Molecular and Cellular Basis of the Internalization of *Bartonella henselae* by Human Endothelial Cells

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1. INTRODUCTION

1. INTRODUCTION

1.1 Biology of the Human Vasculature

This chapter highlights different aspects of the anatomy and physiology of the human vasculature and explains relevant mechanisms for establishment, maintenance and function of the human vasculature in order to provide the reader of this thesis a sound basis for understanding the subsequent discussion on the biology of endothelial cells constituting an integral part of the human vasculature.

Anatomical drawings created by Leonardo da Vinci (1452-1519) depict parts of the blood vessel system including the coronary system of the heart and the circulatory system of the lung (Da Vinci, 1509). Da Vinci was the first scientist to reveal that these "tree-like" structures form the functional connections of the human circulatory system.

In principle, the human circulatory system comprises the (cardiac) heart muscle with elastic efferent (arteries) and afferent (veins) blood vessels which branch into smaller and thinner vessels (arterioles and venules, respectively) and finally pervade visceral organs and muscles as a fine meshwork, termed capillaries or sinusoids (Schmidt and Thews, 1989). The prototype circuit leads away from the heart into the body periphery via arteries to smaller arterioles, further to capillaries or sinusoids, then to venules, to veins and back to the heart, which contracts rhythmically to enable blood perfusion. The fine network of blood vessels which pervades organs and muscles is termed the vasculature.

The vasculature functions in (i) the perfusion of oxygen, nutrients and hormones and (ii) the removal of carbon dioxide, ammonia and other metabolic waste products. Exchange of these solutes between the blood and the surrounding tissues takes place in the capillaries (e.g. in muscles) and sinusoids (e.g. in liver, spleen, bone marrow) (Schmidt and Thews, 1989).

The vasculature is formed during embryogenesis by mesoderm-inducing factors of the fibroblast growth factor (FGF) family in a process termed vasculogenesis from angioblast progenitor cells (Jain, 2003). This process is accompanied in later stages of development by maturation of blood vessels, termed arteriogenesis, and the formation of new blood vessels from pre-existing ones, in a process termed angiogenesis, by sprouting and intussusception (Carmeliet, 2005; Ferrara and Kerbel, 2005) and establishment of the lymphatic vessel system (Alitalo et al., 2005) (see Figure 1). Angiogenesis is of fundamental importance under physiological conditions, e.g. during wound healing or during the menstrual cycle of the woman, and is abberant in tumor formation and growth.

Histology of the vasculature reveals a basic building plan applying to all blood vessels: A blood vessel is a hollow tube of three different cell layers, the intima, the media and the adventitia (Risau, 1995). The intima is the innermost layer comprises the lining of the endothelial cells, termed endothelium. The endothelium is encased and stabilized by a thin basal membrane. The media comprises a lamella of elastic connective tissue, which is enveloped by a layer of vascular smooth muscle cells (SMCs). The adventitia comprises a layer of elastic connective tissue which embeds the blood vessel in the surrounding tissue. In larger vessels, the adventitia itself is is pervaded by nerves that supply the muscular layer along with nutrient capillaries.

Blood vessels dysfunction is relevant for a plethora of medical conditions, especially cardiovascular diseases such as arteriosclerosis, congestive heart failure, coronary artery disease, stroke and hypertension (WHO, 2006). In cancer, the formation of new blood vessels is a tissue response towards the elevated need for oxygen and nutrients in a growing tumour (Bergers and Benjamin, 2003). In inflammation, blood vessel permeability is increased resulting in influx of blood plasma into underlying tissues as a response to infection or irritation, giving rise to oedema and swellings (Fiedler et al., 2006; Imhof and Aurrand-Lions, 2006). Furthermore, inflammation of the blood vessel itself, termed vasculitis, is the consequence of an autoimmune response impairing vascular homeostasis. (Davies, 2005; Pendergraft et al., 2004).

1.2 Biology of Endothelial Cells

This chapter places an emphasis on one specific cell type of the vasculature, namely endothelial cells. The fundamental principles of endothelial cell biology and endothelial cell function in the context of blood vessels system are presented in order to allow the reader of this thesis to understand how bacterial pathogens manage to subvert these functions during the infection process as discussed later.

Endothelial cells (ECs) are flat and thin (squamous), of oblong to spindle-shaped appearance, lining the interior of the blood and lymphatic vessel system from the heart to capillaries. The entity of ECs lining is termed the endothelium. ECs form a slick interface between the blood vessel lumen with the circulating blood and the proper blood vessel wall.

ECs exert diverse biological functions and contribute to vascular homeostasis (Risau, 1995). These functions are highlighted in the following five paragraphs.

(1) ECs control the vascular tone by generating and transducing auto-, para-, and endocrine signals leading e.g. to contraction (vasoconstriction) or relaxation (vasodilatation) of vascular smooth muscle cells (SMCs) to regulate blood pressure. It has been found, that nitric oxide (NO) is a key signalling molecule in this process acting as a powerful vasodilator (Cockcroft, 2005). NO is produced by endothelial nitric oxide synthetase (eNOS) from L-arginine to L-citrulline (Fish and Marsden, 2006). NO activates soluble guanylate cyclases in vascular SMCs in order to lower the vascular tone.

(2) ECs are important target cells in local inflammatory processes as a response to bacterial infections by allowing adhesion, rolling and transmigration of professional phagocytes, e.g. monocytes or polymorphonucleocytes into underlying tissues (Luster et al., 2005). The pro-inflammatory response leads to the secretion of cytokines from ECs. These pro-inflammatory signals are interleukin 1 (IL-1) and tumor necrosis factor α (TNFα). Their release promotes surface expression of adhesion molecules for leukocytes, namely CD62P (P-selectin) and E-selectin (CD62E) for initial leukocyte binding to and rolling on ECs and CD54 (intercellular adhesion molecule 1, ICAM-1) and CD106 (vascular intercellular adhesion molecule-1, VCAM-1) for firm adhesion. Furthermore, the release of IL-1 and TNFα stimulates nuclear factor-κB (NF-κB)-dependent secretion of the chemokine interleukin 8 (IL-8) from ECs and engagement of CD31 (platelet endothelial cell adhesion molecule 1, PECAM-1) to stimulate transmigration of adherent leukocytes through the blood vessels into the underlying tissues and to resolve infection.

(3) Vascular endothelial growth factor (VEGF) is a potent mitogen for ECs and acts in a paracrine fashion on ECs. VEGF activates ECs to migrate and to proliferate and enhances endothelial cell survival in the process of angiogenesis, eventually leading to tube structure formation and vascular remodelling (Carmeliet, 2005; Ferrara and Kerbel, 2005). Furthermore, VEGF stimulates the aforementioned enzyme eNOS leading to increased NO production which contributes to angiogenesis.

(4) ECs control blood coagulation events such as fibrinolysis during the repair of damaged blood vessels or counteract blood vessel clotting by thrombolysis.

(5) ECs act as a selective physical diffusion barrier controlling influx and efflux of gaseous and solute substances between bloodstream and underlying tissues (e.g. in renal glomeruli, in the blood brain barrier or in the placenta).

1.3 Bacterial Interactions with Endothelial Cells

Bacterial pathogens have developed strategies to adhere to and to invade a wide range of human cell types. Establishment of a portal of entry is critical for colonization and subsequent dissemination. Entry sites for bacterial pathogens are mucosal surfaces like the gastrointestinal, the urogenital and the respiratory tract, but as well the conjunctiva, the blood-brain barrier, the maternal placental blood-barrier and the inner lining of blood vessels. These entry sites share the common property that they represent an interface between the external environment and the underlying body tissues.

Both gram-positive (e.g. group B streptococci, *Staphylococcus aureus*, *Listeria monocytogenes*) and gram-negative (*Chlamydia pneunomiae*, enterohaemorrhagic *E. coli*, *Neisseria meningitidis*, *Rickettsia* spp., *Bartonella* spp.) bacteria have been identified to display endothelial host cell tropism and to be able to infect these important target cells.

To exemplify these strategies I chose to present two gram-positive bacterial pathogens and three gram-negative bacterial pathogens. The focus of these strategies centres around the question on how these pathogens are able to adhere and invade ECs to establish successful infections of the vasculature.

1.3.1 Group B Streptococci

The genus *Streptococcus* represents facultative anaerobic, gram-positive cocci growing in chains or pairs with polysaccharide capsules rich in sialic acid, muramic acid and glucosamine. Streptococci are able to colonize the oral cavity, the skin, the intestinal and respiratory tract of humans as part of the normal commensal flora. *S. agalactiae* belongs to group B streptococci (GBS) which are a leading cause of sepsis and meningitis. Meningitis is a serious infection of the central nervous system (CNS) and is frequently associated with newborn infants and immuno-compromised adults. GBS interact with cerebral endothelial cells in the process of bacterial transcytosis to breach the blood-brain barrier (BBB). Subsequent bacterial replication in the CNS provokes a massive inflammatory response leading to meningitis, septicaemia and brain injury. GBS are able to adhere to a variety of host cell surface proteins such as fibronectin (Tamura and Rubens, 1995), laminin (Spellerberg et al., 1999) and cytokeratin (Tamura and Nittayajarn, 2000). GBS invasion of human brain microvascular endothelial cells (BMEC) represents a model for transcytosis of polar BMEC monolayers *in vitro* (Nizet et al., 1997), leading to significant injury and disruption of the BBB at high bacterial concentrations correlated to β-hemolysin activity (Nizet et al., 1997). Additional cellular consequences are the release of the pro-inflammatory chemokine interleukine-8 (IL-8) and of the cytokine interleukine-6 (IL-6), and upregulation of CD54 (intercellular adhesion molecule-1, ICAM-1) that act to orchestrate neutrophil recruitment and activation to sites of bacterial infection (Doran et al., 2003). Pneumonia is often observed with early-onset meningitis in newborns. The primary portal of GBS entry into the fetus occurs over interactions with the lung epithelia by aspiration of infected amniotic fluid and dissemination into the blood-stream (Rubens et al., 1992). Subsequent invasion of pulmonary blood vessels can be mimicked by GBS infection of pulmonary artery endothelial cells (PAEC) and lung microvascular endothelial cells (LMEC) *in vitro* (Gibson et al., 1995) stimulating the realease of inflammatory eicosanoids, which are associated with bacteremia and a clear indication for sepsis that accompanies meningitis *in vivo* (Rubens et al., 1991).

1.3.2 *Staphylococcus aureus*

The gram-positive bacterium *S. aureus* is coagulase-positive and grows facultative anaerobically in grape-like clusters characterized by golden pigmentation. *S. aureus* encodes for a plethora of extracellular enzymes and exotoxins (e.g. α-toxin, leucodicin, exfoliants, entertoxins and toxic shock syndrom toxin) and is a leading cause of human disease, representing two subgroups, invasive infections and proper toxicoses. *S. aureus* has the capability to invade the vascular endothelium (Sinha and Herrmann, 2005). The invasive potential is reflected by the bacterial ability to cause serious endovascular infections, such as endocarditis and vasculitis. The major adhesins known are clumping factor A (ClfA) and fibronecting-binding protein A (FnBPA) (Massey et al., 2001). Subsequent internalization of *S. aureus* into human endothelial cells requires the host cell actin cytoskeleton (Zhang et al., 2002), is dependent on the expression of fibronectin-binding proteins (Sinha et al., 1999), and triggers recruitment of focal contact-associated proteins vinculin, tensin, zyxin and focal adhesion kinase (FAK) to the sites of bacterial attachment and invasion (Agerer et al., 2005). Dominant-negative versions of FAK block integrin-mediated internalization and FAK-deficient cells are severely impaired in their ability to internalise *S. aureus*. Pathogen binding induces tyrosine phosphorylation of several host proteins associated with bacterial attachment sites, including FAK and the Src substrate cortactin (Agerer et al., 2005) *S. aureus* internalization leads to enhanced expression of CD106 (VCAM-1) and CD54 (ICAM-1) but does not alter expression of CD62P (P-selectin), CD62E (E-selectin) and CD31 (PECAM-1). After internalization, *S. aureus* may either persist and escape host defences and antimicrobial agents or multiply and disseminate. Both vacuole-bound bacteria and cytoplasmic bacteria can be found (Peacock et al., 1999). The intracellular fate is dependent on the presence of the α-toxin, which acts as caspase-dependent and Fas-independent (Haslinger-Loffler et al., 2005) apoptotic stimulus for endothelial cells (Menzies and Kourteva, 2000).

1.3.3 *Rickettsia rickettsii*

Rickettsiae are gram-negative α-proteobacteria, exhibiting an obligate intracellular lifestyle. Bacilli are non-motile and of pleomorphic appearance. Rickettsial species are divided into two subgroups, the spotted fever group (SFG) and the typhoid group, and are transmitted to humans by arthropods, e.g. ticks. *R. rickettsii* belongs to the SFG and is the etiological agent of Rocky Mountain spotted fever (RMSF). RMSF is an acute life-threatening, febrile illness, accompanied by a typical rash on the extremities (Dumler and Walker, 2005). *R. rickettsii* spreads and replicates in the cytoplasm of endothelial cells eliciting widespread vascular inflammation (vasculitis), reduced blood perfusion (thrombosis), end-organ damage (by ischemia), which is most dangerous in lungs and brain.

Efficiency of invasion of HUVECs (Silverman, 1984; Silverman and Bond, 1984) by *R. rickettsii* is dependent on the multiplicity of infection (Silverman and Bond, 1984) and on the host cell actin cytoskeleton (Rydkina et al., 2005). Bacterial internalization into ECs leads to activation of protein kinase C (Sahni et al., 1999), which, in turn, leads to activation of NF-κB, as suggested by the activation of IκB kinase (IKBK) and phosphorylation and subsequent proteasomal degradation of the IκB inhibitory subunit (Clifton et al., 2005). This signalling cascade leads to the suppression of apoptosis by *R. rickettsii* in ECs (Joshi et al., 2004).

For the closely related species *R. conorii*, the causative agent of Mediterranean spotted fever, it has been shown, that entry into non-phagocytic cells correlates with the tyrosine phosphorylation of several host proteins, including focal adhesion kinase (FAK), depends on the Arp2/3 complex, which involves the interplay of Cdc42, phosphatidylinositol 3-kinase (PI3K), c-Src and cortactin leading to localized actin rearrangements (Martinez and Cossart, 2004) (see Figure 1).

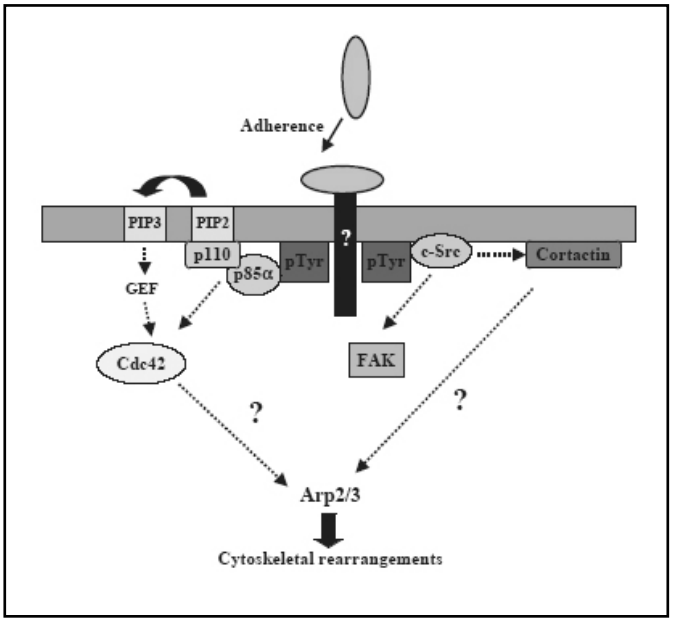


Figure 1

Early signalling events involved in the host cell entry of *Rickettsia conorii*
(Martinez J.J. and Cossart P., Journal of Cell Science, 2005)

1.3.4 *Neisseria meningitidis*

Neisseria meningitidis (or simply the meningococcus) is a gram-negative bacterium, of coccoid shape, which is protected by a polysaccharide capsule, and which belongs to the order of β-proteobacteria. *N. meningitidis* specifically infects humans, there is no animal reservoir known. Serogroups A, B, C and W135 are the most important clinical subtypes of *N. meningitidis*.

Meningococcal meningitis and sepsis frequently affect infants and adolescents, even at epidemic scales. Avirulent meningococci persistently and asymptomatically colonize the nasophrarynx. In contrast, virulent meningococci penetrate mucosal surfaces of the nasopharynx, spread haematogenously, eventually crossing the BBB causing systemic meningitis and sepsis. *N. meningitidis* adherence to and invasion of HUVECs (Virji et al., 1994) and HBMECs (Unkmeir et al., 2002) has been demonstrated.

Adherence of meningococci to epithelial and endothelial cells is mediated by type IV pili (Nassif et al., 1994) and CD46 (membrane cofactor protein, MCP) is considered to constitute the host cell receptor (Kallstrom et al., 1997; Kirchner and Meyer, 2005). In addition to type IV pilus-mediated adhesion, meningococcal attachment to ECs is mediated as well by the bacterial outer membrane protein Opc that binds fibronectin, thereby anchoring the bacterium to the integrin α5 β1-receptor on the endothelial cell surface (Sokolova et al., 2004). Bacterial adhesion results in the formation of cellular protrusions at the site of bacterial attachment. These microvilli-like protrusions are highly enriched for ezrin and moesin, two members of the ERM (ezrin/radixin/moesin) family of actin-binding proteins, whereas the focal adhesion proteins, vinculin and paxillin, are absent (Eugene et al., 2002). Formation of membrane ruffles and subsequent internalisation requires cortical actin polymerization and depends on the activation of the small GTPases Rho and Cdc42, but not of Rac1 (Eugene et al., 2002).

Proper internalisation of *N. meningitidis* into ECs involves the activation of the ErbB2 tyrosine kinase receptor and the c-Src kinase, leading to tyrosine phosphorylation of cortactin (Hoffmann et al., 2001). *N. meningitidis* mutants expressing a deglycosylated lipooligosaccharide (LOS) are poorly invasive. These mutants show structurally altered actin polymerization. Moreover, although they efficiently recruit and activate the kinases ErbB2 and c-Src, these mutants are defective in the recruitment and phosphorylation of cortactin (Lambotin et al., 2005) (see Figure 2).

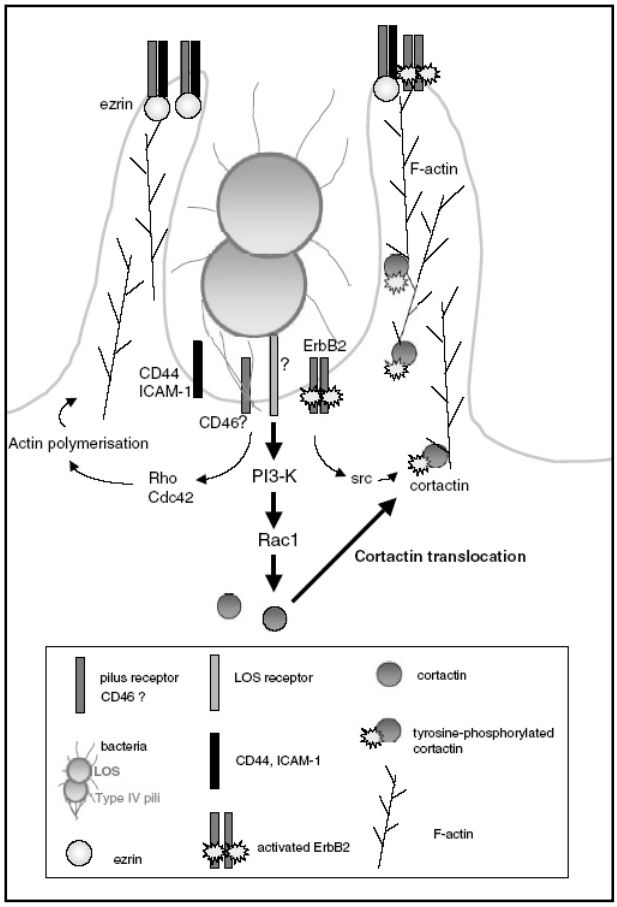


Figure 2

Neisseria meningitidis invasion of endothelial cells

(Lambotin et al., Journal of Cell Science, 2005)

Phosphorylated cortactin controls cortical actin polymerization, which leads to membrane protrusion formation. In addition, cortactin recruitment is dependent on the activation of a PI3K/Rac1-GTPase signalling pathway, which is required for actin polymerization and internalization of *N. meningitides* in ECs, and is not activated by the mutant strains (Lambotin et al., 2005).

Furthermore, it has been shown that binding of meningococci to HBMEC phosphorylates and activates c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2) and p38 mitogen-activated protein kinase (p38 MAPK) as well as their direct substrates c-Jun and MAP kinase activated protein kinase-2 (MAPKAPK-2), respectively (Sokolova et al., 2004). Non-invasive meningococcal strains lacking the *opc* locus still activate p38 MAPK, but fail to activate JNK. Inhibition of JNK1 and JNK2 significantly reduces internalization of *N. meningitidis* by HBMEC without affecting its adherence. Blocking the endothelial integrin $\alpha 5\beta 1$ also decreases *N. meningitidis*-induced JNK activation in HBMEC. These findings indicate the crucial role of JNK signalling pathway in *N. meningitidis* invasion in HBMEC.

In contrast, the p38 MAPK pathway is important for the control of IL-6 and IL-8 release by HBMEC in eliciting a pro-inflammatory response (Sokolova et al., 2004). Genistein, a protein tyrosine kinase inhibitor, decreases both invasion of *N. meningitidis* into HBMEC and IL-6 and IL-8 release, indicating that protein tyrosine kinases, which link signals from integrins to intracellular signalling pathways, are essential for both bacterial internalization and cytokine secretion by HBMEC (Sokolova et al., 2004).

1.3.5 *Bartonella henselae*

Bartonella henselae is a gram-negative, α-proteobacterial zoonotic pathogen and exhibits a strong host cell tropism for ECs. *B. henselae* is able to adhere to and invade ECs and serves as a model organism for vascular colonization. Clinical manifestations, modes of infection, and virulence factors of *B. henselae* involved in pathogenesis are highlighted in the following three subchapters. These introductory chapters provide the knowledge on the basic mechanisms of *B. henselae* host cell interactions relevant for the experimental approaches presented in the results section.

1.3.5.1 Pathogenesis of *B. henselae*

B. henselae is a pleomorphous, rod-shaped bacterium that is fastidious in growth. It causes longstanding intraerythrocytic bacteraemia in its natural reservoir, the cat. Transmission from cat to cat occurs mainly by the cat flea. Transmission from cat to the incidental human host occurs by cat flea or a cat scratch or bite. The infection in cats is asymptomatic or associated with light fever. Importantly, *B. henselae* is responsible for most cases of human bartonellosis (Dehio, 2004) and the outcome of disease in humans is dependent on the immune status of the infected individual (see Figure 3).

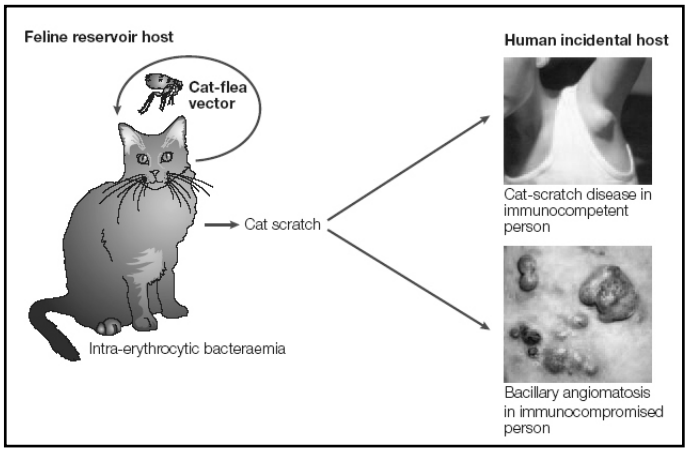


Figure 3
Pathogenesis of *B. henselae*
(Dehio C., Nature Reviews in Microbiology, 2005)

Upon infection with *B. henselae*, immunocompetent individuals establish a clinical condition usually referred to as cat-scratch disease (CSD). CSD is most commonly characterized by a regional inflammation and swelling of lymph nodes, termed lymphadenopathy, which is a self-limiting disease with no adverse consequences. Bacillary angiomatosis (BA) is the most common clinical manifestation of *B. henselae* infection in immunocompromised individuals, especially in AIDS patients. Cutaneous lesions in BA are bacterially-induced, with bacterial aggregates found in intimate contact with proliferating ECs. BA lesions are also infiltrated with macrophages/monocytes and polymorphonuclear neutrophils (PMN). BA lesions provoked by *B. henselae* are indistinguishable from BA lesions elicited by the related species *Bartonella quintana*. However, other than *B. quintana*, *B. henselae* can also cause a similar vasoproliferative disorder in inner organs, bacillary peliosis (BP), which is characterized by vascular proliferation in liver and spleen. BA and BP lesions always contain proliferating ECs and both conditions are characterized by the formation of benign, untransformed, vascular tumours. These tumours are caused by a mitogenic bacterial factor leading to enhanced cell migration and proliferation of ECs (Dehio, 2005). Furthermore, *B. henselae* is a major cause of endocarditis, which represents an inflammation of the endocardium, the inner layer of the heart, usually affecting cardiac valves. Homeless and chronically alcoholic individuals are particularly susceptible to this disease, which can be established as well by the aforementioned species *B. quintana* and several other zoonotic *Bartonella* species (Dehio, 2004). *B. henselae* exhibits a facultative intracellular life-style and is able to infect ECs in the feline reservoir and the incidental human host. Primary human umbilical vein endothelial cells (HUVEC) have been used as an appropriate *in vitro* system to study the interactions of *B. henselae* with the human vascular endothelium, i.e. (i) endothelial cell proliferation (ii) activation of the transcription factor NF-κB and stimulation of a pro-inflammatory response, which both are considered to contribute to *Bartonella*-triggered vasoproliferation, (iii) inhibition of endothelial cell apoptosis, and (iv) cellular invasion by rearrangement of host cell actin cytoskeleton (Schmid et al., 2004).

1.3.5.2 Type IV Secretion Systems in *B. henselae*

Activation of the transcription factor NF-κB and stimulation of a pro-inflammatory response, inhibition of endothelial cell apoptosis, and cellular invasion by rearrangement of the actin cytoskeleton elicited by *B. henselae* can be associated genetically to one virulence determinant of *B. henselae*, the VirB/VirD4 type IV secretion system (T4SS) (Padmalayam et al., 2000; Schmid et al., 2004; Schmiederer and Anderson, 2000; Schulein and Dehio, 2002). The VirB/VirD4 T4SS of *B. henselae* is chromosomally encoded and is closely related to the bacterial conjugation system AvhB/TraG of the *Agrobacterium tumefaciens* cryptic plasmid pAT18 based on the conservation of amino acid identities (see Figure 4).

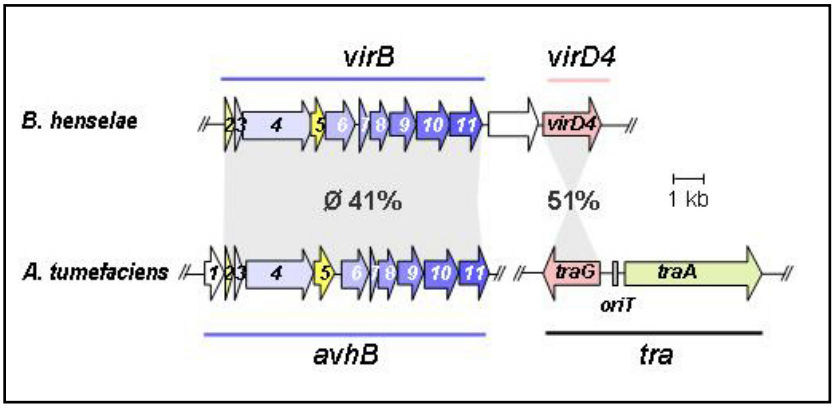


Figure 4

Genetic organisation of the *virB/virD4* loci of *Bartonella henselae* and *avhB/tra* loci of *Agrobacterium tumefaciens*. (adapted from Schröder G. and Dehio C., Trends in Microbiology, 2005)

T4SS are versatile macromolecular secretion machineries found in many gram-negative bacteria, and are evolutionarily derived from conjugation systems involved in horizontal gene transfer. T4SS are either plasmid-borne or chromosomally encoded, genetically organised into operons, usually constituting a proper pathogenicity island.

Upon induction, the T4SS is expressed and assembles into a membrane-spanning multiprotein complex (see Figure 5) competent for the secretion of either singular effector proteins, multiprotein complexes or even nucleoprotein complexes (Cascales and Christie, 2003).

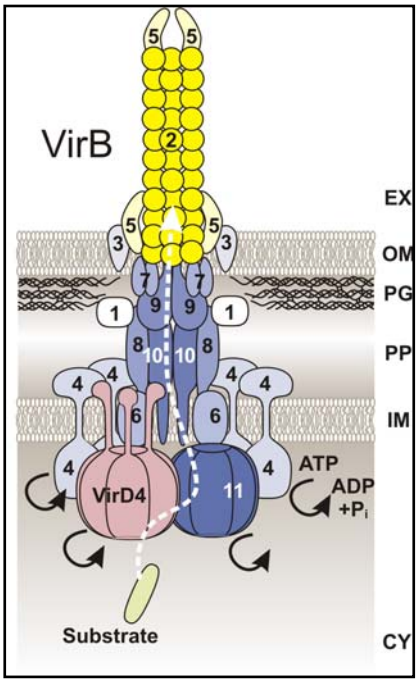


Figure 5

Macromolecular Assembly of the VirB/VirD4 T4SS of *Bartonella henselae*.
(adapted from Schröder G. and Dehio C., Trends in Microbiology, 2005)

The role in pathogenesis of the VirB/VirD4 T4SS has been studied in an appropriate animal model *in vivo* (Schulein and Dehio, 2002). Experimental infection of rats by *Bartonella tribocorum* – a close relative of *B. henselae* - revealed the initial colonization of a yet unidentified niche outside of circulating blood, presumably represented by the vascular endothelium. This primary niche periodically seeds bacteria into the bloodstream, resulting in the invasion and persistent intracellular colonisation of erythrocytes (Schulein et al., 2001).

Introduction of non-polar in-frame deletions in structural components of the VirB/VirD4 T4SS (e.g. *ΔvirB4* or *ΔvirD4*) completely abrogates the capability to cause bacteraemia, whereas complementation with the full-length genes *in trans* restored infectivity, clearly indicating that the VirB/VirD4 T4SS is essential for pathogenesis (Schulein and Dehio, 2002). *B. henselae* harbours a second functional T4SS, termed Trw (Seubert et al., 2003), which is highly similar to the Trw conjugation machinery of the broad-host-range antibiotic resistance plasmid R388. The Trw T4SS is upregulated intracellularly during the interaction of *B. henselae* with HUVECs. However, the contribution to pathogenesis of *Bartonella* on ECs remains to be demonstrated (Seubert et al., 2003).

1.3.5.3 Other Virulence Determinants of *B. henselae*

Further virulence factors of *B. henselae* important for the interaction with ECs are outer membrane proteins, namely (i) HbpA, (ii), Omp43, and (iii) BadA. HbpA (Pap31) has been originally identified as a hemin-binding protein of *B. henselae*, but appears to mediate as well adhesion to fibronection and to heparin on HUVECs (Dabo et al., 2006), while the prototypical β-barrel Omp43 might be an adhesin for HUVECs (Burgess and Anderson, 1998; Burgess et al., 2000).

The non-fimbrial adhesin BadA mediates the binding of *B. henselae* to extracellular matrix proteins and adhesion to ECs and is an immunodominant protein detectable in *B. henselae*-infected patients and rodents (Riess et al., 2004). BadA expression is important for the activation of hypoxia-inducible factor 1 (HIF-1) (Kempf et al., 2005) and the secretion of vascular endothelial growth factor (VEGF), which acts as proangiogenic mediators in EC proliferation induced by *B. henselae* (Kempf et al., 2005).

1.4 Bacterial Subversion of the Host Cell Actin Cytoskeleton Function

Modulation of components of the actin cytoskeleton machinery is frequently observed in the interaction of pathogenic bacteria with host cells. This involves (i) the stimulation of actin polymerization/depolymerization, (ii) the modulation of the activity of actin-tethering/bundling/branching proteins and finally (iii) the modulation of the activity of proteins which itself are in control of actin-dependent processes in response to cellular cues under physiological situations. Control of the dynamics and the assembly of the actin cytoskeleton converge on a specific protein family, the Rho-family of small GTPases (Hall, 1998), that regulate actin-dependent processes and function as molecular switches. Thus, bacterial subversion of the host cell actin cytoskeleton machinery frequently triggers the Rho GTPase switch leading to rearrangement, polymerization or disruption of the host cell actin cytoskeleton in order to gain access into non-phagocytic cells or to prevent uptake into phagocytic cells (Gruenheid and Finlay, 2003).

The following chapters are dedicated to five topics. The first chapter introduces the GTPase switch as a target for bacterial effector proteins. The second chapter introduces the invasion strategy of the *Salmonella typhimurium* to get access into non-phagocytic cells, whereas the third chapter introduces the strategy of *Yersinia enterocolitica* to prevent its own uptake into phagocytic cells. The fourth chapter highlights how *Listeria monocytogenes* not only promotes its own uptake into host cells, but as well how it employs the actin cytoskeleton machinery to spread intra- and intercellulary. The fifth and sixth chapter deal with *Helicobacter pylori* and *B. henselae* and how they gain access to their target cells.

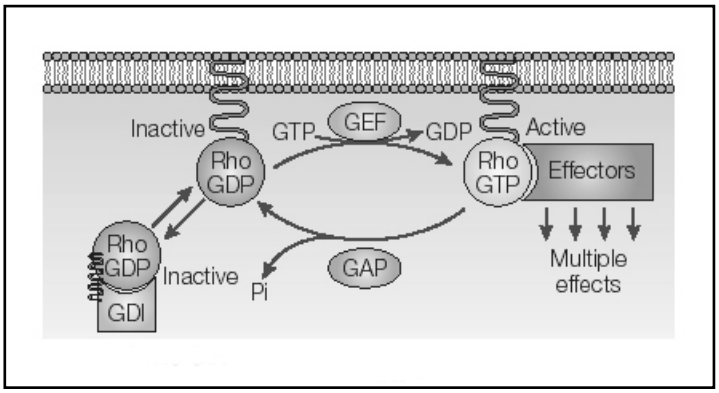
1.4.1 The GTPase Switch

Rho-family small GTPases belong to the Ras superfamily of small GTPases and share a high degree of conservation in their amino acid sequence from yeast to man. Twenty genes encoding different family members have been identified in the human genome (Hall, 1998). Out of these, the most important members are Rho, Rac and Cdc42 (Hall, 2005; Hall and Nobes, 2000). Rho, Rac and Cdc42 each control a signal transduction pathway linking membrane receptors to the assembly and disassembly of the actin cytoskeleton. Rho elicits the formation of stress fibers and focal adhesion contacts, Rac controls the formation of membrane ruffles and lamellipodia, and Cdc42 engages the formation of filopodial cell extensions (Nobes and Hall, 1995). The Rho-family small GTPases comprise versatile molecular players and regulate many cellular events of fundamental importance such as cell growth, cell morphogenesis, cytokinesis, cell movement, and lipid trafficking (Nobes and Hall, 1999).

Rho-family small GTPases act as molecular switches (Jaffe and Hall, 2005). They exist in an inactive GDP-bound and an active GTP-bound form that is able to signal to downstream effector proteins (see Figure 6). Cycling between these two states is regulated by the action of GTPase exchange factors (GEFs) which stimulate exchange of GDP for GTP, whereas GTPase activating proteins (GAPs) stimulate the GTP hydrolysis to GDP. Over 80 GEFs have been identified in mammals, which fall, based on their domain structure into two families. The larger family of Rho GEFs contains a characteristic DH (Dbl homology) domain adjacent to a PH (pleckstrin homology) domain (Rossman et al., 2005), whereas the smaller family of Rho GEFs shares homology with a protein called DOCK180 (dedicator of cytokinesis 180) (Meller et al., 2005).

On the other hand, Rho GAPs are more variable and do not share significant sequence homologies between each other (Bernards and Settleman, 2004; Moon and Zheng, 2003). Finally, GTPase dissociation inhibitors (GDIs) inhibit not only the exchange of GDP for GTP but as well recruitment of C-terminally prenylated Rho-family small GTPases to the plasma membrane, where they excert their biological function (Olofsson, 1999).

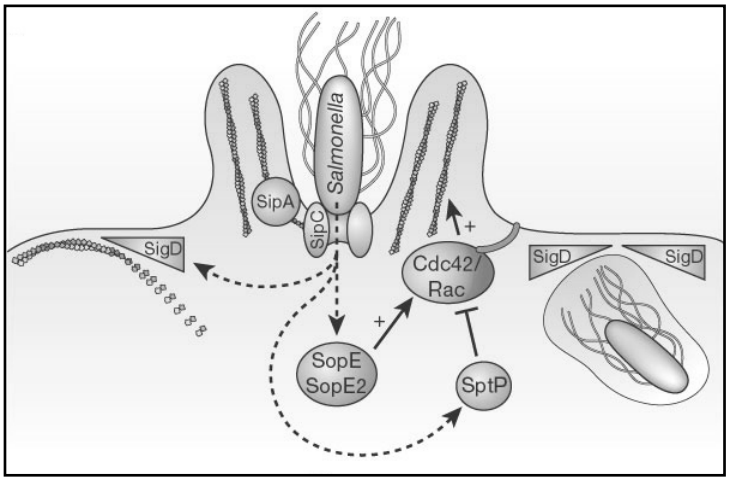
Figure 6
The Rho-family GTPase switch (Aktories P. and Barbieri J.T., Nature Reviews in Microbiology, 2005)



1.4.2 *Salmonella enterica* serovar *Typhimurium*

Salmonella is a ubiquitous gram-negative intestinal pathogen and the causative agent of several food-borne diarrheal diseases. *Salmonella* gains access to the intracellular environment by localized actin polymerization at the cell cortex, leading to extensive membrane ruffling and subsequent uptake (Altmeyer et al., 1993; Pace et al., 1993). This mode of forced cell entry is usually referred to as "trigger"-mechanism (Francis et al., 1993; Jones et al., 1993). Host cell invasion requires the concerted action of several bacterial effector proteins (see Figure 7). Translocation of these proteins from the bacterium into the host cell cytoplasm is accomplished by a specific macromolecular machine, termed injectisome. Injectisomes are found in many gram-negative bacterial pathogens and are collectively referred to as type III secretion system (T3SS). The T3SS of *Salmonella* required for invasion is termed SPI-1 (*Salmonella* Pathogenicity Island 1) (Kaniga et al., 1995; Mills et al., 1995).

Figure 7
Salmonella entry into host cells is mediated by the SPI-1 T3SS and its effectors (Gruenheid S. and Finlay B.B., Nature, 2003)



The following paragraphs introduce the most important effector proteins translocated by the SPI-1 T3SS of *Salmonella*.

The effector proteins SopE/SopE2 stimulate cytoskeletal reorganization during *Salmonella* invasion in a Rac1 and Cdc42 dependent manner, interacts with Rac1 and Cdc42 *in vivo* and stimulates nucleotide exchange of Rac1 and *Cdc42 in vitro* (Hardt et al., 1998; Stender et al., 2000). This action is reversed by SptP which in turn functions as a GAP for Rac1 and Cdc42 (Fu and Galan, 1999). Both, SopE and SptP are delivered early during infection in equal amounts into the host cell cytoplasm. However, SopE is rapidly cleared through proteasomal degradation, while SptP is degraded with much slower kinetics. This regulatory mechanism allows activation and inactivation of Rac1/Cdc42 during invasion in a precisely timed manner (Kubori and Galan, 2003).

The effector protein SigD/SopB is an inositol 3-phosphatase increasing cellular levels of phosphatidylinositol 4,5-bisphosphate leading to indirect activation of Cdc42 (Hernandez et al., 2004; Terebiznik et al., 2002). Activation of Cdc42 and Rac1 lead to recruitment and activation of WASP and Scar/WAVE family proteins together with the Arp2/3 complex involved in initiating actin polymerization (Shi et al., 2005; Unsworth et al., 2004).

The effector protein SipA nucleates and bundles actin filaments (Zhou et al., 1999a; Zhou et al., 1999b) and SipC stabilizes bundles of actin filaments (Hayward and Koronakis, 1999) which are formed during membrane ruffling and bacterial engulfment.

Loss-of-function mutants in one or more of these effector loci result in a significant impairment of invasion. Several other gram-negative bacteria such as *Shigella flexneri* use comparable strategies by injecting effector proteins via their T3SS to promote invasion and to force uptake into non-phagocytic cells.

Intracellular *Salmonella* reside in a membrane-bound compartment permissive for replication, termed *Salmonella*-containing vacuole (SCV) (Cuellar-Mata et al., 2002; Steele-Mortimer et al., 1999). Biogenesis, maturation and maintenance of the SCV is dependent on the *Salmonella* pathogenicity island 2 (SPI-2) T3SS in epithelial cells (Steele-Mortimer et al., 2002). After bacterial uptake, *Salmonella* induces the formation of a fine meshwork of F-actin decorating the SCV established in epithelial cells, fibroblasts and macrophages which is SPI-2 dependent. Treatment of infected cells with actin-depolymerizing drugs inhibits *Salmonella* replication and results in the loss of the SCV membrane and the release of bacteria into the cytoplasm indicating that actin recruitment to the SCV is essential. Intracellular *Salmonella* produce another T3SS-translocated effector protein, termed SpvB, which triggers actin depolymerization in host cells (Lesnick et al., 2001). SpvB is encoded by the *spv* locus, a regulon consisting of the spvABCD structural genes controlled by spvR (Coynault et al., 1992). SpvB is an actin-ADP-ribosylating toxin that modifies several isoforms and mediates disruption of actin around the SCV and at other host cell sites and induces the loss of cytoskeletal integrity.

1.4.3 *Yersinia enterocolitica*

Yersinia enterocolitica is a gram-negative pathogen and the etiological agent of food-borne illnesses, which are usually self-limiting. *Y. enterocolitica* is able to penetrate the intestinal mucosa, to cross the epithelial cell lining and to multiply in the lymphoid tissues of the gastrointestinal tract, such as Peyer's patches (Cornelis, 2002a; Cornelis, 2002b). *Yersinia* has developed two strategies during host cell interaction to modulate the host cell actin cytoskeleton, namely invasion of non-phagocytotic epithelial cells and inhibition of phagocytosis into macrophages and PMNs.

The *Yersinia* outer membrane protein invasin is an adhesin and is chromosomally encoded by the *inv* locus (Isberg et al., 1987). Invasin binds to the heterodimeric transmembrane receptor β1-integrin (Isberg and Leong, 1990). Upon binding, β1-integrins oligomerize into clusters to initiate Cdc42-controlled downstream signalling promoting bacterial entry into specialized intestinal cells, termed M cells, at the phagocytic cup (McGee et al., 2001; Wiedemann et al., 2001). Invasin/β1-integrin interaction is highly similar to the interaction of Intimin/Tir in enteropathogenic *E. coli* which directs actin pedestal formation on host epithelial cells at sites of bacterial attachment (Kenny et al., 1997).

Inhibition of phagocytosis into macrophages and PMNs by *Yersinia* is another example of subversion of the host cell actin cytoskeleton. For that purpose *Yersinia* engages a subset of Ysc T3SS-translocated effector proteins (Michiels and Cornelis, 1991; Michiels et al., 1990), termed YopE, YopT, YopO/YpkA and YopH that are injected intracellularly into phagocytes and paralyze them by inactivation of components of the actin cytoskeleton (see Figure 12). The first three effectors target Rho-family small GTPases and YopH acts as a powerful tyrosine phosphatase.

(1) The effector protein YopE is translocated into host cells by the Ysc T3SS injectisome (Sory et al., 1995; Sory and Cornelis, 1994). YopE is a single-domain protein and functions as a GTPase-activating protein (GAP) (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000) for the small GTPases RhoA, Rac1 and Cdc42 by direct binding and promoting efficient GTP hydrolysis (Andor et al., 2001). YopE-induced disintegration of the actin cytoskeleton causes cell rounding and detachment of infected cells, suggesting cytotoxic effects (Von Pawel-Rammingen et al., 2000).

(2) YopT is a cysteine protease that inactivates RhoA, Rac1 and Cdc42 (Aepfelbacher et al., 2003; Iriarte and Cornelis, 1998; Zumbihl et al., 1999). YopT cleaves small GTPases at a specific C-terminal cysteine which lies in a conserved amino acid motif, termed CAAX (Shao et al., 2002; Shao et al., 2003). This tetrapeptid motif is the site of isoprenylation of Rho-family GTPases which is essential for membrane association. Thus YopT causes membrane release of Rho-family GTPases Rho, Rac and Cdc42 (Sorg et al., 2001) and blocks interaction with GDIs (Zumbihl et al., 1999). The cellular consequence of YopT action is the disruption of actin filaments in professional phagocytes in order to prevent uptake of *Yersinia*, a process termed antiphagocytosis (Grosdent et al., 2002).

(3) The YopO/YpkA is a serine/theronine kinase that becomes autophosphorylated upon contact with F-actin, RhoA and Rac1 (Barz et al., 2000). YopO binds to GDP- and GTP-bound forms of RhoA and Rac1 with similar affinity, but this interaction does not affect GDP/GTP exchange by the GTPase and is independent from the YopO kinase activity. The target of the N-terminal kinase domain remains to be elucidated. During infections, YopO disrupts the actin cytoskeleton of cultured cells and leads to the complete loss of actin stress fibers (Nejedlik et al., 2004).

(4) YopH, the most powerful phosphotyrosine phosphatases (PTPase) known (Green et al., 1995; Persson et al., 1995), is targeted to focal adhesions and to other protein complexes where it dephosphorylates proteins such as the tyrosine kinase FAK, the adaptor protein $p130^{Cas}$ (Black and Bliska, 1997; Persson et al., 1997), the scaffolding protein SKAP-HOM (Black et al., 2000) and Fyn kinase binding protein Fyb (Hamid et al., 1999) which are engaged in the assembly of focal adhesions.

Yersinia lacking one of these four Yops exhibit stronger phagocytosis by PMNs and macrophages, indicating that there is no redundancy between these Yops but rather synergy in establishing the antiphagocytic phenotype (Grosdent et al., 2002).

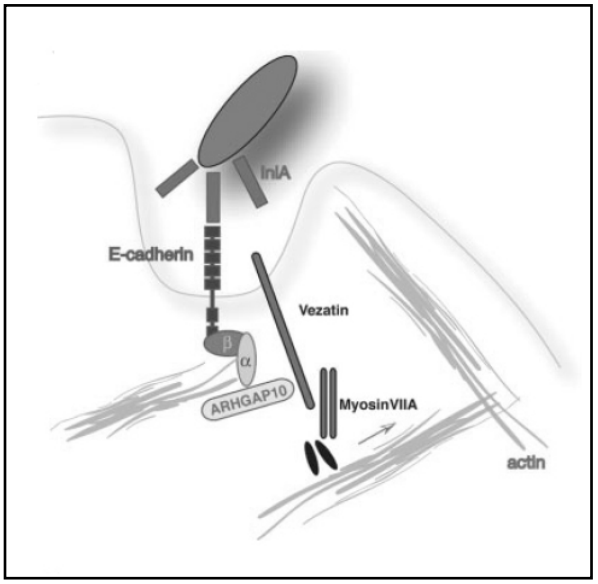
1.4.4 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, opportunistic rod with peritrichous flagella and the etiological agent of listeriolosis, a clinical condition which may lead to severe gastroenteritis in immuno competent individuals and to sepsis or meningoencephalitis in infants, elderly or immuno compromised individuals.

Subversion of the host cell actin cytoskeleton by *L. monocytogenes* serves two purposes, namely uptake into non-phagocytic cells and intracellular movement and cell-to-cell spread (Cossart et al., 2003; Pizarro-Cerda and Cossart, 2006b). Invasion of polarized epithelial cells by *L. monocytogenes* involves distinct changes in the host cell actin cytoskeleton by two alternative pathways, which are dependent on two bacterial surface proteins, internalin A (InlA) and internalin B (InlB) respectively (Cossart et al., 2003).

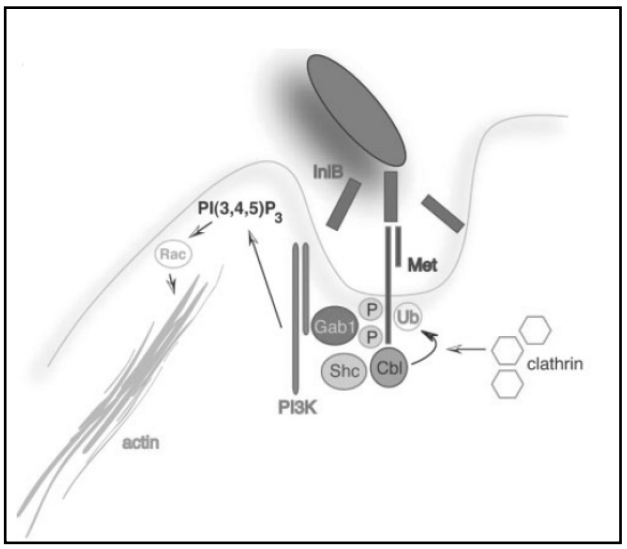
Key steps of the InlA-invasion pathway (Gaillard et al., 1991; Mengaud et al., 1996) are (1) the interaction of InlA with its host cell surface receptor E-cadherin, (2) recruitment of α-and β-catenins, which modulate and stabilize anchoring of the cortical actin cytoskeleton, (3) recruitment of the Rho GAP ARHGAP10, which, in turn, recruits vezatin and the unconventional myosin VIIA (Sousa et al., 2005) (see Figure 8).

Figure 8
InternalinA-dependent host cell entry of *Listeria monocytogenes*
(Pizarro-Cerdo J. and Cossart P., Journal of Pathology, 2006)



Key steps of the InlB-invasion pathway (Dramsi et al., 1995) are (1) the interaction of InlB with its host cell surface receptor, the hepatocyte growth factor receptor, Met (Shen et al., 2000), leading to receptor dimerization and autophosphorylation, (2) recruitment of several kinases such as Shc, Gab1 and Cbl (Ireton et al., 1999), (3) recruitment of type IA PI3Kto the plasma membrane (Ireton et al., 1996), (4) generation of phosphatidylinositol 3,4,5-trisphosphate, (5) subsequent activation of Rac1 leading to (6) the activation of WASP-related proteins, Abi1 and Ena/VASP (Bierne et al., 2005) involved in Arp2/3 complex-mediated actin polymerization (Bierne et al., 2001) (see Figure 9).

Figure 9
InternalinB-dependent host cell entry of *Listeria monocytogenes*
(Pizarro-Cerdo J. and Cossart P., Journal of Pathology, 2006)



Thus, *L. monocytogenes* uptake redirects actin cytoskeletal functions of the host cell to promote entry at sites of bacterial attachment by exploiting the cellular machinery of adherens junctions (InlA-dependent invasion) (Sousa et al., 2005) and receptor ubiquitination and endocytosis (InlB-dependent invasion) (Veiga and Cossart, 2005).

After escape from the membrane-bound vacuole by the action of listerolysin O (Mengaud et al., 1987), *L. monocytogenes* subverts the actin cytoskeletal machinery from within the infected cells to promote its own spread by actin-based motility. ActA (Kocks et al., 1992) is a non-covalently attached cell wall protein, which functions in (1) assembly of a scaffold for actin polymerization by recruitment of the adaptor protein Ena/VASP to one cell pole, (Laurent et al., 1999) (2) subsequent recruitment of the actin monomer-binding protein profilin (Grenklo et al., 2003) and the Arp2/3 complex (Welch et al., 1997), (3) initiation of actin polymerization and assembly into parallel actin filaments (David et al., 1998), and finally the (4) propulsion through the cytoplasm driven by a characteristic "actin comet tail" (see Figure 10).

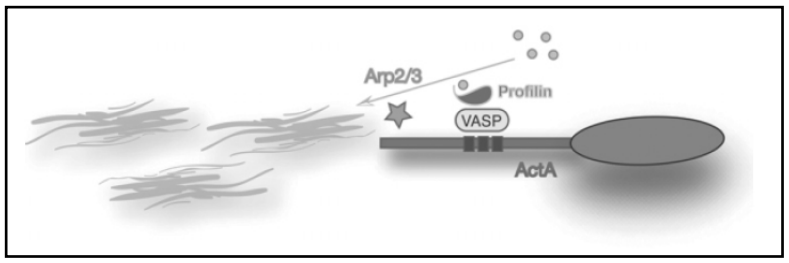


Figure 10
Actin-based motility of *Listeria monocytogenes* (Pizarro-Cerdo J. and Cossart P., Journal of Pathology, 2006)

Thus, *L. monocytogenes* harnesses the machinery that controls cellular actin nucleation under normal physiological conditions such as cell migration or pseudopod extension for the purpose of actin-based motility. Intracellular propulsion by "actin comet tails" can be found in other bacteria such as *Shigella* and *Rickettsia* (Gouin et al., 1999). These intracellular pathogens encode proteins that are functionally similar to ActA, namely IcsA/VirG (Goldberg and Theriot, 1995) and RickA (Gouin et al., 2004), respectively.

1.4.5 *Helicobacter pylori*

Helicobacter pylori is a gram-negative microaerophilic rod with lophotrichous flagella. *H. pylori* is the etiological agent of chronic gastritis, gastric ulcers and adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* invades cultured gastric epithelial cells (AGS cells) (Segal et al., 1996) by a zipper-like mechanism (Kwok et al., 2002), which comprises receptor-mediated internalization by β1-integrins (Segal et al., 1996; Su et al., 1999). Internalisation coincides with *cag* T4SS-dependent translocation of the effector protein CagA into the host cell cytoplasm where it is tyrosine-phosphorylated (Odenbreit et al., 2000; Stein et al., 2000) by the Src family kinases, c-Src and Lyn (Stein et al., 2002).

Invasion of gastric epithelial cells has two outcomes that involve modulation of the host cell actin cytoskeleton, namely (1) stimulation of cellular motility (Churin et al., 2003) and (2) host cell elongation (Higashi et al., 2004; Moese et al., 2004), which collectively result in the induction of the hummingbird phenotype (Segal et al., 1999). CagA interacts with the receptor tyrosine kinase c-Met/HGF in a phospholipase C γ-dependent manner enhancing the motogenic response of AGS cells upon *H. pylori* infection (Churin et al., 2003). Upon activation, PLC γ cleaves its membrane-bound substrate phosphatidylinositolbisphosphate (PIP_2). PIP_2 release stimulates actin-modifiying proteins such as gelsolin, profiling and cofilin, which interact with the cortical actin cytoskeleton to sustain cell motility (Chen et al., 1996). CagA promotes host cell elongation by interaction with a series of host cell proteins to form long actin-rich cell protrusions (Brandt et al., 2005; Higashi et al., 2002; Suzuki et al., 2005). Three pathways are known to participate in this process.

(1) CagA is phosphorylated by the tyrosine kinase c-Src, which, in turn, is inactivated by CagA by a negative feedback loop. c-Src inactivation leads to a dramatic decrease in tyrosine phosphorylation of the c-Src substrates ezrin (Selbach et al., 2004) and cortactin (Selbach et al., 2003). Dephosphorylated cortactin has enhanced actin cross-linking and nucleation activity, thereby modulating actin dynamics and thus contributing to host cell elongation.

(2) CagA stimulates the activation of the small GTPases Rac1 and Cdc42 leading to the subsequent activation of the downstream effector kinase PAK1 (p21-activated kinase) that promotes cytoskeletal changes culminating in host cell elongation (Churin et al., 2001).

(3) Finally, phosphorylation-dependent interaction of CagA with the tyrosine kinase Crk results in various cellular changes, one of which is WAVE-dependent and WASP-independent induction of actin nucleation as observed in actin-rich cell protrusions (Suzuki et al., 2005).

1.4.6 *Bartonella spp.*

B. bacilliformis, *B. quintana* and *B. henselae* are known to be able to induce vasoproliferative lesions in humans as a result of intimate interaction with ECs. All of these species are competent for adhesion to and invasion of HUVECs by an actin-dependent process. Bacterial internalization is reminiscent of conventional phagocytosis. Within few hours, single bacteria adhering to the host cell membrane are engulfed by membrane protrusions in a phagocytic cup and are internalized subsequently. This results in the establishment of perinuclearly located *Bartonella*-containing vacuoles (BCVs) whose nature is poorly understood (Dehio et al., 1997).

Cytoskeletal remodelling during internalisation of single bacilli has been studied at the example of *B. bacilliformis* invading ECs. Invasion of ECs by *B. bacilliformis* is dependent on the Rho-family small GTPases RhoA, Rac1 and Cdc42. Pretreament of HUVECs with C3 exoenzyme, which inactivates small GTPases, blocked bacterial internalization (Verma et al., 2000). RhoA is activated during *B. bacilliformis* invasion of ECs leading to (1) the formation of actin stress fibres orientated in parallel to the long axis of the cells and (2) the establishment of an increased number of focal adhesion contacts, which coincides with reduced cell motility (Verma et al., 2001). Furthermore, *B. bacilliformis* invasion of ECs leads to the formation of membrane ruffles and lamellipodia. Rac1 and Cdc42 are activated and recruited to the plasma membrane to sites of bacterial entry. Inhibiton of Rac1 and Cdc42 function by toxin treatment significantly reduces invasion frequencies of *B. bacilliformis* (Verma and Ihler, 2002).

Uptake of *B. henselae* into ECs induces re-organization of the host cell plasma membrane and re-arrangement of the actin cytoskeleton, resulting in the internalization of single bacilli as aforementioned and in addition uptake of bacterial aggregates, by a unique invasive structure, termed invasome (Dehio et al., 1997).

The process of invasome formation is accompanied by massive rearrangements of the underlying F-actin cytoskeleton and can be inhibited by the use of the drug cytochalasin D (Wakatsuki et al., 2001). Invasome formation of *B. henselae* into primary human umbilical vein endothelial cells (HUVECs) and into the hybridoma cell line Eahy.926 cells (fusion of HUVECs and A549 lung carcinoma cells) has been shown. Typically, an invasome comprises a ring-like basal part of twisted F-actin stress fibres anchored by focal adhesion plaques. The bacterial aggregate resides on top of this structure engulfed by membrane protrusions, which are enriched for cortical F-actin, intercellular adhesion molecule-1, and phosphotyrosines (Dehio et al., 1997) (see Figure 11).

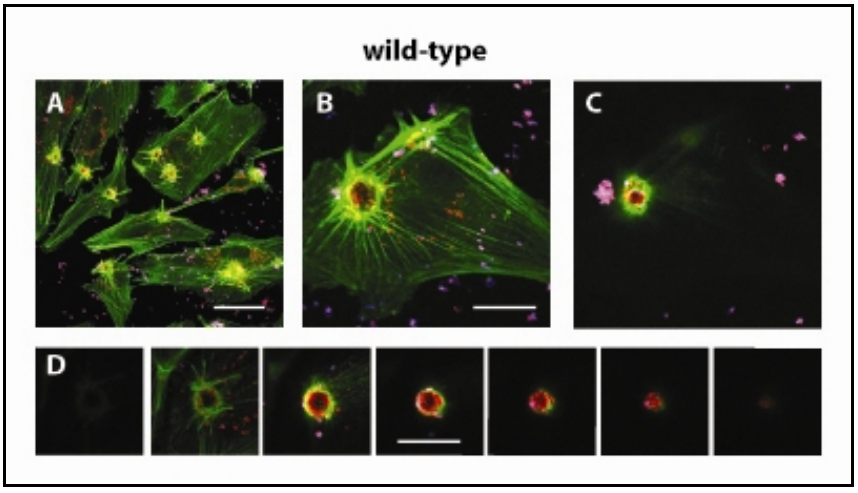


Figure 11

Invasome Formation as seen by confocal laser scanning microscopy. Endothelial cells were infected with *B. henselae* wild-type for 48 hours (MOI=100). (A) bottom view, 40x magnification; (B) bottom view, 126x magnification; (C) top view, 126x magnification; (D) serial cross sections from bottom to top. Scale (20μm).

Invasome formation requires 24 hours for completion and represents a three-step process, which is characterized by initial adherence and accumulation of dozens of bacteria on the cell surface leading to (i) aggregation, followed by (ii) engulfment and (iii) internalization of the bacterial aggregate by membrane protrusions. The relevance of invasome-mediated uptake *in vivo* remains to be demonstrated.

2. AIM OF THE THESIS

2. AIM OF THE THESIS

The aim of my Ph.D. thesis was

(1) to characterize the molecular function of putative VirB/VirD4 T4SS-translocated effector proteins of *B. henselae*,

(2) to analyze the contribution of these effector proteins to invasome-mediated internalization of *B. henselae* by human endothelial cells, and

(3) to identify host cell factors required for invasome formation,

employing bacterial genetics, cell biological assays, microscopy and biochemical methods as appropriate experimental tools.

3. RESULTS

3.1 Original Publications Relevant to the Work Described

3.1.1 Research Article I

A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells.

Schulein R., Guye P., Rhomberg T.A., Schmid M.C., Schroder G., Vergunst A.C., Carena I., Dehio C.

Proc. Natl. Acad. Sci. U. S. A. 2005 Jan 18; 102 (3): 856-61.

Summary

This research article describes the identification of multiple effector proteins of the VirB/VirD4 T4SS, termed Bep (*Bartonella*-translocated effector protein). These effector loci, *bepA-bepG*, are encoded downstream of the *virB/virD4* operon. Taken together these genes comprise a pathogenicity island, termed *virB/virD4/bep*. BepA-BepG are shown to mediate all known VirB/VirD4 T4SS-associated phenotypes including (i) the massive rearrangements of the actin cytoskeleton, resulting in the assembly of bacterial aggregates and their subsequent internalization by the so-called invasome structure, (ii) the inhibition of apoptotic cell death, resulting in enhanced HEC survival, (iii) nuclear factor κB-dependent proinflammatory activation, leading to IL-8 secretion, and cytostatic/cytotoxic effects at high bacterial titers, which interfere with a potent VirB-independent mitogenic activity.

BepA-BepG display a modular domain architecture comprising an N-terminal effector region which is different from Bep to Bep and one to four copies of a novel conserved domain in the C-terminal part, termed BID (Bep-intracellular delivery), T4SS-dependent translocation is demonstrated by BepD, which upon translocation becomes tyrosine-phosphorylated by host cell tyrosine kinases in the N-terminal effector domain and can be detected be immunocytochemical means in a punctuate pattern in the host cell cytoplasmn. Furthermore, this research article describes the delineation of the C-terminal T4S signal of the BepA-BepG. The signal required for T4SS-dependent translocation was analyzed by means of a reporter assay and was found to mediate efficient delivery of into ECs as demonstrated by the fusion of the C-terminal part of BepD including its BID domain. The same T4S signal is found in all Bep proteins and protein translocation was demonstrated accordingly for BepB, BepC and BepF.

The translocation signal was further analyzed and was found to be bipartite, comprising the not only the BID (Bep-intracellular delivery) domain, but as well a series of conserved positively charged amino acids at the very C-terminus of the Bep proteins. Finally, the BID domain was identified by means of a hidden Markov model alignment in other bacterial proteins of α-proteobacterial origin, namely in relaxases of plasmid-borne conjugative DNA-transfer systems. To demonstrate that TraA, wich is the relaxase of the AvhB/TraG conguation system of *A. tumefaciens* plasmid pAtC58, contains a functional BID domain, the C-terminus of harbouring a BID domain and a positively charged tail sequence was tested in the translocation reporter assay and found to efficiently direct VirB/VirD4-dependent heterologous protein translocation form *B. henselae* into ECs. The aforementioned relaxases and other T4S substrates are thought to interact with the coupling proteins (VirD4/TraG) of T4SS during the export of nucleoprotein complexes. A phylogenetic analysis of coupling proteins of representative T4SS revealed that these proteins fall into two clusters based on the conservation of amino acid identity. Interestingly, these clusters distinguish oneselves in the presence or absence of the BID domain in the respective T4S protein substrates of these coupling proteins suggesting coevolution of the coupling protein and the T4S signal. Interestingly, no BID domain was found in T4S substrates of agrobacterial T-DNA transfer systems or in the T4S substrates of the the *H. pylori* Cag system or the *L. pneumophila* Dot/Icm system.

Statement of own contribution

My contribution to this research article was (i) to participate in sequencing of the 23 kb *bep* locus (Figure 1a), (ii) to generate and to validate two mutant strains in *B. henselae*, *ΔvirD4* and *ΔbepB-G*, (Figure 1c), and (iii) to setup infections, produce immunocytochemical stainings and quantify frequencies of invasome formation to assess one of the four VirB/VirD4 T4SS-dependent phenotypes (Supplementary Table 4).

A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells

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Bacterial type IV secretion (T4S) systems mediate the transfer of macromolecular substrates into various target cells, e.g., the conjugative transfer of DNA into bacteria or the transfer of virulence proteins into eukaryotic host cells. The T4S apparatus VirB of the vascular tumor-inducing pathogen *Bartonella henselae* causes subversion of human endothelial cell (HEC) function. Here we report the identification of multiple protein substrates of VirB, which, upon translocation into HEC, mediate all known VirB-dependent cellular changes. These *Bartonella*-translocated effector proteins (Beps) A–G are encoded together with the VirB system and the T4S coupling protein VirD4 on a *Bartonella*-specific pathogenicity island. The Beps display a modular architecture, suggesting an evolution by extensive domain duplication and reshuffling. The C terminus of each Bep harbors at least one copy of the Bep-intracellular delivery domain and a short positively charged tail sequence. This biparte C terminus constitutes a transfer signal that is sufficient to mediate VirB/VirD4-dependent intracellular delivery of reporter protein fusions. The Bep-intracellular delivery domain is also present in conjugative relaxases of bacterial conjugation systems. We exemplarily show that the C terminus of such a conjugative relaxase mediates protein transfer through the *Bartonella henselae* VirB/VirD4 system into HEC. Conjugative relaxases may thus represent the evolutionary origin of the here defined T4S signal for protein transfer into human cells.

conjugative relaxase | effector protein | endothelial cell | protein translocation | antiapoptosis

Bacterial type IV secretion (T4S) systems are versatile transporters ancestrally related to bacterial conjugation machines. Present-day functions of T4S systems include (*i*) DNA transfer into bacterial or plant cells by cell-to-cell contact, (*ii*) protein delivery into mammalian or plant cells by cell-to-cell contact, (*iii*) DNA release to or uptake from the extracellular milieu, and (*iv*) release of multisubunit protein toxins to the extracellular milieu (1, 2). The prototypic T4S system for interkingdom substrate transfer is the VirB apparatus (encoded by *virB1–virB11*) and associated T4S coupling protein VirD4 of the phytopathogen *Agrobacterium tumefaciens* (*At*). This VirB/VirD4 T4S system mediates transfer of all components of the so called T-DNA complex, which is composed of protein substrates (VirD2 and VirE2) and single-stranded DNA (T-DNA), into plant cells (3). Intracellular delivery of solely protein substrates subverting host cell function (effector proteins) is considered to represent the primary function of T4S systems in human pathogenic bacteria (2). Examples include the Cag system of the gastric pathogen *Helicobacter pylori* (*Hp*), which translocates the CagA effector protein into gastric epithelial cells (4), and the Dot/Icm system of the Legionnaires disease agent *Legionella pneumophila* (*Lp*), which translocates multiple effector proteins into infected macrophages (5, 6). Although reporter protein fusions with subdomains of T4S substrates of *At* VirB/VirD4 or *Lp* Dot/Icm have indicated the requirement of C-terminal sequences for interkingdom protein transfer (5, 7, 8), no conserved T4S signal has been defined yet (1, 2).

Bartonella henselae (*Bh*) is a zoonotic pathogen causing a broad range of clinical manifestations in humans, including cat-scratch disease, bacillary angiomatosis-peliosis, bacteremia with fever, and neuroretinitis. Bacillary angiomatosis-peliosis is characterized by the formation of vasoproliferative tumors, which result from bacterial colonization and activation of human endothelial cell (HEC) (9). VirB, a T4S system closely related to conjugative DNA-transfer systems of α-proteobacterial plasmids (10), is a major virulence determinant of *Bh* for subversion of HEC function. VirB-dependent changes of HEC include (*i*) massive cytoskeletal rearrangements resulting in cell-surface aggregation and uptake of large bacterial aggregates by a defined structure termed the invasome; (*ii*) induction of a proinflammatory phenotype by activation of NF-κB, resulting in surface expression of the cell adhesion molecules ICAM-1 and E-selectin and secretion of the proinflammatory cytokine IL-8; (*iii*) increased cell survival by inhibition of early and late events of apoptosis (caspase activation and DNA fragmentation, respectively); and (*iv*) cytostatic or even cytotoxic effects at high infection doses, which interfers with a potent VirB-independent mitogenic activity of *Bh* (11).

Here, we report the identification of the genes encoding the T4S coupling protein VirD4 and seven putative effector proteins [*Bartonella*-translocated effector proteins (Beps) A–G]. We provide evidence that VirD4 and at least one of the effector proteins mediates all VirB-dependent phenotypes in HEC. Furthermore, we exemplarily show BepD to be translocated into HEC in a VirB/VirD4-dependent manner. Based on sequence homology between all seven Beps, we functionally define the signal for VirB/VirD4-dependent protein transfer and propose its evolutionary origin from conjugative relaxases of bacterial conjugation systems.

Materials and Methods

Bacterial Strains, Cell Lines, and Growth Conditions. *Bh* and *Escherichia coli* strains were grown as described in ref. 11, and *At* C58 was grown on plates containing Luria–Bertani medium plus agar at 28°C overnight. Table 1, which is published as supporting information on the PNAS web site, lists all the strains used in this

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Abbreviations: T4S, type IV secretion; Bep, *Bartonella*-translocated effector protein; *Hp*, *Helicobacter pylori*; *Lp*, *Legionella pneumophila*; *At*, *Agrobacterium tumefaciens*; *Bh*, *Bartonella henselae*; HEC, human endothelial cell; HUVEC, human umbilical vein endothelial cell; NLS, nuclear localization signal; CRAFT, Cre recombinase reporter assay for translocation; PAI, pathogenicity island; gpc, GFP-positive cells; BID, Bep intracellular delivery.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ556988).

†R.S. and P.G. contributed equally to this work.

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study. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described in ref. 12. The endothelial cell line Ea.hy926 resulting from a fusion of HUVEC and the lung carcinoma cell line A549 were cultured as reported in ref. 13.

DNA Sequencing and Plasmid Construction. Sequencing of the *bep* region of *Bh* ATCC 49882^T was performed from a cosmid library by using a primer walking strategy, starting with primers used for the sequencing of *virD4* of *Bartonella tribocorum* (10). Details are described in *Supporting Materials and Methods* and Table 2, which are published as supporting information on the PNAS web site. The resulting sequence has been deposited in GenBank under accession no. AJ556988. The nuclear localization signal (NLS)–Cre–Bep fusion protein-expressing vectors (pRS49–pRS124), the Cre-sensor vector (pRS56), and the BepD expression vector (pPG104) were constructed by multiple cloning steps. Sequences of oligonucleotides (Table 2), sources of gene cassettes, and further details of cloning steps are given in *Supporting Materials and Methods*. Briefly, for the expression of NLS–Cre–Bep fusion proteins in the bacteria, we first constructed pRS40, which contains the coding sequence for an NLS–Cre fusion protein under the control of the *taclac* promoter. Sequences of interest of the *bep* genes were amplified from genomic DNA and cloned into the region encoding the C terminus of the NLS–Cre gene in pRS40, providing vectors for inducible expression of NLS–Cre–Bep fusion proteins (pRS49–pRS124). pRS56 was constructed for generation of cell line Ea.hy926/pRS56-c#B1, and it contains the successive arrangement of a *lox*H site, a neomycin phosphotransferase (*neo*) gene followed by a terminator, a *lox*P site, and an *egfp* gene encoding GFP. To express FLAG-tagged BepD, we first constructed a vector containing the coding sequence for the FLAG tag following the starting methionine (MDYKDDDDK) under the control of the *taclac* promoter (pPG100). *bepD* was amplified from genomic DNA and cloned downstream of the FLAG tag in pPG100, which yielded pPG104.

Construction of In-Frame Deletions and Complementation of the Deletion Mutants. In-frame deletion mutants of *Bh* RSE247 were generated by a two-step gene replacement procedure as described in refs. 10 and 11. The Δ*virD4* mutant contains an in-frame deletion of 1.63 kb in *virD4*. The Δ*bepB–G* strain carries a 14.33-kb chromosomal deletion resulting in a 51-bp cryptic ORF composed of a 5′ sequence of *bepB* and a 3′ sequence of *bepG*. To construct the Δ*bepA–G* strain, a 1.49 kb in-frame deletion in *bepA* was introduced into the Δ*bepB–G* strain, which resulted in a remaining 144-bp cryptic ORF composed of 5′ and 3′ sequences of *bepA*. Further details are provided in *Supporting Materials and Methods*.

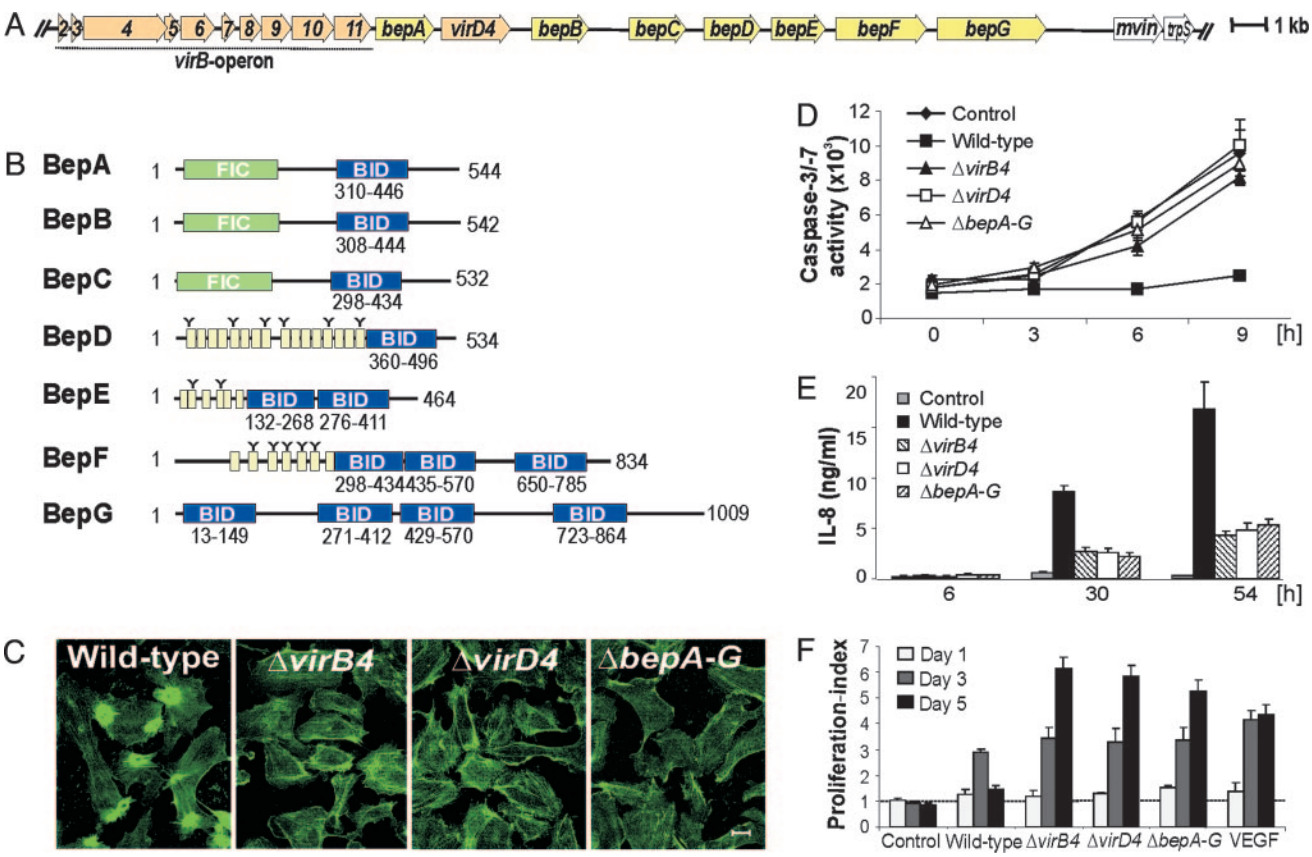

Fig. 1. The Beps mediate VirB/VirD4-dependent invasion, antiapoptotic protection, proinflammatory activation, and control of proliferation of HEC. (*A*) Structure of the *virB/virD4/bep* locus encoding the VirB components (VirB2–VirB11), the T4S coupling protein (VirD4), and seven putative effector proteins (BepA–G). (*B*) Domain structure of BepA–G. Yellow boxes represent tyrosine-containing sequence repeats resembling tyrosine-phosphorylation motifs (indicated by Y). (*C*) VirB4/VirD4/Bep proteins are required for mediating characteristic actin rearrangements, which result in uptake of *Bh* aggregates by means of invasomes. HUVEC infected with the indicated *Bh* strains were stained for F-actin. (Scale bar, 10 μm.) (*D*) VirB4/VirD4/Bep proteins are required for antiapoptosis. Caspase-3/7 activity of HUVEC was measured after infection with the indicated *Bh* strains for 24 h, followed by induction of apoptosis by actinomycin D for the indicated times. (*E*) VirB/VirD4/Bep proteins are required for NF-κB-dependent proinflammatory activation. HUVEC were infected for the indicated time with the indicated *Bh* strains, followed by quantification of IL-8 in the culture medium. (*F*) VirB4/VirD4/Bep proteins are required for controlling *Bh*-stimulated HUVEC proliferation. HUVEC infected with the indicated *Bh* strains were counted at the indicated time points, and proliferation indices were calculated. (*D–F*) Triplicate samples ± standard deviation.

Caspase Activity, IL-8 Secretion, and Proliferation. The infection of HEC and the determination of caspase-3 and caspase-7 activity [multiplicity of infection (moi) = 100], secretion of IL-8 (moi = 300), and cell proliferation (moi = 30) were carried out as described in ref. 11.

Immunocytochemical Stainings and Immunoprecipitation. HEC were infected with *Bh* strains, stained for F-actin, total bacteria, and extracellular bacteria or anti-FLAG M2. To assess the tyrosine phosphorylation of BepD upon translocation by the T4S system, Ea.hy926 cells were infected with *Bh* strains expressing FLAG-tagged BepD. Cells were subsequently lysed, and the FLAG-tagged BepD was immunoprecipitated with anti-FLAG agarose and probed with antiphosphotyrosine antibody in a Western blot. Experimental details are described in *Supporting Materials and Methods*.

Cre Recombinase Reporter Assay for Translocation (CRAFT). CRAFT (7, 8) was used to monitor the translocation of NLS–Cre–Bep fusion proteins from *Bh* into Ea.hy926 cells stably transfected with pRS56 (clone Ea.hy926/pRS56-c#B1). After transport to the nucleus, the fusion protein recombines two *lox* sites in pRS56, thereby excising *neo* and the terminator, which resulted in expression of eGFP. Briefly, Ea.hy926/pRS56-c#B1 were infected with *Bh* strains harboring plasmids containing NLS–Cre–Bep fusions, trypsinized after 120 h, and analyzed by flow cytometry. To monitor the stability of NLS–Cre–Bep fusions in *Bh*, steady-state protein levels in total lysates of bacteria grown on isopropyl β-D-thiogalactoside-containing medium were determined by immunoblotting with anti-Cre antibodies. Experimental details are described in *Supporting Materials and Methods*.

Bioinformatic Analysis. The putative C-terminal transfer domains of *Bh* BepA–G were aligned by CLUSTALW. The alignment was further edited manually, and a hidden Markov model was built thereof. By using this model, we queried the UniProt database (14) as described in *Supporting Materials and Methods*. Sequences of interest were aligned, and a neighbor-joining tree was generated as described in *Supporting Materials and Methods*.

Results

***Bh* Carries a Pathogenicity Island (PAI) Encoding the VirB/VirD4 T4S System and Seven Putative Protein Substrates.** Assuming functional clustering of the operon encoding the previously described VirB apparatus (*virB2–virB11*) (15) with genes encoding further T4S-related functions, we sequenced 23,294 base pairs that were downstream of *virB11* (Fig. 1*A*) (GenBank accession no. AJ556988). Among the 10 genes encoded by this region, only the distal *mviN* and *trpS* are present in the chromosome of related α-proteobacteria, suggesting that these genes belong to the ancestral core genome (16). A cryptic prophage integrase gene upstream of *mviN* indicates that the flanking region may have been acquired by horizontal gene transfer (16). Based on criteria defined by Hacker *et al*. (17), the *virB* operon and the eight downstream-located genes may constitute a PAI. The second gene downstream of *virB11* encodes the T4S coupling protein VirD4. The remaining seven genes of the PAI code for putative VirB/VirD4-translocated effector proteins, which we termed BepA–G. Sequence analysis revealed a modular domain structure for BepA–G (Fig. 1*B*). BepA–C are homologues carrying an N-terminal filamentation induced by cAMP (Fic) domain, which is implicated in bacterial cell division (18) and is conserved in many bacterial species (Fig. 1*B* and Fig. 5, which is published as supporting information on the PNAS web site). The N-terminal regions of BepD–F contain repeated tyrosine-containing peptide sequences that resemble tyrosine-phosphorylation motifs (e.g., EPLYA, Fig. 1*B* and Fig. 6, which is published as supporting information on the PNAS web site). Strikingly, all Beps share at least one copy of a domain of ≈140 aa in their C-terminal region (Fig. 1*B* and Fig. 7, which is published as supporting information on the PNAS web site). This domain was suspected to be involved in Bep translocation and was thus designated the Bep intracellular delivery (BID) domain. In addition to the BID domain, the C termini of BepA–G contain short unconserved tail sequences rich in positively charged residues, each carrying a net positive charge (Table 3, which is published as supporting information on the PNAS web site).

All Known VirB-Dependent Cellular Phenotypes of HEC Require VirD4 and at Least One of the Putative Effector Proteins BepA–G. To test whether VirD4 and BepA–G contribute to VirB-mediated virulence, we generated nonpolar in-frame deletion mutants (Δ*virD4* and Δ*bepA–G*, the latter mutant being constructed by sequential deletion of *bepB–G* and *bepA*) and compared them with the isogenic Δ*virB4* mutant and wild-type strain with respect to known VirB-dependent phenotypes of *Bh*-infected HEC (11). Opposed to wild type, all three deletion mutants were deficient for triggering (*i*) the formation of the characteristic F-actin rearrangements associated with invasome-mediated invasion (Fig. 1*C* and Table 4, which is published as supporting information on the PNAS web site), (*ii*) the inhibition of apoptotic cell death triggered by actinomycin D as measured by caspase-3/7 activity (Fig. 1*D*), (*iii*) the activation of an NF-κB-dependent proinflammatory response determined by quantification of secreted IL-8 in the culture medium (Fig. 1*E*), and (*iv*) cytostatic/cytotoxic effects interfering with the VirB-independent mitogenic activity of *Bh* as measured by cell counting (Fig. 1*F*). We conclude that all known VirB-mediated phenotypes of HEC require the T4S coupling protein

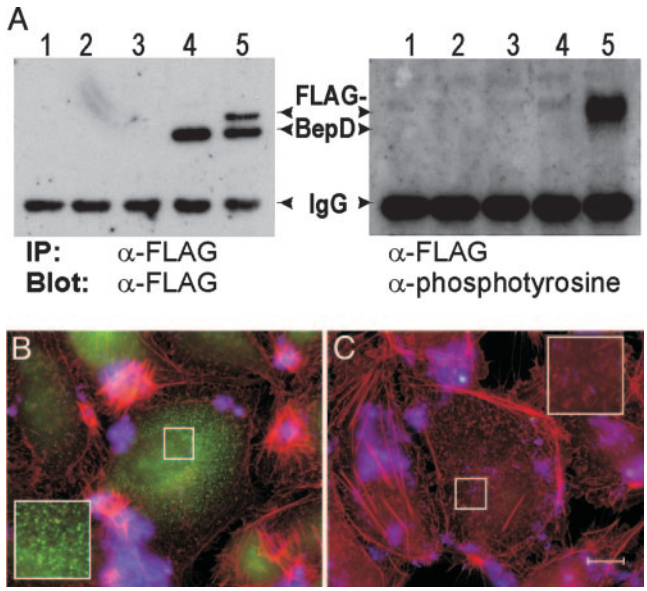

Fig. 2. BepD becomes tyrosine-phosphorylated after VirB4-dependent translocation into HEC. (*A*) VirB4-dependent translocation of BepD into HEC results in tyrosine phosphorylation and a coincident reduction in electrophoretic mobility. Total protein extracts of Ea.hy926 cells uninfected (lane 1) or infected with Δ*virB4* (lane 2), wild type (lane 3), Δ*virB4*/pPG104 (lane 5), or wild type/pPG104 were prepared. FLAG-BepD encoded by pPG104 was immunoprecipitated with anti-FLAG antibodies, separated by SDS/PAGE, and immunoblotted with anti-FLAG (*Left*) or anti-phosphotyrosine antibodies (*Right*). (*B* and *C*) Immunocytochemical detection of FLAG-BepD after VirB/VirD4-mediated translocation into HEC. Ea.hy926 cells were infected with wild-type (*B*) or the Δ*virB4* mutant (*C*), each harboring pPG104. Specimens were immunocytochemically stained for the FLAG epitope (green), F-actin (red), and bacteria (blue). (Scale bar, 10 μm.)

VirD4 and at least one of the putative effector proteins BepA–G. Moreover, these data suggest that most likely all genes encoded by the *virB/virD4/bep* PAI of *Bh* have functions related to T4S.

BepD Is Translocated into HEC in a VirB-Dependent Manner. Next, we tested whether BepD, one of the three putative substrates for host cell tyrosine kinases among the Beps (Fig. 1*B*), becomes tyrosine-phosphorylated during infection of HEC. Phosphorylation by host cell tyrosine kinases was previously used to demonstrate translocation of bacterial proteins into human cells (19). We show that FLAG-epitope-tagged BepD becomes tyrosine-phosphorylated during HEC infection when expressed in wild type but not in the Δ*virB4* mutant (Fig. 2*A Right*). Tyrosine phosphorylation coincided with a prominent shift in electrophoretic mobility (Fig. 2*A Left*), suggesting additional protein modification. Immunocytochemistry revealed a VirB4-dependent punctuate staining pattern of FLAG-BepD in the host cell cytoplasm (Fig. 2 *B* and *C*). Together, these data demonstrate VirB-dependent translocation of BepD into HEC.

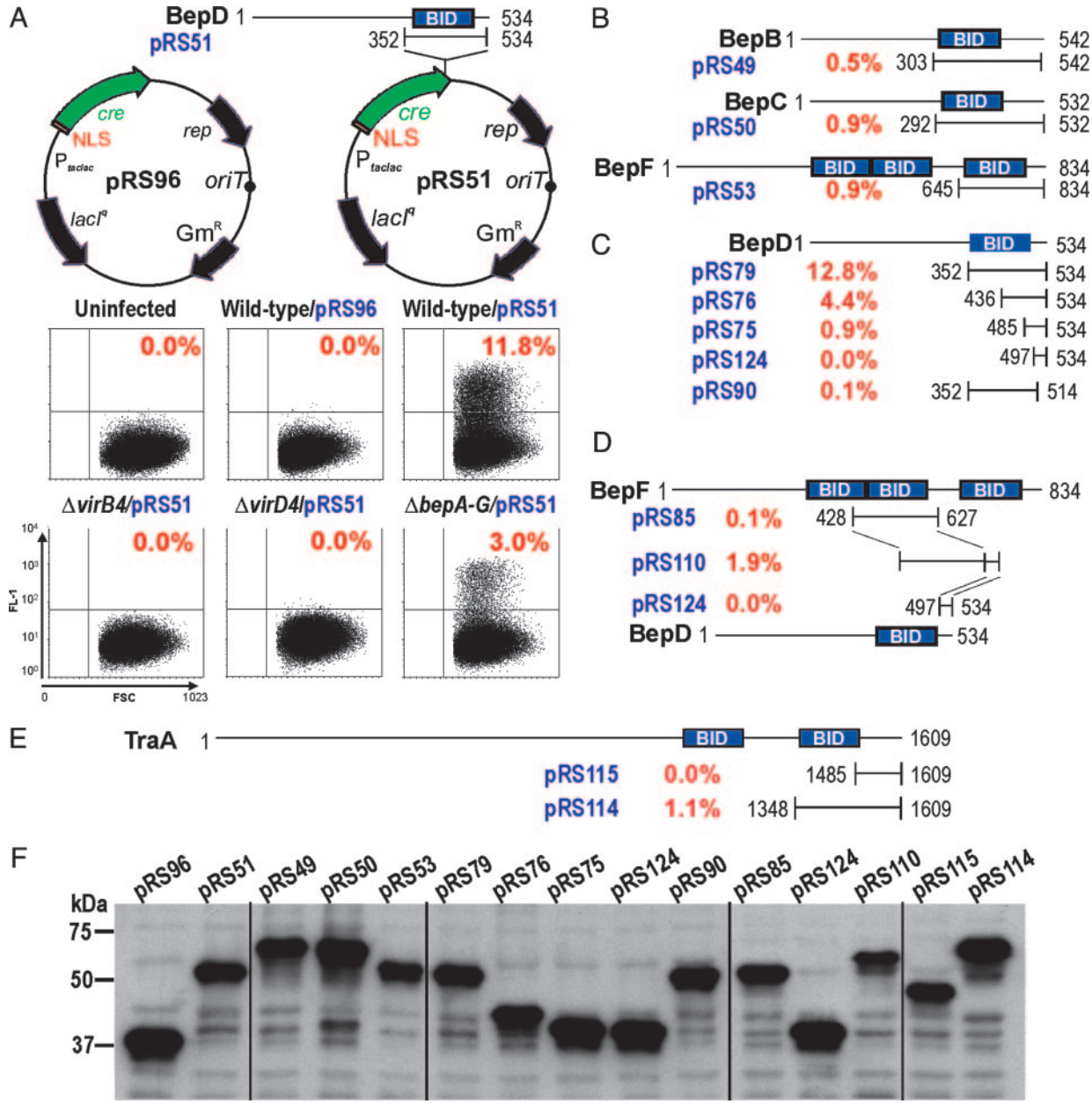

Fig. 3. The C-terminal translocation signal of Beps mediates VirB/VirD4-dependent protein transfer into HEC. Protein transfer was determined by CRAFT. The Cre-tester cell line Ea.hy926/pRS56-c#B1 was infected with the indicated *Bh* strains expressing different NLS–Cre fusion proteins (plasmid names are indicated in blue in *A–E* or black in *F*). The region of a given Bep fused to the C terminus of NLS–Cre is specified by the respective first and last amino acids (except for pRS96, which expresses only NLS–Cre). Percentages of GFP-positive cells as determined by FACS analysis are indicated in red. (*A*) NLS–Cre fused to the C-terminal 183 aa of BepD translocates efficiently into HEC in a VirB/VirD4-dependent manner. Dot blots of forward scatter (FSC) and GFP fluorescence (FL-1) are shown for the indicated *Bh* strains. (*B*) Relative translocation efficiency mediated by the BID domain of BepB, BepC, and BepF. (*C*) The signal for VirB/VirD4-dependent translocation into HEC is bipartite, composed of the BID domain and an adjacent unconserved C-terminal tail. (*D*) Creation of an efficient bipartite translocation signal by fusing a BID domain of BepF and the C-terminal tail of BepD. (*E*) The C terminus of the relaxase TraA of *At* plasmid pATC58 contains a BID domain and mediates efficient protein transfer from *Bh* into HEC. (*F*) Steady-state NLS–Cre fusion protein levels in *Bh* grown on isopropyl β-D-thiogalactoside-containing medium.

Delineation of the Bipartite T4S Signal of the Beps. To delimit the BepD translocation signal and to demonstrate translocation of other Beps, we adapted CRAFT, a reporter assay originally designed to detect translocation of bacterial effector proteins into plant cells (8) (Fig. 3*A* and Fig. 8, which is published as supporting information on the PNAS web site). After infection of the Cre-tester cell line Eahy.926/pRS56-c#B1 with *Bh* strains expressing NLS–Cre–recombinase fusion proteins, the percentage of GFP-positive cells (gpc) as determined by FACS analysis was used as a relative measure for the efficiency of protein transfer from *Bh* into HEC. Expression of an NLS–Cre–recombinase fusion protein in wild-type *Bh* resulted in 0.0% gpc and was thus negative in this assay (Fig. 3*A*, pRS96). In contrast, NLS–Cre fused to the C-terminal 183 aa of BepD (BID domain plus a short positively charged tail sequence, pRS51) was efficiently translocated from wild-type (11.8% gpc) and Δ*bepA–G* (3.0% gpc), whereas no translocation occurred from Δ*virB4* or Δ*virD4* strains (0.0% gpc) (Fig. 3 *A* and *F*). Hence, this heterologous fusion protein was translocated in a VirB4/VirD4-dependent, and essentially Bep-independent, manner. Similar as for BepD, NLS–Cre fusions to the BID domain-containing C terminus of BepB, BepC, and BepF were translocated into HEC, albeit at lower frequency (Fig. 3 *B* and *F*). Taken together, we provide evidence for a functional T4S signal in the C terminus of four Bep proteins (BepB, BepC, BepD, and BepF).

To further delimit the T4S signal contained in the 183-aa C-terminal fragment of BepD, we performed a deletion analysis (Fig. 3 *C* and *F*). C-terminal deletion of 20 aa of the short positively charged C-terminal tail sequence almost completely abolished translocation (0.1% gpc). Stepwise deletion of the BID domain from the N terminus resulted in a gradual reduction of translocation efficiency. Together, these data suggest a bipartite translocation signal at the C terminus, composed of a BID domain and a short positively charged tail sequence. As illustrated in Fig. 3*D*, this notion was supported by the success in creating an efficient translocation signal (1.9% gpc) via fusion of a translocation-inefficient BID domain of BepF (0.1% gpc) with the translocation-deficient positively charged tail of BepD (0.0% gpc). Notably, all NLS–Cre–Bep fusion proteins analyzed by CRAFT displayed comparable steady-state protein levels in bacteria (Fig. 3*F*), indicating that the low translocation efficiency observed for several fusion proteins does not result from protein instability but rather reflects the absence of an appropriate T4S signal.

Identification of BID Domains in Conjugative Relaxases and Demonstration of Their Function As T4S Signal for the *Bh* VirB/VirD4 System. To search for other proteins containing a BID domain, we queried the UniProt database with a hidden Markov model (20) generated from an alignment of all BID domains of BepA–G (Fig. 7 and Table 5, which is published as supporting information on the PNAS web site). Among the 40 top hits are 27 hits within putative T4S substrates. These hits include BepA–G of *Bh* and their homologues in *Bartonella quintana,* annotated as hypothetical proteins in the recently published genome sequences (16), as well as Fic-1, which is a BepA homologue in *Bartonella tribocorum* (10). The other hits in putative T4S substrates are all in relaxases of conjugative plasmids found in various α-proteobacteria. The plasmid-borne conjugation systems associated with these conjugative relaxases are closely related to each other as well as to the *Bh* VirB/VirD4 system (10), as indicated by clustering in one clade of a phylogenetic tree for VirD4/TraG-like T4S coupling proteins (Fig. 4, cluster A). Interestingly, no BID domain was found in protein substrates of agrobacterial T-DNA transfer systems (VirB/VirD4), which cluster in a separate clade of the VirD4/TraG phylogram (Fig. 4, cluster B), or in the T4S substrates of *Lp* or *Hp*. For the AvhB/TraG conjugation system of *At* plasmid pAtC58 (21), we show that the C terminus of its relaxase harbors a BID domain and a positively charged tail sequence, which efficiently directs VirB/VirD4-dependent protein transfer from *Bh* into HEC (Fig. 3*E*, pRS114), whereas the positively charged tail alone did not result in detectable transfer activity (Fig. 3*E*, pRS115). These data suggest that the *Bh* VirB/VirD4/Bep protein transfer system evolved rather recently from one of the wide-spread conjugative plasmid-transfer systems in α-proteobacteria and that the bipartite transfer signals in the substrates of these T4S systems are functionally interchangeable.

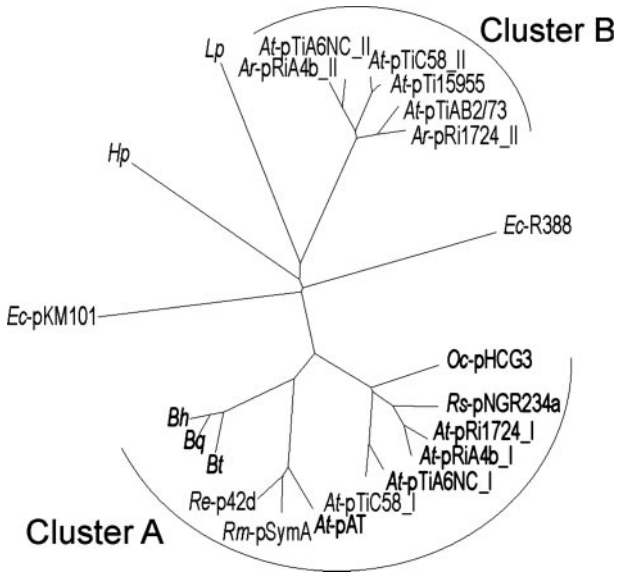

Fig. 4. The coupling proteins (VirD4/TraG) of T4S systems containing a BID domain in their protein substrate(s) form a distinct phylogenetic cluster. This cluster is formed by *Bartonella* VirB/VirD4 systems and α-proteobacterial conjugative DNA transfer systems (cluster A) and does not contain agrobacterial T-DNA transfer systems (cluster B). VirD4/TraG protein sequences were extracted from the Uniprot database and then aligned and diagrammed as an unrooted neighbor-joining radial tree. T4S systems containing a BID domain in one of their substrate(s) are marked in bold (compare with Table 5). The following sequences (with corresponding accession numbers) are included. *At*: plasmid pAT (Q8UKJ4), pTiC58 (Q44346 and P18594), pTiA6NC (Q44360 and P09817), pRi1724 (Q9F5E3 and Q9F585), pTi15955 (Q8VLK3), and pTiAB2/73 (Q8VT85); *Agrobacterium rhizogenes*: pRiA4b (Q93UY7 and P13464); *Bh* (Q6G2A8); *Bartonella quintana* (Q6FYV9); *Bartonella tribocorum* (Q8GJ55); *Escherichia coli*: pKM101 (Q46706) and R388 (Q04230); *Hp* (Q75XB9); *Lp* (Q9RLR2); *Oligotropha carboxydovorans* (Q6LB53); *Rhizobium etli*: p42d (Q8KL68); *Rhizobium meliloti*: pSymA (Q92ZI3); and *Rhizobium* spp.: pNGR234a (P55421).

Discussion

In this study, we characterized a PAI encoding presumably all proteins related to the function of a pathogenesis-related T4S system in *Bh*. In addition to the previously described T4S apparatus VirB (VirB2–VirB11) (11, 15), this PAI encodes the T4S coupling protein, VirD4, and seven T4S substrates termed BepA–G. Deletion of either *virD4* or the complete set of *bep* genes (*bepA–G*) resulted in a similar phenotype as that described for deletion of *virB4* (11); i.e., these mutants are deficient for subverting multiple HEC functions related to the cytoskeleton and to inflammation, apoptosis, and proliferation. The essential role of VirD4 for mediating VirB-dependent host cellular changes is consistent with the proposed function as T4S coupling protein, representing the interface between the T4S apparatus and the translocated substrates (1). The loss of all known VirB/VirD4-dependent HEC changes in the Δ*bepA–G* mutant indicates that BepA–G may comprise the complete set of VirB/VirD4-translocated effector proteins. Preliminary data

from our laboratory suggest that the specific contribution of individual Beps to the complex VirB/VirD4-dependent phenotypic changes of HEC can be assessed by their expression, either alone or in combination, in the effector-free Δ*bepA–G* mutant background (M.C.S., P.G., and C.D., unpublished data).

The recently published comparative genome analysis of *Bh* and *Bartonella quintana* revealed that the *virB/virD4/bep* PAI characterized herein is present in both *Bartonella* genomes but not in any other published genome sequence. However, in contrast to the highly conserved *virB/virD4* loci, the *bep* loci display a high degree of plasticity, including signatures of gene duplication and degradation (data not shown) as well as intragenic domain duplication and intragenic or intergenic domain reshuffling (Fig. 1*B*). As a result, the domain structure of the Beps is highly modular. The N termini of BepA–C are composed of a domain (Fic) conserved in many bacterial species that is considered to be involved in cell division (18). The N termini of BepD–F contain short repeated peptide sequences containing conserved putative tyrosine phosphorylation motifs (i.e., EPLYA) similar to the EPIYA motif of the CagA effector protein of *Hp* known to be phosphorlyated by human Src-family kinases (4, 22). Consistently, we show BepD to become tyrosine-phosphorylated upon T4S-dependent transfer into HEC. Taken together, the N termini of the Beps are highly divergent and may primarily serve effector functions within HEC. In the C-terminal region of all Beps, we could define at least one copy of a 142-aa domain called BID. An unconserved, positively charged tail sequence at the C terminus and the proximal BID domain was shown here to represent a bipartite T4S signal that mediates VirB/VirD4-dependent protein transfer into HEC. This finding is in agreement with a requirement of C-terminal sequences for interkingdom transfer of T4S substrates of *At* and *Lp* (5, 7, 8).

A hidden Markov model of the BID domain alignment from *Bh* allowed us to search for other proteins containing a similar domain. A large proportion of the top hits were indeed within putative T4S substrates, including all Bep homologues of bartonellae as well as the conjugative relaxases of plasmid-borne bacterial conjugation systems present in various α-proteobacteria. Conjugative relaxases direct the transfer of plasmid DNA by first cleaving and covalently attaching to one DNA strand, followed by transport of the resulting protein–DNA conjugate by the plasmid-encoded T4S system (23). In this process, the specific interaction between the relaxase and the T4S coupling protein is thought to initiate the transport through the membrane-spanning T4S channel (24). The BID domain has likely evolved in the relaxases of α-proteobacterial conjugation systems before horizontal transfer occurred into a progenitor of *Bartonella*. A phylogenetic analysis of the T4S coupling proteins (VirD4/TraG) of representative T4S systems indeed revealed that the coupling proteins of T4S systems with a BID domain in their substrate(s) form a distinct cluster. This finding suggests coevolution of the coupling protein and the T4S signal, which is consistent with the finding that coupling proteins and T4S substrates physically interact (24–26). The absence of a BID domain in the substrates of other T4S systems (e.g., of the agrobacterial VirB/VirD4 system, the *Hp* Cag system, and the *Lp* Dot/Icm system) suggests that a different signal mediates protein transfer by these T4S systems.

We show that the BID domain and short positively charged C-terminal tail of the conjugative relaxase (TraA) of the *At* pAtC58 conjugation system AvhB/TraG is functional for mediating VirB/VirD4-dependent protein transfer from *Bh* into HEC. The T4S signals of these related T4S systems involved either in interbacterial DNA transfer or interkingdom protein transfer are thus interchangeable. This finding makes it tempting to speculate that conjugative relaxases are also transported by the *Bh* VirB/VirD4 system into HEC when they are covalently attached to their single-stranded DNA substrate, similar to the interkingdom DNA transfer by the *At* VirB/VirD4 system into plant cells. T4S-mediated DNA transfer from virulence-attenuated *Bh* in human cells could have important applications for gene therapy and vaccination and should thus be an interesting subject for future investigations.

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3.1 Original Publications Relevant to the Work Described

3.1.2 Research Article II

A translocated bacterial effector protein triggers Rac1- and Cdc42-dependent cytoskeletal rearrangements during invasome-mediated uptake of *Bartonella henselae* into human endothelial cells

Rhomberg T.A., Guye P., Ellner Y. and Dehio C.

Manuscript in preparation.

Summary

This research article addresses the molecular mechanism underlying bacterial uptake of *B. henselae* during the interaction with ECs, which culminates in the establishment of a unique actin-dependent structure directing the internalization of bacterial aggregates, termed invasomes.

This work identifies the bacterial effector protein BepG as a *bona fide* translocated substrate of the VirB/VirD4 T4SS of *B. henselae*. BepG is shown to be sufficient to direct the sustained rearrangement of the host cell actin cytoskeleton during invasome formation. Expression of BepG in the effectorless isogenic mutant background of *B. henselae* (*ΔbepA G/bepG*) reproduces essential characteristics of invasome formation observed with *B. henselae* wild-type, namely internalization of bacterial aggregates and establishment of a basal ring-linke structure with protruding F-actin fibers. However, the fine morphology of invasomes formed by the wild-type strain appears to differ with invasomes elicited by effectorless mutant strain expressing BepG (*ΔbepA-G/bepG*). Furthermore, it was found that a non-polar in-frame deletion mutant of *B. henselae* (*ΔbepG*) does not loss its capacity to form invasomes, which indicates the existence of a parallel pathway promoting invasome formation. Upon transfection, BepG appears to co-localize to distinct F-actin structures, namely the cortical actin beneath the plasma membrane, pseudopodial structures in the cell periphery and stress fibers anchoring cells to the substratum, suggesting that BepG is able to interact with the actin cytoskeleton machinery.

On the host cell side, this research article provides evidence that Rho-family small GTPases Cdc42 and Rac1 but not RhoA are involved in the process of BepG-mediated invasome formation. More specifically, Rac1 appears to be the key player in controlling rearrangement of the host cell actin cytoskeleton, since introduction of dominant-negative or constitutively active versions of Rac1 strongly reduces frequencies of invasome formation. This finding indicates that distinct morphological alterions mediated by Rac1 under physiological conditions, namely formation of membrane ruffles and lamellipodia, are crucial in the interaction of adhering bacteria in order to form aggregates on the host cell surface and to induce actin cytoskeletal changes. In addition, it is shown, that rearrangement of the host cell actin cytoskeleton during invasome formation is accompanied by F-actin assembly at sites of invasome formation. F-actin assembly is shown to be mediated by a pathway involving the adaptor proteins Scar/WAVE downstream of Rac1 and recruitment of the Arp2/3 complex to sites of invasome formation. Finally, invasome formation was found to bypass entry of bacterial aggregates into the endocytic-lysosomal pathway by preventing the fusion with Lamp-1 positive, degradative vesicles. This finding indicates a possible mechanism leading to non-fusogenic bacterial internalization allowing the establishment of an intracellular niche in ECs permissive for growth and persistent colonization.

Statement of own contribution

I developed all experimental strategies presented in this research articles and all cellular assays presented, namely infections, transfections and reporter assay for translocation, were performed myself with the kind assistance of Y. Ellner. Picture acquisition by confocal laser scanning microscopy, data evaluations and all quantifications were carried out by myself. Cloning of reporter plasmids and generation of the non-polar in-frame mutant were performed by myself. Strains for single gene complementations were provided by coworkers P. Guye and M. C. Schmid.

A translocated bacterial effector protein triggers Rac1- and Cdc42-dependent cytoskeletal rearrangements during invasome-mediated uptake of *Bartonella henselae* into human endothelial cells

Rhomberg T.A., Guye P., Ellner Y. and Dehio C. *

Manuscript in preparation.

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6899

Words in Summary

156

Summary

The facultative intracellular bacterium *Bartonella henselae* enters human endothelial cells either passively by conventional phagocytosis or actively by a pathogen-triggered process known as invasome-mediated internalization. The latter involves the formation of a cell-surface-associated bacterial aggregate, which is subsequently engulfed by host cell membranes eventually resulting in its complete internalization. Here, we report on the identification and characterization of a translocated bacterial effector protein, BepG, which co-localizes with F-actin and mediates the sustained cytoskeletal changes leading to invasome-mediated internalization. Moreover, we show that invasome-mediated uptake depends on the Rho-family small GTPases Cdc42 and Rac1, but not RhoA. Invasome formation leads both to the rearrangement of pre-existing F-actin fibres and to localized actin polymerization enriched for Arp2/3, which occurs in a Scar1/WAVE-dependent manner. Finally, we demonstrate that after complete internalization the invasome membranes do not fuse with Lamp-1 positive lysosomes, indicating that invasome-mediated invasion represents a novel mechanism allowing the uptake of bacteria without entering the endocytic-lysosomal pathway.

Introduction

Bartonella henselae is a world-wide distributed zoonotic pathogen that infects cats and humans (Dehio, 2004). This gram-negative pathogen exhibits a facultative intracellular life-style and is highly adapted to cause longstanding intraerythrocytic infection in the feline reservoir, among which it is transmitted by infected cat fleas. Transmission to the incidental human host by cat scratches can result in a wide range of clinical symptoms. In immuno-competent patients, the most frequent manifestation is cat-scratch disease, a typically self-limiting disease resulting in local swelling of lymph nodes and fever. Immuno-compromized patients commonly develop bacillary angiomatosis-peliosis, a clinical condition characterized by the formation of benign, vasoproliferative tumors in skin and liver. These tumors arise from bacterial colonization of the vasculature, which results in the activation, migration and proliferation of human endothelial cells (EC) (Dehio, 2005). Vascular colonization by *B. henselae in vivo* can be mimicked by infection of EC *in vitro*, i.e. by using primary human umbilical vein endothelial cells (HUVEC) or the Ea.hy926 cell line originating from the fusion of HUVEC with the lung carcinoma cell line A549 (Dehio et al., 1997; Schulein et al., 2005).

Most of the distinct cell biological outcomes of EC infection by *B. henselae* are dependent on one particular bacterial virulence determinant, the type IV secretion system (T4SS) VirB/VirD4 (Schmid et al., 2004). This macromolecular transporter is known to translocate seven effector proteins, BepA-BepG (*Bartonella*-translocated effector proteins) into infected EC (Schulein et al., 2005). The Beps display a modular domain structure, which is characterized by an N-terminal effector domain and a C-terminal export signal. This signal for T4SS-dependent protein translocation is bipartite, comprising at least one copy of the approximately 140 aa large BID-domain (*Bartonella* Intracellular Delivery) and an adjacent non-conserved C-terminal tail sequence carrying a net positive charge (Schulein et al., 2005).

BepA-BepG mediate all known VirB/VirD4-dependent cellular phenotypes of EC, namely (i) activation of the transcription factor NF-κB and stimulation of a pro-inflammatory response, (ii) protection from apoptosis, and (iii) cell invasion by a unique cellular structure termed the invasome (Schmid et al., 2004). *B. henselae* enters EC also by a T4SS-independent route resembling conventional phagocytosis (Dehio et al., 1997). In the latter case, single bacteria adhering to the host cell plasma membrane are engulfed within minutes by membrane protrusions forming a phagocytic cup, which finally leads to complete bacterial internalization. Subsequently, this results in the establishment of perinuclearly located *Bartonella*-containing vacuoles (BCVs), whose nature is poorly understood.

In contrast, the T4SS-dependent process of invasome formation is much slower and requires at least 16 h for completion in individual EC (Dehio et al., 1997). Invasome-mediated uptake is a three-step process, which is characterized by initial adherence and accumulation of dozens of bacteria on the cell surface, leading to (i) aggregation, followed by (ii) engulfment by membrane protrusions and (iii) eventually complete internalization of the bacterial aggregate. The process of invasome formation is accompanied by local rearrangements of the F-actin cytoskeleton and is inhibited by cytochalasin D (Wakatsuki et al., 2001). The membrane protrusions engulfing the bacterial aggregate are enriched for cortical F-actin, intercellular adhesion molecule-1, and phosphotyrosine. Underneath the engulfed bacterial aggregate, the invasome characteristically comprises stress fibres winded up to a dense ring-shaped F-actin structure (Dehio et al., 1997).

In the present study, we investigated the molecular and cellular basis of invasome-mediated internalization of *B. henselae* into EC. Based on the expression of individual Bep effectors in the effector-deletion mutant *ΔbepA-G*, which is incapable of forming invasomes (Schulein and Dehio, 2002), we genetically defined BepG as a T4SS-effector protein mediating cellular changes characteristic for invasome formation. Furthermore, we provide evidence that invasome-mediated internalization is also mediated by a parallel pathway involving at least two other Bep effectors.

On the host cell side, we identified key factors involved in invasome formation. We demonstrate the involvement of the Rho-family small GTPases Rac1 and Cdc42, but not RhoA, which control actin organization and give rise to the formation of cellular structures like membrane ruffles, filopodia, or stress fibres, respectively (Nobes and Hall, 1999). In addition, we provide evidence for the requirement of factors involved in *de novo* actin polymerization, i.e. the Wiskott-Aldrich Syndrom protein (WASp)-related adaptor protein Scar (Machesky and Insall, 1998). Finally, we show that the Arp2/3 complex mediating F-actin nucleation and branching (Machesky and Gould, 1999) is recruited to the cortical F-actin associated with the invasome structure, indicating that it plays a role in *de novo* F-actin polymerization in conjuction with invasome-mediate uptake of *B. henselae* into EC.

Material and Methods

Bacterial Strains, Growth Conditions, Conjugations. *B. henselae* wild-type strain RSE 247 (Schmid et al., 2004), isogenic mutants Δ*virB4* (RSE 242) (Schmid et al., 2004), *ΔbepA-G* (MSE 150) (Schulein et al., 2005), Δ*bepG* (TRB 223) and mutant strains expressing single Bep proteins, *ΔbepA-G*/p*bepA* (MSE 156), *ΔbepA-G*/p*bepB* (MSE 167), *ΔbepA-G*/p*bepC* (MSE 159), *ΔbepA-G*/p*bepD* (PGD03), *ΔbepA-G*/p*bepE* (PGD10), *ΔbepA-G*/p*bepF* (TRB171), *ΔbepA-G*/p*bepG* (TRB169), *ΔbepG*/p*bepG* (TRB234), wild-type/pRS51 (RSE308), wild-type/pTR1703 (TRB265), and *ΔvirB4*/pTR1703 (TRB293) were cultivated and selected as described (Dehio and Meyer, 1997). *E. coli* strains Novablue (Novagen, Madison) and β2150 (Dehio and Meyer, 1997; Schulein and Dehio, 2002) were cultured following standard procedures (Sambrook et al., 1989), triparental matings were performed as described (Dehio and Meyer, 1997).

Plasmid Construction, Generation of In-frame Deletion Mutant. DNA manipulations were carried out following standard protocols (Sambrook et al., 1989). All parts of DNA constructs generated by the polymerase chain reaction (PCR) were confirmed by sequencing. Shuttle vector pPG100 (Schulein et al., 2005) and derivatives encoding full-length *bepA* (pPG101 = p*bepA*), *bepB* (pMS006 = p*bepB*), *bepC* (pMS007 = p*bepC*) and *bepD* (pPG104 = p*bepD*) have been described (Schulein et al., 2005; Schmid et al., 2006). Accordingly, the coding regions of full-length *bepE* (pPG104 = p*bepE*), *bepF* (pPG106 = p*bepF*) and *bepG* (pPG107 = p*bepG*) were amplified by PCR by primer pairs prPG100 (5'-GGAATTCCAT**ATG**AAAAGAAATC AACCACCC-3')/ prPG101 (5'-GGAATTCCATATGTTAGATGGCGAAAGCTATT GC-3'),prPG102 (5'-GGAATTCCAT**ATG**AAAAAAAACCAACCATCCT-3') / prPG103 (5'-GGAATTCCATATGTTAGAGTGCCAGCACCATTT-3') and prPG131 (5'-CGCGCTTATTAAT**ATG**AAAAAAAAACAACCAGCCC-3')/prPG105 (5'-CGC GCTTATTAATTTATCTACTCATAGAAACTACTT-3'), respectively, and cloned into pPG100 via *NdeI* or *AseI*. pTR1703 was obtained by PCR amplifying the C-terminal part of bepG (corresponding to amino acids 715-1009) by primer pairs prTR078 (5'-ACGCGTCGACTCTTCACTCAAGAAACGCAAAAAAT-3') and prTR076 (5'-CCCCCCGGGTTATCTACTCATAGAAACTACTTT-3'). The resulting 0.9 kb fragment was cleaved with *SalI*/X*maI* and ligated into the plasmid backbone of pRS51 cleaved accordingly. pTR1778 (eGFP-BepG) was obtained by PCR amplification of full-length *bepG* by primer pair prTR079 (5'-TCCCCCCGGGATGAA AAAAAAACAACCAGCCC-3') and prTR076, cutting the resulting 3.05 kb fragment with X*maI* and by its ligation into pWAY21 (eGFP, Molecular Motion, Montana Labs) cut accordingly. pTR1078 was constructed to generate a non-polar in-frame deletion mutant of *bepG*. Upstream (-625 bp of ATG) and downstream (+518 bp of TAA) regions of *bepG* were amplified by primer pairs prTR057 (5'-GCTCTAGAGCTCCGC TTGCTGGCAAG-3') / prTR058 (5'-GGTCGAACTATCTGTTCTACAGGTGTTGA AGGGGCTGG-3') and prTR059 (5'-GTAGAACAGATAGTTCGACC-3')/ prTR060 (5'-GTAGAACAGATAGTTCGACC-3'), giving rise to two fragments (686 kb and 569 bp), which were fused by megaprime PCR, cut by *XbaI* and ligated into pTR1000 which was cut accordingly.

The resulting suicide plasmid pTR1078 gave rise to an internal deletion of 2934 bp in *bepG*. This plasmid (3030bp) was mobilized into RSE 247 by conjugation. Loop-in and loop-out were selected as described and successful generation of the resulting isogenic *ΔbepG* mutant strain TRB223 was confirmed by overspanning PCR.

Cell Lines and Cell Culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultivated as described (Dehio et al., 1997). Ea.hy296 cells and the stably transfected cell line Ea.hy296/pRS56-clone B1 were cultivated in DMEM/10% FCS as described (Schulein et al., 2005). HeLa cells were cultivated in DMEM/10% FCS.

Infection and Transfection Assays. The day before infection, HUVEC (passage 3-9) were seeded onto gelatine-coated cover-slips into 24-well plates. Fresh medium was supplemented 4-6 hours post seeding. Approximately 30’000 cells were infected at a multiplicity of infection (MOI) = 100 in medium M199/10%FCS supplemented with 500 µM IPTG (Promega) and incubated for 48 hours. Cytochalasin D (Sigma) was dissolved in DMSO (Fluka) and added to the final concentrations indicated. Ea.hy926 and HeLa cells were seeded onto cover-slips into 24-well plates at cell densities of 50’000 cells per well. For transfections with Effectene (Qiagen), 200 ng of endotoxin-free high purity DNA (Endotoxin Free Plasmid Maxi Prep, QIAgen or Macherey Nagel) were used according to the protocol of the manufacturer at a ratio of 1:3 for reporter vs. tester plasmid. pRK5 encoding myc-tagged versions of Rho-family small GTPases RhoA, Rac1 and Cdc42 (Olson et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992)as well as pRK5 encoding myc-tagged Scar1 derivatives have been described (Machesky and Insall, 1998). 6 hours post transfection, cells were washed once with phosphate-buffered saline (PBS) and fresh medium M199/10% FCS supplemented with 500 µM IPTG was added. Bacteria were then added at a MOI=100.

To test functionality of dominant negative version of Rac1, Ea.hy926 cells were washed 30 hours post transfection once with PBS and supplemented with fresh serum-free DMEM including D-erythro sphingosine-1-phosphate (Calbiochem, 1 μM) and stimulated for 2 minutes as described (Andor et al., 2001). At the time points indicated, cells were washed once with PBS and fixed in 3.7 % paraformaldehyde (Sigma).

Immunofluorescent (IF) labelling. Indirect IF labelling was performed as described (Dehio et al., 1997). For staining of F-actin, tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma, 100 μg/ml stock solution, final concentration 1:400) was used. Primary antibodies for IF used in this study are (1) serum 2037 (polyclonal rabbit anti-*B. henselae* total bacteria, 1:100), (2) anti-Arp3 (monoclonal mouse clone 4, BD Biosciences Pharmingen, 1:50) and (3) anti-LAMP-1 (monoclonal mouse clone H4A3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, US, 1:100). Secondary antibodies for IF used in this study are (1) Cy5-conjugated goat anti rabbit Ig antibodies and (2) Cy2-conjugated goat anti rabbit Ig antibodies (both Dianova, Hamburg, Germany, 1:100). For triple staining of bacteria, F-actin and a third probe the secondary antibody was goat anti-mouse IgG (H+L) Alexa Fluor 488 (Molecular Probes, 1:100).

Epi-fluorescence and Confocal Laser Scanning Microscopy. For quantification of invasome structures, specimens were examined with a Leica DM-IRBE inverted epi-fluorescence microscope at a magnification of 40x in immersion. Assessment of successive invasome stages was performed as described (Dehio et al., 1997). For confocal laser scanning microscopy, a Leica TCS SP was used.

Recordings were made in one focal plane at 40x or 63x magnification in immersion in the xyz or xzλ mode with image size of 512 x 512 pixels. Channels were assembled and adjusted using Metamorph and Adobe Photoshop, and pictures were arranged and labelled in Adobe CS Illustrator.

CRAfT (Cre Recombinase Reporter Assay For Translocation). CRAfT (Vergunst et al., 2000) was used to monitor translocation of NLS-Cre-BepG fusion proteins from *B. henselae* into the stably transfected reporter cell line Ea.hy296/pRS56 clone B1 as described (Schulein et al., 2005). Approximately 50'000 cells were infected with a MOI=150 for 5 days and the percentage of GFP-positive cells was measured with a FACSCalibur flow cytometer (Becton Dickinson).

Immunoblot analysis. Expression of N-terminal FLAG-tagged Bep fusion proteins was verified by analysis of total cell lysates obtained from Ea.hy926 cells infected with *B. henselae* for 48 hours. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybond, Amersham Biosciences), and examined for the presence of the FLAG epitope using mouse monoclonal anti-FLAG antibody M2 (Sigma, 1:1000). Steady-state levels of NLS-Cre-Bep fusion proteins were analyzed by separation of bacterial cell lysates by SDS-PAGE, transferred onto PVDF membranes (Hybond-P, Amersham Biosciences), and examined for the presence of the Cre fusion protein using polyclonal anti-Cre antibody (EMD Biosciences Inc., Novagen, 1:10000). In both experiments, the secondary horseradish peroxidase-conjugated antibody (HRP, Amersham, 1:2000) was visualized by enhanced chemiluminescence (PerkinElmer).

Results

BepG is the only *Bartonella* effector protein required to trigger invasome formation

Invasome-mediated uptake of *B. henselae* is dependent on the VirB/VirD4 T4SS and at least one of the seven translocated effector proteins BepA-BepG (Schmid et al., 2004; Schulein et al., 2005). Accordingly, the effector-deletion mutant *ΔbepA-G* does not trigger the formation invasomes (Schulein et al., 2005). In an attempt to determine the effector mediating invasome formation we expressed all seven effector proteins individually in this mutant background *in trans* and assayed for phenotypic complementation.

The strain expressing BepG (*ΔbepA-G*/p*bepG*) promoted actin cytoskeletal rearrangements characteristic for the invasome structure, whereas no other strain was found to elicit a similar actin remodelling (Figure 1A). However, the strain expressing BepF (*ΔbepA-G*/p*bepF*) fostered the formation of small F-actin foci at the cell cortex, but these structures were not reminiscent of invasomes (Figure 1B). Single bacteria or bacterial aggregates adhering to the cell surface were observed with all strains, but only the BepG-expressing strain (*ΔbepA-G*/p*bepG*) promoted the complete internalization of these aggregates in concert with rearrangement of the underlying F-actin (Figure 1B). Furthermore, irregardless of the genetic background tested, individual bacteria were found to be taken up by conventional phagocytosis, leading to a perinuclear localization in BCVs (Figure 1B).

Next, the steady state level of expression of all effector constructs used was analysed by western blot analysis. All Beps were apparently stably expressed and displayed an electrophoretic mobility in good agreement with the calculated molecular weight of the recombinant proteins (Figure 1C).

Invasomes triggered by the BepG-expressing strain are similar but not identical to invasomes formed by wild-type bacteria

Next, we set for a detailed analysis of invasome formation promoted by the action of BepG. Invasomes formed by *B. henselae* wild-type and the BepG-expressing strain *ΔbepA-G*/p*bepG* were found to be very similar in appearance (Figure 2A). In both cases, we observed a dramatic rearrangement of the cellular actin cytoskeleton in association with bacterial aggregates, giving rise to the described basal ring-like structure (Figure 2A). Then, we determined the frequency of invasome formation (Figure 2B). In agreement with previously published data (Dehio et al., 1997; Schmid et al., 2004), *B. henselae* wild-type induced at least one invasome in approximately 90 % of cells, while the effector-deletion mutant *ΔbepA-G* did not induce any invasomes. The BepG-expressing strain *ΔbepA*-G/p*bepG* restored the ability to induce at least one invasome in 60 % of cells.

As we had observed different morphologies of invasomes elicited by *B. henselae* wild-type, we defined parameters to quantify these morphological variation to be able to discriminate invasomes elicited by either *B. henselae* wild-type or any mutant strain. These parameters included appearance of the basal ring-like structure (spherical, ellipsoid or distorted) and organization of protruding tangential F-actin fibers (radial, polar or aberrant). We found that wild-type mostly provokes the formation of spherical (51%) and ellipsoid (24%) basal structures associated with predominately polar (46%) and radial (37%) F-actin fibers (Figure 2C). Basal structures elicited by the BepG-expressing strain *ΔbepA*-G/p*bepG* were found to be similar to the ones elicited by the wild-type strain, yet they were more frequently distorted (45% vs. 25%) and less often radial (22% vs. 37%). The organization of F-actin fibers associated to basal structures elicited by the BepG-expressing strain *ΔbepA*-G/p*bepG* was found to be similar, yet less radial fibres were detected (22% vs. 37%).

Next, we analyzed the different steps of invasome formation, namely aggregation, engulfment and internalization of bacterial aggregates as previously described (Dehio et al., 1997). All three stages of invasome formation were observed with almost equal frequencies for wild-type and the BepG-expressing strain *ΔbepA*-G/p*bepG* (Figure 2D).

Taken together, these findings indicate that the action of BepG reproduces cytoskeletal changes characteristic for invasome formation, as suggested by the comparison of invasomes elicted by *B. henselae* wild-type and the BepG-expressing strain *ΔbepA-G*/p*bepG*.

A Δ*bepG* deletion mutant is still capable of triggering invasome structures, indicating redundant ways of invasome formation by the Bep proteins of *B. henselae*

Next, we generated a non-polar in-frame deletion mutant in the *bepG* locus (*ΔbepG*) to assess its phenotypic behaviour and capability to induce invasome formation. To our surprise, the deletion mutant (*ΔbepG*) was still able to promote invasome formation (Figure 3A). As compared to wild-type, the frequency of invasome formation by the *ΔbepG* mutant was only slightly reduced (74% vs. 92%), suggesting only modest attenuation of this mutant (Figure 3B). Compared to wild-type (Figure 3C), basal structures elicited by the *ΔbepG* mutant were found to be less spherical (13% vs. 48%) and more distorted (53% vs. 21%). F-actin fibres were orientated more frequently in a polar fashion (62% vs. 48%) and less often in a radial fashion (22% vs. 39%). Finally, the different stages of invasome formation were found to coincide equally in both the wild-type strain and the deletion mutant (*ΔbepG*) (Figure 3D). The apparent differences of the *ΔbepG* mutant and wild-type regarding the morphological appearance and the frequency of invasomes were restored by expression of BepG *in trans* (*ΔbepG/pbepG*).

This finding leads to the conclusion that two independent pathways dependent on *Bartonella* effectors proteins (BepA-BepG) promote invasome-mediated internalization of *B. henselae*. One of these pathways is governed by the sole action of BepG as shown by the initial screen for invasome formation based on the expression of individual Bep proteins in the effector-deletion mutant background (Figure 1). It has thus to be postulated that at least two other Beps different from BepG govern the parallel pathway of invasome formation, which is evident in the *ΔbepG* deletion mutant.

Finally, we compared the frequency of invasome formation by *B. henselae* wild-type and the BepG-expressing strain *ΔbepA-G*/p*bepG* on HUVEC in the presence of the F-actin-depolymerizing drug cytochalasin D to assess the question to which extent the pathway governed by the action of BepG triggers cellular changes alone compared to the combined action both pathways. In both cases, the total number of invasomes detected regressed in a dose-dependent manner (Figure 4). Host cell viability and morphology appeared unaffected in the range of concentrations tested.

This finding confirmed that the action of BepG alone is sufficient to elicit cellular changes leading to invasome formation, but that the action of BepG alone does not compensate for the action of the parallel pathway, indicating that both pathways could act synergistically in promoting bacterial uptake.

BepG is a T4SS-Translocated Effector Protein

The C-terminus of all Bep effector proteins contain the signature of a bipartite signal for T4SS-dependent protein translocation into EC, while functionality of this signal was thus far demonstrated only for BepB, BepC, BepD and BepF by aid of an appropriate reporter assay, CRAfT (Cre-Recombinase Assay for Translocation) (Schulein et al., 2005). To test for functionality of the putative export signal of BepG, we expressed the C-terminal part (corresponding to aa 715-1009, including the fourth BID-domain, corresponding to aa 723-864) as a fusion to an NLS-Cre reporter protein (pTR1703) in the background of wild-type (functional T4SS) and the *ΔvirB4* mutant (non-functional T4SS).

Following infection of the Cre-reporter cell line Ea.hy926/pRS56-clone B1, the translocation efficiency of the reporter fusion was analyzed by flow cytometic determination of the percentage of GFP-positive cells. As shown in the supplementary Figure 3A, this assay demonstrated T4SS-dependent protein translocation, demonstrating that BepG is a *bona fide* T4SS-translocated effector protein of *B. henselae*. The export signal of *B. henselae* BepD previously shown to mediate translocation of an NLS-Cre reporter fusion (pRS51) served as positive control (Schulein et al., 2005). Immunoblot analysis indicated that the NLS-Cre fusion proteins were stable (Supplementary Figure 2B).

BepG Co-Localizes to Components of the Cellular Actin Cytoskeleton

To analyze the subcellular localization of the effector protein BepG within human cells, we transfected HeLa cells with a eukaryotic expression plasmid encoding an eGFP-BepG fusion protein. Co-staining of F-actin by TRITC-phalloidine allowed us to demonstrate colocalization of eGFP-BepG with distinct components of the actin cytoskeleton. Co-localization was observed both with stress fibres (Supplementary Figure 3B, captation), as well as cortical actin in filopodial cell extensions in the cell periphery (Supplementary Figure 3B) or the cortex underneath the apical plasma membrane (Supplementary Figure 3E). eGFP alone served as control and localized uniformly to the cytoplasm of transfected cells (supplementary Figure 3A, 3D). Non-transfected cells served as negative control (supplementary Figure 3C, 3F). This finding is interesting in regard of the function of BepG in triggering the formation of the actin rearrangements culminating in invasome formation.

Invasome Formation is Dependent on the Small GTPases Rac1 and Cdc42, but not on RhoA

RhoA, Rac1, and Cdc42 are key regulators of the actin cytoskeleton, triggering the formation of stress fibers anchored by focal adhesions, lamellipodia at the leading edge of cells along with membrane ruffles, and filopodial cell extensions, respectively (Hall and Nobes, 2000).

These Rho-family small GTPases have also been shown to be involved in the cellular invasion process of several bacterial pathogens (Gruenheid and Finlay, 2003; Pizarro-Cerda and Cossart, 2006a). To assess their putative involvement in invasome formation, we transfected Ea.hy926 cells with eGFP (as a marker for transfection in individual cells) together with dominant negative versions of RhoA (N19), Rac1 (N17) and Cdc42 (N17). Cells were then infected with either *B. henselae* wild-type strain, the effector-deletion mutant *ΔbepA-G*, or its BepG-expressing derivative *ΔbepA-G*/p*bepG* for 48 hours. Finally, the frequency of invasome formation was determined in GFP-positive cells (Figure 5A). Invasome formation decreased modestly (-25 %) in EC transfected with dominant negative Cdc42, and strongly (-50 to -60 %) in EC transfected with dominant negative Rac1. No change in frequency could be observed in EC transfected with dominant negative RhoA as compared to empty vector or non-transfected EC (Figure 5B).

Sphingosine-1-phosphate is a potent activator of Rac1 and induces formation of lamellipodia and membrane ruffles (Vouret-Craviari et al., 2002). In a control experiment, sphingosine-1-phosphate was used to test for suppression of these structures in Ea.hy926 cells co-transfected with the dominant negative version of Rac1 and eGFP as a reporter (Figure 5B). The formation of membrane ruffles and lamellipodia was indeed strongly reduced in GFP-positive cells, while these structures were formed with normal frequencies in eGFP-negative cells. Thus, in the EC system used in this study, Rac1 activity is effectively inhibited by overexpression of the dominant-negative form (N17) Rac1.

We next tested whether ectopic expression of a constitutive form of Rac1, (L61) Rac1, affects invasome formation. Upon cotransfection of Ea.hy926 cells with eGFP (as transfection marker) and (L61) Rac1, the constitutively active Rac1 elicited the formation of lamellipodial structures (Figure 6A, 6C) and of subcortical membrane ruffles (Figure 6B, 6D). After infection of the transfected Ea.hy926 cells with *B. henselae* wild-type strain, the effector-deletion mutant *ΔbepA-G* and its BepG-expressing derivative *ΔbepA-G*/p*bepG*, the frequencies of invasome formation were determined in GFP-positive cells (Figure 6E).

The empty vector pRK5myc, the wild-type version of Rac1, and non-transfected cells served as controls. Again, we found that Rac1 plays an important role in invasome formation. Expression of a constitutively active version of Rac1 in EC reduced the rate of invasome formation dramatically (-70 % to -80 %). No change in frequency was observed in EC transfected with empty vector or wild-type Rac1.

Invasome Formation involves Scar/WAVE-dependent Activation of the Arp2/3 Complex

Scar/WAVE adaptor proteins are involved in the formation of lamellipodia and membrane ruffles downstream of Rac1 (Eden et al., 2002; Steffen et al., 2004). Scar1 is able to bind and activate the Arp2/3 complex (Machesky and Insall, 1998), which in turn nucleates actin at barbed ends and branching filaments (Machesky and Gould, 1999). To test for the involvement of Scar/WAVE adaptor proteins in invasome formation, we over-expressed full-length Scar1 (Scar-FL) or a truncated version still binding the Arp2/3 complex (Scar-WA) (Martinez and Cossart, 2004). After transfection of Ea.hy926 cells for 48 h with eGFP (as transfection marker) together with Scar-FL (Figure 7A) or Scar-WA (Figure 7B), phalloidine staining demonstrated the deleterious effect of these constructs on the integrity of the actin cytoskeleton. Compared to untransfected cells, only few actin stress fibres were visible.

Infection of Ea.hy926 cells for 48 hours with *B. henselae* wild-type, the effector-deletion mutant strain *ΔbepA-G*, and the isogenic BepG-expressing derivative *ΔbepA-G*/p*bepG* revealed that invasome formation was impaired severely by expression of either Scar-FL or Scar-WA. The frequency of invasome formation dropped in average by 25% (Scar-FL) or 35% (Scar-WA) as compared to empty vector control and non-transfected Ea.hy926 cells (Figure 7C), indicating that Rac1-mediated activation of Scar1 is involved in the process of invasome formation.

Next, we tested whether the Arp2/3 complex is recruited to the sites of F-actin polymerization in the invasome structures. To this end, we stained Ea.hy926 cells infected for 48 h with *B. henselae* for Arp3 by IF. Indeed Arp3 was found to be enriched in the cortical actin of membrane protrusion engulfing the bacterial aggregates within invasomes (Figure 7D), suggesting that *de novo* F-actin polymerization occurs in these membrane protrusions of the invasome structure.

Intracellular Fate of *B. henselae* internalized via the Invasome Structure

Finally, we wanted to study the intracellular fate of bacteria entering EC as aggregates via the invasome structure vs. bacteria entering EC individually by conventional phagocytosis. Laser scanning microscopy revealed that 48 hours post infection, most of single bacteria residing in perinuclearly located BCVs had acquired LAMP-1, a marker protein for phago-lysosomal fusion (Figure 8A, B) (Stuart and Ezekowitz, 2005). Thus, individual bacteria internalized by conventional phagocytosis are not able to interfere with the entry of their phagosome into the endocytic network, resulting in the sequential acquisition of lysosomal proteins. In sharp contrast, the membrane of the invasome structure embedding aggregates of wild-type bacteria did not acquire LAMP-1. This result implies, that establishment of invasomes may serve as an entry mechanism protecting the bacteria from interactions with the endocytic network, thus providing an intracellular escape route from phago-lysosomal fusion.

Discussion

Bacterial pathogens have developed numerous strategies to corrupt, hijack, or mimic cellular processes involved in the modulation of the host cell actin cytoskeleton (Gruenheid and Finlay, 2003; Rottner et al., 2005). In particular, intracellular pathogens subvert host cell cytoskeletal functions to trigger their internalization into non-professional phagocytes, such as epithelial cells and EC (Pizarro-Cerda and Cossart, 2006b). Here, we investigated the molecular and cellular basis of EC invasion by *B. henselae* via a unique structure, the invasome. This invasion process is characterized by aggregation of bacteria on the cell surface, followed by engulfment of the bacterial aggregate by membrane protrusion, eventually resulting in complete internalization. This dynamic invasion process is actin-dependent and associated with massive rearrangements of the actin cytoskeleton (Dehio et al., 1997). In contrast to a parallel entry pathway for individual bacteria via classical phagocytosis, invasome-mediated uptake was previously shown to depend on a functional T4SS, the VirB/VirD4 system (Schmid et al., 2004), and the presence of a genomic region encoding seven VirB/VirD4-translocated *Bartonella* effector proteins (BepA-BepG) (Schulein et al., 2005). Here we demonstrate that these Bep effectors trigger at least two independent pathways of invasome formation. First, the expression of individual effectors in the background of the effector-deletion mutant *ΔbepA-G* identified BepG as the only Bep capable of triggering invasome-like structures in the absence of the other six effectors (Figure 1). BepG thus plays a pivotal role in invasome formation. However, a deletion mutant in *bepG* (Δ*bepG*) was still able to elicit invasome structures, signifying a pathway of BepG-independent invasome formation (Figure 2). The latter pathway should depend on any of the effectors BepA-BepF, however, given that non of these effectors had the capacity of trigger invasomes when expressed individually in the background of the effector-deletion mutant *ΔbepA-G*, this alternative pathway must depend on at least two different Bep effectors. Both the BepG-dependent and BepG-independent processes of invasome formation resemble the morphology of invasome structures triggered by wild-type bacteria. However, a multi-parameter morphometric

analysis (Figure 2B-C and 3B-D) revealed some subtle qualitative and quantitative differences, which may indicate that the parallel pathways of BepG-dependent and BepG-independent invasome formation are not only redundant but also to some extent synergistic.

Rho-family small GTPases control actin organization in diverse biological systems by integrating various cellular cues to downstream actin-dependent signalling processes (Jaffe and Hall, 2005). Bacterial virulence factors involved in mediating cellular invasion commonly target the Rho-family of small GTPases (Aktories and Barbieri, 2005). We show here that the process of invasome formation requires the small GTPase Rac1. Expression of dominant negative (Figure 5) or constitutively active (Figure 6) forms of Rac1 in EC dramatically reduced the efficiency of invasome formation by *B. henselae*. Interestingly, EC transfected with wild-type Rac1 were not affected, indicating that over-expression *per se* is not the cause for decreased rates in invasome formation, but rather specific modulation of its activity, i.e. governed by the action of GTPase exchange factors (GEFs) and GTPase activation proteins (GAPs). In addition to Rac1, Cdc42 was identified in this study to contribute to invasome formation, even to a lesser extent than Rac1. Whether this result mostly reflects the functional crosstalk of these small GTPases with Cdc42 acting upstream of Rac1, or whether other downstream effectors of Cdc42 play a specific role in invasome formation remains to be demonstrated. In contrast to Rac1 and Cdc42, RhoA was found to be dispensable for invasome formation. BepG-dependent invasome formation displayed a similar dependency on these small GTPases than invasome formation by wild-type, indicating that the redundant BepG-dependent and BepG-independent pathways of invasome formation may converge in targeting the same cellular signalling components. In summary, invasome formation by *B. henselae* was shown to depend on Rac1 and to a lesser extend on Cdc42, while RhoA does not seem to play any role in this process. Moreover, modulation of Rac1 activity in time and space appears to be a critical factor for the process of invasome formation.

In addition to the rearrangement of pre-existing actin structures during invasome formation, i.e. by winding up stress fibres around the basal invasome structure resulting in a characteristic ring-like structures, the dense cortical actin network within the

membrane protrusions engulfing the bacterial aggregate on top of this ring-like structure are suggestive for *de novo* synthesis of cortical F-actin (Dehio et al., 1997). Downstream of Rac1, the IRSp53/Scar1/WAVE pathway mediates the polymerization of cortical F-actin via the Arp2/3 complex (Machesky and Insall, 1998), which nucleates actin at barbed ends and branches filaments during the polymerization process (Machesky and Gould, 1999). We thus tested for a role of Scar1/WAVE and the Arp2/3 complex in mediating invasome formation. Using immunocytochemistry for Arp3, we were able to show that the Arp2/3 complex was indeed specifically enriched in the cortical actin at of invasome structures, while the rearranged actin fibres of the basal ring-like structure were not found to be enriched for the Arp2/3 complex (Figure 7D). Moreover, the ectopic expression of dominant-negative constructs of Scar1 interfered with the process of invasome formation, supporting the notion that invasome formation depends on the Rac1/IRSp53/Scar1/WAVE pathway of triggering Arp2/3-dependent cortical actin polymerization. However, it cannot be excluded that actin polymerization in the invasome structure involves as well the Cdc42/N-WASp pathway (Alrutz et al., 2001), which has also been shown to activate the Arp2/3 complex resulting in the subsequent reorganization of the cortical actin cytoskeleton (Rohatgi et al., 1999). The finding that an ectopically expressed eGFP-BepG fusion protein specifically localizes to F-actin structures, both to actin fibres and the cortical actin network (supplementary Figure 3B, E) suggests that this potent effector of invasome formation may recruit host cell signalling molecules to the sites of actin rearrangement. This attractive hypothesis should receive further attention in future experiments to elucidate the molecular mechanism of BepG-dependent invasome formation.

Finally, we provide evidence that the intracellular fate of *B. henselae* entering EC by the invasome route is different from bacteria entering via the parallel pathway of conventional phagocytosis. The mostly individual bacteria entering via phagocytosis were found to localize to perinuclear BCVs, which were enriched for the lysosomal marker Lamp-1, evidencing an intracelluar compartment that is fusogenic with the phago-lysosomal pathway (May and Machesky, 2001). In contrast, the phagosmal membranes encompassing the bacterial aggregate entering EC via the invasome did not acquire any Lamp-1, indicating a non-fusogenic state of the invasomal membranes. Thus, the sustained actin cytoskeletal reorganization during invasome formation might represent a mechanism to bypass phagocytotic uptake and entry into the phago-lysosomal pathoway, therefore establishing an intracellular niche permissive for growth, or egression and dissemination of the bacterium at later stages of infection. Further studies are necessary to clarify to which extent *B. henselae* can replicate in EC *in vitro*. This would also result in a better understanding of the pathophysiological importance of invasome formation and to provide insights, how *B. henselae* is able to persistently infect and colonize the vasculature *in vivo*.

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Figure Legends

Figure 1. Expression of BepG in an effector-deletion mutant background of *B. henselae* is sufficient to trigger invasome-mediated uptake by EC. (A) HUVEC were infected for 48 hours with an MOI=100 of *B. henselae* wild-type, the effector-deletion mutant strain (*ΔbepA-G*), or isogenic strains expressing individual Bep proteins (*ΔbepA-G*/p*bepA, bepB, bepC, bepD, bepE, bepF,* or *bepG*), followed by fixation, immunocytochemical staining and confocal laser scanning microscopy. Extracellular bacteria are represented in purple, intracellular bacteria in red and the F-actin cytoskeleton in green. The scale bar corresponds to 20 μm (B) Synopsis on phenotypic observations during the internalization of *B. henselae* strains by HUVEC. **(C)** Western blot analysis of the steady-state protein levels of Flag-tagged Beps.

Figure 2. Invasomes triggered by *B. henselae* wild-type and a strain expressing BepG in an effector-deletion mutant background display similar actin-dependent cytoskeletal rearrangements. (A) HUVEC were infected for 48 hours with an MOI=100 of *B. henselae* wild-type, the effector-deletion mutant strain (*ΔbepA-G*) or the BepG-expressing derivative (*ΔbepA-G*/p*bepG*), followed by fixation, immunocytochemical staining and confocal laser scanning microscopy. Extracellular bacteria are represented in purple, intracellular bacteria in red and the F-actin cytoskeleton in green. (A) Representative images of invasome formation acquired in xy-plains at bottom and top levels and in an xz-plain on the single cell level. (B) Determination of frequencies of invasome formation (n=50). (C) Morphometric analysis of basal ring-like structures and protruding F-actin fibers characteristic for the invasome structure (n=100). (D) Analysis of different stages of invasome formation (n=100). Results of three independent experiments +/- standard deviation are depicted.

Figure 3. Invasome formation comprises two parallel pathways, one of which is governed by BepG. HUVEC were infected with *B. henselae* wild-type, a mutant carrying a non-polar inframe deletion in *bepG* (*ΔbepG*), and the corresponding strain complemented with *bepG in trans* (*ΔbepG*/p*bepG*) for 48 hours (MOI=100), followed by fixation, immunocytochemical staining and confocal laser scanning microscopy. Extracellular bacteria are represented in purple, intracellular bacteria in red and the F-actin cytoskeleton in green. (A) Representative images of invasome formation acquired in xy-plains at bottom and top levels and in an xz-plain on the single cell level. The scale bar corresponds to 20 μm. (B) Determination of frequencies of invasome formation (n=50). (C) Morphometric analysis of basal ring-like structures and protruding F-actin fibers characteristic for the invasome structure (n=100). (D) Analysis of different stages of invasome formation (n=100). Results of three independent experiments +/- standard deviation are depicted.

Figure 4. Invasome formed by wild-type and the BepG-expressing strain display a similar sensitive to cytochalasin D. HUVEC were infected for 48 hours with an MOI=100 of *B. henselae* wild-type, the effector-deletion mutant strain (*ΔbepA-G*), or the BepG-expressing derivative (*ΔbepA-G*/p*bepG*) in the presence of different concentration of cytochalasin D. Cells were fixed, stained and analyzed for the presence of invasome structures by epifluorescence microscopy. Results of three independent experiments +/- SD are depicted (n=100).

Figure 5. Invasome formation is dependent on Rac1 and Cdc42. Ea.hy926 cells were co-transfected with a plasmid encoding eGFP (pWay21) and a second plasmid encoding either dominant-negative RhoA [(N19) Rho], dominant-negative Rac1 [(N17) Rac1], dominant-negative CDC42 [(N17) CDC42] or with the empty vector pRK5myc (negative control). **(A)** After 6 h, cells were infected with *B. henselae* wild-type, the effector-deletion mutant strain *ΔbepA-G* and its BepG-expressing derivative *ΔbepA-G*/p*bepG* (MOI=100) for 48 h. The frequency of invasome formation was determined as described in Fig. 2B, except that in transfected samples only cells expressing visible amounts of eGFP were considered. Results of four independent experiments +/- standard deviation are depicted (n=50). **(B)** Rac1 was activated by 1 μM sphingosine-1-phosphate, and subsequent to fixation and staining for F-actin the inhibition of membrane ruffling by dominant-negative Rac1 [(N17) Rac1] was demonstrated by laser scanning microscopy in the xy-plain (left) or xz-plain (right). The scale bar corresponds to 10 μm.

Figure 6. Invasome formation is inhibited by constitutively active Rac1. Ea.hy926 cells were co-transfected with a plasmid encoding eGFP (pWay21) and a second plasmid encoding either Rac1 (wild-type), a constitutively active version of Rac1 [(L61) Rac1], or with the empty vector pRK5myc (negative control). **(A-D)** After 48 h, cells were fixed, stained for F-actin with TRITC-phalloidine and analysed by confocal laser scanning microscopy in the (A, C) xy-plain or (B, D) xz-plain plane. The scale bar corresponds to 10 μm. **(E)** After 6 h, cells were infected with *B. henselae* wild-type, the effector-deletion mutant strain (*ΔbepA-G*) or its derivative expressing BepG (*ΔbepA-G*/p*bepG*) for 48 h (MOI=100). The frequency of invasome formation was then determined as described in Fig. 5A. Results of four independent experiments +/- SD are depicted (n=50).

Figure 7. Cytoskeletal remodelling during invasome formation depends on Scar and involves the Arp2/3 complex. (A-C) Ea.hy926 cells were co-transfected with a plasmid encoding eGFP (pWay21) and a second plasmid encoding either (A, C) full-length Scar (Scar-FL), (B, C) a truncated form encoding only the actin-binding domain, or (C) the empty vector pRK5. After 48 h, cells were fixed, stained for F-actin with TRITC-phalloidine and (A, B) analysed by confocal laser scanning microscopy. The scale bar corresponds to 10 μm. **(C)** After 6 h, cells were infected with *B. henselae* wild-type, the effector-deletion mutant strain (*ΔbepA-G*) or its derivative expressing BepG (*ΔbepA-G*/p*bepG*) for 48 h (MOI=100). The frequency of invasome formation was then determined as described in Fig. 5A. Results of four independent experiments +/- SD are depicted (n=50). **(D)** HUVEC infected with *B. henselae* wild-type (MOI=100) for 48 h were fixed, stained for F-actin and Arp3 and analysed by confocal laser scanning microscopy. The scale bar corresponds to 10 μm.

Figure 8. Bacteria entering EC by phagocytosis or invasome-mediated uptake have different intracellular fates. HUVEC were infected with *B. henselae* wild-type (MOI=100) for 48 h, followed by fixation and immunocytochemical staining for F-actin (represented in green), bacteria (represented in red) and the lysosomal marker protein LAMP-1 (represented in blue), followed by laser scanning microscopy. The scale bar corresponds to 5 μm.

Supplemental Figure 1. Modular domain organization of BepG. BepG harbours four BID-domains (BID-G1 to G4) and two sets of repeated domains of unknown function (DUF1a, 1b, 1c, and DUF2a and DUF2b).

Supplemental Figure 2. The C-terminal part of BepG mediates VirB/VirD4-dependent translocation into EC. **(A)** For the Cre-Recombinae Assay for Translocation (CRAfT), the Cre-reporter cell line Ea.hy296/pRS56-clone B1 was infected for 5 days with an MOI=100 of *B. henselae* strains harbouring plasmid pRS51 [encoding NLS-Cre-BepD(aa 352-534)] in the wild-type background, and plasmid pTR1703 [encoding NLS-Cre-BepG(aa 716-1009)] in the wild-type or Δ*virB4* mutant background. Then, the percentage of GFP-positive cells was determined by flow cytometric analysis. **(B)** Steady state levels of expression of NLS-Cre reporter fusion proteins in plate grown bacteria of the different strains used for CRAfT. Total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-FLAG antibodies.

Supplemental Figure 3. Ectopic expression of an eGFP-BepG fusion in EC results in co-localization of the fusion protein with components of the actin cytoskeleton. HeLa cells were not transfected (negative control), or transfected with plasmids encoding eGFP alone (positive control) or eGFP fused C-terminally to full-length BepG (eGFP-BepG) for 48 hours and analyzed by confocal laser scanning microscopy in the **(A-C)** xy-plain or **(D-F)** xz-plain. Colocalization of the eGFP-BepG fusion protein is shown for **(B)** stress fibres or **(E)** cortical F-actin. The scale bar corresponds to 10 μm.

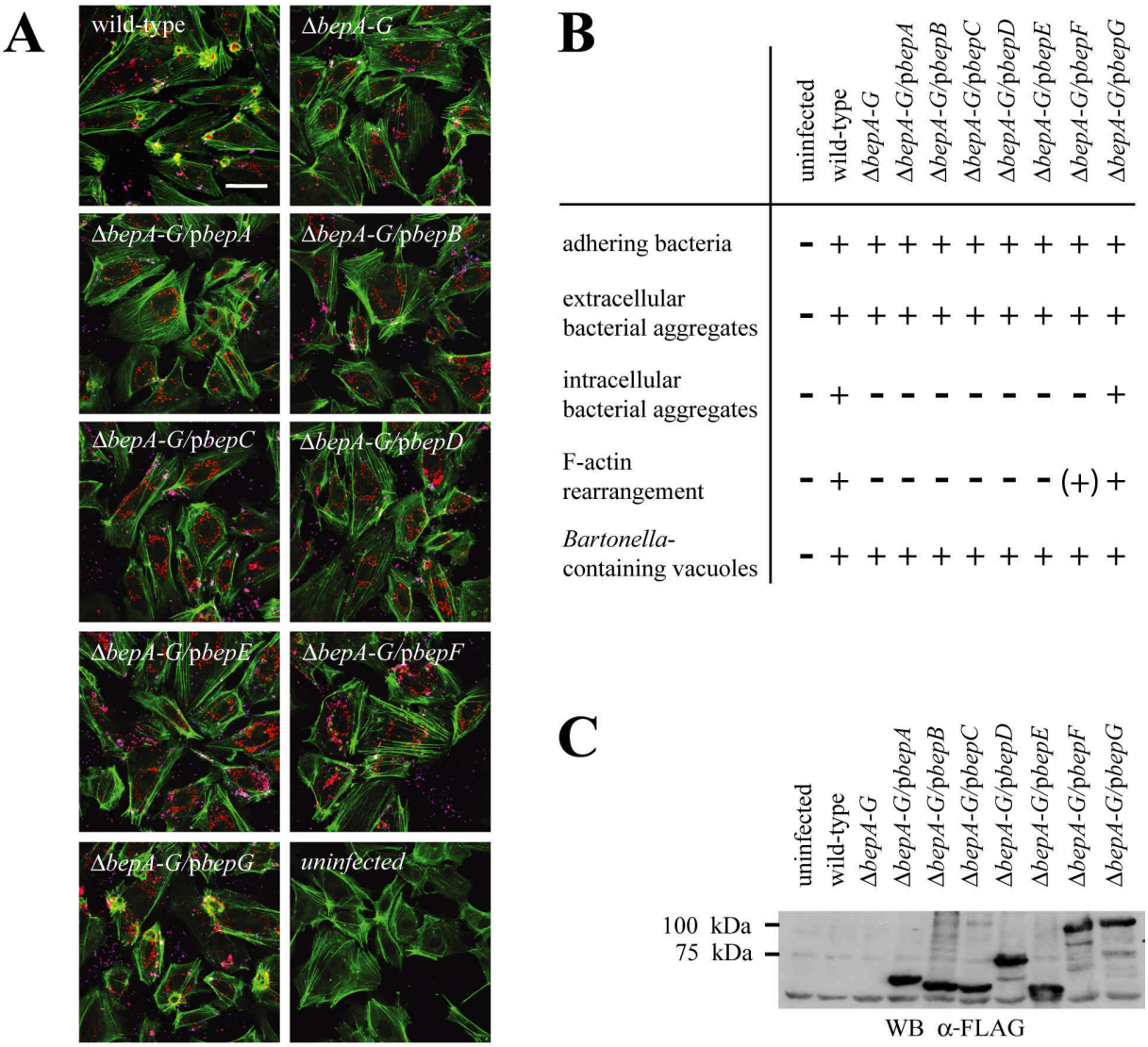


Figure 1
(Rhomberg *et al.*)

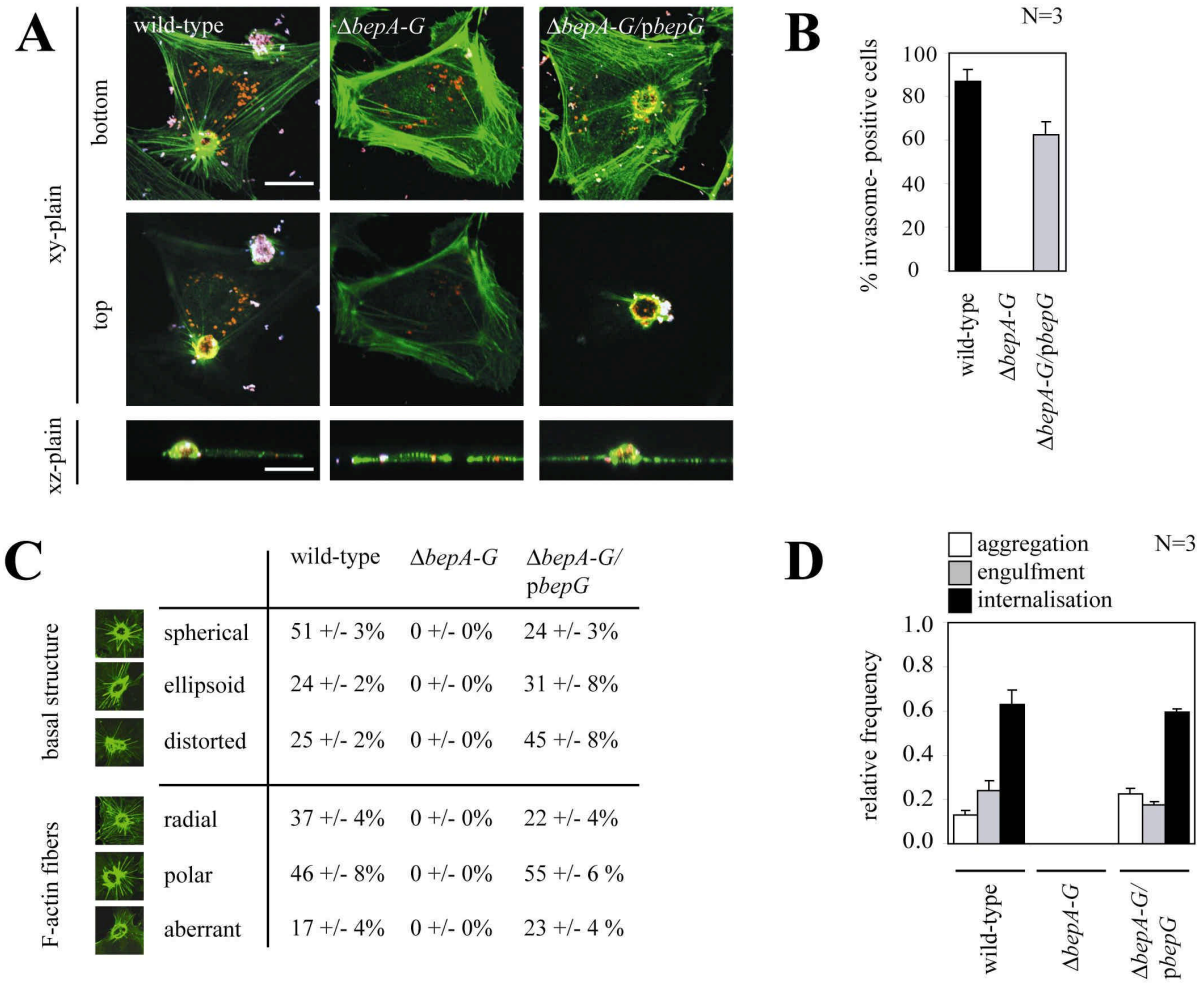
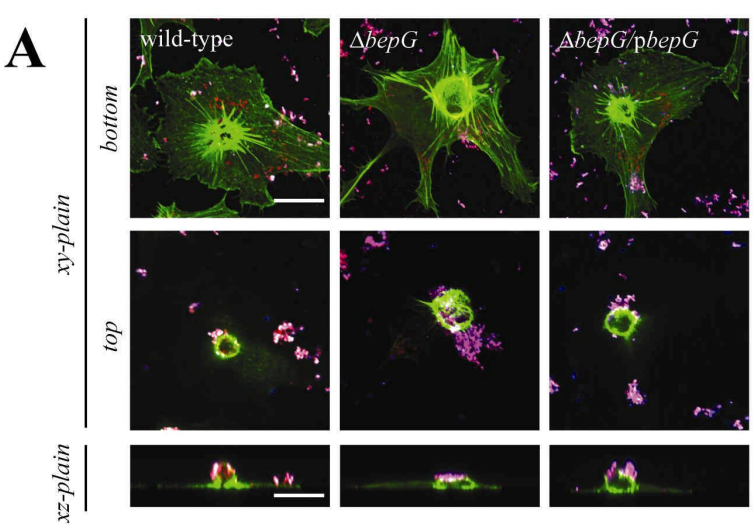
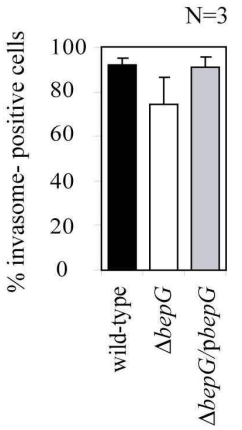


Figure 2
(Rhomberg *et al.*)





C

		wild-type	ΔbepG	ΔbepG/ pbepG
basal structure	spherical	48 +/- 8%	13 +/- 2%	46 +/- 8%
	ellipsoid	31 +/- 10%	34 +/- 7%	26 +/- 1%
	distorted	21 +/- 2%	53 +/- 7%	28 +/- 9%
F-actin fibers	radial	39 +/- 8 %	22 +/- 4%	42 +/- 5 %
	polar	48+/- 5%	62 +/- 6%	46+/- 7%
	aberrant	13 +/- 6%	16 +/- 4%	12 +/- 3%

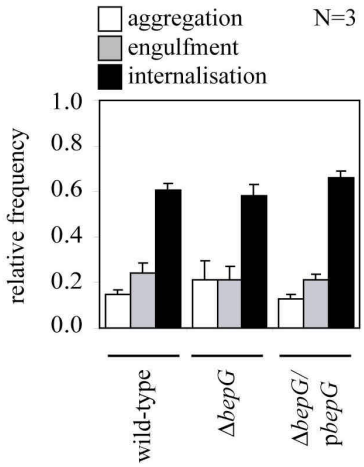


Figure 3
(Rhomberg *et al.*)

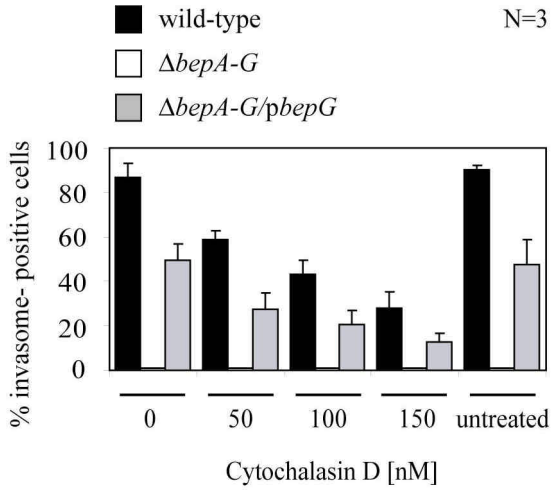


Figure 4
(Rhomberg *et al.*)

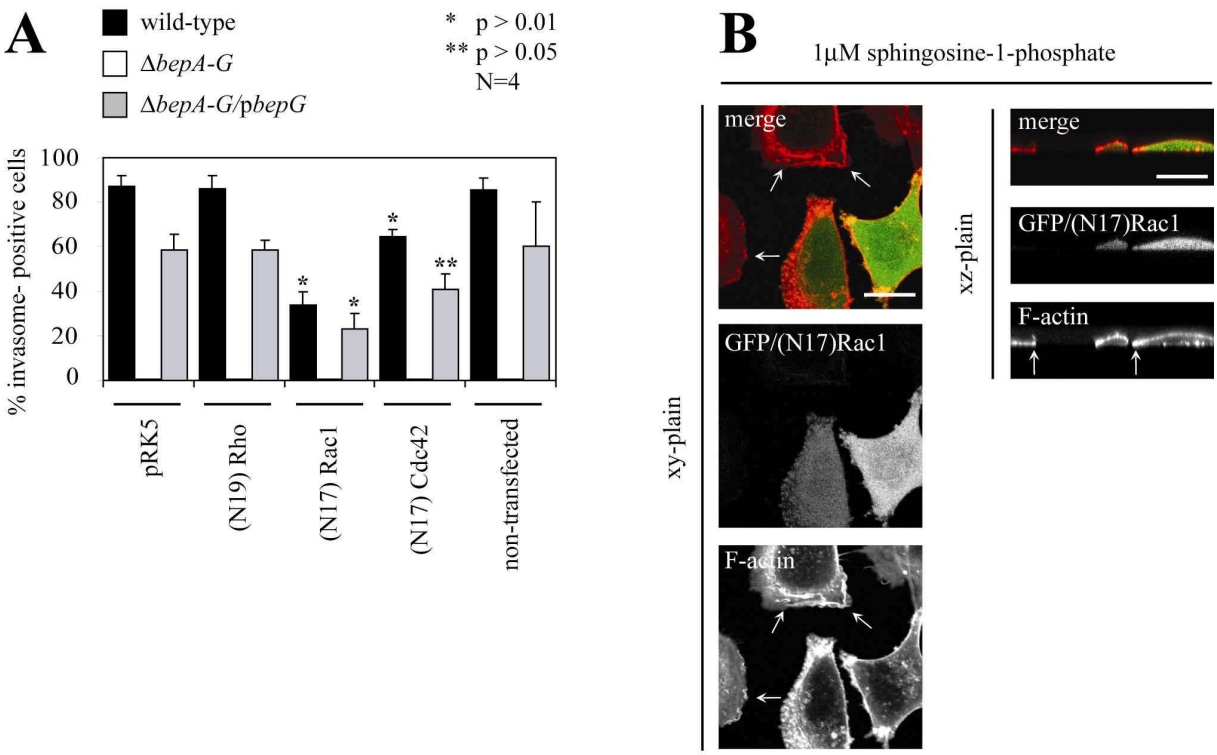


Figure 5
(Rhomberg *et al.*)

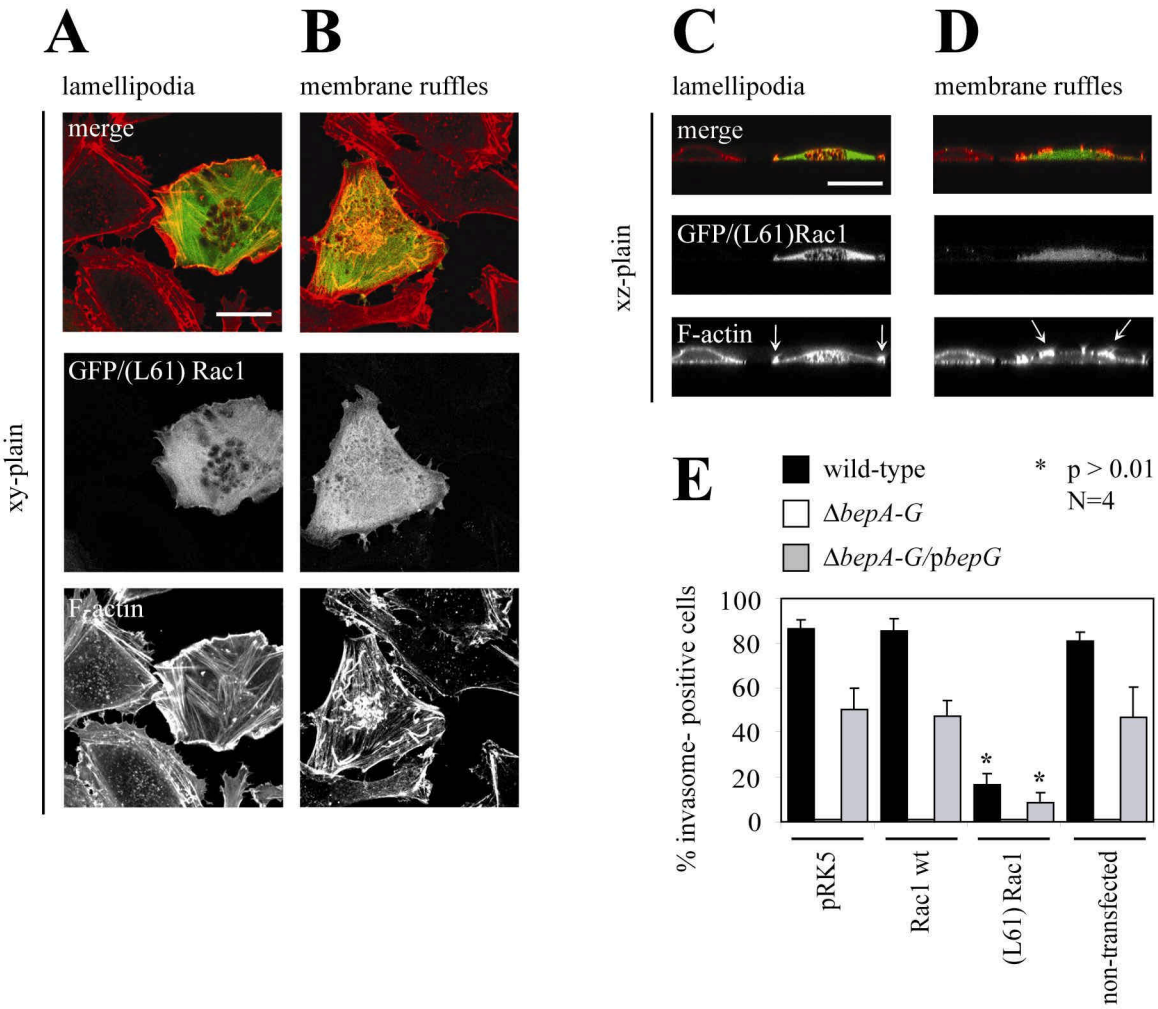


Figure 6
(Rhomberg *et al.*)

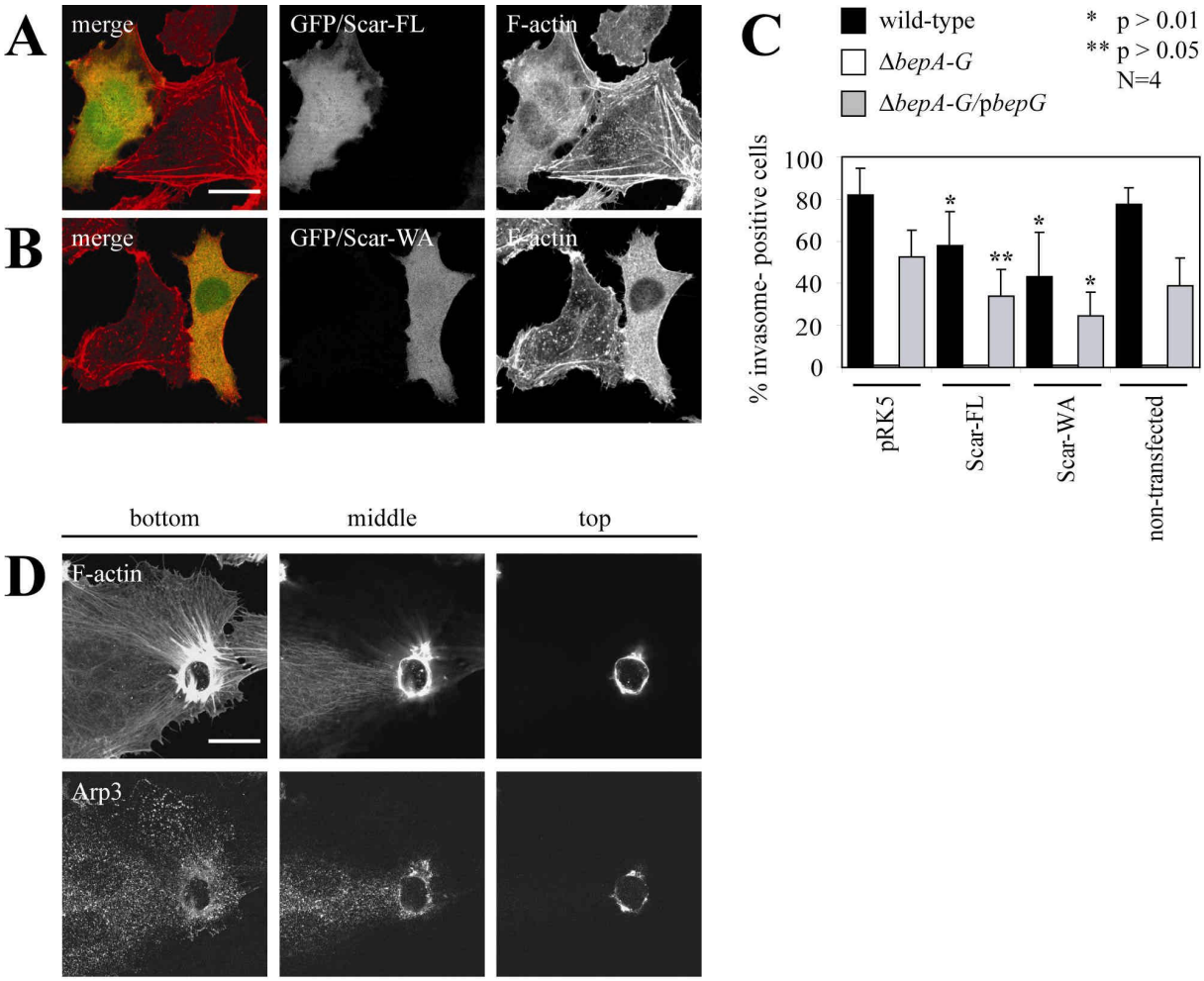


Figure 7
(Rhomberg *et al.*)

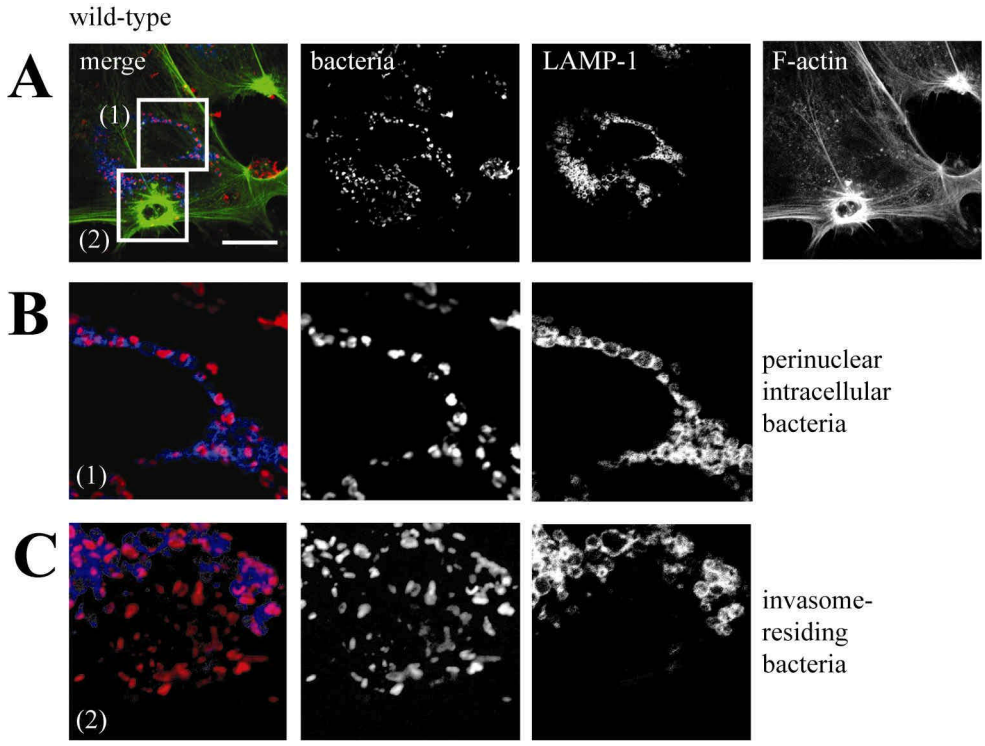


Figure 8
(Rhomberg *et al.*)

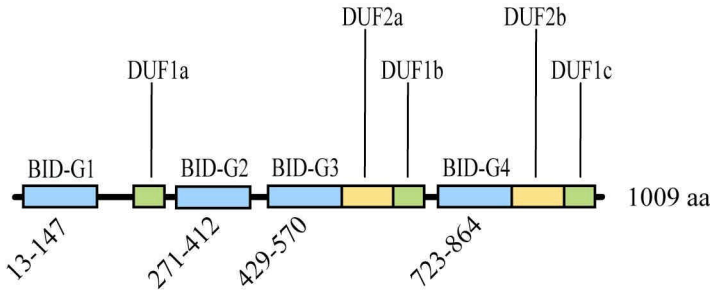


Figure S1
(Rhomberg *et al.*)

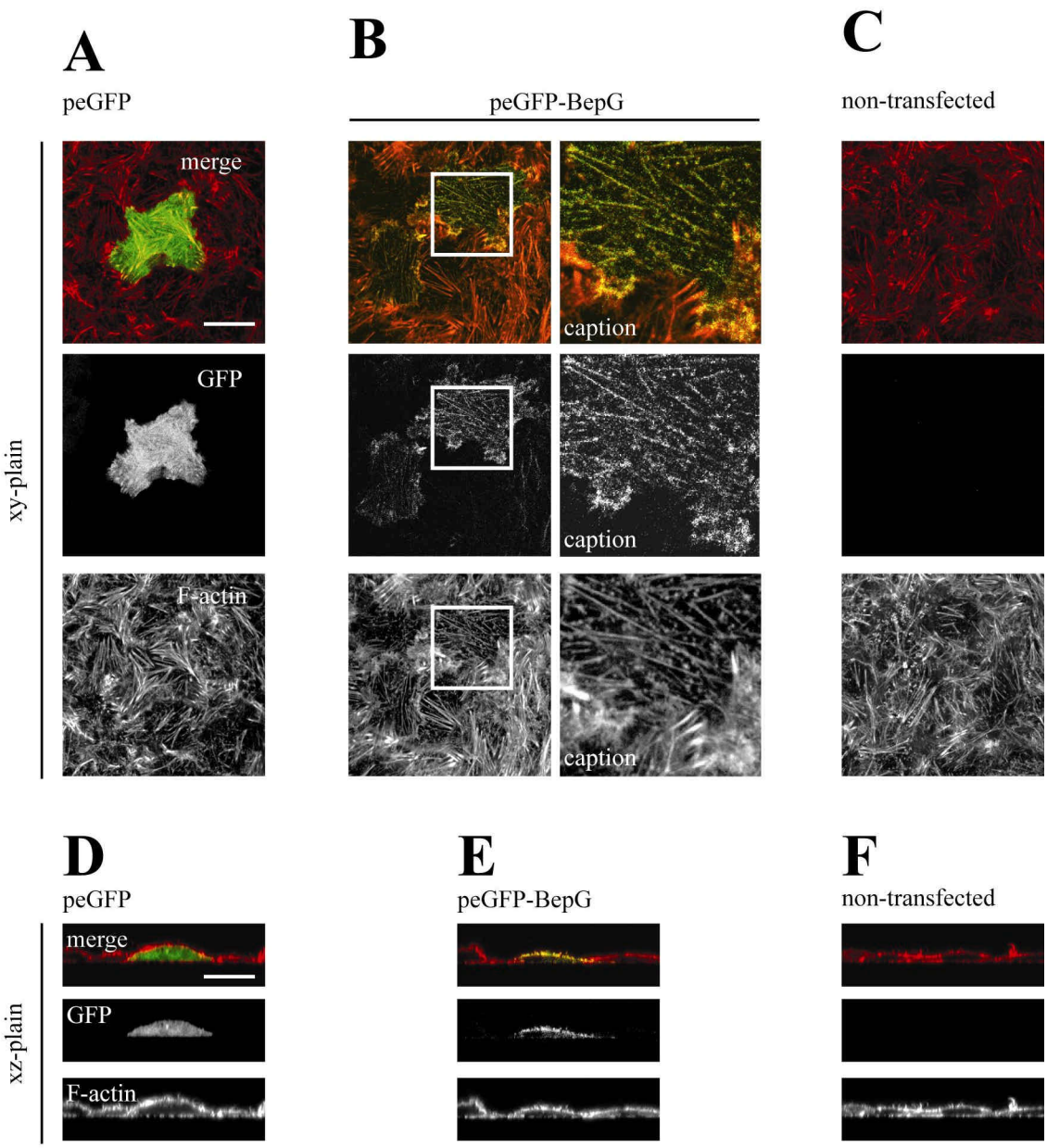


Figure S3
(Rhomberg *et al.*)

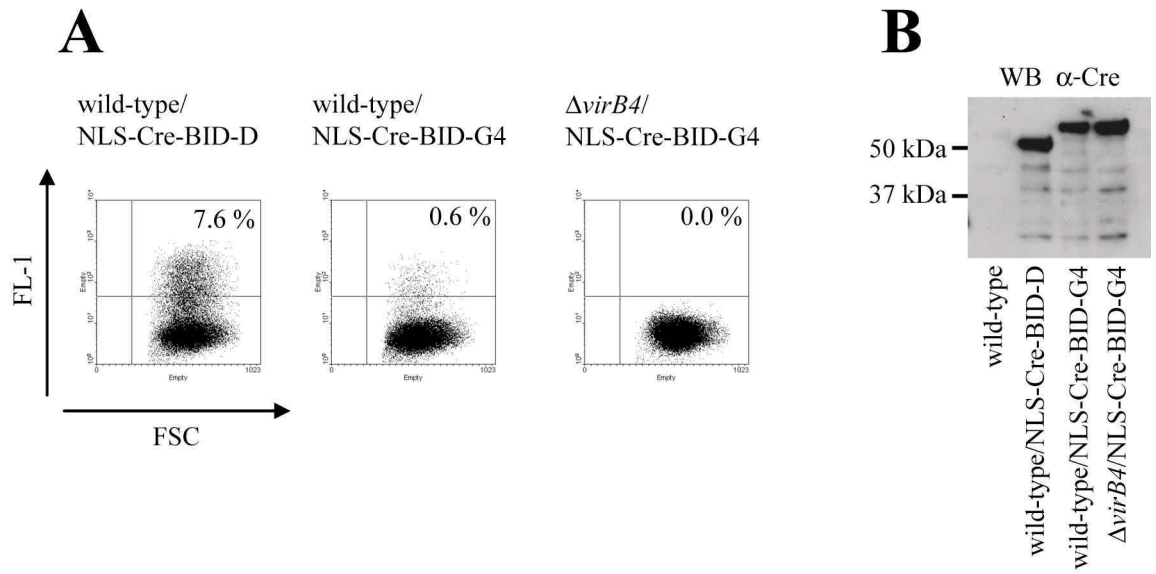


Figure S2
(Rhomberg *et al.*)

3.2 Unpublished Results Relevant to the Work Described

3.2.1 BepG is not a WxxxE-Family Protein

Only recently, a novel family of T3SS-translocated bacterial effector proteins interfering with the host cell actin cytoskeleton has been identified in *Salmonella*, *Shigella*, and enteropathogenic *E. coli* (EPEC) (Alto et al., 2006). Members of this family subvert host cell function by mimicking the signalling properties of Rho-family small GTPases. The *S. flexneri* effector protein IpgB2 stimulates cellular responses analogous to active form of RhoA, whereas the effector proteins IpgB1 from *S. flexneri* and Map from EPEC function as the active forms of Rac1 and Cdc42, respectively. Interestingly, these bacterial effector proteins do not bind guanine nucleotides and do not have sequence homologies to the small GTPase domain, suggesting that they are functional but not structural mimics. The activities of IpgB2, IpgB1, and Map are dependent on an invariant WxxxE-motif in their respective effector domains. Single amino acid substitutions introduced into IpgB2 by site directed mutagenesis (W62A and E66A) abolishes the biological function of IpgB2 completely whereas single amino acid substitutions outside of this motif do not affect the effector function (S72A and N76A) (Alto et al., 2006).

The T4SS-translocated effector proteins BepG and BepF of *B. henselae* both harbour a WxxxE-motif in their respective N-termini. To assess the relevance of this sequence motif for the observed function of BepG (This work), nucleotide exchanges leading to single amino acid substitutions (W77A and E81A) were introduced into the vector p*bepG* (pPG107) by site-directed mutagenesis (Promega QuickChange) following the manufacturer's protocol.

The derivatives of p*bepG* were then introduced into the effectorless mutant strain *ΔbepA-G* by conjugation and the ability to trigger invasome-mediated uptake of *B. henselae* into HUVECs was tested by infections (Figure 12).

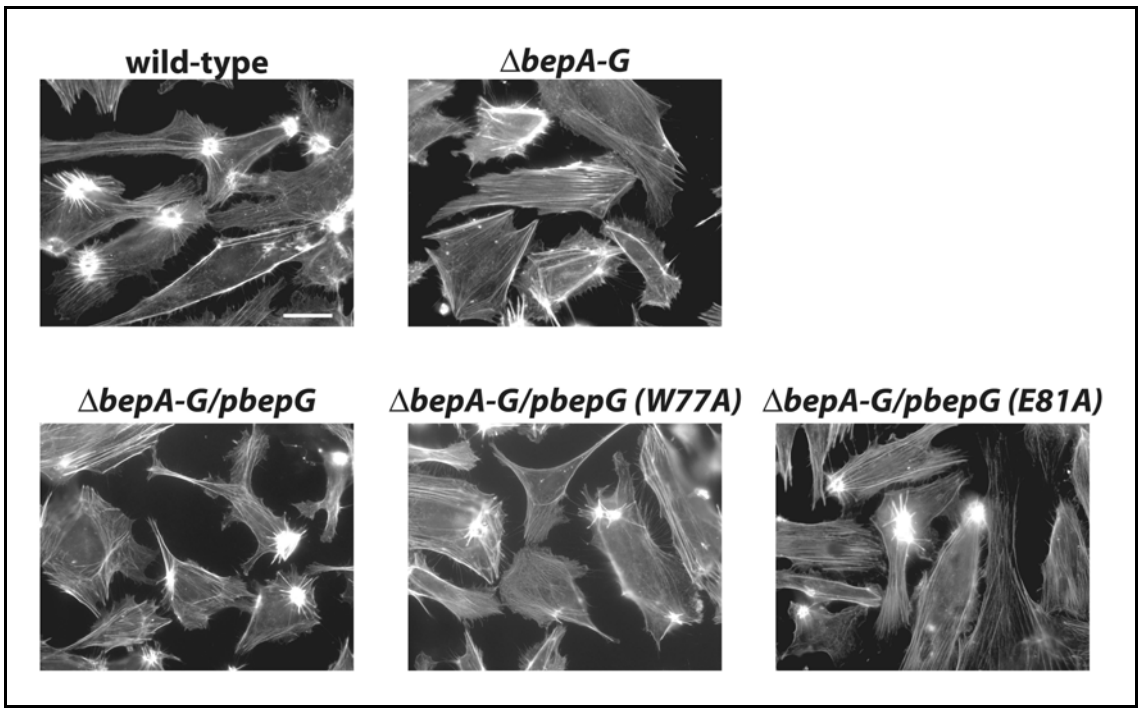


Figure 12

Amino acid substitutions introduced into the WxxxE-motif of the effector protein BepG do not abolish invasome formation. HUVECs were infected with *B. henselae* strains expressing different versions of the T4SS-translocated effector protein BepG (MOI=100, 48 hours). Cells were fixed subsequently and the actin cytoskeleton was stained with TRITC-Phalloidin. Pictures were acquired by epifluorescence microscopy. Scale bar=20μm.

Site-directed mutagenesis of *pbepG* (W77A and E81A) did not appear to cause any detectable impairment in BepG-mediated invasome formation. Rather, the strains *ΔbepA-G/*p*bepG*, *ΔbepA-G/*p*bep (W77A)* and *ΔbepA-G/*p*bepG (E81A)* were all able to induce the prototypical basal ring-like F-actin structure with protruding F-actin fibers characteristic for invasomes which appeared to be of the same quality. Next, the frequencies of invasome formation were determined (Table 1).

genotype	% of invasome-positive cells
wild-type	90 +/- 5 %
Δ*bepA-G*	0 +/- 0 %
Δ*bepA-G/pbepG (wt)*	61 +/- 3 %
Δ*bepA-G/pbepG (W77A)*	54 +/- 4 %
Δ*bepA-G/pbepG (E81A)*	59 +/- 3 %

Table 1

Amino acid substitutions introduced into the WxxxE-motif of the effector protein BepG do not decrease frequencies of invasome formation. HUVECs were infected with different genotypes of *B. henselae* of the T4SS-translocated effector protein BepG (MOI=100, 48 hours). Cells were then fixed, stained and analyzed by epifluorescence microscopy and the number of invasome-positive cells was determined. Results of three independent experiments are depicted +/-SD.

Clearly, rates of invasome formation by the two strains expressing derivatives of BepG (*ΔbepA-G/*p*bepG (W77A)* and *ΔbepA-G/*p*bepG (E81A)*) were only slightly reduced as compared to the the strain expressing the wild-type version of BepG (*ΔbepA-G/*p*bepG*). Frequencies of invasome formation in the positive control strain (wild-type) and of the negative control strain (*ΔbepA-G*) were in the range of rates determined in previous experiments (see chapter 3.1.2).

Taken together, these data suggest that BepG is not a WxxxE-family protein since single amino acid exchanges introduced did not affect the biological function of BepG in regard to invasome formation. In consequence, one can exclude the possibility that BepG mediates F-actin rearrangements leading to invasome formation by molecular mimicry of Rho-family small GTPases.

3.2.2 BepG-mediated Invasome Formation does not directly activate Rac1

GTPase pulldown assays are frequently used to determine the activation status of Rho-family small GTPases and allow specific affinity precipitation of GTP-loaded/ activated small GTPases in a given protein sample, which is compared to the total amount of the respective small GTPase. This ratio is indicative for direct or indirect stimulation of this small GTPase in response to an external stimulus. Protein samples are then separated by SDS-PAGE, transferred to PVDF membranes, probed with monoclonal Rac1 antibody and visualized by enhanced chemiluminescence.

As described, BepG triggers Rac1- and Cdc42-dependent uptake of *B. henselae* into ECs. To assess the question whether BepG might be able to modulate the activity of Rac1 during infection, Rac1 activation pulldown assays were performed by means of a commercial kit (Pierce, EZ Rac1 Activation Assay). To obtain appropriate protein samples, infection assays employing three different genotypes of *B. henselae* (wildtype, *ΔbepA-G*, *ΔbepA-G/*p*bepG*) were performed. Uninfected cells served as negative control. Two sets of uninfected cells were treated with either GTPγS or GDP before harvesting in order to either fully activate or to fully deactive Rac1 to assess maximal and minimal response to stimulation served as positive controls. Infections of HUVECs were performed as described (this work) with the following two modifications. The concentration of fetal calf serum (FCS) in the endothelial cell-culture medium (M199) was reduced from 10 % to 1 % to minimize the possible interference of growth hormones in the serum with the activation status of Rac1. Moreover, the multiplicity of infection was increased from MOI=100 to MOI=300 to maximize the number of invasome-positive HUVECs 24 hours post infection when protein samples were obtained.

One representative result out of four independent experiments is depicted in Figure 13.

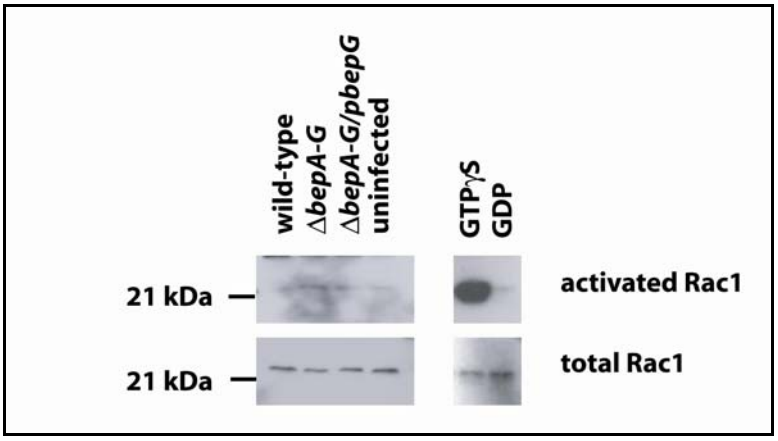


Figure 13

Rac1 is not activated in HUVECs in response to invasome-mediated internalization of *B. henselae* 24 hours post infection (MOI=300). Western blot analysis of affinity precipitated activated Rac1 (upper panels) compared to the total amount of cellular Rac1 (lower panels). GTPγS and GDP serve as positive and negative controls.

Clearly, it was not possible to detect significant activation of Rac1 in response to internalisation of *B. henselae* during invasome formation. As expected, preloading of Rac1 with GTPγS induced massive activation of Rac1 whereas preloading of Rac1 with GDP resulted deactivation of Rac1 to basal levels as suggested by the amount of precipitated Rac1, implying that the experimental setup was appropriately chosen. The basal level of activation in the GDP-preloaded sample is comparable to the activation level of uninfected cells and equally to infected cells, suggesting that Rac1 is not activated at this time point of infection.

3.2.3 BepC contributes to BepG-mediated Invasome Formation

As discussed in chapter 3.1.2, invasome formation of the effectorless mutant strain expressing BepG (*ΔbepA-G/*p*bepG*) and the wild-type strain are similar but not identical, suggesting the contribution of other Bep proteins to the process of BepG-mediated invasome formation.

To study the putative contribution of BepA-BepF to BepG-mediated invasome formation of *B. henselae*, a screen for extracellular complementation was set up. For this purpose, mixed infections employing different strain combinations were assayed. Six strains (*ΔbepA-G/*p*bepA, ΔbepA-G/*p*bepB, ΔbepA-G/*p*bepC, ΔbepA-G/*p*bepD, ΔbepA-G/*p*bepE, ΔbepA-G/*p*bep F,* see chapter 3.1.2) were individually mixed with the effectorless mutant strain expressing BepG (*ΔbepA-G/*p*bepG*) at a 1:1 ratio and used for infections of HUVECs for 48 hours (MOI=100). As a control an effectorless mutant strain harbouring the empty expression vector was included in the setup (*ΔbepA-G/*p*empty*). Only one infection setup, comprising *ΔbepA-G/*p*bepC* mixed with *ΔbepA-G/*p*bepG*, markedly enhanced invasome formation both qualitatively (Figure 14) and quantitatively (Table X) while the remaining five setups did not elicit significant changes (data not shown). In the initial screen for invasome formation (see chapter 3.1.2, Figure 1), BepC exhibited no significant effect on the host cell actin cytoskeleton when expressed in an effectorless mutant background (*ΔbepA-G/*p*bepC*). However, upon mixed infections (*ΔbepA-G/*p*bepG+ΔbepA-G/*p*bepC*, MOI=50+50), it becomes evident, that BepC – in concert with BepG – markedly enhances the level of F-actin rearrangement leading to establishment of invasomes. More spherical, evenly shaped basal ring-like structures, frequently decorated with regularily arranged radial F-actin fibers, are detected when compared to infections employing only the effectorless mutant strain expressing BepG (*ΔbepA-G/*p*bepG*, MOI=100) or compared to mixed infections employing the latter strain together with the control strain harbouring only the control plasmid (*ΔbepA-G/*p*bepG+ΔbepA-G/*p*empty*, MOI=50+50).

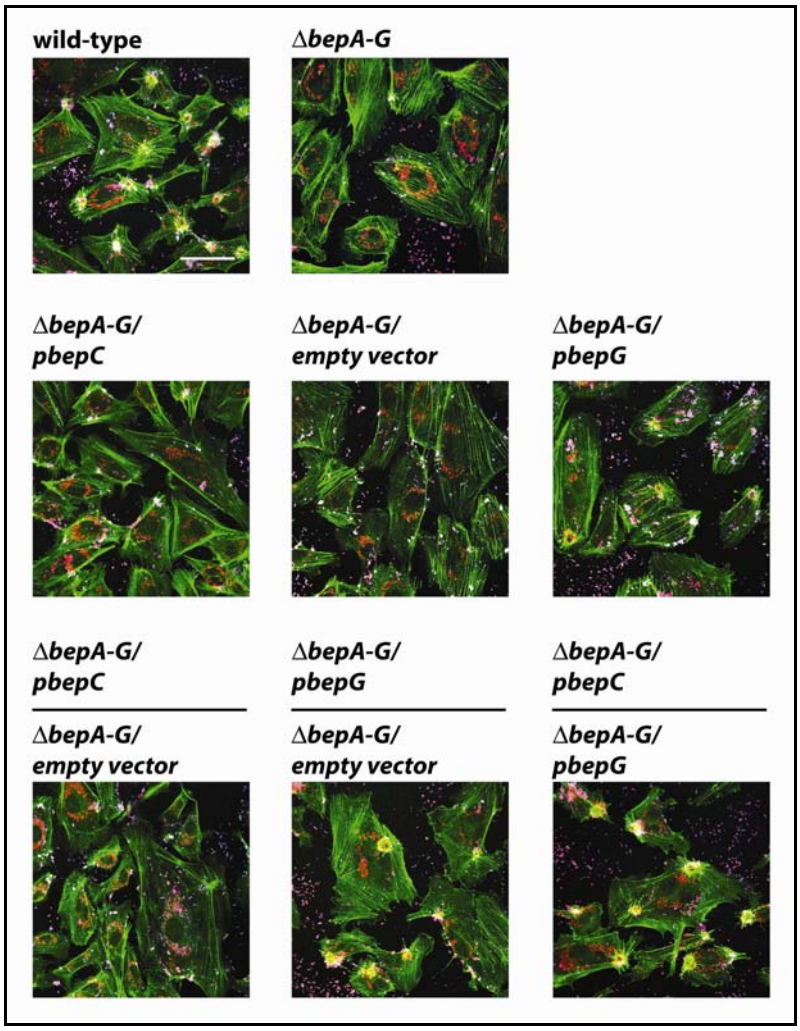


Figure 14

BepC is not sufficient for invasome formation, but contributes synergistically to BepG-mediated invasome formation. HUVECs were infected with either one or two strains of *B. henselae* expressing either BepG, BepC or no effector protein (MOI=100, MOI=50+50, 48 hours). Cells were fixed subsequently and stained for F-actin (green), intracellular bacteria (red) and extracellular bacteria (purple). Pictures were acquired by confocal laser scanning microscopy. Scale bar=20μm.

On the quantitative level, the contribution of BepC to BepG-mediated invasome formation is reflected in equal terms (Table 2) as suggested by the increased number of invasomes observed.

genotype	% of invasome-positive cells
wild-type (MOI=100)	88 +/- 7 %
Δ*bepA-G* (MOI=100)	0 +/- 0 %
Δ*bepA-G/empty vector* (MOI=100)	0 +/- 0 %
Δ*bepA-G/pbepC* (MOI=100)	0 +/- 0 %
Δ*bepA-G/pbepG* (MOI=100)	52 +/- 5 %
Δ*bepA-G/empty vector* (MOI=50) + Δ*bepA-G/pbepG*(MOI=50)	42 +/- 6 %
Δ*bepA-G/empty vector* (MOI=50) + Δ*bepA-G/pbepC* (MOI=50)	0 +/- 0 %
Δ*bepA-G/pbepG* (MOI=50) + Δ*bepA-G/pbepC* (MOI=50)	77 +/- 6 %

Table 2

The action of BepC and BepG induces invasomes at higher frequencies than BepG alone. HUVECs were infected with either one or two strains of *B. henselae* expressing either BepG, BepC or no effector protein (MOI=100, MOI=50+50, 48 hours). Cells were then fixed, stained and analyzed by epifluorescence microscopy and the number of invasome-positive cells was determined. Results of three independent experiments are depicted +/-SD.

To study the contribution of BepC to BepG-mediated invasome formation in more detail, two novel non-polar in-frame deletion mutants were generated, namely the mutant *ΔbepC* in the background of *B. henselae* wild-type and the double mutant *ΔbepCG* in the background of the pre-existing mutant *ΔbepG*. These mutants were complemented with p*bepC* and p*bepC* or p*bepG*, respectively, used for infections and analyzed for the capability to form invasomes (Figure 15).

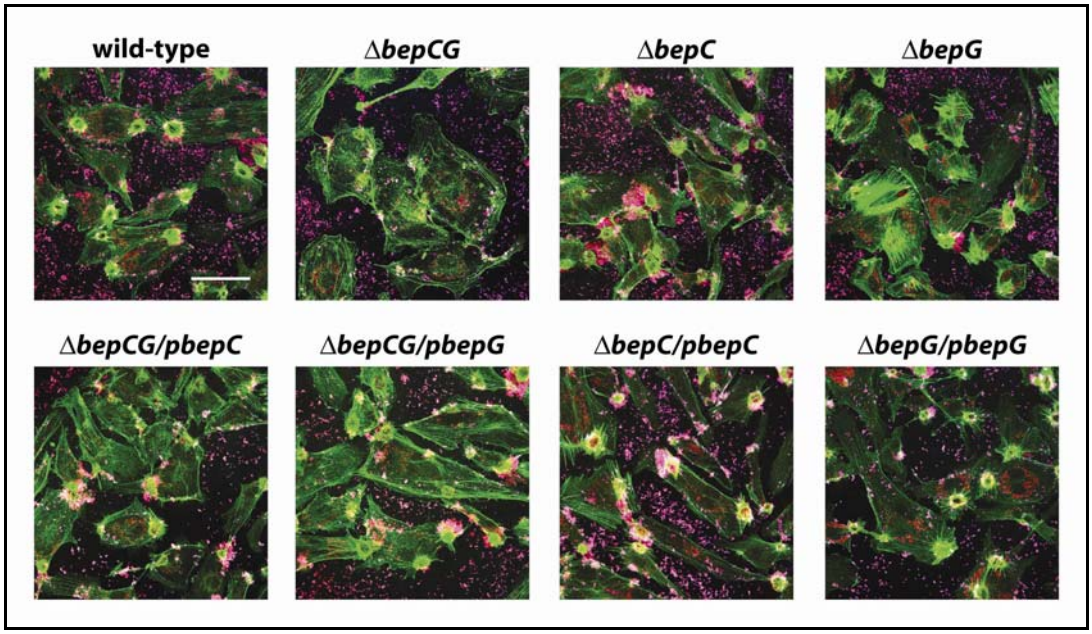


Figure 15

The combined action of BepG and BepC is a prerequisite for invasome formation, whereas the action of either BepG or BepC alone is dispensible. HUVECs were infected with different strains *B. henselae* (MOI=100, 48 hours). Cells were fixed subsequently and stained for F-actin (green), intracellular bacteria (red) and extracellular bacteria (purple). Pictures were acquired by confocal laser scanning microscopy. Scale bar=20μm.

The deletion mutant *ΔbepC* was found to be still able to form invasomes, yet the mutant appeared to be attenuated as suggested by minor differences in the morphological appearance of the basal ring-like structure as compared to the ones elicited by the wild-type strain. In contrast, the basal ring-like structures elicited by the deletion mutant *ΔbepG* differ more dramatically to the ones elicited by the wild-type strain (see chapter 3.1.2). Complementation of the two deletion mutants appeared to be highly effective, since basal structures elicited by the complemented strains (*ΔbepG*/p*bepC* and *ΔbepG*/p*bepG)* were practically indistinctible from the ones elicited by the wild-type strain.

Clearly, the double deletion mutant *ΔbepCG* exhibited a null phenotype for invasome formation as suggested by the absence of the F-actin rearrangements charactersistic for invasomes. Complementation of the double deletion mutant *ΔbepCG* with either p*bepG* or p*bepC* restored the capability to form invasomes. These morphological observations are nicely reflected when determining the frequencies of invasome formation (Table 3).

genotype	% of invasome-positive cells
wild-type	92 +/- 4 %
Δ*bepG*	81 +/- 6 %
Δ*bepG/pbepG*	94 +/- 5 %
Δ*bepC*	66 +/- 12 %
Δ*bepC/pbepC*	86 +/- 4 %
Δ*bepCG*	0 +/- 0 %
Δ*bepCG/pbepG*	51 +/- 6 %
Δ*bepCG/pbepC*	78 +/- 6 %

Table 3

The synergism of the interaction of BepG and BepC is dominated by BepC. HUVECs were infected with different genotypes of *B. henselae* for 48 hours (MOI=100). Cells were then fixed, stained and analyzed by epifluorescence microscopy and the number of invasome-positive cells was determined. Results of two independent experiments performed in duplicates are depicted +/-SD.

Furthermore, it was found that the deletion mutant *ΔbepC* is more severly attenuated than the deletion mutant *ΔbepG* in regard of invasome formation as suggested by an apparent reduction of invasomes observed. Interestingly, the complementation of the double deletion mutant *ΔbepCG* appeared to be more effective when expressing BepC (*ΔbepCG*+p*bepC*) than when expressing BepG (*ΔbepCG*+p*bepG*) as suggested by a difference of number of invasomes observed.

3.2.4 BepF Mediates the Formation of Small Actin Foci and Becomes Tyrosine-Phosphorylated upon Translocation into Host Cells

In the initial screen for invasome-mediated internalization, a subset of five strains (i.e. *ΔbepA-G/pbepA/B/C/D/E*) did not appear to elicit any detectable effect on the host cell actin cytoskeleton integrity or assembly indicating that their solitary action is not sufficient to induce F-actin rearrangements leading to invasome formation. In contrast, one strain (i.e. *ΔbepA-G/bepF*) exhibited the formation of small actin foci upon interaction with human ECs at contact sites with the host cell membrane (Figure 16) that were or were not associated with bacteria (data not shown). However, these nodular microstructures are clearly not reminiscent to invasomes as suggested by their morphology, size and the apparent lack of large bacterial aggregates associated to them. BepF triggers local F-actin rearrangements, but the function of these actin foci for bacterial internalization remains elusive.

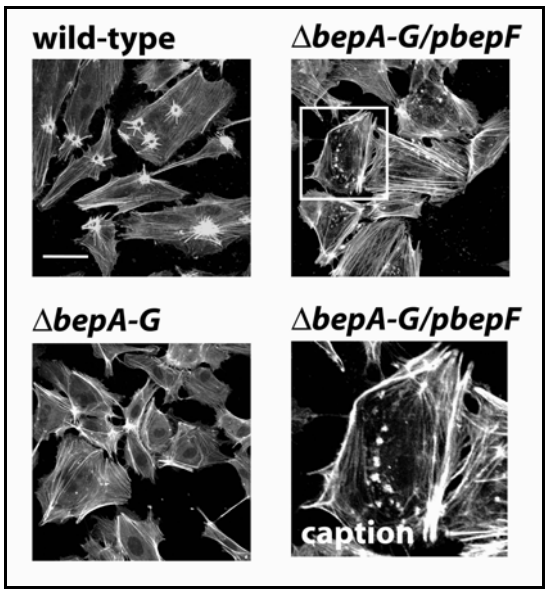


Figure 16

BepF promotes the formation of small actin foci. HUVECs were infected with different genotypes of *B. henselae* (MOI=100, 48 hours). Cells were fixed subsequently and the actin cytoskeleton was stained with TRITC-Phalloidin. Pictures were acquired by confocal laser scanning microscopy. Scale bar=20μm.

The effector proteins BepD, BepE and BepF of *B. henselae* encode short repeated peptide sequences in their N-terminal effector domain similar to tyrosine phosphorylation motifs present in various eukaryotic proteins, which allow protein-protein interactions in a phosphorylation-dependent manner. So far, only BepD has been shown to be tyrosine-phosphorylated upon translocation (Schulein et al., 2005). Likewise, BepF was analyzed by means of immunoprecipitation and western blot analysis as described (Schulein et al., 2005) (Figure 17).

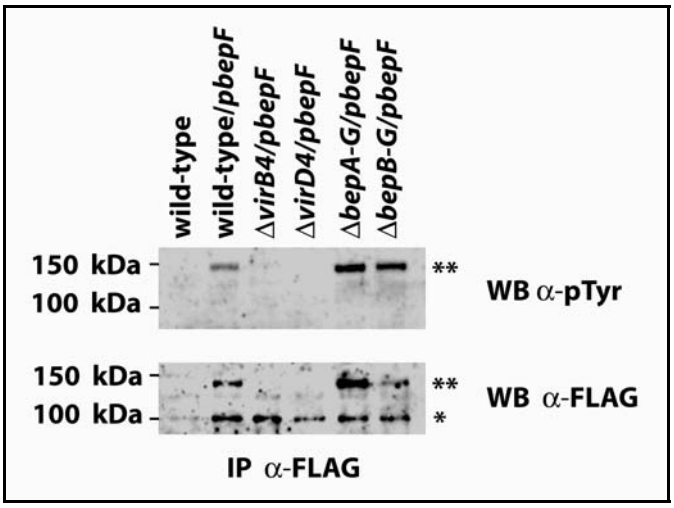


Figure 17

BepF is is tyrosine-phosphorylated upon VirB/VirD4 T4SS-mediated translocation into human ECs. Immunoprecipitation of BepF from EA.hy 926 cell lysate infected with different genotypes of *B. henselae* (MOI=100, 48 hours) was followed by western blot analysis probing with α-phospho-tyrosine and α-FLAG monoclonal antibodies. (*) BepF, un-phosphorylated, (**) BepF, tyrosine-phosphorylated.

Indeed, BepF was found to be tyrosine-phosphorylated upon translocation in a VirB/VirD4 T4SS-dependent manner indicating that BepF is able to interact with eukaryotic protein tyrosine kinases. In addition, other secondary posttranslational modifications of BepF do occur resulting in a prominent increase in the molecular size and a lower electrophorphoretic mobility of the tyrosine-phosphorylated species of BepF.

3.3 Further Publications

3.3.1 Research Article III

Molecular Mimicry of Inhibitory Immune Receptors by the Bacterial Pathogen *Bartonella*

Guye P., Schein H., Rhomberg T.A., Jenö P. and Dehio C.

Manuscript in preparation.

Summary

This research article presents the identification and characterization of the effector protein BepE of the vascular tumor-inducing pathogen *B. henselae*. BepE contains putative tyrosine-phosphorylation sites in its N-terminus, and two C-terminal BID domains thought to mediate the translocation of this protein from the bacteria into the host cells in a VirB/VirD4 T4SS-dependent manner.

In this study, BepE is shown to be tyrosine-phosphorylated in its N-terminus upon secretion into host cells, to acquire a membrane-proximal localization, and additionally to co-localize with VE-Cadherin at cell-cell contacts in human umbilical vein endothelial cells (HUVECs). In addition, the kinase c-Src is shown to be able to tyrosine-phosphorylate purified BepE *in vitro*.

Furthermore, five putative tyrosine-phosphorylation motifs in the N-terminus of this protein are identified and characterized. The first motif is similar to a known binding site for the C-terminal c-Src kinase (Csk) in VE-Cadherin. This motif is followed by two tandems of immunotyrosine inhibitory and immunotyrosine-based switch motifs (ITIMs, ITSMs). These motifs are widely present in the intracellular domain of inhibitory immune receptors of mammals. By recruiting the phosphatases like SHP1, SHP2 and SHIP or the kinase Csk to these motifs, these inhibitory receptors inhibit the activation in almost all cells of the immune system. Co-immunoprecipitation experiments suggest that the effector protein BepE indeed interacts with both Csk and SHP2 in a tyrosine-phosphorylation dependent manner.

Systematic amino acid exchanges of tyrosines to phenylalanines in these N-terminal five motifs of BepE reveal that binding Csk to BepE occurs exclusively at the first motif. In contrast, binding of SHP2 to BepE is possible at both ITIM-ITSM tandems. To abrogate binding of Csk to BepE completely, both ITIM-ITSM tandems have to be mutated.

Taken together, these data suggest that BepE is translocated in to host cells where it acts as a molecular mimickry of inhibitory immune receptors that interfere with host cell signalling, thus representing a novel paradigm for subversion of host cell function by a bacterial pathogen.

Statement of own contribution

My contribution to this research article is restricted to the cloning of the pTR1773 encoding full-length BepE under the control of a CMV promotor which is the basic construct for transfection, immunoprecipitation and protein tyrosine phosphorylation assays presented in this study.

4. SUMMARY

4. SUMMARY

The facultative intracellular bacterium *Bartonella henselae* enters human endothelial cells either passively by conventional phagocytosis or actively by a pathogen-triggered process known as invasome-mediated internalization. The latter involves the formation of a cell-surface-associated bacterial aggregate, which is subsequently engulfed by host cell membranes eventually resulting in its complete internalization.

Recent work indicated that invasome formation of *B. henselae* depends on its VirB/VirD4 T4SS.

This work describes that the VirB/VirD4 T4SS of *B. henselae* injects a cocktail of seven effector proteins into endothelial host cells to subvert cellular functions and that one of these translocated effector proteins, BepG, mediates the sustained cytoskeletal changes leading to invasome formation. Moreover, this work indicates the existence of two non-redundant pathways to promote invasome formation, one of which is governed by the action of BepG and another one, involving further Bep proteins, which is BepG-independent.

On the host cell side, Rho-family small GTPases Cdc42 and Rac1, but not RhoA are shown to be required for invasome-mediated internalization. Furthermore, it is shown that invasome formation leads both to the rearrangement of pre-existing F-actin fibers and to localized actin polymerization enriched for Arp2/3, which occurs in a Scar1/WAVE-dependent manner. Finally, this work provides evidence that after complete internalization the invasome membranes do not fuse with Lamp-1 positive lysosomes, indicating that invasome-mediated invasion represents a novel mechanism allowing the uptake of bacteria without entering the endocytic-lysosomal pathway.

Importantly, this is the first report, which attributes the orchestrated action of more than one effector protein of *B. henselae* to a known VirB/VirD4 T4SS-dependent phenotype, namely invasome formation, which represents a multifacetted example for the complexity of host cell subversion by a bacterial pathogen.

5. DISCUSSION

5. DISCUSSION

The Molecular Basis of Internalization of *B. henselae* by Human Endothelial Cells

In this study, I have started the genetic analysis of the internalization of *B. henselae* by human ECs. For this purpose, non-polar in-frame deletions mutants and corresponding complementants expressing individual Bep proteins were generated and analyzed. In this chapter, I discuss these findings and try to integrate experimental data and observations into a molecular model of invasome-mediated internalization.

The genetic basis of invasome-mediated internalization of *B. henselae.*

Initial experiments demonstrated that invasome-mediated internalization by human ECs requires a functional VirB/VirD4 T4SS. Deletion of either structural components of the T4S apparatus (i.e. *ΔvirB4*), the T4S coupling protein (i.e. *ΔvirD4*), or the corresponding effector proteins (i.e. *ΔbepA-G*) completely abrogates invasome-mediated internalization of *B. henselae* by human ECs (Schulein et al., 2005). This finding indicated that invasome-mediated internalization requires one or more of the seven known VirB/VirD4 T4SS-translocated effector proteins of *B. henselae*, BepA-BepG.

The pivotal role of BepG in invasome formation.

In the initial screen for invasome-mediated internalization, I observed that the solitary action of BepG is sufficient to promote invasome formation in the absence of BepA-BepF (i.e. *ΔbepA-G/pbepG*). This finding points out the central role of BepG in the process of invasome formation. BepG promotes F-actin rearrangements and internalization of bacterial aggregates characteristic for invasomes.

BepG is the largest of the Bep proteins (1009 amino acids in length), comprises a total of four BID domains (BID-G1 to BID-G4) and two sets of repeated domains of unknown function (termed DUF1a, 1b, 1c and DUF2a, 2b) that do not share significant amino acid sequence homology to known bacterial or eukaryotic proteins at all (see chapter 3.1.2, Supplementary Figure 1). Furthermore, BepG contains as a short sequence motif in BID-G1 (i.e. -W(77)-QIS-E(81)-) similar to a novel family of T3SS-translocated bacterial effector proteins (Alto et al., 2006), termed WxxxE-proteins, which interfere with the host cell actin cytoskeleton by functioning as a molecular mimickry of activated Rho-family small GTPase to promote cytoskeletal changes (see chapter 3.2.1). However, BepG does not belong to this WxxxE-protein family, since single amino acid changes introduced in this motif do not abolish the function of BepG, indicating that BepG promotes cytoskeletal changes by a distinct mechanism. Finally, by means of a reporter assay (CRAfT, see chapter 3.1.2, Supplementary Figure 2) it was possible to demonstrate that the BID-G1 of BepG is sufficient to mediate VirB/VirD4 T4SS-dependent translocation of a reporter protein from the bacterium into the host cell cytoplasm, indicating that full-length BepG is translocated accordingly in a VirB/VirD4 T4SS-dependent manner.

Towards a model of invasome formation by *B. henselae*.

The deletion of the effector locus *bepG* (i.e. *ΔbepG*) did not abolish invasome-mediated internalization (see chapter 3.2.1, Figure 3). Intriguingly, this finding highly suggests that there is more than one possibility to promote invasome-mediated internalization. I conclude from this, that the action of BepG must be paralleled by the action of other effectors, namely BepA-BepF, in order to sustain invasome formation as seen in the deletion mutant of BepG (i.e. *ΔbepG*, see chapter 3.2.1, Figure 3). Furthermore, this finding indicates that more than just one Bep protein must participate in this parallel process, since none of the effector BepA-BepF alone (i.e. *ΔbepA-G/pbepA/B/C/D/E/F*) elicited cellular changes leading to invasome formation at all (see chapter 3.1.2, Figure 1).

These findings can be integrated into a preliminary model for the action of effector proteins in the process of invasome-mediated internalization of *B. henselae* by human ECs (see Figure 18).

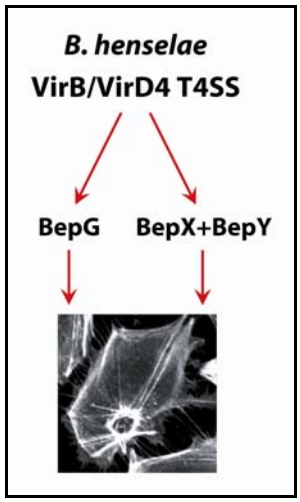


Figure 18

Invasome formation encompasses (at least) three VirB/VirD4 T4SS-translocated effector proteins organized in two separate pathways, one of which is governed by the action of BepG

This model encompasses two parallel pathways including (at least) three VirB/VirD4 T4SS-translocated effector proteins. One pathway comprises BepG, whereas the other pathway comprises (at least) two other Bep proteins, termed here, for convenience, BepX and BepY. As a matter of fact, one cannot exclude that even more Bep proteins participitate to promote invasome formation of course. However, the minimal non-redundant set of effectors necessary is two. Therefore, I will continue with this terminology as of here.

This prompted us to extend our investigations and to analyze the putative contribution of BepX and BepY to the action of BepG in order to find arguments for the correctness of this preliminary model.

The role of BepC in invasome formation.

Follow-up experiments then allowed us to identify BepC as an effector protein contributing synergistically to invasome-mediated internalization of *B. henselae* by human ECs. BepC is a *bona fide* VirB/VirD4 T4SS-translocated effector protein of *B. henselae* and encodes a BID-domain for protein translocation at its C-terminus (see chapter 3.1.1). The N-terminal, putative effector domain of BepC shares high similarity to a protein domain termed FIC (filamentation induced by cAMP), which is found as well in the effector proteins BepA and BepB of *B. henselae* (Schulein et al., 2005). However, the functional contribution of the FIC domain in BepA-BepC to the pathogenesis of *B. henselae* remains elusive. Originally described as a protein domain implicated in cell septation in *E. coli* (Kawamukai et al., 1988), this domain has been later identified in other proteins of gram-negative and gram-positive bacteria, e.g. partners in toxin/anti-toxin systems (RelE, ParE, Doc) from post-segregational cell killing systems (Anantharaman and Aravind, 2003), and even eukaryotic proteins, such as human huntingtin associated protein E (HYPE). All of these proteins contain a short characteristic sequence motif (i.e. -HPFxxGNG-), whose significance is not understood.

In the initial screen for invasome formation, the action of BepC alone (i.e. *ΔbepA-G/pbep*C) did not result in any apparent effect on the host cell actin cytoskeleton. The action of BepC only becomes evident in the presence of BepG as suggested by means of extracellular complementation, since the combined action of BepC and BepG promote invasome formation more efficiently than BepG alone (i.e. *ΔbepA-G/pbepG+ΔbepA-G/pbepC* as compared to *ΔbepA-G/pbepG*) both on qualitative (see chapter 3.2.3, Figure 14) and a quantitative level (see chapter 3.2.3, Table 2). Moreover, no beneficial contributions other than BepC to BepG-mediated invasome formation were detected, which means the action of neither BepA-BepB nor BepD-BepF improved invasome formation qualitatively or quantitatively, indicating that they do not interact with or contribute to the action of BepG.

Thus, BepC may represent one of the two postulated effector proteins, BepX and BepY, being involved in the pathway parallel to the one governed by the action of BepG. The central role of BepC for invasome formation is highlighted by another set of experiments. Interestingly, the double deletion of the *bepC* and the *bepG* loci (i.e. *ΔbepCG*) completely abolishes invasome formation (see chapter 3.2.3, Figure 15 and Table 3). This finding is in agreement with the preliminary model I proposed. The double mutant (i.e. *ΔbepCG*) targets two of the three effectors postulated in both pathways at a time and therefore leads to the observed null phenotype. Moreover, this finding indicates that the interaction of BepC and the second postulated Bep protein, BepY, is indeed essential – in accordance to the proposed model - since the BepY alone is not able to promote invasome formation alone in this background (i.e. *ΔbepCG*), which, in turn, is in agreement with the null phenotype observed in the initial screen (i.e. *ΔbepA-G/pbepA/B/D/E/F*).

The role of BepC in invasome-mediated internalization is further substantiated by the following fact. Deletion of the *bepC* locus (i.e. *ΔbepC*) results in a stronger reduction of invasome formation than deletion of the *bepG* locus (i.e. *ΔbepG*) as suggested by a decrease of -26 % and a decrease of -11 % as compared to the isogenic wild-type strain (see chapter 3.2.3, Table 3). Similarily, complementation of the double mutant (i.e. *ΔbepCG*) with a plasmid expressing BepG (i.e.*ΔbepCG/pbepG*) does not restore the capability of invasome formation as efficient as complementation of the double mutant (i.e. *ΔbepCG*) with a plasmid expressing BepC (i.e.*ΔbepCG/pbepC*) as suggested by the increase of invasome formation of +51 % compared to an increase of +78 %.

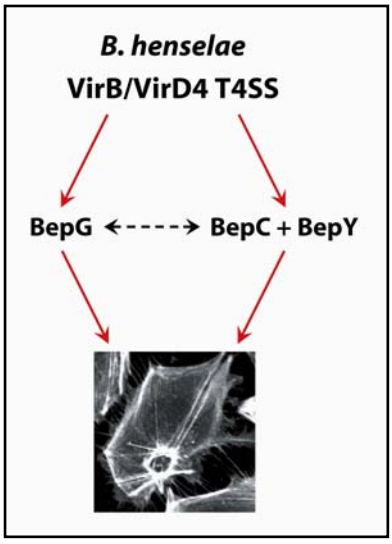
Interpretation of this finding is rather difficult. So far, it is clear that both pathways postulated act in concert and in parallel. Moreover it might be possible that the pathway involving BepC and a second Bep protein is more efficient in promoting invasome formation than the pathway involving BepG alone as suggested by these data.

However, the interpretation of this observation has to be further validated and requires more experimental data. Once BepY, interacting with BepC, is identified, the generation of the corresponding double mutant (e.g. *ΔbepCY*), assessment of its capability to promote invasome formation and direct comparison to the deletion mutant in the *bepG* locus (i.e. *ΔbepG*) will further substantiate this argument.

A refined model of invasome formation by *B.henselae.*

Taken together, these additional findings make it necessary to integrate this knowledge into the preliminary model proposed beforehand. Importantly, all the data gathered so far indicate that the original model is correct and appropriate, as no results were found, which would contradict this model. Thus, the most up-to-date model looks as follows (Figure 19).

Figure 19
Invasome formation encompasses (at least) three VirB/VirD4 T4SS-translocated effector proteins organized in two parallel pathways, that act synergistically.



Two parallel non-redundant pathways promote invasome-mediated internalization of *B. henselae* by human ECs. One pathway is governed by the solitary action of BepG, whereas the other pathway is governed by the combined action of BepC and BepY. Both pathways act synergistically to promote these cytoskeletal changes as suggested by a crosstalk of BepG and BepC (see chapter 3.2.3).

Invasome formation in the genus *Bartonella.*

Interestingly, the human-specific species *B. quintana*, which is *nota bene* closely related to *B. henselae*, exhibits a strong endothelial host cell tropism, is being internalized by human ECs, but has not been reported to promote invasome formation. During the course of my Ph.D. thesis, the genomes of *B. quintana* and *B. henselae* have been sequenced and annotated (Alsmark et al., 2004) allowing a direct comparison of the VirB/VirD4 T4SS along with the corresponding effector proteins (Figure 20). Intriguingly, *B. quintana* does not encode for a protein product similar to BepG. Similarily, BepB and BepD are missing in *B. quintana*, whereas BepC is present in both species. Therefore, it is likely, that the inability of *B. quintana* to promote invasome-mediated internalization might be explained by these apparent genetic differences according to the model I have proposed (Figure 19).

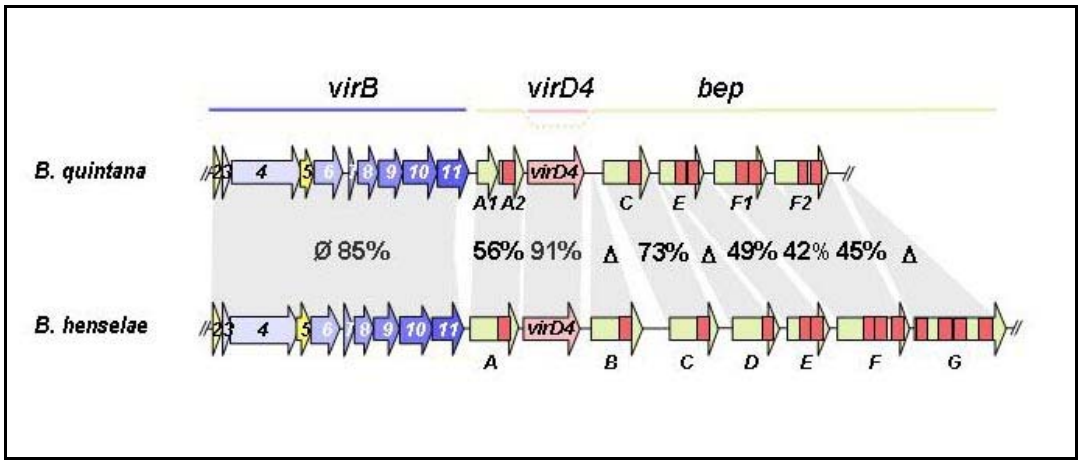


Figure 20

Comparison of the genetic organisation of the virB/virD4/bep loci of *Bartonella henselae* and *Bartonella quintana* reveals a high degree of synteny and sequence homology. Numbers indicate the percentage of amino acid identities shared between the gene products of both organism (adapted from Schröder G. and Dehio C., Trends in Microbiology, 2005)

In consequence, this might indicate that BepB and BepD, which both are absent in *B. quintana*, might represent the unknown effector BepY in *B. henselae* according to our model. At this stage, however, it is difficult to argue about these possibilities. Nevertheless, both species are uniquely adapted to interact with human ECs and the obvious differences in the mode of uptake must be determined in the array of Bep proteins both species encode for.

The orchestrated action of bacterial effector proteins.

The combined action of several bacterial effector proteins triggering bacterial internalization, as proposed for invasome-mediated internalization of *B. henselae* by human ECs, is not unusual amongst bacterial pathogens.

The gram-positive bacterial pathogen *L. monocytogenes* promotes its own uptake into non-phagocytic cells as well by two independent pathways, namely by the action of two surface-exposed proteins, Internalin A (InlA) (Gaillard et al., 1991) and internalin B (InlB) (Dramsi et al., 1995). Both molecules are proper invasion molecules and are required to trigger invasion into polarized epithelial cells (Lingnau et al., 1995) subverting the host cell actin cytoskeletal machinery independently and with different outcomes (see chapter 1.4.4).

Likewise, the coordinated action of several T3SS-translocated effector proteins enable the internalization of the gram-negative bacterial pathogens *Salmonella* (e.g. SipC, SopE/E2, SopB, SipA, see chapter 1.4.2) or *Shigella* (e.g. IpaC, VirA, IpgD, IpaA) (Nhieu et al., 2005) into non-phagocytic cells. In contrast multiple T3SS-translocated effector proteins of the gram-negative bacterial pathogen *Yersinia* (e.g. YopE, YopH, YopO, YopT, see chapter 1.4.3) prevent uptake into phagocytic cells.

The Cellular Basis of Internalization of *B. henselae* by Human Endothelial Cells

Adherence to and invasion of host cells by bacterial pathogens is a recurrent motif in bacterial infection processes (Pizarro-Cerda and Cossart, 2006a). Bacterial pathogens have developed numerous strategies to corrupt, to hijack, or to mimic cellular processes involved in the modulation of the host cell actin cytoskeleton to either foster uptake into non-phagocytic cells or to inhibit uptake into phagocytic cells (Gruenheid and Finlay, 2003; Rottner et al., 2005).

Well described gram-negative pathogens targeting the host cell actin cytoskeleton for these purposes are *Salmonella* (Patel and Galan, 2005), *Shigella* (Nhieu et al., 2005; Sansonetti, 2001), *Yersinia* (Cornelis, 2002b), and EPEC (Celli et al., 2000; Vallance and Finlay, 2000), all employing injectisomes (T3SSs, see chapter 1.2.2 and chapter 1.2.3) in order to deliver bacterial effectors into host cells for redirecting the actin cytoskeletal machinery.

In contrast to the above mentioned examples, the *B. henselae* translocates its effector proteins by a VirB/VirD4 T4SS into the host cell cytoplasm to foster its own uptake by a unique actin-dependent structure, termed invasome.

In this study, I have started the cell biological analysis of invasome-mediated internalization of *B. henselae* by human ECs with the aim to identify host cell proteins required for invasome formation. In this chapter, I discuss these findings and try to integrate experimental data and observations into a cellular model of invasome formation.

Subcellular localization of BepG.

Upon transfection, BepG localizes with distinct components of the actin cytoskeleton. Co-localization was observed both with F-actin stress fibers, as well as cortical F-actin in filopodial cell extensions in the cell periphery or the cortex underneath the apical plasma membrane (see chapter 3.1.2, Supplementary Figure 3). This finding is interesting in regard of the function of BepG in triggering the formation of the actin rearrangements culminating in invasome formation. This prompted us to perform actin-binding assays to analyze the putative binding of BepG to F-actin (data not shown). However, it was not possible to prove this hypothesis. Nevertheless, the apparent interference of BepG with the host cell actin cytoskeleton upon infection suggests that putative cellular interaction partners of BepG belong to protein families, which are in control of the dynamics and the assembly of the actin cytoskeletal machinery under physiological conditions.

The pivotal role of Rho-family small GTPases during invasome formation.

Rho-family small GTPases, RhoA, Rac1 and Cdc42 are master regulators of actin cytoskeleton (see chapter 1.4.1). Therefore, the role of RhoA, Rac1 and Cdc42 was analyzed in the process of invasome-mediated internalization. For this purpose, plasmid-borne, dominant negative versions of these proteins were delivered by transfection into human ECs, which were subsequently infected with different genotypes of *B. henselae*, whose capacity to promote invasome formation was assessed quantitatively (see chapter 3.1.2, Figure 5A). Whereas RhoA was found to play no role in the establishment of these actin-dependent structures, Cdc42 and Rac1 were found to be essential, yet by differing extents. Expression of dominant-negative Cdc42 exhibited a milder effect than expression of dominant-negative Rac1 as suggested by a reduction of frequencies of invasome formation by 25 % and by 50% respectively. This finding reflects the hierarchy of action, since Cdc42 elicits cellular changes upstream of Rac1 (Hall, 1998) and points out a major role of Rac1 in the process of invasome-mediated internalization of *B. henselae* by human ECs.

To further analyze the involvement of Rac1 in invasome-mediated internalization, human ECs were transfected with a constitutively active version of Rac1, infected and evaluated for invasome formation accordingly. Interestingly, expression of a constitutively active version of Rac1 had a strong deleterious effect on invasome-mediated internalization as suggested by a reduction of frequencies of invasome formation by 70 % (see chapter 3.1.2, Figure 6B). In contrast, overexpression of wild-type Rac1 did not exhibit any detrimental effect, indicating that modulation of the activity of Rac1, rather than the sheer abundance of this small GTPase by overexpression, is critical. Thus, the control of the activity of the small GTPase Rac1 in time and space is an important factor for the establishment of invasomes during the interaction of *B. henselae* with human ECs.

Under physiological conditions, Rac1 governs the formation of distinct actin cytoskeletal structures, such as lamellipodia and membrane ruffles (see chapter 1.2.1). As a matter of control, a series of experiments were performed to assess whether the dominant-negative derivate of Rac1 could inhibit formation of and membrane ruffles and whether constitutively active derivatives of Rac1 promoted these structures in human ECs.

For this purpose, the activity of endogenous Rac1 in human ECs, transfected with a dominant-negative version of Rac1, was stimulated with the phospholipid sphingosine-1-phosphate, comparing the cellular response of transfected versus non-transfected cells. Whereas non-transfected cells were found to form both lamellipodia and membrane ruffles, transfected cells did not, validating the appropriateness of the assay system used (see chapter 3.1.2, Figure 5B). On the other hand, human ECs were analyzed for their capacity to form lamellipodia and membrane ruffles after transfection with a constitutively active version of Rac1. *Vice versa*, lamellipodia and membrane ruffles were detected in high frequency in transfected, whereas non-transfected cells did not exert this behaviour likewise, again proving the appropriateness the assay system used (see chapter 3.1.2, Figure 6A).

Next, the question was addressed whether Rac1 is localized to sites of invasome formation. For this purpose, immunocytochemical stainings of endogenous Rac1 of cells after infection employing a monoclonal Rac1 antibody were performed (data not shown). By this methodology, it was not possible to reliabily detect or localize Rac1 intracellularly, implying that Rac1 is not abundantly expressed or that anti Rac1 antibodies used are innapropriate. Therefore, further attempts may employ plasmid encoded eGFP-Rac1 fusion proteins to localize Rac1 intracellulary upon infection.

Next, the status of activation of endogenous Rac1 in human ECs as a response to invasome-mediated internalization of *B. henselae* was assessed (see chapter 3.3.2). No evidence for bacterially-induced activation of Rac1 at the time point of choice, namely at 24 hours post infection, was gained. This time point had been chosen for two reasons. First, at time points earlier than 24 hours, only few invasomes are established, thus making it rather unlikely to detect the putative activation of Rac1 earlier than 24 hours post infection in respect to invasome formation. Second, we wanted to make sure that Rac1 was not activated in response to bacterial internalization by regular phagocytosis which mainly occurs early during infection.

This is of great importance, since activation of Rac1 and Cdc42, as a consequence of internalization of *B. bacilliformis* by regular phagocytosis into HUVECs, has been demonstrated (Verma and Ihler, 2002). Herein, activation of Rac1 was detected after 1 hour until 24 hours post infection with a maximum of activation at 8 hours post infection (Verma and Ihler, 2002).

Since there are no means to uncouple these two parallel entry routes and since the kinetics of internalization by invasomes and by regular phagocytosis appear to be overlapping, it may prove to be difficult to track activation of Rac1 as a strict consequence of invasome-mediated internalization.

The requirement for Rho-family small GTPases during the process of internalization by mammalian host cells varies. For instance, the invasion of human ECs by *N. meningitidis* and Group B streptococci requires the action of Rho and Rac1, but not Cdc42 (Eugene et al., 2002; Shin and Kim, 2006), whereas the invasion of human ECs by *R. conorii* requires Cdc42, but not Rac1 (Martinez and Cossart, 2004). In contrast, invasion of human ECs by *E. coli* K1 requires Rac1 but not Cdc42.

In turn, invasome-mediated internalization of *B. henselae* by human ECs requires the action of Rac1 – and to a lower extent of Cdc42 – but not of RhoA. Internalization of *B. bacilliformis* by HUVECs has been shown to require and activate equally all Rho-family small GTPases, RhoA, Rac1 and Cdc42 (Verma et al., 2000; Verma and Ihler, 2002). Thus, many variations of the same theme are observed, reflecting different strategies and modes of subversion of the host cell actin cytoskeleton in the process of internalization.

The role of cellular effector proteins downstream of Rac1 for invasome formation.

Next, actin-dependent signalling events necessary for invasome-mediated internalization downstream of Rac1 were analyzed. GTP-loaded, activated Rac1 governs various cellular effector proteins in control of actin dynamics and assembly. Three major pathways participating in the organization of the actin cytoskeleton downstream of Rac1 have been identified.

The first pathway is controlled by p21-activated kinase (PAK1), which, on one hand, regulates contractility of the actino-myosin cytoskeleton through the engagement of myosin light chain kinase II (Zeng et al., 2000) and, on the other hand, regulates the activity of LIM kinase (Lin et al., 2003), which inihibits cofilin, an actin depolymerising protein, by phosphorylation (Dawe et al., 2003).

The second pathway is controlled directly by Rac1 (Eden et al., 2002) engaging actin adaptor proteins Scar/WAVE (Machesky and Insall, 1998), in order to activate and recruit the Arp2/3 complex (Machesky and Gould, 1999) which nucleates actin at branching filaments and at barbed ends of filaments (Bailly et al., 2001).

The third pathway is controlled by phosphatidylinositol-4 phosphate-5 kinase (PIPK5) (Oude Weernink et al., 2004), which engages ERM (ezrin/moesin/radixin) proteins, controlling actin organization at the cell cortex (Bretscher et al., 2002).

Involvement of the first and the second pathway for invasome formation have been further analyzed so far.

The involvement of PAK1 in the process of invasome-mediated internalization was analyzed by means of western blot analysis. Upon interaction with activated Rac1 or Cdc42, but not RhoA, PAK1 becomes autophosphorylated at position Ser144 and Thr423 in order to promote its own kinase activity (Bokoch et al., 1998; Zenke et al., 1999). To test, whether PAK1 is activated during bacterial internalization, HUVECs were infected with different genotypes of *B. henselae* (as described for Rac1 pulldown assays, see chapter 3.2.2) and total cell lysates were prepared, separated by SDS-PAGE, electrotransferred to nitrocellulose membranes and probed with polyclonal anti (phospho-Ser144) PAK1 sera (data not shown). However, phosphorylation of Ser144 was not detected under these experimental conditions, indicating that Rac1-induced activation of PAK1 was not occuring.

The involvement of Scar/WAVE adaptor proteins in the process of invasome-mediated internalization was analyzed by means of transfection followed by infection. SCAR (suppressor of cAMP receptor)/WAVE [WASP (Wiskott-Aldrich syndrome protein)-family verprolin homology protein] proteins are members of the conserved WASP family of cytoskeletal regulators (Eden et al., 2002; Steffen et al., 2004), which play a critical role in actin dynamics by triggering Arp2/3 (actin-related protein 2/3)-dependent actin nucleation (Machesky and Insall, 1998) at barbed ends and branching filaments (Machesky and Gould, 1999). Scar1 is able to bind and activate the Arp2/3 complex, which promotes *de novo* polymerization of F-actin.

Plasmids encoding full-length Scar1 (Scar-FL) and an Arp2/3-binding derivative of Scar1 (Scar-WA) (Martinez and Cossart, 2004) were delivered into human ECs by transfection. Overexpression of these proteins titrate endogenous Arp2/3 complexes, thereby impairing actin polymerization. Both, Scar-FL and Scar-WA, elicited a deleterious effect on the integrity of the actin cytoskeleton with only few stress fibers visible, which are otherwise typically found in these cells *in vitro* (see chapter 3.1.2, Figure 7A and Figure 7B), proving that these variants of Scar were functional in the cellular system used.

Subsequent infections with different genotypes of *B. henselae* (MOI=100, 48 hours) revealed that invasome-mediated internalization is severly impaired as suggested by a decrease of frequencies of invasome formation of -25% (Scar-FL) and -35% (Scar-WA) (see chapter 3.1.2, Figure 7C). To test whether the Arp2/3 complex is recruited to sites of invasome formation in response to Scar1-mediated activation immunocytochemical stainings were performed. Indeed, the Arp2/3 complex was found to be enriched at sites of invasome formation, namely the basal ring-like structure and the meshwork of F-actin engulfing the bacterial aggregates, as suggested by the detection of the Arp3 subunit with monoclonal antibodies (see chapter, 3.1.2, Figure 7D). This finding highlights that internalization involves both rearrangement of preexisting F-actin fibers as well as *de novo* actin polymerization driven by the Arp2/3 complex, whose recruitment and activation is dependent on the action of Rac1 and Scar/WAVE.

During cellular invasion of *Salmonella*, both Rac1 and Cdc42 get activated. In the case of *Salmonella*, actin polymerization by the Arp2/3 complex is triggered by two pathways in parallel, one involving Rac1/Scar/WAVE (Criss and Casanova, 2003; Shi et al., 2005; Stender et al., 2000) and one involving Cdc42/N-WASP (Rohatgi et al., 1999) to potentiate the host cell response regarding actin assembly during invasion (Unsworth et al., 2004). Similarily, *B. henselae* may trigger this parallel pathway of actin polymerization as well, as suggested by the parallel requirement of Rac1 and Cdc42 for invasome-mediated internalization of *B. henselae* (see chapter 3.1.2, Figure 5).

Mechanistic aspects of invasome formation.

Bacterial adhesion to and invasion of non-phagocytic cells occurs by means of two dedicated mechanisms, usually referred to as "trigger"- or "zipper"-type of induced phagocytosis (Cossart and Sansonetti, 2004).

The trigger mechanism of uptake is characterized by extensive membrane ruffles being erected by host cells in response to translocated effector proteins, causing membrane ruffles to fold over and to trap adhering bacteria. Enteroinvasive bacteria such as *Salmonella* or *Shigella* employ the trigger mechanism for internalization.

The zipper mechanism of uptake is characterized by receptor-mediated, firm adhesion of the bacterium to the cell surface which is followed by modest actin polymerization only in the immediate neighborhood of bacterial sites of attachement leading to engulfment and internalization. *Listeria* and *Yersinia* promote their own uptake by the trigger mechanism, employing cell surface molecules InlA/B and invasin respectively (see chapter 1.4.3 and 1.4.4).

Based on the fact that extensive membrane ruffles engulfing the bacterial aggregate are thrown up by the host cell in the course of internalization and that internalization is accompanied by massive F-actin rearrangement and polymerization, suggests that invasome formation represents rather a "trigger" than a "zipper" mechanism of induced phagocytosis. However, there is one major difference between induced phagocytosis of *Salmonella* or *Shigella* and *B. henselae*. In the first two cases only single bacteria are internalized, whereas in the latter case bacterial aggregates are internalized.

The intracellular fate of *B. henselae*.

Independently of their capacity to form invasomes all strains of *B. henselae* employed in this study were also found to be internalized by regular phagocytosis into BCVs suggesting that this mode of uptake is independent from the respective bacterial genotype. Assessment of the intracellular fate of *B. henselae* revealed that the sustained actin cytoskeletal reorganization during invasome-mediated internalization might represent a mechanism to bypass phagocytotic uptake (May and Machesky, 2001) by establishment of an intracellular niche which is not fusogenic with the phago-lysosomal pathway. BCVs perinuclearily located in the host cell cytoplasm were found to acquire the prototype marker protein of lysosomes, Lamp-1, which is indicative for the entry of BCVs into the degradative endocytic pathway.

On the other hand, the membranes engulfing bacterial aggregates contained in invasomes did not acquire this marker protein. This might indicate that invasome formation might protect bacterial aggregates from harmful interactions with the degradative endocytic pathway. Herein, the role of the extensive F-actin meshwork, surrounding the membrane-wrapped bacterial aggregate, remains to be elucidated. It may be hypothesized, that invasomes could serve as a putative protective niche providing an intracellular reservoir permissive for growth or egress and dissemination of the bacterium in later stages of infection.

Several gram-negative bacterial pathogens such as *Legionella pneumophila* (Kagan and Roy, 2002; Kagan et al., 2004; Nagai et al., 2002) or *Brucella abortus* (Celli et al., 2003; Celli et al., 2005; O'Callaghan et al., 1999) employ their T4SS and their effector proteins to redirect vesicular trafficking of macrophages to establish an intracellular niche for replication. However, there is a marked difference between the mechanisms of these pathogens and *B. henselae* how to escape phago-lysosomal fusion.

Initially, wild-type and T4SS-deficient *Legionella* or *Brucella* are internalized equally into the same vacuoles. However, only wild-type bacilli are able to redirect vesicular trafficking subsequently and to prevent fusion with the phago-lysosomal pathway, whereas T4SS-deficient bacilli enter this pathway being unable to redirect vesicular trafficking. In contrast, *B. henselae* wild-type appears to lack this intrinsic capacity to prevent phago-lysosomal fusion once they are internalized by regular phagocytosis, as suggested by single wild-type bacilli residing in BCVs, which are fusogenic with Lamp-1 positive vesicles. Yet, by employing its VirB/VirD4 T4SS and by promoting invasome formation while being still extracellular, *B. henselae* is able to bypass the process of regular phagocytosis and to direct its own uptake into a specialized intracellular compartment, which is not fusogenic.

***In vivo* relevance of invasome formation.**

Invasome-mediated internalization of *B. henselae* by human ECs has not been demonstrated *in vivo*. This is mainly due to the fact, that there is no appropriate experimental animal model available for mimicking *B. henselae* infection of the incidental human host. Nevertheless, it might be speculated that the observed bacterial aggregates associated to BA or BP lesions of diseased individuals (Kostianovsky and Greco, 1994; Manders, 1996) may represent invasomes established during the infection *in vivo*. Clumps of bacteria are found in intimate contact with proliferating ECs and infiltrates of leukocytes characteristic for these lesions. Evidence for invasome formation *in vivo* would highlight the importance of this process of host cell subversion under pathophysiological conditions and provide insights into how *B. henselae* is able to persistently infect and colonize the vasculature.

6. OUTLOOK

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This chapter highlights open questions regarding the internalization of *B. henselae* by human ECs and describes experimental approaches, which appear to be applicable.

BepA-BepG.

A strategy to identify BepY might be again by extracellular complementation. Mixed infections of strains expressing exclusively BepC (i.e. *ΔbepA-G/*p*bepC*) or the the BepY candidate (i.e. *ΔbepA-G/*p*bepA/B/D/E/F*) should make it possible to restore the capability to promote F-actin rearrangements characteristic for invasome formation. Another possibility might be co-expression of two compatible plasmids in the background of the effectorless strain (e.g. *ΔbepA-G/*p*bepC/*p*bepY*).

A prime candidate for the enigmatic BepY might be BepF, for the simple reason, that it exhibits a modulatory effect on the host cell actin cytoskeleton during infection (see chapter 3.2.4). Clearly, further studies are necessary to understand this phenomenon in more detail and to be able to attribute a function of these actin foci in the process of internalization of *B. henselae* and maybe even to invasome formation. Whether tyrosine-phosphorylation and the observed effect of BepF on the host cell actin cytsoskeleton correlate has to be clarified in the future accordingly. Interestingly, BepF harbours an N-terminal WxxxE-motif (i.e. -W(362)-EVS-E(366)-), as does BepG. Whether this WxxxE-motif is functional or not, may be assessed accordingly to BepG, by introduction of single amino acid exchanges in the open reading frame of BepF (e.g p*bepF (W362A)* and p*bepF (W366A)* and by subsequent infection of human ECs by appropriate tester strains (e.g. *ΔbepA-G/*p*bepF (W362A)* and *ΔbepA-G/*p*bepF (W366A)*).

Likewise, the conserved sequence motif of BepC (i.e. –HPFxxGNG-) might be mutated and analyzed. Furthermore, the N-terminally encoded FIC domain of BepC might be truncated or deleted and the function of these derivatives tested by mixed infections (see chapter 3.2.3) and to assess the function of this putative effector domain.

To further elucidate the role of the four BID domains, BID-G1 to BID-G4, (see chapter 3.1.2, Supplementary Figure 1) and to delineate the effector domain of BepG, one might attempt the approach of truncations of the open reading frame of BepG. Starting from the N-terminus, one might systematically delete one module after the other, e.g. deletion of the most N-terminal domain BID-G1 (p*bepGΔ*$_{1–148}$) or consecutive deletion of a BID-G1 and DUF1a (p*bepGΔ*$_{1–260}$), and assess the ability of these truncated versions of BepG to promote invasome formation (e.g. *ΔbepA-G/* p*bepGΔ*$_{1–148}$ or *ΔbepA-G/* p*bepGΔ*$_{1–260}$). Likewise, this type of analysis may be extended by the introduction of internal deletions into the open reading frame of BepG, by erasing single BID domains (e.g. BID-G2, p*bepGΔ*$_{271–412}$) or DUF domains (e.g. DUF1a, p*bepGΔ*$_{217–260}$) to assess essential or redundant domains necessary for the function of BepG. Another possibility would be to introduce these truncations or internal deletions into the eGFP-reporter plasmid of BepG (peGFP-BepG, pTR1778) and assess the effect on the subcellular localization to the actin cytoskeleton, such as F-actin stress fibers, filopodial cell extensions or the submembraneous cortical F-actin (see chapter 3.1.2, Supplementary Figure 3).

Host cell proteins upstream of Rac1 with a putative role in invasome formation.

Future attempts in elucidating host cell signalling events involved in the process of invasome formation might include the study of mediators in the control of the host cell actin cytoskeleton integrating cellular signalling upstream of Rac1, namely PI3K (Schulte et al., 1998) and Src-family kinases (Dehio et al., 1995; Dumenil et al., 2000), which play important roles during the invasion process of many gram-negative bacterial pathogens.

PI3-K is engaged during the invasion of endothelial host cells by *N. menigitidis* (Eugene et al., 2002; Lambotin et al., 2005), *R. conorii* (Martinez and Cossart, 2004) and by *E. coli* K1 (Chen et al., 2002; Reddy et al., 2000; Sukumaran et al., 2003; Sukumaran and Prasadarao, 2002) promoting the direct activation of Rac1.

c-Src is required for the invasion of endothelial host cells by *N. menigitidis* (Hoffmann et al., 2001; Lambotin et al., 2005) and *R. conorii* (Martinez and Cossart, 2004). c-Src is a pivotal tyrosine kinase mediating signals from receptor tyrosine kinases to downstream molecules leading to the engagement of Rac1.

Frequently, experimental approaches analyzing the involvement of these signalling components employ cell-permeable drugs to block these kinases (i.e. wortmannin to block PI3K or genistein to block tyrosine kinases). However, this attempt may be hampered by the fact that they are either quickly catabolized or have a cytotoxic effects over prolonged incubation times in cellular assays. Therefore, it might be applicable – due to the prolonged time of coculture of *B. henselae* and human ECs necessary for invasome formation - to transfect dominant-negative versions of these proteins in order to titrate endogenous signalling, in analogy to the example of Rho-family small GTPases. Alternatively, the possibility exists to employ post-transcriptional gene silencing by RNA intereference for the depletion of selected target proteins (Hannon and Rossi, 2004; Meister and Tuschl, 2004), which might prove to be instrumental for the study host cell proteins required for invasome formation.

Other host cell proteins with a putative role in invasome formation.

Cortactin has been shown to be involved in the uptake of *N. menigitidis* by human ECs (Lambotin et al., 2005) and is engaged as well during the uptake of *H. pylori* into gastric epithelial cells in a CagA-dependent manner (Backert and Selbach, 2005; Naumann, 2005; Selbach et al., 2003) in order to promote cortical actin rearrangements leading to the formation of membrane ruffles. The engulfment of bacterial aggregates during internalization of invasomes might likewise require the action of this adaptor protein, which can stimulate actin polymerization by direct interaction with the Arp2/3 complex.

FAK has been found to be engage during internalization of *R. conorii* (Martinez and Cossart, 2004) and *S. aureus* (Agerer et al., 2005) by human ECs. FAK is triggered by the action of integrins and controls – in conjunction with c-Src - potential substrates such as tensin, zyxin, paxillin and p130cas involved in the assembly and disassembly of focal adhesion complexes. Modulation of focal adhesion dynamics may be of importance during the rearrangement of F-actin fibers in the course of invasome formation of *B. henselae*, since these F-actin stress fibers are twisted and spooled up to give rise to the basal ring-like structure characteristic for invasomes.

Interaction partners.

On the molecular level, all these questions and approaches converge on the putative interaction of bacterial effector proteins and host cell proteins. Identification of interaction partners is essentail to further extend the knowledge of the mechanisms underlying the internalization of *B. henselae* by human ECs. This may be attempted by biochemical approaches such as co-immunoprecipitation assays, crosslinking assays, pulldown assays or genetic approaches such as yeast two hybrid screen for interaction partners.

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Refereed Publications

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