (Fig. 2f). Collectively, our conclusions from using PFO as a T3S inhibitory tool were that: (1) PFO interrupted T3S effector translocon function but not membrane insertion, differing from the effects of M β CD; and (2) neither binding to nor oligomerization of membrane cholesterol, or the redistribution of lipid rafts by PFO, appeared to be sufficient to

interfere with *Pa*-T3S. Rather, alterations in membrane properties associated with intact PFO were required to inhibit *Pa*-T3S in HT-29 cells.

To summarize, studies of T3S-sensitive cells provided evidence that cellular properties linked to membrane structure were involved in host cell *Pa*-T3S sensitivity, and that membrane cholesterol influenced T3S translocon insertion and function. What remained unclear was the mechanism underlying the role of membrane structure in *Pa*-T3S sensitivity, although M β CD or PFO treatment showed cholesterol or lipid raft reorganization to be insufficient to interrupt host cell *Pa*-T3S sensitivity.

Induction of *Pa*-T3S sensitivity in HL-60 cells

Promyelocytic HL-60 cells are innately resistant to *Pa*-T3S (rHL-60 cells), but can be induced to become T3S-sensitive by treatment with the differentiation agent TPA (dHL-60 cells) (Rucks & Olson, 2005). It has also been observed that HL-60 cells acquire sensitivity to *Pa*-T3S in culture after multiple passages (sHL-60 cells). We therefore used HL-60 cells as an alternative model system to study host cell properties involved in *Pa*-T3S by monitoring cellular alterations in rHL-60, sHL-60 and dHL-60 cells. Although the stimulus that induces sHL-60 cell *Pa*-T3S sensitivity is unknown, TPA-induced differentiation of HL-60 cells has been well studied and causes HL-60 cells to differentiate from round, non-adherent promyelocytes to adherent macrophages that exhibit properties of polarized migrating cells (Collins *et al.*, 1978; Leglise *et al.*, 1988). TPA differentiation of HL-60 cells is known to involve activation of protein kinase C (PKC β) signalling pathways, which induce early (after 15 min), intermediate (2–3 h) and late (12–24 h) patterns of gene expression (Slosberg *et al.*, 2000; Tonetti *et al.*, 1994; Zheng *et al.*, 2002). Relating temporal patterns of TPA-induced gene expression to induction of T3S sensitivity in turn provided another means of gaining insight into host cell properties involved in *Pa*-T3S sensitivity.

When HL-60 cells were exposed to 20 nM TPA and then co-cultured with *Pa*-ExoS-HA, induction of T3S sensitivity occurred after a 12–24 h exposure to TPA, as assayed based on ExoS translocation and ADP ribosylation of RalA (Fig. 3a). In examining the relationship between induction of T3S sensitivity and T3S translocon function, ExoS membrane translocation was found to coincide first with PopB and then with PopD translocon protein membrane insertion (Fig. 3a). This finding indicated that HL-60 cell resistance to *Pa*-T3S related to membrane properties that affected T3S translocon insertion, which, similar to the situation in HT-29 cells, identified a role for membrane properties in T3S translocon insertion and function. The requirement for a 12–24 h exposure to TPA for induction of T3S sensitivity indicated that expression of late TPA response genes, which include actin binding and adherence-related proteins, were involved in cellular alterations affecting T3S sensitivity.

Fig. 3. Examining the induction of *Pa*-T3S sensitivity in HL-60 cells. (a) *Pa*-T3S-resistant rHL-60 cells were treated with 20 nM TPA for the indicated time, prior to co-culture with *Pa*-ExoS-HA. Cells were lysed and fractionated, and the membrane fraction was resolved by SDS-PAGE and immunoblotted for ExoS and RalA to assay T3S effector translocation and function, and for PopB and PopD to assess T3S translocon membrane insertion. sn, *P. aeruginosa* culture supernatant used as molecular mass markers for ExoS, PopB and PopD. (b) The morphology of rHL-60 cells, T3S-sensitive sHL-60 cells and TPA-differentiated dHL-60 cells (differentiated for 12, 18 or 24 h) was examined by IF staining for tubulin (green) and actin (red). MT radiations developed during the early stages of *Pa*-T3S sensitivity and continued to develop in dHL-60 cells in association with actin-enriched projections and induction of *Pa*-T3S sensitivity. Images are representative of common phenotypes observed under the indicated experimental conditions in two or more experiments. Bar, 10 μ m.

To further understand host cell properties involved in *Pa*-T3S sensitivity, we monitored alterations in HL-60 cell architecture relative to induction of T3S sensitivity by staining for the cytoskeletal proteins actin and tubulin. *Pa*-T3S-resistant rHL-60 cells were characterized by dense cortical actin staining and weak tubulin staining that lacked pronounced MT radiations (Fig. 3b). sHL-60 cells main-

tained a round, non-adherent morphology, indistinguishable from that of rHL-60 cells by phase-contrast microscopy. However, upon staining for actin and tubulin, sHL-60 cells exhibited pronounced MT radiations, which identifies an early stage in the induction of directional migration in HL-60 cells. MT radiations were also evident in a small population of HL-60 cells exposed to TPA for

12 h, but the frequency of this phenotype was not sufficient to detect effector translocation by immunoblot analysis. After 18 and 24 h exposure to TPA, dHL-60 cells exhibited extensive tubulin radiations and actin-enriched projections that defined a mature migratory phenotype. At this stage in TPA differentiation, dHL-60 cells were highly sensitive to *Pa*-T3S, as documented by immunoblot analyses at 24 h (Fig. 3a). To summarize, in studies of HL-60 cell architecture it became evident that MT radiations and actin-rich projections, characteristic of polarized migrating cells, occurred in conjunction with induction of T3S sensitivity.

Association of eukaryotic cell adhesion processes with *Pa*-T3S sensitivity

The complex nature of bacterial–host cell interactions during T3S has made it experimentally difficult to determine how eukaryotic cell properties contribute to T3S translocon function. The serendipitous finding in studies of *P. aeruginosa* that helped bridge this understanding was that eukaryotic cells acquire or lose adherence to tissue culture wells in conjunction with the gain or loss of *Pa*-T3S sensitivity. TPA induces HL-60 cells to differentiate from non-adherent promyelocytes to adherent macrophages (Collins *et al.*, 1978; Leglise *et al.*, 1988). In relating HL-60 cell adherence to *Pa*-T3S sensitivity, approximately 70% of HL-60 cells became adherent to tissue culture wells following a 24 h exposure to TPA. To further examine this relationship, TPA induction of HL-60 cell T3S sensitivity was directly compared with induction of HL-60 cell adherence in samples run in parallel, using ExoS translocation and ADP ribosylation of RalA to quantify the efficiency of T3S. In these studies, a positive correlation ($r=0.8146$, determined using Pearson's correlation coefficient) was detected between the percentage of adherent HL-60 cells and the efficiency of T3S effector translocation (shown in Supplementary Fig. S1a). Studies of TPA-differentiated HL-60 cells thus provided an initial indication that the relationship between eukaryotic cell adherence mechanisms and *Pa*-T3S was statistically relevant.

We also observed that HT-29 epithelial cells lost adherence to tissue culture wells in association with the inhibitory effects of M β CD and PFO on *Pa*-T3S (Figs 1a and 2a). To determine whether a reverse correlation between inhibition of *Pa*-T3S and loss of adherence was evident, we used a similar approach to examine adherence and T3S sensitivity in HT-29 cells. In these studies, a positive correlation was observed between the loss of HT-29 cell adherence to tissue culture wells and the inhibitory effects of M β CD ($r=0.8651$) and PFO ($r=0.7836$) on *Pa*-T3S sensitivity (Supplementary Fig. S1b, c). The finding that inhibition of HT-29 cell *Pa*-T3S sensitivity correlated with loss of adherence to tissue culture wells, and conversely that induction of HL-60 cell T3S sensitivity correlated with acquisition of adherence, provided evidence that eukaryotic cell adherence mechanisms and *Pa*-T3S sensitivity

might somehow be functionally linked. The importance of this finding to our studies was that it directed our attention to the use of eukaryotic cell adherence mechanisms in further analyses of host cell involvement in *Pa*-T3S.

Examining the type of adhesion mechanism involved in host cell *Pa*-T3S sensitivity

Wounded epithelial barriers, which are sensitive to *P. aeruginosa* infections and *Pa*-T3S, undergo cell migration in response to the wound healing process. Fig. 4(a) shows the organizational structure of LE proteins that drive cell movement. Rac1 mediates adherence at the LE through the formation of dynamic, focal complexes (Nobes & Hall, 1995). Focal complexes can mature into more stable Rho-mediated focal adhesions (as shown in Supplementary Fig. S2a) that form behind focal complexes and at the rear of cells, where they serve as traction points in cell migration (Ridley *et al.*, 2003). Based on evidence that host cell adherence to tissue culture wells correlated with *Pa*-T3S sensitivity, we next used inhibitors that target proteins within focal complexes or focal adhesions to examine whether these adhesion mechanisms were involved in *Pa*-T3S.

Inhibitors that were used to test the involvement of focal complexes in *Pa*-T3S included: (1) NSC23766, which inhibits Rac1 function by blocking Rac1 interaction with its GEF (Gao *et al.*, 2004); and (2) secriamine A, which interferes with Cdc42 binding to membranes in a RhoGDI-dependent manner (Pelish *et al.*, 2006; Xu *et al.*, 2006). When HT-29 cells were treated with Rac1 or Cdc42 inhibitors, prior to co-culture with *Pa*-ExoS-HA, dose-dependent decreases in effector translocation were observed (Fig. 4a, black bars), with significant decreases in T3S ($P<0.05$ – 0.025) occurring at higher drug concentrations. Treatment with both Rac1 and Cdc42 inhibitors together did not augment inhibitory effects on *Pa*-T3S (results not shown). Inhibitors used to test the role of focal adhesions in *Pa*-T3S included: (1) Y-27632, an ATP-competitive inhibitor of p160ROCK (Davies *et al.*, 2000); and (2) PP2, a Src kinase inhibitor. Unlike treatments that affect focal complexes, ROCK inhibitor at four times its inhibitory concentration, and Src kinase inhibitor at concentrations as high as 80 μ M, caused no dose-dependent or significant inhibition of *Pa*-T3S (Supplementary Fig. S2b, black bars). None of the inhibitors affected *P. aeruginosa* growth or secretion of T3S proteins.

The correlation between cell adherence and *Pa*-T3S sensitivity led us to examine whether eukaryotic cell adherence processes stabilize T3S translocon function. To test this possibility, HT-29 cells were treated with Rac1, Cdc42, ROCK or Src kinase inhibitors, as described above, but inhibitors were added at the time of HT-29 cell seeding, prior to establishment of adherence. When treated with inhibitors before adherence, HT-29 cells remained non- or loosely adherent during co-culture with *Pa*-ExoS-

Fig. 4. Examining adherence mechanisms and LE properties involved in host cell *Pa*-T3S sensitivity. (a) Diagram of major eukaryotic cell proteins mediating LE focal complex adherence. Proteins shown in solid lined boxes were found to mediate *Pa*-T3S sensitivity based on inhibition studies. To examine the role of focal complexes in *Pa*-T3S, HT-29 cells were seeded and allowed to adhere for 48 h (black bars), before treatment with Rac1 inhibitor (NSC23766) or Cdc42 inhibitor (secramine A), or were treated with inhibitors in the same manner immediately following seeding prior to establishment of adherence (grey bars). Cells were then co-cultured with *Pa*-ExoS-HA in the presence of inhibitors, and *Pa*-T3S sensitivity was quantified in cell lysates based on the efficiency of ExoS ADP ribosylation of RalA. The mean $\pm$ SEM of three independent experiments is presented. Significant decreases relative to non-drug-treated controls are indicated by * ($P < 0.05$) or ** ($P < 0.025$). (b) To examine the role of LE properties in *Pa*-T3S sensitivity, HT-29 cells were treated with the indicated inhibitors, either 48 h after (black bars) or immediately following seeding (grey bars) and co-cultured with *Pa*-ExoS-HA and assayed for *Pa*-T3S sensitivity as in (a). Inhibitor treatments included: LatB, to disrupt actin; nocodazole, combined with incubation at 4 °C for 3 h, to destabilize/disrupt MTs; AS605240, to inhibit PI3K γ ; and LY294002, to inhibit all PI3K isoforms. Drug concentration relative to the efficiency of *Pa*-T3S effector translocation is shown as in (a), and the mean $\pm$ SEM of five to seven independent experiments are represented. Significant decreases in RalA ADP ribosylation relative to non-drug-treated controls are indicated by * ($P < 0.02$) or ** ($P < 0.002$).

HA. As shown in Fig. 4(a) (grey bars), both Rac1 and Cdc42 inhibitors caused a greater inhibition of T3S effector translocation when applied to non-adherent as compared with adherent HT-29 cells. In contrast, no significant or dose-dependent inhibition of T3S effector translocation

was detected when ROCK or Src kinase inhibitors were applied prior to establishment of adherence (Supplementary Fig. S2b, grey bars). Additional evidence supporting the lack of involvement of focal adhesions in *Pa*-T3S included: (1) the inability of antibodies that block

integrin signalling to interfere with *Pa*-T3S (results not shown), and (2) the finding that SYF fibroblastic cells that are deficient for all three Src kinases, Src, Fyn and Yes (Klinghoffer *et al.*, 1999), were sensitive to *Pa*-T3S (results not shown). Together these studies favoured a role of LE focal complexes, over that of focal adhesions, in host cell *Pa*-T3S sensitivity, and also provided evidence that host cell adherence processes stabilize *Pa*-T3S secretion.

Examining the relationship between host cell LE properties and *Pa*-T3S

If LE focal complexes contribute to host cell *Pa*-T3S sensitivity, other LE associated proteins, as shown in Fig. 4(a), might be predicted to influence T3S effector translocation. To allow for a stabilizing effect of cell adherence on *Pa*-T3S, HT-29 cells were treated at the time of seeding, prior to establishment of adherence, or 48 h after adherence with: (1) LatB to inhibit actin polymerization; (2) nocodazole, combined with incubation at 4 °C, to destabilize and inhibit MT formation; (3) LY294002 to inhibit all PI3K isoforms; or (4) AS605240 to inhibit the γ -isoform of PI3K involved in cell motility (Barber *et al.*, 2005; Camps *et al.*, 2005). Following drug treatment, cells were co-cultured with *Pa*-ExoS-HA, and host cell T3S sensitivity was quantified based on the efficiency of ExoS translocation and ADP ribosylation of RalA. As shown in Fig. 4(b) (black bars), and consistent with the data in Supplementary Table S1, most of these treatments caused no significant decrease in T3S effector translocation when applied to adherent HT-29 cells. The one exception was 100 μ M LatB, which caused a significant ($P<0.02$) decrease in T3S effector translocation. When applied to non-adherent HT-29 cells, both 10 and 100 μ M LatB caused a significant decrease ($P<0.002$) in T3S effector translocation (Fig. 4b, grey bars), as did 10 μ M nocodazole ($P<0.02$), implicating a role of MTs in *Pa*-T3S. The role of PI3K in *Pa*-T3S was less evident, with 200 μ M LY294002 causing a decrease in effector translocation, but this did not attain statistical significance. The finding that the roles of actin, MTs and PI3K in T3S effector translocation paralleled their organizational structure at the LE provided further validation of the role of LE properties in *Pa*-T3S. Also, the increased effectiveness of inhibitors when applied to non-adherent HT-29 cells was again supportive of LE adherence exerting a stabilizing role in *Pa*-T3S translocation.

IQGAP1 localizes to the LE, where it functions as a scaffolding protein during cell migration, binding directly to F-actin, Cdc42 and Rac1, and indirectly capturing MT plus ends during cell migration (Bashour *et al.*, 1997; Fukata *et al.*, 2002; Hart *et al.*, 1996). The role of LE IQGAP1 in cell motility has been confirmed in IQGAP1 knockdown (siRNA), which led to suppressed cell migration (Mataraza *et al.*, 2003). To further examine whether LE properties associated with cell migration contributed to host cell *Pa*-T3S sensitivity, transient IQGAP1 siRNA knockdown studies were performed in

HT-29 cells, and IQGAP1 expression was related to the efficiency of *Pa*-T3S effector translocation following co-culture with *Pa*-ExoS-HA. When IQGAP1 expression was quantified by densitometry in siRNA dose-response studies, a significant decrease in IQGAP1 was detected at 5 nM IQGAP1 siRNA ($P<0.001$), with non-targeting siRNA studies requiring 20-fold higher concentrations to decrease IQGAP1 expression (see Supplementary Fig. S3). Significant decreases ($P<0.001$) in T3S effector translocation relative to non-targeting siRNA occurred at 100 nM IQGAP1 siRNA. The fact that transient siRNA expression studies were performed on adherent HT-29 cells, yet still a significant reduction in *Pa*-T3S was detected, is supportive of IQGAP1 playing a role in *Pa*-T3S.

Collectively, the model developing for host cell involvement in *Pa*-T3S was that membrane properties linked to eukaryotic cell adhesion mechanisms played a stabilizing role in T3S effector translocation. In studies examining the type of adhesion mechanism involved, a hierarchical order of protein involvement reflecting the architecture of the LE was observed with: (1) actin, Rac1 and Cdc42 playing a more direct role in *Pa*-T3S, (2) MTs and IQGAP1 playing secondary roles, and (3) PI3K playing a more distant role in *Pa*-T3S.

Examining host cell architecture associated with *Pa*-T3S sensitivity

The LE is a functional assemblage of proteins that leads to cortical actin polymerization, which exerts a force on the plasma membrane to drive cell movement. Because of its dependence on assembly for function, the preferred method for examining LE function is through IF microscopy of LE architecture. The ability to alter *Pa*-T3S sensitivity in both HL-60 cells and HT-29 cells provided systems to monitor alterations in LE architecture relative to induction or inhibition of host cell T3S sensitivity.

HL-60 cell model

HL-60 cells allow LE architecture to be examined relative to stages in induction of *Pa*-T3S sensitivity, as defined by rHL-60, sHL-60 and dHL-60 cells. Host cell proteins that were visualized by IF staining for their cellular localization relative to alterations in *Pa*-T3S sensitivity included: (1) LE-associated Rac, IQGAP1 and PI3K, (2) focal adhesion-associated Rho, and (3) actin. Supplementary Fig. S4(a) shows representative images from T3S-resistant rHL-60 cells, which, consistent with their undifferentiated state, exhibited limited motility and LE properties, and lacked actin-enriched membrane structures, such as lamellipodia or filopodia. Rac distribution was cytosolic and did not co-localize with actin. IQGAP1 localized to the cell periphery, and as a scaffolding protein, co-localized with cortical actin (represented by yellow staining in the merged image). Localization of PI3K, which serves as a marker for

polarization, resembled that of Rac1. Rho exhibited a polarized cytosolic staining pattern and did not co-localize with actin. In comparing staining patterns of rHL-60 with sHL-60 cells, the major differences observed in sHL-60 cells were that: (1) Rac1 staining became co-localized with actin, and (2) PI3K staining became more polarized (Supplementary Fig. S4a, b, indicated by arrows). Co-localization of Rac1 with actin and the polarized localization of PI3K serve as markers for the induction of functional LE properties. The finding that Rho localization was not altered in sHL-60 cells indicated that focal adhesion properties were not being modulated in conjunction with induction of *Pa*-T3S sensitivity.

Binding of *P. aeruginosa* to LE-associated Rac, IQGAP1 and actin in T3S-sensitive dHL-60 cells is shown in Fig. 5. HL-60 cells treated with TPA for 18 h developed mature LE structures, as evident from Rac and IQGAP1 staining patterns, which co-localize with actin in association with lamellipodia and cell motility projections. *P. aeruginosa* was found to bind to actin-rich LE projections at a 44.8 % frequency and at sites of high-intensity Rac staining at a 78.5 % frequency, when compared with *P. aeruginosa* association with dHL-60 cells in general. (Frequencies of *P. aeruginosa* association with LE regions were derived from 13 co-culture experiments and observation of 289 bacterial–eukaryotic cell associations.) Our conclusions from studies of LE architecture in HL-60 cells were that: (1) induction and development of Rac1, IQGAP1 and actin LE structures coincide with induction of T3S sensitivity; and (2) LE cell properties preferentially underlie *P. aeruginosa* binding in T3S cells.

HT-29 cell model

HT-29 cells provided a means of monitoring alterations in LE architecture in relation to loss of *Pa*-T3S sensitivity

following treatments with M β CD or PFO. To relate LE architecture to previous *Pa*-T3S sensitivity studies, HT-29 cells were treated with increasing concentrations of M β CD or PFO, then co-cultured with *Pa*-ExoS-HA, and stained for Rac1 or PI3K in relation to cortical actin. Similar studies were performed using *P. aeruginosa* expressing an ExoS-GAP/ADPRT mutant to assess how ExoS activity influenced these studies.

HT-29 cells maintain properties of normal epithelial cells, including the ability to undergo collective migration, as occurs during wound healing (Huet *et al.*, 1987). Consistent with these properties, LE migration of HT-29 cells occurred at the periphery of cell clusters (Supplementary Fig. S5, 0 M β CD or PFO). In monitoring the effects of M β CD or PFO on LE properties in the presence of *Pa*-ExoS-HA, intact LE properties were evident at sub-T3S-inhibitory concentrations of M β CD (0.5 mM) or PFO (0.02 μ g ml⁻¹), as observed by the close underlying association of Rac1 or PI3K with cortical actin in the direction of cell migration (Supplementary Fig. S5, marked by arrowheads). Alterations in LE architecture became apparent in some cells at 1.0 mM M β CD or 0.1 μ g PFO ml⁻¹ by the disruption or loss of actin staining relative to Rac1 or PI3K (Supplementary Fig. S5, marked by arrows). Extensive loss of LE actin became apparent at T3S inhibitory concentrations of M β CD (3 mM) or PFO (0.2 μ g ml⁻¹). To summarize, studies using M β CD or PFO to inhibit T3S sensitivity found disruption of LE actin to closely coincide with interference with *Pa*-T3S.

Inhibitory effects of M β CD or PFO on *Pa*-T3S also coincided with alterations in LE properties when cells were co-cultured with *P. aeruginosa* expressing enzymically inactive ExoS. However, consistent with ExoS targeting LE-associated Rac1 and Cdc42 (Fraylick *et al.*, 2001; Pederson *et al.*, 1999; Rocha *et al.*, 2005), phenotypic

Fig. 5. *P. aeruginosa* binding to LE of T3S-sensitive dHL-60 cells. HL-60 cells were differentiated with TPA for 18 h, then co-cultured with *P. aeruginosa* and stained for *P. aeruginosa* (blue) relative to LE-associated Rac or IQGAP1 (both green) and actin (red). Images show LE properties underlying *P. aeruginosa* binding and are representative of staining patterns obtained from two or more independent experiments. Bar, 10 μ m.

alterations occurring in association with enzymically inactive ExoS were different. Rather than observing severe disruption or loss of LE actin upon inhibition of *Pa*-T3S, LE protein architecture underlying actin was disrupted in M β CD- or PFO-treated HT-29 cells in association with *P. aeruginosa* expression of an ExoS-GAP/ADPRT mutant (results not shown). These results are of interest because they highlight the potential for T3S effectors, such as ExoS, to target host cell properties predicted to facilitate T3S translocon function. The effect of ExoS on LE actin also provides an explanation of why the inhibitory effects of M β CD and PFO on *Pa*-T3S were augmented by ExoS.

In examining *P. aeruginosa* association with LE properties in HT-29 cells, LE architecture was found to underlie *P. aeruginosa* binding in 54% of bacterial-HT-29 cell associations under conditions of T3S sensitivity. (Frequencies of *P. aeruginosa* binding to HT-29 cells were determined based on eight independent co-culture experiments and greater than 500 *P. aeruginosa*-HT-29 cell enumerated associations.) This association is represented in Fig. 6 (T3S-sensitive panels), where IQGAP1, actin and *P. aeruginosa* staining were used to visualize *P. aeruginosa* binding to the LE of HT-29 cells. *P. aeruginosa* did not preferentially associate with the LE of HT-29 cells after inhibition of T3S by treatment with 0.2 μ g PFO ml⁻¹, but rather *P. aeruginosa* binding became more random and occurred at intercellular junctions (Fig. 6, T3S-resistant panel).

To summarize, studies examining the relationship between LE architecture and *Pa*-T3S sensitivity found that acquisition of LE architecture coincided with induction of *Pa*-T3S sensitivity in HL-60 cells. Conversely, disruption of LE architecture coincided with loss of *Pa*-T3S sensitivity in HT-29 cells. LE architecture was also found to underlie *P. aeruginosa* binding in both HL-60 and HT-29 cells under

conditions of *Pa*-T3S sensitivity. As bacterial binding to host cells is integral to T3S translocon function, the preferential binding of *P. aeruginosa* to the LE of HL-60 and HT-29 cells under conditions of T3S sensitivity is consistent with the notion that the LE region serves as the host cell site of *P. aeruginosa* binding during T3S translocon insertion and function. Together, these findings provide evidence of a close relationship or a role of eukaryotic cell LE properties in *Pa*-T3S sensitivity.

DISCUSSION

Studies support the suggestion that T3S effectors are translocated into eukaryotic cells through a bacterially produced pore or channel in host cell membranes. Once internalized, T3S effectors manipulate eukaryotic cell function in a manner that contributes to the establishment of infections. The purpose of our studies was to gain insight into the possible role of host cell properties in T3S translocon function and effector translocation. We chose *P. aeruginosa* as a model system to study this problem, because as an opportunistic pathogen, eukaryotic cell lines that are sensitive (HT-29 cells) or resistant (HL-60 cells) to *Pa*-T3S were available for mechanistic comparisons (McGuffie *et al.*, 1999; Rucks & Olson, 2005). Addressing how host cells function in *Pa*-T3S required a series of progressive studies, but eventually studies of HT-29 and HL-60 cells converged to reveal a relationship between host cell LE properties and *Pa*-T3S translocon function.

When T3S-sensitive HT-29 cells were used to study host cell properties involved in *Pa*-T3S, it proved experimentally difficult to inhibit *Pa*-T3S. Of the many agents tested, only M β CD and PFO were found to inhibit *Pa*-T3S sensitivity in the absence of effects on *P. aeruginosa*. M β CD and PFO have the common property of modulating

Fig. 6. *P. aeruginosa* binding to LE of HT-29 cells. *P. aeruginosa* binding to T3S-sensitive or T3S-resistant HT-29 cells was assessed following co-culture with *Pa*-ExoS-HA and staining for IQGAP1 (green), actin (red) and *P. aeruginosa* (blue). Left panel (lower magnification) shows the frequency of *P. aeruginosa* association with the LE of clusters of T3S-sensitive HT-29 cells. Bar, 20 μ m. Middle panel (higher magnification) shows *P. aeruginosa* association with LE lamellipodia. Right image shows the more random and intercellular association of *P. aeruginosa* with HT-29 cells treated with 0.2 μ g PFO ml⁻¹ to cause loss of T3S sensitivity. Results are representative images obtained in two or three independent experiments. Bars, 10 μ m.

membrane properties in a cholesterol-dependent manner. However, in examining the mechanism underlying their inhibition of T3S, neither modulation of membrane cholesterol or lipid rafts appeared sufficient to inhibit *Pa*-T3S sensitivity. Rather, the eukaryotic cell property found to correlate most closely with loss of *Pa*-T3S sensitivity was loss of HT-29 cell adherence to tissue culture wells.

Promyelocytic HL-60 cells are non-adherent and lack directional polarity and, along with polarized epithelial monolayers, are the only other cell line identified to be resistant to *Pa*-T3S (Rucks & Olson, 2005). HL-60 cells can be experimentally induced to become *Pa*-T3S-sensitive by treatment with the differentiating agent TPA (Rucks & Olson, 2005). TPA-differentiated HL-60 cells also serve as a model system to study neutrophil migration in response to chemoattractants (Hauert *et al.*, 2002; Servant *et al.*, 2000). In using HL-60 cells to study alterations in cellular properties that occur upon induction of *Pa*-T3S sensitivity, again a direct correlation was observed between HL-60 cell adherence to tissue culture wells and *Pa*-T3S sensitivity. It was further recognized in HL-60 cells that resistance to *Pa*-T3S was related to the lack of PopB and PopD translocon protein insertion into the cellular membrane.

Recognition of a direct correlation between eukaryotic cell adherence mechanisms and *Pa*-T3S translocon function provided an important missing link in understanding host cell involvement in *Pa*-T3S, and led us to explore the functional relationship between eukaryotic cell adhesion mechanisms and *Pa*-T3S. Focal complexes form dynamic cell adherence structures at the LE and can be distinguished from more stable focal adhesions based on Rac/Cdc42, rather than Rho, mediating their adherence mechanisms. In using Rac1 or Cdc42 inhibitors to examine the role of focal complexes in T3S, both agents were found to inhibit T3S effector translocation. This was in contrast with ROCK or Src kinase inhibitors that target focal adhesions, which caused no significant inhibition of *Pa*-T3S. Notably, Rac1 and Cdc42 inhibitors were more efficient in inhibiting *Pa*-T3S if added prior to adherence of HT-29 cells to tissue culture wells. Together, these studies provided evidence that LE adherence properties were facilitating and stabilizing *Pa*-T3S effector translocation.

The relationship between eukaryotic cell LE properties and *Pa*-T3S sensitivity was corroborated in studies of HL-60 cells. sHL-60 cells gained sensitivity to *Pa*-T3S in culture after multiple passages but remained phenotypically indistinguishable from rHL-60 cells relative to a round, non-adherent morphology. In comparing LE properties of rHL-60 and sHL-60 cells, differences observed in sHL-60 cells included enhanced MT projections, Rac co-localization with actin and the polarized localization of PI3K. These observations are consistent with sHL-60 cells acquiring LE architecture in conjunction with induction of *Pa*-T3S sensitivity.

As an opportunistic pathogen, *P. aeruginosa* infects disrupted epithelial barriers, whereas intact epithelial

barriers that maintain apical-basolateral polarity are resistant to *P. aeruginosa* infections (Fleiszig *et al.*, 1997; Kazmierczak *et al.*, 2004; Plotkowski *et al.*, 1999). Evidence showing that host cell LE properties direct cell migration function in *Pa*-T3S sensitivity is highly consistent with *P. aeruginosa* infections, which target disrupted epithelial barriers undergoing wound-healing cell migration. In migrating epithelial cells, polarity is mediated by inter-linked feedback loops that involve Rac, Cdc42, Rho, PI3K, integrins and MTs. In examining host cell proteins contributing to *Pa*-T3S sensitivity, a hierarchical order of protein function was recognized with first, actin, Rac1 and Cdc42, then MTs and IQGAP1, and to a lesser degree PI3K functioning in *Pa*-T3S. Notably, the hierarchical order of proteins affecting *Pa*-T3S sensitivity paralleled the functional assembly of cellular proteins at the LE. Because of the dependence of the LE on protein assembly, LE function was examined by IF staining and microscopic analysis of LE architecture. In using both HL-60 and HT-29 cell models to examine the relationship between LE cell architecture and *Pa*-T3S, the integrity of LE architecture closely coincided with *Pa*-T3S sensitivity. Collectively, identification of: (1) a relationship between host cell adherence mechanisms and *Pa*-T3S sensitivity; (2) evidence for a role of focal complexes, but not focal adhesions, in *Pa*-T3S; (3) a hierarchical order of protein involvement in *Pa*-T3S function that reflected LE structure; and (4) the close correlation between LE cell architecture and *Pa*-T3S sensitivity all point to eukaryotic cell LE properties playing a role in sensitivity to *Pa*-T3S.

Eukaryotic membranes are structured to resist channel formation, as occurs during T3S, but can be modulated through localized alterations in membrane composition or mechanical forces to favour channel formation. *Pa*-T3S translocon formation in eukaryotic membranes requires translocator proteins, PopB, PopD and PcrV. Beyond this, a role of host cell properties in *Pa*-T3S became apparent from the identification of *Pa*-T3S-resistant cells (McGuffie *et al.*, 1999; Rucks & Olson, 2005). Studies presented here provide evidence and lead us to hypothesize that LE properties are the host cell factor required for *Pa*-T3S translocon insertion and effector translocation. Mechanistically, the role of LE properties in facilitating T3S translocon membrane insertion can be explained by alterations in membrane properties that occur at the LE. As shown in the model of host cell involvement in *Pa*-T3S in Fig. 7, activated Rac1/Cdc42-mediated actin polymerization at the LE drives cell membrane lamellipodia or filopodia protrusions, which introduce forces on plasma membranes that could facilitate PopB/PopD membrane insertion. Actin filaments must be tethered to the cell membrane to exert forces that drive membrane protrusions, and LE adherence mechanisms secure and stabilize these actin-membrane associations. The relationship between host cell adherence and *Pa*-T3S can be explained by the stabilizing role that LE adherence processes have on actin-membrane associations predicted to facilitate trans-

Fig. 7. Model for the role of eukaryotic cell properties in *Pa*-T3S. Our model predicts that alterations in membrane properties associated with F-actin polymerization at the LE induce forces on membranes that are required for PopB (B) and PopD (D) translocon insertion and function. V, PcrV, (PscF) T3S needle protein. The role of specific LE proteins in *Pa*-T3S is described in the text. *Pa*, *P. aeruginosa*.

locon insertion. The subtlety of alterations in cell architecture required for *Pa*-T3S sensitivity became evident in sHL-60 cells, in which induction of MT radiations and polarized co-localization of Rac with actin were observed to coincide with induction of *Pa*-T3S sensitivity. Once T3S is established, host cell properties involved in *Pa*-T3S translocon function appear quite stable, as evidenced by the requirement for agents such as M β CD and PFO, which disrupt cell polarity and LE architecture, to inhibit *Pa*-T3S sensitivity. Retrospectively, the inability of most agents in Supplementary Table S1 to inhibit *Pa*-T3S can be explained by their inability to cause severe alterations in cell polarity. The notion that M β CD and PFO interfere with *Pa*-T3S by disrupting cell polarity and LE architecture is consistent with recent studies which have found M β CD to alter the polarized redistribution of cellular factors mediating cell migration (Rose *et al.*, 2008). Cholesterol is known to accumulate at the LE of migrating cells, where it has been hypothesized to alter plasma membrane viscosity to allow the dynamic actin network to push the membrane forward (Mañes & Martínez-A, 2004). The correlation between inhibition of *Pa*-T3S sensitivity by M β CD and PFO with loss of LE structure attests to the possibility that the function of membrane cholesterol in *Pa*-T3S relates to its polarization to the LE and its facilitation of actin-induced membrane protrusions. Important to an understanding of host cell involvement in *Pa*-T3S is that loss or gain of LE architecture in HT-29 or HL-60 cells provides an explanation for alterations in *Pa*-T3S sensitivity and for the stabilizing role of LE adherence in translocon function.

The opportunistic nature of *P. aeruginosa* infections and reliance on a predisposing host cell factor were essential to our identifying host cell properties involved in *Pa*-T3S. However, the opportunistic nature of *P. aeruginosa* infections likely adds a dimension to T3S translocon function not apparent in obligate Gram-negative pathogens. For example, our model predicts that cortical actin polymerization at the LE is required for *Pa*-T3S translocon function, which closely aligns with recent studies in *Yersinia* that have found T3S translocon function to also rely on actin polymerization (Mejia *et al.*, 2008). *Yersinia* is known to engage host cell integrin receptors in association with T3S effector translocation, and this leads to Rho activation and actin polymerization, which are proposed to trigger YopB/YopD translocon function. *P. aeruginosa*, as an opportunistic pathogen, is unable to engage integrin or other actin polymerization mechanisms, and as a result is dependent on LE properties that trigger actin polymerization for the PopB/PopD translocon function. The role of actin polymerization in *Yersinia* T3S, and our studies which identify a role for actin polymerization in *Pa*-T3S, draw attention to a potential central role of host cell cortical actin in T3S translocon function.

To summarize, the complexity of bacterial–eukaryotic cell interactions during T3S has made it difficult to understand how eukaryotic cell properties might contribute to T3S translocon function. By using cell culture models that are sensitive or resistant to *Pa*-T3S, we were able to identify a relationship between LE properties and host cell *Pa*-T3S sensitivity, and this has led us to hypothesize that cortical actin-induced alterations in membrane properties at the LE facilitate T3S translocon insertion and function. The integral and complex role of LE properties in eukaryotic cell survival attests to the highly co-evolved nature of *Pa*-T3S relative to eukaryotic cell function. The use of the LE in *Pa*-T3S is highly consistent with the opportunistic lifestyle of *P. aeruginosa*, which targets wounded epithelial barriers undergoing cell migration. The requirement of LE properties for *Pa*-T3S is predicted to differ from that of obligate pathogens, such as *Yersinia*, which are able to engage integrin to induce actin polymerization required for translocon function. The ability of cortical actin dynamics to alter membrane properties to favour T3S translocon insertion or function has the potential to be a common theme for host cell involvement in T3S. The precise characterization of host involvement in T3S is anticipated to provide insight into the design and development of novel therapeutic approaches to inhibit this stage in the infectious process of Gram-negative pathogens.

ACKNOWLEDGEMENTS

We wish to thank Dara Frank and Joseph Barbieri (Medical College of Wisconsin) for providing *P. aeruginosa* strains, the ExoS-HA construct, and PopB and PopD antibodies. We also wish to thank Rodney Tweten (University of Oklahoma Health Sciences Center) for providing PFO and PFO mutants, and Thomas Kirchhausen (Harvard Medical School) and Gerald B. Hammond (University of

Louisville) for providing secramine A. We would also like to acknowledge the technical assistance of Jennifer Salazar and Yermie Canizales in these studies, and the statistical assistance of Gerry Hobbs, West Virginia University (WVU) Department of Statistics. All IF images were taken at the WVU Imaging Facility with the assistance of Karen Martin. These studies were supported by Public Health Services Grant NIH NIAID 45569, by the Mary C. Babb Cancer Center Foundation Fund 2V882, and by the WVU HSC internal grants funding program.

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Edited by: W. Bitter