The impact of *Yersinia enterocolitica* (Schleifstein and Coleman, 1939) virulence factors on Rho GTPases in host cells

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As a native English speaker, I confirm that the language used in this thesis is, to the best of my knowledge, correct.

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A. Abstract

Pathogenic bacteria of the genus *Yersinia* (*Y. pestis, Y. pseudotuberculosis and Y. enterocolitica*) have evolved numerous ways to manipulate the activity of Rho GTPases and establish a successful infection. Rho GTPases are activated by *Yersinia* adhesins to trigger invasion and inactivated by *Yersinia* effector Yops (i.e. YopE, YopT, YpkA/YopO) to suppress immune cell functions. Using biochemical assays and fluorescence imaging, RhoG, a Rac-related protein that can act as an upstream regulator of other Rho GTPases, was found to be initially activated and then deactivated during *Yersinia* infection of cells. The ß1-integrin-clustering protein, invasin, on the bacterial surface, was identified as being crucial for RhoG activation, thereby promoting cell invasion.

On the other hand, the type III secretion system effector YopE downregulated active RhoG by acting as a Rho GTPase activating protein. While non-virulent yersiniae were internalized by cultured cells within only a few minutes, internalization of a virulent *Yersinia* strain could last up to 23 minutes. Under these conditions spatiotemporally organized activation and deactivation of RhoG could be visualized at the contact site of virulent *Y. enterocolitica* and host cells.

YopE localization to the Golgi apparatus and endoplasmatic reticulum, mediated by its membrane localization domain, determined its substrate specificity for RhoG and other selected Rho GTPases. RhoG was found to be responsible for invasin-stimulated Rac1 activation and inactivation of RhoG was entirely responsible for downregulation of endogenously active Rac1 by YopE. In addition, direct Rac1 activation by the guanine nucleotide exchange factor Tiam1 could be inhibited but not abrogated by YopE. These experiments show that RhoG is a central target of both, stimulatory and inhibitory *Yersinia* virulence factors. By controlling RhoG, *Yersinia* abolishes some specific Rac1 activation pathways but leaves intact, at least partially, alternative Rac1 activation pathways. Thus, by targeting of RhoG, *Yersinia* can fine tune Rac1 activity in host cells, which adds another level of complexity by which *Yersinia* modulates Rho GTPase signaling networks.

These results provide novel information on molecular mechanisms of *Yersinia* infection and gives new insights into the pathogenicity of *Yersinia*.

B. Introduction

1. The Genus *Yersinia*

Yersiniae are gram-negative, rod-shaped bacteria that belong to the family *Enterobacteriaceae*. They were initially called *Pasteurella pestis*, after Louis Pasteur, until 1964 when they were re-named after Alexandre Yersin, who first identified and isolated *Yersinia pestis* in 1894 (Drancourt and Raoult, 2002).

The genus *Yersinia* consists of eleven species, three of which are pathogenic to humans: *Yersinia pestis, Yersinia pseudotuberculosis and Yersinia enterocolitica*, *Y. pestis*, the causal agent of the plague, *Y. pseudotuberculosis*, the causal agent of mesenteric adenitis and septicemia and *Y. enterocolitica* the causal agent of acute enteritis and mesenteric lymphadenitis (Bleves and Cornelis, 2000). While *Y. pestis* seems to have evolved from *Y. pseudotuberculosis* 1,500-20,000 years ago, as shown by population genetic studies (Achtman et al., 1999), *Y. enterocolitica* comprises a biochemically and genetically heterogeneous group of organisms (Wren, 2003).

Y. pestis has a complex life cycle including a mammalian reservoir (rodents) and a flea vector. Humans are usually infected when bitten by a flea. The bacteria then disseminate from the subcutaneous site of the flea bite to adjacent lymphoid tissues. Purulent abscesses in the latter are known as bubonic plague. If the infection progresses to the lungs, pneumonic plague develops, which is highly infectious (Wren, 2003). *Y. pseudotuberculosis* and *Y. enterocolitica* are food-borne pathogens that are found widely in the environment (e.g. soil) and humans are normally infected after the consumption of contaminated food or water. After ingestion, bacteria reach the small intestine where they translocate across the intestinal epithelium through Peyer's patches and migrate to mesenteric lymph nodes (Lian et al., 1987). The symptoms of gastroenteritis arise when the bacteria proliferate and inflammation occurs. In spite of different infection routes, yersiniae share a common tropism for lymphoid tissues and a common capacity to resist non-specific immune responses, in particular phagocytosis and killing by both macrophages and polymorphonuclear leukocytes (Cornelis et al., 1998). Multiple chromosomal and plasmid-associated factors (see table 1) are responsible for *Yersinia* virulence and resistence in mammalian hosts and flea vectors (Brubaker, 1991; Straley and Perry, 1995; Wren, 2003). While the virulence plasmid pYV is common to all three pathogenic yersiniae, *Y. pestis* has acquired two more plasmids that contribute to virulence: being firstly pPla – a plasmid that encodes the

plasminogen activator Pla and secondly pMT1 (also called pFra) – encoding Phospholipase D and the F1 capsule. Pla has been suggested to be important for dissemination of *Y. pestis* after subcutaneous injection into mammalian hosts (Sodeinde et al., 1992) and Phospholipase D has been shown to be required for survival in and colonization of fleas (Hinnebusch et al., 2002).

Table 1: Plasmids important for virulence of pathogenic yersiniae (Wren, 2003 with modifications)

Plasmid name	virulence determinants	size (kb)	role in disease
Virulence plasmid pYV	*Yersinia* outer proteins, Type III secretion	70.3	avoidance of immune system, toxicity
Plasmid-encoding murine toxin, pMT1 (pFra)	Phospholipase D, F1 capsule-like antigen	96.2	bacterial transmission by fleas
Plasminogen-activating plasmid pPla	Plasminogen activator	9.6	dissemination from intra-dermal sites of infection

2. *Yersinia enterocolitica*

2.1 Classification

Y. enterocolitica is divided in 6 biogroups that can be differentiated by biochemical tests: 1A (non pathogenic), 1B (highly pathogenic), 2, 3, 4 and 5 (weakly pathogenic). Serologically they can be seperated into approximately 60 serogroups based on the variability of the O side chain (O antigen) of LPS. 11 serogroups have been associated with human infections (Bottone, 1999), with the majority being caused by serogroups O:3, O:9, O:5,27 and O:8. More rarely encountered virulent serogroups are O:4,32, O:13, O:18, O:21 (Skurnik and Toivanen, 1993).

Table 2: Association of *Y. enterocolitica* with biogroup, serogroup and ecological/ geographical distribution (Bottone, 1999 with modifications)

Associated with human infections	Biogroup	Serogroup	Ecological/Geographical distribution
No	1A	O:5, O:6,30 O:7,8, O;18, O:46	Environment, pig, food, water, global
Yes	1B	O:8, O:4, O:13a,b O:18, O:20, O:21	Environment, USA, Japan, Europe
Yes	2	O:9, O:5,27	Pig, Europe (O:9), USA, Japan (O:5,27)
Yes	3	O:1,2,3, O:5,27	Chinchilla (O:1,2,3), pigs (O:5,27)
Yes	4	O:3	Pig, Europe, USA, Japan, South Africa, Canada
Yes	5	O:2,3	Hare, Europe

Y. enterocolitica is widely distributed in nature in aquatic and animal reservoirs, with pigs serving as a major reservoir for the human pathogenic strains. The majority of non-porcine-isolated bacteria are of the non-pathogenic group 1A (Bottone, 1997).

2.2 Pathogenesis

Y. enterocolitica is usually ingested with contaminated food or water and must undergo a temperature adaption in the human host prior to the initiation of infection. To achieve this, the bacteria make use of several chromosomal and plasmid-associated factors that are regulated in a temperature-dependent manner. Important virulence genes that are expressed below 28 °C are the chromosomally encoded invasin (Inv) or genes that are responsible for motility (*fleABC*) (Straley and Perry, 1995). Once adaption to 37 °C has occurred, other factors that contribute to virulence are expressed: the chromosomally encoded attachment invasion locus (Ail) and proteins that are encoded on the virulence plasmid pYV, which is absent in avirulent strains (Bottone, 1997; Bottone, 1999).

After oral ingestion, yersiniae adhere to the mucosa of the small intestine and cross the intestinal epithelial barrier through M-cells in the follicle-associated epithelium of the Peyer's patches. The surface protein invasin appears to be particularly important in this early stage of infection by triggering outside-in β_1-integrin signaling leading to bacterial internalization (Grassl et al., 2003). After translocation to the Peyer's patches, *Yersinia* proliferates extracellulary and disseminates into other organ sites (Trulzsch et al., 2007). To evade the non-specific immune response of the host, the virulence plasmid pYV plays a major role in yersinial virulence at this later stage of infection. Expression of the adhesin YadA, the type III secretion system and the *Yersinia* outer proteins (Yops) contribute to the antiphagocytic and immunosuppressive effects by injecting Yops into host cells via the type III secretion system in order to interfere with various signaling processes inside the cell (Cornelis, 2002).

2.3 Clinical picture

Y. enterocolitica causes a wide array of gastrointestinal syndromes such as enteritis, enterocolitis, acute mesenteric lymphadenitis and terminal ileitis depending partly on the age and condition of the host and serogroup of bacterial strain. Acute enteritis with fever and inflammatory diarrhea is the most frequent occurence in children, while acute terminal ileitis and mesenteric lymphadenitis, often mimicking appendicitis, is more common in young adults (Lee et al., 1990; Chandler and Parisi, 1994). Illness may last for 3 – 28 days in infants and 1 to 2 weeks in adults. Septicemia may occur in immunosuppressed hosts or those with an underlying disorder especially when an iron overload was induced (Blei and Puder, 1993). Infection with *Y. enterocolitica*, predominantly serogroup O:3, can also lead to secondary immunologically-induced sequelae such as arthritis, erythema nodosum, glomerulonephritis or myocarditis (Laitenen et al., 1972). In most cases, *Y. enterocolitica* infections and their sequelae are self-limiting.

3. Chromosomally encoded virulence factors

3.1 High Pathogenicity Island (HPI)

The HPI is a mobile element of about 40kb, which leads to biosynthesis and transport of yersiniabactin (Ybt), a catechol and thiazoline residue-containing siderophore, which provides the bacteria with iron, especially during multiplication within the mammalian host. It was shown that a major difference between high- and low-pathogenicity *Yersinia* lies in their ability to take up iron, which is necessary for systemic infection. In the absence of a high-affinity iron-chelating compound, pathogenic *Yersinia* causes only local symptoms of moderate intensity (Carniel, 2001; Schubert et al., 2004).

3.2 Attachment Invasion Locus (Ail)

Ail is a 17 kDa outer membrane protein that was first identified together with invasin in a screen for *Y. enterocolitica* invasive phenotypes that could be transferred to *E. coli*. A non-invasive *E. coli* strain harbouring the *ail* gene was able to adhere to and invade CHO and Hep-2 cells (Miller and Falkow, 1988). *Ail* sequences were only found in pathogenic *Yersinia* strains, but a functional copy of *ail* from a pathogenic strain introduced into non-pathogenic strains did not result in an invasive phenotype. However, these recombinant strains became resistant to killing by human serum (Pierson and Falkow, 1993). In contrast, the *ail* gene of *Y. pseudotuberculosis* did not lead to *E. coli* showing the attachment and invasion phenotype but did confer a high level of serum resistance, which suggests that these two functions are separated from each other (Yang et al., 1996). Ail expression is both temperature and growth phase-dependent. While invasin is generally expressed at low temperatures, Ail was shown to be expressed at 30 °C in logarithmically growing cells and at 37 °C in the stationary phase. Thus invasin being expressed under conditions likely to be encountered during an infection (Pierson and Falkow, 1993). In a mouse infection model, Ail played only a minor role in virulence of *Y. enterocolitica* compared to the other adhesins invasin and YadA (Pepe et al., 1995).

3.3 Invasin (Inv)

The invasin protein of *Y. enterocolitica* consists of 835 amino acid residues and shares homology to proteins of the intimin family. Intimins can trigger reorganization of the host-cell actin

cytoskeleton into pedestal-like pseudopods and therefore promote efficient colonization of the intestine by enteropathogenic and enterohaemorrhagic *E. coli* (Hayward et al., 2006). While intimins bind to a bacterial receptor, which is first translocated through a type III secretion system into host cells and subsequently inserted into the host cell plasma membrane, invasin directly binds to β_1 chain integrin receptors on the cell surface, which are normally involved in attachment to the extracellular matrix and cell-cell contacts (Wong and Isberg, 2005). It was shown that invasin can bind to the five integrin receptors $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_V\beta_1$ (Isberg and Leong, 1990). The binding affinity of invasin to integrin is about 100-fold higher than the binding to its natural ligand fibronectin and this strong binding activates key regulators of the actin cytoskeleton leading to efficient internalization of the bacteria (Van Nhieu and Isberg, 1991). Recently the crystal structure of the C-terminal 497 amino acids, the extracellular part of *Y. pseudotuberculosis* invasin, has been solved. It was shown that these amino acids form an 18 nm long rod that comprises five domains. Domain D1 to D4 are structurally related to immunoglobulin superfamily domains and D5 is similar to C-type lectin-like domains of proteins (Hamburger et al., 1999).

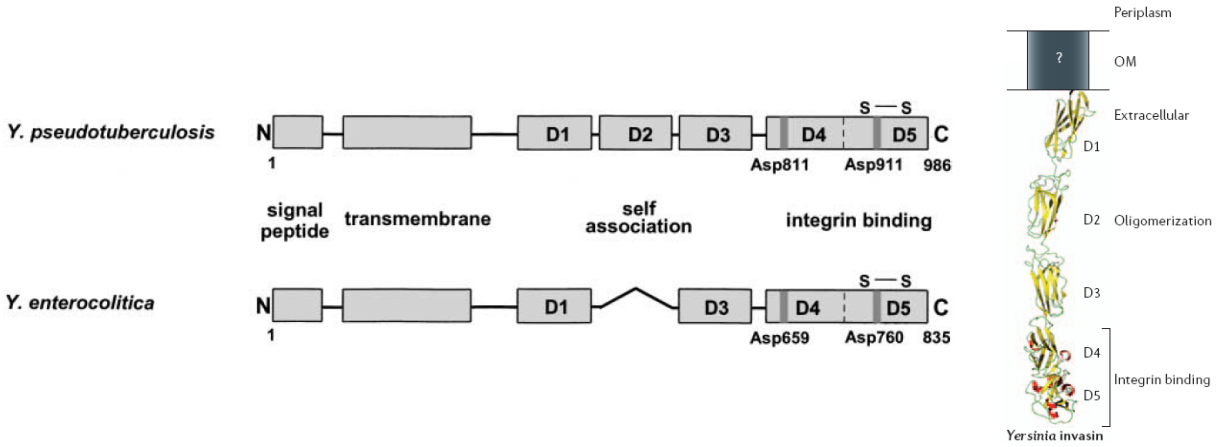

Figure 1. Functional domains of *Yersinia* invasin (Grassl et. al., 2003; Hayward et. al., 2006 with modifications).
The protein is anchored in the outer membrane with its amino-terminus. *Y. pseudotuberculosis* invasin contains five extracellular domains with D4 and D5 containing integrin-binding features. Aspartic acid residues Asp811 and Asp911 are critical for integrin binding. Invasin molecules homomultimerize using domain D2, which is missing in *Y. enterocolitica*. Asp659 and Asp760 correspond to amino acids Asp811 and Asp911.

A signaling response to the actin cytoskeleton requires engagement of several integrin receptor molecules simultaneously (Schlaepfer et al., 1997) and binding to multimeric substrates is much more efficient than binding to monomeric substrates (Stupack et al., 1999). To this end, the D2 domain, which is absent in *Y. enterocolitica,* is able to homomultimerize and lead to clustering of the integrin receptors, promoting a more efficient uptake. Hence *Y. enterocolitica* is less efficient in its ability to be internalized (Dersch and Isberg, 2000). Mutational and competitive-inhibition studies indicate that invasin recognizes a site that is either identical to or overlaps that of the naturally bound substrate fibronectin (Van Nhieu and Isberg, 1991). Genetic studies revealed that aspartic acid Asp1495 of the Arg-Gly-Asp (RGD) sequence motif was the most significant contributor to the binding of fibronectin to integrin $\alpha_5\beta_1$ (Aota et al., 1994), while in invasin aspartic acid residue Asp911 in the C-terminal domain D5 was most critical for receptor binding. Although invasin and fibronectin seem to recognize similar residues on the integrin receptor, invasin has a significantly higher affinity to the receptor and is a competitive inhibitor of the binding of fibronectin to $\alpha_5\beta_1$ integrins. This high affinity binding allows invasin to compete efficiently with other ligands for integrin receptors and allows stable contact between the host and bacteria, promoting engulfment of the bacteria by a zipper mechanism (Isberg and Barnes, 2001). Invasin-mediated adhesion of *Yersinia* to target cells induces a variety of intracellular signaling molecules such as tyrosine kinases (Rosenshine et al., 1992), phosphoinositide 3-kinase (Mecsas et al., 1998) and MAP kinase cascades (Fincham et al., 2000). It also induces expression of proteins that are directly associated with actin polymerization including Wiskott-Aldrich syndrome protein (WASP) and Arp2/3 complex (Alrutz et al., 2001; McGee et al., 2001; Wiedemann et al., 2001). Rho GTPase family members, including Rac1, Cdc42 and RhoA were also shown to be important for internalization. Latex beads coated with invasin stimulated the activation of Rac1 and while a dominant negative variant of Rac1 inhibited, a constitutively active Rac1 mutant stimulated the uptake of *Yersinia* (Alrutz et al., 2001). In a different study, Cdc42, Rac1 and RhoA were required for mediating phagocytosis of invasin-coated latex beads by macrophages (Wiedemann et al., 2001). Furthermore, analysis of different Yops that were secreted into the host cell gave insight into the signaling proteins involved in invasin-dependent uptake. YopE, which has GTPase activating protein (GAP) activity, appeared to inhibit invasin-mediated uptake by depleting activated Rho GTPases and blocking their interaction with downstream effectors (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000). Another Yop, YopH is a tyrosine phosphatase and was shown to dephosphorylate focal adhesion kinase

(FAK) and p130Cas, two proteins that regulate the association of integrins with the actin cytoskeleton (Black and Bliska, 1997; Persson et al., 1997). In addition, the cysteine protease YopT contributes to antiphagocytosis by modification of RhoA and disruption of the actin cytoskeleton (Zumbihl et al., 1999). Invasin not only mediates internalization into epithelial cells, but was also demonstrated to induce activation of nuclear factor κB (NF-κB) and production of proinflammatory cytokines, which was dependent on Rac1 and MAP kinase cascades (Grassl et al., 2003).

Expression analyis of *inv* showed that it is maximally expressed at 26 °C, pH 8 or at 37 °C, pH 5.5 but only weakly expressed at 37 °C, pH 8 (Revell and Miller, 2000). These findings are consistent with the model that invasin plays an important role in the early phase of intestinal *Yersinia* infection, while the bacteria grow at ambient temperature before oral ingestion. Bacteria that have not undergone sufficient biosynthesis at 37 °C to allow for maximal Yop expression remain uptake-competent during the encounter with M-cells (Schulte et al., 2000). This assumption is further supported by a mouse infection model, where a *Y. enterocolitica inv* mutant was severely impaired in its ability to cross the intestinal epithelium, but was not affected at later stages of disease when mouse infection was established (Pepe and Miller, 1993).

4. *Yersinia* virulence plasmid pYV

4.1 General properties

The *Yersinia* virulence plasmid pYV is approximately 70 kb long and the genetic maps of the pYV plasmids from various *Yersinia* species are relatively similar except for the presence of some transposons and reshuffling that has occured during evolution. The most striking difference between *Y. enterocolitica* and *Y. pseudotuberculosis* pYV plasmids is a large inversion of almost half the plasmid (Biot and Cornelis, 1988). The virulence plasmid encodes virulence proteins (Yops), a type III secretion system, the adhesin YadA and various regulatory proteins and is responsible for the so-called low calcium response (lcr), which refers to a complex response to in vitro growth conditions of 37 °C and low extracellular calcium concentration. Under these conditions, pathogenic *Yersinia* shifts from vegetative growth to the production and secretion of virulence proteins. These in vitro conditions could probably mimic a signal in the mammalian

host where the lcr results in antiphagocytosis and suppression of cell-mediated immunity (Cornelis et al., 1998).

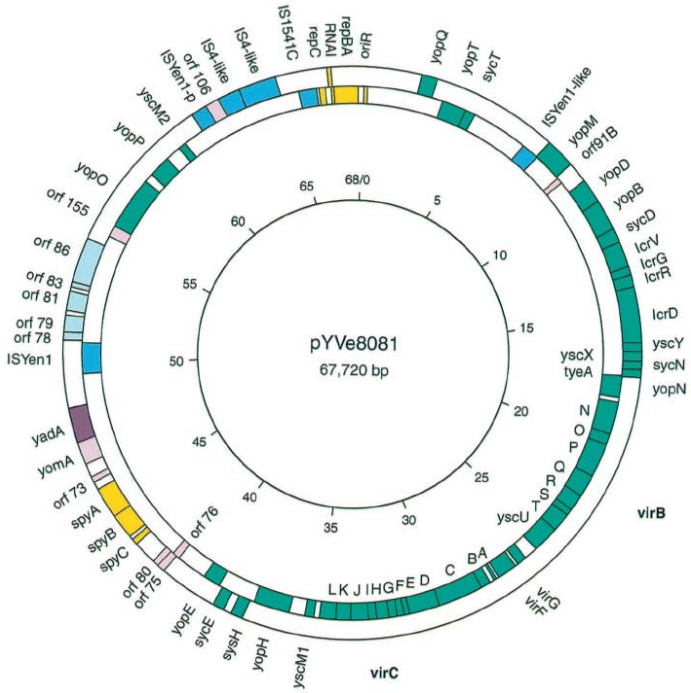

Figure 2. Map of *Yersinia* virulence plasmid pYVe8081 (Snellings et al., 2001).
Map of the virulence plasmid of *Y. enterocolitica* serogroup O:8, showing significant genes, IS elements, and replication and partition regions. The direction of transcription is clockwise for genes shown inside the circle and counterclockwise for genes shown outside the circle. Green boxes indicate genes comprising lcr stimulon, the purple box indicates YadA, yellow boxes indicate genes with replication and partition functions and pink and light blue boxes indicate genes of unknown functions. The inner circle shows the scale in kilobase pairs.

4.2 *Yersinia* adhesin A (YadA)

YadA from *Y. enterocolitica* and *Y. pseudotuberculosis* is expressed as a homotrimeric 200-240 kDa outer membrane protein that is dependent on the transcriptional activator VirF/LcrF. It is expressed only at 37 °C and unlike the other VirF/LcrF-regulated genes, it is Ca^{2+}-independent (Bolin et al., 1982; Lambert de Rouvroit et al., 1992; Skurnik and Toivanen, 1992). YadA is not expressed in *Y. pestis*, despite the pYV plasmid encoding the *yadA* gene. However, it has a single base-pair-deletion, that results in a frameshift mutation, leading to a reduced mRNA half-life and so the YadA protein is not produced (Rosqvist et al., 1988; Skurnik and Wolf-Watz, 1989). YadA is expressed as a preprotein and during translocation to the outer membrane, a 25 amino acid signal peptide is cleaved off (Bolin and Wolf-Watz, 1984). Recently an electron microscopy

analysis described the structure of YadA as 'lollipop'-shaped with an overall lenght of about 23 nm, which consists of an N-terminal head domain, a rod-like stalk-neck domain and a C-terminal membrane anchor. The C-terminal part seemed to be crucial for general stability, oligomerization and anchoring to the cell surface, the outermost N-terminal tip involved in neutrophil binding and the more proximal part of this domain responsible for interaction of the molecules, mediating autoagglutination and binding of extracellular matrix (ECM) proteins. The stalk was predicted to form a right-handed coiled-coil structure (Tamm et al., 1993; Roggenkamp et al., 1995; Hoiczyk et al., 2000).

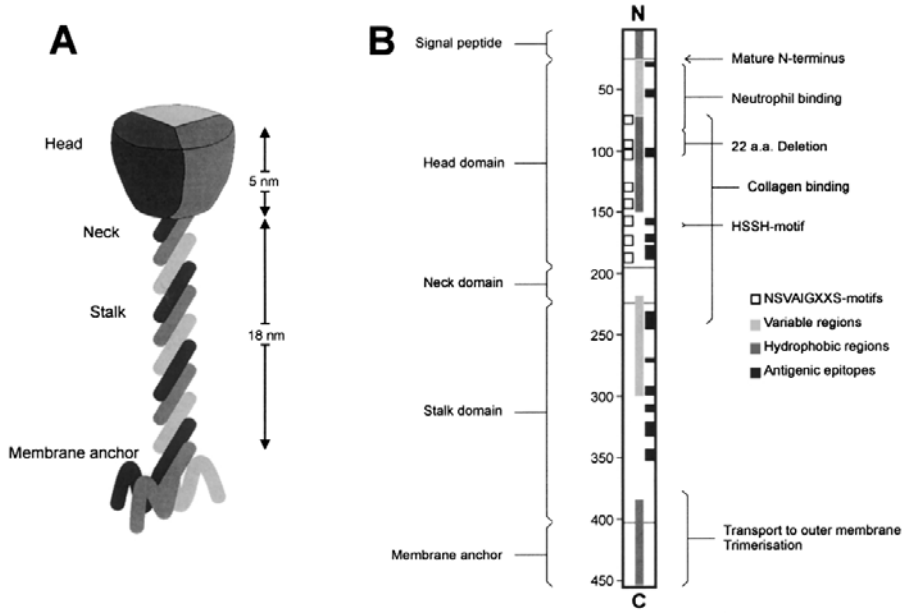

Figure 3. Structure of YadA (El Tahir and Skurnik, 2001).
(A) Hypothetical 'lollipop' structure of YadA trimer. The three intertwined polypeptides are drawn with different shades of grey and structural domains with their dimensions are indicated. (B) Schematic diagram of one 455-residue $YadA_{YEO3}$ polypeptide. Scale indicates positions of amino acid residues. Structural domains of YadA are indicated on the left and functions and properties of the different regions at the right.

While in *Y. enterocolitica*, YadA is an important mediator of virulence, it seems to be redundant in *Y. pseudotuberculosis*. The first mouse infection model showed no difference in virulence of a *yadA* mutant strain and a wild-type strain while another group reported only a small difference in virulence between these two strains (Bolin and Wolf-Watz, 1984; Han and Miller, 1997). In contrast, in a mouse intestinal colonization assay, a *Y. enterocolitica* O:9 *yadA* mutant strain was

cleared more efficiently from the intestine than the wild-type strain (Kapperud et al., 1987) and a *Y. enterocolitica* O:8 *yadA* mutant strain was completely avirulent in mice (Tamm et al., 1993). YadA is a multifunctional protein with various properties that contribute to virulence. It promotes binding to epithelial cells, professional phagocytes and extracellular matrix proteins such as collagen, laminin and fibronectin, expression of YadA causes bacterial aggregation and induces specific agglutination of erythrocytes, it protects the bacteria against defensins and confers resistance to bactericidal activity of serum complement (Balligand et al., 1985; Kapperud et al., 1987; Tertti et al., 1992; Roggenkamp et al., 1996). Recently it was reported that YadA of *Y. pseudotuberculosis* was able to anchor the bacterium via fibronectin to β_1-integrins on eukaryotic cells and promote efficient uptake into host cells independently of invasin (Eitel and Dersch, 2002). Furthermore YadA was able to trigger the production and release of proinflammatory cytokines, similar to the invasin protein (Eitel et al., 2005).

4.3 Type III secretion system

The type III secretion system (TTSS), which is structurally and evolutionary related to the flagella system, is a complex protein export pathway. It is used by numerous gram-negative pathogens to secrete effector proteins directly into infected eukaryotic host cells (Macnab, 2004; Gerlach and Hensel, 2007) in order to interfere with various signaling processes and exert antiphagocytic and immunosuppressive effects. This is thought to enable survival and extracellular multiplication of bacteria in lymphoid tissue and dissemination to other organs (Viboud and Bliska, 2005). The TTSS of *Yersinia* consists of six effector proteins (Yops), their chaperones, and proteins responsible for secretion/translocation of the effector Yops, the so-called injectisome (Cornelis, 2002). The injectisome is a complex structure that spans the two bacterial membranes, the extracellular space and the host cell membrane. The basal body of the injectisome spans the two bacterial membranes and the peptidoglycan layer and is topped by a needle-like structure that protrudes from the bacterium. The basal body shows homology to proteins of the flagellum and contains an ATPase, YscN, which resembles the α and β subunits of the F_0F_1 proton translocase (Woestyn et al., 1994). The external part, YscC, which connects to the needle, is not related to the flagellum and is a homomultimeric ring-shaped structure with a central pore of about 50 Å (Koster et al., 1997). The single monomers of YscC are related to filamentous phage proteins that are inserted in the bacterial outer membrane to allow their extrusion. The needle is formed by polymerization of monomers of the YscF protein and has a

hollow centre of about 20 Å (Hoiczyk and Blobel, 2001). To translocate effector Yops successfully into host cells, a continuous conduit between the bacterial cytosol and the eukaryotic plasma membrane is needed (Cornelis, 2002). The translocator Yops that are implicated in the formation of this channel are YopB, YopD and LcrV. YopB and YopD contain hydrophobic regions that are predicted to function as transmembrane domains and LcrV, which lacks a predicted transmembrane domain, exists as a soluble dimer in solution. Together they form a translocation pore in the host cell membrane, through which the effector Yops are secreted into the cytoplasm (Viboud and Bliska, 2005). A study that supports this observation was published recently, where YopB and YopD were able to induce pores in artificial liposomes and form ion-conducting channels (Tardy et al., 1999).

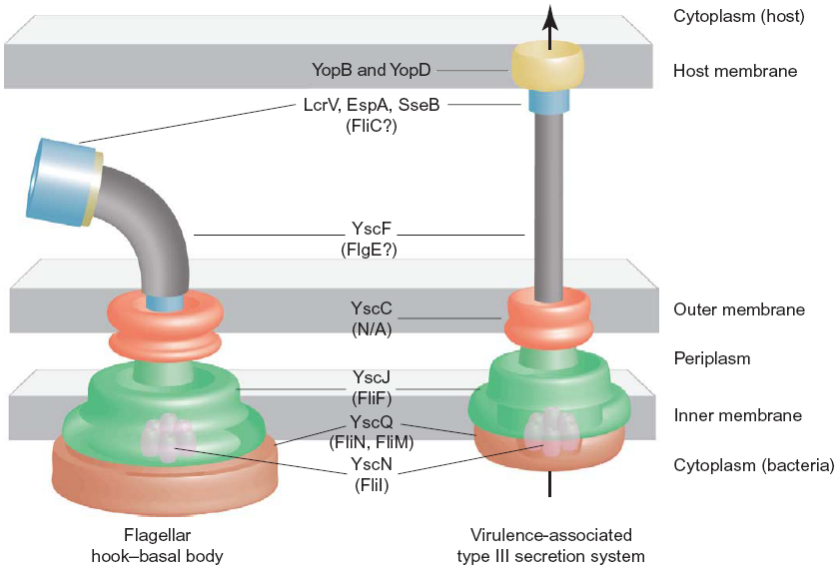

Figure 4. Morphology and organization of bacterial flagellar apparatus and TTSS (Yip and Strynadka, 2006 with modifications).
The overall morphologies of the two supramolecular complexes are highly similar: each consists of inner membrane (green and brown) and outer membrane (red) ring structures, a membrane associated ATPase (pink) and helical extracellular structures (grey). The TTSS translocates bacterial proteins directly into the cytoplasm of eukaryotic host cells (arrow), a process that requires LcrV (blue) and the translocation pore, formed by YopB and YopD (yellow). Also shown are selected protein components of the TTSS with their counterparts in the flagellar system given in parentheses.

In a different study it was shown that purified LcrV could also form channels in lipid bilayers (Holmstrom et al., 2001).

Although under physiological conditions, secretion of Yops is not induced in a cell free environment and physical contact of yersiniae with eukaryotic cells, as accomplished by adhesins, is necessary to trigger this process (Rosqvist et al., 1994), secretion into culture medium can be triggered artificially by Ca^{2+}-chelation. Effector Yops that get translocated through the injectisome do not possess a classical signal sequence but instead have a signal at the N-terminus of the protein that is needed for proper secretion (Sory et al., 1995). In addition, translocation of Yops involves the Syc proteins (specific Yop chaperone), a family of small cytosolic chaperones. They only bind to a specific partner Yop and in their absence secretion and translocation can be severely impaired (Wattiau et al., 1994). They are small acidic proteins with little or no sequence similarity among each other or to ATP-dependent chaperones such as heat shock protein 70. Various studies have analyzed the possible functions of Syc proteins during the translocation process: It was proposed that they inhibit folding of their respective Yop to prevent occurrence of catalytically active forms of the protein inside the bacterium (Stebbins and Galan, 2001) or provide an additional three dimensional secretion signal (Birtalan et al., 2002) helping to orchestrate secretion of effectors in a defined order (Boyd et al., 2000; Wulff-Strobel et al., 2002). Another study suggested that chaperones keep the Yops partially unfolded because the needle diameter of the injectisome is too small to allow passage of proteins in a folded state (Cornelis, 2002).

Y. enterocolitica secretes six effector Yops into eukaryotic host cells that interfere with signaling proteins: YopT, YopO (YpkA in *Y. pseudotuberculosis*), YopH, YopM, YopP (YopJ in *Y. pestis* and *Y. pseudotuberculosis*) and YopE.

4.4 YopT – a cysteine protease

YopT, a 35 kDa protein is a member of the CA clan of cysteine proteases. While the enzymatic activity resides in the C-terminus of the protein where three conserved amino acid residues are essential (Cys-139, His-258 and Asp-274) (Shao et al., 2002), Rho GTPase binding involves the very N-terminal amino acids (Sorg et al., 2003). It was shown that YopT is able to displace active Rho GTPases from membranes into the cytosol. This occurs by cleavage of the lipid anchor (geranylgeranyl isoprenoid moiety) at the C-terminal cysteine that is linked to the prenyl group, thus inactivating the Rho GTPases (Cornelis, 2002; Shao et al., 2003). YopT has in vitro activity

towards RhoA, Rac1 and Cdc42 but in vivo, RhoA seems to be the preferred target, which is consistent with the finding that actin stress fibres were disrupted by YopT (Iriarte and Cornelis, 1998; Aepfelbacher et al., 2003). After secretion into host cells, YopT translocates to the plasma membrane, where it binds and cleaves RhoA, and in addition it can cleave guanine nucleotide dissociation inhibitor (GDI) bound RhoA, which then accumulates in the cytosol (Aepfelbacher et al., 2003). YopT-modified RhoA may also exert dominant negative effects in the cell, as was shown for RhoA, where the C-terminal region was cleaved by calpain (Kulkarni et al., 2002).
By modification of Rho GTPases, YopT inhibits phagocytosis of opsonized and non-opsonized yersiniae by neutrophils and macrophages (Grosdent et al., 2002). In human macrophages, actin reorganization in phagocytic cups and additionally podosomal adhesion structures required for chemotaxis, were disrupted by YopT-expressing yersiniae (Aepfelbacher, 2004).
Although YopT may act preferentially on RhoA in vivo, other Rho GTPases like Rac1 may also be modified, depending on the cell type and experimental conditions. A recent study described how YopE and YopT cooperate to manipulate the activity of transfected Rac1 (Wong and Isberg, 2005). It was shown that both Yops apparently competed for plasma membrane-bound GTP-loaded Rac1, resulting in a pool of deprenylated active Rac1 in the nucleus and a pool of prenylated inactive Rac1 in the cytoplasm. How this mechanism may contribute to virulence is not known (Aepfelbacher et al., 2007).

4.5 YopO – a serine/threonine kinase

YopO (YpkA in *Y. pseudotuberculosis* and *Y. pestis*) is a 80 kDa protein with multiple functional domains. The N-terminal half consists of a secretion/translocation/membrane-binding domain and a serine/threonine catalytic domain. The C-terminal half of the protein contains a Rho-binding domain (RBD), which is structurally and functionally similiar to guanine dissociation inhibitors (GDIs). The last 21 amino acid residues display some homology to the actin bundling protein coronin (Dukuzumuremyi et al., 2000; Juris et al., 2000; Aepfelbacher et al., 2007).
YopO is autophosphorylated upon binding to monomeric G-actin, which is necessary for YopO's kinase activity and subsequent phosphorylation of external substrates. Kinase activity is dependent on two critical amino acid residues: Asp-267 and Lys-269. Mutation of these amino acids abrogates kinase activity. Removal of 21 amino acids at the C-terminus abolishes actin binding and autophosphorylation (Dukuzumuremyi et al., 2000; Juris et al., 2000; Trasak et al., 2007). Recently the first physiological substrates of YopO were identified as Otubain1 (Ovarian

tumor domain ubiquitin aldehyde binding protein 1), a deubiquitinating enzyme implicated in immune cell clonal anergy (Juris et al., 2006) and Gαq, a heterotrimeric G-protein subunit participating in a variety of cellular signaling pathways (Navarro et al., 2007).

In a yeast two hybrid assay and immunoprecipitation experiments, YpkA was shown to interact with RhoA and Rac1 and this binding was independent of the activation state, although one group reported a more efficient binding to the GDP-bound form of RhoA. HeLa cells infected with *Y. pseudotuberculosis* showed a reduced level of active GTP-bound RhoA (Barz et al., 2000; Dukuzumuremyi et al., 2000). This is consistent with the crystal structure analysis of the C-terminal part of YpkA, which showed intriguing similarities to Rho GDIs. Like eukaryotic Rho GDIs, YpkA inhibits nucleotide exchange in RhoA and Rac1. Mutations of three amino acid residues (Tyr-591, Asn-595 and Glu-599) in the GDI-like domain completely abolish Rho GTPase binding (Prehna et al., 2006).

HeLa cells infected with a *Y. pseudotuberculosis* strain overexpressing YpkA showed cell rounding and pronounced retraction fibres. Additionally, mutants that were deficient in one or more functional domains demonstrated that kinase activity and Rho-binding activity can act synergistically. Cell rounding was due to kinase activity and actin filament disruption could be conferred to GDI-like activity (Prehna et al., 2006; Aepfelbacher et al., 2007; Trasak et al., 2007).

YopO contributes with other Yops to the antiphagocytic activity of *Y. enterocolitica* towards neutrophils and macrophages (Grosdent et al., 2002; Aepfelbacher, 2004) but mouse infection studies do not give a clear picture as to the in vivo role of YopO (Aepfelbacher et al., 2007).

While a *Y. enterocolitica* O:8 YopO mutant strain was not impaired in its ability to colonize the small intestine and Peyer's patches (Trulzsch et al., 2004), a *Y. pseudotuberculosis* strain, expressing a kinase-inactive mutant of YpkA was greatly attenuated in virulence (Wiley et al., 2006). Earlier studies with mouse oral infection experiments showed that mutation of either the catalytic domain or the C-terminal domain of YpkA resulted in avirulence of *Y. pseudotuberculosis* (Galyov et al., 1993; Galyov et al., 1994).

4.6 YopH – a phosphotyrosine phosphatase

YopH, a 50 kDa protein, is a highly active phosphotyrosine phosphatase (PTP) with defined N- and C-terminal domains linked together by a proline-rich sequence (Viboud and Bliska, 2005). The N-terminal domain consists of a secretion/translocation domain and a substrate targeting region, while the C-terminal domain contains the catalytic site, which is related to

phosphotyrosine phosphatase catalytic domains found in eukaryotic cells (Zhang, 2003; Viboud and Bliska, 2005). Mutation of a critical cysteine residue (Cys-403) in the catalytic domain abolishes catalytic activity and leads to a substrate-trapping protein that localizes to focal adhesion complexes (Black and Bliska, 1997; Persson et al., 1997; Aepfelbacher et al., 2007).

Many YopH substrates play a role in regulating the interaction between the actin cytoskeleton and extracellular matrix-binding integrins (Brakebusch and Fassler, 2003). These substrates include proteins such as p130Cas, focal adhesion kinase (Fak), paxillin, Fyn-binding protein (FyB) and the scaffolding protein SKAP-HOM, that are dephosphorylated by YopH in different cell types (Black and Bliska, 1997; Persson et al., 1997; Hamid et al., 1999). These signaling components, which form so-called focal complexes and focal adhesions, are also targets of YopH during invasin-mediated phagocytosis. The cytoskeletal uptake structures formed upon contact with *Yersinia* adhesins with cellular integrins may resemble focal adhesions and disruption by YopH may explain its antiphagocytic activity (Aepfelbacher et al., 2007).

Mouse infection studies with *Y. enterocolitica* showed that a YopH mutant was attenuated in virulence (Trulzsch et al., 2004) and in a lung infection model, a *Y. pseudotuberculosis* YopH mutant was severely impaired in lung colonization. However, deletion of any other single Yop did not attenuate virulence (Fisher et al., 2007). In vitro studies suggest that YopH is responsible for up to 50 % of the antiphagocytic activity of *Yersinia* towards neutrophils and J774 macrophages (Fallman et al., 1995; Ruckdeschel et al., 1996; Aepfelbacher et al., 2007).

Besides interfering with signaling pathways activated by phagocytic mechanisms, YopH counteracts other types of immune response pathways in host cells (Viboud and Bliska, 2005), including inhibition of the production of macrophage chemoattractant protein 1 (Sauvonnet et al., 2002) and inhibition of T- and B-lymphocyte activation (Yao et al., 1999; Viboud and Bliska, 2005). It is not yet known whether these effects are dependent on YopH dephosphorylation of known substrates or if unknown targets are involved (Aepfelbacher et al., 2007).

4.7 YopM – a leucine-rich protein

YopM consists of varying numbers of a 19 amino acid leucine-rich-repeat (LRR) motif, together forming a protein of approximately 42 kDa, dependent on the *Yersinia* strain from which it was isolated. YopM is the only known Yop effector that exerts no obvious enzymatic activity (Aepfelbacher et al., 2007). The LRR repeats are important for protein-protein interaction and both the first three LRR repeats and the 32 C-terminal residues constitute a nuclear localization

signal (NLS) (Benabdillah et al., 2004). YopM has been shown to migrate to the nucleus via a vesicle-associated pathway (Skrzypek et al., 1998) and in coimmunoprecipitation experiments two cytoplasmic kinases were identified as direct interaction partners: PRK2 (protein kinase C-like 2) and RSK1 (ribosomal S6 protein kinase 1) (McDonald et al., 2003). These proteins have assumed functions in regulating expression of genes involved in cell growth and the cell cycle, which is consistent with a microarray analysis on macrophages infected with *Y. enterocolitica*, where YopM seems to regulate genes involved in the cell cycle and cell growth (Sauvonnet et al., 2002). Although YopM has no obvious antiphagocytic function, it is clearly required for virulence of *Yersinia* in mice (Leung et al., 1990; Aepfelbacher, 2004).

4.8 YopP – an enzyme that modulates inflammatory signaling

YopP (YopJ in *Y. pestis* and *Y. pseudotuberculosis*) is a 34 kDa protein and functions as a potent inhibitor of the MAPK and NF-κB signaling pathways of host cells, which results in suppression of cytokine production and induction of macrophage apoptotic death (Orth, 2002; Ruckdeschel, 2002; Viboud and Bliska, 2005). YopP/YopJ associates with members of the MAPK kinase (MKK) superfamily, which represent upstream activators of MAPK and it binds and inhibits the IκB kinase-β (IKK- β), which is the major activator of NF-κB (Orth, 2002; Aepfelbacher, 2004). It was suggested that YopP/YopJ cleaves ubiquitin residues from signaling complexes that are associated with MAPK and NF-κB activation, such as TNF-receptor-associated factor 2 (TRAF2) or TRAF6 (Orth et al., 2000; Zhou et al., 2005). Ubiquitination of these molecules normally regulates the assembly of functional signaling complexes and by removing the ubiquitin moiety, YopP/YopJ could negatively regulate MAPK and NF-κB signaling (Chen, 2005). Recently it was shown that YopJ could act as an acetyltranferase, modifying critical serine and threonine residues in the activation loop of MAPKK6, a MAPK activator, thereby blocking phosphorylation. The acetylation directly competes with phosphorylation, preventing activation of the modified protein (Mukherjee et al., 2006).

In addition, it was shown that the modulation of inflammatory signaling in host cells triggers apoptosis in infected macrophages and dendritic cells. MAPK and NF-κB pathways normally provide protection against apoptosis and repression of these pathways by YopP/YopJ was found to cooperate with the induction of an LPS-induced, TLR4-, and TRIF-mediated proapoptotic response, triggering macrophage apoptosis (Ruckdeschel et al., 2004; Aepfelbacher et al., 2007). In a mouse infection model YopP/YopJ was found to be important for virulence of

enteropathogenic yersiniae (Trulzsch et al., 2004), while in *Y. pestis*, YopJ was dispensible for virulence following intravenous infection (Perry and Fetherston, 1997; Viboud and Bliska, 2005).

4.9 YopE – a GTPase activating protein

YopE is a 25 kDa protein (219 amino acids) that exhibits GTPase activating protein (GAP) activity for Rho GTP binding proteins (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000; Andor et al., 2001). Like its homologous domains within exoenzyme S (ExoS) from *Pseudomonas aeruginosa* and SptP from *Salmonella typhimurium*, YopE shows no sequence homology to eukaryotic GAPs, but shares a critical arginine residue (Arg-144), the so-called arginine finger motif, that is known to be essential for GAP activity (Scheffzek et al., 1998; Evdokimov et al., 2002). While the catalytic RhoGAP domain, containing Arg-144, ranges from amino acids 96-219, the N-terminal residues 1-50 are required for bacterial secretion and translocation of YopE into target cells (Cornelis, 2002; Aepfelbacher et al., 2007). Recently a membrane localization domain (MLD) ranging from amino acids 54 to 74 was identified and shown to be necessary and sufficient for targeting of YopE to a perinuclear membrane region in host cells (Krall et al., 2004).

Figure 5. Domain organization and functional regions of YopE (Aepfelbacher et al., 2007 with modifications).
N-terminal amino acid residues 1 to 50 are required for secretion and translocation of YopE into target cells. The RhoGAP domain with the critical arginine 144 ranges from amino acids 96 to 219. The membrane localization domain ranges from amino acids 50 to 75 and contains two lysine residues that are polyubiquitinated.

YopE causes disruption of actin filaments, cell rounding and inhibition of phagocytosis in infected cells and it has been shown to inactivate Rac1, RhoA and Cdc42 in vitro (Black and Bliska, 2000). In a different study, it was demonstrated with pull-down experiments that YopE can deactivate Rac1 and RhoA in vivo. Rac1 was inactivated within 5 minutes of infection, while

RhoA inactivation did not occur before 30 minutes, which might indicate an indirect action of YopE on RhoA. In most cases, the cellular effects of YopE can be explained by inactivation of these GTPases, but a direct interaction by coimmunoprecipitation has not been shown (Aili et al., 2006; Aepfelbacher et al., 2007). Furthermore, Rho GTPases other than Rac1, RhoA and Cdc42 have not been tested for deactivation by YopE.

Activity of GAPs in cells depends on many variables, such as their subcellular localization, the expression of target GTP-binding proteins and the signaling state of a given cell (Moon and Zheng, 2003; Aepfelbacher et al., 2007). Rho GTPases are often interconnected, with one GTPase activating or inactivating other GTPases (Van Aelst and D'Souza-Schorey, 1997). Hence, YopE might act in a compartmentalized fashion, directly and indirectly influencing members of the large family of Rho GTPases. The specific perinuclear membrane localization supports this notion (Aepfelbacher, 2004; Krall et al., 2004). However this localization of YopE in cells is not the preferred localization of its presumed interaction partner Rac1 (Michaelson et al., 2001; Aepfelbacher et al., 2007). A different study in endothelial cells showed that YopE was able to inhibit Rac1-regulated actin structures, in particular blocking Cdc42-dependent Rac1 activation but not direct Rac1 activation by sphingosine-1-phosphate (Andor et al., 2001).

A YopE GAP mutant, in which arginine 144 was replaced by alanine, was avirulent in mice and unable to induce cytotoxic effects in HeLa cells (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000; Aili et al., 2006). In studies where single amino acids between residues 178 and 183 were mutated, in vitro GAP activity was abolished but not cellular cytotoxicity of YopE, suggesting additional targets of YopE within cells that are dependent on the arginine 144 residue but not on GAP activity (Aepfelbacher, 2004; Aili et al., 2006).

Other YopE effects that could be assigned to inactivation of Rho and Rac in immune cells were inhibition of caspase-1-mediated maturation and release of interleukin-1β, blockage of phagocytosis and also the pore-forming activity of the injectisome (Viboud and Bliska, 2001; Schotte et al., 2004). The fate of *Y. enterocolitica* O:8-translocated YopE in target cells is determined by the host proteasome degradation pathway. YopE was found to be polyubiquitinated at N-terminal lysine residues (K62 and K75) and subjected to degradation by the ubiquitin-proteasome pathway inside the host cell. In contrast, YopH and YopP were not influenced by the degradation pathway (Ruckdeschel et al., 2006).

SycE, the chaperone specific for YopE, is necessery for efficient translocation into target cells and amino acid residues 15 to 50 of YopE are sufficient for chaperone binding (Feldman et al.,

2002). The crystal structure of the N-terminal region of YopE bound to sycE, however, showed that the region of YopE interacting with sycE extends to amino acid residue 78 and a YopE mutant lacking amino acids 50 to 77 was shown to be secreted without sycE (Boyd et al., 2000; Birtalan et al., 2002). Thus, the region comprising amino acids 50 to 77 somehow exerts an inhibitory effect on the secretion of YopE in the absence of sycE and one role of sycE might be to mask this region inside the bacteria (Boyd et al., 2000).

YopE is an important *Yersinia* virulence factor and contributes to the antiphagocytic activity of *Y. enterocolitica* and *Y. pseudotuberculosis* in cooperation with other Yops. Phagocytosis of a *Y. enterocolitica* YopE mutant strain by macrophages and neutrophils was more efficient than in the parental strain (Grosdent et al., 2002) and a *Y. pseudotuberculosis* strain lacking YopE was clearly attenuated in general virulence/lethality (Rosqvist et al., 1988; Aepfelbacher et al., 2007). In a mouse infection model, however, a *Y. pseudotuberculosis* YopE mutant strain showed only minor defects in persistence in intestinal and lymph tissue, but the absence of YopE and YopH together almost eliminated all tissue colonization (Logsdon and Mecsas, 2003). A recent study also showed how YopE and YopT cooperate to manipulate the activity of Rho GTPases (see YopT). These findings suggest that YopE is a major determinant of *Yersinia* pathogenicity and extensively synergizes with other Yops in complex ways during *Yersinia* infection (Aepfelbacher et al., 2007).

5. Rho GTPases

5.1 The Rho family of proteins

Rho GTPases belong to the superfamily of Ras-related small GTPases and can be divided into six subfamilies that exhibit similar, but not identical, properties: RhoA-related subfamily (RhoA, RhoB and RhoC), Rac1-related subfamily (Rac1, Rac2, Rac3 and RhoG), Cdc42-related subfamily (Cdc42, TC10, TCL, Chp/Wrch-2 and Wrch-1), Rnd subfamily (Rnd1, Rnd2 and RhoE/Rnd3), RhoBTB subfamily (RhoBTB-1, RhoBTB-2 and RhoBTB-3) and Miro subfamily (Miro-1 and Miro-2). The other Rho GTPases, RhoD, Rif and TTF/RhoH do not fall into any of these subfamilies (Wennerberg and Der, 2004; Jaffe and Hall, 2005).

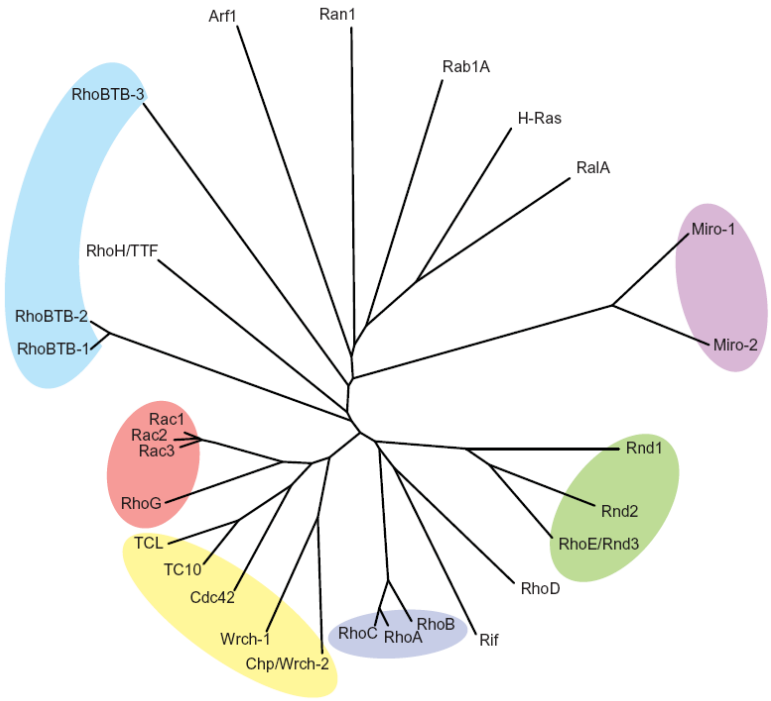

Figure 6. Phylogenetic tree of the Rho family GTPases (Wennerberg and Der, 2004). Phylogenetic tree of Rho GTPases and representatives of other Ras-superfamily GTPases. Phylogenetic analysis of the amino acid sequences of the Rho domains of the 22 Rho-family members made with ClustalW. The family can be divided into six major branches: RhoA-related (blue), Rac-related (red), Cdc42-related (yellow), Rnd proteins (green), RhoBTB proteins (turquoise) and Miro proteins (purple).

Rho GTPases act as molecular switches that are inactive when bound to GDP and active when bound to GTP. The cycling between these two states is mediated by three different groups of regulatory proteins: guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP to GTP and therefore activation of the GTPase, GTPase activating proteins (GAPs) increase the intrinsic rate of GTP hydrolysis and thereby promote formation of the inactive GDP-bound protein and Guanine dissociation inhibitors (GDIs) bind to the hydrophobic isoprenoid moiety at the C-terminus of Rho GTPases and inhibit spontaneous nucleotide exchange (Bishop and Hall, 2000; Wennerberg and Der, 2004).

In the active GTP-bound state, the Rho proteins adopt a conformation that allows interaction and stimulation of a variety of downstream effector proteins such as protein kinases, lipid kinases, phospholipases and various adaptor proteins (Van Aelst and D'Souza-Schorey, 1997).

Recent studies indicate that Rho GTPase activity is not only regulated by the nucleotide switch but also in several other ways such as lipid modification, subcellular localization, transcriptional regulation or differential degradation (Bustelo et al., 2007).

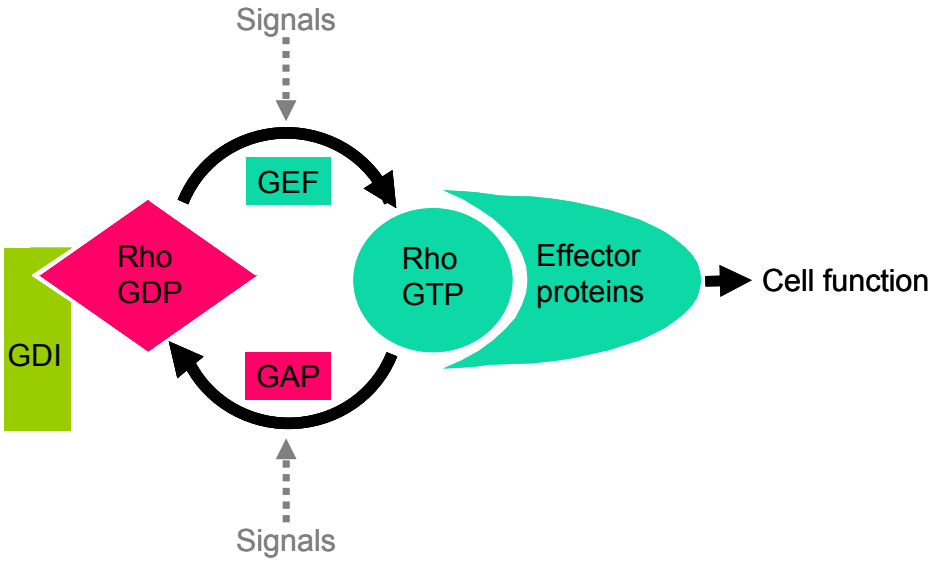

Figure 7. Activation cycle of Rho GTPases.
Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. GEFs catalyze the exchange of GDP to GTP and GAPs stimulate the intrinsic GTPase activity to inactivate the GTPase. GDIs bind to the C-terminus and block spontaneous activation. In an active state GTPases bind to downstream effector proteins, which regulate a variety of cellular functions.

RhoA, Rac1 and Cdc42 are the best characterized members of the Rho protein family. A major contribution to the elucidation of Rho GTPase function was brought about by constitutively active and dominant negative mutants of the GTPases, which can be achieved by substitution of specific amino acid residues. Active mutants prevent intrinsic and GAP-induced GTP hydrolysis and the proteins therefore stay in a constitutively GTP-bound state, while dominant negative mutants compete with the corresponding endogenous GTPase for binding to cellular GEFs. Because the dominant negative mutants do not release the GEFs, this leads to non-productive complexes unable to generate downstream responses (Bishop and Hall, 2000).

The major function of Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton but they also play a role in cellular processes that are dependent on the actin cytoskeleton such as cytokinesis, phagocytosis, morphogenesis, membrane trafficking, axon guidance or cell migration. In addition to these effects, they also have a variety of other functions,

regulating nuclear factor κB (NF-κB), transcription factors, phagocytic NADPH oxidase complex, G1 cell-cycle progression, cell polarity, secretion by mast cells, p38 mitogen-activated protein kinase pathways and cell transformation (Bishop and Hall, 2000).

5.2 Rho GTPases and the actin cytoskeleton

In eukaryotic cells the dynamic process of G-actin polymerization to filamentous actin (F-actin) is mediated through the coordinated activity of monomeric G-actin and two major polymerization factors: Actin-related protein 2/3 complex (Arp2/3 complex) and Formins. Recently two additional polymerization factors were discovered: Cordon-Bleu (Cobl) and Spire, which were shown to have roles in axis patterning in developmental processes (Renault et al., 2008). Rac1 and Cdc42 initiate actin polymerization through the Arp2/3 complex but lead to different morphologically protrusions at the plasma membrane, lamellipodia and filopodia respectively. RhoA stimulates actin polymerization through the diaphanous-related formin mDia1 (and possibly mDia2) and leads to the formation of stress fibres and focal adhesions (Jaffe and Hall, 2005). In addition, RhoA can bind Rho-kinase (ROK) and stimulate activation of LIM-kinase (LIMK) and regulation of Cofilin, which stabilizes filamentous actin structures (DesMarais et al., 2005).

Activation of the Arp2/3 complex by Cdc42 is mediated indirectly through neuronal Wiskott-Aldrich syndrome protein (N-WASP) or its closely related, hematopoetic-specific WASP, while Rac1 activates Arp2/3 via the WASP family Verprolin-homologous protein (WAVE). Cofilin can also be regulated by Rac1 and Cdc42 via activation of p21-activated kinase (PAK) and LIMK (Jaffe and Hall, 2005).

5.3 Rho GTPases and phagocytosis

Phagocytosis is a process driven by the host cell actin cytoskeleton that results in internalization of particles or apoptotic cells and Rho GTPases have been shown to play an essential role in this mechanism. In general, phagocytosis is accomplished by professional phagocytes such as macrophages or dendritic cells and mediated mainly by two different mechanisms (Caron and Hall, 1998; Ernst, 2000). Immunoglobulin receptor (FcγR)-mediated phagocytosis is controlled by Rac1 and Cdc42 and is initiated for example through binding of IgG-coated particles to the Fc-receptor. This induces activation of cellular signaling cascades including multiple tyrosine

kinases, which lead to stimulation of actin nucleation and uptake of the target particle (Niedergang and Chavrier, 2005). Internalization via the Fc-receptor is accompanied by an increase in the production of reactive oxygen species, which is mediated by the multicomponent membrane-associated NADPH oxidase complex leading to killing of phagocytosed bacteria and was shown to be dependent on Rac proteins (Bokoch and Diebold, 2002; Aktories and Barbieri, 2005). Complement receptor (CR)-mediated phagocytosis is initiated by binding of complement fragment C3bi-coated particles to the cellular C3bi (CR3) receptor but in contrast to FcR-mediated phagocytosis, activation of CR3 requires additional extracellular stimuli such as inflammatory cytokines or attachment to the extracellular matrix (May and Machesky, 2001; Niedergang and Chavrier, 2005). RhoA, but not Rac1 or Cdc42 is responsible for CR-initiated phagocytosis and the RhoA effector Rho-kinase and its target myosin II seem to be responsible for F-actin assembly. In addition, internalization does not usually lead to an inflammatory response or oxydative burst through NADPH (May and Machesky, 2001).

Uptake of apoptotic cells is normally carried out by professional phagocytes as well as neighboring non-professional cells and is not followed by an inflammatory response (Niedergang and Chavrier, 2005). In a recent study, it was shown that the two Rac-related proteins Rac1 and RhoG seem to be involved in this uptake mechanism (Henson, 2005).

Invasive bacteria such as *Y. enterocolitica* can actively induce their own uptake by phagocytosis into normally non-phagocytic cells, which also involves Rho GTPases and activation of cytoskeletal components. These bacteria use cell-adhesion proteins to bind to eukaryotic surface receptors that are normally involved in cell-matrix or cell-cell adhesion, such as integrins. Binding to these cellular receptors leads to efficient downstream signaling and cytoskeletal rearrangements, which promotes bacterial uptake (Cossart and Sansonetti, 2004).

5.4 Rho GTPases and bacterial infections

Rho GTPases are important regulators of gene transcription and cytokine expression during a bacterial infection. NF-κB, for example, an important regulator of many inflammatory and anti-apoptotic factors, gets activated through Rac1, RhoA and Cdc42 via different mechanisms (Cammarano and Minden, 2001).

However Rho GTPases are also involved in regulatory mechanisms of adaptive immunity such as B- and T-cell motility, regulation of transcription factors of B- and T-cells or expression of cytokines (Dreikhausen et al., 2001; Croker et al., 2002; Hao et al., 2003).

In order to survive the hostile environment during the immune response, bacteria have evolved numerous ways of manipulating host cell functions. Many bacterial cytotoxins specifically target GTP-binding proteins, especially low-molecular-weight GTPases of the Rho protein subfamily, which play crucial roles in regulation of the organization of the actin cytoskeleton, phagocytosis, vesicle transport, gene transcription and the cell cycle (Aktories and Barbieri, 2005). Many cytotoxins are enzymes (ADP-ribosyl-transferases, glucosyltransferases, proteases or deamidases) that modify their target irreversibly in a catalytic manner but various bacterial proteins have developed mechanisms to reversibly modulate target proteins and functions of eukaryotic regulatory proteins and mimic eukaryotic GEFs or GAPs (Aktories and Barbieri, 2005). In addition some pathogens (e.g. *Shigella, Salmonella, Yersinia*) express several virulence factors that control an overlapping set of Rho GTPases by distinct mechanisms, suggesting that it is a major goal of the bacteria to orchestrate Rho protein signaling networks and cascades (Aepfelbacher, 2004).

5.5 RhoG – a Rac-related protein

RhoG belongs to the subfamily of Rac-related GTPases and was originally described as a late-response gene, induced after serum stimulation of starved fibroblasts, suggesting an involvement in the regulation of the cell cycle (Vincent et al., 1992). It was shown to be expressed in multiple tissues such as lung, heart and brain, but also in lymphocytes (Vincent et al., 1992; Vigorito et al., 2004). RhoG has been characterized as a regulator of cytoskeletal rearrangements, but the signaling pathways responsible for these are controversial. Some studies indicate that RhoG regulates the actin cytoskeleton by activating Rac1 and Cdc42 (Gauthier-Rouviere et al., 1998; Blangy et al., 2000) while others suggest that it signals independently and in parallel to them (Wennerberg et al., 2002; Prieto-Sanchez and Bustelo, 2003). In a recent study, however, it was clearly shown that RhoG can activate Rac1 via the proteins Elmo and Dock180. Active RhoG can directly interact with Elmo and form a ternary complex with Dock180 to induce activation of Rac1, leading to changes in the cytoskeleton such as ruffling and neurite outgrowth (Katoh et al., 2000; Katoh and Negishi, 2003; Katoh et al., 2006). Rac1 activation through RhoG also played a role in the uptake of apoptotic cells (deBakker et al., 2004). On the other hand, RhoG may also have Rac1-independent effects on the cytoskeleton as demonstrated in a recent paper (Meller et al., 2008).

Other studies have shown the importance of RhoG in various other cellular functions such as caveolar endocytosis, trans-endothelial migration of leukocytes and activation of NADPH oxidase (Condliffe et al., 2006; Prieto-Sanchez et al., 2006; van Buul et al., 2007).

The signaling cascade involving RhoG and Rac1 was also shown to play a role in host-pathogen interactions. The *Shigella* effector IpgB1 mimics RhoG to activate Elmo/Dock180 and Rac1, which induces membrane ruffling and bacterial invasion (Handa et al., 2007). Another pathogen *Salmonella enterica* was reported to activate RhoG via SH3-containing guanine nucleotide exchange factor (SGEF) and thereby stimulate actin remodeling (Patel and Galan, 2006).

6. Aim of this study

Rho GTP-binding proteins in host cells serve different functions during an infection with *Yersinia enterocolitica*. They are activated by *Yersinia* adhesins to trigger bacterial internalization and inactivated by *Yersinia* effector proteins to suppress immune cell functions. The effects on the well-characterized Rho GTPases Rac1, RhoA and Cdc42 have been extensively studied and Rac1 has been identified as a putative central target of both, *Yersinia* adhesins and Yops. However, additional effects of *Yersinia* factors on less characterized Rho GTPases are highly likely.

RhoG, a close relative of Rac1 has previously been implicated in having a role in phagocytosis and bacterial invasion and was shown to act as an upstream regulator of Rac1 by controlling the Elmo/Dock180 module.

First it was necessary to determine if *Y. enterocolitica* is able to modulate RhoG activity in host cells during an infection. After initial results showed that RhoG is in fact a target of *Y. enterocolitica* virulence factors, a number of questions arose:

- Which bacterial proteins are involved in the modulation of RhoG?
- What is the biochemical mechanism of RhoG modulation?
- What is the cellular impact?
- Is there an influence on bacterial invasion or an immunosuppressive effect?
- Does the modulation of RhoG affect its downstream effector Rac1?
- How does it fit in the virulence strategy of *Yersinia*?

Answering these questions will provide a better insight into the complex processes by which *Yersinia* modulates Rho GTPase networks and signaling cascades.

C. Results

1. Differential modulation of RhoG activity by *Y. enterocolitica*

1.1 Elmo2NT used as a probe to monitor active RhoG

In order to investigate whether RhoG activity is modulated by *Y. enterocolitica* during an infection, a faithful biosensor for monitoring active GTP-bound RhoG in cells was required. Recently it has been reported that RhoG can activate Rac1 via the proteins Elmo and Dock180. The armadillo repeats at the N-terminus of Elmo (Elmo2NT) were shown to contain a binding domain for GTP-bound RhoG but not for other Rho GTPases (Katoh and Negishi, 2003). Several assays were employed to assure usability of Elmo2NT as a specific probe for active RhoG. A pull-down assay confirmed that GST-Elmo2NT binds to constitutively active RhoGV12 but not to dominant negative RhoGN17. All other constitutively active Rho GTPases that were tested (Rac1L61, Cdc42L61, RhoAL63, TC10L75 and TCLL79) failed to show any binding to GST-Elmo2NT, except for Rac1, which showed a very slight interaction. This might be due to Rac1's very close relatedness to RhoG (Figure 8).

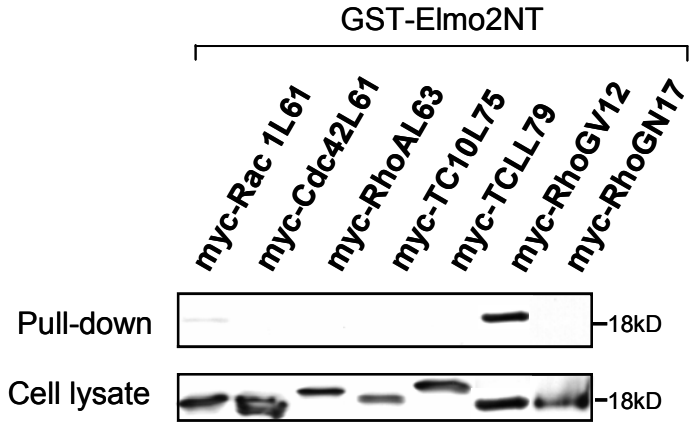

Figure 8. GST-Elmo2NT specifically binds to active RhoG.
Cos-7 cells were transfected with indicated myc-tagged Rho GTPase constructs, lysed and subjected to pull-down using GST-Elmo2NT. Proteins from pull-down and in cell lysates were detected by Western Blotting using anti-myc antibody.

To monitor cellular localization of active RhoG, a GFP-tagging construct of Elmo2NT was created and the distribution of GFP-Elmo2NT and hence, active RhoG was investigated in human umbilical vein endothelial cells (HUVEC). HUVEC that were transfected with only a vector expressing GFP-Elmo2NT showed a diffuse cytoplasmic and nuclear staining. However, upon coexpression with constitutively active RhoGV12, GFP-Elmo2NT redistributed to the perinuclear area of HUVEC, where it colocalized with myc-RhoGV12. In contrast, coexpression with dominant negative myc-RhoGN17 did not alter the normal diffuse cellular distribution of GFP-Elmo2NT (Figure 9).

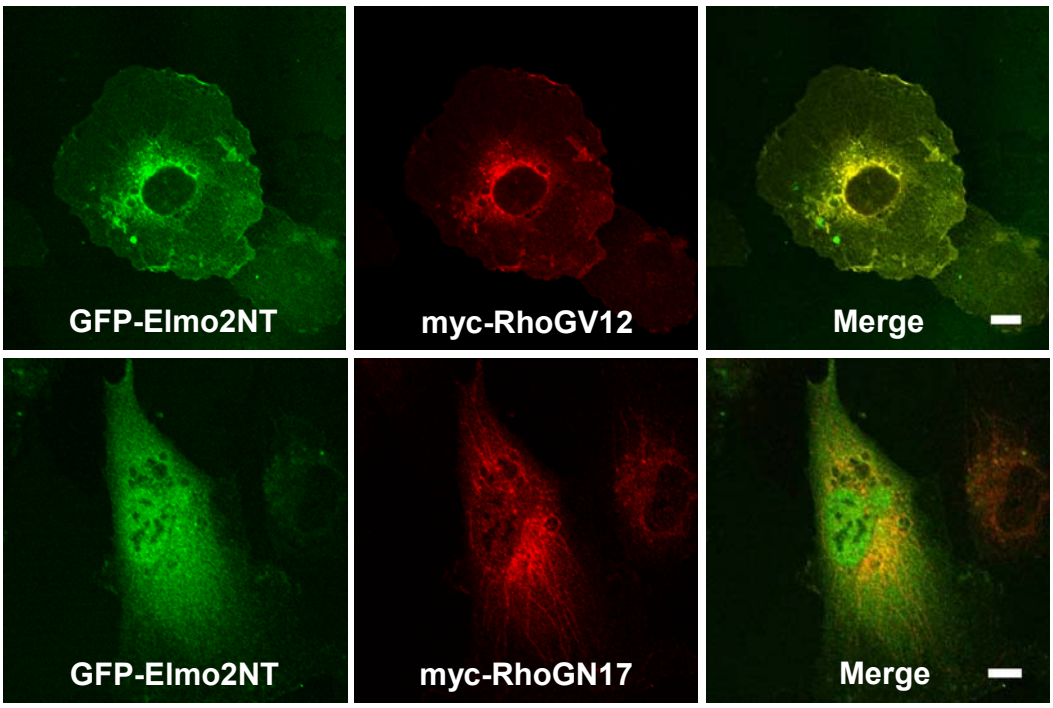

Figure 9. Specific intracellular redistribution of GFP-Elmo2NT by active RhoG.
Confocal fluorescence images of HUVEC cotransfected with GFP-Elmo2NT and myc-RhoGV12 or myc-RhoGN17. Merge represents overlays of green and red fluorescence channel resulting in yellow color. Myc-tagged constructs were stained with anti-myc antibody. GFP-Elmo2NT colocalizes with constitutively active RhoG but not with dominant negative RhoGN17. Bars represent 10 µm.

Two different RhoG-specific GEFs were described recently: TrioD1 and SGEF. These GEFs have been shown to specifically mediate GTP-loading and activation of RhoG in vitro and in cells. Furthermore it was demonstrated that they induce dorsal ruffles similar to those generated by constitutively active RhoG (Blangy et al., 2000; Ellerbroek et al., 2004).

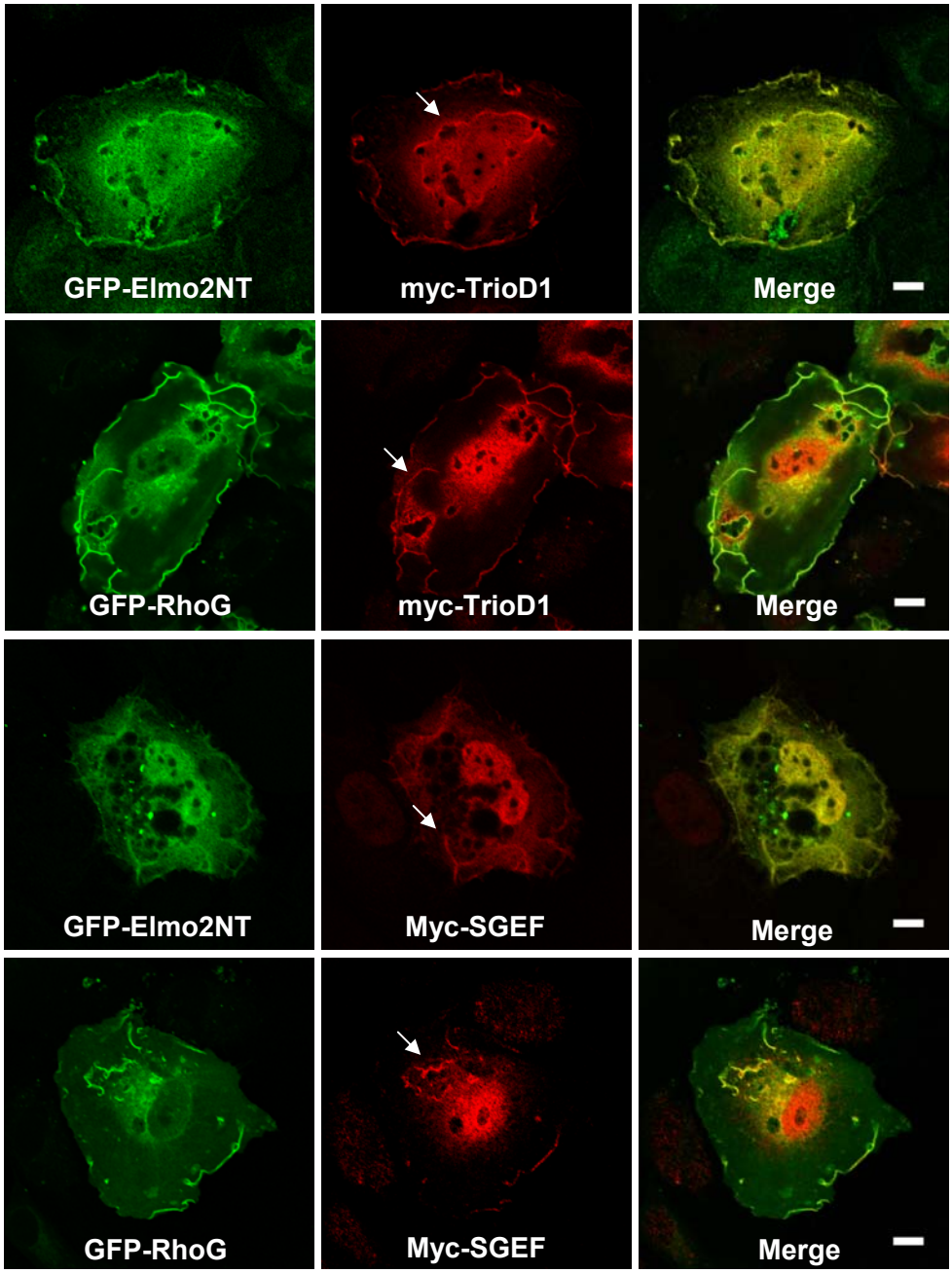

Figure 10. RhoG and Elmo2NT are recruited to SGEF- and TrioD1-induced dorsal ruffles. HUVEC were transfected with myc-SGEF or myc-TrioD1 and either GFP-RhoG or GFP-Elmo2NT and immunostained using anti-myc antibody. RhoG and Elmo2NT colocalize with SGEF and TrioD1 in dorsal ruffles. Arrows indicate dorsal ruffle formation induced by TrioD1 and SGEF. Bars represent 10 µm.

Dorsal ruffles are transient actin-rich membrane protrusions that are formed from the dorsal surface of cells in vitro and are implicated in macropinocytosis (Dowrick et al., 1993; Ladwein and Rottner, 2008). Consistent with previously published results, transfection of TrioD1 or SGEF clearly stimulated dorsal ruffle formation in HUVEC (Blangy et al., 2000; Ellerbroek et al., 2004), as indicated by arrows in Figure 10. Cotransfection of myc-TrioD1 or myc-SGEF with GFP-RhoG or GFP-Elmo2NT caused translocation of these proteins to dorsal ruffles (Figure 10). These experiments indicate that GFP-Elmo2NT faithfully monitors the localization of active RhoG in cells.

1.2 *Yersinia* activates RhoG by invasin-triggered β1-integrin signaling

Yersinia enterocolitica strain 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 via its TTSS. The non-virulent *Yersinia* strain WA-C is a plasmid cured strain and is devoid of the virulence plasmid pYV. Thus, without YadA, the TTSS and the effector Yops, *Yersinia* WA-C interacts with host cells mostly via invasin, although the contribution of other adhesins (e.g. Ail) cannot be excluded.

In order to investigate RhoG activation during cellular infection, HUVEC were transfected with GFP-Elmo2NT and recruitment to bacterial contact sites was analyzed. Infection with *Yersinia* resulted in prominent Elmo2NT-enriched phagocytic cups, which were quantified at two different time-points. After 10 minutes of infection, GFP-Elmo2NT was recruited by about 10 % of *Yersinia* WA-C bacteria but only by about 1 % of *Yersinia* WA-314 bacteria. After 45 minutes of infection, recruitment to WA-C bacteria was still about 5 % and to WA-314 about 0.5 %. Representative cells in Figure 11 show GFP-Elmo2NT recruitment to the contact site with WA-C or WA-314. The percentage of recruiting bacteria was determined microscopically by counting GFP-Elmo2NT cups of total cell-associated bacteria.

This data suggests that either active RhoG is directly recruited to the bacterial contact site by *Yersinia* or that RhoG is activated subsequently after recruitment. However, this data also indicates that virulent yersiniae inhibit or downregulate RhoG activation, most likely through one or more TTSS-translocated Yops.

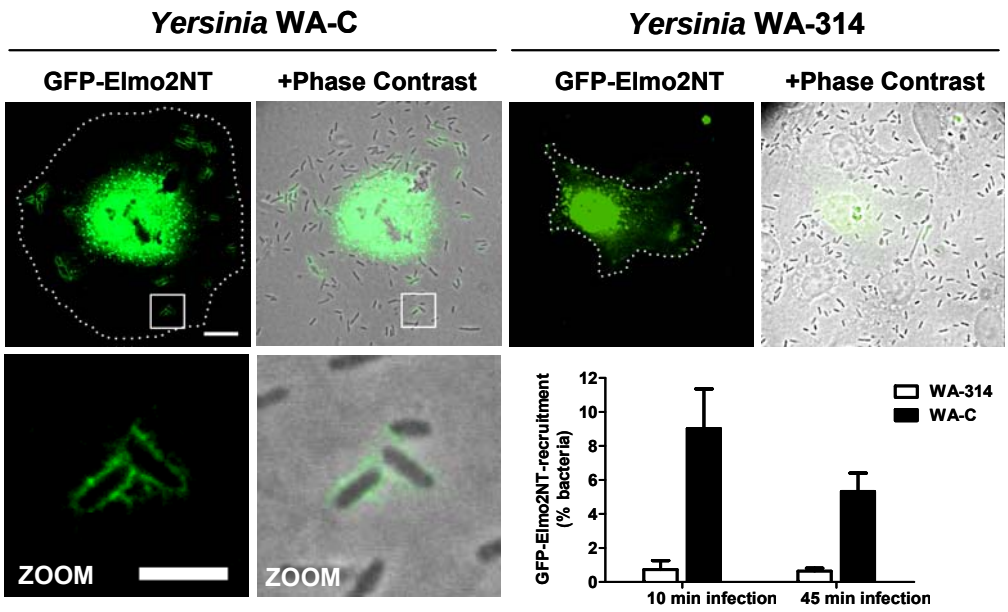

Figure 11. Differential recruitment and activation of RhoG by *Yersinia enterocolitica*. HUVEC were transfected with GFP-Elmo2NT and then infected with *Yersinia* WA-C or *Yersinia* WA-314 for 10 minutes. Images show representative cells that either display a high and a low level degree of recruitment of GFP-Elmo2NT. Merged images represent overlays of green fluorescence and phase-contrast channel. White boxes indicate areas that were enlarged about 8-fold (Zoom). Dotted lines outline the cell borders. The graph shows quantification of GFP-Elmo2NT recruitment by indicated strains after 10 and 45 minutes of 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 3 different experiments with at least 33 cells analyzed per experiment. Bars represent 10 µm (upper panel) or 3 µm (lower panel).

To further verify activation of RhoG and to investigate the role of *Yersinia* adhesins therein, GST-Elmo2NT pull-down assays were performed. Cos-7 cells were transfected with GFP-RhoG and infected with non-virulent WA-C or virulent WA-314 yersiniae for 30 minutes. Infection with *Yersinia* WA-C but not *Yersinia* WA-314 resulted in an increased level of GTP-bound GFP-RhoG (Figure 12A, left panel).

Invasin, which plays an important role in the early phase of intestinal infection, mediates internalization into non-phagocytic cells by triggering outside-in β_1-integrin signaling (Grassl et al., 2003). YadA is a matrix-binding adhesin that has been shown to interact with and activate $\alpha5\beta1$ integrin indirectly via a fibronectin bridge (El Tahir and Skurnik, 2001). To determine its role in RhoG activation, the C-terminal 397 amino acids of invasin, containing the integrin-binding domain fused to GST, were coupled to fluorescence microspheres (latex beads) and Cos-

7 cells were treated with these beads. Latex beads coated with the C-terminus of *Yersinia* invasin were shown to be rapidly internalized by epithelial cells and this type of engulfment resembles the zipper mechanism of viable invasin-expressing *Yersinia* (Dersch and Isberg, 1999; Wiedemann et al., 2001). As a control, beads coated with GST alone were used. In addition, Cos-7 cells were infected with *E. coli* strains that constitutively express either invasin or YadA on their surface. Activation of GFP-RhoG was detected in Cos-7 cells treated with GST-Invasin397 and *Invasin-E.coli* but not with *YadA-E.coli* or GST-beads, respectively (Figure 12A, middle and right panel). Activation of endogenous RhoG could be detected in HeLa cells, which expressed relatively high levels of endogenous RhoG, after infection with *Yersinia* WA-C but not WA-314. Endogenous RhoG was also activated by *Invasin-E.coli* in these cells (Figure 12B). These pull-down assays further confirm that in *Yersinia* infected cells RhoG is activated by invasin-induced signaling.

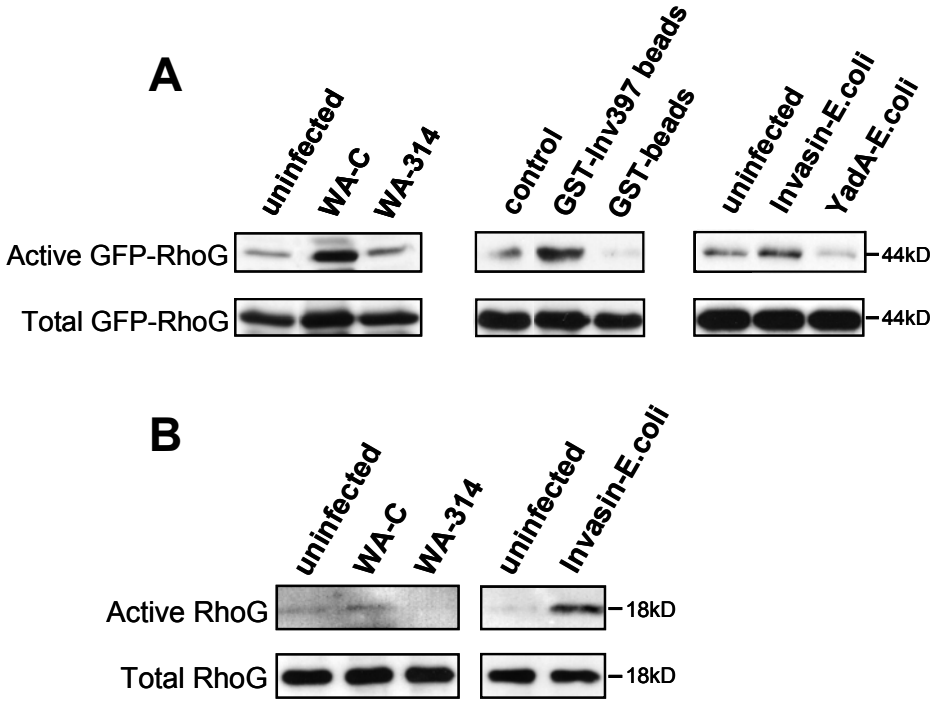

Figure 12. RhoG is activated by *Yersinia* invasin.
(A) Cos-7 cells were transfected with GFP-RhoG and infected with *Yersinia* WA-C or WA-314 (left panel), incubated with beads coated with GST-Invasin397 or GST (middle panel) or infected with *Invasin-E.coli* or *YadA-E coli* for 30 minutes (right panel). 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 using anti-RhoG antibody.

1.3 Spatiotemporal dynamics of RhoG activation and deactivation

The previous experiments indicated that RhoG is recruited and activated very quickly upon activation of β1-integrin receptors by *Yersinia*. However, in cells infected with virulent *Yersinia* WA-314, RhoG is subsequently deactivated by a TTSS-translocated effector Yop. To visualize the spatiotemporal dynamics of RhoG activation and deactivation, live-cell imaging was employed. HUVEC were transfected with GFP-RhoG or GFP-Elmo2NT, infected with either WA-C or WA-314 and recruitment of these fluorescent proteins to bacterial contact sites was investigated.

The recordings showed 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 Figure 13A, upper panel). In sharp contrast to the fast and continuous recruitment of GFP-RhoG by WA-C bacteria, GFP-RhoG appeared to alternately advance and recede along the rods of *Yersinia* WA-314. This back and forth movement process could take up to 23 minutes (mean 14.6 ± 8.5 minutes, n=3; single frames of a representative movie are shown in Figure 13A, lower panel), although in all cases recorded it led to formation of a closed phagocytic cup. Such a disordered and protracted phagocytic cup assembly could also be seen in HUVEC, transfected with GFP-Elmo2NT (Figure 13B). This data shows subversion of RhoG activation at the bacterial cell contact site. To evaluate timing of RhoG activation, HUVEC were cotransfected with GFP-Elmo2NT and mCherry-RhoG and infected with *Yersinia* strain WA-C. This experiment revealed that GFP-Elmo2NT and mCherry-RhoG were translocated in parallel to *Yersinia* WA-C. However, whereas GFP-Elmo2NT stayed at the phagocytic cup only until phagocytic cup closure, GFP-RhoG remained there for extended time periods (Figure 13C). This suggested that active RhoG is recruited by the bacteria and deactivated shortly after phagocytic cup closure but then remains at the nascent phagosome.

In addition, endogenous RhoG recruitment by *Yersinia* strain WA-C was investigated. Prominent endogenous RhoG-enriched cups at the bacterial contact site were detected with an anti-RhoG antibody after infection with *Yersinia* strain WA-C for 30 minutes (Figure 14A). To confirm the specificity of staining, HUVEC were cotransfected with RhoG siRNA and pmCherry and stained with anti-RhoG antibody. To identify RhoG siRNA transfection, only cells which displayed red fluorescence were examined and these cells showed a reduced number of phagocytic RhoG cups at the bacterial contact site (Figure 14B).

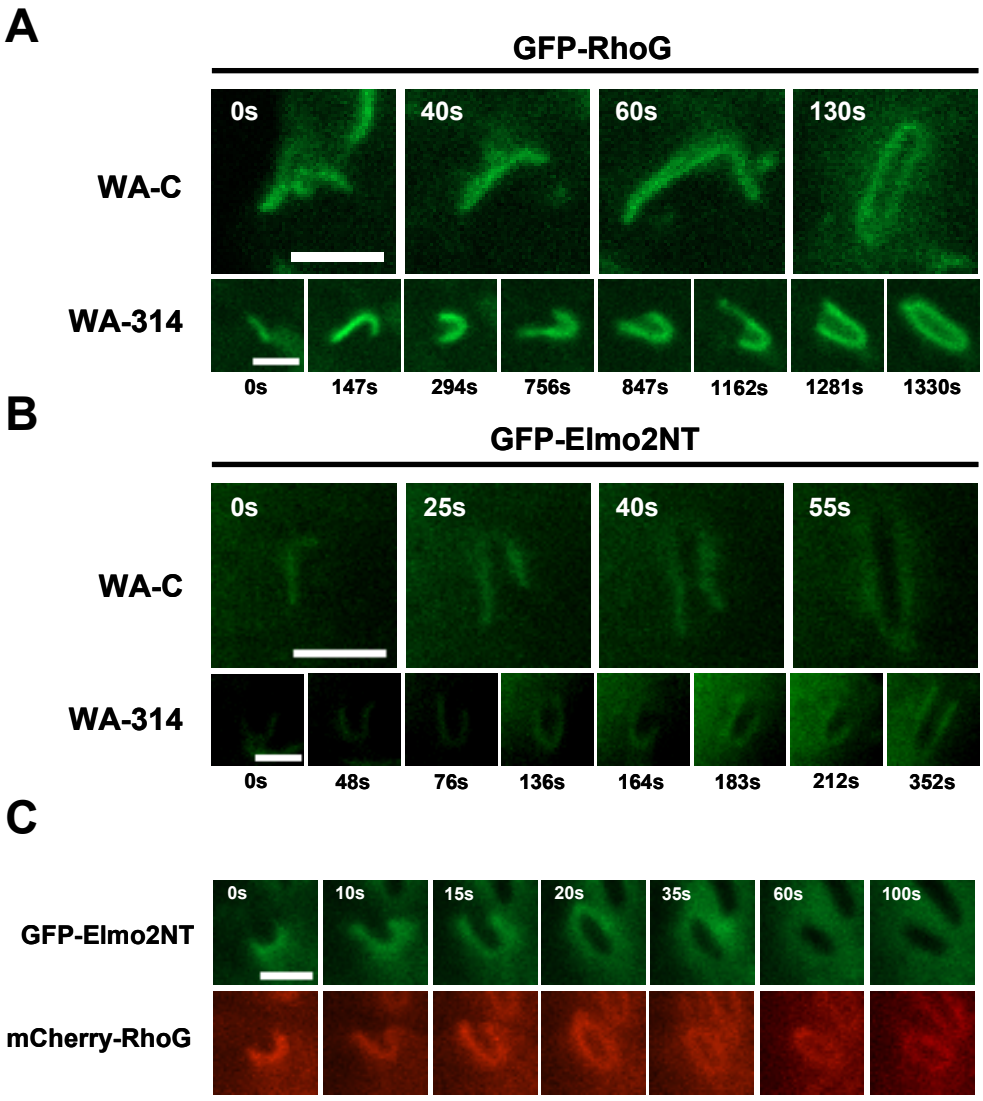

Figure 13. Spatiotemporal dynamics of RhoG activation and deactivation.
(A) HUVEC were transfected with GFP-RhoG and then infected with *Yersinia* WA-C or WA-314. GFP-RhoG recruitment was visualized using live-cell imaging. Depicted still images were taken from representative movies at indicated time points. Cells infected with WA-C show rapid closure of GFP-RhoG phagocytic cups. In contrast, cells infected with WA-314 show severely impaired GFP-RhoG recruitment to phagocytic cups with alternating back and forth movements.
(B) As described in A, but depicting HUVEC transfected with GFP-Elmo2NT.
(C) HUVEC were cotransfected with GFP-Elmo2NT and mCherry-RhoG and then infected with *Yersinia* WA-C. Depicted still images were taken from representative movie at indicated time points. After phagocytic cup closure, mCherry-RhoG but not GFP-Elmo2NT remains at the phagosome. Bars represent 2 µm.

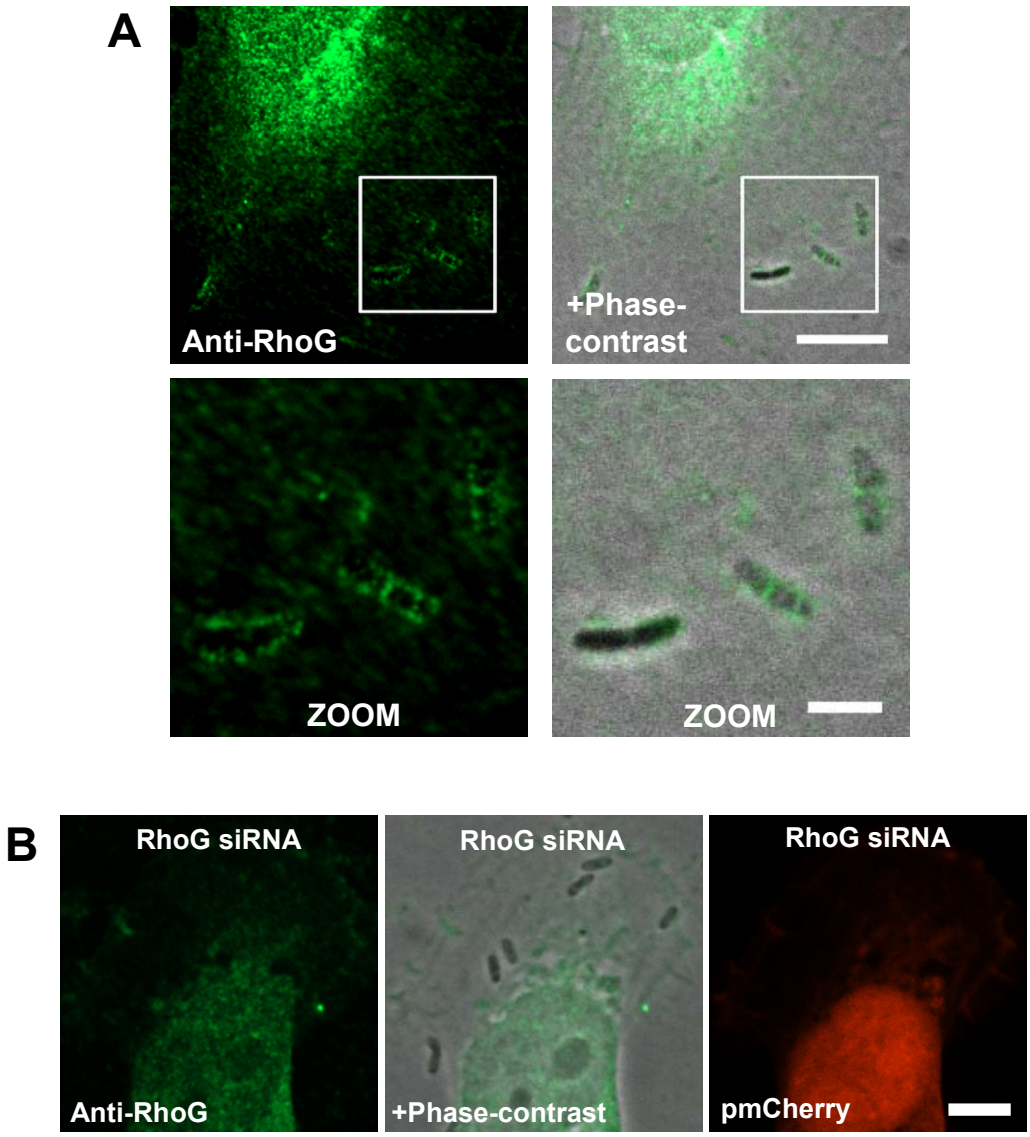

Figure 14. Endogenous RhoG is recruited to *Yersinia* cell contact site.
(A) HUVEC were infected with *Yersinia* strain WA-C for 30 minutes and endogenous RhoG was detected with immunofluorescence using anti-RhoG antibody (green fluorescence). Merged images represents overlay of green fluorescence and phase-contrast. Boxes indicate areas that were enlarged about 3-fold (Zoom). Bar represents 6 µm (upper panel) or 2 µm (lower panel).
(B) HUVEC were cotransfected with RhoG siRNA and pmCherry and immunostained with anti-RhoG antibody (green fluorescence). Single cells transfected with RhoG siRNA were identified by red fluorescence of mCherry expressing cells. Cells with RhoG siRNA show decreased RhoG recruitment to bacteria. Merged image represents overlay of green fluorescence channel and phase-contrast. Bars represent 6 µm.

2. RhoG is deactivated by *Yersinia* YopE

2.1 TTSS-translocated YopE inactivates RhoG

Yersinia enterocolitica translocates six effector Yops via its TTSS into host cells. In order to identify the Yop(s) responsible for downregulation of RhoG, a cell system that exhibits strong RhoG activity was established. Because the basal level of active RhoG was very low in all cell-lines tested, an exchange factor was used to stimulate RhoG activity.

TrioD1 has been shown to specifically activate RhoG and as shown in Figure 10, it caused translocation of GFP-RhoG to TrioD1-induced dorsal ruffles. To confirm RhoG stimulation by TrioD1 in a GST-Elmo2NT pull-down assay, myc-TrioD1 and myc-RhoG were cotransfected in Cos-7 cells. As a control, Tiam1, a Rac1-specific exchange factor, was transfected (Habets et al., 1994). The experiment demonstrated that, while RhoG was strongly activated by TrioD1, Tiam1 had no effect on its activation status (Figure 15). This indicated that cotransfection of myc-TrioD1 and myc-RhoG is a suitable method to strongly and reproducibly activate RhoG, which then could serve to study RhoG deactivation mechanisms.

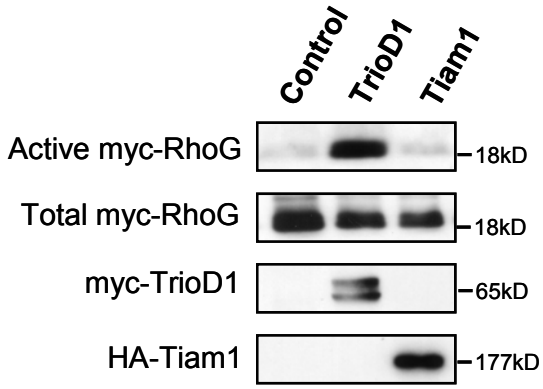

Figure 15. TrioD1 specifically activates RhoG.
Cos-7 cells were cotransfected with myc-RhoG and either control plasmid (left lane), myc-TrioD1 (middle lane) or HA-Tiam1 (right lane). Active myc-RhoG was precipitated with GST-Elmo2NT and analyzed by Western Blotting. Myc-tagged constructs were detected with anti-myc antibody and HA-Tiam1 with anti-HA antibody. TrioD1, but not Tiam1 produced a strong RhoG activation.

To determine the Yop responsible for RhoG deactivation, different *Yersinia* mutant strains deficient in individual Yops (ΔE, ΔH, ΔM, ΔO, ΔP or ΔT) were used. Each of these strains has a single Yop rendered inactive by the insertion of an antibiotic resistance cassette and therefore translocates only the remaining five effector proteins into host cells (for description of strains see Materials and Methods). As a positive control, cells were infected with wild-type *Yersinia* strain WA-314 to ensure RhoG inactivation.

To achieve robust RhoG activation, Cos-7 cells were cotransfected with vectors expressing myc-RhoG and myc-TrioD1 and then infected with aforementioned *Yersinia* strains for 2 hours.

As demonstrated in Figure 16, *Yersinia* WA-314 and all mutant strains downregulated TrioD1-stimulated RhoG activity, except for the *Yersinia* strain lacking YopE (WA-314ΔYopE).

To confirm that YopE is necessary and sufficient to downregulate RhoG activity, a *Yersinia* strain, WA-C(pTTSS+pYopE), which solely translocates YopE into host cells was used. Cos-7 cells were cotransfected with myc-RhoG and myc-TrioD1 to stimulate RhoG activity. Upon infection with *Yersinia* YopE strain, RhoG activity was completely abolished (Figure 17A, middle lane). These experiments show that YopE and no other Yop is crucially involved in RhoG deactivation.

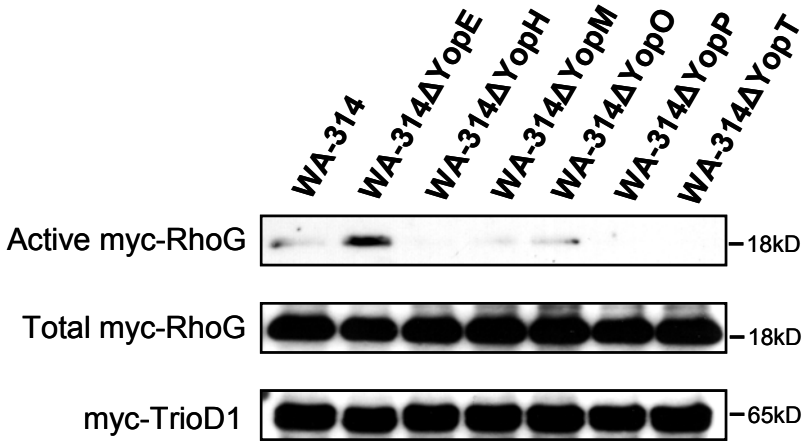

Figure 16. RhoG is inactivated by YopE.
Cos-7 cells were cotransfected with myc-RhoG and myc-TrioD1. After 24 hours cells were infected with indicated *Yersinia* strains for 2 hours, lysed and subjected to GST-Elmo2NT pull-down. RhoG is downregulated by all strains except ΔYopE strain.

2.2 Deactivation by YopE is dependent on its GAP activity

YopE is a TTSS-translocated effector protein that contains a RhoGAP domain and has been shown to deactivate Rac1, RhoA and Cdc42 in vitro (Black and Bliska, 2000). Like eukaryotic GAPs, the enzymatic activity of YopE is absolutely dependent on the so-called arginine finger motif, a critical arginine residue. In vitro GAP activity is abolished by replacing the arginine residue (Arg-144) with alanine (Black and Bliska, 2000).

To test if RhoG deactivation by YopE is dependent on its GAP activity, a *Yersinia* strain that exclusively translocates a GAP-defective YopE was constructed, WA-C(pTTSS+pYopER144A). TrioD1-stimulated cells were infected for 2 hours with this strain and active RhoG was pulled down with GST-Elmo2NT (Figure 17A, right lane). The results showed that GAP-defective YopE had no effect on RhoG activity.

To directly demonstrate GAP function of YopE for RhoG, an in vitro GTP-hydrolysis assay was performed. RhoG and YopE were bacterially expressed, purified as GST-fused proteins and then coincubated for 20 minutes with GTP. As a positive control, the catalytic domain of p50RhoGAP was used. p50RhoGAP has been shown to also deactivate Rac1 in vitro (Zhang et al., 1998).

The experiments demonstrate that YopE stimulates the intrinsic GTPase activity of RhoG as efficiently as that of Rac1, but had no effect on Ras (Figure 17B).

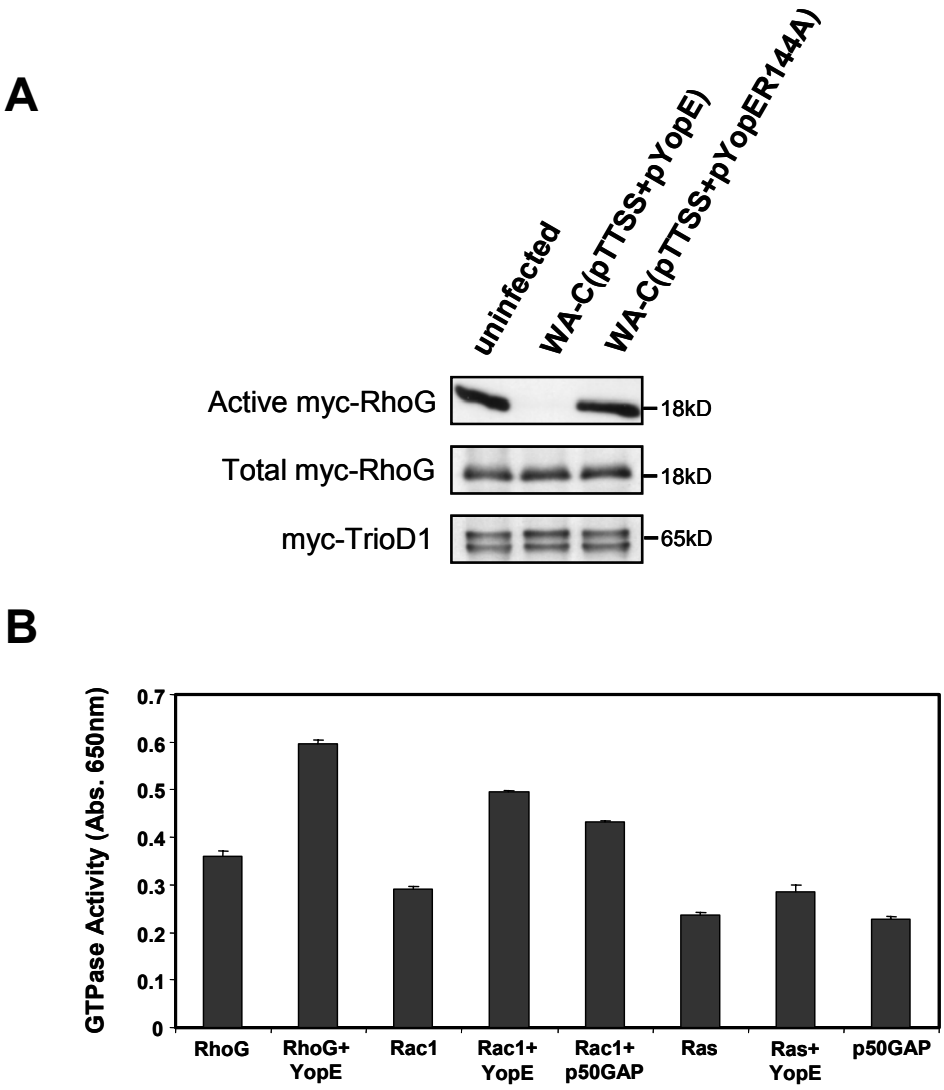

Figure 17. Inactivation of RhoG is dependent on YopE GAP function.
(A) Cos-7 cells were cotransfected with myc-RhoG and myc-TrioD1. After 24 hours, cells were infected with indicated *Yersinia* strains for 2 hours, lysed and subjected to GST-Elmo2NT pull-down.
(B) In vitro GAP assay. GTP-hydrolysis was measured at 650 nm absorbance after coincubation of indicated proteins for 20 minutes. YopE stimulated the GTPase activity of RhoG and Rac1 but not of Ras. The catalytic domain of p50RhoGAP was used as a positive control. Values are mean ± s.d. (error bars) of 3 independent experiments.

2.3 RhoG recruitment to *Yersinia* is reduced by YopE

To test whether YopE is sufficient to inhibit RhoG recruitment by *Yersinia*, HUVEC were transfected with GFP-RhoG and infected with *Yersinia* WA-C(pTTSS+pYopE) or WA-C(pTTSS+pYopER144A). GFP-RhoG recruitment to the bacterial contact site was evaluated. Phagocytic cups enriched in GFP-RhoG were quantified after 15 and 45 minutes of infection. After 15 minutes of infection with the YopE GAP-deficient strain, about 65 % of cell-associated bacteria showed GFP-RhoG recruitment. In contrast, cells infected with a strain that translocates wild-type YopE only, showed 45 % GFP-RhoG recruitment. After 45 minutes of infection, an even more pronounced reduction could be detected (Figure 18).

In conclusion, YopE was shown to be a specific and highly effective inhibitor of RhoG activity and recruitment during *Yersinia* infection.

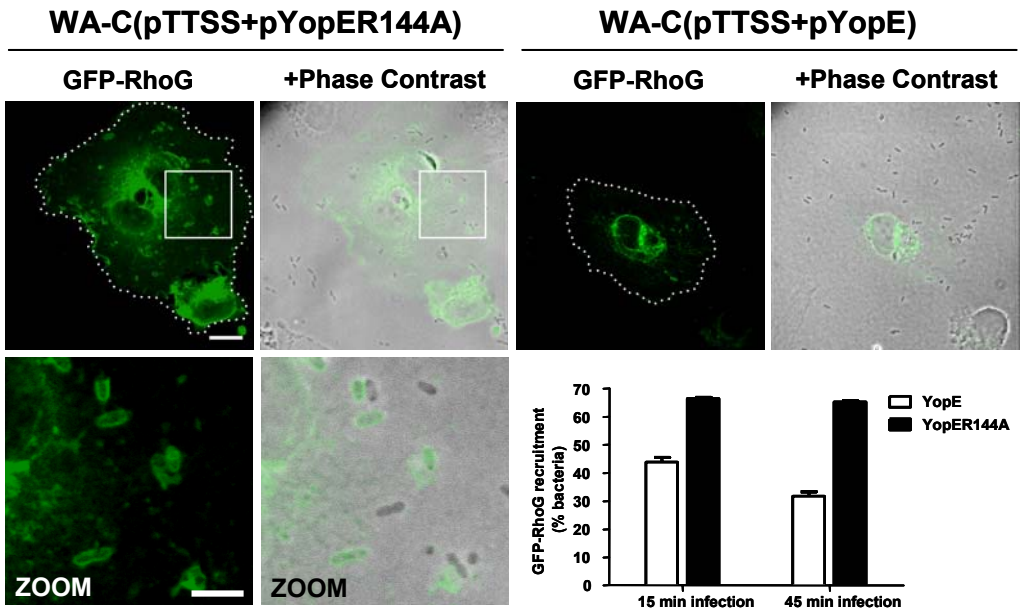

Figure 18. YopE reduces RhoG recruitment to *Yersinia*.
HUVEC were transfected with GFP-RhoG and then infected with *Yersinia* WA-C(pTTSS+pYopE) or *Yersinia* WA-C(pTTSS+pYopER144A). Images show GFP-RhoG distribution after 15 minutes of infection (green fluorescence). Merged images represent overlays of green fluorescence channel and phase contrast. White boxes indicate areas that were enlarged about 4-fold. Dotted lines outline the cell borders. For the bar graph GFP-RhoG recruitment by indicated strains was quantified after 15 and 45 minutes of infection. Each bar represents mean ± s.d. (error bars) of 3 different experiments with at least 33 cells analyzed per experiment. Bars represent 10 µm (upper panel) or 3 µm (lower panel).

3. Intracellular localization determines substrate specificity of YopE

3.1 YopE localizes to perinuclear structures

YopE has been described to localize to perinuclear membrane compartments and this localization is mediated by its membrane localization domain (Krall et al., 2004). However, the exact intracellular membranes or organelles to which YopE is localizing had not been characterized.

To investigate intracellular localization of YopE, HUVEC were infected with a *Yersinia* strain translocating YopER144A. After 60 minutes, cells were fixed and stained with anti-YopE antibody and YopE localization to perinuclear structures was evaluated (Figure 19, left image). YopE localization studies were performed with the GAP-defective mutant, because wild-type YopE causes severe morphological alteration of cells, preventing visualization of intracellular structures. To determine if transfected, vector-expressed YopER144A had the same localization properties, HUVEC were transfected with GFP-YopER144A. As shown in Figure 19, bacterially translocated and vector-expressed YopER144A localized to the same perinuclear structures, validating transfected GFP-YopER144A as a suitable tool to study intracellular localization.

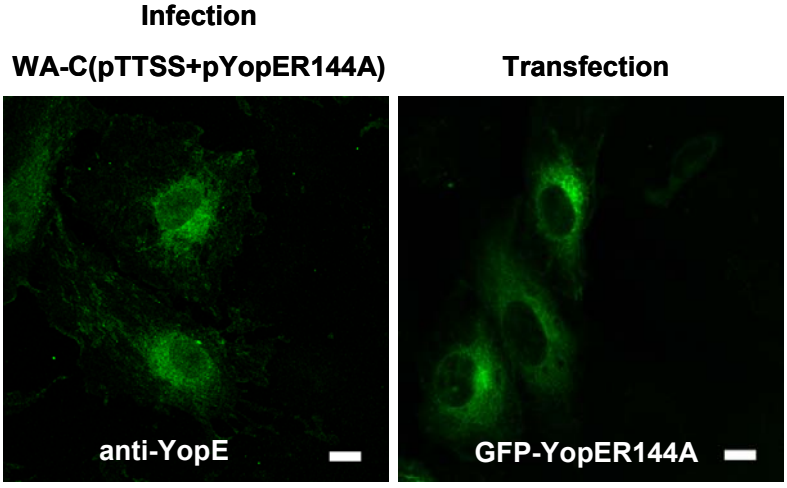

Figure 19. Intracellular localization of YopE.
Bacterially translocated and vector-expressed YopER144A localize to the same compartments in HUVEC. Cells were either infected with *Yersinia* strain WA-C(pTTSS+pYopER144A) for 60 minutes and then immunostained for YopE or transfected with GFP-YopER144A. Bars represent 10 µm.

In order to obtain detailed information about intracellular localization, HUVEC were transfected with GFP-YopER144A and immunostained with antibodies that visualize subcellular compartments normally located in the vicinity of the nucleus, endoplasmatic reticulum (ER) and the Golgi apparatus. Calreticulin, a protein that resides in the ER, and GS27, an integral membrane protein of the Golgi apparatus were used as specific markers for these organelles.
Strong colocalization of YopE with the Golgi marker GS27 and weak colocalization with the ER marker could be observed, as indicated by yellow color in merged images (Figure 20A).
To further confirm Golgi localization, GFP-YopER144A-transfected HUVEC were incubated with Brefeldin A for 60 minutes to disrupt the Golgi apparatus. Brefeldin A is a lactone antibiotic, produced in fungi, which specifically interferes with anterograde protein transport from the ER to the Golgi apparatus, leading to a redistribution of the Golgi into the ER (Klausner et al., 1992).
As shown in Figure 20B, Brefeldin A treatment destroyed the Golgi apparatus and redistributed it into the ER. Consequently the Golgi-specific localization of YopER144A was abolished, while the ER localization was unaffected (compare to Figure 20A).
These data showed that intracellular YopE localizes to the Golgi apparatus and to a lesser extent to the ER.

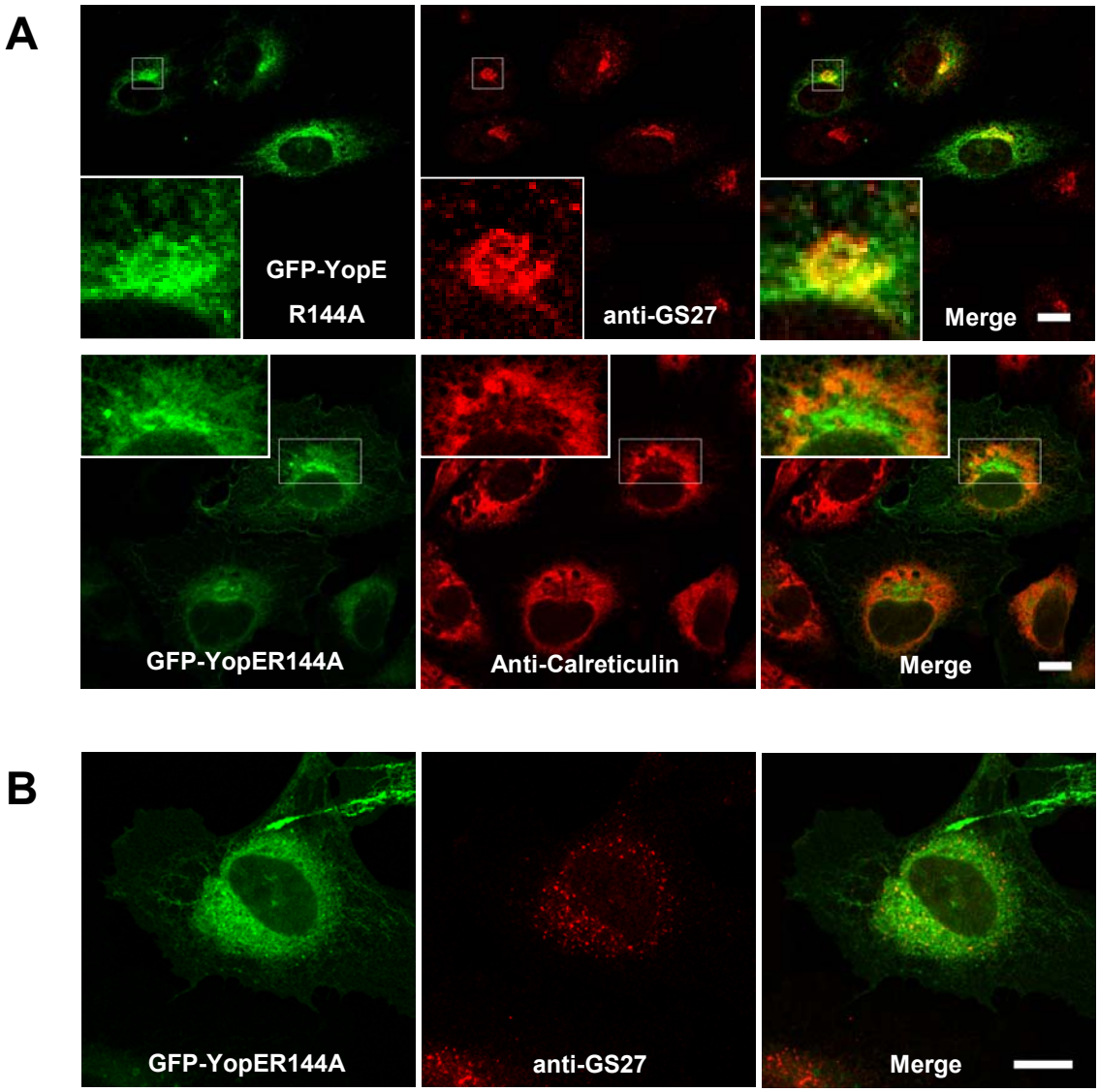

Figure 20. YopE localizes to the ER and Golgi.
(A) Confocal fluorescence images of HUVEC transfected with GFP-YopER144A. ER was immunostained with anti-Calreticulin antibody and Golgi with anti-GS27 antibody. Merged images represent overlays of green and red channel resulting in yellow color. Boxes indicate areas that were enlarged 2-3-fold and shown in insets. Bars represent 10 µm.
(B) For disruption of the Golgi apparatus, Brefeldin A was added to GFP-YopER144A transfected HUVEC for 60 minutes and Golgi was immunostained with anti-GS27 antibody. GS27 staining was dispersed and the Golgi-specific localization of YopE was abolished (compare with A). Merged image is the overlay of the green and red channel. Bar represents 10 µm.

3.2 The membrane localization domain determines intracellular localization

The membrane localization domain (MLD) of YopE was recently identified as being amino acid residues 54-75 and was shown to mediate intracellular membrane binding (Krall et al., 2004). To determine the impact of this region on localization, a YopE mutant lacking the MLD was constructed (GFP-YopER144AΔMLD) and subcellular localization was investigated. In addition, the arginine residue responsible for GAP activity was replaced by alanine because, similar to native YopE, YopEΔMLD displayed severe cellular abnormalities making it impossible to follow intracellular localization.

As demonstrated in Figure 21, GFP-YopER144AΔMLD showed a cytoplasm-like fluorescence and no signs of Golgi/ER localization.

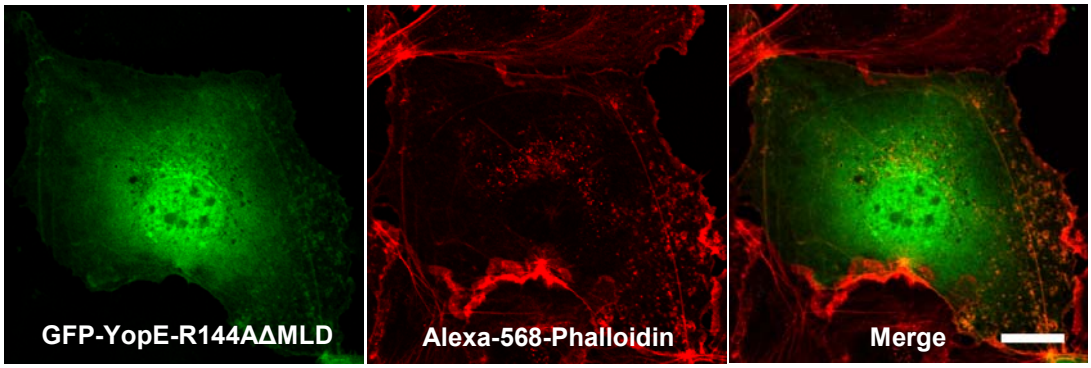

Figure 21. Intracellular localization of YopEΔMLD.
HUVEC were transfected with GFP-YopER144AΔMLD and stained for F-Actin with Alexa-568-labeled phalloidin to visualize the cell border. Merged image is the overlay of the green and red channel. Bar represents 10 µm

3.3 YopE and RhoG colocalize in the ER and Golgi

The relationship between YopE activity and localization inside the host cell had not been studied. To find out if the substrate-specificity of YopE is dependent on its localization, different experimental set-ups were tested. At first, colocalization of YopE with various Rho GTPase family members was investigated.

To this end HUVEC were cotransfected with a vector expressing mCherry-YopER144A and GFP-fused constructs of various Rho GTPases (GFP-RhoG, GFP-Rac1, GFP-Cdc42 and GFP-TC10).

The Golgi- and ER-localized mCherry-YopER144A showed extensive colocalization with GFP-RhoG and GFP-Cdc42 but only limited colocalization with GFP-Rac1 and GFP-TC10 (Figure 22). The intense colocalization with RhoG is consistent with the previous finding that YopE inactivates RhoG. In contrast, the supposed primary target of YopE, Rac1, did not show any colocalization in the ER/Golgi region, as it was distributed throughout the cytoplasm. The strong colocalization with Cdc42 is also consistent with previously published results showing that YopE inactivates Cdc42 in vitro (Aili et al., 2006). Effects on the less-characterized Rho GTPase TC10 have not been described.

It therefore appears that YopE associates with its primary target RhoG and also Cdc42, but only at a low level with Rac1 and TC10 at the ER and Golgi of host cells.

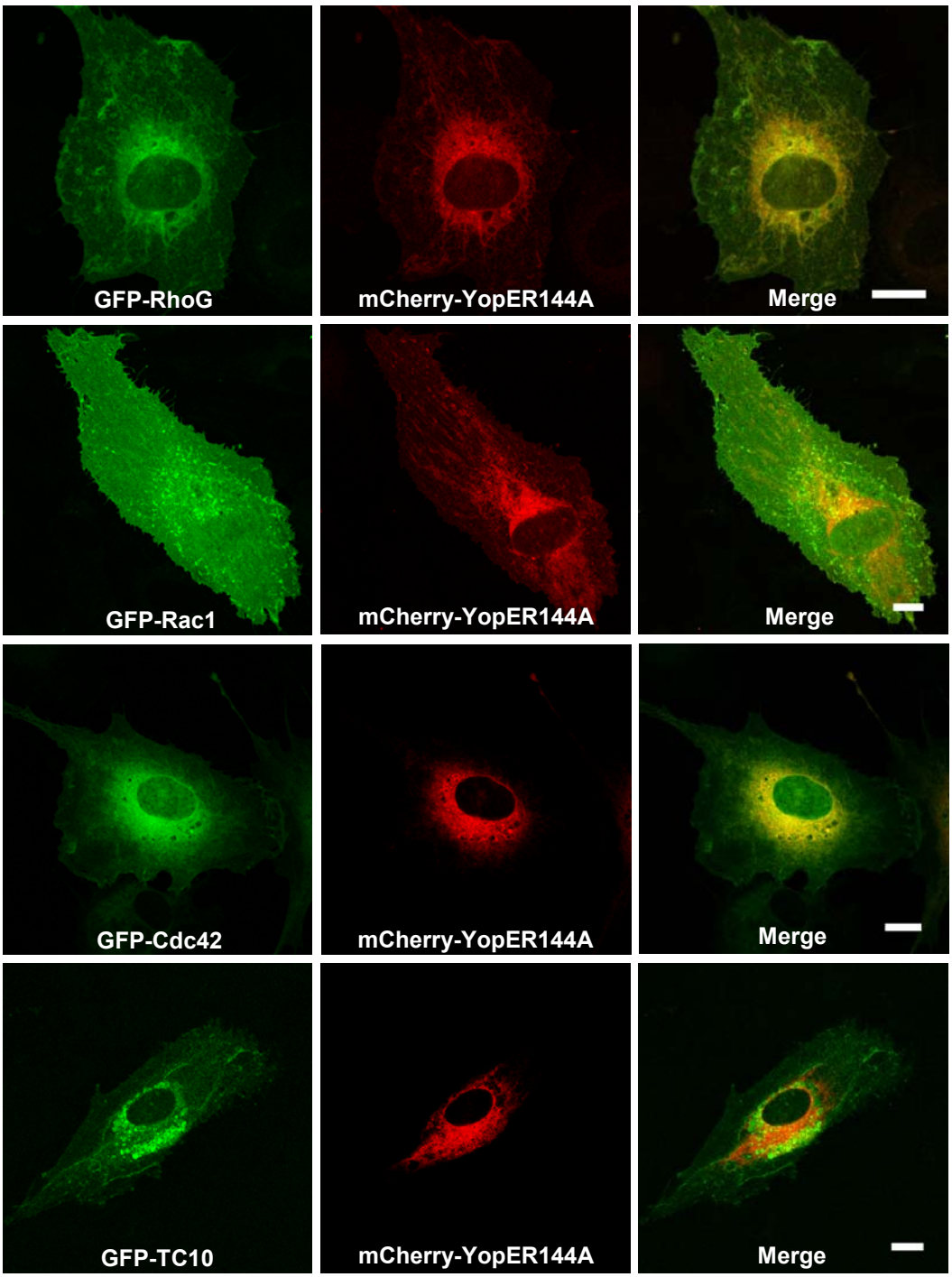

Figure 22. Colocalization study of YopE and different Rho GTPases.
Confocal fluorescence images of HUVEC cotransfected with mCherry-YopER144A and GFP-RhoG, GFP-Rac1, GFP-Cdc42 or GFP-TC10. Merge represents overlays of green and red fluorescence channel. Bars represent 10 µm

3.4 Substrate range of YopE is determined by its localization

Recently the MLD of the YopE homologue in *Pseudomonas aeruginosa*, ExoS, was characterized. ExoS was shown to have a restricted substrate range upon complete removal of the MLD or replacement of single amino acid residues within the MLD (Zhang et al., 2007).

To further examine the relationship between YopE localization and function, a *Yersinia* strain, WA-C(pTTSS+YopEΔMLD) was constructed, which translocates a MLD-deficient YopE. The activation status of Rho GTPases was quantified using pull-down assays with different GST-fused constructs. Active Rac1, Cdc42 and TC10 were measured using GST-PAK-CRIB and active RhoA was measured with GST-Rhotekin. These GST-fused constructs specifically pull down the abovementioned active GTPases from cell lysates.

Cos-7 cells were infected with the YopEΔMLD *Yersinia* strain and activation of Rho GTPases was compared to cells that were infected with a strain translocating native YopE. As shown in Figure 23A, YopEΔMLD and wild-type YopE were equally effective in downregulating TrioD1-stimulated RhoG (compare to Figure 17A). As a control, *Yersinia* strain WA-314ΔYopE was used. Endogenous Cdc42 was also equally well deactivated upon infection with *Yersinia* strains translocating YopEΔMLD or YopE (Figure 23B). In cells infected with the GAP-defective strain WA-C(pTTSS+pYopER144A) no effect on Cdc42 is seen.

In contrast, YopEΔMLD effectively deactivated GFP-TC10, while YopE had only a minor effect on GFP-TC10. An even more pronounced effect was detected on RhoA. Whereas YopEΔMLD abrogated baseline RhoA activity in Cos7 cells completely, YopE had almost no effect on RhoA (Figure 23C and D).

Taken together, these data suggest that the substrate range and activity of YopE is crucially determined by its compartmentalization in the Golgi/ER. Unlike ExoS from *Pseudomonas aeruginosa*, the substrate range of which is restricted upon removing the MLD, YopE seems to broaden its specificity towards Rho GTPases when membrane localization is prevented.

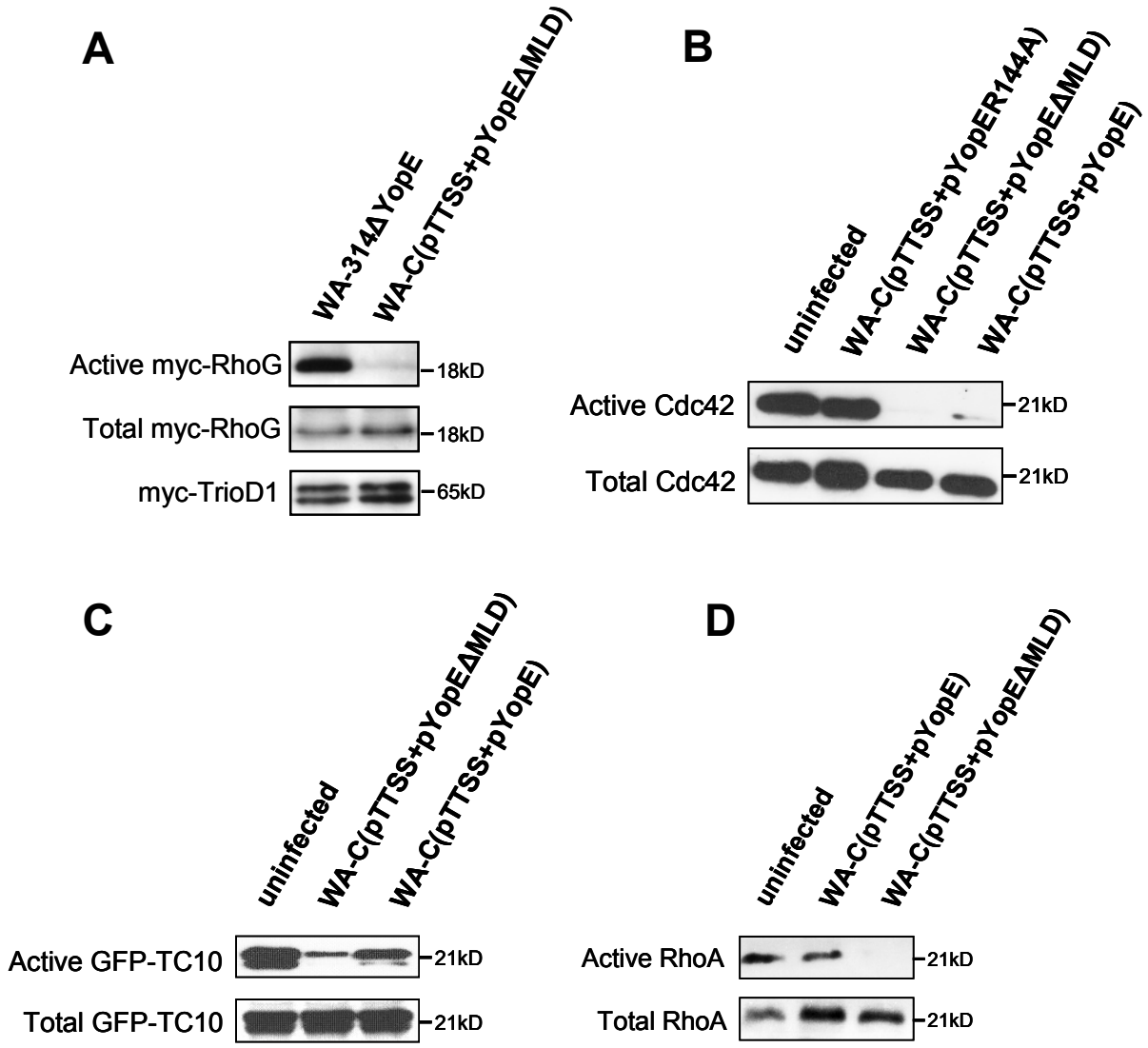

Figure 23. Unspecific inactivation of Rho GTPases by YopEΔMLD.
(A) Cos-7 cells were cotransfected with myc-RhoG and myc-TrioD1. After 24 hours, cells were infected with indicated *Yersinia* strains for 2 hours, lysed and subjected to GST-Elmo2NT pull-down. Active myc-RhoG and myc-TrioD1 were detected with anti-myc antibody.
(B) Cos-7 cells were infected with indicated *Yersinia* strains for 2 hours and subjected to GST-PAK-CRIB pull-down assay. Endogenous Cdc42 was detected with anti-Cdc42 antibody.
(C) Cos-7 cells were transfected with GFP-TC10 and then infected with indicated *Yersinia* strains for 2 hours. Active GFP-TC-10 was pulled down with GST-PAK-CRIB and detected by Western Blotting using anti-GFP antibody.
(D) 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. RhoA is inactivated by YopEΔMLD but not native YopE.

4. Modulation of RhoG by *Yersinia* controls Rac1 activity

4.1 Rac1 activity is inhibited by RhoG siRNA

Rac1 has been described as a preferred target of *Y. enterocolitica* (Aepfelbacher et al., 2007) and recently RhoG has been shown to activate Rac1 via the proteins Elmo and Dock180 (Katoh and Negishi, 2003). The question arose if effects of *Yersinia* are in fact due to direct action on Rac1 or are rather due to effects on RhoG which then regulates Rac1. To test this notion, a pool of four siRNAs directed against RhoG was used. Efficiency of siRNA was tested 48 and 72 hours after transfection. While an almost complete knock-down of endogenous RhoG could be obtained, there was no effect on Rac1 protein level after 48 hours (Figure 24A). All further experiments with RhoG siRNA were therefore carried out 48 hours after siRNA transfection, as the result indicated that RhoG protein level increases again after 72 hours. To determine the impact of RhoG knock-down on Rac1, Cos-7 cells were transfected with RhoG siRNA and active Rac1 was pulled down with GST-PAK-CRIB. As a control, a pool of four non-targeting (nt) siRNAs was used. Activity of Rac1 could be abrogated almost completely with RhoG siRNA, while nt siRNA had no effect (Figure 24B). These data show that RhoG is an important mediator of baseline Rac1 activity in Cos-7 cells.

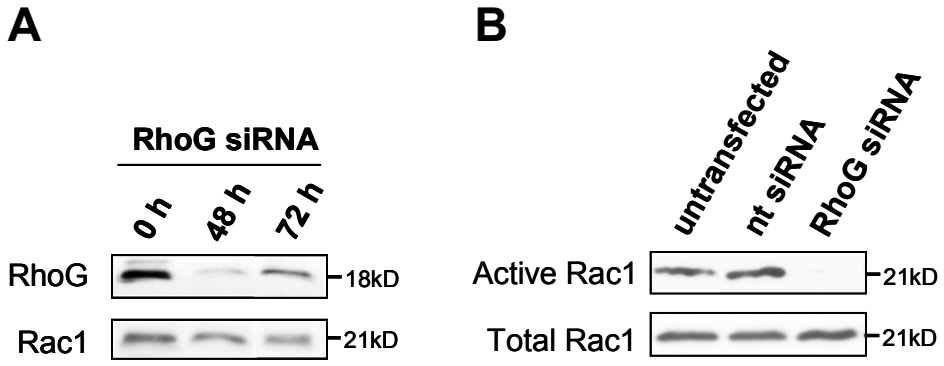

Figure 24. RhoG siRNA inhibits Rac1 activity.
(A) Cos-7 cells were transfected with siRNA for RhoG and at indicated time points equal amounts of protein were analyzed by Western Blotting using anti-RhoG and anti-Rac1 antibody. RhoG siRNA effectively reduced the level of endogenous RhoG protein at 48 hours but had no effect on Rac1 protein level.
(B) Cos-7 cells were transfected with 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.

4.2 Invasin stimulates Rac1 activity via RhoG

Yersinia enterocolitica adheres to β1-integrins of host cells via invasin. Within only a few minutes, signaling cascades are activated, leading to rapid internalization of the bacteria (Isberg et al., 2000). Rac1 has been described to be activated subsequent to β1-integrin activation by invasin (Alrutz et al., 2001). To confirm this, HUVEC were infected with *Invasin-E.coli* for 15 minutes and Rac1 activation was measured by GST-PAK-CRIB pull-down. Compared to uninfected cells, an increased level of active Rac1 was observed (Figure 25, lane 1 and 2). To test if the activation of Rac1 was RhoG-dependent, HUVEC were transfected with RhoG siRNA for 48 hours and infected with *Invasin-E.coli* for 15 minutes. Knock-down of RhoG blocked invasin-stimulated Rac1 activation completely (Figure 25, lane 3 and 4).

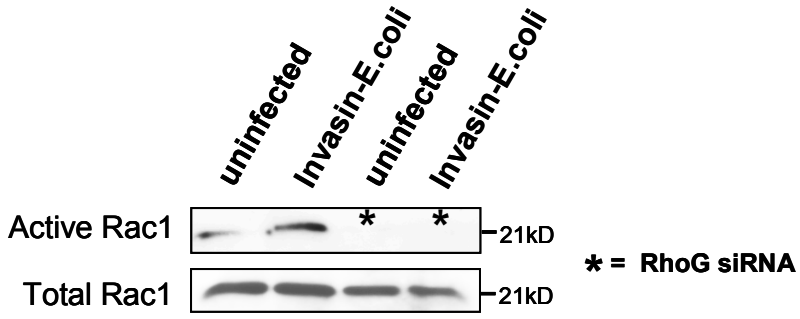

Figure 25. Invasin stimulates Rac1 activity via RhoG.
HUVEC were left untransfected or transfected with RhoG siRNA (indicated by asterisks) for 48 hours and then infected with *Invasin-E.coli* for 15 minutes. The level of active Rac1 was determined by GST-PAK-CRIB pull-down and Western Blotting using anti-Rac1 antibody.

4.3 Internalization of *Yersinia* is reduced by RhoG siRNA

In order to prove functional significance of the RhoG signal pathway during cell invasion of *Yersinia*, the internalization ability of *Yersinia* strain WA-C was investigated in RhoG siRNA-transfected cells. A double fluorescence staining method that discriminates between internalized and extracellular bacteria was used (see Materials and Methods).

HUVEC were transfected with RhoG siRNA or nt siRNA for 48 hours and then inoculated with *Yersinia* WA-C for 60 minutes. Confocal images in Figure 26A show representative cells that display internalization of bacteria. Internalized bacteria are shown in green, while extracellular bacteria are stained in yellow. Statistical evaluation of internalization experiments demonstrated that invasin-mediated uptake of bacteria was reduced by approximately 50 % in the presence of RhoG siRNA compared to cells that were transfected with nt siRNA (Figure 26B).

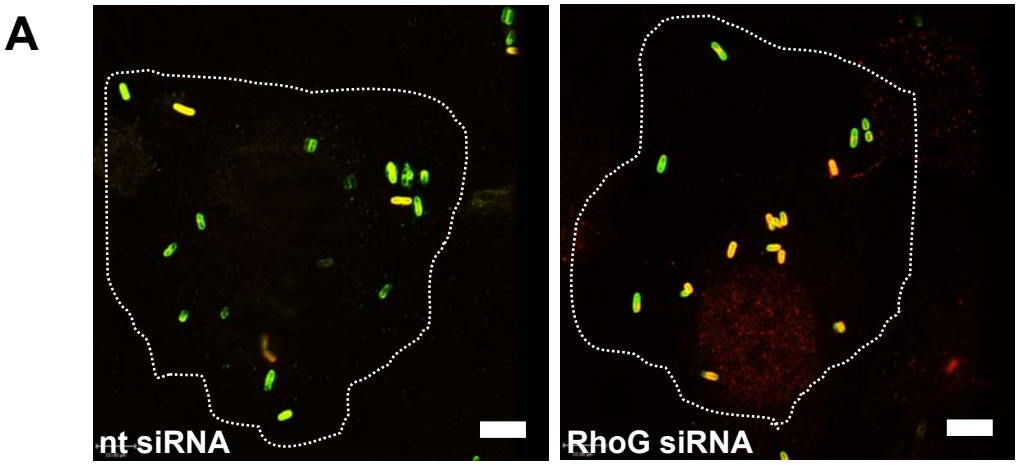

Figure 26. Cell invasion of *Yersinia* WA-C is reduced by RhoG siRNA.
(A) HUVEC were transfected with RhoG siRNA or non-targeting siRNA for 48 hours and then infected with *Yersinia* WA-C for 60 minutes. Bacteria were stained with anti-invasin antibody and with AlexaFluor 568- (red) and AlexaFluor 488- (green) labeled secondary antibody. Inside/outside staining identifies internalized bacteria (green) and extracellular bacteria (yellow). Dotted lines outline the cell borders. Bars represent 10 µm.

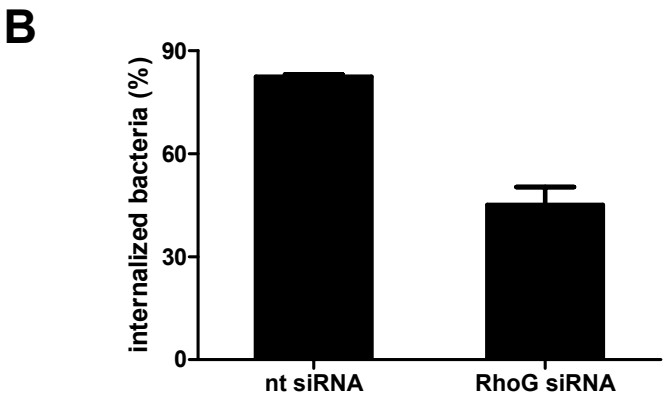

Figure 26. Cell invasion of *Yersinia* WA-C is reduced by RhoG siRNA.
(B) Statistical analysis of *Yersinia* WA-C cell invasion into HUVEC, transfected with RhoG siRNA or nt siRNA after 60 minutes of infection. Internalization was quantified by immunofluorescence staining, which distinguishes between intracellular and total cell-associated bacteria. Bars represent mean ± s.d. (error bars) of 3 independent experiments with at least 60 cells analyzed per experiment.

4.4 YopE inhibits Rac1 activity via RhoG

YopE has been reported to downregulate Rac1 (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000) and to inhibit formation of Rac1-dependent actin-structures in infected cells (Andor et al., 2001). To what extent YopE-induced Rac1 inhibition is in fact mediated by downregulation of RhoG and the Elmo/Dock180 module was tested using two different strategies. First, the RhoG signal pathway to Rac1 was blocked by Elmo-T618. Elmo-T618 lacks its C-terminal domain, and is thus unable to bind to its downstream effector Dock180, blocking downstream signaling to Rac1 (Katoh and Negishi, 2003). Secondly, Rac1 was activated directly with an exchange factor and inhibition by YopE was examined.

Infection of myc-Rac1 transfected Cos-7 cells with a *Yersinia* strain translocating native YopE resulted in reduced baseline activation level of myc-Rac1 (Figure 27A, lane 1 and 2).

If dominant negative Elmo-T618 was transfected, the activation level of Rac1 was reduced to a similar extent than with transfection of RhoG siRNA. However, infection of Elmo-T618 expressing cells with *Yersinia* strain WA-C(pTTSS+pYopE) did not produce a further reduction of Rac1 activity (Figure 27A, lane 3 and 4). These data suggest that Rac1 is inactivated by YopE for the most part through inhibition of RhoG.

As demonstrated in Figure 17B, Rac1 was inactivated by YopE in vitro. To determine whether YopE can directly inactivate Rac1 in cells, direct Rac1 activation was induced by Tiam1 in HUVEC, which showed very low basal Rac1 activity.

Upon infection of Tiam1-expressing HUVEC with WA-C(pTTSS+pYopE), Rac1 activity was inhibited but not fully abrogated (Figure 27B). These experiments showed that YopE can directly inhibit Rac1 activity in cells by competition with Rac1 exchange factors.

Taken together, YopE was shown to inactivate Rac1 by direct and indirect mechanisms. YopE can compete with Rac1 GEFs and directly stimulate GTP-hydrolysis in Rac1. Furthermore YopE can induce GTP-hydrolysis in RhoG and thereby downregulate Elmo/Dock180 signaling to Rac1.

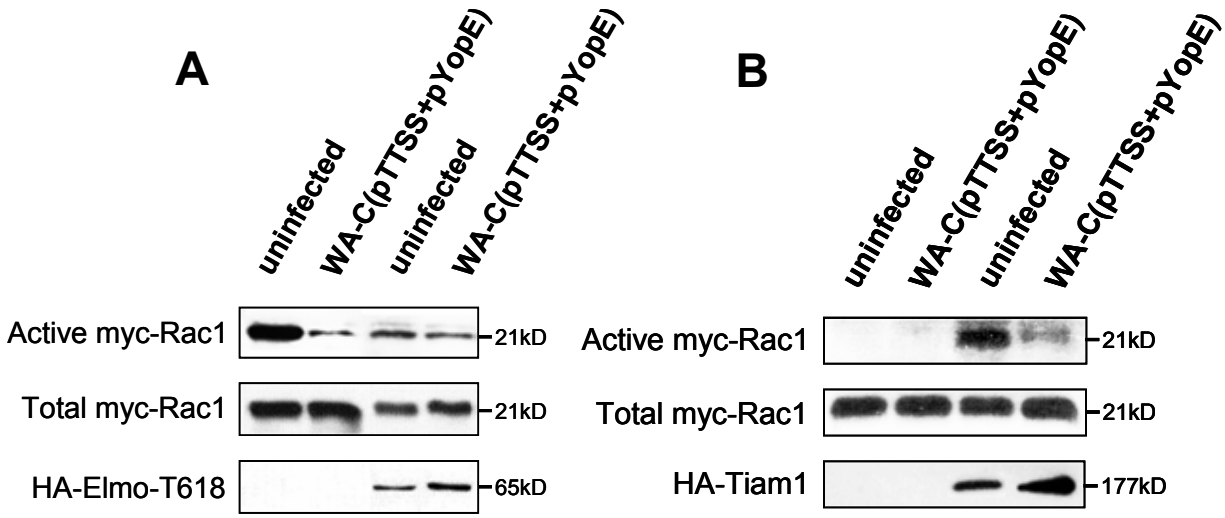

Figure 27. YopE inhibits Rac1 activity via RhoG.
(A) Cos-7 cells were transfected with myc-Rac1 (lane 1 and 2) or cotransfected with myc-Rac1 and HA-Elmo-T618 (lane 3 and 4) and then infected with WA-C(pTTSS+pYopE) for 2 hours. The level of active myc-Rac1 was determined by GST-PAK-CRIB pull-down and Western Blotting using anti-myc antibody. HA-Elmo-T618 was detected with anti-HA antibody.
(B) 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 A.

D. Discussion

Invasin-mediated RhoG activation

During the early stage of infection, *Yersinia enterocolitica* adheres to M-cells of the small intestine, leading to rapid internalization and subsequent transport to the Peyer's patches. Multiple signaling proteins inside the host cell are involved in this bacterial zipper-like uptake mechanism.

In this work RhoG, a Rac-related protein, was shown to be recruited to the bacterial contact site, which resulted in activation of this Rho GTPase. Localized activation of RhoG was measured using the N-terminal domain of Elmo (Elmo2NT), a downstream effector protein of RhoG. Several assays indicated that Elmo2NT faithfully monitors active RhoG. GST-Elmo2NT specifically precipitated active RhoGV12 but not dominant negative RhoGN17 or a panel of constitutively active Rho GTPases from cell lysates. Transfection of constitutively active RhoGV12 but not dominant negative RhoGN17 caused redistribution of GFP-Elmo2NT to the Golgi and ER. Furthermore, two RhoG-specific GEFs induced dorsal ruffle formation and translocation of RhoG and Elmo2NT to these actin structures. Using siRNA-mediated knock-down it could be shown that RhoG is responsible for mediating bacterial internalization.

RhoG has previously been implicated in phagocytosis and bacterial invasion. A signal 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 remodeling, leading to bacterial uptake (Patel and Galan, 2006). Finally, the *Shigella* TTSS effector IpgB1 has been reported to mimic RhoG and activate the Elmo/Dock180 complex. The activation induces membrane ruffling and subsequent bacterial invasion. Thus, RhoG has evolved as a central target of bacterial virulence factors promoting host cell invasion. As the results in this study show, RhoG activation leading to bacterial uptake was triggered by invasin, which is in agreement with the fact that invasin plays an important role in the initial step of infection, when yersiniae adhere to β1-integrins of M-cells, resulting in their uptake (Grassl et al., 2003). Several factors have been demonstrated to be important in promoting invasin-mediated uptake, including a number of Rho GTPase family members (Wiedemann et al., 2001). Rac1 and its downstream effectors seem to be especially required for internalization (Alrutz et al., 2001). Recently, the Elmo/Dock180

module was described as a Rac1-specific GEF, activated by RhoG (Katoh and Negishi, 2003). Hence, it seemed reasonable to assume that invasin-stimulated uptake might involve the RhoG/Elmo/Dock180 pathway, acting upstream of Rac1. In fact, the results demonstrated that invasin-induced RhoG activation was entirely responsible for Rac1 activation. However, whether RhoG acts exclusively through Elmo/Dock180 and Rac1 to mediate bacteria-induced internalization, or if RhoG might also have different functions in bacterial invasion, needs to be elucidated. Recently, Rac1-independent RhoG effects on the actin cytoskeleton were described (Meller et al., 2008). Furthermore, an involvement of RhoG in macropinocytosis, caveolar endocytosis and transendothelial migration was reported (Ellerbroek et al., 2004; Prieto-Sanchez et al., 2006; van Buul et al., 2007). The observation that RhoG remained at the phagosome for extended time periods supports the notion of it having other, yet unknown functions during phagocytosis, such as phagosome maturation.

Transfection of RhoG siRNA reduced internalization of non-virulent *Yersinia* by only about 50 %, although, as shown in Figure 24A, an almost complete knock-down of endogenous RhoG could be obtained after 48 hours. Residual RhoG might be responsible for this relatively minor effect. Alternatively, the loss of RhoG expression may have been compensated for by other Rho GTPases. Such functional compensation could recently be demonstrated for two closely related Rab GTPases (Wasmeier et al., 2006).

In macrophages, individual inhibition of Rac1, RhoA or Cdc42 also resulted in approximately 50 % reduction of invasin-mediated uptake (Wiedemann et al., 2001). These data indicate that in all cases, multiple Rho GTPases seem to be involved in uptake mechanisms and to obtain complete inhibition of internalization, several GTPases concurrently need to be blocked.

YadA, the virulence plasmid-encoded adhesin, did not contribute to RhoG activation during uptake. Previously published results demonstrated that YadA from *Yersinia pseudotuberculosis* was also able to induce internalization via extracellular matrix-dependent bridging to host cell β1-integrin receptors in the absence of invasin (Eitel and Dersch, 2002). However, YadA from *Y. enterocolitica* did not induce any detectable uptake in this study. Hence, *Y. enterocolitica* YadA may lack features that are important for the *Y. pseudotuberculosis* YadA-triggered mechanism (Eitel and Dersch, 2002).

To what extent RhoG might be involved in the *Y. pseudotuberculosis* YadA-dependent uptake pathway needs to be examined but activation of RhoG seems highly likely as well, since the same cellular receptor, β1-integrin, is exploited by YadA and invasin.

RhoG deactivation by YopE

In non-virulent *Yersinia* strain WA-C, active RhoG was recruited by the bacteria immediately upon adhesion and was deactivated shortly after a full phagocytic cup was formed and bacteria were internalized. During infection with virulent *Yersinia* strain WA-314, RhoG was inhibited, its recruitment was severely altered and bacterial internalization was inhibited.

In later stages of infection, the type III secretion system translocates six effector Yops into host cells. TTSS-translocated YopE was identified as the sole RhoG inhibitor of virulent yersiniae in this dissertation work. YopE has been demonstrated to exert multiple effects on host cells, such as inhibition of phagocytosis, inhibition of caspase-1-mediated maturation and release of interleukin-1β, and superoxide anion production (Black and Bliska, 2000; Schotte et al., 2004; Aepfelbacher et al., 2007). In addition, YopE affected translocation of other Yops by regulating the TTSS pore (Viboud and Bliska, 2001; Mejia et al., 2008). The Rho GTPases Rac1, RhoA and Cdc42 are inactivated by YopE in vitro. However, only Rac1 and RhoA were reported to be inactivated in vivo (Aili et al., 2006). Most of above cellular YopE effects have been attributed to inhibition of Rac1. Rac1 controls a variety of cell functions associated with immune defense and it plays a crucial role in a number of Rho GTPase signaling networks (Burridge and Wennerberg, 2004). Actin polymerization via WAVE and the Arp2/3 complex is regulated by Rac1 (Jaffe and Hall, 2005), as well as immunoglobulin receptor-mediated phagocytosis (Niedergang and Chavrier, 2005). In contrast, regulation of the translocation pore was described as both, Rac1- and RhoA-dependent (Mejia et al., 2008).

Thus, it seems obvious that Rac1 is a preferred target of *Yersinia*, because its inactivation may contribute to a large degree to the antiphagocytic and immunosuppressive effects of these pathogenic bacteria.

The results presented in this study confirmed that Rac1 is directly inhibited by YopE. YopE was shown to inactivate Rac1 in vitro in a GTP-hydrolysis assay and in vivo by competing with a Rac1-specific GEF. However, a second major mechanism by which YopE can modulate Rac1 activity could be identified in this dissertation. Rac1 was inhibited indirectly by blocking RhoG and the Elmo/Dock180 signaling pathway.

Previous findings showed that some signaling pathways to Rac1 are not blocked by YopE, that cooperation of YopE and YopT can lead to differentially activated and localized pools of Rac1 in cells and that YopE of some *Yersinia* serotypes is degraded in host cells by the proteasome leading to reactivation of Rac1 (Andor et al., 2001; Wong and Isberg, 2005; Ruckdeschel et al.,

2006; Hentschke et al., 2007). Furthermore, YopO has been reported to act as a dissociation inhibitor for Rac1 (Prehna et al., 2006). Hence, YopE, T and O might cooperate to produce differentially activated and compartmentalized subsets of Rac1 and potentially other Rho GTPases in other pathways. This strategy might allow Rho GTPase activation in selective cell compartments and signaling pathways and in parallel downregulation of other Rho GTPase pathways. For instance, Rac1 activity may be allowed for leaving open the bacterial translocation pore whereas, in parallel, immune cell responses may be inhibited. However, relevance of such strategies for the in vivo infection process of *Yersinia* is merely speculative and needs to be examined in future studies.

Although RhoG-dependent Rac1 inhibition seems to be an important mechanism, Rac1-independent effects cannot be excluded. RhoG has been described to exert various cell functions that are important for an immune response during a bacterial infection besides phagocytosis (see above) and that are suppressed by *Yersinia*. NADPH-oxidase, which is important during the innate immune response for production of reactive oxygen species, was reported to be activated by RhoG (Condliffe et al., 2006). A different study demonstrated an influence of RhoG on gene transcription of B- and T-cells, in particular in the regulation of the interferon-gamma promoter and transcription of nuclear factor of activated T-cells (NFAT) in lymphocytes (Vigorito et al., 2004). Finally, RhoG might have an impact on the microtubule system via kinectin and facilitate microtubule-dependent lysosomal transport (Vignal et al., 2001). An influence on lysosomal transport supports the results observed in this study where RhoG remained at the phagosome after phagocytic cup closure.

RhoG was found to be highly expressed in lymphocytes. However, its functional significance has to be investigated further, as studies in RhoG knock-out mice have shown (Vigorito et al., 2004). These mice lack a strong phenotype and have a normal health status, suggesting functional redundancy or compensation by other Rho GTPases. It has to be emphasized, however, that these mice were kept in a pathogen-free environment and no bacterial infections were performed (Vigorito et al., 2004). Rac1 knock-out mice were shown to be lethal in early embryogenesis (Sugihara et al., 1998), but Rac1 conditional knock-out mice may help to further understand the individual mechanisms and particular functions of the two Rho GTPases (Heasman and Ridley, 2008).

Furthermore, the question arises why RhoG is activated by *Yersinia enterocolitica* in the later stages of infection when invasin expression is suppressed. YadA was able to induce

internalization of *Yersinia* via fibronectin and β1-integrin, which might involve RhoG activation. After translocation through M-cells, the temperature shift to 37°C is responsible for a change in the expression pattern. YadA is expressed, but residual invasin may be present at the bacterial surface that stimulates RhoG activation. Furthermore, and what most likely happens is that RhoG activation is triggered by the phagocytosis program of cells of the innate immune system.
All of the described YopE effects have been assigned to its GAP function. The results presented in this work have also shown that GAP activity of YopE is solely responsible for its inhibitory effect on RhoG. Although GAP-independent effects on other proteins have not been described, they cannot be excluded since one other study suggested that in vitro GAP activity is not absolutely required for YopE-induced cell cytotoxicity (Aili et al., 2003).

Localization of YopE inside the host cell

Yersinia TTSS-translocated and vector-expressed YopE localized to a perinuclear region, which could be identified as the Golgi apparatus and endoplasmatic reticulum. Similar to YopE, ExoS, its homologue in *Pseudomonas aeruginosa*, was described to localize to a perinuclear region (Krall et al., 2004). After type III delivery, ExoS has been shown to traffic via an endocytic route involving Rab proteins, along microtubules, directly to the ER, bypassing the Golgi. The majority of ExoS was associated with stationary vesicles, but it was able to recycle back to the plasma membrane from ER (Deng et al., 2007). Furthermore, ExoS was shown to inhibit vesicle trafficking and endocytosis (Deng and Barbieri, 2008). However, ExoS is a bifunctional type III cytotoxin with an additional ADP-ribosyltransferase domain and if YopE transport in cells acts in a similar fashion is unknown.
In this study, YopE was only detected at the Golgi/ER and no other vesicular membrane localization could be identified. The question came up, how YopE traffics to the Golgi after type III delivery and where inactivation of Rho GTPases takes place. Neither a distinct localization of YopE at the bacterial contact site, nor any trafficking to the plasma membrane or along microtubules was observed (not shown in this work). However, transport of YopE starting from the very time point of its delivery was not investigated in this study and the localization of transfected YopE might not reflect the processes occuring during type III delivery.
On the other hand, live-cell imaging of activation and deactivation indicate that RhoG is inhibited directly at the contact site of host cells and virulent *Yersinia*. Thus, YopE might act on RhoG

directly after TTSS delivery, before translocation to Golgi/ER occurs. Alternatively, RhoG might be inhibited by YopE while trafficking to the plasma membrane.

The membrane localization domain of YopE was completely responsible for its Golgi and ER localization and removing it caused redistribution of YopEΔMLD to the cytoplasm.

A connection between localization and function could be demonstrated with GST pull-down assays, investigating the activation status of different Rho GTPases upon infection with *Yersinia* translocating YopEΔMLD. YopEΔMLD was as least as active as wild-type YopE in inhibiting RhoG and Cdc42. In addition, YopEΔMLD newly acquired the ability to efficiently inhibit RhoA and TC10. In this regard, YopE behaves differently to its homologue ExoS, the substrate range of which is restricted upon removing the MLD (Zhang et al., 2007). The catalytic activity of eukaryotic GAPs is regulated by a variety of mechanisms, such as phosphorylation, protein-protein interactions, proteolysis and most notably their interaction with lipids. Particular phospholipids can substantially alter the substrate specificity of various GAPs (Ligeti and Settleman, 2006). Similar to eukaryotic GAPs, different lipid environments of membrane-bound YopE vs. cytoplasmic YopEΔMLD may influence their Rho GTPase specificities.

In this work, RhoG was identified as a major target of YopE. However, Cdc42 and TC10 were also shown to be inhibited by native YopE. RhoA has been reported to be inactivated by YopE from *Y. pseudotuberculosis* in vivo, although, inactivation occured after 30 minutes of infection, suggesting an indirect effect on RhoA (Aili et al., 2006). In this study, only YopEΔMLD efficiently deactivated RhoA, while native YopE barely had any effect. The reason for this difference is not clear, but it is possible that YopE from *Y. pseudotuberculosis* has slight functional differences compared to *Y. enterocolitica.* Alternatively, properties of different cell-lines used in the studies account for the observed phenotype.

Certainly, in vivo relevance and functional significance of individual Rho GTPase inactivation have to be investigated further.

In conclusion, the results of this work can be summarized in two different models (Figure 28). During the invasive phase of *Yersinia* infection when Yops are not yet expressed, invasin-triggered RhoG activation mediates bacterial internalization, and this occurs by stimulating Rac1 via the Elmo/Dock180 module. During the subsequent antiphagocytic and immunosuppressive phase of the infection, RhoG is activated by *Yersinia*, but at this point RhoG and Rac1 are downregulated by YopE in a compartmentalized and differential fashion.

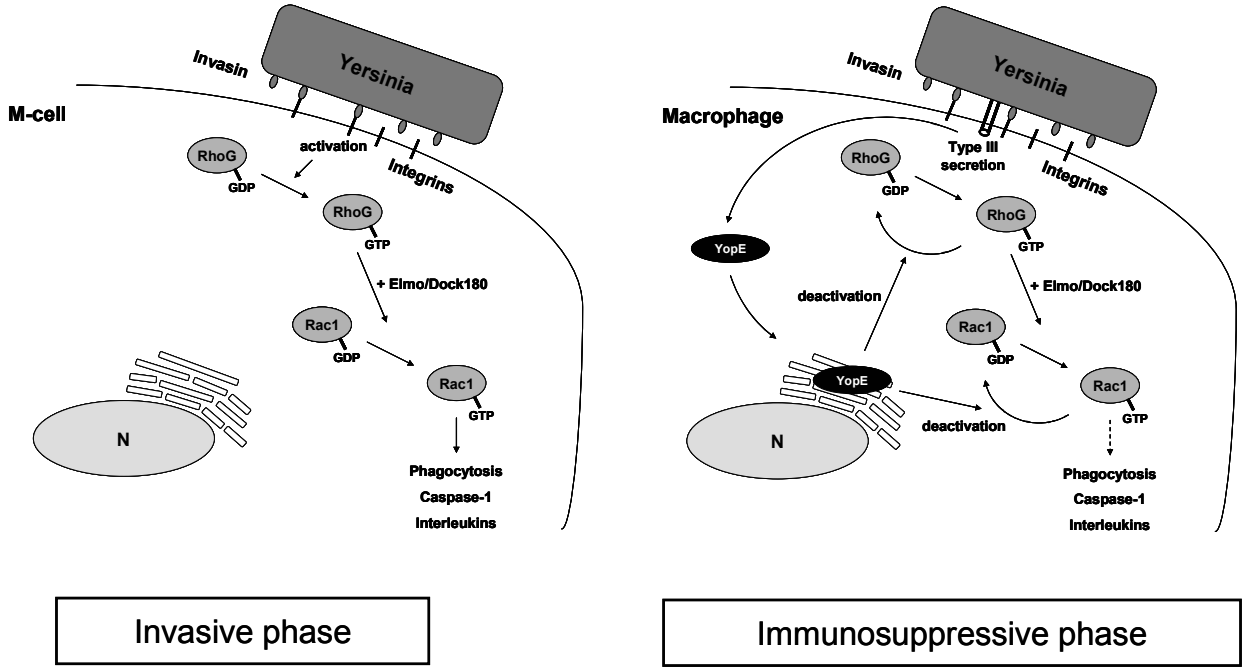

Figure 28. Model for modulation of RhoG activity by *Yersinia*.
During the invasive phase of infection, *Yersinia* binds to integrins via invasin, which triggers activation of RhoG and stimulation of Rac1 through the Elmo/Dock180 module. Activation of RhoG and Rac1 leads to cellular responses, such as 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.

E. Materials and Methods

1. Equipment and material

1.1 General equipment

Cassette for film exposure	Hartenstein, Würzburg, Germany
Cell Transfection	Amaxa, Nucleofector II, Cologne, Germany
Centrifuge	Thermo Scientific: Sorvall RC-5B, Sorvall RC 28S, Rockford, USA; Eppendorf table centrifuges: 5417R, 5810R, Hamburg, Germany; Sigma, Sigma centrifuge 3-18K, Osterode, Germany
Electrophoresis	Agarose gels: Roth, Karlsruhe, Germany SDS-PAGE: BioRad, Mini-Protean II Cell and Western Blot apparatus, Munich, Germany
Electroporation	BioRad, GenePulser II, Munich, Germany
Gel Dryer	BioRad, Gel-dryer 543, Munich, Germany
Glass bottom culture dishes	MatTek, Ashland, USA, Ibidi, Munich, Germany
Glass coverslips, round (12mm diameter)	Hartenstein, Würzburg, Germany
Incubator	Heraeus, BBD 6220; Binder, CB series, Tuttlingen, Germany
Incubator (shaking)	Sartorius, Certomat BS-1, Göttingen, Germany; Eppendorf Thermomoxer comfort / compact, Hamburg, Germany
Magnetic stirrer	RCT-basic, IKA-Labortechnik, Staufen, Germany
Microplate reader	Tecan, Infinite M200, Männedorf, Switzerland
Microscope (binocular)	Zeiss, Axiovert 25, Jena, Germany
Microscope slides	Marienfeld, Lauda-Königshofen, Germany
Microwave	Panasonic
PCR-Cycler	MWG-Biotech, Primus 96, Ebersberg, Germany; Eppendorf, Mastercycler gradient

pH Meter	Mettler-Toledo, Seven easy, Giessen, Germany
Photometer	GE Healthcare Europe, Ultrospec 3100 pro, Munich, Germany
Pipettes	Gilson, (10, 20, 100, 200, 1000 µl) Pipettes, DenHaag, Netherlands; Brand, Accu-jet pro, Wertheim, Germany
Power supply	BioRad, Power Pac 200 / universal
Processor for X-ray development	Agfa, Curix 60, Mortsel, Belgium
Refrigerator	Liebherr, profi line
Balance	Kern 440-47N
Scanner	Canon, CanoScan N67OU, Amsterdam, Netherlands
Sonifier	Branson, digital Sonifier 250-D, Danbury, USA
Transilluminator	Hartenstein, Vilber Lourmat
UV-Transilluminator	BioRad, ChemiDoc XRS
Vortex	Heidolph, Reax top
Water bath	GFL, GFL Typ 1013, Burgwedel, Germany

1.2 Microscopy equipment

For confocal fluorescence images, two different microscope settings were used. For live-cell imaging, a spinning-disk confocal system from Improvision (Coventry, England) was used. It was equipped with the following components:

Microscope	Zeiss Axiovert 200M
Objectives	Plan-Neofluar 10x/0.3
	Plan-Neofluar 20x/0.5
	Plan-Apo 63x/1.4 oil immersion
Confocal unit	Spinning disk CSU22 (Yokogawa, Japan)
Camera	Digital camera EM-CCD C9100-02 (Hamamatsu, Japan)
Laser	Cobolt Calypso CW 491nm, Cobolt Jive 561nm (Stockholm, Sweden)
Emmision filters	ET 525/50 (green), ET 620/60 (red) (Chroma Technology, Rockingham, USA)
UV lamp	X-cite series with Hg-lamp (EXFO, Ontario, Canada)

Incubation chamber	Solent Scientific, (Regensworth, England)

Fixed images were aquired with a confocal laser scanning microscope from Leica (Wetzlar, Germany) equipped with the following:

Microscope	Leica DM IRE2
Objectives	Plan-Apo 63x/1.3 water
	Plan-Apo 63x/1.4 oil
Confocal unit	Leica TCS SP2 AOBS confocal point scanner
Laser	Ar, Ar/Kr (488nm, 514nm), HeNe (543nm, 594nm, 633nm)

Images were aquired and processed with: Volocity (Improvision), Leica TCS software (Leica) and Adobe Photoshop.

2. Chemicals and enzymes

2.1 Chemicals and antibiotics

All chemicals and antibiotics were obtained from: Amersham/GE Healthcare (Munich, Germany), Biozym (Oldendorf, Germany), Dianova (Hamburg, Germany), Fermentas (St. Leon-Rot, Germany), Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), PAA (Pasching, Austria), PromoCell (Heidelberg, Germany), Roche (Mannheim, Germany) Roth (Karlsruhe, Germany) and Sigma-Aldrich (Munich, Germany).

The following antibiotic solutions were used for *Y. enterocolitica* and *E. coli* strains:

Antibiotic	dissolved in	final concentration
Ampicillin (Amp)	H_2O	100 µg/µl
Kanamycin (Kan)	H_2O	50 µg/µl
Tetracycline (Tet)	70 %C_2H_5OH	20 µg/µl
Spectinomycin (Spec)	H_2O	50 µg/µl
Chloramphenicol (Cm)	70 %C_2H_5OH	20 µg/µl
Nalidixic acid (Nal)	0.5 % NaOH	50 µg/µl

2.2 Kits and enzymes

Kits	
QIAprep spin Miniprep Kit	Qiagen GmbH, Hilden, Germany
EndoFree Plasmid Maxi Kit	Qiagen GmbH, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen GmbH, Hilden, Germany
QIAEX II Gel Extraction Kit	Qiagen GmbH, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen GmbH, Hilden, Germany
QuickChange II Site directed Mutagenesis Kit	Stratagene, Amsterdam, Netherlands
Phusion Site directed Mutagenesis Kit	Finnzymes, Espoo, Finland
RhoGAP assay Biochem Kit	Cytoskeleton, Denver, USA
HUVEC Nucleofector kit	Amaxa, Cologne, Germany
ExGen 500	Fermentas, St.Leon-Rot, Germany
X-treme Gene siRNA transfection reagents	Roche, Mannheim, Germany
ECL Western blotting detection reagents	GE Healthcare, Munich, Germany
SuperSignal West Femto detection reagents	Thermo Scientific, Rockford, USA
BioRad Protein Assay	BioRad, Munich, Germany
Complete Protease Inhibitor Cocktail Tablets	Roche, Mannheim, Germany

Enzymes	
BamHI	Fermentas, St.Leon-Rot, Germany
EcoRI	Fermentas, St.Leon-Rot, Germany
HindIII	Fermentas, St.Leon-Rot, Germany
NotI	Fermentas, St.Leon-Rot, Germany
SalI	Fermentas, St.Leon-Rot, Germany
XhoI	Fermentas, St.Leon-Rot, Germany
T4 DNA Ligase	Roche, Mannheim, Germany
Taq DNA Polymerase	Fermentas, St.Leon-Rot, Germany
Triple Master PCR System	Eppendorf, Hamburg, Germany

3. Bacterial strains

3.1 *E. coli* strains

strain	relevant characteristic	reference
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	(Grant et al., 1990)
BL21	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ	(Moffatt and Studier, 1987)
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)

3.2 *Yersinia enterocolitica* strains

strain	relevant characteristic	reference
WA-314	Wild-type strain; serogroup O:8; clinical isolate harbouring 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 O:9; clinical isolate harbouring the virulence plasmid pYV40	(Sory et al., 1995)
WA-CpTTSS	Strain WA-C harbouring plasmid pTTSS, Encoding for secretion and translocation	(Trulzsch et al., 2003)

	apparatus of WA-314	
WA-C(pTTSS+ pYopE)	WA-CpTTSS complemented with wild-type *yopE* from strain E-40	(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 was deleted (aa 54-75)	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*	(Trulzsch et al., 2004)
WA-314ΔyopO	Wild-type strain with insertional inactivation of *yopO*	(Trulzsch et al., 2004)
WA-314ΔyopP	Wild-type strain with insertional inactivation of *yopP*	(Trulzsch et al., 2004)
WA-314ΔyopT	Wild-type strain with insertional inactivation of *yopT*	(Zumbihl et al., 1999)

4. Plasmids

4.1 Bacterial expression

plasmid	description	reference
pGEX-4T	prokaryotic protein expression, empty vector	Amersham
GST-Inv397	pGEX4T-3-inv397; GST-Invasin	(Wiedemann et al., 2001)

	prokaryotic protein expression for in vitro use	
GST-RhoG	pGEX4T-2-RhoG; GST-RhoG prokaryotic protein expression	this study
GST-PAK-CRIB	pGEX4T-2-PAK-CRIB; GST-PAK-CRIB prokaryotic protein expression	(Sander et al., 1998)
GST-Rhotekin	pGEX4T-Rhotekin; GST-Rhotekin prokaryotic protein expression	(Sander et al., 1998)
GST-YopE	pGEX4T-2-YopEwt; GST-YopEwt prokaryotic protein expression	this study
GST-Elmo2NT	pGEX4T-2-Elmo2NT; GST-Elmo2NT prokaryotic protein expression	this study
pACYC184	Low copy vector, Cm^R, Tet^R	N.E.Biolabs

Plasmids for expression of YopE mutants in *Y. enterocolitica*

To create *Y. enterocolitica* strains WA-C(pTTSS+pYopER144A) and WA-C(pTTSS+pYopEΔMLD), the genes encoding YopER144A and YopEΔMLD were subcloned together with SycE into vector pACYC184. The resulting plasmids were then transformed into *Y. enterocolitica* strain WA-CpTTSS.

4.2 Mammalian expression

plasmid	description	reference
pEGFP-N	enhanced green fluorescence protein mammalian expression vector; MCS N-terminal of GFP	BD Clontech
pEGFP-C	enhanced green fluorescence protein mammalian expression vector; MCS C-terminal of GFP	BD Clontech

pmCherry	GFP was exchanged with mCherry in pEGFP-C1	G. Zenner
pRK5myc	myc-tag mammalian expression vector	BD Clontech

The following plasmids were kindly provided:

Myc-RhoGV12, mycRac1L61, myc-Cdc42L61, myc-TC10L75 and myc-TCLL79: Dr. Pontus Aspenström, Uppsala University, Uppsala, Sweden

GFP-RhoG, GFP-RhoGN17 and myc-TrioD1: Dr. Anne Blangy, CRBM, Montpellier, France

GFP-Elmo2NT: Dr. Xose Bustelo, University of Salamanca, Salamanca, Spain

Myc-SGEF: Dr. Keith Burridge, University of North Carolina, Chapel Hill, USA

HA-Tiam1: Dr. John Collard, Netherlands Cancer Institute, Amsterdam, Netherlands

HA-Elmo2-T618: Dr. Hironori Katoh, Kyoto University, Kyoto, Japan

GFP-Rac1 and GFP-Cdc42: Dr. Klaudia Giehl, University of Ulm, Ulm, Germany

GFP-TC10: Dr. David Michaelson, New York University School of Medicine, New York, USA

RhoG, RhoGN17, Rac1 and Cdc42 were subcloned into mammalian expression vector pRK5myc.

Plasmids for mammalian expression of YopE

To accomplish mammalian expression, the gene encoding YopE was amplified by PCR from *Y. enterocolitica* serotype O9 strain E40 and cloned into mammalian expression vectors pEGFP-N1 and pmCherry.

To create single amino acid substitution, YopER144A, QuickChangeII site directed mutagenesis kit was used. To create MLD deletion mutant, YopEΔMLD, Phusion site directed Mutagenesis kit was used.

5. Primers

Primers to amplify YopE from *Y. enterocolitica* strain E40

YE89-Xho-fwd	5’ CCAAGGGAATAACTCGAGATGAAAATATC 3’
YE9-Eco-rev	5’ CTTGTTTTTGAATTCAGCACATCAATG 3’

Mutagenesis primers to create single amino acid substitution YopER144A

YE-R144A-fwd 5’ GCGGCCCTCTTGCTGGCAGTATCACTCAATGC 3’

YE-R144A-rev 5’ GCATTGAGTGATACTGCCAGCAAGAGGGCCGC 3’

Mutagenesis primers to create MLD deletion mutant, YopEΔMLD

dMLD-fwd 5’ CGCATGTTCTCGGAGGGGAGCCATAAA 3’

dMLD-rev 3’ GGAACCCTGAGGGCTTTCAGTGCG 3’

Primers to clone YopE into vector pmCherry

YE-Xho-red 5’ CAGCTCGAGAGATGAAAATATCATTT 3’

YE-Eco-red 5’ GCGAATTCCGTCACATCAATGACAGTA 3’

Primers to clone YopER144A and YopEΔMLD into vector pACYC184

YopE-O8/9for 5’ GCCGAAGCTTGATATTGCTGGCACCACAAA 3’

YE9-Eco-rev 5’ CTTGTTTTTGAATTCAGCACATCAATG 3’

Primers to clone RhoG into vector pRK5myc

RhoG-Bam-fwd 5’ CTGGGATCCATGCAGAGCATCAAG 3’

RhoG-Eco-rev 5’ AGAATTCGATCACAAGAGGATGCA 3’

Primers to clone Elmo2NT into vector pGEX4T-2

Elmo-fwd 5’ CAGCTTCGAATTCCACCTCCGTCG 3’

Elmo-rev 5’ GGCCCCTCGAGCCTACAAGGCTGG 3’

6. Antibodies

6.1 Primary antibodies

The following primary antibodies were used either for immunofluorescence or Western Blot analysis:

antigen	species	supplier
GS27	mouse	BD Biosciences
Calreticulin	mouse	Stressgen
Myc	mouse	Cell Signaling
GFP	mouse	BD Biosciences

HA	rat	Roche
Cdc42	mouse	Cell Signaling
Rac1	mouse	BD Biosciences
RhoA	mouse	Santa Cruz
Y. enterocolitica	rabbit	Sifin
YopE	rabbit	(Ruckdeschel et al., 2006)
RhoG	mouse	(Meller et al., 2008)

Actin was stained with AlexaFluor® 568 labeled Phalloidin (Invitrogen).

6.2 Secondary antibodies

The following secondary antibodies were used for immunofluorescence or Western Blot analysis:

description	supplier
AlexaFluor® 488 goat anti mouse	Invitrogen
AlexaFluor® 568 goat anti mouse	Invitrogen
AlexaFluor® 488 goat anti rabbit	Invitrogen
AlexaFluor® 568 goat anti rabbit	Invitrogen
Anti-mouse IgG, horseradish peroxidase linked	GE Healthcare
Anti-rabbit IgG, horseradish peroxidase linked	GE Healthcare
Anti-rat IgG, horseradish peroxidase linked	GE Healthcare

7. Molecular Biology Methods

7.1 DNA digestions

DNA restriction enzymes recognize specific palindromic sequences within a DNA molecule and cut it by hydrolyzing two phosphodiester bonds, generating “sticky” (5’ and 3’ overhangs) or “blunt” (no 5’ or 3’ overhangs) ends.

DNA digestions were performed with restriction enzymes purchased from Fermentas. Digestions were performed at temperatures recommended by the manufacturer (normally 37°C) in a thermo

block or incubator for 1 h to overnight with an appropriate buffer, supplemented with BSA (10 mg/ml). Usually 1 unit of enzyme was sufficient to cut 1 µg of DNA in 1 hour.

Digestion reaction:

DNA: 100 ng to 1 µg

Buffer 10x: 2 µl to 7.5 µl

Enzyme: 0.2 µl to 1 µl

ddH_2O: ad 20 µl to 75 µl

7.2 DNA ligations

To ligate vector DNA with the desired insert DNA, both need to be digested with the same or compatible set of restriction enzymes. Ligation reactions were performed with T4-DNA ligase from Roche at 16°C overnight.

Ligation reaction:

Vector DNA: 10 ng to 100 ng

Insert DNA: 3x molar ratio of vector DNA

10x ligase buffer: 1 µl

T4-DNA Ligase: 1 µl

ddH_2O: ad 10 µl

1 to 5 µl of the ligation reaction was directly transformed into competent *E. coli*.

7.3 PCR

Polymerase chain reactions (PCR) were performed in 0.5 ml reaction tubes in a volume of 20 µl for screening purposes or in 75 µl for preparative gels, respectively. For screening purposes, *Taq*-DNA polymerase (Fermentas) was used. If the PCR fragments were to be used subsequently for cloning, the Triple Master PCR system was used (Eppendorf).

Typical PCR reaction setup:

DNA template: 1 µl (10 ng – 100 ng)

dNTP-mix: 1 µl (10 mM each)

Reaction buffer (10x): 2 µl – 7.5 µl

5’ Primer: 1 µl (10 pmol/µl)

3’ Primer: 1 µl (10 pmol/µl)

Polymerase: 0.2 µl – 1 µl

ddH_2O: ad 20 µl – 75 µl

After mixing the components on ice, the reaction tubes were placed in the PCR machine and run with the following protocol. The annealing temperature was chosen dependent on the primer pair and elongation time was chosen dependent on the length of the PCR fragment.

Initial denaturation: 94°C, 5 min

15-30 cycles: Denaturation: 94°C, 30 sec

Annealing: 45-70°C, 30 sec

Elongation: 72°C, 1-3 min

Final elongation: 72°C, 5 min

Hold at 4°C

PCR products were analyzed by agarose gel electrophoresis.

7.4 DNA Mutagenesis

Single amino acid substitutions were introduced with the QuickChangeII Site directed Mutagenesis kit (Stratagene) according to the manufacturer instructions. In brief, mutagenic primers with single amino acid substitutions were designed and used in a PCR to incorporate the mutation of interest into newly synthesized DNA. The mutagenic strands were transformed into competent *E. coli* and transformants were checked for the desired mutation by DNA sequencing.
To make the deletion mutant of YopE, YopEΔMLD, the Phusion Site directed Mutagenesis Kit (Finnzymes) was used according to the protocol of the manufacturer. Primer pairs that annealed adjacent to the desired deletion fragment were designed and used in a PCR to delete the fragment of interest. The PCR reaction was transformed into bacteria and transformants were analyzed by DNA sequencing.

7.5 Agarose Gel Electrophoresis

Agarose gels were prepared by mixing an appropriate proportion of agarose with 1x TAE buffer (40 mM Tris/Acetate, pH=8.3, 10 mM EDTA). Gel concentrations varied between 0.7 % and 2 %, depending on the size of the DNA molecule. The mixture was boiled in a microwave, cooled down and poured into agarose gel chambers. DNA was mixed with 5x DNA loading buffer, loaded into the gel and separated by application of voltage (50-120 V). After separation, the gel

was stained in ethidium bromide solution and DNA was visualized under ultraviolet light. The size of DNA molecules was estimated by comparison with a ready to load "1kb ladder" purchased from Fermentas.

7.6 DNA extractions from agarose gels

In order to isolate PCR fragments or digested DNA fragments, the desired DNA was excised from the gel and extracted either with the QIAquick Gel Extraction Kit or the QIAEX II Gel Extraction Kit according to the protocol of the manufacturer (Qiagen).

7.7 Preparation of Plasmid DNA

To obtain analytical quantities of DNA, plasmids were isolated with the QIAprep spin Miniprep Kit. For isolation of large quantities of plasmid DNA, the EndoFree Plasmid Maxi Kit was used. Endofree plasmids were used for transfection of mammalian cells while plasmids isolated with the Miniprep kit were used for screening and cloning purposes.

7.8 DNA Sequencing

DNA sequencing was performed in the MWG-Biotech sequencing lab (Martinsried, Germany) using 1 – 2 µg of plasmid DNA. The resulting sequences were analyzed with BLASTN or BLASTX provided by NCBI (National Center for Biotechnology Information)

7.9 Determination of DNA concentration and purity

Concentration of isolated DNA was measured with a spectrophotometer at a wavelength of 260 nm (Ultraspec 3001). To determine purity grade, DNA was measured at a wavelength of 260 nm and 280 nm. An $A_{260/280}$ coefficient < 1.8 and > 2 indicated contamination of the DNA.

7.10 Preparation of competent bacteria

Preparation of calcium-competent *E. coli* for heat shock transformation:

- 50 ml LB were inoculated with 1 ml of an overnight culture and incubated with vigorous shaking at 37°C to an OD600 of 0.6
- Cells were centrifuged for 10 min at 5500 g at 4°C

- The pellet was resuspended in 25 ml of ice cold 0.1 M $CaCl_2$ and incubated for 30 min on ice
- Cells were centrifuged for 10 min at 5500 g at 4°C
- The pellet was resuspended in 5 ml of ice cold 0.1 M $CaCl_2$/20 % glycerol
- Bacteria were aliquoted, frozen in liquid nitrogen and stored at -80°C

Preparation of electrocompetent *Y. enterocolitica* for electroporation:

- 100 ml LB were inoculated with 2 ml of an overnight culture and incubated with shaking at 27°C to an OD600 of 0.5 - 0.8
- Cells were centrifuged for 10 min at 8000rpm at 4°C
- Pellets were washed 2x with 10 ml of ice cold ddH_2O
- Pellets were washed 1x with 5 ml of ice cold ddH_2O/10 % glycerol
- Pellets were resuspended in 600 µl ddH_2O/10 % glycerol
- Cells were divided into 60 µl aliquots, frozen in liquid nitrogen and stored at -80°C

7.11Transformation of bacteria

Heat shock transformation of calcium-competent *E. coli*:

- 0.5 – 50 ng of plasmid DNA or 1 – 10 µl of a ligation reaction were added to 100 µl competent cells and incubated on ice for 30 min
- Cells were subjected to a heat shock at 42°C for 2 min and afterwards incubated for 3 min on ice
- 900 µl of antibiotic free LB or SOC medium was added and incubated for 60 min with shaking at 37°C
- Bacterial cells were centrifuged for 3 min at 5500rpm and the pellet resuspended in 200 µl LB
- 20 – 150µl of the bacteria were plated out on selective LB-agar plates and incubated at 37°C

Electroporation of electrocompetent *Y. enterocolitica*:

- 0.5 µg of plasmid DNA was added to 60 µl competent cells and transferred to an electroporation cuvette
- Bacteria were electroporated in a BioRad GenePulser II with the following settings: 100 low range, 50µF, 1,8 kV
- 500 µl LB or SOC medium was added and cells were incubated with shaking at 27°C for 50 min
- 10 – 200 µl of the bacterial cells were plated out on selective LB-agar plates and incubated at 27°C

8. Biochemical Methods

8.1 Protein expression and purification

The principle of expression and purification of GST fused proteins is based on the high affinity of Glutathione S-transferase (GST) for glutathione. The protein of interest is fused to the C-terminus of GST in a bacterial expression vector. Expression of the fusion protein can be induced and the protein then purified from the bacteria with a Glutathione Sepharose 4B matrix (GE Healthcare).
GST-fused constructs were transformed into *E. coli* BL21. For protein expression, overnight bacterial cultures were diluted 1:20 in fresh LB containing the selective antibiotic and grown at 37°C to an OD600 of 0.5 – 0.7. To induce protein expression, 0.4 mM IPTG (isopropyl-b-D-thiogalactopyranoside) was added and bacteria were incubated at 20 – 37°C (dependent on the protein) for 3-4 hours. Cells were harvested (6000rpm for 15 min at 4°C), the pellet resuspended in 5 ml lysis buffer (PBS or 50 mM Tris pH7.5 + 0.1 mM PMSF) and sonicated 10x for 10 sec at 18 %. Cell debris was centrifuged for 30 min at 14000rpm and GST-fused proteins in the supernatant were purified with Glutathione Sepharose 4B. After several washing steps with lysis buffer, proteins were eluted with lysis buffer containing reduced glutathione (30 mM glutathione, 50 mM Tris pH7.5). Where necessary, proteins were dialyzed overnight against PBS or 50 mM Tris pH7.5 at 4°C.

8.2 Determination of protein concentrations

Concentration of solubilized protein was measured with the BioRad protein assay, based on the method of Bradford. As a protein standard bovine serum albumine (BSA) in different concentrations was used (20 µg/ml, 15 µg/ml, 10 µg/ml, 5 µg/ml and 1 µg/ml). To determine protein concentration, 800 µl of sample or 800 µl standard solution was mixed with 200 µl of assay reagent. After vortexing and an incubation of 5 min at room temperature, the absorbance at 595 nm was measured.

8.3 SDS-PAGE

In a SDS-PAGE (Sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis) proteins are seperated on the basis of their molecular weights as they migrate through a gel. SDS is an anionic detergent, which denatures secondary and tertiary structures and applies a negative charge to proteins relative to their mass. Protein samples were mixed with 5x Laemmli SDS loading buffer (with a 5 % final concentration of β-mercapto-ethanol), boiled for 5 min at 95°C and subjected to electrophoresis at a constant voltage of 100V. To estimate approximate molecular weights, a protein marker (prestained protein marker IV, PeqLab, Erlangen, Germany) was additionally loaded onto the gel.

5x SDS loading buffer

0.5 M Tris/HCl pH 6.8	5 ml
Glycerol	2 ml
SDS	0.8 g
0.5 % Bromphenol blue	1 ml
ddH_2O	2 ml

Stacking gel buffer

Tris/HCl	0.5 M (15.1 g)
SDS	1 g
ddH_2O	250 ml, pH 6.8

Resolving gel buffer

Tris/HCl	1.5 M (45.3 g)
SDS	1 g

ddH_2O	250 ml, pH 8.8

Stacking gel

ddH_2O	1.55 ml
Stacking gel buffer	625 µl
Acrylamide	325 µl
10 % ammonium persulfate (APS)	12.5 µl
TEMED	5 µl

Resolving gel	**10 %**	**12.5 %**
ddH2O	2.09 ml	1.6 ml
Resolving gel buffer	1.25 ml	1.25 ml
Acrylamide	1.67 ml	2.08 ml
10 % ammonium persulfate (APS)	25 µl	25 µl
TEMED	2.5 µl	2.5 µl

SDS-PAGE running buffer (10x)

Tris-Base	0.025 M (30.3 g)
Glycine	0.192 M (144 g)
SDS	0.1 % (10 g)
ddH_2O	1 l

8.4 Coomassie staining

To stain proteins directly in the gel after SDS-PAGE, the gel was incubated for 15 min in Commassie staining solution (0.1 % Commassie Brilliant Blue, 25 % methanol and 10 % glacial acetic acid). For destaining, the gel was incubated in destaining solution (25 % methanol and 10 % glacial acetic acid).

8.5 Western Blot

Proteins that were seperated by SDS-PAGE were transferred ("blotted") onto a polyvinylidene fluoride (PVDF) membrane (Immobilion-P, Millipore, Schwalbach, Germany) by electrophoretic transfer with a semi-dry Western Blot. For this, the SDS gel was placed directly on a piece of

methanol-activated PVDF membrane and between two filter papers soaked in Anode buffer 1, one filter paper soaked in Anode Buffer 2 and three filter papers soaked in Cathode buffer. The transfer stack was then placed between two electrodes and proteins blotted for 1 hour at 1.2 mA/cm^2.

Anode buffer 1

Tris/HCl	0.3 M (7.26 g)
Methanol	10 % (20 ml)
ddH_2O	200 ml, pH 10.4

Anode buffer 2

Tris/HCl	25 mM (0.605 g)
Methanol	10 % (20 ml)
ddH_2O	200 ml, pH 10.4

Cathode buffer

Tris/HCl	25 mM (0.605 g)
6-amino-n-caproic acid	40 mM (1.0494 g)
Methanol	10 % (20 ml)
ddH_2O	200 ml, pH 9.4

After the transfer, the membrane was blocked for 1 h at room temperature or overnight at 4°C, in TBST / 2 % BSA.

TBST 10x

Tris/HCl	20 mM (24.2 g)
NaCl	150 mM (87.6 g)
ddH2O	1 l, pH 7.4
Tween 20	0.3 %

The membrane was incubated with primary antibody for 1 h at room temperature (or 4°C, overnight), washed three times with TBST for 5 minutes followed by incubation with horseradish peroxidase linked secondary antibody for 1 hour (1:10.000 to 1:100.000). After three more washing steps with TBST, detection solution was added to the membrane according to

manufacturers' instructions (SuperSignal West Femto). Emitted light was detected by exposing the membrane to an X-ray film (Fuji, Düsseldorf, Germany) and developed in a processor for X-ray film development (Agfa, Curix 60).

8.6 Rho GTPase activity assay

In order to measure activity of specific Rho GTPases, GST-pull down assays were performed. Individual active Rho GTPases can be pulled down from cell lysates with specific GST-fused proteins. These proteins are usually effector proteins of the Rho GTPases and bind only to their activated form. The GST-fused proteins were recombinantly expressed and purified from *E. coli*.
To measure active Rac1, Cdc42 and TC10, GST-PAK-CRIB was used. To measure active RhoA, GST-Rhotekin was used and to pull down active RhoG, GST-Elmo2NT was used.
Glutathione-Sepharose 4B beads were washed three times with PBS and then incubated for 1 hour at 4°C with 2-80 µg of GST-fused proteins, followed by another three washing steps with PBS (with centrifugation steps: 3 min at 500 g)
Cells were lysed in lysis buffer, centrifuged for 10 min at 10.000 g and supernatants were incubated for 1 hour at 4°C with GST-fused proteins bound to glutathione-Sepharose 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 and then immunoblotted.

Lysis buffer: Rac1, Cdc42, TC10 (GST-PAK-CRIB)

Tris pH 7.4	50 mM
NaCl	150 mM
$MgCl_2$	5 mM
Sodiumdeoxycholate	0.5 mM
SDS	0.1 %
Triton X-100	1 %
Protease inhibitor cocktail	

Lysis buffer: RhoA (GST-Rhotekin)

Tris, pH 7.4	50 mM
NaCl	100 mM
$MgCl_2$	2 mM
Glycerol	10 %

Triton X-100	1 %
Protease inhibitor cocktail	

Lysis buffer: RhoG (GST-Elmo2NT)

Tris pH 7.4	20 mM
NaCl	150 mM
$MgCl_2$	5 mM
Sodiumorthovanadate	0.1 mM
DTT	1 mM
Triton X-100	1 %
Protease inhibitor cocktail	

8.7 In vitro GAP assay

For in vitro GAP activity, measurements the RhoGAP assay Biochem kit from Cytoskeleton was used according to the protocol of the manufacturer. RhoG and YopE were expressed and purified as GST-fused proteins and recombinant Rac1, Ras and p50RhoGAP were part of the kit. Proteins were incubated at 37°C with 200 mM GTP. After 20 min the reaction was stopped and green color development of free phosphate was measured with a microplate-reader at 650 nm.

9. Cell Culture and Cell Biological Methods

9.1 Cell culture

Cells and cell lines

name	description
HUVEC	Human umbilical vein endothelial cells, primary cells
HeLa	Human cervix carcinoma cell line
Cos-7	African green monkey kidney cells, fibroblast-like cell line

All cells were cultured in a 37°C incubator with 5 % CO_2 and 90 % humidity.

HUVEC

Human Umbilical Vein Endothelial Cells were isolated from human umbilical veins and cultivated in Endothelial Cell Growth Medium (ECGM, PromoCell, Heidelberg, Germany) supplemented with 2 % Fetal Calf Serum (FCS), 50 µg/ml Gentamicin and 0.05 µg/ml Amphotericin B. Cells were used from passage 2 to 6.

HeLa

Epithelial-like cell line that was established from an epitheloid cervix carcinoma of a 31-year old woman in 1951. Cells were cultured in RPMI 1640 + L-glutamine (Invitrogen, Karlsruhe, Germany) supplemented with 10 % FCS and 100 µg/ml penicillin/streptomycin.

Cos-7

Fibroblast-like cell line that was derived from CV-1, a simian cell line, by transformation with an origin-defective mutant of SV-40. Cells were cultured in DMEM + L-glutamine (Invitrogen, Karlsruhe, Germany) with 10 % FCS and 100 µg/ml penicillin/streptomycin.

9.2 Isolation of HUVEC

Human umbilical vein endothelial cells (HUVEC) were obtained by trypsin treatment of human umbilical cord veins by a method adapted from Jaffe (Jaffe et al., 1973).

The cord was inspected and all areas with clamp marks were cut off. The umbilical vein was cannulated at one end with a blunt 2 cm long needle and the needle was secured by clamping the cord over the needle with two compressors. After perfusing the umbilical vein with sterile PBS to wash out the blood, the other end of the vein was clamped shut and 0.1 % α-chymotrypsin in PBS was infused into the umbilical vein. The umbilical cord was incubated at 37°C for 20 minutes. After incubation, the chymotrypsin solution was flushed from the cord by perfusion with sterile PBS. The effluent was collected in a sterile 50 ml conical centrifuge tube (BD Bioschiences) containing 2 ml of FCS to stop the trypsinization. The cells were sedimented at 130 g for 10 min at 25°C, resuspended in endothelial cell growth medium containing 2 % FCS, transferred to collagen-coated culture flasks (Nunc, Wiesbaden, Germany) and incubated at 37°C, 5 % CO_2 and 90 % humidity. After 3 h, non-adherent cells were removed by replacement of culture medium. Cell culture medium was replaced every 2-3 days.

9.3 Passaging of cells

To passage adherent cells, growth medium was removed, cells washed with PBS and Trypsin-EDTA (0.05 % Trypsin, 0.53 mM EDTA x 4Na; Invitrogen, Karlsruhe, Germany) was added for 5 min. Trypsin activity was stopped by addition of culture medium with FCS and then cells were pelleted for 5 min at 1000rpm at 21°C and resuspended in fresh growth medium. Cells were counted with a Hemocytometer (Neubauer improved) and transferred either onto coverslips or into new cell culture flasks.

9.4 Freezing and thawing of cells

Freezing of cells

Cells were trypsinized from cell culture flasks, centrifuged for 5 min at 1000rpm and the cell pellet was resuspended in sterile-filtered pre-cooled freezing medium (growth medium + 10 % dimethylsulfoxide, DMSO; Sigma-Aldrich, Munich, Germany). Cells were transferred to 1.5 ml Cryo-vials, incubated for 10 min on ice and subsequently transferred to -80 °C.

Thawing of cells

Cryo-vials were removed from -80 °C and quickly thawed in a 37 °C water bath. Thawed cells were transferred into 5 ml of warm growth medium, centrifuged for 5 min at 1000rpm, re-suspended in growth medium and transferred to cell culture flasks.

9.5 Coating of coverslips and culture flasks

To facilitate adhesion of endothelial cells to glass coverslips and cell culture flasks, they were treated with a 0.2 % solution of gelatine in PBS (Gelatine solution TypeB from bovine skin, 2 %; Sigma-Aldrich, Munich, Germany). Gelatine was applied to coverslips or culture flasks and allowed to dry for at least 3 hours. Residual gelatine was removed and flasks stored at 4°C.

9.6 Transfection with Nucleofector

To induce transient expression of proteins in HUVEC, transfection of plasmid DNA was performed with the Amaxa Nucleofector system, which is a combination of electroporation and lipofection, and resulted in rapid and high cellular expression. Transfection was performed according to the protocol of the manufacturer. In brief, cells were trypsinized, pelleted and 0.5 –

1 million cells were then transfected with 2 µg of DNA. Transfected cells were transferred to glass coverslips or cell culture flasks and incubated at 37 °C until experiments were performed.

9.7 Transfection with ExGen 500

Cos-7 cells were transiently transfected with ExGen 500 (Fermentas, St.Leon-Rot, Germany), a cationic polymer transfection reagent. On the day before transfection, 2.5 x 10^6 cells were seeded in culture flasks. After 24 hours, transfection reagent – DNA mixture was added (3.3 µl ExGen per 1 µg DNA) and centrifuged onto the cells for 5 min at 280 g. Experiments were performed the next day.

9.8 Transfection of siRNA

A pool of 4 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 and Cos-7 cells were transfected with X-treme Gene siRNA transfection reagent (Roche, Mannheim, Germany) according to instructions of the manufacturer.

9.9 Coating of fluorescent beads

Fluorescent polystyrene microspheres (beads, diameter 1 µm, blue, Ex./Em.=350/440 nm) with sulfate groups on the surface for passive adsorption of proteins were purchased from Molecular Probes (Invitrogen, Karlsruhe, Germany). 200 µl beads slurry were washed with 1 ml PBS and purified GST or GST-Inv397 (Wiedemann et al., 2001) were added at a concentration of 1 mg/ml. Proteins were allowed to adsorb to the beads for 3 h at room temperature or overnight at 4 °C, respectively. Beads were pelleted, resuspended in 500 µl 1 % BSA in PBS and incubated for another 1 h at room temperature. Beads were washed in BSA/PBS and stored in 0.2 % BSA in PBS at 4 °C. Coupling efficiency was determined by measuring protein concentration before and after coating of beads.

9.10 Immunofluorescence methods

For immunofluorescence staining of cells, four different fixation/permeabilization methods were used, depending on the antibody and the assay that was used.

Formaldehyde/Acetone fixation – permeabilization

Cells seeded on coverslips were fixed in 3.7 % [v/v] formaldehyde in PBS for 10 minutes at room temperature and subsequently permeabilized in ice-cold acetone at -20 °C for 5 minutes.

Formaldehyde/Triton fixation – permeabilization

Cells seeded on coverslips were fixed in 3.7 % formaldehyde in PBS for 10 min and permeabilized with 0.1 % Triton X-100 in PBS for 10 min at room tmperature.

Methanol fixation – permeabilization

Cells seeded on coverslips were fixed and permeabilized in a one step procedure by incubating in ice-cold methanol for 60 seconds at -20 °C.

Paraformaldehyde fixation

For minimal disruption of cell membranes, cells were fixed with freshly made 4 % paraformaldehyde in PBS. 4 % paraformaldehyde solution was prepared by dissolving 2 g of paraformaldehyde in 25 ml of ddH_2O (NaOH and heating was required for dissolving). Finally 25 ml of PBS was added to produce a 4 % paraformaldehyde solution. Cells were fixed in paraformaldehyde for 10 min at room temperature. Paraformaldehyde fixation was used for bacterial internalization assay to distinguish between intra- and extracellular bacteria.

Indirect immunofluorescence staining

Indirect immunofluorescence staining of cells seeded on glass coverslips was performed in a wet chamber on a layer of parafilm (American National Can, Menasha, USA). 30 µl of the solution was placed on the parafilm and glass coverslips were applied with the cellular side oriented downwards. After fixation and permeabilization cells were blocked with 1 % BSA in PBS for 15 min and then incubated with primary antibody for 45 min. Cells were washed three times with 1 % BSA/PBS and incubated with secondary antibody for 30 min. For specific staining of F-actin, AlexaFluor 568-phalloidin was added instead of, or additionally to a secondary antibody. After three washing steps with PBS, coverslips were air dried, mounted on a drop of Mowiol (including *p*-Phenylendiamin as antibleach) and sealed with nail polish.

Inside/Outside staining (double fluorescence staining)

To distinguish between extracellular (cell-attached) and intracellular (ingested) bacteria or particles, a double fluorescence staining method was used that is based upon the fact that cell membranes are impermeable to antibodies after fixation with paraformaldehyde but permeable after treatment with acetone. Thus, extracellular bacteria were stained before permeabilization with primary and secondary antibody and intracellular bacteria (together with extracellular bacteria) after permeabilization with acetone. This results in internalized bacteria stained in one color and extracellular bacteria stained with two colors. By comparison of fluorescence channels, numbers of extracellular and intracellular bacteria can be determined microscopically.

9.11 Brefeldin A assay

Brefeldin A is an antibiotic produced by fungal organisms, which interferes with the protein transport from the endoplasmatic reticulum to the Golgi apparatus. It leads to a breakdown of the Golgi apparatus and its redistribution into the ER. Golgi tubules are no longer visible and Golgi markers show punctate distribution (Klausner et al., 1992). Brefeldin A (Sigma-Aldrich, Munich, Germany) was added to cells for 60 min at a concentration of 5 µg/ml. Cells were then fixed and the Golgi immunostained with anti-GS27 antibody.

9.12 Cell infection

For cell infection with *Y. enterocolitica* strains overnight bacterial cultures grown at 27 °C in LB containing the appropriate antibiotics were diluted 1:20 in fresh LB and grown for an additional 90 min to allow 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 h at 37 °C. Bacterial cultures were centrifuged for 15 min at 6000 g, washed in PBS and OD_{600} was adjusted to 0.36. To synchronize infection, bacteria were attached to cells by centrifugation at 200 g for 2 min at a ratio of 10 to 100 bacteria per cell. Latex beads coated with GST-Inv397 or GST were centrifuged onto the cells at 200 g for 3 min 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.

9.13 Live-cell imaging

For confocal time-lapse microscopy, HUVEC were transfected with desired constructs and seeded onto glass-bottomed dishes (MatTek, Ashland, USA) one day before imaging. During live-cell imaging, cells were kept in a microscope chamber at 37 °C and 5 % CO_2, and bacteria were pipetted into the medium and allowed to sediment onto cells.

9.14 Bacterial internalization assay

Bacterial internalization was determined in HUVEC transfected with siRNA against RhoG or with non-targeting siRNA, respectively. Cells were transfected with siRNA for 48 h and then infected with *Yersinia* strain WA-C for 60 min. After washing, cells were subjected to an inside/outside staining (see above) to differentiate between extracellular and intracellular bacteria. Invasion of bacteria was determined by counting the bacteria using a fluorescence microscope.

F. Abbreviations

°C	degree Celsius
A	adenine
A	alanine
aa	amino acid
ab	antibody
Ail	attachment invasion locus
Amp	ampicillin
APS	ammoniumpersulfate
ATP	adenosintriphosphate
bp	basepairs
BSA	bovine serum albumine
C	cytosine
cDNA	complementary DNA
Cm	chloramphenicol
CR	complement receptor
CRIB	Cdc42-Rac1 interaction binding
Da	Dalton
dd	double distilled
DMEM	Dulbecco's Modified Eagle Medium
DNA	desoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
DTT	dithiothreitol
ECGM	endothelial cell growth medium
EDTA	ethylendiamin-N,N,N',N'-tetra acetic acid
ER	endoplasmatic reticulum
EtOH	ethanol
g	gram
G	Guanine
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	GTP exchange factor
GFP	green fluorescent protein
GST	Glutathione-S-Transferase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
HPI	high pathogenicity island
HUVEC	human umbilical vein endothelial cells
Ig	Immunoglobulin
IPTG	1-Isopropyl-β-D-1-thiogalactoside
K	lysine
Kan	kanamycin
kb	kilo bases
kDa	kilo Dalton
l	liter
LB	Luria-Bertani
Lcr	low calcium response
LPS	lipopolysaccharide
m	milli
m	meter
M	molar
min	minute
MLD	membrane localization domain
n	nano
Nal	nalidixic acid
nt	non-targeting
o/n	over night
OD	optical density
p	plasmid
PAGE	poly-acrylamide gel electrophoresis
PAK	p21-activated kinase
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PMSF	phenylmethanosulfonylfluoride
PTP	protein tyrosine phosphatase
PVDF	polyvinylidenfluoride
pYV	*Yersinia* virulence plasmid
RNA	ribonucleic acid
rpm	revolutions per minute
R	arginine
RT	room temperature
s.d.	standard deviation
SDS	sodiumdodecylsulfate
SGEF	SH3-containing GEF
si	small interfering
Sm	streptomycin
T	thymine
T	threonine
TAE	Tris/Acetate/EDTA
TE	Tris/EDTA
TEMED	N,N,N',N'-tetramethylethylendiamine
Tet	tetracycline
Tris	Tris(hydroxymethyl)aminomethane
TTSS	type III secretion system
U	uracil
V	volt
W	watt
WASP	Wiskott Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein
wt	wild-type
Yop	*Yersinia* outer protein
µ	micro

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