Yersinia enterocolitica differentially modulates RhoG activity in host cells

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Summary

Pathogenic bacteria of the genus *Yersinia* (*Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*) have evolved numerous virulence factors (termed a stratagem) to manipulate the activity of Rho GTPases. Here, we show that *Y. enterocolitica* modulates RhoG, an upstream regulator of other Rho GTPases. At the contact site of virulent *Y. enterocolitica* and host cells, we could visualise spatiotemporally organised activation and deactivation of RhoG. On the one hand, the β1-integrin clustering protein Invasin on the bacterial surface was found to activate RhoG and this promoted cell invasion. On the other hand, active RhoG was downregulated by the type III secretion system effector YopE acting as a GTPase-activating protein (GAP). YopE localised to Golgi and endoplasmic reticulum, and this determined its specificity for RhoG and other selected Rho GTPases. RhoG and its downstream effector module Elmo/Dock180 controlled both Rac1 activation by Invasin and Rac1 deactivation by YopE. We propose that RhoG is a central target of the *Yersinia* stratagem and a major upstream regulator of Rac1 during different phases of the *Yersinia* infection cycle.

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Key words: *Yersinia enterocolitica*, RhoG, YopE, RhoGAP, Elmo, Dock180, Invasin

Introduction

Yersinia pestis, *Y. enterocolitica* and *Y. pseudotuberculosis* are widespread bacterial pathogens that elicit plaque, gastroenteritis and enteric lymphadenitis in humans (Naktin and Beavis, 1999; Putzker et al., 2001). After oral ingestion, enteropathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* reach distal parts of the small intestine where they invade and cross the mucosa. In the submucosal Peyer-Plaques, the yersiniae proliferate extracellularly and from there they can spread systemically (Trülzsch et al., 2007). To elicit and maintain infection, *Yersinia* has evolved ingenious ways of manipulating host cell functions (Cornelis, 2006; Heesemann et al., 2006; Navarro et al., 2005). Central to the invasive phase of enteropathogenic *Yersinia* infection is the bacterial surface protein Invasin which triggers outside-in β1-integrin signalling, leading to bacterial permeation of intestinal cells (Wong and Isberg, 2005a). In the subsequent infection phases, yersiniae employ a sophisticated organelle, the type III secretion system (TTSS), to exert anti-phagocytic and immunosuppressive effects. The TTSS delivers a collection of effector proteins (Yersinia outer proteins; Yops) into host cells in order to interfere with and reorient various signalling processes (Aepfelbacher et al., 2007; Cornelis, 2006; Viboud and Bliska, 2005).

A great variety of bacterial toxins and modulins target Rho GTP-binding proteins, which play pivotal roles in cell regulation (such as organisation of the actin cytoskeleton). The actin cytoskeleton has crucial functions in the infection biology of pathogens, and in cells of innate and adaptive immunity (Aktories and Barbieri, 2005; Ivetic and Ridley, 2004; Vicente-Manzanares and Sanchez-Madrid, 2004). It is well established that the *Yersinia* stratagem modulates the activity of Rho GTP-binding proteins both for tissue invasion and subversion of the host immune system (Aepfelbacher et al., 2007). The Rho family members RhoA, Rac1 and Cdc42 are activated by Invasin-triggered integrin signalling and mediate cytoskeletal reorganisation, IL-8 release and bacterial invasion (Grassl et al., 2003; Wiedemann et al., 2001; Wong and Isberg, 2005a). By contrast, the cysteine protease YopT inactivates RhoA, Rac1 and Cdc42 by removing their C-terminal isoprenoid moiety (Shao et al., 2002). Furthermore, the serine/threonine kinase YopO/YpkA inhibits the activity of Rac1 and RhoA via a domain that structurally resembles guanine nucleotide dissociation inhibitors (GDIs) for Rho GTPases (Prehna et al., 2006). Finally, YopE mimics a GTPase activating protein (GAP) for Rho GTPases and has been reported to downregulate Rac1, RhoA and Cdc42 in infected cells (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000). The fact that several *Yersinia* virulence factors control an overlapping set of Rho GTPases by distinct mechanisms suggests that it is a major goal of the *Yersinia* stratagem to orchestrate Rho GTPase signalling networks and cascades.

It has been established that downregulation of Rac (Rac1 and presumably Rac2) mediates various immunosuppressive effects of YopE, such as inhibition of phagocytosis, IL-1β production and superoxide anion production (Aepfelbacher et al., 2007; Cornelis, 2002; Schotte et al., 2004; Viboud and Bliska, 2005). We have reported previously that the ability of YopE to inhibit Rac regulated actin structures depends on the signal pathway by which Rac is activated (Andor et al., 2001). Recent work shows that the combined action of YopT and YopE produces two distinct pools of Rac1: an active nuclear pool and an inactive cytosolic pool (Wong and Isberg, 2005b). Notably, YopE of some *Yersinia* serotypes can also be degraded by the host cell proteasome, which causes reactivation of Rac1 (Ruckdeschel et al., 2006). Together, these findings indicate that Rac is the preferred Rho GTPase target of the *Yersinia*

stratagem. This is comprehensible because Rac controls a myriad of cell functions and plays a crucial role in a number of Rho GTPase signalling cascades (Burridge and Wennerberg, 2004). One recently recognised way of upstream control of Rac1 is exerted through RhoG and the Elmo/Dock180 module. It has been established that binding of active RhoG to a complex of Elmo and Dock180 stimulates the GEF activity of Dock180, leading to GTP-loading of Rac1 (deBakker et al., 2004; Katoh and Negishi, 2003; Lu and Ravichandran, 2006). Interestingly, RhoG has been implicated in various cell functions known to be subdued by *Yersinia*, such as integrin-mediated phagocytosis, transendothelial migration and activation of NADPH oxidase (Condliffe et al., 2006; Nakaya et al., 2006; van Buul et al., 2007).

Here, we report that RhoG activity is modulated by *Y. enterocolitica*. We were able to visualise the spatiotemporal dynamics of RhoG activation and deactivation at the contact site of host cells and virulent *Yersinia*. The β1-integrin clustering protein Invasin was identified as RhoG activator and the Rho GTPase activating protein YopE as the specific RhoG inhibitor. Modulation of RhoG activity produced parallel changes in Rac1 activity. Thus, our data demonstrate a yin and yang of RhoG modulation by the *Yersinia* stratagem that may be adapted to the different phases of the *Yersinia* infection cycle in vivo.

Results

Differential modulation of RhoG activity by *Yersinia enterocolitica*

In order to investigate whether RhoG is regulated by *Y. enterocolitica*, we established a biosensor for monitoring active GTP-bound RhoG in cells. It has been reported that the armadillo repeats at the N-terminus of Elmo (Elmo2NT) constitute a binding domain for GTP-bound RhoG but not for other Rho GTPases (deBakker et al., 2004; Katoh and Negishi, 2003). We confirmed by pulldown that GST-Elmo2NT binds to constitutively active RhoGV12 but not to other constitutively active Rho GTPases or to dominant-negative RhoGN17 (Fig. 1). Upon co-expression with RhoGV12, GFP-Elmo2NT redistributed to the perinuclear area of human umbilical vein endothelial cells (HUVEC) where it colocalised with RhoGV12 (Fig. 2). By contrast, co-expression with RhoGN17 did not alter the normally diffuse cellular distribution of GFP-Elmo2NT (Fig. 2). Moreover, the RhoG guanine nucleotide exchange factors (GEFs) TrioD1 and SH3-containing guanine nucleotide exchange factor (SGEF) caused translocation of GFP-RhoG and GFP-Elmo2NT to dorsal ruffles in HUVEC (Fig. 2; see Fig. S1 in the supplementary material). TrioD1 and SGEF are RhoG-specific GEFs that have been shown to induce GTP-loading and activation of RhoG in vitro and in cells (Blangy et al., 2000; Ellerbroek et al., 2004). Rac1V12 and the Rac-specific GEF Tiam1 did not induce GFP-Elmo2NT redistribution in HUVEC (B.R., A.R., M.H., K.R. and M.A., unpublished). Together, these experiments indicate that GFP-Elmo2NT faithfully monitors the localisation of active RhoG in cells.

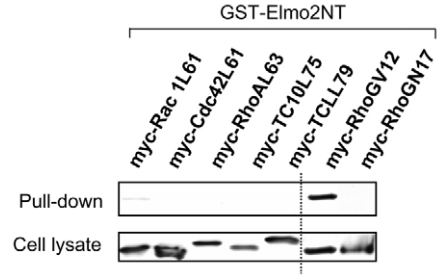


Fig. 1. GST-Elmo2NT specifically binds to active RhoG. Cos-7 cells were transfected with indicated myc-tagged Rho GTPase constructs, lysed and subjected to pull-down using GST-Elmo2NT. Proteins from pull-down and in cell lysates were detected by western blotting using anti-Myc antibody.

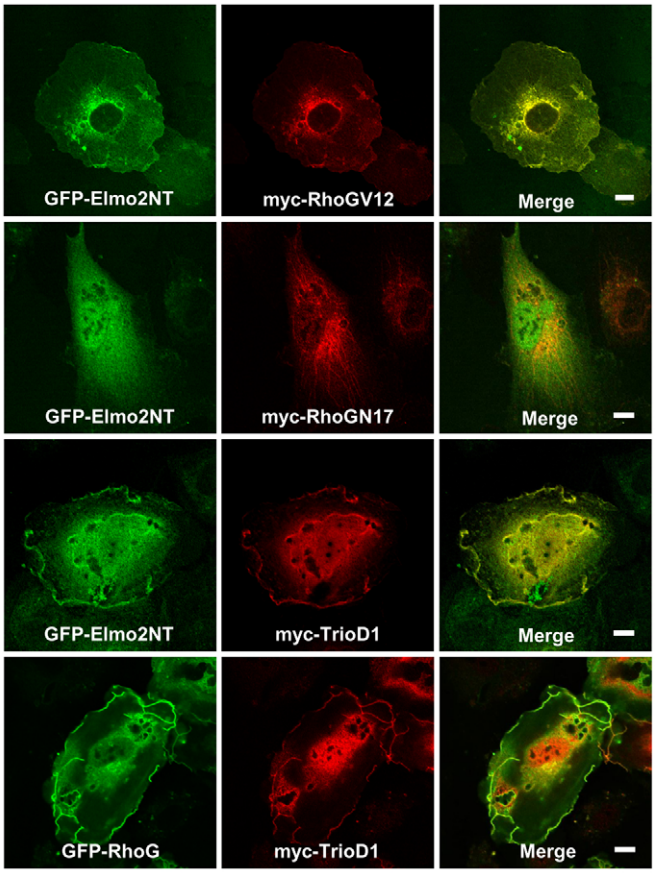
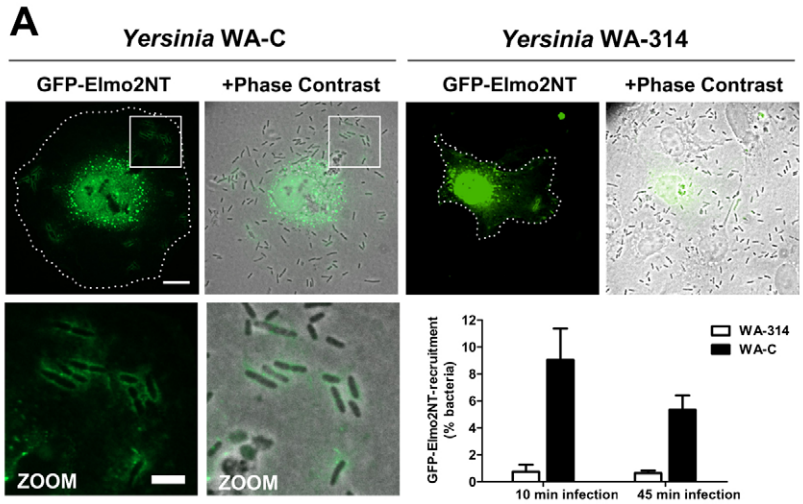


Fig. 2. Specific intracellular redistribution of GFP-Elmo2NT by active RhoG. Confocal fluorescence images of HUVEC co-transfected with GFP-Elmo2NT and myc-RhoGV12, myc-RhoGN17 or myc-TrioD1. GFP-RhoG was co-transfected with myc-TrioD1. Merge represents overlays of green and red fluorescence channel resulting in yellow colour. Myc-tagged constructs were stained with anti-myc antibody. GFP-Elmo2NT colocalises with constitutively active RhoGV12 but not with dominant-negative RhoGN17. GFP-Elmo2NT and GFP-RhoG translocate to TrioD1-induced dorsal ruffles. Bar, 10 μm.

On this basis, we analysed GFP-Elmo2NT redistribution in HUVEC infected with virulent *Yersinia* WA-314 or non-virulent *Yersinia* WA-C. *Yersinia* WA-314 adheres to host cells mainly via the β1-integrin binding protein Invasin and the extracellular matrix binding protein YadA, and translocates six effector Yops (YopH, YopO, YopP, YopE, YopM and YopT) via its TTSS. By comparison, *Yersinia* WA-C interacts with host cells via Invasin but is devoid of YadA, the TTSS and Yops (Table 1). GFP-Elmo2NT was recruited by about 10 and 5% of *Yersinia* WA-C bacteria but only by about 1 and 0.5% of *Yersinia* WA-314 bacteria after 10 and 45 minutes of infection, respectively (Fig. 3A). An Invasin-negative WA-C strain (WA-C inv–; Table 1) did not produce GFP-Elmo2NT cups and conversely an *E. coli* strain that heterologously expresses Invasin (*Invasin-E.coli*; Table 1) produced prominent GFP-Elmo2NT phagocytic cups. Furthermore, no GFP-Elmo2NT cups

Table 1. Bacterial strains

Strain	Relevant characteristic	Reference
Yersinia		
WA-314	Wild-type strain; serogroup O8; clinical isolate harboring the virulence plasmid pYVO8	(Heesemann and Laufs, 1983)
WA-C	Plasmidless derivative of strain WA-314	(Heesemann and Laufs, 1983)
WA-C inv-	WA-C with insertional inactivation of *inv*	(Ruckdeschel et al., 1996)
E40	Wild-type strain; serogroup O9; clinical isolate harboring the virulence plasmid pYV40	(Sory et al., 1995)
WA-CpTTSS	Strain WA-C harboring plasmid pTTSS, encoding for secretion and translocation apparatus of WA-314	(Trülzsch et al., 2003)
WA-C(pTTSS+pYopE)	WA-CpTTSS complemented with wild-type *yopE* from strain E40	(Hentschke et al., 2007)
WA-C(pTTSS+pYopER144A)	WA-CpTTSS complemented with *yopE* in which arginine 144 was replaced by alanine	(This study)
WA-C(pTTSS+pYopEΔMLD)	WA-CpTTSS complemented with *yopE* in which MLD (aa 54-75) was deleted	(This study)
WA-314ΔyopE	Wild-type strain with insertional inactivation of *yopE*	(Zumbihl et al., 1999)
WA-314ΔyopH	Wild-type strain with insertional inactivation of *yopH*	(Gaede and Heesemann, 1995)
WA-314ΔyopM	Wild-type strain with insertional inactivation of *yopM*	(Trülzsch et al., 2004)
WA-314ΔyopO	Wild-type strain with insertional inactivation of *yopO*	(Trülzsch et al., 2004)
WA-314ΔyopP	Wild-type strain with insertional inactivation of *yopP*	(Trülzsch et al., 2004)
WA-314ΔyopT	Wild-type strain with insertional inactivation of *yopT*	(Zumbihl et al., 1999)
E. coli		
Invasin-E.coli	HB101-Inv+ *E.coli*, constitutively expressing invasin on bacterial surface	(Schulte et al., 1998)
YadA-E.coli	DH5α-YadA+ *E.coli*, constitutively expressing YadA on bacterial surface	(Roggenkamp et al., 1995)



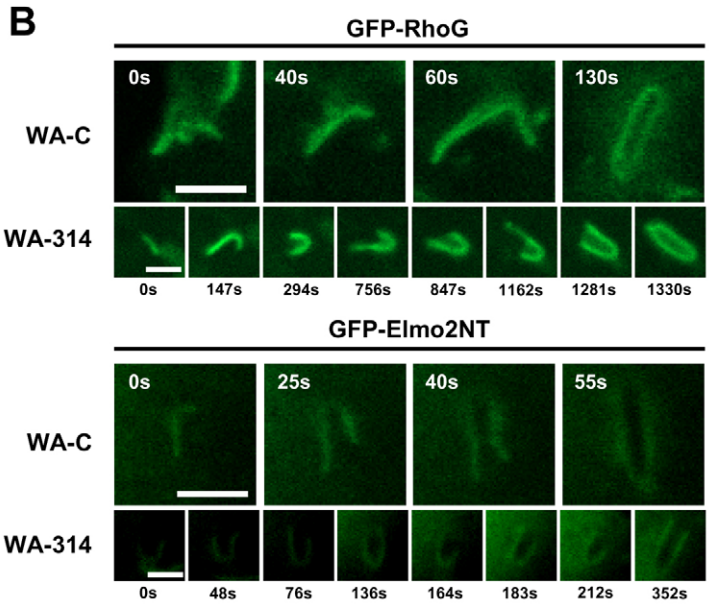


Fig. 3. Differential recruitment and activation of RhoG by *Y. enterocolitica*. (A) HUVEC were transfected with GFP-Elmo2NT and then infected with *Yersinia* WA-C or *Yersinia* WA-314 for 10 minutes. Images show representative cells that display recruitment and essentially no recruitment of GFP-Elmo2NT, respectively. The WA-314-infected cell shows severe morphological alteration owing to Yop action. Merged images represent overlays of green fluorescence and phase-contrast channel. White boxes indicate areas that were enlarged about fourfold. Broken lines outline the cell borders. The graph shows quantification of GFP-Elmo2NT recruitment by indicated strains 10 and 45 minutes after infection. Percentage of total cell-associated bacteria showing GFP-Elmo2NT enriched at phagocytic cups was determined microscopically. Each bar represents mean±s.d. (error bars) of three different experiments with at least 33 cells analysed per experiment. Bars, 10 μm (upper panel) and 3 μm (lower panel). (B) HUVEC were transfected with GFP-RhoG (upper panel) or GFP-Elmo2NT (lower panel), and then infected with *Yersinia* WA-314 or WA-C. GFP-RhoG or GFP-Elmo2NT recruitment was visualised using live-cell imaging. Depicted still images were taken from representative movies at indicated time points (see also supplementary material Movie 1). Cells infected with WA-C show rapid closure of GFP-RhoG- and GFP-Elmo2NT phagocytic cups. By contrast, cells infected with WA-314 show severely impaired GFP-RhoG and GFP-Elmo2NT recruitment to phagocytic cups with alternating back and forth movements of the respective fluorescent proteins. Bars, 2 μm.

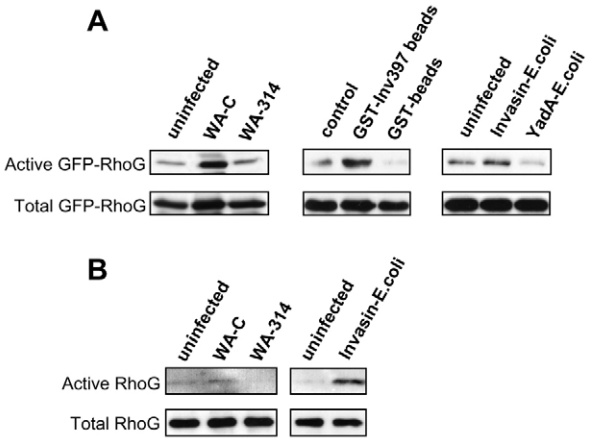


Fig. 4. RhoG is activated by *Yersinia* Invasin. (A) Cos-7 cells were transfected with GFP-RhoG and infected with *Yersinia* WA-C or WA-314 (left panel), reacted with beads coated with GST-Invasin397 or GST (middle panel) or infected with *Invasin-E. coli* or *YadA-E. coli* for 30 minutes (right panel). Active GFP-RhoG was precipitated with GST-Elmo2NT and detected by western blotting using anti-GFP antibody. (B) HeLa cells were infected with *Yersinia* WA-C or WA-314 (left panel) or *Invasin-E. coli* (right panel) for 30 minutes, and active endogenous RhoG was precipitated with GST-Elmo2NT and detected by western blotting.

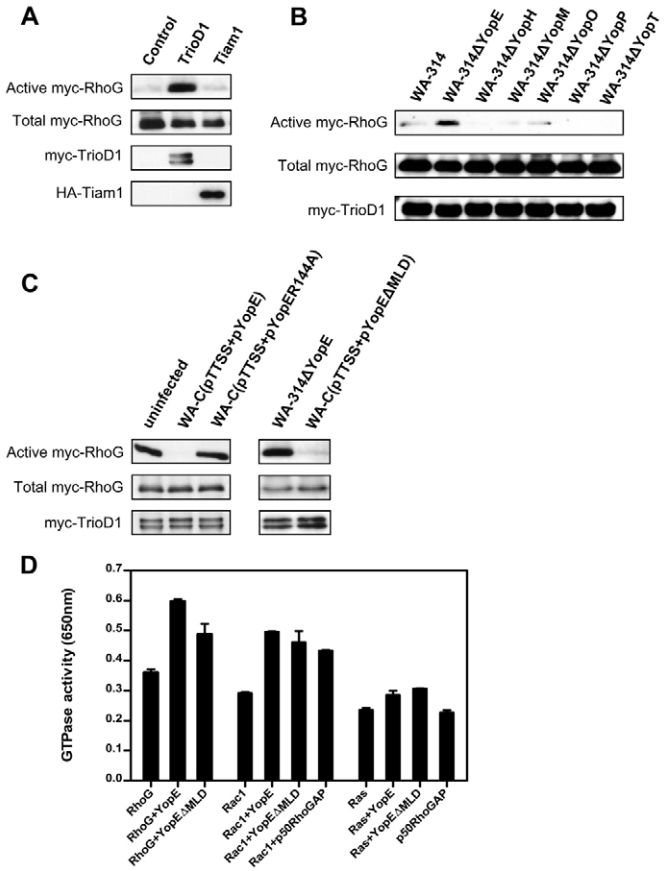


Fig. 5. RhoG is inactivated by YopE. (A) Cos-7 cells were co-transfected with myc-RhoG and either control plasmid (left lane), myc-TrioD1 (middle lane) or HA-Tiam1 (right lane). Active myc-RhoG was precipitated with GST-Elmo2NT and analysed by western blotting. Myc-RhoG and myc-TrioD1 were detected with anti-myc antibodies and HA-Tiam1 with anti-HA antibody. TrioD1 but not Tiam1 produced a strong RhoG activation. (B) Cos-7 cells were co-transfected with myc-RhoG and myc-TrioD1. After 24 hours, cells were infected with indicated *Yersinia* strains for 2 hours, lysed and subjected to GST-Elmo2NT pull-down. RhoG is downregulated by all strains except by ΔYopE strain. (C) Experiments were performed as in B but cells were infected with indicated bacterial strains. (D) In vitro GAP assay. GTP-hydrolysis was measured at 650 nm absorbance after co-incubation of indicated proteins for 20 minutes. YopE and YopEΔMLD stimulated the GTPase activity of RhoG and Rac1 but not of Ras. p50RhoGAP was used as a positive control. Values are mean±s.d. (error bars) of three independent experiments.

could be seen in β1-integrin$^{-/-}$ GD25 cells but in β1-integrin recomplemented GD25-β1A cells (B.R., A.R., M.H., K.R. and M.A., unpublished) (Wennerberg et al., 1996).

Thus, we conclude that *Yersinia* activates RhoG at the bacteria cell contact site by Invasin-triggered β1-integrin signalling. Our data further indicate that virulent yersiniae suppress RhoG, most probably through one or more TTSS translocated Yops.

To visualise the spatiotemporal dynamics of RhoG activation, we performed live imaging of *Yersinia*-infected HUVEC expressing fluorescent RhoG and/or Elmo2NT. The recordings indicate that GFP-RhoG rapidly and continuously encloses single bacterial rods of *Yersinia* WA-C. Formation of a full circumferential phagocytic cup took about 1-2 minutes (mean 70±54 seconds, n=3) (single frames of a representative movie are presented in Fig. 3B). Endogenous RhoG also translocated to the *Yersinia* WA-C cell contact site (supplementary material Fig. S2A). In sharp contrast to the fast and continuous recruitment of GFP-RhoG by the WA-C bacteria, GFP-RhoG appeared to alternatively advance and recede along the rods of *Yersinia* WA-314. This back and forth process could take up to 23 minutes (14.6±8.5 minutes, n=3) (single frames of a representative movie are shown in Fig. 3B) (see also supplementary material Movie 1), although in all cases recorded it eventually led to formation of a closed phagocytic cup. Such a disordered and protracted phagocytic cup assembly could also be seen with GFP-Elmo2NT (Fig. 3B). These data therefore visualise subversion of RhoG activation at the bacteria cell contact site. Further experiments revealed that mCherry-RhoG and GFP-Elmo2NT translocated in parallel to *Yersinia* WA-C. Whereas GFP-Elmo2NT stayed at the phagocytic cups only until phagocytic cup closure, GFP-RhoG remained there for extended time periods (B.R., A.R., M.H., K.R. and M.A., unpublished). We conclude that RhoG is recruited by the bacteria in its activated form, is deactivated shortly after phagocytic cup closure but then remains at the nascent phagosome.

To further verify regulation of RhoG activity by *Y. enterocolitica*, we performed GST-Elmo2NT pull-down assays. As demonstrated in Fig. 4A, infection of GFP-RhoG transfected Cos-7 cells with *Yersinia* WA-C, but not *Yersinia* WA-314, produced an increased level of GTP-bound GFP-RhoG. Activation of GFP-RhoG was also detected in Cos-7 cells treated with GST-Invasin-397 beads or *Invasin-E. coli* but not with *YadA-E. coli* (Fig. 4A). YadA is a matrix binding adhesin of enteropathogenic yersiniae (Roggenkamp et al., 1995). In HeLa cells, which express relatively high levels of endogenous RhoG, we could show activation of endogenous RhoG by Yersinia WA-C but not WA-314. Endogenous RhoG was also activated by *Invasin-E. coli* in these cells (Fig. 4B). Together these experiments indicate that in *Yersinia* infected cells RhoG is activated by Invasin-induced signalling but then downregulated by a TTSS-translocated Yop effector.

RhoG is deactivated by *Yersinia* YopE

To identify the Yop(s) responsible for downregulation of RhoG, we sought a cell system that displays robust RhoG activity. For this, we co-transfected Cos-7 cells with vectors expressing RhoG (myc-RhoG) and TrioD1 (myc-TrioD1). GST-Elmo2NT pull-down proved that TrioD1 strongly activated RhoG, whereas the Rac-specific exchange factor Tiam1 (HA-Tiam1) did not (Fig. 5A). Next, RhoG activity was determined in cells infected with *Yersinia* WA-314 or *Yersinia* mutants deficient in individual Yops (ΔE, ΔH, ΔM, ΔO, ΔP or ΔT; see Table 1). *Yersinia* WA-314 and all *Yersinia* mutants, except for the ΔYopE mutant, downregulated TrioD1-stimulated RhoG activity (Fig. 5B). Thus, we conclude that YopE is crucially involved in RhoG deactivation.

To test whether YopE is sufficient to inactivate RhoG and whether this depends on its GAP function, we constructed and employed *Yersinia* strains WA-C(pTTSS+pYopE) or WA-C(pTTSS+pYopER144A). These strains do not possess YopH, YopO, YopP, YopM or YopT, and solely translocate YopE or the GAP-defective YopER144A (Trülzsch et al., 2003). The results showed that WA-C(pTTSS+pYopE) abolished TrioD1-stimulated RhoG activity, whereas WA-C(pTTSS+pYopER144A) had no effect (Fig. 5C, left panel). Together, these data show that YopE is both necessary and sufficient to downregulate RhoG activity in *Y. enterocolitica*-infected cells.

YopE has not formally been shown to operate as a GAP for RhoG, although our results are consistent with this notion. To demonstrate that YopE stimulates the intrinsic GTPase activity of RhoG, we performed an in vitro GTP-hydrolysis assay. The data presented in Fig. 5D clearly demonstrate that YopE is a bona fide GAP for RhoG and acts as efficiently on RhoG as on Rac1 in vitro.

Having identified YopE as a potent deactivator of RhoG, we next tested whether YopE is sufficient to inhibit RhoG recruitment by *Yersinia*. HUVEC-expressing EGFP-RhoG were infected with WA-C(pTTSS+pYopE) or WA-C(pTTSS+pYopER144A), and GFP-RhoG recruitment to phagocytic cups was evaluated. A time-dependent 30 to 50% reduction of RhoG recruitment by the *Yersinia* strain translocating YopE when compared with the strain translocating YopER144A was detected (Fig. 6). Consistently, recruitment of GFP-Elmo2NT was reduced by more than 50% by the YopE-translocating versus the YopER144A-translocating *Yersinia* strain (supplementary material Fig. S5). In summary, we conclude that YopE is a specific and highly effective inihibitor of RhoG activity during *Yersinia* infection.

Intracellular localisation determines substrate specificity of YopE

YopE has been reported to localise to a perinuclear compartment identified as endoplasmatic reticulum (ER) (Krall et al., 2004). By testing colocalisation of GFP-YopER144A with markers of intracellular membrane compartments, we verified that a fraction of intracellular YopE colocalises with the ER marker calreticulin (Fig. 7A). Yet, we also detected colocalisation of YopE with the Golgi markers GS27 and p230 (Fig. 7A; B.R., A.R., M.H., K.R. and M.A., unpublished). Sensitivity to Brefeldin-A further confirmed the Golgi localisation of YopE (supplementary material Fig. S3A). The localisation of bacterially translocated YopER144A was similar to that of vector expressed GFP-YopER144A (supplementary material Fig. S3B). These experiments were performed with the GAP-defective YopE mutant YopER144A because native YopE causes severe morphological alteration of cells hampering visualisation of intracellular structures. We next tested for colocalisation of mCherry-YopER144A with GFP-RhoG, -Rac1, -RhoA, -TC10 or -Cdc42. Both the Golgi- and ER-localised mCherry-YopER144A colocalised extensively with GFP-RhoG (Fig. 7B). A limited colocalisation of mCherry-YopER144A with GFP-Rac1 was observed (Fig. 7B). Furthermore, we found an extensive colocalisation of mRFP-YopER144A with GFP-Cdc42, but essentially no colocalisation with GFP-TC10 or GFP-RhoA (Fig. 7B; B.R., A.R., M.H., K.R. and M.A., unpublished). It therefore appears that YopE associates with its primary target RhoG, with Cdc42, but only barely with Rac1 at the ER and Golgi of host cells.

Previously, a region comprising amino acid residues 54-75 (termed membrane localisation domain; MLD) was found to mediate intracellular membrane binding of YopE (Krall et al., 2004). Consistently, a YopE construct lacking the MLD (GFP-YopER144AΔMLD) displayed a cytoplasm-like fluorescence and no signs of Golgi/ER localisation (supplementary material Fig. S3C). Recombinant YopEΔMLD showed the same specificity and activity as native YopE in the in vitro GAP assay (Fig. 5D). In order to clarify the function of YopE compartmentalisation, we tested the effect of WA-C(pTTSS+pYopEΔMLD) on TrioD1-activated RhoG in Cos-7 cells. As demonstrated in Fig. 5C, right and left panels, YopEΔMLD and YopE were equally effective in downregulating RhoG. Furthermore, Cdc42 was also equally well deactivated by YopE and YopEΔMLD (supplementary material Fig. S4A). However, whereas YopEΔMLD abrogated baseline RhoA activity in Cos-7 cells YopE did not have an effect on RhoA (Fig. 8).

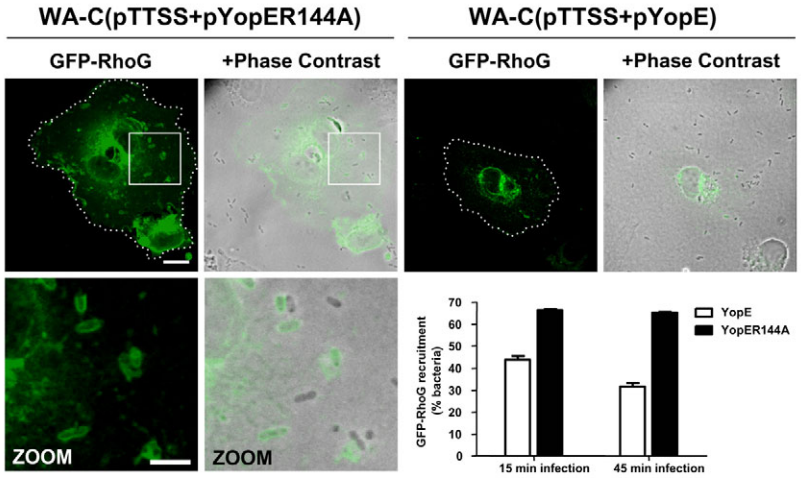


Fig. 6. YopE reduces RhoG recruitment to *Yersinia*. HUVEC were transfected with GFP-RhoG and then infected with *Yersinia* WA-C(pTTSS+pYopER144A) or *Yersinia* WA-C(pTTSS+pYopE). Images show GFP-RhoG distribution (green fluorescence) after 15 minutes of infection. Merged images represent overlays of green fluorescence channel and phase contrast. White boxes indicate areas that were enlarged about fourfold. Broken lines outline the cell borders. For the bar chart, GFP-RhoG recruitment by indicated strains was quantified after 15 and 45 minutes of infection. Each bar represents mean±s.d. (error bars) of three different experiments with at least 33 cells analysed per experiment. Bars, 10 μm (upper panels) or 3 μm (lower panels).

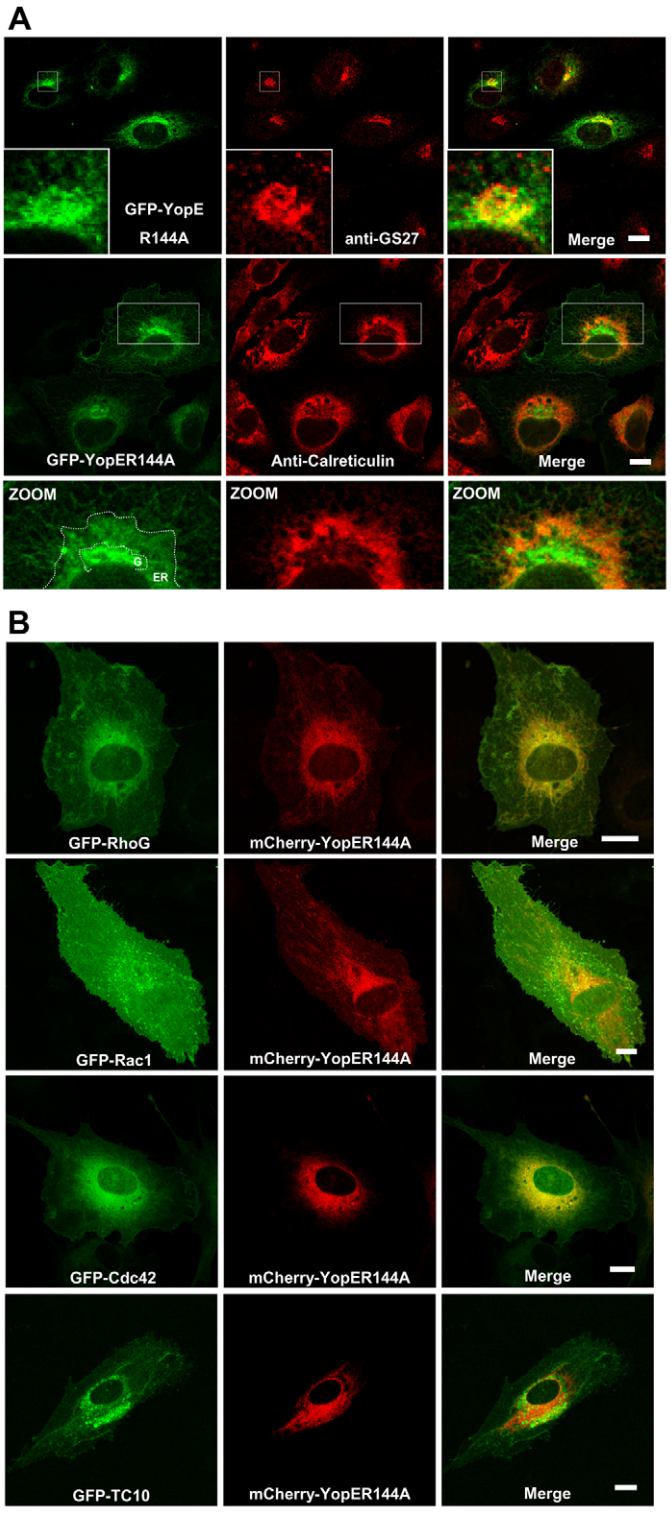


Fig. 7. YopE and RhoG colocalise in ER and Golgi. (A) Confocal fluorescence images of HUVEC transfected with GFP-YopER144A. Golgi was immunostained with anti-GS27 antibody and ER with anti-calreticulin antibody. Merged images represent overlays of green and red channels, resulting in yellow colour. Boxes indicate areas that were enlarged two- to threefold and shown in insets or bottom panels (ZOOM). In the bottom-left panel, the putative Golgi- and ER localisation of YopER144A is outlined. Bars, 10 µm. (B) Confocal fluorescence images of HUVEC co-transfected with mCherry-YopER144A and GFP-RhoG, GFP-Rac1, GFP-Cdc42 and GFP-TC10. Merges represent overlays of green and red channel. Bars, 10 µm.

Similarly, YopEΔMLD could effectively deactivate TC10, whereas YopE had only a minor effect on TC10 (supplementary material Fig. S4B). Together, these experiments suggest that the substrate range and activity of YopE is crucially determined by its compartmentalisation in the Golgi/ER.

RhoG controls activation of Rac1 by Invasin and deactivation of Rac1 by YopE

The question arises of how does modulation of RhoG fit in the virulence strategy of *Yersinia*? It is noticeable that RhoG can control Rac1 via the Elmo/Dock180 module and that Rac1 is the preferred Rho GTPase target of *Yersinia* (Aepfelbacher et al., 2007; Katoh and Negishi, 2003). We thus asked whether RhoG mediates the effects of the *Yersinia* stratagem on Rac1. To answer this question, Cos-7 cells in which RhoG was knocked down by RNAi were infected with WA-C or *Invasin- E. coli* and Rac1 activity was determined by GST-PAK-CRIB pulldown. Knock down of RhoG not only inhibited baseline Rac1 activity but also blocked Invasin-stimulated Rac1 activation almost quantitatively (Fig. 9A,B). Inhibition of baseline and Invasin-stimulated Rac activation could also be obtained by expression of dominant-negative Elmo-T618 in Cos-7 cells (B.R., A.R., M.H., K.R. and M.A., unpublished) (Katoh and Negishi, 2003). The finding that cell invasion of *Yersinia* WA-C was reduced by RhoG RNAi proves the functional significance of the RhoG signal pathway (Fig. 9C).

We next investigated to what extent YopE-induced Rac1 inhibition is in fact mediated by downregulation of RhoG and the Elmo/Dock180 module. Infection of Cos-7 cells with WA-C(pTTSS+pYopE) largely reduced the baseline activation level of Rac1 (Fig. 9D). As already found for knock down of RhoG by RNAi (Fig. 9A), expression of Elmo-T618 reduced the baseline level of activated Rac1 in Cos-7 cells (Fig. 9D). Thus, YopE, RhoG RNAi and dominant-negative Elmo-T618 induce a similar degree of Rac1 inhibition. Notably, infection of the Elmo-T618-expressing cells with WA-C(pTTSS+pYopE) did not produce a further reduction of Rac1 activity (Fig. 9D). We propose that under our experimental conditions YopE downregulates baseline Rac1 activity for the most part through inhibition of RhoG. By comparison, RhoA activity was neither affected by YopE nor by Elmo-T618 (B.R., A.R., M.H., K.R. and M.A., unpublished).

To clarify whether or not YopE can directly inactivate Rac1, we induced Rac1 activation in HUVEC by expression of the Rac-specific exchange factor Tiam1. Infection with WA-C(pTTSS+pYopE) caused inhibition but not abrogation of Tiam-induced Rac1 activity (Fig. 9E). These data clearly show that YopE

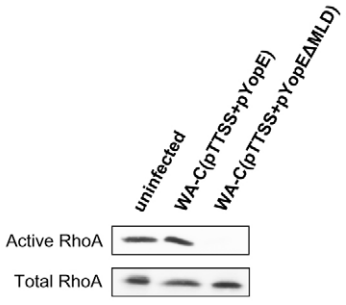


Fig. 8. YopEΔMLD but not native YopE inactivates RhoA. Active RhoA was precipitated by GST-Rhotekin from Cos-7 cells infected with *Yersinia* strains WA-C(pTTSS+pYopE) or WA-C(pTTSS+pYopEΔMLD) for 2 hours. RhoA was detected by western blotting using anti-RhoA antibody.

can directly inhibit Rac1 activity by competition with Rac exchange factors.

We conclude that YopE inactivates Rac1 by direct and indirect mechanisms. Clearly, YopE can compete with Rac GEFs and can directly stimulate GTP-hydrolysis in Rac1. In addition, YopE can induce GTP-hydrolysis in RhoG and thereby downregulate Elmo/Dock180 signalling to Rac1, a mechanism that appears to play a prominent role in *Yersinia* infection.

Together these experiments suggest that by regulating RhoG *Yersinia* can exert an additional way of control over the Rac GTPase.

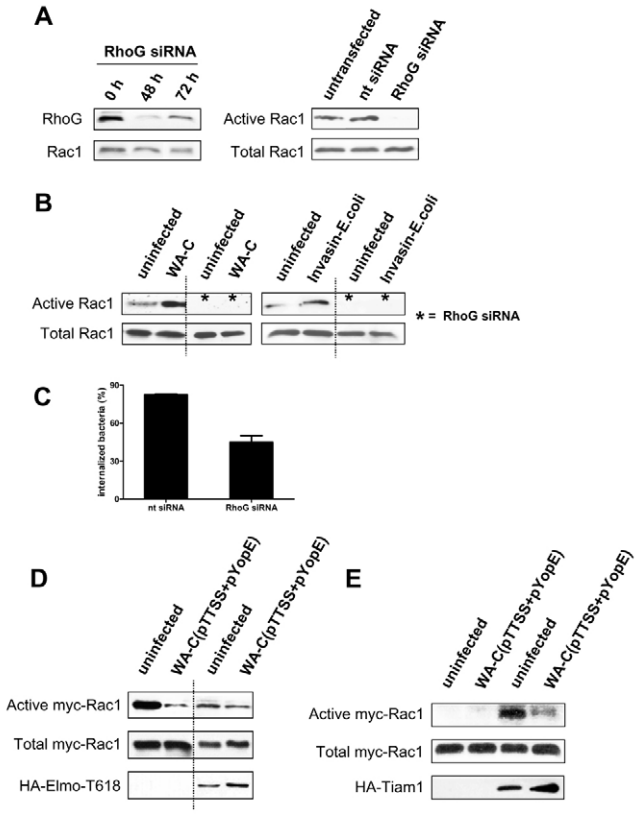


Fig. 9. Invasin stimulates and YopE inhibits Rac1 activity via RhoG. (A) Cos-7 cells were transfected with siRNA for RhoG and at indicated time points equal amounts of protein were analysed by western blotting using anti-RhoG and anti-Rac1 antibody. RhoG siRNA effectively reduced the level of endogenous RhoG protein at 48 hours but had no effect on Rac1 protein level (left panel). Cos-7 cells were transfected with non-targeting (nt) siRNA or with RhoG siRNA for 48 hours and then subjected to GST-PAK-CRIB pull-down assay. Rac1 was detected by western blotting using anti-Rac1 antibody (right panel). (B) HUVEC were left untransfected or transfected with RhoG siRNA (indicated by stars) for 48 hours and then infected or not with WA-C or *Invasin-E. coli* for 15 minutes. The level of active Rac1 was determined by GST-PAK-CRIB pull-down and western blotting using anti-Rac1 antibody. (C) HUVEC were transfected with RhoG siRNA or non-targeting (nt) siRNA for 48 hours and then infected with *Yersinia* WA-C for 60 minutes. Invasion of bacteria was determined using a double fluorescence method (see Materials and Methods). Bars represent mean±s.d. (error bars) of three independent experiments with at least 60 cells analysed per experiment. (D) Cos-7 cells were transfected with myc-Rac1 (lane 1 and 2) or co-transfected with myc-Rac1 and HA-Elmo-T618 (lane 3 and 4) and then infected with WA-C(pTTSS+pYopE) for 2 hours. The level of active myc-Rac1 was determined by GST-PAK-CRIB pull-down and western blotting using anti-myc antibody. HA-Elmo-T618 was detected with anti-HA antibody. (E) HUVEC were transfected with plasmids expressing myc-Rac1 (lane 1 and 2) or myc-Rac1 and HA-Tiam1 (lane 3 and 4), and infected with *Yersinia* strain WA-C(pTTSS+pYopE) for 2 hours. The level of active Rac1 was determined by GST-PAK-CRIB pull-down as described in D.

This most certainly will affect multiple host cell processes during *Yersinia* infection.

Discussion

We report here that *Y. enterocolitica* differentially regulates the activation level of the Rho GTPase RhoG. RhoG becomes activated by Invasin-triggered β1-integrin signalling and this mediates bacterial invasion. RhoG has previously been implicated in phagocytosis and bacterial invasion. A signalling pathway involving RhoG, its GEF Trio and the Rac GEF-complex Elmo/Dock180 was shown to regulate phagocytosis of apoptotic cells (deBakker et al., 2004). The TTSS effector SopB of *Salmonella enterica* was reported to activate RhoG via SGEF and thereby stimulate actin remodelling (Patel and Galan, 2006). Furthermore, the *Shigella* effector IpgB1 mimics RhoG to activate Elmo/Dock180, which induces membrane ruffling and bacterial invasion (Handa et al., 2007). Similarly, our data suggest that Invasin-triggered integrin signalling causes activation of the RhoG/Elmo/Dock 180 module. Hence, RhoG evolves as a central target of bacterial virulence factors promoting host cell invasion. Clearly, besides the RhoG/Rac1 signalling system, RhoA- and Cdc42-regulated pathways can also contribute to bacterial invasion (Wiedemann et al., 2001; Patel and Galan, 2006). RhoG may exclusively act through its close relative Rac1 for mediating bacteria-induced cell responses. However, RhoG may also have an individual function in bacterial invasion given the reported Rac1-independent RhoG effects on the cytoskeleton (Meller et al., 2008) and on intracellular vesicle transport (Prieto-Sanchez et al., 2006; Tanaka et al., 2007).

For visualising the spatiotemporal dynamics of RhoG activation at the *Yersinia* cell contact site, we employed spinning disc microscopy of GFP-Elmo2NT. Several pieces of evidence indicate that localisation of intracellular GFP-Elmo2NT properly monitors active RhoG. Elmo2NT binds to active RhoG but not to other Rho GTPases. Constitutively active, but not dominant-negative, RhoG induced GFP-Elmo2NT redistribution to Golgi and ER, and two RhoG-GEFs stimulated its redistribution to dorsal ruffles. GFP-Elmo2NT and mCherry-RhoG were co-recruited to phagocytic cups. The finding that, shortly after phagocytic cup closure, GFP-Elmo2NT vanished whereas mCherry-RhoG remained at the phagosome, supports the notion that Elmo2NT associates with an active subfraction of RhoG that functions in the early phases of phagocytosis/invasion (B.R., A.R., M.H., K.R. and M.A., unpublished).

Live cell imaging revealed that virulent *Y. enterocolitica* considerably protracts phagocytic cup assembly and causes an artificial alternation of RhoG activation and deactivation. We could identify YopE as the sole RhoG inhibitor of virulent yersiniae. YopE reportedly has multiple effects on host cell regulation, such as inhibition of phagocytosis, IL-1β production and superoxide anion production (Aepfelbacher et al., 2007; Cornelis, 2002; Schotte et al., 2004; Viboud and Bliska, 2005), and it also affects Yop translocation by regulating the TTSS pore (Mejia et al., 2008). Although most YopE effects have been attributed to inhibition of Rac1 (Aepfelbacher et al., 2007; Cornelis, 2002; Schotte et al., 2004; Viboud and Bliska, 2005) and we in fact confirmed that YopE can directly inhibit Rac1, we demonstrate here that YopE abrogates baseline- and Invasin-stimulated Rac1 activity by inhibition of RhoG. Hence, there are at least two mechanisms by which YopE can modulate Rac1 activity in host cells: by direct downregulation of Rac1 and by blocking RhoG and the ensuing Elmo/Dock180 signalling to Rac1. Together with the previous findings that: (1)

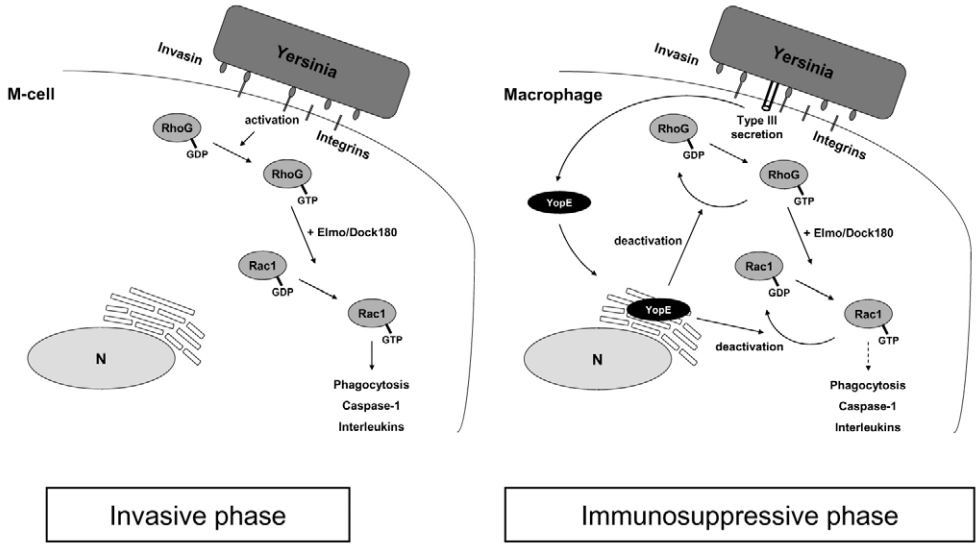


Fig. 10. Model for modulation of RhoG activity by *Yersinia*. During the invasive phase of infection, *Yersinia* binds to integrins via Invasin, which triggers activation of RhoG and stimulation of Rac1 through the Elmo/Dock180 module. Activation of RhoG and Rac1 leads to cellular responses, such as invasion, phagocytosis, caspase 1 activation and production of interleukins. During the subsequent immunosuppressive phase, *Yersinia* injects effector Yops through its TTSS. The effector YopE acting as a GAP inactivates RhoG and Rac1, and reverses the specific cellular responses.

some signalling pathways to Rac are not blocked by YopE; (2) cooperation of *Yersinia* YopE and YopT can lead to differentially activated and localised pools of Rac in cells; and (3) YopE of some *Yersinia* serotypes is degraded in host cells leading to reactivation of Rac1, a highly sophisticated strategy evolves by which *Yersinia* manipulates the Rac1 signalling network (Andor et al., 2001; Hentschke et al., 2007; Ruckdeschel et al., 2006; Wong and Isberg, 2005b).

We also identified a function for the Golgi and ER localisation of YopE. YopE lacking its membrane localisation domain (YopEΔMLD) was at least as active as the wild-type YopE in inhibiting RhoG and Cdc42, and newly acquired the capability to inhibit RhoA. In this regard, YopE behaves differently from its homolog ExoS from *Pseudomonas aeruginosa*, for which the substrate range is restricted upon removing the MLD (Zhang et al., 2007). Interestingly, phospholipids can switch the substrate specificity of GAPs (Ligeti et al., 2004). Thus, the different lipid environments of membrane-bound YopE versus cytoplasmic YopEΔMLD may also influence their Rho GTPase specificities.

In summary, we propose that during the invasive phase of *Yersinia* infection, when Yops are not yet expressed, Invasin-triggered RhoG activation mediates bacterial internalisation, and this occurs at least partly by stimulating Rac1 via the Elmo/Dock180 module. During the subsequent antiphagocytic and immunosuppressive phase of the infection, RhoG also becomes activated by *Yersinia* but this may be triggered by a phagocytosis program of cells of innate immunity. At this point, RhoG and Rac1 are downregulated by YopE in a compartmentalised and differential fashion (Fig. 10). Rac1, which has pleiotropic functions in the biology of *Yersinia* infection and in immune cells, is accessorily modulated by YopT and YopO. It is likely that YopE, YopT and YopO synergise in producing differentially active and compartmentalised subsets of Rac1 and potentially of other Rho GTPases in host cells. Using this strategy, *Yersinia* may allow Rho GTPase activation in selective cell compartments and signalling pathways, and in parallel downregulate Rho GTPases in other pathways. For example, by leaving open the bacterial translocation pore, Rac1 activity may be admitted whereas, in parallel, Rac1 pathways that regulate immune cell responses are inhibited. The relevance of such strategies for the in vivo infection process of *Yersinia* is speculative at the moment but needs to be investigated in future studies.

Materials and Methods

Cell culture and transfection

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously (Essler et al., 2003) and cultured in endothelial cell growth medium (PromoCell, Heidelberg, Germany) containing 2% FCS at 37°C with 5% CO_2. HeLa and Cos-7 cells were cultured in either RPMI medium (GIBCO, Karlsruhe, Germany) or DMEM medium (GIBCO, Karlsruhe, Germany) supplemented with 10% FCS (Sigma-Aldrich, Munich, Germany). GD25 cells and GD25-β1A cells were cultured in DMEM medium supplemented with 10% FCS with 10 μg/ml puromycin added to the GD25-β1A cells (Wennerberg et al., 1996). HUVEC were transiently transfected with Amaxa Nucleofector system II according to the manufacturer instructions (Amaxa, Cologne, Germany). HeLa-, Cos-7-, GD25- and GD25-β1A cells were transiently transfected with ExGen500 as specified by the manufacturer (Fermentas, St Leon-Rot, Germany).

Bacterial strains and cell infection

For cell infection with *Y. enterocolitica* strains (Table 1), overnight bacterial cultures grown at 27°C in Luriani-Bertani broth (LB) containing the appropriate antibiotics were diluted 1:20 in fresh LB and grown for additional 90 minutes at 37°C to allow for expression of the type III secretion machinery and Yops. For infection with *E. coli* HB101 inv+ and DH5α-yadA+, bacteria were grown overnight at 37°C in LB containing the appropriate antibiotics, diluted 1:20 in fresh LB and grown for additional 2 hours at 37°C. To synchronize infection, bacteria were attached to cells by centrifugation at 200 ***g*** for 2 minutes at a ratio of 10 to 100 bacteria per cell. Latex beads (1 μm diameter, sulphate microspheres, Invitrogen, Karlsruhe, Germany) were coated with GST or GST-Inv397 as described (Wiedemann et al., 2001) and centrifuged onto the cells at 200 ***g*** for 3 minutes at a bead to cell ratio of 100:1. At designated time points, cells were washed twice in ice-cold PBS and processed for immunofluorescence or pull-down assays. For live-cell imaging, bacteria were pipetted into the medium and allowed to sediment onto cells.

Plasmids and expression of proteins

We gratefully acknowledge the gift of plasmids. Plasmids encoding myc-RhoGV12, myc-Rac1L61, myc-Cdc42L61, myc-RhoAL63, myc-TC10L75 and myc-TCLL79 were kindly provided by Dr Pontus Aspenström (Uppsala University, Uppsala, Sweden); GFP-RhoG, GFP-RhoGN17 and myc-TrioD1 by Dr Anne Blangy (Research Center of Macromolecular Biochemistry, Montpellier, France); GFP-Elmo2NT by Dr Xose Bustelo (University of Salamanca, Salamanca, Spain); myc-SGEF by Dr Keith Burridge (University of North Carolina, Chapel Hill, USA); HA-Tiam1, GST-PAK-CRIB and GST-Rhotekin by Dr John Collard (Netherlands Cancer Institute, Amsterdam, Netherlands); HA-Elmo2-T618 by Dr Hironori Katoh (Kyoto University, Kyoto, Japan); GFP-Rac1 and GFP-Cdc42 by Dr Klaudia Giehl (University of Ulm, Ulm, Germany); and GFP-TC10 by Dr David Michaelson (New York University School of Medicine, New York, USA). The genes encoding RhoG and Elmo2NT were subcloned into pGEX4T-2 (GE Healthcare, Munich, Germany) and RhoG, RhoGN17, Rac1 and Cdc42 into vector pRK5myc (Clontech, St.-Germain-en-Laye,

France). The gene encoding YopE was amplified by PCR from *Y. enterocolitica* serotype O9 strain E40 (Hentschke et al., 2007) and cloned into vectors pEGFP-N1 (Clontech, St.-Germain-en-Laye, France), pGEX4T-2 (GE Healthcare, Munich, Germany) and pmCherry (gift of Dr Marek Drab, Max-Planck Institute for Infection Biology, Berlin, Germany). YopER144A was generated using QuikChangeII site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) and YopEΔMLD using Phusion site directed Mutagenesis kit (Finnzymes, Espoo, Finland). The genes encoding YopER144A and YopEΔMLD together with SycE were subcloned into vector pACYC184 (N.E. Biolabs, Frankfurt, Germany) and transformed into *Y. enterocolitica* strain WA-C(pTTSS), resulting in strains WA-C(pTTSS+pYopER144A) and WA-C(pTTSS+pYopEΔMLD), respectively (Table 1). All GST-tagged proteins were purified from BL21 *E. coli* using glutathione-Sepharose 4B (GE Healthcare, Munich, Germany) as described elsewhere (Trasak et al., 2006). Where necessary, proteins were dialysed against PBS overnight at 4°C.

RhoGTPase activity assays

GST-Elmo2NT was generated and expressed as described above. Cells were lysed in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM DTT, 0.5% Triton X-100 and protease inhibitor cocktail), centrifuged for 10 minutes at 10,000 ***g*** and supernatants were incubated for 1 hour at 4°C with 20-80 μg GST-Elmo2NT prebound to glutathione-Sepharose 4B beads. Beads loaded with GTP-bound Rho GTPases were washed three times with lysis buffer and equal amounts of beads and total cell lysates were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobilion-P, Millipore, Schwalbach, Germany) and immunoblotted. Blots were developed with chemiluminescence reagent (Supersignal WestFemto, Pierce Chemical, Rockford, USA). The following antibodies were used: anti-myc (Cell Signaling, Frankfurt, Germany), anti-GFP (BD-Biosciences, Heidelberg, Germany), anti-HA (Roche, Mannheim, Germany), anti-Cdc42 (Cell Signaling, Frankfurt, Germany), anti-RhoA (Santa Cruz, Heidelberg, Germany), anti-Rac1 (BD-Biosciences, Heidelberg, Germany) and anti-RhoG (gift of Dr Martin Schwartz).

Rac1, Cdc42 and TC10 activity assays were performed with GST-PAK-CRIB pulldown, and the RhoA activity assay was performed with GST-Rhotekin pulldown essentially as described for RhoG pull-down, except that lysis buffers contained 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100 and protease inhibitor cocktail for GST-PAK-CRIB pull-down and 50 mM Tris (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 10% Glycerol and protease inhibitor cocktail for GST-Rhotekin pull-down.

In vitro GAP assay

In vitro GAP assay was performed with the RhoGAP assay Biochem kit (Cytoskeleton, Denver, USA). RhoG, YopE and YopEΔMLD were expressed and purified as GST-fused proteins, recombinant Rac1, Ras and p50RhoGAP were part of the kit. Proteins were incubated at 37°C with 200 mM GTP. After 20 minutes the reaction was stopped and green colour development of free phosphate was measured at 650 nm.

Immunofluorescence and live cell imaging

Cells were fixed in 3.7% formaldehyde in PBS for 10 minutes and permeabilized with ice-cold acetone for 5 minutes. Actin was stained with Alexa 568-labeled phalloidin (Molecular Probes, Karlsruhe, Germany), Golgi apparatus with anti-GS27 (BD Biosciences, Heidelberg, Germany) and anti p230 (BD-Biosciences, Heidelberg, Germany), endoplasmatic reticulum with anti-calreticulin (Stressgen, Vancouver, Canada), myc-tagged constructs with anti-myc (Cell Signaling, Frankfurt, Germany) and GFP-tagged constructs with anti-GFP (BD-Biosciences, Heidelberg, Germany). The anti-RhoG antibody was a generous gift from Dr Martin Schwartz (University of Virginia, Charlottesville, VA). Specificity of endogenous RhoG immunostaining using this antibody was verified in siRNA RhoG knock down cells (supplementary material Fig. S2B).

YopE was stained with a rabbit polyclonal antibody (Ruckdeschel et al., 2006). Secondary antibodies were Alexa-488- or Alexa-568-labeled goat anti-mouse or goat anti-rabbit IgG (Molecular Probes, Karlsruhe, Germany). Coverslips were mounted in MOWIOL (Calbiochem, Darmstadt, Germany) containing 0.18% p-phenylendiamine (Sigma-Aldrich, Munich, Germany) as anti-fading reagent and sealed with clear nail polish. Images of fixed samples were acquired with a confocal laser-scanning microscope (Leica DM IRE2 with a Leica TCS SP2 AOBS confocal point scanner) equipped with an oil-immersion plan Apo 63× NA 1.4 objective. Acquisition and processing of images was performed with Leica Confocal Software (Leica, Wetzlar, Germany). For the internalization assay, bacteria were quantified with a double fluorescence staining method as described (Schröder et al., 2006). For confocal time-lapse microscopy, HUVEC were transfected and seeded onto glass-bottomed dishes (MatTek, Ashland, USA) 1 day before the experiment. For live imaging, cells were kept in a microscope chamber at 37°C and 5% CO_2. For assessing the kinetics of phagocytic cup formation, t=0 was defined as the first visible accumulation of a GFP-protein at the bacterium and phagocytic cup closure is defined as the time point when a completely closed cup can be seen for the first time. Images were acquired with a spinning disc confocal system (Spinning disc CSU22, Yokogawa, Japan) fitted on a Zeiss Axiovert 200M microscope with a temperature- and CO_2-controllable environmental chamber (Solent Scientific, Regensworth, UK), oil immersion plan Apo 63× NA 1.4 objective and a CCD camera (EM-CCD C-9100-2, Hamamatsu, Japan). Acquisition and processing of images was performed with Volocity Software (Improvision, Coventry, UK).

siRNA knock down

A pool of four siRNAs against RhoG and a pool of siControl nontargeting siRNAs were obtained from the Dharmacon siRNA collection (Lafayette, USA). HUVEC were transfected with the Amaxa Nucleofector system. Cos-7 were transfected with X-treme Gene siRNA transfection reagent (Roche, Mannheim, Germany).

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