A bacterial type III secretion system inhibits actin polymerization to prevent pore formation in host cell membranes

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The bacterial pathogen *Yersinia pseudotuberculosis* uses type III secretion machinery to translocate Yop effector proteins through host cell plasma membranes. A current model suggests that a type III translocation channel is inserted into the plasma membrane, and if Yops are not present to fill the channel, the channel will form a pore. We examined the possibility that Yops act within the host cell to prevent pore formation. Yop⁻ mutants of *Y. pseudotuberculosis* were assayed for pore-forming activity in HeLa cells. A YopE⁻ mutant exhibited high levels of pore-forming activity. The GTPase-downregulating function of YopE was required to prevent pore formation. YopE⁺ bacteria had increased pore-forming activity when HeLa cells expressed activated Rho GTPases. Pore formation by YopE⁻ bacteria required actin polymerization. F-actin was concentrated at sites of contact between HeLa cells and YopE⁻ bacteria. The data suggest that localized actin polymerization, triggered by the type III machinery, results in pore formation in cells infected with YopE⁻ bacteria. Thus, translocated YopE inhibits actin polymerization to prevent membrane damage to cells infected with wild-type bacteria.
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Introduction

A number of bacteria that are pathogenic for animals or plants use type III secretion systems to translocate virulence factors into host cells (Galán and Bliska, 1996; Hueck, 1998). Type III secretion systems produced by *Salmonella typhimurium* and *Shigella flexnerii* have been purified and visualized by electron microscopy (Blocker *et al.*, 1999; Kubori *et al.*, 2000; Tamano *et al.*, 2000). These assemblages exhibit a basal body that spans the periplasm and a hollow projection that extends out from the bacterial surface like a needle. The basal body likely functions to allow protein transport from the bacterial cytoplasm across the inner and outer membranes. The 'needle' is thought to allow protein translocation into the host cell cytoplasm.

Members of the genus *Yersinia* that are pathogenic for humans (*Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) contain a plasmid-encoded type III secretion system that is required for virulence (Galán and Bliska, 1996; Cornelis *et al.*, 1998). The type III

system is activated when *Yersinia* binds to the surface of a host cell (Cornelis *et al.*, 1998). In the enteropathogenic *Yersinia* spp. (*Y. enterocolitica* and *Y. pseudotuberculosis*) this tight contact can be achieved by a high affinity interaction between the adhesive protein invasin and $\beta 1$ integrin receptors displayed on the eukaryotic host cell (Isberg and Leong, 1990; Isberg and Tran Van Nhieu, 1994). Upon activation of the secretion system, a set of at least six Yop effector proteins [YopE, H, J, M, O (YpkA) and T] are translocated into the cytoplasm of the cell. The Yops act to inhibit several key innate defense mechanisms, including phagocytosis, superoxide production and cytokine synthesis. By this method, *Yersinia* evade host defense mechanisms while replicating extracellularly in lymphoid tissues (Galán and Bliska, 1996; Cornelis *et al.*, 1998).

YopE and YopT interfere with actin dynamics in host cells (Rosqvist *et al.*, 1991; Iriarte and Cornelis, 1998). Following translocation of YopE or YopT, cultured cells lose actin filaments, round up, and eventually detach from the extracellular matrix (Bliska, 2000). In addition, YopE has been shown to have a potent antiphagocytic effect (Rosqvist *et al.*, 1990, 1991). YopE is a GTPase-activating protein (GAP) for the Rho family of small GTP-binding proteins (RhoA, Rac1 and Cdc42) (Black and Bliska, 2000; Von Pawel-Rammingen *et al.*, 2000). When bound to GTP, RhoA, Rac1 and Cdc42 stimulate different modes of actin polymerization (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). YopE switches RhoA, Rac1 and Cdc42 off by accelerating GTP hydrolysis. YopT appears to specifically inactivate RhoA, but by a mechanism distinct from that used by YopE. Evidence indicates that YopT covalently modifies RhoA, resulting in the displacement of RhoA from the host membrane to the cytoplasm (Zumbihl *et al.*, 1999).

YopB, YopD and LcrV are thought to be central components of a translocation channel that is inserted in the host cell plasma membrane by the type III system (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Holmström *et al.*, 2001). Several studies have demonstrated that YopB, YopD and LcrV are required for effector translocation into host cells (Persson *et al.*, 1995; Håkansson *et al.*, 1996; Sarker *et al.*, 1998; Pettersson *et al.*, 1999; Holmström *et al.*, 2001). In addition, a pore-forming and lytic activity associated with expression of the type III system requires YopB, YopD and LcrV (Neyt and Cornelis, 1999; Tardy *et al.*, 1999). Osmoprotection experiments have estimated the size of the pore generated by the *Yersinia* type III system to be 1.2–3.5 nm (Håkansson *et al.*, 1996). Interestingly, mutants that are defective for multiple Yop effectors (multi-Yop⁻ mutants) have higher pore-forming activity than wild-type *Yersinia* strains (Håkansson *et al.*, 1996; Neyt and Cornelis, 1999). For example, Håkansson *et al.* (1996) found that HeLa

Fig. 1. Infection of HeLa cells with a multi-Yop⁻ mutant of *Y.pseudotuberculosis* results in membrane blebbing and EtdBr uptake. HeLa cells grown on coverslips were infected with a wild-type strain (YP126) or a YopEHJ⁻ mutant (YP27). After 3 h, coverslips were inverted onto a 5 μ l drop of staining solution (25 μ g/ml EtdBr and 5 μ g/ml AO in PBS) placed on the surface of a glass slide. Infected cells were analyzed immediately by phase contrast (A and C) or epifluorescence (B and D) microscopy using a 40 $\times$ objective. Images were recorded using a digital camera. The arrows in (C) and (D) point to a large bleb on a HeLa cell.

cells exhibited membrane blebbing and an inability to exclude Trypan Blue after they were infected with a *Y.pseudotuberculosis* multi-Yop⁻ mutant but not with the wild-type strain. In addition, lytic activity in red blood cells was reduced when YopE was overproduced in the multi-Yop⁻ mutant (Håkansson *et al.*, 1996). On the basis of these results it was proposed that Yop effectors prevent formation of a pore, and therefore cell lysis, by occupying the translocation channel (Håkansson *et al.*, 1996). In this study, we investigated the mechanism by which Yop effectors prevent pore formation in host cells. We found that YopE plays a crucial role in preventing pore formation in HeLa cells by *Y.pseudotuberculosis*. A catalytically inactive form of YopE was unable to prevent pore formation, indicating that pore formation by YopE⁻ mutant *Yersinia* involves activation of Rho GTPases and actin polymerization. Our results rule out the possibility that YopE prevents pore formation simply by filling translocation channels. A model is presented whereby translocated YopE downregulates Rho GTPases to prevent damage to the plasma membrane during *Yersinia*–host cell interactions.

Results

A multi-Yop⁻ mutant of *Y.pseudotuberculosis* has a YopB-dependent pore-forming activity on HeLa cells

To investigate the role of Yop effectors in preventing pore formation, we infected HeLa cells at a multiplicity of infection of 100 with a wild-type strain of *Y.pseudo-*

tuberculosis (YP126) or an isogenic mutant (YP27) defective for expression of YopE, YopH and YopJ (YopEHJ⁻ mutant). At different time points post infection we examined the morphology of the infected HeLa cells using phase contrast microscopy. HeLa cells infected with the YopEHJ⁻ mutant exhibited dramatic membrane blebbing that could be detected within 1 h of infection (Figure 1C). In contrast, cells infected with the wild-type strain displayed the characteristic rounded morphology associated with YopE-mediated cytoskeletal disruption, but no membrane blebbing (Figure 1A). To assess membrane damage, infected cells were stained with ethidium bromide (EtdBr) and acridine orange (AO) and observed by fluorescence microscopy. AO penetrates cells with intact membranes and stains them green. EtdBr enters cells with perforated membranes and stains their nuclei red. The majority of cells infected with the YopEHJ⁻ mutant displayed red nuclear staining, whereas cells infected with the wild-type strain stained only green (Figure 1D and B, respectively).

To quantify membrane damage, we assayed for release of the cytoplasmic enzyme lactate dehydrogenase (LDH) from HeLa cells infected with the wild-type strain or the YopEHJ⁻ mutant. In a time course experiment, LDH release was detected within 1 h of infection with the YopEHJ⁻ mutant (Figure 2A). LDH release increased linearly with time, reaching 30–40% of the total amount of LDH in uninfected cells after 5 h (Figure 2A). The amount of LDH released from HeLa cells infected with the YopEHJ⁻ mutant after 3 h was ~10-fold higher than that released from cells infected with the wild-type strain

Fig. 2. Release of LDH by HeLa cells infected with *Y. pseudotuberculosis*. (A) Kinetics of LDH release after infection with a wild-type strain (YP126), a YopEHJ⁻ mutant (YP27) or a YopE⁻ mutant (YP6). Culture supernatants were collected from wells containing HeLa cells at indicated time points after infection. LDH in culture supernatants was measured using a CytoTox 96 assay kit (Promega). The percentage of LDH release was calculated by dividing the amount of LDH released from infected cells by the amount of LDH released from uninfected cells that were lysed by a freeze-thaw cycle. Error bars represent the standard deviation of the mean values obtained from triplicate samples. (B) YopB is required for LDH release. HeLa cells were infected with a wild-type strain (YP126), a YopEHJ⁻ mutant (YP27), a YopEHJB⁻ mutant (YP29) or a YopEHJB⁻ mutant complemented with wild-type *yopB*⁺ (YP29/pYopB). Culture supernatants were collected 3 h post infection and assayed for LDH activity as described above.

(Figure 2B). Similar amounts of LDH were released from HeLa cells infected with a YopEHJKOM⁻ mutant (data not shown). As shown in Figure 2B, HeLa cells infected with a mutant (YP29) deficient in YopB (YopEHJB⁻ mutant) did not release significant amounts of LDH. When a plasmid that encodes YopB was introduced into YP29 (YopEHJB⁻/YopB⁺), lytic activity was restored (Figure 2B).

Osmoprotection experiments were performed to determine whether the membrane damage and cell lysis we observed was due to pore formation. Dextran 6000 (6000 Da), which is too large to diffuse efficiently through a small pore, should act as an osmoprotectant and prevent LDH release if membrane damage is due to pore formation (Kirby *et al.*, 1998; Neyt and Cornelis, 1999; Holmström *et al.*, 2001). However, Dextran 6000 should not interfere with the uptake of EtdBr, which is small enough (394.3 Da) to pass through pores. Release of LDH was diminished to background levels when HeLa cells were infected with the YopEHJ⁻ mutant in the presence of 30 mM Dextran 6000 (Figure 3). In contrast, Dextran 6000 had no effect on EtdBr uptake (Figure 4D). Interestingly, Dextran 6000 did not prevent membrane blebbing, but did reduce the size of

Fig. 3. Effect of Dextran 6000 on release of LDH from HeLa cells infected with *Y. pseudotuberculosis*. HeLa cells were infected with a YopEHJ⁻ mutant (YP27) or a YopE⁻ mutant (YP6) in the presence or absence of 30 mM Dextran 6000 or 30 mM sucrose. LDH release 3 h post infection was determined as described in the legend to Figure 2.

the blebs (Figure 4, compare A and C). As a control, we also carried out infections in the presence of 30 mM sucrose (342 Da). Addition of sucrose did not prevent LDH release (Figure 3) or EtdBr uptake (Figure 4B). These results confirm that multi-Yop⁻ mutants of *Y. pseudotuberculosis* have a YopB-dependent pore-forming activity in HeLa cells, which results in osmotic lysis (Håkansson *et al.*, 1996). LDH release was used in subsequent experiments as a quantitative assay of osmotic lysis resulting from pore formation.

YopE plays a crucial role in preventing pore formation

To determine whether pore-forming activity required inactivation of multiple effectors, we assayed for LDH release by HeLa cells infected with *Y. pseudotuberculosis* strains deficient for only one Yop (YopE⁻, YopH⁻ or YopJ⁻ mutants). As shown in Figure 5, HeLa cells infected for 3 h with a YopH⁻ mutant or a YopJ⁻ mutant did not release significant levels of LDH. However, high levels of LDH were detected in the culture supernatants of HeLa cells infected with the YopE⁻ mutant (Figure 5). The kinetics of LDH release by HeLa cells infected with the YopE⁻ mutant were comparable to those observed in cells infected with the YopEHJ⁻ mutant (Figure 2A). Similarly, LDH released from HeLa cells infected with the YopE⁻ mutant was prevented by addition of Dextran 6000 (Figure 3). Thus, YopE plays a critical role in preventing pore formation.

YopE GAP activity is required to inhibit pore formation

If YopE prevents pore formation by occupying translocation channels, it should not matter whether the protein is active or inactive. To examine this possibility, plasmids that encode either wild-type YopE (pYopE) or catalytically inactive YopE (pYopER144A) were introduced into the YopE⁻ mutant (YP6). The resulting strains were tested for lytic activity in HeLa cells using the LDH release assay. YP6/pYopER144A and a control strain (YP6/pvector) stimulated similar levels of LDH release (Figure 6A). In contrast, YP6/pYopE had a low level of lytic activity that was comparable to the wild-type strain (Figure 6A).

Fig. 4. Effect of Dextran 6000 on membrane blebbing and uptake of EtdBr. HeLa cells were infected for 3 h with a YopEHJ⁻ mutant (YP27) in the presence of 30 mM sucrose (A and B) or 30 mM Dextran 6000 (C and D). Images were obtained as described in the legend to Figure 1.

To determine whether YopE and YopER144A were translocated into HeLa cells at equivalent levels, a Triton X-100 solubility assay was performed. HeLa cells infected as above for 2 h were lysed in 1% Triton X-100 and the lysates were centrifuged to separate translocated YopE (soluble fraction) from non-translocated YopE (insoluble fraction). Comparable amounts of YopE and YopER144A were detected in the soluble fractions by immunoblotting (Figure 6B, lanes 3 and 4). As a control, infections were also carried out with a strain deficient for YopB (YP19/pYopE). In this case, YopE was found only in the insoluble fraction (Figure 6B, lane 5). As YopE and YopER144A were translocated at similar levels, and only active YopE prevented pore formation, it is unlikely that YopE prevents pore formation by occupying translocation channels. Rather, it appears that YopE must enter the host cell and inactivate one or more Rho GTPases to inhibit pore formation.

YopT inhibits pore formation

As YopT has been shown to inhibit RhoA activity (Zumbihl *et al.*, 1999), we examined whether YopT could prevent pore formation in HeLa cells infected with *Y.pseudotuberculosis*. YopT is not produced by our wild-type serogroup III strain YP126, but is produced by strains of other serogroups (our unpublished results). A segment of DNA encoding YopT and its chaperone SycT was isolated from a serogroup II strain and inserted into an expression vector (Materials and methods). The resulting plasmid (pYopT) was introduced into the YopE⁻ mutant YP6. LDH released by HeLa cells infected with YP6/pYopE or YP6/pYopT was measured 3 h post infection. As shown in the first three columns of Figure 7A, similar

Fig. 5. YopE plays a critical role in preventing pore formation. HeLa cells were infected for 3 h with a wild-type strain (YP126), a YopEHJ⁻ mutant (YP27), a YopE⁻ mutant (YP6), a YopH⁻ mutant (YP15), or a YopJ⁻ mutant (YP26). LDH release was determined as described in the legend to Figure 2.

amounts of LDH were released from cells infected with YP6/pYopE or YP6/pYopT, indicating that YopE and YopT have comparable anti-pore-forming activities.

Constitutively activated forms of RhoA or Rac1 rescue pore-forming activity in the presence of YopE or YopT

The above results encouraged us to ask whether Rho GTPases are directly involved in pore formation. Toward this end, constitutively activated forms of RhoA (RhoA-V14) or Rac1 (Rac1-V12) were produced in HeLa cells. HeLa cells were transfected with a control vector

Fig. 6. YopE GAP activity is required to inhibit pore formation. HeLa cells were infected with a wild-type strain (YP126), a YopE⁻ mutant harboring an empty vector (YP6/vector), a YopE⁻ mutant complemented with *yopE*⁺ (YP6/pYopE), a YopE⁻ mutant complemented with *yopER144A* (YP6/pYopER144A), or a YopEHB⁻ mutant complemented with *yopE*⁺ (YP19/pYopE). (A) LDH released from HeLa cells infected for 3 h was measured as described in the legend to Figure 2. (B) HeLa cells infected for 2 h were lysed in a buffer containing 1% Triton X-100. The lysates were separated by centrifugation into soluble and insoluble fractions. Samples of protein equivalent to 1×10^4 HeLa cells (soluble fraction) or 3×10^5 HeLa cells (insoluble fraction) were separated by SDS-PAGE and analyzed by immunoblotting with anti-YopE antibody.

(pCGT) or vectors producing either RhoA-V14 or Rac1-V12. Twenty-four hours post transfection, HeLa cells were infected with YP6/pvector, YP6/pYopE or YP6/pYopT. LDH release was assayed 3 h post infection. In cells infected with YP6/pYopE or YP6/pYopT, release of LDH was increased 2-fold in the presence of RhoA-V14 and 3-fold in the presence Rac1-V12 (Figure 7A). In addition, the lytic activity of YP6/pvector increased slightly in cells containing Rac1-V12, but not in cells containing RhoA-V14 (Figure 7A). Non-infected HeLa cells producing RhoA-V14 or Rac1-V12 did not release more LDH than non-transfected cells (data not shown). Thus, production of either RhoA-V14 or Rac1-V12 in HeLa cells resulted in a partial rescue of pore-forming activity in the presence of YopE or YopT.

To determine the level of RhoA-V14 and Rac1-V12 expression in transfected cells, HeLa cells were lysed in detergent. Samples of the lysates were analyzed by immunoblotting using a monoclonal antibody that recognizes the T7 epitope fused to RhoA-V14 and Rac1-V12 (Figure 7B). The cell extracts were also analyzed by immunoblotting with a monoclonal antibody specific for the Raf kinase, to control for sample loading. As shown in Figure 7B both proteins were produced, although Rac1-V12 was produced at higher levels than RhoA-V14 (lanes 2 and 3). The higher expression level of Rac1-V12 may explain the increased rescue of pore-forming activity by Rac1-V12 as compared with RhoA-V14 (Figure 7A).

Fig. 7. Constitutively active forms of RhoA and Rac1 rescue pore-forming activity in the presence of YopE or YopT. HeLa cells were not transfected (non transf.) or were transfected with an empty vector (pCGT), pCGTRhoA-V14 (pRhoA-V14) or pCGTRac1-V12 (pRac1-V12). (A) LDH released from HeLa cells infected with YP6/vector, YP6/pYopE or YP6/pYopT for 3 h was determined as described in the legend to Figure 2. (B) Expression of RhoA-V14 and Rac1-V12 in HeLa cells was evaluated 24 h post transfection by immunoblotting. Transfected cells were lysed in detergent and samples of soluble protein equivalent to 1×10^4 HeLa cells were analyzed by immunoblotting with a monoclonal antibody that recognizes the T7 epitope tag appended to RhoA-V14 and Rac1-V12. Duplicate samples of protein were analyzed by immunoblotting with a monoclonal antibody specific for the Raf kinase, to control for loading.

Inhibition of actin polymerization prevents pore formation and cell lysis

As RhoA and Rac1 are key regulators of actin polymerization, we investigated the role of actin polymerization in pore formation by *Y.pseudotuberculosis*. HeLa cells were exposed to the actin polymerization inhibitor cytochalasin D (3.9 μ M) or vehicle alone (DMSO), for 2 h prior to and during infection with the wild-type *Y.pseudotuberculosis* strain or the YopE⁻ mutant. LDH release was assayed 3 h post infection. As shown in Figure 8A, release of LDH from cells infected with the YopE⁻ mutant was significantly reduced in the presence of cytochalasin D. Cytochalasin D had no effect on LDH release in non-infected cells (mock) or in cells infected with the wild-type strain (Figure 8A). Analysis of infected cells by fluorescence microscopy after staining with EtdBr/AO showed that cytochalasin D prevented uptake of EtdBr by cells infected with the YopE⁻ mutant (data not shown). From these results and from those described above we conclude that actin polymerization stimulated by Rho GTPases is required for pore formation by YopE⁻ mutant *Y.pseudotuberculosis*. To examine further the role of actin polymerization in pore formation, we tested the effect of two other actin modifying agents, latrunculin B and jasplakinolide. The marine toxin latrunculin B forms a 1:1 complex with actin monomers and inhibits actin

Fig. 8. Actin polymerization inhibitors prevent pore formation in HeLa cells infected with YopE⁻ mutant *Y.pseudotuberculosis*. HeLa cells were exposed to the different actin modifying agents dissolved in DMSO or to DMSO only for 2 h prior to and during infection. Cells were either left uninfected (mock) or infected with a wild-type strain (YP126) or a YopE⁻ mutant (YP6) for 3 h. Culture supernatants were assayed for LDH as described in the legend to Figure 2. **(A)** Cells were treated with 3.9 μ M cytochalasin (+CD) or DMSO only (–CD). **(B)** Cells were treated with 5 μ M latrunculin B (Lat), 100 nM Jasplakinolide (Jas) or DMSO only.

nucleation. In contrast, jasplakinolide enhances the rate of actin filament nucleation, inducing polymerization of monomeric actin *in vivo*. As predicted from the results with cytochalasin D, 5 μ M latrunculin B had a strong inhibitory effect on LDH release by cells infected with the YopE⁻ mutant (Figure 8B). Jasplakinolide (10 nM) had no significant effect on LDH release (Figure 8B). The lack of effect with jasplakinolide may indicate that actin polymerization must occur by a specific mechanism or in a specific location to trigger pore formation.

Bacterial internalization is not required for pore formation

We next considered the possibility that invasin-dependent bacterial entry into HeLa cells, which is dependent upon Rho GTPases and actin polymerization, was required for pore formation. Inhibitors of phosphatidylinositol (PI) 3-kinase antagonize uptake of *Y.pseudotuberculosis* by HeLa cells (Mecas et al., 1998). PI 3-kinase inhibitors, such as wortmannin, impair closure of the phagocytic cup, but not actin polymerization *per se* (Araki et al., 1996; Cox et al., 1999). HeLa cells were treated with 100 nM wortmannin or vehicle alone (DMSO), for 2 h prior to and during infection with wild-type *Y.pseudotuberculosis* or

Fig. 9. Wortmannin inhibits bacterial internalization but not LDH release. HeLa cells were exposed to 100 nM wortmannin in DMSO (+Wort.) or to DMSO alone (–Wort.) for 2 h prior to and during infections. Cells were infected with a wild-type strain (YP126) or a YopE⁻ mutant (YP6). **(A)** Bacterial internalization (%) at 1 h post infection was measured by a gentamicin protection assay as described in Materials and methods. **(B)** LDH release at 3 h post infection was assayed as described in the legend to Figure 2.

the YopE⁻ mutant. The effect of the inhibitor on bacterial uptake or LDH release was determined 1 or 3 h post infection, respectively. As determined by a gentamicin protection assay, wortmannin reduced entry of the YopE⁻ mutant into HeLa cells by ~2.5-fold (Figure 9A). However, wortmannin did not diminish LDH release from HeLa cells infected with the YopE⁻ mutant (Figure 9B). Thus, bacterial uptake into HeLa cells did not appear to be required for pore formation.

Detection of F-actin at sites of contact between multi-Yop⁻ mutants and HeLa cells

As actin polymerization appeared to stimulate pore formation, we looked for evidence of actin polymerization at sites of contact between HeLa cells and multi-Yop⁻ mutants of *Y.pseudotuberculosis*. HeLa cells were infected with either the YopEHJ⁻ mutant or the YopEHJB⁻ mutant. At 30, 60, 90 and 120 min post infection, the cells were fixed and stained with rhodamine-phalloidin to label F-actin. The samples were then observed by epifluorescence microscopy. Intensely staining F-actin structures surrounding what appeared to be cell-associated bacteria were detected at all time points in cells infected with the YopEHJ⁻ mutant, but not in cells infected with the YopEHJB⁻ mutant (Figure 10, arrows, and data not shown). These ‘halos’ of F-actin were also observed in HeLa cells infected with the YopE⁻ mutant, but not in cells

Fig. 10. Formation of F-actin halos in HeLa cells infected with YopEHJ⁻ mutant *Y.pseudotuberculosis*. HeLa cells grown on coverslips were infected with a YopEHJ⁻ mutant (YP27) (A and C) or a YopEHJB⁻ mutant (YP29) (B and D). At 60 (A and B) or 90 (C and D) min post infection, the cells were fixed, permeabilized, and stained with rhodamine-phalloidin to label F-actin. F-actin was detected by epifluorescence microscopy using a 100× objective. Images were captured with a digital camera. Arrows in (A) and (C) point to F-actin halos. ‘Clumps’ of concentrated F-actin observed in HeLa cells infected with the YopEHJB⁻ mutant are morphologically distinct from the F-actin halos observed in cells infected with the YopEHJ⁻ mutant.

Fig. 11. Bacteria co-localized with F-actin halos. HeLa cells grown on coverslips were exposed to 100 nM wortmannin in DMSO for 2 h prior to and during infection. The HeLa cells were infected with a YopEHJ⁻ mutant (YP27) for 60 min. The infected cells were fixed, permeabilized, and bacteria were labeled by sequential incubation with rabbit anti-*Yersinia* primary antibody and FITC-conjugated goat anti-rabbit IgG secondary antibody. F-actin was labeled with rhodamine-phalloidin. Cells were observed by epifluorescence microscopy using a 100× objective and filters to detect rhodamine (A) or FITC (B) fluorescence. Images were captured with a digital camera. A superimposition of (A) and (B) is presented in (C). Arrows point to F-actin halos in (A) and (C).

infected with the wild-type strain (data not shown). Treatment with wortmannin did not prevent formation of the halos (Figure 11A, arrows). Therefore, Rho GTPase activity, but not PI 3-kinase activity, was required for assembly of F-actin halos. Treatment with cytochalasin D prevented formation of the F-actin halos in HeLa cells infected with YopE⁻ mutant bacteria (data not shown). The

infected HeLa cells were also stained with rabbit anti-*Yersinia* antibody and FITC-conjugated anti-rabbit IgG antibody to label bacteria (Figure 11B). As shown in Figure 11C, F-actin halos co-localized with a subset of cell-associated bacteria. These results indicate that YopE⁻ mutant strains of *Y.pseudotuberculosis* stimulate actin polymerization at sites of bacterial–host cell contact.

Discussion

Several lines of evidence suggest that Yop effectors are translocated into host cells through a channel introduced into the eukaryotic cell plasma membrane (Håkansson *et al.*, 1996; Neyt and Cornelis, 1999; Tardy *et al.*, 1999; Holmström *et al.*, 2001). If such a channel is formed, it must be carefully gated because wild-type strains of *Yersinia* exhibit very low levels of pore-forming activity. Based on the observation that multi-Yop⁻ mutants display high levels of pore-forming activity, it was suggested that Yop effectors prevent pore formation by filling translocation channels (Håkansson *et al.*, 1996). In this work we examined the mechanism by which Yop effectors prevent pore formation.

Using osmoprotectants and assays that measure membrane damage (EtdBr/AO uptake) or cell lysis (LDH release) we confirmed that a multi-Yop⁻ mutant of *Y.pseudotuberculosis* has a YopB-dependent pore-forming activity that leads to osmotic lysis of HeLa cells (Håkansson *et al.*, 1996). Previously it was thought that pore-forming activity in *Yersinia* required the inactivation of multiple effectors (Håkansson *et al.*, 1996; Neyt and Cornelis, 1999). However, Håkansson *et al.* (1996) showed that overproduction of YopE alone in a multi-Yop⁻ mutant reduced lytic activity by 50%, suggesting that YopE plays a crucial role in preventing pore formation, possibly due to its ability to occupy translocation channels. We found that the lytic activity of a YopE⁻ mutant was comparable to that of a YopEHJ⁻ mutant or a YopEHJKMO⁻ mutant, and that the GAP activity of YopE was required to prevent pore formation. In addition, the pore-forming activity of a YopE⁻ mutant was reduced by ectopic expression of YopT. These data argue that the pore-forming activity of multi-Yop⁻ mutants results from their inability to downregulate Rho-related GTPases.

Which Rho-related GTPases are involved in this pore-forming activity? YopE displays GAP activity towards RhoA, Rac1 and Cdc42 (Black and Bliska, 2000; Von Pawel-Rammingen *et al.*, 2000), while YopT appears to specifically inactivate RhoA (Zumbühl *et al.*, 1999). Therefore, it seems likely that RhoA is a key factor. However, we found that overproduction of Rac1-V12 in HeLa cells partially rescued pore-forming activity in the presence of YopT or YopE. These data suggest that activation of either Rac1 or RhoA can stimulate pore-forming activity. Determination of which GTPases are activated in HeLa cells infected with the YopE⁻ mutant may help to clarify this issue.

As Rho family GTPases are responsible for stimulating actin polymerization, we examined the possibility that actin polymerization was involved in pore formation. Pore-formation was significantly reduced when HeLa cells were treated with the actin polymerization inhibitors cytochalasin D or latrunculin B. Actin polymerization inhibitors do not interfere with Yop translocation (Sory and Cornelis, 1994) and therefore should not reduce channel formation. However, inhibitors of actin polymerization do block invasin-mediated uptake of *Yersinia* by host cells, raising the possibility that pore formation requires bacterial entry. To address this issue, we used the PI 3-kinase inhibitor wortmannin, which antagonizes invasin-mediated entry of *Y.pseudotuberculosis* into

HeLa cells, but does not block actin polymerization. Wortmannin decreased bacterial entry 2.5-fold, but had no effect on pore formation, indicating that bacterial entry is not required for pore formation. One problem with this interpretation is that wortmannin did not block entry completely. Thus, an extremely low level of bacterial internalization may be sufficient to trigger pore formation.

The most surprising finding in this work is that YopE⁻ mutant strains stimulate formation of F-actin structures, which we term 'halos', at sites of contact with HeLa cells. Why the F-actin assembly is associated with only a subset of the cell-attached bacteria remains unclear. One possibility is that it is a transient process, and that at any given time only a subset of the cell-associated bacteria are stimulating actin polymerization.

Assembly of F-actin halos was dependent upon YopB, suggesting that channel formation is required for this process. YopE prevented formation of F-actin halos, indicating that Rho GTPases are required as well. We believe that the process leading to formation of F-actin at sites of bacterial attachment is somehow associated with, or responsible for, pore formation. One possibility is that local F-actin assembly drives uncontrolled membrane movement in the vicinity of the attached bacterium. If the bacterium assembles some type of needle that forms a stable conduit between the bacterial surface and the translocation channel, uncontrolled membrane movement could lead to breakage of the conduit. This could lead to pore formation if the translocation channel remains inserted in the membrane in the absence of an intact needle. In support of this concept, it has recently been shown that the type III sytem of *Y.enterocolitica* assembles needle structures during bacterial-host cell interaction (Hoiczky and Blobel, 2001).

One important question raised by these findings is the nature of the stimulus responsible for triggering F-actin assembly at sites of host cell contact with YopE⁻ mutant bacteria. As the process is dependent upon YopB, one possibility is that a bacterial protein that moves through the translocation channel is responsible for stimulating Rho GTPases and F-actin assembly. None of the known effectors, including YopO (YpkA) or YopM, appears to be involved in this process. An unidentified effector protein could function to activate Rho GTPases. Alternatively, one of the proteins involved in channel formation could perform this function. There is evidence that YopD and LcrV gain access to the host cell cytosol (Francis and Wolf-Watz, 1998; Fields and Straley, 1999). The *Shigella* IpaC protein, which is similar in overall structure to YopD, has been shown to induce actin polymerization through activation of Cdc42 and Rac1 (Tran Van Nhieu *et al.*, 1999). If a translocated bacterial protein is responsible for activating this type of signaling, it is at present unclear what role this activity might play in the type III secretion/translocation mechanism. A second possibility is that introduction of the putative translocation channel into the plasma membrane stimulates a stress response at the membrane that leads to activation of Rho GTPases. This could be viewed as an unwanted side effect of the translocation channel.

In summary, our data suggest that the *Yersinia* type III secretion/translocation system triggers a signaling pathway in HeLa cells resulting in activation of Rho GTPases.

Under normal conditions, GTPase activation is rapidly terminated following the delivery of YopE. In the absence of YopE, local F-actin assembly is triggered, leading to pore formation. It is interesting to draw a comparison between these series of events and the process of *S.typhimurium* invasion into epithelial cells. *Salmonella typhimurium* triggers local F-actin assembly through the actions of translocated type III effector proteins (Galán and Zhou, 2000). Dramatic membrane ruffling ensues, leading to bacterial internalization by macropinocytosis (Galán and Zhou, 2000). Following bacterial entry, F-actin assembly is downregulated by translocated SptP, a GAP sharing structural and functional similarity to YopE (Fú and Galán, 1999). Thus, in addition to its antiphagocytic function, YopE may be part of a mechanism that minimizes overt damage to the plasma membrane during the translocation of Yops into host cells.

Materials and methods

Bacterial strains and infection conditions

The wild-type serogroup III *Y.pseudotuberculosis* strain YP126 (Bölin *et al.*, 1982) and the mutants derived thereof [YP15 (YopH⁻), YP6 (YopE⁻), YP26 (YopF⁻), YP27 (YopEHJ⁻), YP29 (YopEHJB⁻) and YP19 (YopEHB⁻)] have been described (Black and Bliska, 1997; Palmer *et al.*, 1998, 1999). Construction of YP37 (YopEHJKOM⁻; our unpublished data) will be described elsewhere. YP126 and its derivatives carry a naturally occurring deletion in virulence plasmid which inactivates the *yopT* gene and are thus devoid of YopT activity (our unpublished data). Prior to infection of HeLa cells the bacteria were grown in Luria-Bertani (LB) broth at 37°C under conditions that stimulate Yop expression (Palmer *et al.*, 1998).

Plasmids

The plasmids pYopE and pYopER144A have been described previously (Black and Bliska, 2000). The plasmid pYopER144A encodes an inactive form of YopE due to conversion of the essential Arg at position 144 to Ala (R144A). Construction of pYopT (our unpublished data) will be described elsewhere. Plasmids were introduced into *Y.pseudotuberculosis* by conjugation as described previously (Bliska and Black, 1995). The eukaryotic expression plasmids pCGT, pCGT-Rac1-V12 and pCGT-RhoA-V14 were obtained from Dr D.Bar-Sagi (SUNY, Stony Brook, NY) and were purified by CsCl₂/EtdBr gradient centrifugation.

Cell culture and transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) and 1 mM sodium pyruvate in a 5% CO₂ humidified incubator at 37°C. For standard infection experiments 1 × 10⁵ HeLa cells were seeded into wells of a 24-well tissue culture plate 24 h prior to assay. For experiments involving transfected cells 5 × 10⁴ HeLa cells were seeded into wells of a 24-well tissue culture plate. Approximately 24 h later, the cells were transiently transfected with 0.5 µg of the indicated plasmid per well using FuGene 6 (Boehringer Mannheim) as specified by the manufacturer. Infections with *Y.pseudotuberculosis* were performed 24 h after transfection. For microscopic assays the HeLa cells were seeded onto glass coverslips in 24-well dishes and cultured as above.

Infection conditions

For experiments carried out in the presence of inhibitors, HeLa cells were preincubated for 2 h in the presence of 3.9 µM cytochalasin D (Sigma), 100 nM wortmannin (Sigma), 5 µM latrunculin B (Calbiochem) or 10 µM jasplakinolide (Molecular Probes). HeLa cells were also exposed to the appropriate concentration of vehicle alone (DMSO) as a control. Bacterial infections were performed in fresh media containing the same concentration of inhibitors or vehicle. Bacteria were used to infect HeLa cells at a multiplicity of infection of 100. The plates containing the infected cells were centrifuged for 5 min at 83 g and incubated at 37°C with 5% CO₂ for different periods of time to allow bacterial–host cell interaction. In some cases, the medium was replaced 5 min post infection with fresh medium containing Dextran 6000 or sucrose at a final concentration of 30 mM.

EtdBr/AO uptake assay

After 3 h of infection, coverslips containing infected HeLa cells were inverted onto a 5 µl drop of 25 µg/ml EtdBr and 5 µg/ml AO in phosphate-buffered saline (PBS) placed on the surface of a glass slide. The cells were immediately analyzed by phase contrast or epifluorescence microscopy using a Zeiss AxioPlan 2 microscope equipped with a 40× objective (NA 0.75). Images were captured with a Diagnostic Instruments Spot digital camera and processed using Adobe Photoshop 5.5.

LDH assay

Samples of culture media from wells containing infected cells were collected at different time points post infection. Levels of LDH were assayed using the CytoTox 96 assay kit (Promega) according to the manufacturer's instructions. After a 30 min incubation with the substrate, the reaction was stopped and absorbance at 490 nm was determined using an MRX microplate reader (Dynatech Laboratories). Total LDH was determined by assaying supernatants from uninfected cells that had been lysed by a freeze–thaw cycle. The percentage of LDH release was calculated by dividing the amount of LDH released from infected cells by the amount of total LDH.

Gentamicin protection assay

HeLa cells pretreated with wortmannin dissolved in DMSO, or with DMSO alone, were incubated with bacteria as described above. After 1 h of infection, the cells were exposed to 100 µg/ml gentamicin for 1 h. After washing with PBS, the cells are solubilized by treatment with 0.1% Triton X-100 for 5 min at room temperature. The number of viable bacteria was determined by plating serial 10-fold dilutions of the lysates on LB agar plates. Percentage bacterial internalization was calculated as the number of bacteria surviving gentamicin per well divided by the number of bacteria inoculated per well.

Immunoblotting

Infected HeLa cells were washed three times with ice-cold PBS containing 1 mM Na₃VO₄ and 10 mM NaF. To each well, 0.2 ml of cold 1% Triton X-100 buffer (10 mM Tris pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na₃VO₄, 10 mM NaF, 200 µM AEBSF, 20 µM leupeptin and 1 µM pepstatin) were added, and the plates were incubated for 15 min on ice with occasional rocking. The cells from three wells were scraped into a single microcentrifuge tube and the tube was centrifuged for 10 min at 12 000 g at 4°C. Supernatants (soluble fractions) were transferred to new tubes, and protein concentrations were determined using the Bio-Rad protein assay. Pellets (insoluble fractions) were carefully washed in lysis buffer, resuspended in 10 µl of Laemmli sample buffer and boiled. Samples of the fractions containing equivalent amounts of protein (or sample volume for insoluble fractions) were separated by SDS–PAGE under reducing conditions and transferred electrophoretically onto nitrocellulose. Immunoblotting was performed as described previously (Black and Bliska, 1997) using an affinity-purified polyclonal anti-YopE antibody (Black and Bliska, 2000) diluted 1:2000. Transfected cells in 24-well dishes were processed as above except that insoluble fractions were not analyzed, and immunoblotting was performed with either monoclonal anti-T7 antibody (Novagen) or monoclonal anti-Raf (Transduction Laboratories) antibody. Secondary anti-rabbit IgG and anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP) were purchased from Sigma.

Rhodamine–phalloidin staining and immunofluorescence assay

HeLa cells on coverslips were washed with PBS, fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 10 min. After washing, coverslips were incubated for 20 min with rhodamine–phalloidin (Molecular Probes) at a final concentration of 50 U/ml in PBS, to label F-actin. To label F-actin and bacteria, the fixed cells were first incubated with polyclonal anti-*Yersinia* antibody SB349 (Black and Bliska, 2000) diluted 1:1000 in PBS containing 3% BSA. After 40 min of incubation, the coverslips were washed three times with PBS containing 1% BSA. The fixed cells were then incubated for 40 min with FITC-conjugated goat anti-rabbit IgG (Jackson Laboratories) diluted 1:250 in PBS containing 3% BSA. After washing with PBS, the fixed cells were incubated with rhodamine–phalloidin at a final concentration of 0.2 U/ml in PBS for 20 min. Coverslips were washed with PBS before mounting in 10% Airvol (Air Products) dissolved in 100 mM Tris pH 8.5, 25% glycerol and 2.5% DABCO (Sigma). Samples were analyzed by epifluorescence microscopy using a Zeiss AxioPlan 2 microscope equipped with a 100× (NA 1.3) oil immersion objective. Images were captured with a Diagnostic Instruments Spot digital camera and processed using Adobe Photoshop 5.5.

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