Subversion of endothelial cell functions
by the human pathogen *Bartonella henselae*

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My thesis is written in a cumulative format. It consists of a synopsis about a topic related to my work (Apoptosis and its modulation by bacterial pathogens), followed by chapters, each presenting a manuscript with a short summary. Finally, I resume the major findings of my thesis in the section concluding remarks.

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Apoptosis and its modulation by bacterial pathogens

General introduction

The term apoptosis was introduced in 1972 by John Kerr and is derived from the Greek meaning "falling off". It describes a distinct form of cell death whereby cells die in a tightly regulated fashion. In contrast to apoptosis, also known as programmed cell death, necrosis is a degenerative process in which cells swell and lyse after irreversible tissue injury (Hengartner, 2000). Apoptosis is characterized by changes including nuclear and cytoplasmic condensation, membrane budding, internucleosomal DNA fragmentation and the formation of apoptotic bodies (Kerr et al., 1972). Apoptosis acts as a homeostatic mechanism for controlling cell proliferation, because excessive death may lead to compromised development or degenerative disease, while the lack of cell death could result in proliferative disorders (Reed, 2003). In this synopsis, I will concentrate on bacterial pathogens that employ different strategies to modulate the programmed cell death of the infected host cell. In general, the specific modulation of the host cell fate enhances bacterial spreading and enables bacterial survival in the infected host.

The first part of this synopsis introduces the basic and relevant molecular mechanisms of apoptosis. The second part discusses the mechanisms by which bacterial pathogens induce and/or block apoptosis, and the implications of these modulations on pathogenesis and diseases.

Molecular mechanisms of apoptosis

Caspases: the central executioners

The central component of the apoptotic machinery is a family of cysteine proteases. These proteases called caspases (cysteine aspartyl-specific proteases) (Thornberry, 1998) belong to a family of intracellular enzymes that cleave their substrate at aspartic acid (Asp) residues. Caspases are often categorized as either upstream "initiator" or downstream "effector" caspases (Salvesen and Dixit, 1997). Upon induction of apoptosis, initiator caspases are activated. The active initiator caspases trigger then the activation of the effector caspases resulting in a caspase cascade (Salvesen and Dixit, 1999). The targets of activated effector caspases

appear to be crucial in maintaining the cell architecture, in RNA splicing, and in DNA repair (Table 1).

Human caspase-1, -4, and -5 are classified in a different subgroup, the so called inflammatory caspases. Although there is evidence that these caspases are involved in apoptosis, their primary function is the regulation of inflammatory processes. They mediate the processing of pro-inflammatory cytokines, particularly pro-Interleukin-1β (pro-IL-1β) and pro-IL-18 (Martinon and Tschopp, 2004).

Table 1. *Classification of the caspases.*

Caspase (Cysteine Aspartate Specific Protease)		
Class	*Characteristics*	*Name*
Initiators	First to be activated. Cleave and activate the effector caspases.	Caspase-2 Caspase-8 Caspase-9 Caspase-10
Effectors	Cleave cellular substrates.	Caspase-3 Caspase-6 Caspase-7
Inflammatory	Process cytokines.	Caspase-1 Caspase-4 Caspase-5

Death receptors

Two major pathways have been described to induce apoptosis, an extrinsic and an intrinsic mechanism (Figure 1, pA-3). Both result in the activation of caspases. The extrinsic pathway is initiated by an extrinsic signal and mediates the activation of the tumor necrosis factor receptors (TNFR) superfamily , also known as the death receptors. Two well-characterized death receptors are Fas and the tumor necrosis factor receptor 1 (TNFR1). In their cytoplasmic tails, they carry the so-called "death domains" (DD) (Ashkenazi, 2002). Upon ligand binding to the extracellular domain, the DDs interact with adapter proteins like Fas-associated DD (FADD) protein and TNFR-associated DD (TRADD) for transduction of the apoptotic signal. These adapter proteins then interact and active the initiator caspases (Ashkenazi, 2002).

Figure 1*. The intrinsic and extrinsic apoptotic pathway.* The intrinsic pathway is initiated by the permeabilization of the mitochondrial membrane. The extrinsic pathway is triggered after ligand binding of the death receptors. Both result in the activation of executioner caspases.
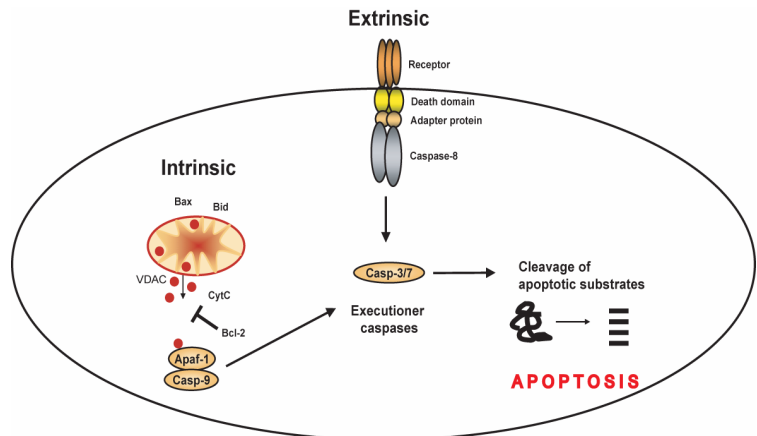

Mitochondrial control of cell death

The intrinsic pathway to induce apoptosis is controlled by the mitochondrium. The mitochondrium is able to release a potent cocktail of pro-apoptotic proteins into the cytoplasm of the cell. The main player among these proteins is cytochrome c. Once cytochrome c is released from the mitochondrium to the cytosol, it associates with Apaf-1 (apoptotic protease activating factor 1) and pro-caspase-9 to form a complex known as the Apoptosome (Li et al., 1997). The exact mechanism, how cytochrome c manages to cross the mitochondrial outer membrane is not yet known. Nevertheless, it is proposed that pore-forming proteins like the voltage-dependent anion channel (VDAC) and members of the Bcl-2 family are involved in this process (Shimizu et al., 1999) (Figure 1, pA-3).

The Bcl-2 family

The Bcl-2 family consists of pro-apoptotic and anti-apoptotic Bcl-2 proteins controlling the release of cytochrome c from the mitochondrium (Figure 2, pA-4). The pro-apoptotic proteins like Bax and Bak might operate as pore formers or ion channels and induce the release of cytochrome c through the outer mitochondrial membrane, whereas the anti-apoptotic proteins Bcl-2 and Bcl-X_L suppress this release (Kuwana and Newmeyer, 2003). The Bcl-2-family proteins share defined homology between each other. This homology is found in one to four regions designated the Bcl-2 homology (BH) domains BH1-4. Some pro-apoptotic family members, such as Bad, Bid, and Bim, contain only one homology domain, the BH3 region. This group is also defined as BH3-only proteins. Several BH3-only proteins

can inactivate anti-apoptotic Bcl-2 family members and thereby induce apoptosis (Kelekar and Thompson, 1998).

Figure 2. *Balance of apoptosis*. The amount of pro- and anti-apoptotic Bcl-2 family members controls the permeabilization of the outer mitochondrial membrane and the release of cytochrome c.
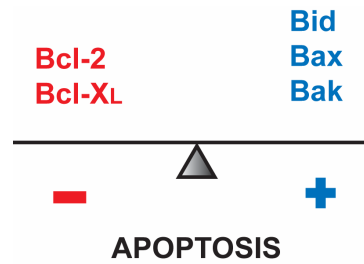
The "inhibitor of apoptosis" gene family

The inhibitor of apoptosis (IAP) proteins family was initially identified in the baculoviral genome. They all contain baculovirus IAP repeat (BIR) domains, which are essential for the anti-apoptotic properties (Fesik, 2000). Although IAP-family members like XIAP, cIAP1, cIAP2, and survivin possess other functions, most of them bind and potentially inhibit activated caspases. Usually the effector caspase-3 and -7, and/or the initiator caspase-9 are inhibited by IAPs (Deveraux and Reed, 1999). This inhibition of caspases by IAP differs from the Bcl-2 family related apoptotic suppressors. Whereas Bcl-2 proteins prevent initiation of apoptosis by inhibiting the sequestering of pro-apoptotic signals from the mitochondrium, IAPs are able to inhibit already activated caspases.

Modulation of apoptosis by bacterial pathogens

Bacterial control of apoptosis

Bacterial pathogens have developed different strategies to modulate the apoptotic pathway of their host cell (Dockrell, 2001; Hueffer and Galan, 2004; Zychlinsky and Sansonetti, 1997). Pathogen-induced activation of the host cell-death pathway may serve to eliminate key immune cells or evade host defenses. Several bacterial pathogens induce apoptosis by inducing caspase activation, which also can be involved in inflammation. The elicited accompanied inflammation process at the place of infection leads to disruption of the surrounding tissue barriers. This might facilitate the initiation of infection and secures an efficient microbial spread in the host. In contrast, inhibition of apoptosis might be essential for intracellular pathogens to protect their invaded host cells from collapsing. Thereby they facilitate their replication and persistence in the infected host. Activation and inhibition of apoptosis by bacterial pathogens thus plays an important role in a variety of infectious diseases.

Induction of apoptosis by bacterial pathogens

To induce apoptosis, a number of pathogens possess virulence factors, which can activate the death pathway of the host cell or interfere with survival pathways (Navarre and Zychlinsky, 2000; Weinrauch and Zychlinsky, 1999). These bacterial virulence factors induce apoptosis by a variety of mechanisms such as (i) pore-forming toxins, which interact with the host cell membrane and permit the leakage of cellular components, (ii) toxins inhibiting host-cell protein synthesis, (iii) direct injection of effector proteins into the host-cell cytoplasm via a type III secretion system, (iv) effectors of the type IV secretion system, (v) superantigens targeting immune cells, and (vi) other effectors.

(i) Pore-forming toxins

Staphylococcus aureus is a Gram-positive coccus that causes a number of diseases, including carbuncles, wound infections, toxic shock syndrome, and food poisoning (gastroenteritis) (Manders, 1998). *S. aureus* mediates apoptosis either by a pore-forming α-toxin or by superantigens as discussed later. The α-toxin (or α-hemolysin) of *S. aureus* belongs to the channel-forming toxins and is active

against erythrocytes as well as a range of other cells including epithelial cells and endothelial cells (Menzies and Kourteva, 2000; Wesson et al., 2000). Two mechanisms are proposed for the induction of apoptosis by α-toxin. The first one suggests that secretion of α-toxin at low levels results in its association to the eukaryotic cell membrane and the formation of small ion-permeable pores which initiate cell death (Jonas et al., 1994). The second one suggests that intracellular *S. aureus* uses α-toxin to escape from the phagosome into the cytoplasm. Upon evasion of the phagosome apoptosis is induced by targeting the pore-forming α-toxin to the mitochondrial membrane, resulting in the release of cytochrome c (Figure 3, p. A-14) (Bantel et al., 2001; Essmann et al., 2003)

Pore-forming toxins are also found in **Listeria monocytogenes**, a Gram-positive rod. *L. monocytogenes* is an opportunistic food-borne human and animal pathogen responsible for serious infections predominantly in immunocompromised individuals, pregnant women, and neonates (Portnoy et al., 1992). After internalization, *L. monocytogenes* lyses the phagosomal membrane and escapes into the cytoplasm. This process is mediated by listeriolysin O (LlyO), a bacterial secreted pore-forming toxin (Guzman et al., 1996). Intracellular *L. monocytogenes* can induce apoptosis in a variety of cells *in vivo* and *in vitro*, but a bacterial escape of the phagosome is required. It is thought that the cytoplasmic LlyO permeabilizes mitochondrial membranes in a similar way as the α-toxin from *S. aureus* resulting in cytochrome c release (Figure 3, p. A-14) (Rogers et al., 1996).

Another bacterial pore-forming toxin, which was described to induce apoptosis, is the outer membrane protein PorB of **Neisseria gonorrhoeae**. *N. gonorrhoeae* is a Gram-negative, human-specific intracellular bacterial pathogen that colonizes the genital or rectal mucosa, causing the sexually transmitted disease gonorrhea (Naumann et al., 1999). It is thought that apoptosis of mucosal epithelial cells supports the access of the gonococci to deeper tissues during infection. It has been proposed that PorB translocates by a yet undefined mechanism from the outer bacterial membrane to the mitochondrium, where it forms a pore in the membrane. These pores lead to an efflux of cytochrome c from the mitochondrium into the cytosol resulting in the induction of the intrinsic apoptotic pathway (Figure 3, p. A-14) (Muller et al., 2000). PorB shares structural and functional homology with the mitochondrial VDAC, suggesting that it may represent an analogue or precursor of this putative central regulator of apoptosis (Muller et al., 2002). The same authors

have previously shown that PorB also translocates to the cytoplasmic cell membrane, leading to an influx of calcium, and then calpain and caspase-3 activation (Muller et al., 1999). Importantly, PorB of *N. meningitidis* was also described as an anti-apoptotic factor, contradicting observations in *N. gonorrhoeae*. This will be discussed in more detail in the section "inhibition of apoptosis by bacterial pathogens".

The Gram-negative pathogenic bacterium **Helicobacter pylori** is the causing agent of chronic gastritis, which may develop to a lymphoma of the stomach. Beside the Cag pathogenicity island (Cag-PAI), encoding a type IV secretion system (T4SS), the vacuolating cytotoxin VacA has been reported to be an important pathogenic factor, although its precise role in *H. pylori* virulence is still unknown (Monack et al., 2004; Reyrat et al., 1999). VacA is a secreted protein that, among other functions, induces cellular vacuolization in epithelial cells. Ectopic expression of VacA as well as external application of VacA to human tumor cell lines induces apoptosis. VacA is targeted to the mitochondrium, where it initiates the release of cytochrome c. This effect can be inhibited by co-expressing Bcl-2, a known inhibitor of mitochondrial membrane permeabilization (Galmiche et al., 2000). Induction of apoptosis by VacA *in vivo* is based on the disruption of the epithelial barrier and the leakage of nutrients into the place of *H. pylori* infection (Monack et al., 2004).

(ii) Protein synthesis inhibitors

The diphtheria toxin (DT) produced by **Corynebacterium diphtheriae**, a Gram-positive extracellular pathogen, is the causative agent of the respiratory disease diphtheria (Popovic et al., 2000). DT can induce apoptosis in several epithelial and myeloid cell lines (Morimoto and Bonavida, 1992). Expression of DT by *C. diphtheriae* requires an integrated bacteriophage genome encoding the toxin gene (Cianciotto and Groman, 1997). DT belongs to the family of secreted A-B toxins. The B subunit mediates attachment to the cell through a host receptor and facilitates the delivery of the catalytic A subunit to the cytoplasm. The A subunit of DT causes ADP-ribosylation of the elongation factor 2 (EF2), an essential co-factor of the protein synthesis machinery. ADP-ribosylation of EF2 blocks the protein synthesis of the eukaryotic cell and leads to cell death (Chang et al., 1989; Holmes, 2000). Other studies indicate that DT-mediated apoptosis involves additionally a cellular apoptosis susceptibility protein (CAS) (Brinkmann et al., 1995).

Other bacterial pathogens inducing apoptosis by protein synthesis inhibitors are **Shigella dysenteriae** and enterohemorrhagic **Escherichia coli** *(EHEC)*, the etiological agents of dysenteric syndromes. These bacteria produce similar A-B toxins, called Shiga toxin (ST) and Shiga-like toxin (SLT) (Karmali, 2004). The catalytic A subunit cleaves eukaryotic rRNA and thereby disrupts eukaryotic ribosome function and protein synthesis. The B subunit, mediating the internalization of the A subunit into the host cell, binds to a host cell receptor specifically expressed on cells from the kidney and in the central nerve system (CNS). Significantly, macrophages do not express this receptor and are not susceptible to ST or STL cytotoxicity (Herold et al., 2004).

(iii) Type III secreted proteins

Type III secretion systems (T3SS) are specialized organelles of Gram-negative bacterial pathogens that deliver proteins to host cell membranes and the cytosol (Hueck, 1998). The T3SS apparatus is a needle-like structure which spans the inner and outer membranes of the bacterial envelope and secretes translocon and effector proteins. The structure of the needle is similar to that of the flagellar basal body. Translocon proteins mediate pore formation in the host cell membrane and translocated effector proteins subvert different aspects of host cell physiology, thereby promoting bacterial virulence (Waterman and Holden, 2003).

Shigella spp. are the causative agents of bacillary dysentery and bloody diarrhea (Jennison and Verma, 2004). Induction of apoptosis of macrophages by *Shigella* is mediated by the plasmid-encoded invasion plasmid antigen B (IpaB) (Hilbi et al., 1998). Upon phagocytosis by the macrophage, *Shigella* escapes from the phagosome into the cytoplasm. There, *Shigella* injects the pro-apoptotic IpaB effector protein into the cytoplasm of the macrophage by its T3SS. After translocation, IpaB binds to and activates caspase-1, resulting in macrophage apoptosis (Figure 3, p. A-14) (Zychlinsky et al., 1992). Caspase-1 activation and apoptotic death of *Shigella*-infected macrophages causes the release of mature IL-1β, which results in an inflammatory response. This leads to the recruitment of leukocytes, which compromises the integrity of the epithelial barrier during transmigration. The induction of an inflammatory response might be essential for the spreading of the bacteria into the deeper epithelium of the intestine (Dockrell, 2001; Zychlinsky and Sansonetti, 1997).

Salmonella spp. cause a variety of clinical syndromes, including a self-limiting gastroenteritis in humans (Galan, 2001; House et al., 2001). *Salmonella* induces rapidly apoptosis of macrophages during its logarithmic-growth phase depending on the T3SS that is encoded by the *Salmonella* pathogenicity island 1 (SPI-1). Upon phagocytosis by the macrophage, *Salmonella* resides and replicates within an acidified phagosome. Once *Salmonella* has escaped from this phagosome, it secretes the *Salmonella* invasive protein B (SipB) into the cytoplasm of the host cell by using its T3SS. SipB is a homologue of IpaB and binds directly to and activates caspase-1 (Figure 3, p. A-14) (Hersh et al., 1999; Hueffer and Galan, 2004). However, *Salmonella* can also induce macrophage cell death that occurs in a delayed manner. In that situation *Salmonella* uses a different T3SS that is encoded by a second pathogenicity island, SPI-2 (van der Velden et al., 2000). Besides the DNA fragmentation reported in this study, additional biochemical events are still unknown.

Yersinia spp. harbors a T3SS like *Shigella* and *Salmonella* and is able to induce apoptosis in macrophages. *Y. pestis* is the causative agent of bubonic plague, *Y. pseudotuberculosis* and *Y. enterocolitica* can cause gastrointestinal symptoms and septicemia (Cornelis, 2000). In contrast to *Shigella* and *Salmonella,* which both induce apoptosis of macrophages after invasion, *Yersinia* is able to induce apoptosis without being internalized by the macrophage. Upon adherence, *Yersinia* injects by its T3SS *Yersinia* effector proteins (Yop) across the bacterial and host cell membrane into the cytoplasm of the macrophage (Mills et al., 1997). Apoptosis is caused by the effector YopP/J and is accompanied by the activation of the pro-apoptotic Bcl-2 family protein BID, the release of cytochrome c, and the activation of caspase -3/-7 (Denecker et al., 2001) (Figure 3, p. A-14). In the cytoplasm, YopP/J represses the activation of transcription factor NFκB (Schesser et al., 1998). NFκB is known to induce the expression of genes which are involved in a pro-inflammatory response and in the inhibition of apoptosis. This defines the NFκB pathway also as a survival pathway of the cell (Karin and Lin, 2002). In addition YopP/J interferes with the mitogen-activated protein kinase (MAPK) signaling pathway by binding to multiple MAPK kinases (MAPKKs) (Orth et al., 1999). It was also shown that YopP/J can act as an ubiquitin-like protein (SUMO-1) protease, thereby deregulating post-translational protein modification (Orth et al., 2000). Apoptosis induced by YopP/J

might thus be a result of the activation of a death mechanism and/or due to the inhibition of the survival pathway NF-κB (Cornelis, 2002; Ruckdeschel et al., 2001).

(iv) Type IV secreted proteins

Similar to the previously described T3SS, the T4 secretion machineries allow the translocation of bacterial macromolecules across bacterial and host cell membranes. T4SS are evolutionary related to the conjugal transfer system. Bacterial pathogen use T4SS to deliver effector molecules to eukaryotic target cells during infection. Upon translocation, bacterial effector proteins subvert the host cell function and they can contribute in different ways to the infection process (Cascales and Christie, 2003).

Legionella pneumophila is a Gram-negative facultative intracellular bacterium. It is the causative agent of Legionnaire's disease, and invades and replicates within alveolar macrophages, monocytes, and possibly alveolar epithelial cells (Bitar et al., 2004). Inside the cell, *L. pneumophila* resides within a specialized phagosome that does not acidify and fails to fuse with the lysosomes. Induction of apoptosis of these cells is mediated by the activation of the executioner caspase-3. The expression of apoptosis-inducing factor(s) by *L. pneumophila* might be regulated by the Dot/Icm T4SS, as several secretion deficient *dot/icm* mutants fail to induce apoptosis (Gao and Abu Kwaik, 1999). Other reports indicate that *Legionella*-induced cytotoxicity may be caused by osmotic lysis upon pore formation in the macrophage cytoplasmic membrane (Bitar et al., 2004; Kirby and Isberg, 1998) or by the activation of the intrinsic mitochondrial death pathway (Neumeister et al., 2002). As factors encoded by the *dot/icm* gene cluster of *Legionella spp*. are essential for altering phagosome properties and are as well required for pore formation, it can be speculated that *Legionella*-induced apoptosis might be due to pore formation upon translocation of unknown pro-apoptotic effector proteins through the Dot/Icm secretion system.

(v) Superantigens

Beside the already described pore-forming α-toxin, **Staphylococcus aureus** harbors as additional virulence factors superantigens (Manders, 1998). Bacterial superantigens are proteins that interact with immune cells and stimulate potent immune responses. Superantigens activate T cells by directly binding to major

histocompatibility complex (MHC) class II molecules on antigen-presenting cells and to the T-cell receptor (TCR) on T cells. Activation of the TCR through superantigens like staphylococcal exotoxin (SE) A and B induces apoptosis in T cells. The precise pathway how this activation results in apoptosis is not yet known (Baker and Acharya, 2004; Goodyear and Silverman, 2004). A possible mechanism for SEB mediated cell death is that SEB induces the expression of the death receptor Fas on normal human T lymphocytes which results in Fas-ligand-triggered apoptosis (Ettinger et al., 1995; Sohn et al., 2003).

(vi) Other effectors

Mycobacterium tuberculosis is the etiologic agent of the tuberculosis. Macrophages infected with *M. tuberculosis* undergo increased rates of apoptosis which is accompanied by the release of the cytokines TNF-α and IL-10 (Rojas et al., 1999). The production of TNF-α by macrophages and the induction of apoptosis can be mediated by binding of bacterial cell wall components and/or lipoproteins to the Toll-like receptor-2 (TLR-2) (Quesniaux et al., 2004). A putative pro-apoptotic candidate is a cell-associated and secreted 19-kDa glycoprotein which is able to induce apoptosis dependent on TLR-2 (Lopez et al., 2003). In addition to the pathway described above, downregulation of expression of the anti-apoptotic protein Bcl-2 has also been shown in *M. tuberculosis*-infected macrophages (Figure 3, p. A-14) (Klingler et al., 1997). Paradoxically, *M. tuberculosis* can also protect cells against apoptosis through a TLR-2-dependent mechanism, as discussed in the following section.

Chlamydia spp. is also a bacterial genus that is able to modulate the apoptotic pathways of its host cell in two opposing directions (Fischer et al., 2004; Perfettini et al., 2002). *Chlamydia* are obligate intracellular pathogens causing a broad range of diseases in humans, including trachoma, sexually transmitted diseases, and pneumonia (Byrne and Ojcius, 2004). The pro- and anti-apoptotic effect of *Chlamydia* is thought to be dependent on (i) the stage of the infection, (ii) the infected host cell, and (iii) the different biovars (Byrne and Ojcius, 2004). It is proposed that induction of apoptosis during the late state of infection facilitates bacterial spread and re-infection.

Inhibition of apoptosis by bacterial pathogens

Many bacteria live within the cytoplasm or the endocytotic compartment of the cell. Inside the cytoplasm, the pathogenic bacteria have unlimited access to the host cell metabolites, which serves as nutritional source. In addition, hidden inside the host cell, the bacterial pathogens are protected from the immune system. Bacterial pathogens use different strategies to inhibit cell death of their host cell: (i) hijacking a pro-survival pathway of the host cell, (ii) secretion of anti-apoptotic effector proteins through a T4SS, and (iii) other mechanisms.

Several bacteria described for their abilities to induce apoptosis in the previous section will appear in this section again, because they are also able to inhibit apoptosis at a different stage of infection.

(i) Hijacking a pro-survival pathway

The pathogen **M. tuberculosis**, capable of inducing apoptosis in macrophages, was in few reports described to also inhibit apoptotic cell death in macrophages by two different pathways. Firstly, through enhancement of the production of a soluble death receptor sTNFR2. The sTNFR2 binds surrounding TNF-α, which leads to the neutralization of the pro-apoptotic activity of TNF-α (Balcewicz-Sablinska et al., 1998). The release of sTNFR2 by infected macrophages is regulated by the cytokine IL-10. Secondly, bacterial lipoproteins binding to TLR-2 might activate the transcription factor NF-κB and thereby induce the NF-κB survival pathway (Figure 3, p. A-14) (Means et al., 2001; Toossi et al., 1997).

A second bacterial pathogen that shows modulation of pro-survival and pro-apoptotic host cell signals is **Chlamydia** (Byrne and Ojcius, 2004). The anti-apoptotic activity of *Chlamydia spp*. is believed to be important during the initial stage of invasion where it relies on the host cell integrity and metabolism for its restricted intracellular lifestyle. *Chlamydia*-infected host cells are resistant to a variety of pro-apoptotic stimuli. It has been shown that the activation of NF-κB during *C. pneumoniae* infection is associated with the protection of macrophages against apoptosis (Wahl et al., 2001). A recent study revealed a additional protection mechanism by which *C. pneumoniae* inhibits host cell apoptosis by the degradation of pro-apoptotic BH3-only proteins (Figure 3, p. A-14) (Fischer et al., 2004). Interestingly, genes encoding a T3SS have been identified in *Chlamydia spp*. (Hsia

et al., 1997). Whether the bacteria use this putative secretion system to mediate their anti- and pro-apoptotic effects remains to be elucidated.

Rickettsia rickettsii is an obligate intracellular bacterium that causes Rocky Mountain spotted fever. During *in vivo* infection, the vascular endothelial cell is the primary target cell for *R. rickettsii* invasion and replication (Silverman and Bond, 1984). *In vitro*, *R. rickettsii* protects endothelial cells from apoptotic cell death by activating the NFκB pro-survival signaling pathway (Figure 3, p. A-14) (Clifton et al., 1998; Joshi et al., 2004). NFκB-mediated inhibition of apoptosis and enhanced host cell survival during the course of infection likely facilitates the growth and multiplication of the intracellular rickettsiae.

(ii) Type IV secreted proteins

Bartonella henselae is a Gram-negative zoonotic pathogen, capable of infecting humans as incidental hosts. *B. henselae* is the causing agent of cat-scratch disease in immunocompetent patients (Dehio, 2004). In immunocompromised patients, *B. henselae* has the property to induce vascular tumor formation, described as bacillary angiomatosis (Dehio, 2003). *B. henselae*-infected endothelial cells are protected from apoptotic cell death (Kirby and Nekorchuk, 2002). It is proposed, that inhibition of apoptosis by *B. henselae* contributes to the observed vascular tumor formation (Kirby, 2004; Kirby and Nekorchuk, 2002). As described in chapter 2 (p. C-3), inhibition of apoptotic cell death by *B. henselae* requires a functional VirB/VirD4 T4SS (Schmid et al., 2004). The identification of *Bartonella* effector proteins (Bep), translocated into the host cell via the VirB/VirD4 T4SS, is reported in chapter 3 (p. D-3). In chapter 4 (p. E-3), we identified the *Bartonella* effector protein A (BepA) mediating the anti-apoptotic activity during endothelial cell infection. During HUVEC-infection, BepA induces an increased intracellular cAMP level in the host cell and a transcriptional activation of cAMP-responsive genes. In HUVEC, it is known that an elevated cAMP level can protect the cells from apoptotic cell death (Hippenstiel et al., 2002). Hence, we propose a model, in which BepA protects endothelial cells from apoptosis by increasing the cAMP level in the host cell (Figure 3, p. A-14).

(iii) Other Mechanisms

In contrast to PorB from *Neisseria gonorhoeae*, which induces apoptosis, PorB from **N. meningitidis** was described to act as anti-apoptotic factor. Although both molecules share a similar structure and are both targeted to the mitochondrium, the outcome of this interaction seems to be oppositional and is a matter of debate (Massari et al., 2000; Muller et al., 2000).

The anti-apoptotic effect of PorB is thought to be mediated through protein-protein interactions with the voltage-dependent anion channel (VDAC) of the mitochondrium. PorB is assumed to lead to closure of the VADC is closed and thereby inhibiting the release of cytochrome c (Figure 3, p. A-14) (Massari et al., 2000).
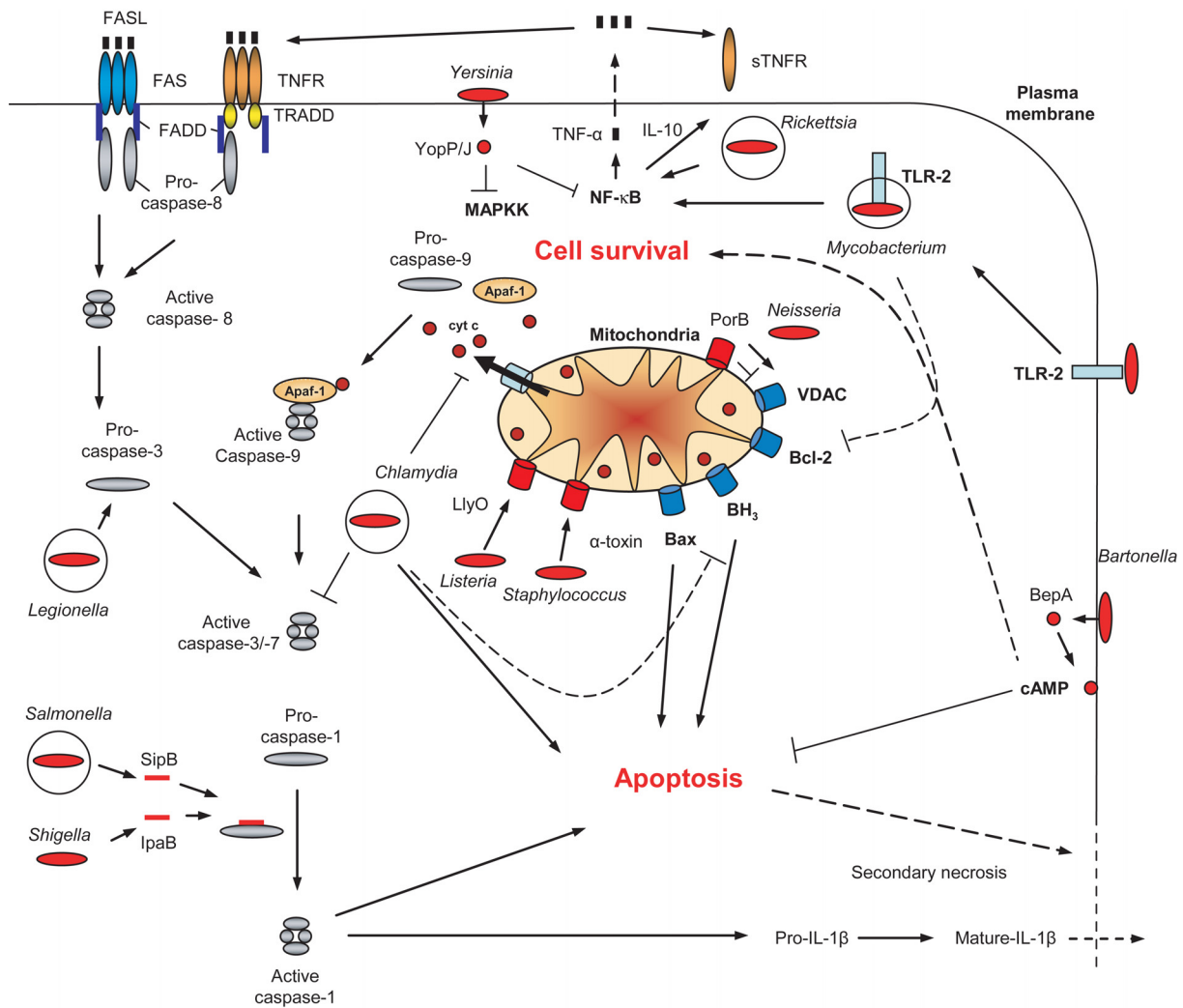
Figure 3. *The modulation of host cell apoptosis by bacterial pathogens*. Details of the various mechanisms are given in the text. Adapted from "Bacterial Disease Mechanism", Cambridge.

Conclusions

Numerous pathogens were shown to modulate apoptosis. The mechanisms to interfere with the apoptotic machinery differ between different pathogens. This includes (i) the alteration of the host cytoplasmic- or mitochondrial membrane by pore forming toxins (*Neisseria, Listeria, Staphylococcus, Helicobacter*) (ii) inhibition of protein synthesis (*Corynebacterium*) (iii) injection of pro- or anti-apoptotic effector molecules into the host cell via secretion systems (*Shigella, Salmonella, Yersinia, Bartonella*) (iv) activation or inhibition of the NFκB survival pathway (*Rickettsia, Chlamydia*) and (v) unbalancing pro- and anti-apoptotic effectors in the host (*Chlamydia, Mycobactrium*). It is difficult to draw a sharp line between the classified mechanisms, which often overlap. In addition, several bacterial pathogens use more than one strategy to modulate host cell apoptosis. Such modulation can lead to elimination of key defense cells that are necessary to eradicate the pathogen, or can facilitate intracellular trafficking of the bacteria, enhance intracellular bacterial replication, release intracellular bacteria after termination of replication, and facilitate bacterial spread. The majority of the here-described pathogens have facultative intracellular life cycles. They are highly adapted to infect a distinct part of the host, including mucosal layers, the endothelium, Peyer's-patches, and the stomach. To survive and replicate in the host, they depend on the preservation of their niche. The interference with host cell apoptosis must therefore be fine-tuned to ensure transmission to new hosts.

Although the subversion of the host cell fate by interfering with the apoptotic pathway is an important virulence factor for bacterial pathogens, only a few mechanisms are understood on the molecular level. The best-characterized mechanisms are the one of *Shigella* and *Salmonella*, where the bacterial factor as well as the eukaryotic response is identified. In these species, induction of apoptosis is a major process during the physiological infection course. In other bacteria, either the bacterial factor or the eukaryotic effector molecules are known. Like in the case of *Yersinia*-induced apoptosis, where the bacterial effector protein, its translocation, and its interaction with the host cell machinery is well established, the apoptosis inducing mechanism still needs further investigation. In contrast, in *Chlamydia*-inhibited apoptosis, where the cell death protection mechanism of the eukaryotic cell has recently been elucidated, the bacterial factors are still unknown.

In our work with *Bartonella*, we identified the anti-apoptotic bacterial factor BepA, which is translocated into the host cell by a T4SS. Furthermore, we propose a putative protection mechanism through which the host cell circumvents apoptosis. Based on our studies, we are able to introduce a second anti-apoptotic bacterial factor to the already controversially described PorB of *Neisseria*. Thereby BepA represents the first T4SS substrate modulating the apoptotic pathway of the host cell.

In summary, modulation of apoptosis reveals further insights into the complex relationship between hosts and bacterial pathogens and is a challenging topic in the field of bacterial pathogen-host interaction. A better understanding of the molecular mechanisms of the pro- and anti-apoptotic activities may help to prevent and cure bacterial infections.

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Bartonella henselae induces NFκB-dependent upregulation of adhesion molecules in cultured human endothelial cells: possible role of outer membrane proteins as pathogenic factors

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Bartonella are unique among bacterial pathogens in stimulating endothelial cell proliferation, which results in the formation of vascular tumors (Dehio, 1999). In these vascular tumors, called vasoproliferative lesions, *B. henselae* localizes around, and within endothelial cells, indicating that the vascular endothelium represents a target tissue for extra- and intracellular colonization *in vivo*. The vasoproliferative lesions are typically infiltrated by polymorphonuclear neutrophiles (PMN) and monocytes, which is indicative of an inflammatory response (Manders, 1996). The pro-inflammatory response is mediated by endothelial cells, which upon activation induce expression of adhesion molecules and release of cytokines. These events promote binding and extravasation of PMN, respectively.

Here we report the invasion and pro-inflammatory activation of human umbilical vein endothelial cells (HUVEC) upon infection with the *B. henselae* strain *Berlin-1*, which was isolated from a bacillary angiomatosis lesion of an immunodeficient patient. First, we investigated the ability of *Berlin-1* to colonize and invade endothelial cells. We infected HUVEC and monitored the invasion process of the bacteria by immunocytochemistry. As previously shown for other *B. henselae* isolates, the *Berlin-1* strain was able to invade HUVEC by two distinct routes (Dehio et al., 1997): (i) by the classical endocytotic pathway, whereby intracellular bacteria localize after invasion in a peri-nuclear endosomal compartment and (ii) by the formation of bacterial aggregates, which are internalized by a unique cellular structure, termed as invasome. Next, we tested whether *Berlin-1* infection of HUVEC results in the activation of a pro-inflammatory response. The pro-inflammatory activation was determined by monitoring the rolling and adherence of PMN on the HUVEC surface in a flow chamber. After 8 h of infection, HUVEC showed enhanced rolling and adhesion of PMN. During a pro-inflammatory response, the rolling and

adhesion of PMN is mediated by the adhesion molecules E-selectin and ICAM-1 (Cook-Mills and Deem, 2005). To test whether the observed enhanced rolling and adhesion of PMN upon *Berlin-1* infection is also dependent on these adhesion molecules, we applied different antibodies to block receptor-bacteria interaction. By blocking the E-selectin adhesion molecules, we abolished PMN rolling and adherence. This indicates that E-selectin is central for PMN-endothelium interaction. In contrast, blocking of the adhesion molecules ICAM-1 and VCAM-1 did not inhibit PMN–HUVEC interaction at 8 h post-infection. Next, we used ELISA to analyze the expression of adherence molecules in the course of bacterial infection. During the first 12 h of bacteria-host cell interaction, E-selectin expression was strongly increased, whereas at later time points (around 24 h) the expression of ICAM-1 was elevated. These increased expression of E-selectin and ICAM-1 correlated with transcriptional upregulation at the according time points, as shown by Northern-blot analysis.

Adhesion molecules involved in a pro-inflammatory response are transcriptionally regulated by nuclear factor κB (NFκB) (Karin and Lin, 2002). To determine the activation of NFκB upon *Berlin-1* infection, we used a bandshift assay. Infection of HUVEC resulted in the activation of NFκB, which was independent of the viability of the bacteria and independent of bacterial LPS. Inhibition of NFκB during *Berlin-1* infection abolished the increased expression of E-selectin and ICAM-1, indicating that NFκB mediates the pro-inflammatory response triggered by *Berlin-1* during HUVEC infection.

To identify putative *Bartonella* effectors mediating the activation of endothelium, the outer membrane protein (OMP) fraction of *Berlin-1* was purified. These OMPs were sufficient to activate endothelial cells in a dose-dependent manner as monitored by ICAM-1 and E-selectin expression.

Statement of the own participation

I contributed to this publication by the following data: To demonstrate that the *B. henselae* strain *Berlin-1* adheres to and invades human endothelial cells, I performed HUVEC-infection assays followed by immunocytochemical analyses (Fig. 1).

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Bartonella henselae Induces NF-κB-Dependent Upregulation of Adhesion Molecules in Cultured Human Endothelial Cells: Possible Role of Outer Membrane Proteins as Pathogenic Factors

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The endothelium is a specific target for *Bartonella henselae*, and endothelial cell infection represents an important step in the pathogenesis of cat scratch disease and bacillary angiomatosis. Mechanisms of *Bartonella*-endothelial cell interaction as well as signaling pathways involved in target cell activation were analyzed. *B. henselae* strain Berlin-1, isolated from bacillary angiomatosis lesions of a human immunodeficiency virus-infected patient, potently stimulated human umbilical cord vein endothelial cells (HUVEC), as determined by NF-κB activation and enhanced adhesion molecule expression. These effects were accompanied by increased PMN rolling on and adhesion to infected endothelial cell monolayers, as measured in a parallel-plate flow chamber assay. Monoclonal antibodies against E-selectin significantly reduced PMN rolling and adhesion. In our hands, *B. henselae* Berlin-1 was substantially more active than the typing strain *B. henselae* ATCC 49882. E-selectin and ICAM-1 upregulation occurred for up to 9 days, as verified by Northern blotting and cell surface enzyme-linked immunosorbent assay. Induction of adhesion molecules was mediated via NF-κB activation and could be blocked by a specific NF-κB inhibitor. Additional studies indicated that *B. henselae*-induced effects did not require living bacteria or *Bartonella* lipopolysaccharides. Exposure of HUVEC to purified *B. henselae* outer membrane proteins (OMPs), however, reproduced all aspects of endothelial cell activation. In conclusion, *B. henselae*, the causative agent of cat scratch disease and bacillary angiomatosis, infects and activates endothelial cells. *B. henselae* OMPs are sufficient to induce NF-κB activation and adhesion molecule expression followed by enhanced rolling and adhesion of leukocytes. These observations identify important new properties of *B. henselae*, demonstrating its capacity to initiate a cascade of events culminating in a proinflammatory phenotype of infected endothelial cells.

Bartonella henselae, a gram-negative, fastidious, rod-shaped bacterium, can cause several human diseases, the most prominent being cat scratch disease, a persistent, necrotizing lymphadenitis (2, 4, 38). The domestic cat is the main reservoir of *B. henselae*, and the cat flea, *Ctenocephalides felis*, has been established as the vector in cat-to-cat transmission (15). An increasing number of other clinical manifestations such as endocarditis, osteolytic lesions, pulmonary nodules, neuroretinitis, and fever of unknown origin have been documented (1, 7, 11, 24, 43). In immunocompromised patients *B. henselae* infection can result in bacillary peliosis hepatitis (BPH). Another typical *Bartonella*-related disease is bacillary angiomatosis (BA), which is characterized by lesions resembling those produced by Kaposi's sarcoma (27–29).

Judged from the histology of BPH and BA lesions bacteria are in direct contact with the endothelium, probably promoting endothelial cell proliferation and angiogenesis. Therefore, endothelial cells appear to be a specific and unique target of the genus *Bartonella* (17). A detailed analysis of bacteria-endothelial cell interaction is vital for understanding the pathophysiology of these *Bartonella*-induced emerging diseases.

Exposure of endothelial cells to the bacterium has been shown to result in aggregation, engulfment, and subsequent internalization of the rods, forming a singular and well-defined host cellular structure, the invasome (18; for a review see reference 19), but uptake by phagocytosis has also been described (8, 18). *B. henselae*-related virulence factors and the target cell reaction upon infection are poorly defined (5, 9, 39).

Rolling and adhesion of circulating polymorphonuclear leukocytes (PMN) to endothelial cells, an early step in an acute inflammatory reaction, are governed by several adhesion molecules such as selectins and intercellular adhesion molecule 1 (ICAM-1) (12, 32). Multiple receptor-ligand pairs act sequentially and in an overlapping manner to effect initial attachment, rolling, firm adhesion, and transmigration of leukocytes. Several receptor-ligand interactions are involved in PMN adhesion to activated endothelial cells: interactions between E-selectin and sialyl Lewis carbohydrate-containing molecules

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(34, 47), P-selectin and P-selectin glycoprotein ligand 1, and ICAM-1 and β_2-integrins (23).

A prerequisite for expression of E-selectin and other proinflammatory mediators is activation and subsequent translocation of NF-κB, which is involved in the transcription of genes associated with the immune response (33, 45, 50).

The main objective of this study was to assess the ability of *B. henselae* to infect and to activate cultured human endothelial cells. For this purpose we used the first European *B. henselae* isolate obtained from BA lesions of a human immunodeficiency virus (HIV)-infected patient (Berlin-1) (3). We compared this isolate with highly passaged phenotypically and genotypically indiscernible typing strain ATCC 49882 (Houston-1). We focused on endothelial signal transduction resulting in enhanced adhesion molecule expression and PMN-endothelial cell interaction. To further characterize effects induced by *B. henselae* (Berlin-1), we also made use of enriched outer membrane protein (OMP) preparations.

The data presented indicate that *B. henselae* bacteria as well as *B. henselae*-derived OMPs induce an NF-κB-dependent upregulation of E-selectin and ICAM-1 in endothelial cells, which in turn results in enhanced PMN rolling and adhesion.

(This work was done in partial fulfillment of the requirements for a Ph.D. by O. Fuhrmann.)

MATERIALS AND METHODS

Materials. Tissue culture plastic ware was obtained from Becton Dickinson (Heidelberg, Germany) and Nunc (Wiesbaden, Germany). MCDB 131 medium, Hanks balanced salt solution, phosphate-buffered salt solution (PBS), trypsin-EDTA solution, HEPES, and fetal calf serum (FCS) were from GIBCO (Karlsruhe, Germany). Collagenase (CLS type II) was purchased from Worthington Biochemical Corp. (Freehold, N.J.). Specific inhibitor of NF-κB translocation caffeic acid phenethyl ester (CAPE) (40) was obtained from Biomol (Hamburg, Germany). Tumor necrosis factor alpha (TNF-α) was from R&D Biosystems (Wiesbaden, Germany). Lipopolysaccharide (LPS) from *Salmonella enterica* serovar Abortus equi was a kind gift from C. Galanos, Freiburg, Germany. All other reagents were purchased from Sigma Chemical Co. (Munich, Germany).

Abs. Purified freeze-dried monoclonal antibodies (MAbs) directed against E-selectin (1.2B6), vascular cell adhesion molecule 1 (VCAM-1) (1G11), and ICAM-1 (15.2) were from Dianova (Hamburg, Germany), and horseradish peroxidase-conjugated polyclonal sheep anti-mouse immunoglobulin G (IgG) antibodies (Abs) were from Amersham Pharmacia (Freiburg, Germany). All Abs used were azide free.

Rabbits were inoculated with 3.5×10^7 CFU of heat-inactivated *B. henselae* Houston-1 (ATCC 49882) in 1 ml of saline intravenously. Blood was collected 4 weeks postinoculation (p.i.), and serum samples were prepared according to standard protocols. The titer of *Bartonella*-specific IgG Abs was measured by indirect immunofluorescence test of the preinoculation and p.i. serum samples and found to be <100 (detection limit) and 10,000, respectively. The hyperimmune serum did not show any cross-reaction at a dilution of 1:100 with other bacterial species tested.

Preparation of HUVEC. Cells were isolated from umbilical cord veins and identified according to the method of Jaffe et al. (25). Briefly, cells obtained from collagenase digestion were washed, resuspended, and cultivated in MCDB 131–10% FCS and seeded into flasks. Confluent monolayers were split once, and first-passage human umbilical cord vein endothelial cells (HUVEC) were used.

Isolation of human PMN. Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient to yield a PMN fraction of >97% purity as previously described (31).

Bacterial strains and growth media. *B. henselae* strain Berlin-1, isolated from bacillary angiomatosis lesions of an HIV-infected patient in Germany, has been characterized previously (3). Low-passage bacteria were grown to logarithmic phase in brucella broth supplemented with 7% Fildes (Unipath Ltd., Basingstoke, United Kingdom) and 250 μg of hemin (EGA-Chemie, Steinhausen, Germany)/ml.

B. henselae strains ATCC 49882 and ATCC 49793 were obtained from the American Type Culture Collection (ATCC; Manassas, Va.). They had originally been isolated from blood of HIV-infected patients. All ATCC *Bartonella* strains were grown on Columbia agar (Difco Laboratories, Detroit, Mich.) supplemented with 7% defibrinated sheep blood (Oxoid, Wesel, Germany) in a humidified atmosphere at 37°C and 5% CO_2.

***B. henselae* OMP preparation.** OMPs were enriched by sarcosyl treatment of total-membrane preparations (20, 49). Bacteria were grown in supplemented brucella broth and harvested during the logarithmic phase by 30 min of centrifugation at $3,000 \times g$ at 4°C. After being washed twice with ice-cold PBS, bacteria were resuspended in 30 ml of PBS. DNase and RNase (1 mg each) were added to the solution, followed by sonication (15 cycles at 2 min each; Branson Sonifier; duty cycle, 50%; output, 5) on ice. To remove residual bacteria, the suspension was centrifuged for 15 min at $3,000 \times g$, followed by additional centrifugation of the clarified supernatant at $20,000 \times g$ for 30 min at 4°C (Beckman; J2–21, JA 20 rotor). The pellet was then resuspended in 1 ml of PBS. After that, the suspension was mixed on ice with 1 ml of sarcosyl (2% in PBS)/ml of pellet. Incubation for 1 h on ice was followed by centrifugation at $20,000 \times g$ for 30 min (Beckman; J2–21, JA 20 rotor). The final membrane pellet, containing enriched OMPs, was then resuspended in 1 ml of PBS and stored in aliquots at -70°C. Protein concentration was determined using the Bradford protein assay (6). Ten micrograms of protein in an equal volume of sample buffer was heated at 95°C for 3 min and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

***Bartonella* infection assay.** Prior to infection with *B. henselae*, HUVEC (passage 1) were washed three times with MCDB 131 medium without supplements or antibiotics. Bacterial concentrations were adjusted to 10^8 bacteria/ml, and appropriate dilutions were prepared. For infection, bacteria were added in a bacteria-to-eukaryotic-cell ratio of 50:1 and centrifuged on a HUVEC monolayer at $1,500 \times g$ for 5 min. After 2 h plates were washed extensively with plain medium and subsequently incubated in MCDB 131–2% FCS. Medium was replaced every 24 h. At times indicated in the figure legends cells were processed for NF-κB electrophoretic mobility shift assay (EMSA), Northern blotting for E-selectin and ICAM-1, cell surface enzyme-linked immunosorbent assay (ELISA), and neutrophil rolling and adhesion assays under defined flow conditions. Where indicated in the figure legends, NF-κB-specific inhibitor CAPE was added 1 h before bacterial infection. In some experiments, inactivation of *B. henselae* was achieved by heating (65°C for 30 min). LPS was inactivated by addition of 50 μg of polymyxin B/ml immediately before exposure to HUVEC.

Immunofluorescence analysis of *B. henselae* infection. After stimulation of HUVEC grown on glass chamber slides (Falcon CultureSlide; Becton Dickinson, Rutherford, N.J.) with 50 bacteria/endothelial cell for the appropriate time, cells were fixed with 4% paraformaldehyde for 15 min. Permeabilization of the cell membrane was achieved by addition of 0.1% Triton X-100 in PBS for 5 min. The primary polyclonal rabbit anti-*B. henselae* Ab (1:200) was added for 30 min. Thereafter cells were washed three times with PBS and exposed to an ALEXA$_{488}$-conjugated goat anti-rabbit Ig Ab and/or ALEXA$_{568}$-phalloidin-conjugated anti-human F-actin Ig Ab (Molecular Probes, Eugene, Oreg.) for 15 min. After the cells were washed three times with PBS, coverslips were sealed and examined in an IMT-2 fluorescence microscope (Olympus Optical; equipped with an Olympus OM-4 camera) with an Olympus 60× objective.

Confocal laser scanning microscopy. Confocal laser scanning microscopy was performed as described by Dehio et al. (18). The samples stained for immunofluorescence were viewed with a TCS NT confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with an argon-krypton mixed-gas laser. In triple stainings, the three channels were recorded simultaneously. The corresponding images were digitally processed with Photoshop, version 5.0 (Adobe Systems, Mountain View, Calif.).

Preparation of nuclear extracts and EMSA. Nuclear proteins were extracted as described by Jonat et al. (26). For the NF-κB EMSA, nuclear proteins (1 μg) were incubated with a ^{32}P-labeled double-stranded consensus oligonucleotide probe 5′-AGT TGA GGG GAC TTT CCC AGGC-3′ (sense strand). Briefly, 0.07 pmol of NF-κB consensus oligonucleotides in 10 mM HEPES (pH 7.9)–50 mM KCl–0.2 mM EDTA–2.5 mM dithiothreitol–50 pmol of salmon sperm DNA–10% glycerol–0.05% NP-40 was incubated with 1 μg of soluble HUVEC nuclear proteins. The binding reaction was performed for 30 min at room temperature. Protein-DNA complexes were resolved on 7% native acrylamide gels run in 5 mM Tris (pH 8.3)–38 mM glycine before vacuum drying and autoradiography. Specificity was shown by addition of a 20-fold excess of unlabeled competitor consensus oligonucleotides.

Northern blot analysis. RNA was extracted using the guanidinium isothiocyanate method described by Chomczynski and Sacchi (14). Total RNA was quantified by measuring absorbance at 260 nm with a Uvikon 860 spectrophotometer (Kontron, Neufahrn, Germany). RNA samples (10 μg/lane) were electropho-

resed on denaturing 1% formaldehyde-agarose gels and transferred onto a Magna nylon membrane (MSI, Westborough, Mass.) and fixed by exposure to UV radiation using a Hoefer UVC 500 cross-linker.

cDNA probes were labeled with [α-^{32}P]dCTP (>3,000 Ci/mmol) by random priming (Rediprime DNA labeling system; Amersham Pharmacia) and added to the prehybridization chambers at 10^6 cpm/ml and incubated for 12 to 16 h at 42°C. The E-selectin cDNA probe was a kind gift from D. Simmons (Institute of Molecular Medicine, Oxford, United Kingdom). The ICAM-1 cDNA probe was prepared by reverse transcription-PCR with custom-designed primer pair 5′-A AAGGATGGCACTTTCCCAC-3′ and 5′-TTCCCCTCTCATCAGGCTAGA C-3′ (Amersham Pharmacia). The 598-bp cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained as previously described (22). Following hybridization, the filters were washed to a stringency of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 30 min at 55°C. Membranes were autoradiographed overnight at −70°C by exposure to Hyperfilm MP (Amersham Pharmacia). After exposure, blots were stripped in 50% formamide–10 mM NaH$_2$PO$_4$ for 1 h at 70°C before subsequent rehybridization. To account for a difference in loading or transfer of the RNA, hybridization was performed with a ^{32}P-labeled GAPDH cDNA probe.

Cell surface ELISA for E-selectin and ICAM-1 expression. E-selectin and ICAM-1 expression on endothelial cells preincubated with *B. henselae* was determined by cell surface ELISA (46). Confluent pretreated HUVEC monolayers in 96-well flat-bottom microtiter plates were washed and finally fixed with 4% paraformaldehyde for 15 min. Human Ig was used to reduce nonspecific binding, and primary Abs were added for 30 min. Thereafter, cells were washed three times and exposed to a horseradish peroxidase-conjugated rabbit anti-mouse Ig Ab for 30 min. After the cells were washed, *o*-phenylenediamine was added for 5 min. Data are indicated as optical densities at 492 nm.

Neutrophil rolling and adhesion assay under flow conditions. Leukocyte rolling and adhesion were determined using a parallel-plate flow chamber as described by Lawrence and Springer (32) and were evaluated as described previously (30). Confluent endothelial monolayers grown on coverslips (Thermanox; 22 by 60 mm; Nunc) were preincubated with live *B. henselae* as described above. A suspension of 3×10^6 leukocytes/ml was perfused through the chamber at a constant wall shear stress of 1.0 dyne/cm^2 (syringe pump sp100i; WPI, Sarasota, Fla.). The field of observation was chosen randomly, and interactions were visualized using a phase-contrast video microscope (with a KP-C551 charge-coupled device color camera; Hitachi, Rodgau, Germany) and videotaped (HR-S7000EG; JVC, Friedberg, Germany) during the entire time period of leukocyte perfusion. Images were recorded in real time and played back at six- or ninefold-slower speed. The tape was paused to mark the locations of cells, and the displacements of the centers of individual cells were measured 2 to 4 s later. Rolling was expressed as the number of rolling cells/high-power field during a 3-min observation period (47). Leukocytes were considered to be adherent after 30 s of stable contact with the monolayer. Adhesion was determined after 5 min of perfusion by analysis of 10 to 12 high-power (40×) fields from videotape (32).

Statistical methods. Depending on the number of groups and number of different time points studied, data were analyzed by a two-way analysis of variance. Main effects were then compared by an *F* probability test. A *P* value of <0.05 was considered significant.

RESULTS

Infection of HUVEC by *B. henselae*. During BA, bacteria are in contact with the endothelium, apparently promoting endothelial cell proliferation and angiogenesis. We exposed HUVEC to recently described *B. henselae* strain Berlin-1 (3). This isolate is phenotypically and genotypically indiscernible from typing strain ATCC 49882 but differs with respect to its low passage number and growth conditions. Exposure of HUVEC to *B. henselae* Berlin-1 resulted in bacterial adhesion and internalization. Singular bacteria appeared to be internalized by phagocytosis within 2 h (Fig. 1a). After 24 h of exposure, perinuclear formation of internalized bacteria was observed (Fig. 1b). By 7 days p.i., the internalized bacteria form a dense area around the nucleus (Fig. 1c), clearly demonstrating the ability of *B. henselae* to replicate within endothelial cells, whereas lysis of cells was not observed. Confocal laser scanning microscopy (Fig. 1d) clearly demonstrated the intra-

cellular localization of the bacteria and, furthermore, gave evidence for the internalization of *B. henselae* Berlin-1 by an invasome-like mechanism, as described for *B. henselae* ATCC 49882 and ATCC 49793 (18).

***B. henselae* induced rolling and adhesion of PMN on HUVEC.** Exposure of HUVEC to *B. henselae* resulted in a profound cell activation with subsequently enhanced PMN rolling and adhesion at 8 h p.i. as determined under flow at a shear rate of 1 dyne/cm^2. *B. henselae* turned out to be almost as potent as TNF-α (20 ng/ml), which was used as a positive control (Fig. 2). In the presence of an anti-E-selectin MAb, PMN rolling and adhesion on *B. henselae*-stimulated cells were reduced by 90%, suggesting that this endothelial adhesion molecule is central for PMN-endothelium interaction in the early phase of *B. henselae* infection. Experiments with anti-ICAM-1 and anti-VCAM-1 antibodies indicated no decrease of rolling and adhesion of PMN upon blockade of ICAM-1 or VCAM, underscoring the important role of E-selectin at 8 h p.i.

Bartonella strains ATCC 49882 and ATCC 49793 which have been passaged frequently induced only a marginal endothelial cell activation. Similarly, *B. henselae* Berlin-1 in passage 20 was only 30 to 40% as active as its low-passage-number parents (data not shown), suggesting that a high passage number is accompanied by loss of rolling- and adhesion-inducing properties. Therefore, low-passage-number *B. henselae* Berlin-1 was used in the following experiments.

***B. henselae* increased E-selectin and ICAM-1 expression in HUVEC.** Enhanced rolling and adhesion of PMN on *B. henselae*-infected HUVEC were accompanied by an increased expression of adhesion molecules on the cell surface (Fig. 3a). E-selectin expression on *B. henselae*-exposed HUVEC increased at 6 h p.i., peaked at 7 to 12 h, and declined to baseline after 20 to 24 h. Significant ICAM-1 expression occurred at 20 to 26 h p.i. and persisted for up to at least 6 days after initial contact with the bacteria. In contrast, control cells showed no increase in adhesion molecule expression.

Northern blot analysis was performed to study mRNA up-regulation for E-selectin and ICAM-1 (Fig. 3b). E-selectin mRNA peaked at 4 to 5 h after *B. henselae* stimulation, declined to almost baseline after 10 h, and was detectable again after 36 h. ICAM-1 mRNA increased by 4 h p.i. and remained elevated for as long as 216 h (9 days).

***B. henselae* induced NF-κB-dependent adhesion molecule expression in HUVEC.** NF-κB is important for regulation of the transcriptional activities of E-selectin and ICAM-1, and multiple NF-κB binding sites have been located in the promoters of the genes for both (21, 36). NF-κB activation and translocation were demonstrated by bandshift assays, which indicated the enhanced capacity of NF-κB to bind to the corresponding consensus oligonucleotides (Fig. 4a). TNF-α was used as a positive control. Exposure of HUVEC to *B. henselae* resulted in NF-κB activation and translocation within 10 min. This signal transiently peaked at 40 min p.i. and remained elevated for at least 72 h (Fig. 4b). Interestingly, HUVEC stimulated with heat-inactivated bacteria in the presence of polymyxin B displayed undiminished NF-κB activation, suggesting that live bacteria and *Bartonella* LPS are not central for NF-κB activation in HUVEC (Fig. 4a). Control experiments with a 2- to 20-fold molar excess of cold NF-κB consen-
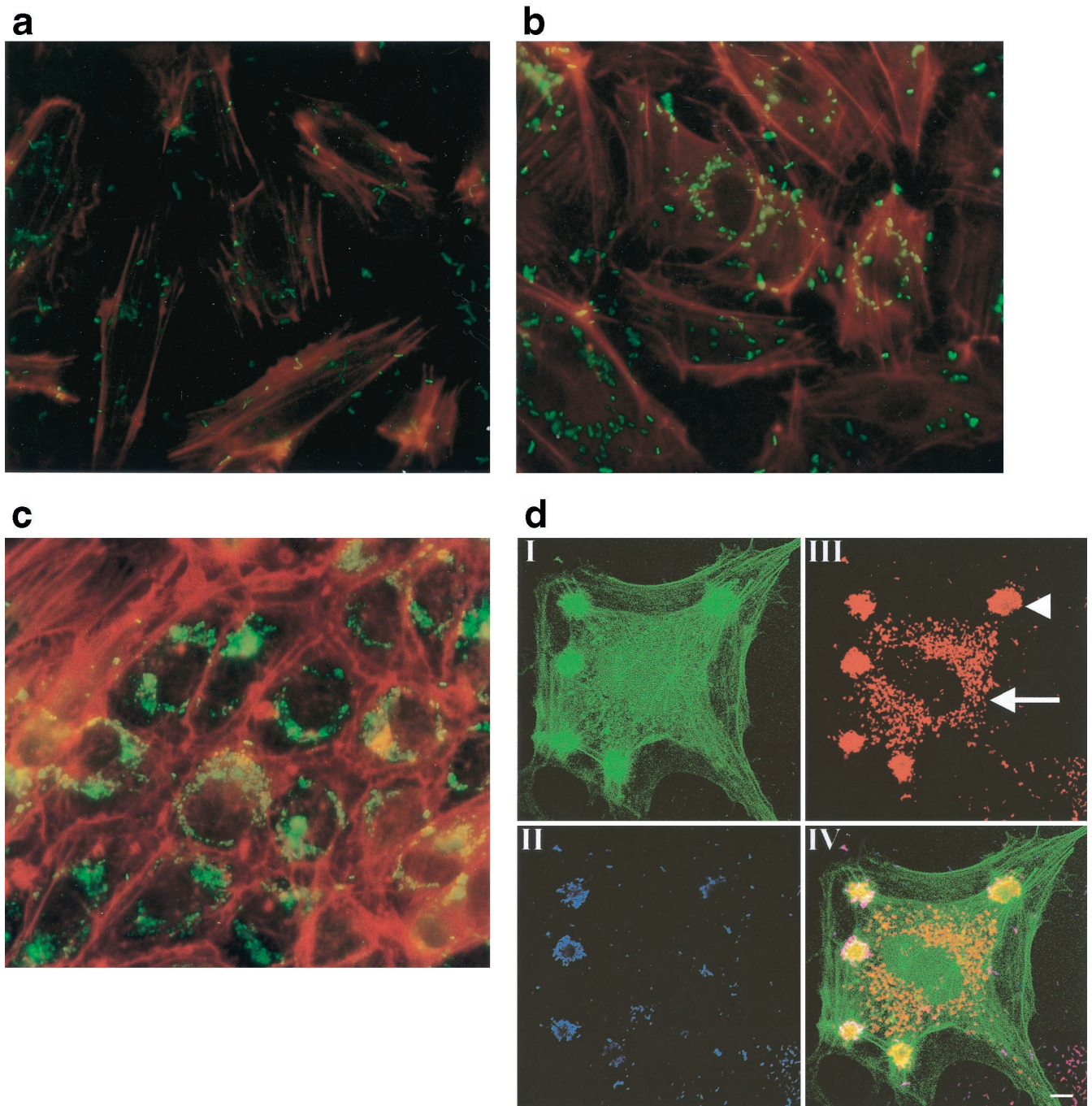
FIG. 1. Infection of HUVEC by *B. henselae* Berlin-1 is shown in double-immunofluorescence experiments. HUVEC were grown on glass chamber slides and exposed to *B. henselae* in a bacteria/cell ratio of 50:1. After the appropriate time, cells were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Anti-human F-actin MAbs and anti-*B. henselae* Abs were added; this was followed by incubation with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated secondary Abs. At 2 h p.i., singular bacteria were phagocytosed (a). At 24 h p.i. perinuclear localization of internalized bacteria was evident (b). At 7 days p.i. dense areas of bacteria were visible (c). Representative pictures from five independent experiments are shown. Magnification, ×540. Specimens were immunocytochemically stained for extracellular bacteria, intra-cellular bacteria, and the actin cytoskeleton and analyzed by confocal laser scanning microscopy (d). Prior to the permeabilization of the host cell membranes, extracellular bacteria were labeled with anti-*B. henselae* antiserum and Texas red-conjugated secondary Abs (II; blue). Following permeabilization, all bacteria were labeled with anti-*B. henselae* antiserum and Cy5-conjugated secondary Abs (III; red). The actin cytoskeleton was stained with FITC-labeled phalloidin to indicate the location of cellular structures (I; green). IV, overlay of all three channels. Intracellular bacteria appear red due to the absence of a signal in the blue channel. In contrast, extracellular bacteria appear purple as a result of the superimposition of signals in both the red and blue channels. Arrow, perinuclear localized bacteria; arrowhead, bacterial aggregate within the invasome. Bar, 10 μm.
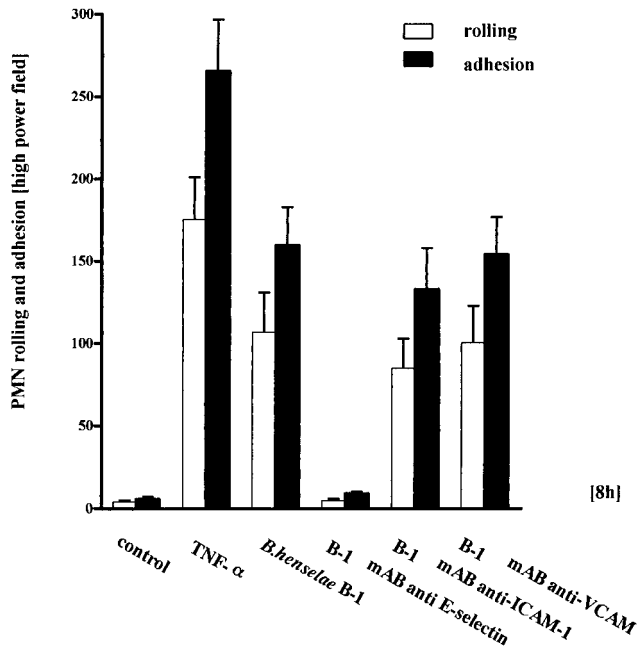
FIG. 2. PMN rolling and adhesion on *Bartonella*-exposed HUVEC under flow at a shear rate of 1 dyne/cm². Bacteria were centrifuged on endothelial cells in four-well plates containing rectangular coverslips. After 8 h coverslips were processed for a laminar-flow adhesion assay. PMN (3 × 10⁶ per ml) were injected into the flow system and perfused over endothelial cell monolayers for 5 min using a high-precision syringe pump. When indicated, cells were preincubated with Abs against E-selectin, ICAM-1, or VCAM-1 120 min before measurement. TNF-α (20 ng/ml) was used as a positive control. Rolling PMN (for a definition see Materials and Methods) were counted over a 3-min observation period. Adherent PMN were determined by counting 10 to 12 random high-power fields (see Materials and Methods). *B. henselae* Berlin-1 was almost as effective as TNF-α. Note that PMN rolling and adhesion were almost completely blocked by an anti-E-selectin MAb. Data are means ± standard errors of the means of three separate experiments.

sus oligonucleotide demonstrated the specificity of the shift (Fig. 4c).

Preincubation of HUVEC with NF-κB-specific inhibitor CAPE dose dependently reduced E-selectin mRNA and protein expression in *B. henselae*-exposed cells (Fig. 4d). The E-selectin protein level in cells preincubated with CAPE (5 and 25 μM) for 1 h was reduced dose dependently. Similar results were obtained for ICAM-1, demonstrating that NF-κB is also a pivotal transcription factor in adhesion molecule expression in *B. henselae*-exposed endothelial cells.

Preparation of *B. henselae* OMPs. The immediate endothelial cell activation by *B. henselae*, even by dead bacteria, as well as the data obtained with polymyxin B pointed to OMPs of *Bartonella* as key initiators of target cell stimulation.

OMPs of *B. henselae* Berlin-1 were purified according to a method described by Welch et al. (49). The sarcosyl fraction and a total-membrane preparation were analyzed by SDS-PAGE. Prominent bands of 92, 43, 32, 30, 28, and 23 kDa were identified (Fig. 5). These OMPs compared very well with those purified by Burgess and Anderson (9). These authors also demonstrated specific binding of *B. henselae* OMPs of 43, 32,

and 28 kDa to endothelial cells, suggesting that these OMPs represent *B. henselae* adhesins.

***B. henselae* OMPs activate endothelial cells.** *B. henselae* Berlin-1 OMPs in the range of 10 pg/ml to 1 μg/ml activated HUVEC dose dependently, as shown by increased E-selectin and ICAM-1 protein expression (Fig. 6a). Data on OMP-induced PMN rolling and adhesion resembled the data obtained for live bacteria (data not shown). OMPs in a concentration of 500 ng/ml were as potent as living *B. henselae* (compare Fig. 6 and 3) and almost as effective as TNF-α, clearly indicating that OMPs are a potent stimulus and that intracellular infection of HUVEC by *B. henselae* is not required for induction of adhesion molecule expression. Effects of *B. henselae* OMPs were not due to LPS, as indicated by Northern blot experiments and cell surface ELISAs, because polymyxin B was very efficient in blocking LPS- but not OMP-induced adhesion molecule expression (Fig. 7a). Furthermore, OMP-related endothelial cell activation was dose dependently reduced by CAPE, suggesting that OMPs also acted via NF-κB (Fig. 7a). Bandshift assays clearly indicate that OMP (500 ng/ml) induced NF-κB activation and translocation, while LPS (*S. enterica* serovar Abortus equi LPS; 50 μg/ml) had only a minor effect. In these experiments TNF-α (20 ng/ml) was used as a positive control (Fig. 7b).

DISCUSSION

This study demonstrates that *B. henselae* infects and activates human endothelial cells. *B. henselae* strain Berlin-1, isolated from a BA lesion of an HIV-infected patient, was used and was shown to upregulate, via NF-κB translocation, endothelial adhesion molecule expression, followed by increased rolling and adhesion of human PMN. Endothelial cell activation was reproduced by exposure to enriched *B. henselae* OMPs. These observations identify important new properties of this pathogen, demonstrating its capacity to initiate a cascade of events culminating in the proinflammatory phenotype of infected endothelial cells.

B. henselae strain Berlin-1 has previously been characterized extensively (3). Infection of HUVEC resulted in internalization by phagocytosis and subsequent perinuclear localization of *B. henselae*. Bacteria seem to slowly replicate within their host cells, forming a dense area around the nucleus. Lysis of the endothelial cells could never be observed, even at 7 days p.i. In addition to phagocytosis of bacteria, we observed invasome-mediated mechanisms of *B. henselae* Berlin-1 uptake, as demonstrated by confocal laser scanning microscopy (18). With respect to endothelial cell infection, Berlin-1 grown in supplemented brucella broth turned out to be substantially more effective in our hands than plated subcultures or typing strains ATCC 49882 and 49793 on agar plates (data not shown).

Avoidance of high passage number and of growth on solid media, which may be responsible for potential loss of pathogenicity, contributed to the high efficiency of *B. henselae* Berlin-1. In addition, centrifugation of bacteria on HUVEC monolayers turned out to be necessary, since exposure without prior centrifugation stimulated adhesion molecule expression by only 30%. Moreover, strict use of first-passage human endothelial cells contributed to a reproducible infection and activation of HUVEC.
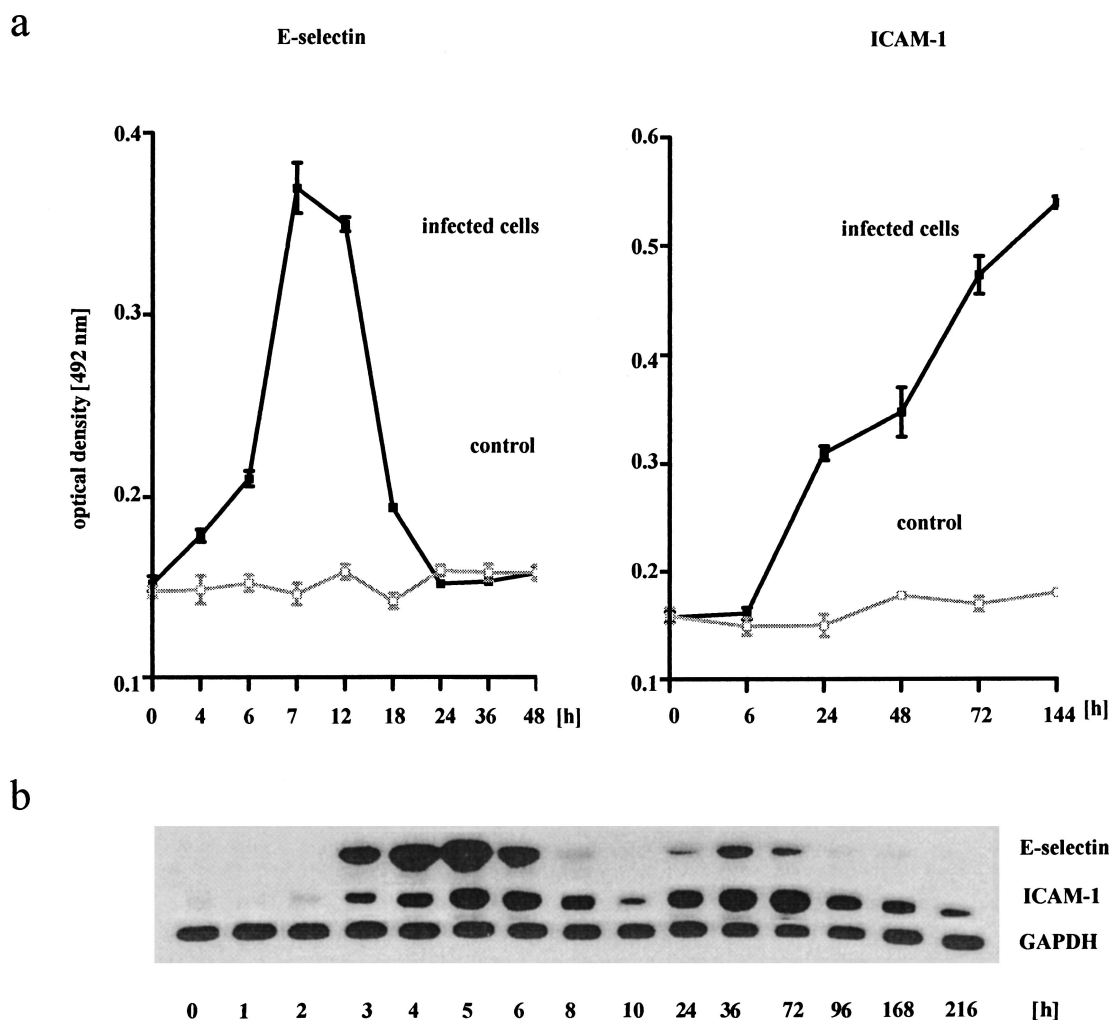
a

b

FIG. 3. (a) Enhanced expression of E-selectin and ICAM-1 in *B. henselae*-infected HUVEC. Bacteria were added to HUVEC monolayers in 96-well plates by centrifugation at 1,500 × *g* in a bacteria-to-cell ratio of 50:1. After 2 h plates were washed three times with fresh medium. After incubation for another 2 to 144 h, cells were processed for E-selectin or ICAM-1 cell surface ELISA. Note that E-selectin expression peaked at 7 to 12 h, while ICAM-1 increased up to 144 h. Data are means ± standard errors of the means of four separate experiments. (b) Northern blot showing increase of E-selectin and ICAM-1 mRNA in *B. henselae*-infected HUVEC. Total endothelial RNA was isolated, and levels of E-selectin-, ICAM-1, and GAPDH mRNA were quantitated. Note different time patterns of mRNA expression after *B. henselae* infection for E-selectin and ICAM-1 and the remarkably long upregulation of ICAM-1. A representative gel (of four independent experiments) is shown.

PMN are known to be important for the control of the early phase of a bacterial infection. Hence, it is conceivable that PMN also contribute to the nonspecific resistance to *Bartonella*, although no studies of *Bartonella*-infected PMN-depleted animals have been published.

We analyzed the PMN-endothelial cell interaction at a defined shear rate of 1 dyne/cm^2 using a parallel-plate flow chamber. This approach allowed the study of PMN rolling and adhesion under physiological conditions (23, 47). At least two separate receptor-ligand pairs appear to be involved in the PMN interaction with infected endothelial cells (23, 34). E-selectin and ICAM-1 on the endothelium bind to E-selectin ligand 1 and leukocyte β_2-integrins, respectively. These adhesion molecules act sequentially and in an overlapping manner to effect leukocyte attachment and adhesion (12). At 8 h after *B. henselae* infection the PMN-HUVEC interaction was dominated by E-selectin, as shown by E-selectin upregulation and

by anti-E-selectin MAb studies (Fig. 2). No significant decrease of rolling and adhesion of PMN was observed upon blockade of ICAM-1 or VCAM-1. In the later phase of infection, an indirect PMN stimulation by *Bartonella*-exposed endothelial cells via E-selectin binding and subsequent upregulation of β_2-integrin, which in turn binds to ICAM-1, is conceivable. This aspect requires further study as does the question of whether *B. henselae* directly stimulates PMN. Moreover, *B. henselae*-exposed endothelial cells likely support adhesion of other circulating leukocytes (lymphocytes and monocytes) which contribute to the specific host defense. Additional studies to address the role of VCAM-1 and different interleukins in the leukocyte-endothelial cell interaction are required.

B. henselae virulence factors leading to activation and reorganization of the endothelial cells are of major interest, but unfortunately knowledge about the pathogenic role of *Bar-*
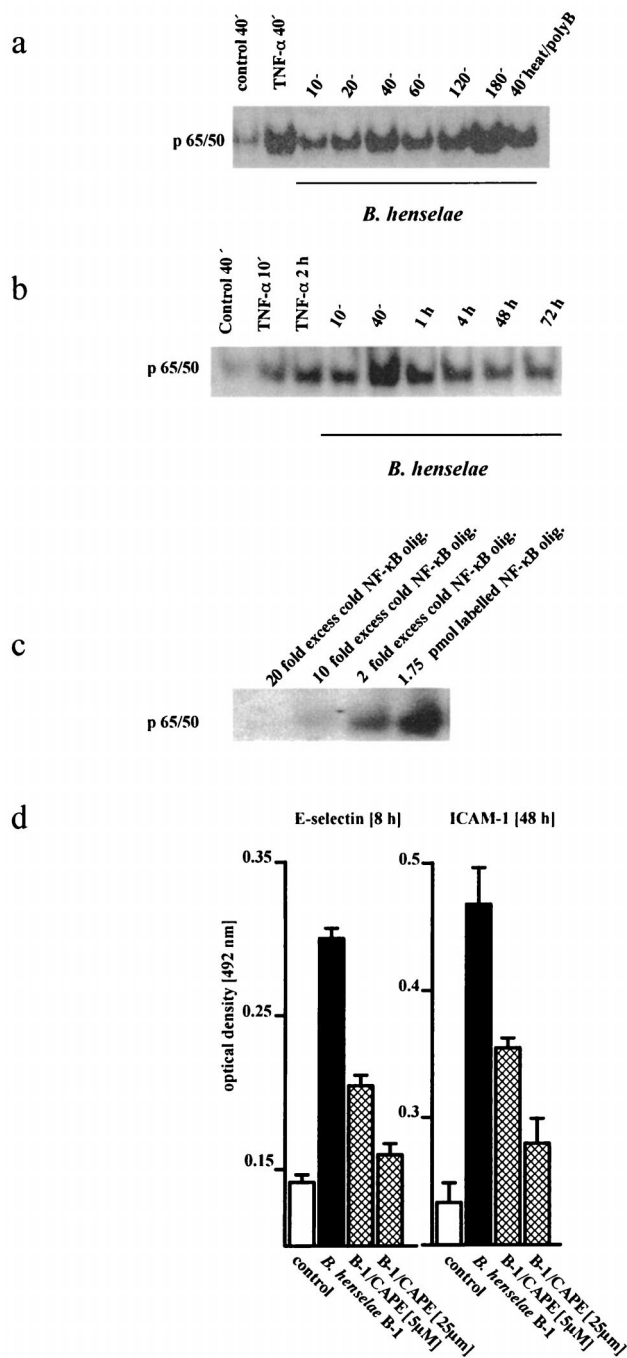
a

p 65/50

B. henselae

b

p 65/50

B. henselae

c

p 65/50

d

E-selectin [8 h]   ICAM-1 [48 h]

FIG. 4. (a) *B. henselae*-induced translocation of transcription factor NF-κB in HUVEC. An EMSA using a ^{32}P-labeled double-stranded NF-κB consensus oligonucleotide was performed. Nuclear proteins were extracted 10 to 180 min p.i. TNF-α was used as a positive control. Note biphasic NF-κB translocation in *B. henselae*-exposed HUVEC. Moreover, note the undiminished NF-κB signal in HUVEC treated with heat-inactivated polymyxin B (50 μg/ml)-exposed bacteria (right lane), suggesting that living bacteria and LPS are not central to NF-κB-translocation. A representative gel (of five independent experiments) is shown. (b) NF-κB upregulation was detected even at 72 h p.i. (c) Control experiments with a 2- to 20-fold molar excess of an NF-κB consensus oligonucleotide clearly demonstrate the specificity of the shift. (d) *B. henselae*-induced E-selectin and ICAM-1 expression was dose dependently reduced by NF-κB-specific inhibitor CAPE, as demonstrated by cell surface ELISA. Data are means ± standard errors of the means of three separate experiments.
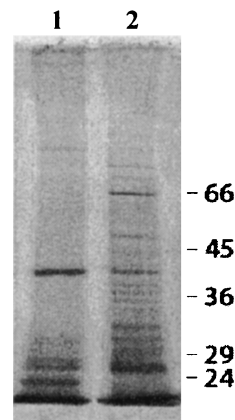
FIG. 5. OMP fractions of *B. henselae* were characterized by SDS–10% PAGE and silver staining. Lane 1, sarcosyl-insoluble outer membrane fraction of *B. henselae*; lane 2, total protein fraction of *B. henselae*.

tonella-specific endotoxin, exotoxins, and pili is very fragmentary (5, 39). Conley et al. (17) localized a stimulus for endothelial cell proliferation in the particulate fraction of *B. henselae* lysates. In contrast, Maeno et al. (36) suggested a

a

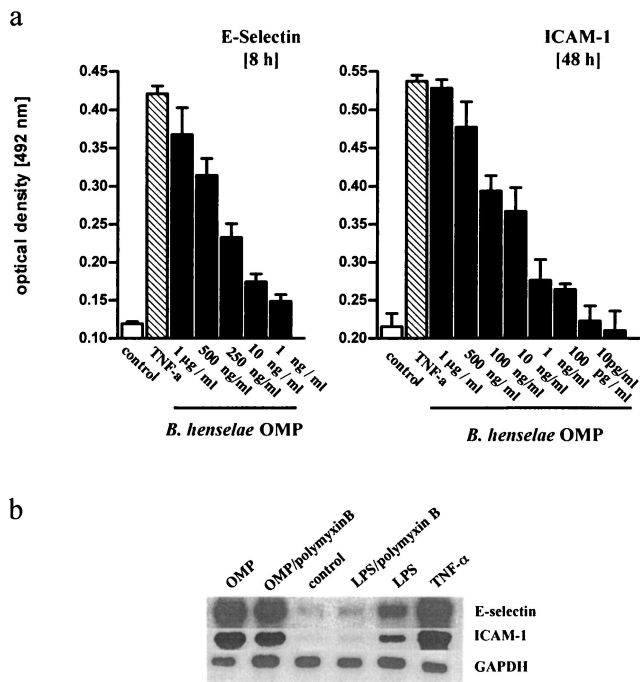E-Selectin [8 h]   ICAM-1 [48 h]

b

FIG. 6. (a) *B. henselae* Berlin-1 OMPs in the range of 10 pg/ml to 1 μg/ml dose dependently enhanced E-selectin and ICAM-1 expression in HUVEC. OMPs were added to cell monolayers in 96-well plates by centrifugation at 1,500 × g. After 8 (E-selectin) or 48 h (ICAM-1) cells were processed for cell surface ELISA. Data are means ± standard errors of the means of three separate experiments. (b) Northern blot showing increase of E-selectin and ICAM-1 mRNA in HUVEC exposed to OMP, TNF-α (10 ng/ml), and LPS (100 ng/ml) after 5 h of stimulation. OMPs were added in a concentration of 500 ng/ml. When indicated, polymyxin B (50 μg/ml) was added 5 min prior to exposure to OMPs or LPS. A representative blot (from one of three independent experiments) is shown.
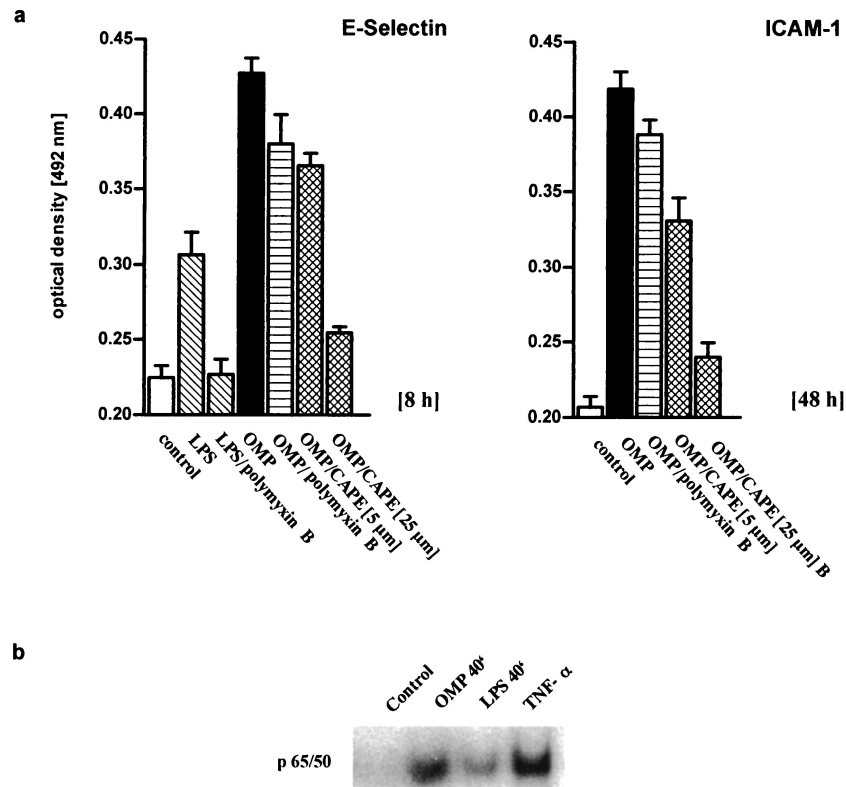
FIG. 7. (a) OMP-induced endothelial adhesion molecule expression was NF-κB dependent and unrelated to LPS. Effects of OMPs (500 ng/ml) on E-selectin and ICAM-1 expression were compared to those for untreated cells. When indicated, cells were pretreated with 5 or 25 μM CAPE 1 h before exposure to OMPs; this resulted in a dose-dependent reduction of adhesion molecule expression. When indicated, polymyxin B was added to OMPs or LPS in a concentration of 50 μg/ml. Data are means ± standard errors of the means of three separate experiments. (b) OMP induced translocation of transcription factor NF-κB in HUVEC. An EMSA was performed as described in Materials and Methods. Nuclear proteins were extracted 40 min p.i. TNF-α was used as a positive control. Note the weak NF-κB signal in LPS-exposed HUVEC compared to that for OMP-treated cells. A representative gel (from three independent experiments) is shown.

soluble *B. henselae*-derived growth factor. Garcia et al. (21) reported on a heat-labile (56°C, 30 min) mitogenic factor of *Bartonella bacilliformis* extract.

Burgess et al. (10) demonstrated a 43-kDa OMP to be the major *B. henselae*-derived adhesin that specifically interacts with HUVEC.

Our observation that heat-inactivated *B. henselae* in the presence or absence of polymyxin B was still able to induce NF-κB activation and adhesion molecule expression in HUVEC suggested the sufficiency of dead *B. henselae* and the requirement for a component other than LPS. This study demonstrates for the first time that a purified and enriched outer membrane fraction of *B. henselae* can mimic the effects of live bacteria, indicating a prominent role for OMPs as a pathogenic factor in *B. henselae*-induced endothelial cell activation.

At least nine proteins (28, 30, 35, 43, 58, 61, 79, 92, and 171 kDa) were reported to occur in the sarcosyl-insoluble fraction of *B. henselae* lysates (9). Five of them (28, 32, 43, 52, and 58 kDa) have been shown to attach to HUVEC, with the 43-kDa OMP being the predominant adhesin (18). Very recent sequence analysis of the 43-kDa OMP by Burgess et al. (10) revealed a 38% identity to *Brucella* spp. OMP2b porin.

Sarcosyl fractionation of *B. henselae* Berlin-1 resulted in the resolution of proteins with similar molecular masses (23, 28,

30, 43, and 92 kDa). The differences observed are possibly due to different purification protocols and/or to *Bartonella*-specific aspects with regard to piliation, strain, passage number, and growth and culture conditions. Further studies to characterize the biological effects of the *B. henselae* Berlin-1 43-kDa OMP and the role of pili in activation of endothelial cells are in progress.

Colonization of host tissues usually is mediated by microbial adhesins, which facilitate recognition of and binding to specific receptors of host cells (35, 42, 44). Use of purified OMPs will help identify endothelial binding sites. ICAM-1 accumulation at the tips of the endothelial protrusions formed during bacterial engulfment was shown by Dehio et al. via confocal microscopy (18). In this context we propose ICAM-1 as a receptor candidate for the 43-kDa OMP. For rhinovirus, NF-κB-mediated induction of its own receptor ICAM-1 has been demonstrated (41).

Data presented indicate that *B. henselae* triggered immediate signal transduction cascades in HUVEC. Bacterial attachment was sufficient to initiate this endothelial response; uptake appeared not to be required. NF-κB is an important regulatory element in endothelial cell adhesion molecule expression (37, 45). Induction of E-selectin requires NF-κB binding to at least three of the four positive regulatory domains in the E-selectin

promoter region, a situation similar to that for the ICAM-1 promoter (16, 48). Our study, aimed at identifying the possible intracellular signaling steps involved, indicated that NF-κB activation and translocation occurred within 10 to 40 min after *B. henselae* exposure to HUVEC. In addition, a specific NF-κB inhibitor significantly blocked adhesion molecule expression on the transcriptional level, proving the importance of NF-κB in the early phase of *Bartonella*-induced endothelial cell activation. Moreover, enriched OMPs (500 ng/ml) induced NF-κB activation and translocation, while LPS (*S. enterica* serovar Abortus equi LPS; 50 μg/ml) had only a minor effect. Further experiments must include *B. henselae* LPS, which unfortunately has not been purified yet.

In vivo, endothelial cells appear to be a specific target of *B. henselae.* In diseases such as BA and BPH bacteria are in contact with the endothelium, thereby promoting endothelial cell proliferation and angiogenesis (13). In this context our observation of prolonged (up to 9 days) upregulation of endothelial adhesion molecule expression might be relevant. Permanently adhering leukocytes and release of angiogenic factors possibly contribute to the vascular lesions described.

The interpretation of our study is limited to cultured human large-vessel endothelial cells. For a detailed analysis of *B. henselae*-related alterations of endothelial function in clinical disorders it would be desirable to study human microvascular endothelial cells of different organs, especially of lymph nodes and dermal capillaries. The isolation and culture of these cells in sufficient quantities, however, are difficult, and therefore the applicability of the data presented to clinical disorders such as cat scratch disease and BA must be verified in further studies.

In conclusion, infection of HUVEC with *B. henselae* resulted in internalization and reproduction of bacteria without subsequent lysis of the host cell. Bacterial contact immediately led to NF-κB-dependent upregulation of adhesion molecule expression and subsequently increased PMN rolling and adhesion. The proinflammatory endothelial cell phenotype was reproduced by exposure of cells to purified *Bartonella* OMP. The data presented suggest that *B. henselae*-induced endothelial cell activation is an important event in the pathogenesis of *Bartonella*-related diseases.

ACKNOWLEDGMENTS

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Chapter 2

The VirB type IV secretion system of *Bartonella henselae* mediates invasion, proinflammatory activation and anti-apoptotic protection of endothelial cells

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The Type IV secretion system (T4SS) VirB of *Bartonella* was recently identified as an essential pathogenicity factor, required to establish intraerythrocytic infection in the mammalian reservoir host (Schulein and Dehio, 2002). In this work, we investigated the role of the VirB T4SS of *B. henselae* during endothelial cell infection in the incidental human host, using HUVEC as an *in vitro* model.

First, we generated a *B. henselae* mutant lacking a functional VirB T4SS. A nonpolar inframe deletion mutant in *virB4* (*ΔvirB4)* was constructed and complemented *in trans* with full-length *virB4.* The *ΔvirB4* mutant was tested for putative attenuation in the capacity to grow in co-culture with, invade into, or survive within endothelial cells compared to the wild-type strain. This was monitored by gentamicin-protection assays after HUVEC infection with either wild-type or the *ΔvirB4* mutant. Thereby the *ΔvirB4* mutant did not show any attenuation. Next, the invasion process of the different *B. henselae* strains into HUVEC was analyzed by immunocytochemistry. Bacteria were internalized by the endocytotic pathway independently of the VirB T4SS. In contrast, invasome formation and the rearrangement of the actin cytoskeleton were restricted to strains harboring a functional VirB T4SS. The efficiency of invasome formation mediated by VirB increased with the multiplicity of infection. To test the ability of the different strains to activate a pro-inflammatory response after HUVEC infection (Fuhrmann et al., 2001), we quantified the activation of the nuclear transcription factor κB (NFκB), the expression of the surface adhesion molecule ICAM-1, and the release of the cytokine IL-8 during infection. All three markers of the pro-inflammatory pathway showed a strong response mediated by the VirB T4SS. However, a slight activation of the pro-inflammatory pathway was also observed in a VirB-independent manner.

B. henselae is able to protect HUVEC from apoptotic cell death (Kirby and Nekorchuk, 2002). To show inhibition of apoptosis by *B. henselae* during endothelial

cell infection, we monitored several apoptotic markers including executioner caspases -3/-7 activity, loss of membrane asymmetry, and DNA-fragmentation. Inhibition of apoptosis by *B. henselae* was strictly dependent on a functional VirB T4SS. Finally, we investigated the role of the VirB T4SS in *B. henselae*-stimulated HUVEC proliferation. During a 5-day proliferation assay, both wild-type and the *ΔvirB* mutant induced initially HUVEC proliferation. At later time points, the VirB T4SS mediated cytostatic and cytotoxic effects at high bacterial titers. In contrast, the *ΔvirB* mutant induced a strong HUVEC proliferation, which was even more potent than that observed with vascular endothelial growth factor included as a positive control. We conclude that the potent mitogenic activity of *B. henselae* is VirB-independent and that the VirB-mediated cytotoxic effect interferes with the mitogenic activity of *B. henselae* in an infection-dose dependent manner.

Statement to the own participation

Dr. R. Schulein constructed the nonpolar *ΔvirB4* deletion mutant of *B. henselae* and complemented this strain *in trans* with full-length *virB4*. Dr. G. Denecker and Dr. M. Dehio provided advice in cellular aspects of apoptosis and the pro-inflammatory response. All reported data were generated by myself.

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The VirB type IV secretion system of *Bartonella henselae* mediates invasion, proinflammatory activation and antiapoptotic protection of endothelial cells

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Summary

Bartonella henselae* is an arthropod-borne zoonotic pathogen causing intraerythrocytic bacteraemia in the feline reservoir host and a broad range of clinical manifestations in incidentally infected humans. Remarkably, *B. henselae* can specifically colonize the human vascular endothelium, resulting in inflammation and the formation of vasoproliferative lesions known as bacillary angiomatosis and bacillary peliosis. Cultured human endothelial cells provide an *in vitro* system to study this intimate interaction of *B. henselae* with the vascular endothelium. However, little is known about the bacterial virulence factors required for this pathogenic process. Recently, we identified the type IV secretion system (T4SS) VirB as an essential pathogenicity factor in *Bartonella*, required to establish intraerythrocytic infection in the mammalian reservoir. Here, we demonstrate that the VirB T4SS also mediates most of the virulence attributes associated with the interaction of *B. henselae* during the interaction with human endothelial cells. These include: (i) massive rearrangements of the actin cytoskeleton, resulting in the formation of bacterial aggregates and their internalization by the invasome structure; (ii) nuclear factor κB-dependent proinflammatory activation, leading to cell adhesion molecule expression and chemokine secretion, and (iii) inhibition of apoptotic cell death, resulting in enhanced endothelial cell survival. Moreover, we show that the VirB system mediates cytostatic and cytotoxic effects at high bacterial titres, which interfere with a potent VirB-independent mito-

genic activity. We conclude that the VirB T4SS is a major virulence determinant of *B. henselae*, required for targeting multiple endothelial cell functions exploited by this vasculotropic pathogen.

Introduction

Bartonella henselae is a zoonotic pathogen of growing medical importance. In the feline reservoir host, this arthropod-borne bacterium causes intraerythrocytic bacteraemia. Transmission to humans occurs by cat scratch or bite or the bite of an infected cat flea. Depending on the immune status of the infected individual, *B. henselae* can cause a variety of clinical manifestations. Immunocompetent patients develop cat scratch disease (a necrotizing lymphadenopathy with fever), endocarditis or neuroretinitis. In immunocompromised patients, *B. henselae* causes vasoproliferative lesions, which result in the formation of tumours of the skin or inner organs (bacillary angiomatosis) or blood-filled cysts in the liver and spleen (bacillary peliosis) (Karem *et al.*, 2000). Among all human pathogenic bacteria, this remarkable capacity to trigger vasoproliferative tumour growth is limited to *Bartonella* spp. (Dehio, 2003).

Within vasoproliferative lesions *B. henselae* is found in close association with proliferating endothelial cells. Clearance of infection by antibiotic treatment results in complete regression of vascular lesions. These findings suggest that *B. henselae* specifically colonizes the vascular endothelium and produces a mitogenic factor that acts locally and temporarily. Primary human umbilical vein endothelial cells (HUVEC) are used as *in vitro* system to study this interaction with the vascular endothelium (Dehio, 2001; 2003). *Bartonella henselae* invades and colonizes HUVEC by two distinct routes, either as individual bacteria through a classical endocytotic pathway, or as bacterial aggregates which are formed on the cell surface, followed by their engulfment and internalization via the invasome structure. Invasome-formation and internalization is an actin-dependent process resulting in massive cytoskeletal rearrangements (Dehio *et al.*, 1997). Remarkably, *B. henselae* can stimulate the proliferation and migration of HUVEC even without direct contact, suggesting that bacteria secrete a vascular mitogen (Maeno

et al., 1999). Endogenous growth factors (e.g. vascular endothelial cell growth factor, VEGF) released by *B. henselae*-infected macrophages *in vitro* may also contribute to endothelial cell proliferation *in vivo* (Kempf *et al.*, 2001; Resto-Ruiz *et al.*, 2002; Dehio, 2003). The proinflammatory activation described for *B. henselae*-infected endothelial cells (Fuhrmann *et al.*, 2001) could contribute to the recruitment of macrophages and other inflammatory cells competent for secretion of VEGF, which typically infiltrate vasoproliferative lesions. The nuclear factor κB(NF-κB)-dependent proinflammatory response of *B. henselae*-infected HUVEC is characterized by cell surface expression of cell adhesion molecules (i.e. ICAM-1 and E-selectin), which result in increased neutrophil rolling and adherence (Fuhrmann *et al.*, 2001). Finally, *B. henselae* protects HUVEC from apoptotic cell death as shown by the suppression of caspase activation and DNA fragmentation. The resulting increased endothelial cell survival is considered to contribute to the formation of vasoproliferative lesions *in vivo* (Kirby and Nekorchuk, 2002).

Recently, we have identified the VirB type IV secretion system (T4SS) as an essential pathogenesis factor in the related species *B. tribocorum* (Schulein and Dehio, 2002). T4SS are multicomponent transporters of Gram-negative bacteria with functions as diverse as the delivery of effector proteins into eukaryotic target cells in pathogenesis or DNA transfer in bacterial conjugation (Christie, 2001). The highly conserved VirB T4SS of *B. tribocorum* and *B. henselae* are encoded by operons of 10 genes (*virB2-10*) (Padmalayam *et al.*, 2000; Schulein and Dehio, 2002). Unlike wild-type *B. tribocorum*, mutants deleted for *virB4* are unable to cause intraerythrocytic bacteraemia (Schulein and Dehio, 2002), which is the hallmark of infection in the mammalian reservoir host (Schulein *et al.*, 2001). Further analysis revealed that the VirB T4SS is required at an early infection stage before the onset of intraerythrocytic bacteraemia (Schulein and Dehio, 2002). Based on the observation that the *virB* operon of *B. henselae* is induced during infection of endothelial cells *in vitro* (Schmiederer *et al.*, 2001), we reasoned that the VirB system may be involved in mediating endothelial interaction in both the mammalian reservoir and the incidental human host. Here, we used genetic analysis of the VirB system of *B. henselae* in combination with cell assays to study the role of this T4SS during HUVEC infection. We demonstrate that most of the known physiological changes associated with *B. henselae* infection of HUVEC, i.e. actin remodelling, and the induction of a proinflammatory and antiapoptotic response are dependent on the VirB system. Moreover, we show that VirB mediates cytostatic or even cytotoxic effects at high bacterial titres, which interfere with the VirB-independent mitogenic activity of *B. henselae*. Together, our data dem-

onstrate that the VirB T4SS represents a major virulence determinant of *Bartonella* mediating subversion of multiple vascular endothelial cell functions.

Results

Mutagenesis of the VirB T4SS of B. henselae

As a genetic basis for analysing the role of VirB in mediating *B. henselae*–HUVEC interaction, we constructed an apolar in frame deletion in the *virB4* gene and complemented the resulting *ΔvirB4* mutant with full-length *virB4 in trans* (*ΔvirB4*/p*virB4*). The isogenic wild-type, *ΔvirB4* and *ΔvirB4*/p*virB4* strains were used for parallel infection in cell assays described below.

Course of bacterial growth in endothelial cell co-culture, cell invasion, and intracellular survival of wild-type and ΔvirB4 *mutant bacteria*

We first tested whether the *ΔvirB4* mutant is attenuated in the capacity to grow in co-culture with, invade into, or survive within endothelial cells. Therefore HUVEC were infected with a multiplicity of infection (MOI) of 100 bacteria per cell (MOI = 100) for 0 h, 6 h, 30 h, 54 h and 78 h. To quantify the total number of cultivatable bacteria in HUVEC co-culture, the infected cell monolayers were lysed, and colony forming units (cfu) were determined by plating of serial dilutions. Figure 1A shows a parallel course of growth of wild-type and *ΔvirB4* mutant bacteria over the entire time-course. The number of bacteria changed little during the first 30 h of infection, but a strong increase followed at later time points (54 h and 78 h). A parallel experiment, in which gentamicin was used to kill extracellular bacteria (gentamicin-protection assay), determined the course of cell invasion (for time points 0 h, 6 h and 30 h) and intracellular survival (for time-points 30 h, 54 h and 78 h). Figure 1B, lower panel, shows a similar number of intracellular wild-type and *ΔvirB4* mutant bacteria at 6 h of infection. The number of intracellular bacteria strongly increased at 30 h of infection, however, with significantly more *ΔvirB4* mutant than wild-type bacteria. To follow the course of intracellular survival of bacteria after 30 h of infection, extracellular bacteria were killed during a 2 h incubation period with gentamicin (30 to 32 h), followed by washing to remove gentamicin and incubation with normal medium for the remaining period of infection (total of 54 h or 78 h). The effectiveness of gentamicin treatment for killing extracellular bacteria in these samples is shown in the upper panel of Fig. 1B (total number of bacteria at 54 h and 78 h). The lower panel illustrates that wild-type and *ΔvirB4* mutant bacteria show an equal drop in the number of cultivable intracellular organisms during one day (1.6-fold reduction at 54 h versus 30 h), and a further drop till 78 h of infection.
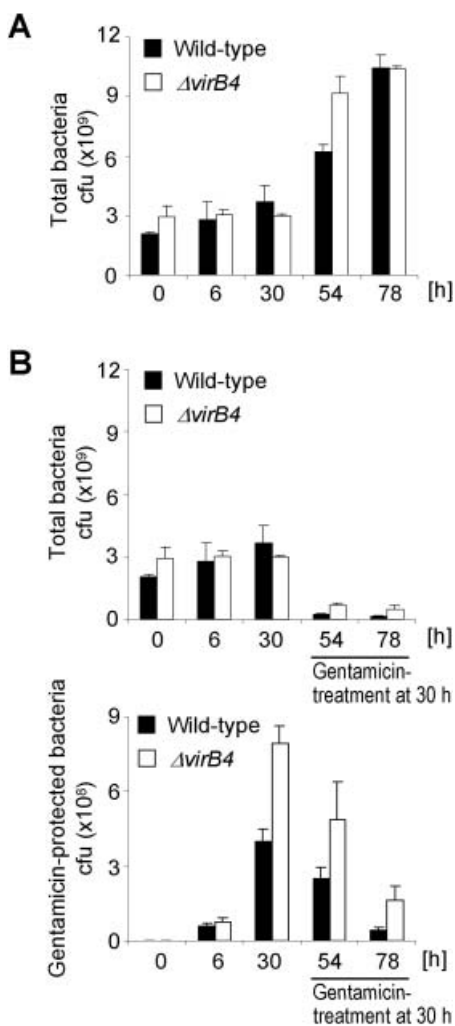
Fig. 1. Course of bacterial growth in endothelial cell co-culture, cell invasion and intracellular survival of wild-type and *ΔvirB4* bacteria. Human umbilical vein endothelial cells were infected with wild-type or *ΔvirB4* mutant bacteria. (A, B, upper panel) Total cfu or (B, lower panel) gentamicin-protected cfu (only intracellular bacteria) were determined after the indicated period of infection. (B) To follow the intracellular survival after 30 h of infection, extracellular bacteria were killed by gentamicin treatment for 2 h, followed by washing steps and further incubation in normal medium for the remaining time of the indicated infection period. Mean values ± SD of triplicate samples are presented for one out of two similar experiments.

Taken together, quantification of cfu during HUVEC infection did not provide any indication of attenuation of the *ΔvirB4* mutant as compared to wild-type bacteria. The course of growth in co-culture with HUVEC and of intracellular survival was similar for both genotypes. Notably, the *ΔvirB4* mutant displayed even an enhanced capacity for cell invasion compared to wild-type bacteria.

VirB is required for invasome-mediated cell entry and the associated massive actin rearrangements

We next investigated whether VirB is required for bacterial

internalization into HUVEC by either of two described pathways, via (i) endocytosis of individual bacteria or via (ii) the invasome-mediated engulfment of large bacterial aggregates formed on the cell surface (Dehio *et al.*, 1997). Human umbilical vein endothelial cells infected for 48 h were differentially stained for intra- and extracellular bacteria and for F-actin and examined by confocal microscopy (Fig. 2). Bacteria of all isogenic strains were internalized by conventional endocytosis as shown by intracellular bacteria localizing to a perinuclear position (Fig. 2B–D, bottom plane, red-coloured bacteria in the overlay). In contrast, invasome structures containing a bacterial aggregate (Fig. 2B and D, top plane) and the associated ring-shaped actin rearrangements (Fig. 2B and D, bottom plane) were exclusively triggered by wild-type and *ΔvirB4*/p*virB4* bacteria. Infection with *ΔvirB4* did not result in any obvious cytoskeletal rearrangement compared to the actin cytoskeleton of uninfected cells (Fig. 2A and C). Quantification of invasome-positive cells, infected with three different infection doses (MOI = 30, 100, and 300) of the three isogenic strains revealed a strict dependency of invasome formation on a functional VirB system (Table 1). Invasome formation by the complemented mutant strain *ΔvirB4*/p*virB4* was indistinguishable from wild-type at higher infection doses, whereas phenotypic restoration was reduced for the lower bacterial titres (i.e. MOI = 30, Table 1).

Notably, the impairment of *ΔvirB4* mutant bacteria to accumulate in an invasome structure resulted in increased endocytic uptake into perinuclear localizing phagosomes (Fig. 2B–D, bottom plane, red-coloured bacteria in the overlay). Given the different time-frames of endocytosis (minutes) and invasome-mediated invasion (up to one day) (Dehio *et al.*, 1997), this finding may explain the increased number of gentamicin-protected (intracellular) *ΔvirB4* mutant versus wild-type bacteria at the 30 h time-point of infection (Fig. 1C).

Taken together, we observed an absolute requirement of VirB for invasome formation and the associated massive actin rearrangements.

VirB mediates an NF-κB-dependent proinflammatory response

We next analysed whether VirB mediates the proinflammatory response activated in *B. henselae*-infected

Table 1. Quantification of invasome formation.

Strain	% invasome-positive cells ± SD		
	MOI = 30	MOI = 100	MOI = 300
Wild-type	66 ± 3	96 ± 2	98 ± 2
ΔvirB4	0 ± 0	0 ± 0	0 ± 0
ΔvirB4/p*virB4*	26 ± 4	80 ± 3	95 ± 3
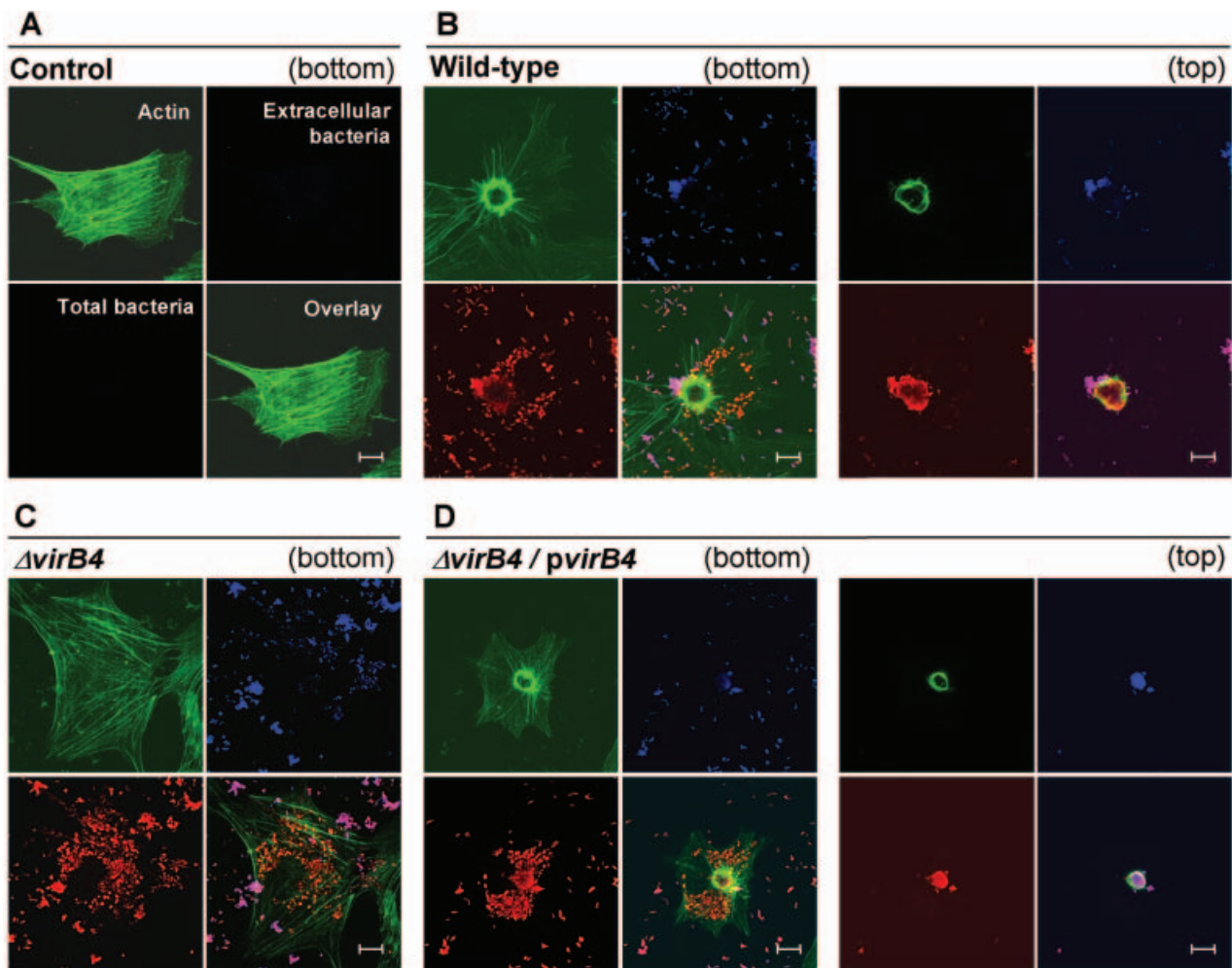
Fig. 2. VirB mediates massive actin rearrangements resulting in invasome-mediated uptake of bacterial aggregates. Human umbilical vein endothelial cells were infected for 48 h (A) without bacteria (control) or with either (B) wild-type (C) *ΔvirB4,* or (D) ΔvirB4/p*virB4* bacteria. Cells were immunocytochemically stained for F-actin (upper left quadrant, green), extracellular bacteria (upper right quadrant, blue), and total bacteria (lower left quadrant, red). The overlay of all three channels is presented in the lower right quadrant, distinguishing extracellular bacteria (appearing in pink as a mixture of red and blue) from intracellular bacteria (appearing in red). Confocal pictures were taken (A–D) in a plane just above the cell substratum where actin stress fibres localize (bottom) or (B, D) in a parallel plane 3 μm above (top). The scale bar represents 10 μm.

HUVEC (Fuhrmann *et al.*, 2001). As typical markers of an NF-κB-dependent proinflammatory response, the cell surface expression of ICAM-1 and the secretion of the chemokine IL-8 into the culture medium were quantified. Compared to uninfected cells, IL-8 secretion was elevated by *ΔvirB4* infection in a time-dependent (30 h and 54 h) and infection-dose dependent manner (MOI = 30, 100 or 300) (Fig. 3A). Infection with wild-type displayed the same time- and infection dose-dependency, whereas IL-8 levels were always significantly increased relative to *ΔvirB4*. The complemented mutant displayed partial phenotypic restoration (Fig. 3A), as in results reported above for invasome formation. Surface expression of ICAM-1 measured at 54 h of infection with a MOI = 100 (Fig. 3B) was in full accordance with the data obtained for IL-8 secretion. Consistent results were also obtained for the transcriptional

activity of NF-κB in HEK293T cells measured by a transfected luciferase reporter plasmid after 30 h of infection with a MOI = 100 (Fig. 3C).

Together, these data show that the strong NF-κB-dependent proinflammatory response triggered by *B. henselae* infection is mediated primarily by VirB, whereas also VirB-independent processes contribute to this response.

VirB mediates suppression of apoptosis

We next tested whether the VirB system mediates suppression of apoptosis in *B. henselae*-infected HUVEC (Kirby and Nekorchuk, 2002). We measured the activity of executioner caspases-3 and -7 as early apoptotic markers by using a specific fluorogenic substrate. The basal
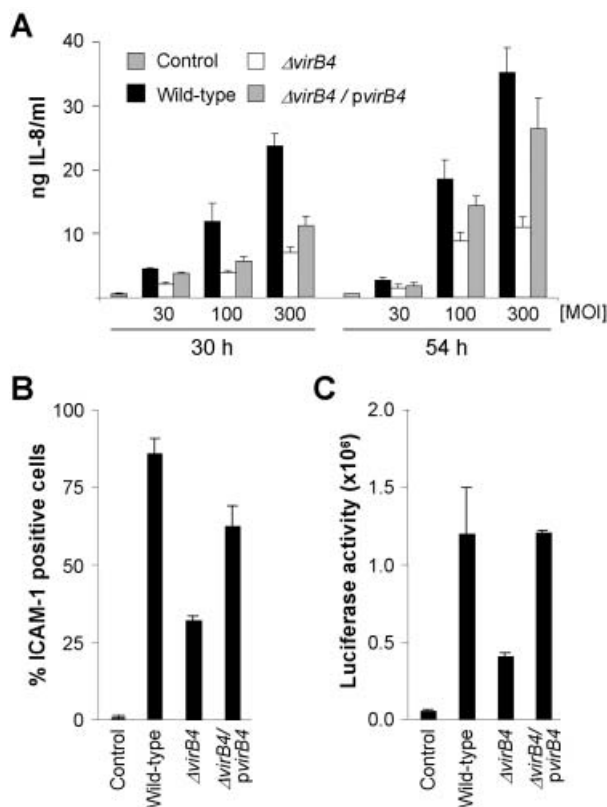
Fig. 3. VirB mediates an NF-κB-dependent proinflammatory pheno-
type. (A, B) HUVEC or (C) HEK293T cells were infected for (A) 30 h
or 54 h (B) 54 h or (C) 30 h without bacteria (control) or with either
wild-type, *ΔvirB4,* or *ΔvirB4*/p*virB4* bacteria with (A) a MOI as speci-
fied (B) MOI = 300 or (C) MOI = 100.
A. IL-8 in the culture supernatants was quantified by ELISA.
B. Cell surface expression of ICAM-1 was determined by flow
cytometry.
C. NF-κB activity was determined by luciferase activity expressed
from a transfected NF-κB reporter plasmid. All assays were repeated
at least three times. Mean values ± SD of triplicate samples are
presented for one representative experiment.

activity of these caspases in uninfected cells, which likely
accounts for a low level of spontaneous apoptosis, was
suppressed by infection with all three bacterial strains at
a MOI = 100 (Fig. 4A). Treatment with the apoptotic
inducer actinomycin D (Kirby and Nekorchuk, 2002)
resulted in an equal increase in caspase-3 and -7 activity
in cells which were either uninfected or infected with
ΔvirB4. In contrast, infection with wild-type or *ΔvirB4*/
p*virB4* resulted in a complete suppression of actinomycin
D-induced caspase activation (Fig. 4B). Suppression of
caspase activation by wild type, but not by the *ΔvirB4*
mutant, was consistently seen for the tested range of
infection doses (MOI = 30, 100 and 300, see Fig. 4C).
Consistent with the partial phenotypic restoration of the
complemented mutant *ΔvirB4*/p*virB4* reported above, this
strain suppressed caspase activation only at higher infec-
tion doses (MOI = 100 and 300, see Fig. 4C). The analy-

sis of loss of lipid membrane asymmetry (Fig. 4D) and
DNA-fragmentation (Fig. 4E) as late apoptotic markers
also demonstrated that wild-type and *ΔvirB4/*p*virB4*, but
not *ΔvirB4,* efficiently suppress actinomycin D-induced
apoptosis.

We conclude that VirB is strictly required for the anti-
apoptotic activity of *B. henselae* on HUVEC.

*VirB mediates cytostatic and cytotoxic effects at high
bacterial titres, which interfere with HUVEC proliferation
stimulated in response to a potent bacterial mitogen*

Finally, we investigated whether VirB is involved in
B. henselae-stimulated HUVEC proliferation (Conley
et al., 1994; Maeno *et al.*, 1999). During a 5-day prolifer-
ation assay in serum-containing culture medium deprived
for specific growth factors, the number of uninfected cells
dropped slightly (Fig. 5A and B), likely due to spontaneous
apoptosis. Unexpectedly, we observed that for all infection
doses tested (MOI = 10, 30 or 100) *ΔvirB4* strongly stim-
ulated HUVEC proliferation, resulting in a ~threefold
increase in cell number already on day 3. On day 5 the
~eightfold increase in cell number even surpassed the
effect caused by the potent mitogen VEGF (Fig. 5A and
B). In contrast, infection with wild type or *ΔvirB4*/p*virB4* at
the lowest dose tested (MOI = 10) resulted in only a ~two-
fold increase in cell number on day 3 without further
increase till day 5. This indicates that VirB mediates a
cytostatic effect at the elevated bacterial titre reached in
the extended course of this co-cultivation experiment
(compare to Fig. 1A). The higher infection doses
(MOI = 30 or 100) also resulted in a ~twofold increase in
cell number on day 3, whereas cell numbers sharply
dropped at day 5, indicating that at very high bacterial
titres VirB can even mediate cytotoxicity (Fig. 5B). These
data clearly show: (i) that the potent mitogenic activity of
B. henselae is VirB-independent, reaching maximal activ-
ity already at a low infection dose (MOI = 10); (ii) that the
cytostatic and cytotoxic effects caused by *B. henselae* at
high titre are entirely dependent on VirB, and (iii) that the
VirB-mediated cytostatic/cytotoxic effects interfere with
the activity of the VirB-independent mitogen in an infection
dose-dependent manner.

Discussion

Vasoproliferative tumour lesions developed by patients
infected with *B. henselae* are the result of a remarkable
interaction between the pathogen and vascular endothe-
lial cells. The use of HUVEC as *in vitro* model allowed to
differentiate between four prominent changes of endothe-
lial cell function in response to *B. henselae* infection
(Dehio, 2003), which we have shown here to be all mod-
ulated by the T4SS VirB – the data are summarized sche-
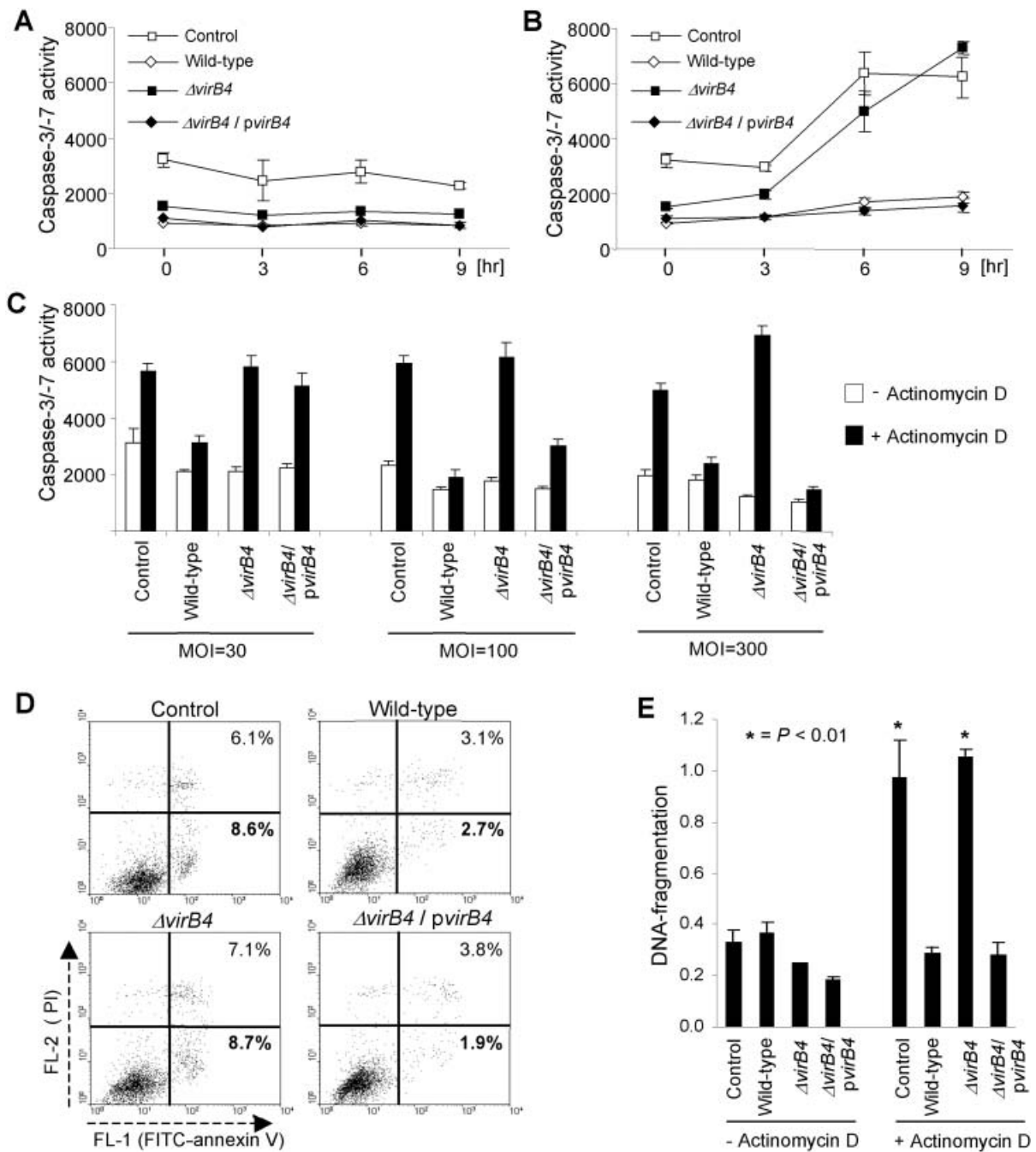
Fig. 4. VirB mediates protection of endothelial cells from apoptosis. (A–E) HUVEC were infected for 24 h without bacteria (control) or with wild-type, *ΔvirB4,* or *ΔvirB4*/p*virB4* bacteria with an MOI = 100 if not specified differently. Cells were then incubated (B, C, D, right panel of E) with actinomycin D to trigger apoptosis or (A, left panel of E) without actinomycin D as control. (A, B) After the time-points indicated or (C–E) after 12 h the assay was terminated and various markers of apoptosis were assayed. (A–C) Caspase-3 and -7 activities were measured with a specific fluorogenic peptide substrate. (D) The loss of membrane asymmetry was quantified by flow cytometry analysis of FITC-annexin V-stained cells. Counterstaining with PI allowed differentiation of necrotic cells (upper right quadrant of the dot plot) and apoptotic cells (lower right quadrant). The percentages of cells localizing to these quadrants are indicated. (E) DNA fragmentation was measured by ELISA. (A–C, E) Mean values ± SD of triplicate samples are presented for one representative out of three replica experiments. (E) Actinomycin D-treated samples differing statistically significantly from untreated control samples are marked by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$).
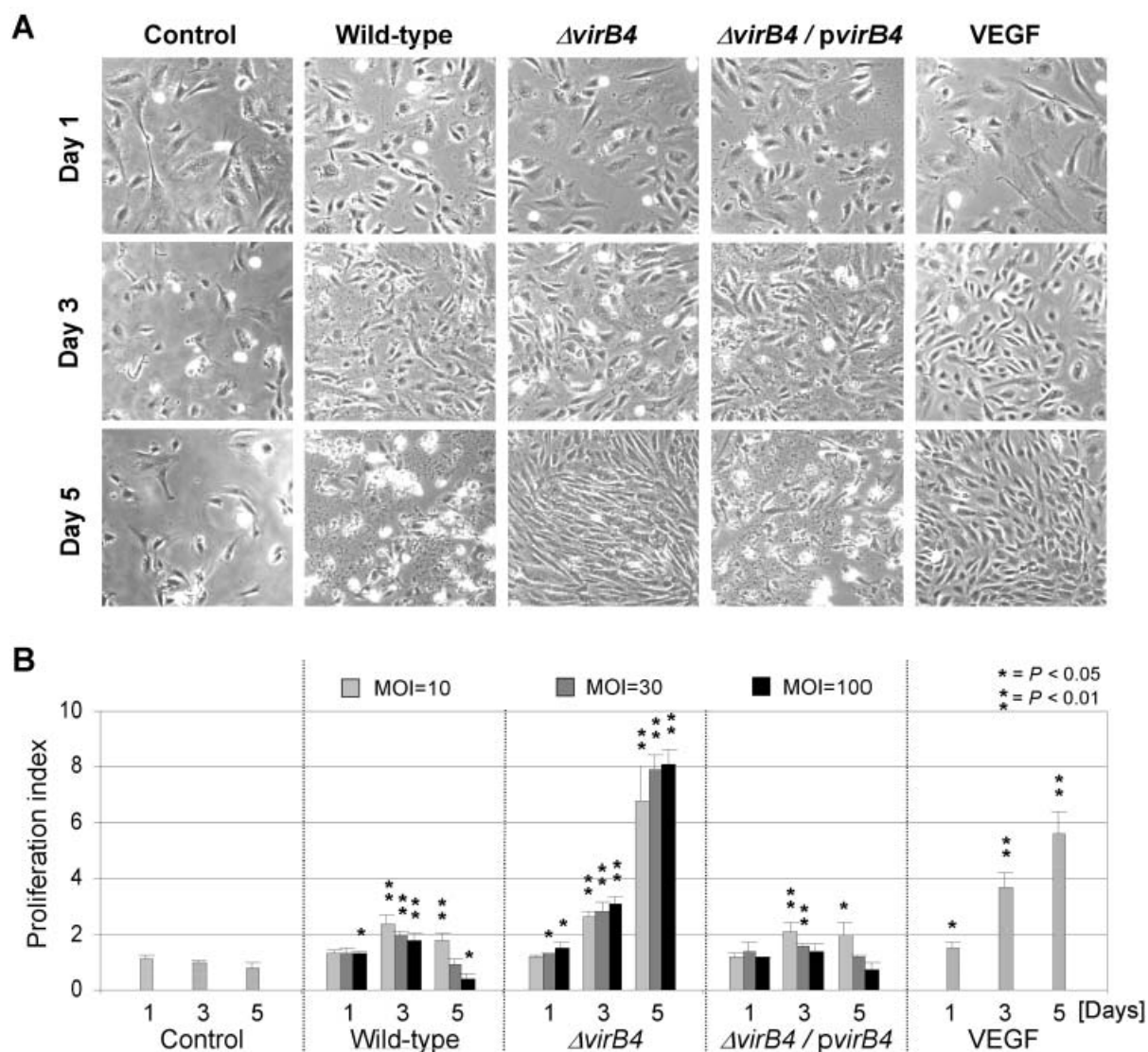
Fig. 5. VirB mediates cytostatic/cytotoxic effects at high bacterial titres, which interfere with HUVEC proliferation stimulated by a potent VirB-independent bacterial mitogen. HUVEC in serum-containing medium deprived for specific growth factors were infected for 5 days without bacteria (control) or with either wild-type, $\Delta virB4$ or $\Delta virB4$/p$virB4$ at the indicated MOI. VEGF (50 ng ml^{-1}) was used as a positive control.
A. Digital pictures of phase-contrast microscopic fields are illustrated for day 1, 3 and 5 (MOI = 30 for bacterial infections).
B. Graph of the proliferation indices (= cell number on given day relative to day 0, mean ± SD of triplicate samples) for day 1, 3 and 5 is illustrated for one representative out of three replica experiments. Proliferation indices which differ statistically significantly from control (day 1) are marked by asterisks (one for $P < 0.05$, two for $P < 0.01$).

matically in Fig. 6. (i) The massive cytoskeletal rearrangements resulting in invasome-mediated uptake of bacterial aggregates (Dehio *et al.*, 1997) are entirely dependent on VirB. This unique invasion process competes with bacterial internalization by conventional endocytosis (Dehio *et al.*, 1997), therefore the $\Delta virB4$ mutant impaired in invasome-mediated invasion displayed increased internalization by the classical endocytic pathway. Internalization via the VirB-dependent invasome represents a novel paradigm for the invasion of bacteria into host cells and may serve as a cellular colonization mech-

anism for endothelial cells associated with the formation of vasoproliferative lesions (Dehio, 2003). (ii) The NF-κB-dependent proinflammatory response (Fuhrmann *et al.*, 2001), considered to contribute indirectly to vasoproliferative growth (Kempf *et al.*, 2001; Resto-Ruiz *et al.*, 2002; Dehio, 2003), is activated to a low level by VirB-independent factors (e.g. LPS), whereas the majority of this phenotype is clearly contributed by VirB. (iii) The inhibition of apoptosis, which results in increased endothelial cell survival and thereby directly contributes to vasoproliferative tumour formation (Kirby and Nekorchuk, 2002), is
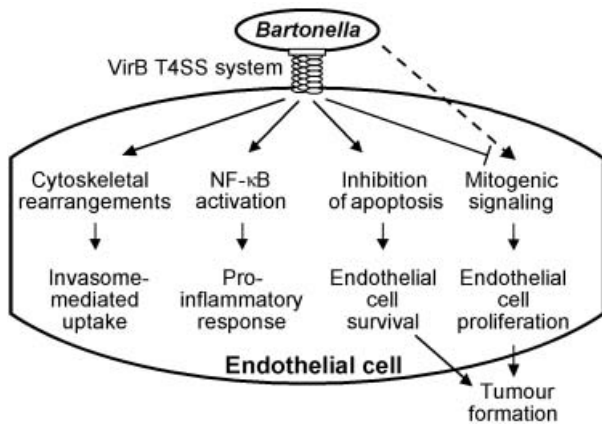
Fig. 6. Scheme of the role of the VirB T4SS in mediating *B. henselae* – vascular endothelial cell interaction.

strictly dependent on VirB. (iv) Mitogenic signalling resulting in endothelial cell proliferation (Dehio, 2003) was the only known physiological change triggered by *B. henselae* infection that we could not assign to VirB. However, VirB turned out to interfere with this proliferative response in an unexpected manner. Remarkably, *ΔvirB4* elicited a proliferative response at least as strong as that by the potent mitogen VEGF. HUVEC proliferation was maximal already at the lowest bacterial infection dose tested (MOI = 10), whereas even the highest dose (MOI = 100) did not cause any sign of cytotoxicity. In sharp contrast, both wild type and *ΔvirB4*/p*virB4* caused cytostatic or even cytotoxic effects in an infection dose-dependent manner, which masked most of the potent mitogenic activity of *B. henselae.* VirB-mediated cytotoxicity was only observed for elevated bacterial titres reached rather late in the course of a 5-day proliferation assay, which was performed as a co-cultivation experiment as described before (Conley *et al.*, 1994; Maeno *et al.*, 1999). Modification of the assay conditions by either daily replacement of the culture medium to remove an excess of bacteria or antibiotic killing of bacteria on day 2 abolished VirB-mediated cytotoxicity and resulted in a similar proliferation response elicited by all three isogenic strains (data not shown). Whether VirB-mediated cytostatic and cytotoxic effects represent an *in vitro* artifact because of elevated bacterial titres or whether they have a physiological role *in vivo* (e.g. to control an excessive proliferation response) needs to be addressed further.

Collectively, our data demonstrate that the VirB T4SS of *B. henselae* modulates several fundamentally important endothelial cell functions *in vitro*, indicating that it represents a central virulence factor for establishing chronic infection of the human vascular endothelium. Our previous work in the *B. tribocorum*/rat model showed that VirB is also required for establishing intraerythrocytic bacteraemia – the hallmark of *Bartonella*-infection in the mam-

malian reservoir (e.g. in the cat for *B. henselae*) (Schulein and Dehio, 2002). In this case, VirB is essential for colonizing a yet undefined primary niche in which the pathogen gains competence for the subsequent erythrocyte infection process (Schulein and Dehio, 2002). Circumstantial evidence suggests that endothelial cells represent a major constituent of this primary niche (Dehio, 2003). Thus, it appears likely that the VirB system represents an essential virulence determinant for vascular endothelial cell infection both in mammalian reservoir hosts (i.e. for establishing intraerythrocytic infection) and in incidentally infected humans (i.e. for establishing the prominent vascular pathologies). Together with surface expression of VirB components, such as the immunogenic 17 kDa antigen (Anderson *et al.*, 1995; Padmalayam *et al.*, 2000), this T4SS system represents an essential surface structure for pathogenicity and therefore a promising target for vaccine development.

T4SS of other human pathogens have been recognized to mediate important virulence features: the Cag system of *Helicobacter pylori* stimulates proinflammatory activation and a motile phenotype of gastric epithelial cells, the Dot/Icm system of *Legionella pneumophila* and the VirB system of *Brucella* spp. are required for the establishment of an intracellular replication niche in macrophages (Nagai and Roy, 2003). However, none of these T4SS has been associated with an equally wide range of physiological changes in target cells as we have reported here for the *B. henselae* VirB system in vascular endothelial cells. Crucial for the molecular understanding of VirB function will be the characterization of effector molecules translocated by this T4SS into infected endothelial cells. Whereas the VirB system shares striking sequence conservation with plasmid conjugal-transfer systems (Schulein and Dehio, 2002), we have recently identified several effector proteins translocated by this T4SS into infected endothelial cells (unpublished data). The knowledge of VirB-translocated effectors and their molecular function in modifying endothelial cell processes related to the cytoskeleton, inflammation, apoptosis, and proliferation would improve our understanding of the infection biology of the vasculotropic pathogen *B. henselae.* Moreover, these bacterial effectors could provide valuable tools for the specific manipulation of endothelial cells, and may allow to uncover novel aspects in vascular biology.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* NovaBlue was used for cloning steps and the *dap⁻ E. coli* strain β2150 for plasmid mobilization to *B. henselae* (Dehio and Meyer, 1997). *Escherichia coli* strains were grown at 37°C overnight in Luria–

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Reference or source
B. henselae strains		
ATCC 49882[T]	'Houston-1', isolated from a bacteraemic HIV-patient	Regnery *et al.* (1992)
RSE242	Δ*virB4* mutant of RSE247	This work
RSE247	Spontaneous Sm[R] strain of ATCC 49882[T]	This work
RSE364	RSE242 containing p*virB4*	This work
E. coli strains		
β2150	*F′ lacZDM15 lacI*[q] *traD36 proA + B + thrB1004 pro thi strA hsdS lacZ*Δ*M15* Δ*dap*A::erm (Erm[R]) *pir*	(Dehio and Meyer, 1997)
NovaBlue	*endA1 hsdR17*(r K12–m K12 +) *supE44 thi-1 recA1 gyrA96 relA1 lac*[*F′ proA + B + lacI*[q]*Z*Δ*M15*::T*n10* (Tc[R])]	Novagen, Madison
Plasmids		
pRS14	*ori*T, *ori*[ColE1], *gfp*[mut2], *lacI*[q], *rpsL*, Km[R], mutagenesis vector for generating a Δ*virB4* in-frame deletion in *B. tribocorum*	Schulein and Dehio (2002)
pRS20	tra[−] mob[+] Gm[R], expression vector for *virB4* of *B. tribocorum*	Schulein and Dehio (2002)
pRS25	Derivative of pRS14 used to generate a Δ*virB4* in frame deletion in *B. henselae*	This work
pRS100	pRS20 containing a 0.4 kb fragment carrying the putative *virB* promoter of *B. henselae*	This work
p*virB4*	Derivative of pRS100 containing full length *virB4* of *B. henselae*	This work

Bertani broth, supplemented with 50 mg l^{−1} kanamycin, 25 mg l^{−1} gentamicin, or 1 mM diaminopimelic acid. *Bartonella henselae* ATCC 49882[T] (Regnery *et al.*, 1992) and its derivatives were grown for 3–7 days on Columbia agar containing 5% defibrinated sheep blood (CBA agar) in a humidified atmosphere with 5% CO_2 at 35°C. The spontaneous streptomycin-resistant mutant RSE247 obtained by selection of *B. henselae* ATCC 49882[T] on 100 mg l^{−1} streptomycin served as wild-type strain. Kanamycin (50 mg l^{−1}) or gentamicin (10 mg l^{−1}) were added to select for transconjugants after mating (Dehio and Meyer, 1997), and 0.5 mM IPTG and 100 mg l^{−1} streptomycin to select for loss of cointegrates of the mutagenesis vector (Schulein and Dehio, 2002).

Generation of a VirB in frame deletion mutant and complementation in trans

DNA manipulations were carried out by standard procedures (Sambrook *et al.*, 1989). Plasmid pRS25 used for generating a Δ*virB4* mutant was constructed as follows: the *Bam*HI insert of pRS14 (Schulein and Dehio, 2002) was replaced by a 1060 bp *Bam*HI fragment of the *virB* locus containing a 2103 bp in frame deletion in *virB4*. This fragment was constructed by megaprime PCR from two PCR products. Product 1 of 0.56 kb was amplified with primers prRS07 and prRS08

and contained the first 123 bp of the *virB4* gene and upstream sequences. Product 2 of 0.50 kb was amplified with primers prRS09 and prRS10 and contained the last 129 bp of the *virB4* gene and downstream sequences. Megapriming and PCR amplification with primers prRS07 and prRS10 were performed as described (Schulein and Dehio, 2002).

pRS25 was used to generate the Δ*virB4* mutant RSE242 in the RSE247 wild-type background by a two-step gene replacement procedure as recently described for *B. tribocorum* (Schulein and Dehio, 2002). Polymerase chain reaction with primers prRS285 and prRS286 priming in *virB2* and *virB5*, respectively, was used to confirm the chromosomal deletion of *virB4* in RSE242.

The *virB4* complementation plasmid p*virB4* was constructed as follows: a 0.40 kb fragment containing the putative *virB* promoter upstream of *virB2* was amplified with primers prRS198 and pRS199. This fragment was digested with *Cla*I, filled-in, and ligated with the 4.8 kb vector backbone of plasmid pRS20 (Schulein and Dehio, 2002) after digestion with *Xho*I and *Cla*I and fill-in, giving pRS100. A 2.39 kb PCR fragment containing the complete *virB4* gene was amplified by primers prRS200 and pRS201, digested with *Xho*I, and ligated with *Xho*I-digested pRS100, resulting in p*virB4*. p*virB4* was conjugated into RSE242, resulting in the *trans*-complemented mutant strain Δ*virB4/*p*virB4* (RSE364). For details of the oligonucleotides used see Table 3.

Table 3. Oligonucleotides used in cloning steps and verification of chromosomal deletion in *B. henselae*.

Name	Sequence[a]	Restriction site
prRS07	CG<u>GGATCCC</u>GTGTAGGTATTGGCTGGATGT	*Bam*HI
prRS08	TACACCTTCAACAACCATCAC	
prRS09	TGATGGTTGTTGAAGGTGTAAATGATGAGATCGCCGTC	
prRS10	CG<u>GGATCCC</u>GCTCTTCACGCAATTCTTCT	*Bam*HI
prRS198	TAGC<u>ATCGAT</u> <u>GGATCC</u>GTTTCATTGCCCTTTCGTATT	*Cla*I, *Bam*HI
prRS199	AAG<u>GATCGAT</u> <u>CTCGAG</u>TTATCCTGGATATAGTGTCTGTCAT	*Cla*I, *Xho*I
prRS200	AAGG<u>CTCGAG</u>GAGGAACTAAACAGGTGAAAC	*Xho*I
prRS201	AAGG<u>CTCGAG</u>TCATTGATTTTCTCTCCTTTG	*Xho*I
prRS285	TTGCTCTTCCATGACAGACACTATATCCAG	
prRS286	TTGCTCTTCGTTAAAGTCGGACATCAGATTTC	

a. Restriction endonuclease cleavage sites are underlined.

Cell lines and cell culture

HUVEC were isolated as described (Dehio *et al.*, 1997) and cultured in EGM medium (PromoCell, Heidelberg, Germany). The human embryonic kidney cell line HEK293T was cultured in DMEM medium with Glutamax (Gibco, Carlsbad, CA) containing 10% fetal calf serum (FCS, Life Technologies, Rockville, MD). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Infection assay

Unless otherwise indicated, HUVEC (passage 3–7) were plated at a density of $2–3 \times 10^4$ cells/cm^2 in EBM medium complemented with the 'Supplement Pack' except for gentamicin and ampho B (PromoCell). The next day, cells were washed twice with medium M199 with Earls salts (M199, Gibco) supplemented with 10% FCS. Unless stated differently, cells were infected with a MOI = 100 bacteria per cell (Kirby and Nekorchuk, 2002) in M199/10% FCS and incubated for the indicated time. If specified, 100 nM actinomycin D (Sigma-Aldrich, St Louis, MO) was added to trigger apoptosis as described before (Kirby and Nekorchuk, 2002).

Bacterial growth in HUVEC co-culture and gentamicin protection assay

Human umbilical vein endothelial cells seeded in 24-well plates were infected with a MOI = 100 for the time indicated and immediately centrifuged at 1200 *g* for 5 min to associate bacteria with the cellular surface. To determine the total number of cultivatable bacteria, samples were directly lysed with a final concentration of 1% saponin (Roth, Karlsruhe, Germany) in PBS, followed by incubation for 15 min at 37°C. Serial dilutions of the cell lysates were plated on CSB-agar and colony forming units (cfu) were determined. To determine intracellular bacteria, an incubation with gentamicin sulphate (125 µg ml^{-1} in culture medium) for 2 h at 37°C and two washing steps were added before lysis with 1% saponin. To monitor intracellular survival, extracellular bacteria were killed after 30 h of infection by incubation for 2 h with gentamicin sulphate (125 µg ml^{-1} in M199/10% FCS), followed by two washing steps and further incubation in fresh culture medium.

Immunocytochemistry and quantification of invasome-positive cells

Human umbilical vein endothelial cells were infected for 48 h, fixed, and stained for F-actin, extracellular, and total bacteria as described (Dehio *et al.*, 1997), except that TRITC-phalloidine was used to label F-actin, and Cy2- and Cy5-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany) to label extracellular and total bacteria respectively. A Leica TCS NT confocal laser scanning microscope equipped with an argon/krypton/HeNe mixed gas laser (Leica Lasertechnik, Heidelberg, Germany) was used for confocal microscopy.

To quantify invasome-positive cells, stained specimen were examined under a Leica DM-IRBE inverted fluorescence microscope using a 10 × objective. Fifty cells for each of three random microscopic fields were examined for the presence of invasome structures based on the massive actin-rearrangments and the associated bacterial aggregates and the mean percentage of invasome-positive cells ± SD was calculated.

Determination of IL-8 secretion and ICAM-1 expression

Human umbilical vein endothelial cells seeded in 6-well plates were infected with a MOI = 300 for the indicated time. IL-8 in the culture supernatant was quantified by the human IL-8 DuoSet ELISA kit (R and D Systems, Minneapolis, MN). Adherent cells were harvested by mild trypsinization and resuspended in PBS containing 3% FCS and stained for 1 h at 4°C with FITC-conjugated mouse anti-human ICAM-1 polyclonal antibodies (R and D Systems). Stained cells were analysed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Caspase activity assay

After infection of HUVEC in 24-well plates for 24 h apoptosis was induced for the indicated time. Subsequently, a fluorogenic assay with the caspase-3 and -7 substrate Ac-DEVD-amc (Peptide Institute, Osaka, Japan) was carried out as described (Vercammen *et al.*, 1998). The release of fluorescent 7-amino-4-methylcoumarin (amc) was measured by fluorometry (excitation at 355 nm, and emission at 460 nm).

Annexin V assay

After infection of HUVEC for 24 h in 6-well plates apoptosis was induced for 12 h. Cells were then collected by mild trypsinization and briefly centrifuged together with the culture supernatant. The cell pellet was washed, resuspended, and stained with Annexin V Alexa Fluor 488 (Molecular Probes, Eugene, OR). Propidium iodide (PI, 1 µg ml^{-1}) was added to counterstain necrotic cells and samples were then analysed with a FACSCalibur flow cytometer (Becton Dickinson).

Cell death detection ELISA

After infection of HUVEC for 24 h in 6-well plates apoptosis was induced for 12 h. Then, cells were lysed and the cytoplasmic histone-associated DNA oligonucleosome fragments were quantified by a cell death detection ELISA (Roche Diagnostics, Indianapolis, IN).

Measurement of nuclear factor κB activity

HEK293T cells were seeded at 1×10^4 cells/cm^2 and on the next day co-transfected via the calcium phosphate precipitation method with the NF-κB reporter plasmid pconaLuc (LMBP3248 from the LMBP collection, http://www.dmb.rug.ac.be/lmbp), containing a luciferase gene under control of a consensus binding site of NF-κB, and the control plasmid pEF*lacZ* (Invitrogen). After 16 h of incubation, the medium was replaced and cells were infected with a MOI = 100. Thirty hours later cells were lysed with NP-40 buffer (Denecker *et al.*, 2001) and NF-κB activity was determined by measuring the luciferase activity present in the cell extracts by a luminescence reader in counts per second

(Janssens *et al*., 2002). Luciferase values were normalized for differences in transfection efficiency on the basis of β-galactosidase activity in the same extracts.

Proliferation assay

Human umbilical vein endothelial cells were plated at 4×10^3 cells/cm^2 in 6-well plates and infected with different MOI as indicated. For the following 5 days, the cell number in each well was determined by capturing digital images (MicroMAX camera from Princeton Instruments, Trenton, NJ, with MetaMorph2 software) of three random microscopic fields per well using the $10 \times$ phase-contrast objective of a Leica DM-IRBE inverted microscope. Cell numbers were counted for each image and the mean and standard deviation were calculated for the three microscopic fields taken per well. The proliferation index was calculated by dividing the cell number by the mean of the cell number counted in the same well at the beginning of the experiment (day 0).

Statistical analysis

All experiments were repeated at least three times with triplicate samples. Statistical significance was determined using Student's *t*-test.

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Chapter 3

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

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The VirB type IV secretion system (T4SS) of *Bartonella henselae* mediates subversion of endothelial cell function upon bacterial infection (Schmid et al., 2004). In this report, multiple protein substrates of the VirB T4SS were identified and shown to mediate all known VirB-dependent cellular changes. In addition, we found a bipartite signal conserved in all the substrates, which enables translocation via the VirB T4SS into the host cell. By sequencing downstream of the *virB* locus (*virB2-virB11*), we identified a region encoding a putative T4SS coupling protein (*virD4*) and seven assumed translocated effector proteins *(ΔbepA-G)*. Together, the *virB/virD4/bep* locus represents a novel pathogenicity island (PAI) encoding 18 proteins with functions related to the type IV secretion. To asses the contribution of VirD4 and the BepA-G to VirB-mediated subversion of host cell function, two nonpolar deletion mutants *(ΔbepA-G* and *ΔvirD4)* were generated. Deletion of *bepA-G* or *virD4* abolished all previously described VirB-dependent cellular phenotypes. These include (i) massive rearrangement of the actin cytoskeleton during invasome formation, (ii) anti-apoptotic protection of endothelial cells, (iii) activation of a pro-inflammatory response, and (iv) cytostatic/cytotoxic effects upon high infection doses.

To demonstrate translocation of one of the newly identified Bep substrates into HUVEC, we monitored the phosphorylation of putative tyrosine–phosphorylation motifs found in BepD. Phosphorylation by a host cell tyrosine kinase upon infection with *B. henselae* was only observed for bacteria harboring a functional VirB/VirD4 T4SS. Next, we delimited the BepD translocation signal using a Cre-recombinase reporter assay for translocation (CRAFT) by flow cytometry. Several different BepD-Cre fusion proteins were constructed and analyzed in the CRAFT assay. The 183 aa C-terminus of BepD was efficiently translocated in a VirB/VirD4-dependent manner. The data revealed a bipartite translocation signal at the C-terminus, which

we defined as Bep intracellular delivery (BID) domain and a short positively charged tail sequence. This VirB/VirD4 T4SS translocation signal was found to be conserved among all seven Beps, and translocation could be demonstrated for BepB, C, D, and F. Moreover, a Hidden-Markov model generated from the alignment of all BID domains allowed us to identify several conjugative relaxases of α-proteobacterial origin which carry such a bipartite translocation signal. Using CRAFT, we exemplary confirmed for the relaxase AvhB/TraG, which is part of the T4SS-related conjugation system of *Agrobacterium tumefaciens*, that its bipartite signal is still able to direct VirB/VirD4-dependent protein transfer from *B. henselae* into HUVEC.

Statement of the own participation

I contributed to this publication by the following data: On the basis of the *ΔbepB-G* deletion mutant, I generated the substrate-free *ΔbepA-G* mutant of *B. henselae*. I also performed most of the experiments showing the subversion of host cell function by the *Bartonella* effector proteins, i.e. the determination of caspase-3/-7 activity (Fig. 1D), cytokine IL-8 secretion (Fig. 1E), and monitoring of the cytotoxic/cytostatic effect (Fig. 1F).

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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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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*.
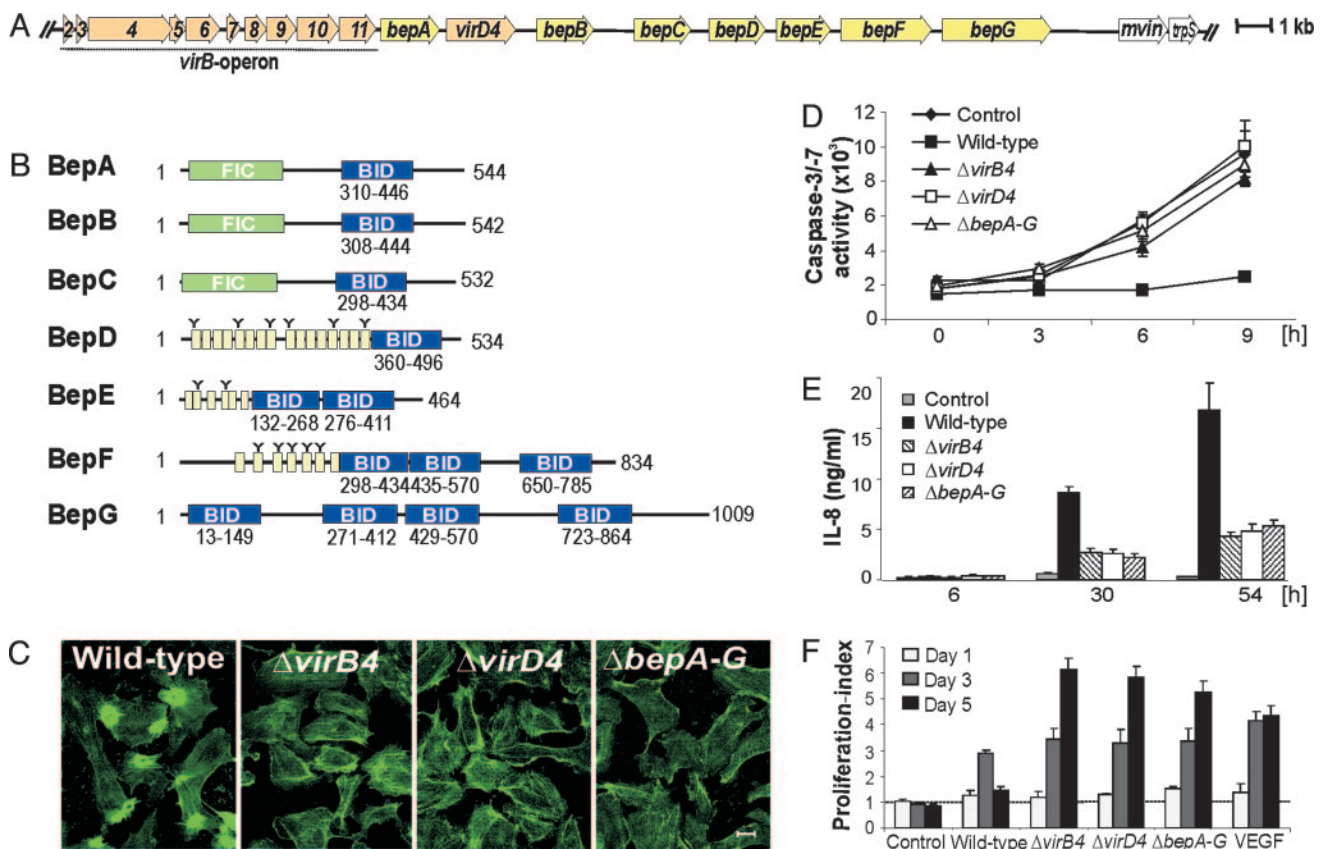
Fig. 1. The Beps mediate VirB/VirD4-dependent invasion, antiapoptotic protection, proinflammatory activation, and control of proliferation of HEC. (*A*) Structure of the *virB/virD4/bep* locus encoding the VirB components (VirB2–VirB11), the T4S coupling protein (VirD4), and seven putative effector proteins (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.

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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
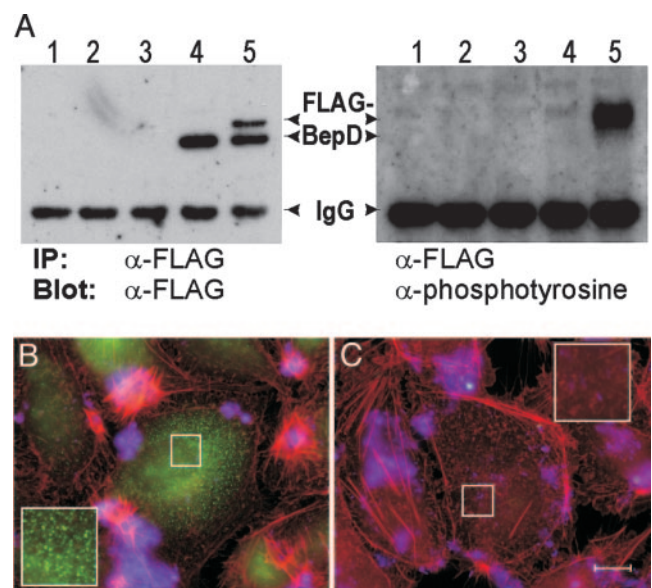
Fig. 2. BepD becomes tyrosine-phosphorylated after VirB4-dependent translocation into HEC. (*A*) VirB4-dependent translocation of BepD into HEC results in tyrosine phosphorylation and a coincident reduction in electrophoretic mobility. Total protein extracts of Ea.hy926 cells uninfected (lane 1) or infected 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.)

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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.
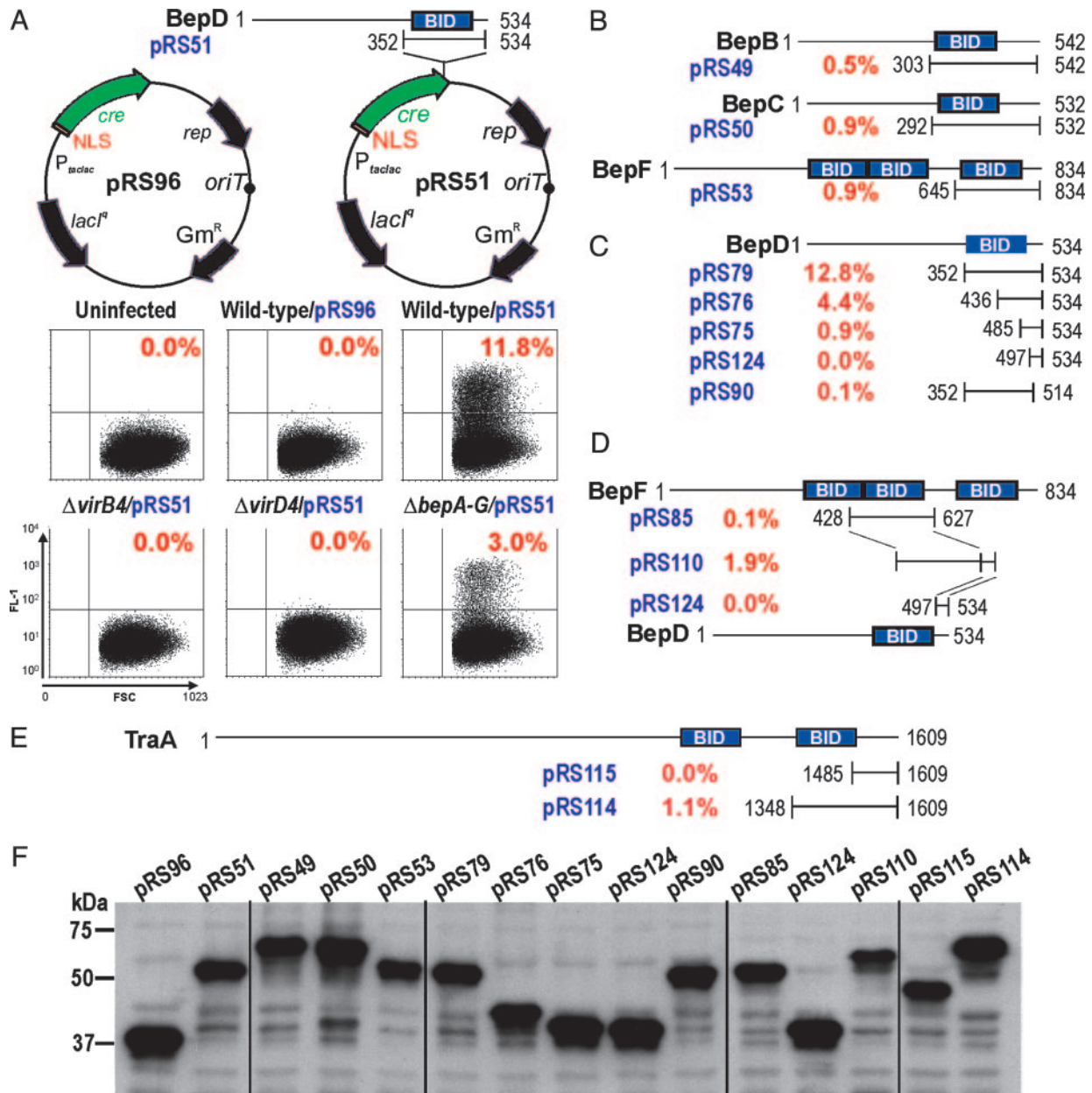
Fig. 3.   The C-terminal translocation signal of Beps mediates VirB/VirD4-dependent protein transfer into HEC. Protein transfer was determined by CRAFT. The Cre-tester cell line Ea.hy926/pRS56-c#B1 was infected with the indicated *Bh* strains expressing different NLS–Cre fusion proteins (plasmid names 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.

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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
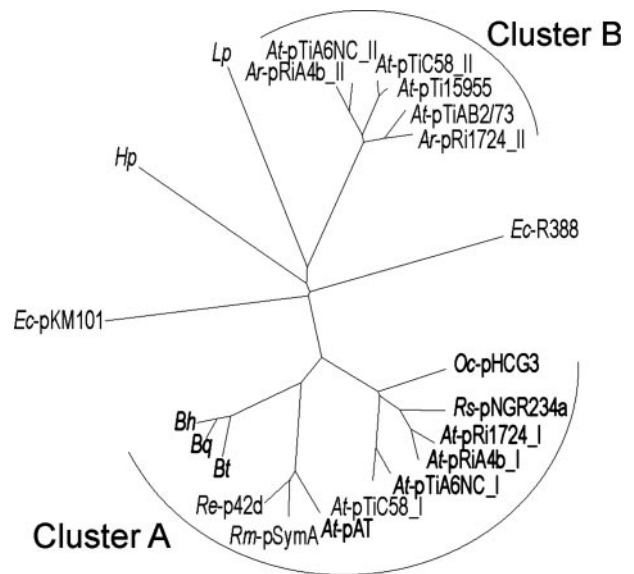
Fig. 4. The coupling proteins (VirD4/TraG) of T4S systems containing a BID domain in their protein substrate(s) form a distinct phylogenetic cluster. This cluster is formed by *Bartonella* VirB/VirD4 systems and α-proteobacterial conjugative DNA transfer systems (cluster A) and does not contain agrobacterial 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).

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.

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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MICROBIOLOGY

Chapter 4

A translocted protein of the vascular-tumor inducing pathogen *Bartonella* protects human vascular endothelial cells from apoptosis

M. C. Schmid, M. Dehio, F. Scheidegger, N. Devaux, B. Biedermann and C. Dehio

The antiapoptotic activity of *Bartonella henselae* during endothelial cell infection is dependent on a functional VirB/VirD4 T4SS (Schmid et al., 2004). In this study, the *Bartonella* effector protein A (BepA) was identified as a VirB/VirD4-translocated substrate that mediates the inhibition of apoptosis in HUVEC. To demonstrate this, a nonpolar *ΔbepA* mutant was constructed, which no longer showed the known anti-apoptotic effect. We were able to restore this phenotype by complementation *in trans*. BepA homologues of different *Bartonella* species were cloned into complementation plasmids and their ability to inhibit apoptosis was monitored by caspase-3/-7 assays. Thereby, only BepA homologues from *Bartonella* species known to induce angioproliferative tumor formation were able to protect endothelial cells from apoptosis. To demonstrate translocation of the two putative effector proteins BepA from *B. henselae* and *B. quintana* into HUVEC, we used the calmodulin-dependent adenylate cyclase (*cya*)-reporter assay. Translocation of the anti-apoptotic BepA from *B. henselae* and *B. quintana* into the host cell was confined to the C-terminus and was dependent on a functional VirB/VirD4 T4SS.

To delineate the functional domain of BepA required to inhibit apoptosis, we constructed different GFP-BepA fusion proteins. These fusion proteins were ectopically expressed in HUVEC and the apoptotic cell population was then determined. We identified a 140 aa domain of BepA, which is sufficient to inhibit apoptosis in HUVEC. This domain corresponds to the conserved BID domain of BepA. Next, we investigated the subcellular localization of BepA in the host cell. Different GFP-BepA fusion constructs were expressed in HUVEC and samples were analyzed by immunocytochemistry. We found that BepA associates to the cytoplasmic membrane of the host cell. Interestingly, only anti-apoptotic GFP-BepA fusion constructs associated to the membrane.

To enlighten the mechanism of inhibition of apoptosis by BepA, we incorporated microarray data obtained for the transcriptome of HUVEC infected with wild-type versus the *ΔvirB4* mutant. These revealed a VirB-dependent upregulation of NFκB and the cAMP/CREM/CREB regulon in infected endothelial cells. We tested whether these pathways were specifically activated by anti-apoptotic BepA homologues. The NFκB pathway did not show any specific activation by anti-apoptotic homologues of BepA. However, the upregulation of cAMP-regulated gene expression and the increased intracellular cAMP level correlated with the property of BepA to inhibit apoptosis. As artificially elevated cAMP levels protected endothelial cells from apoptosis, these data indicate that BepA mediates anti-apoptosis of endothelial cells by heightening cAMP levels by a plasma membrane-associated mechanism. Furthermore, expression of BepA in endothelial cells resulted in a significantly increased resistance to cytotoxic T-lymphocytes mediated cell death. Our results indicate that BepA, a novel T4S effector protein, plays a crucial role during the formation of vascular tumors.

Statement of the own participation

Dr. M. Dehio provided unpublished microarray data and the experimental setup for real time PCR, allowing to test for transcriptional signatures of putative survival pathways activated by BepA. All other data reported in this manuscript were generated by myself.

Reference

Schmid, M.C., Schulein, R., Dehio, M., Denecker, G., Carena, I. and Dehio, C. (2004) The VirB type IV secretion system of Bartonella henselae mediates invasion, proinflammatory activation and antiapoptotic protection of endothelial cells. *Mol Microbiol*, **52**, 81-92.

A translocated protein of the vascular tumor-inducing pathogen

Bartonella protects human vascular endothelial cells

from apoptosis

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Condensed title: **A bacterial protein mediating anti-apoptosis**

Abstract

The modulation of host cell apoptosis by bacterial pathogens is of critical importance for the outcome of the infection process. The capacity of *Bartonella henselae* (*Bh*) and *Bartonella quintana* (*Bq*) to cause vascular tumor formation in immunocompromized patients is linked to the inhibition of vascular endothelial cell (EC) apoptosis. Here, we show that translocation of BepA, a type IV secretion (T4S) substrate is necessary and sufficient to inhibit EC apoptosis. Ectopic expression in EC allowed mapping the anti-apoptotic activity of BepA to the BID domain, which as part of the signal for T4S is conserved in other T4S substrates. The anti-apoptotic activity appeared to be limited to BepA orthologs of *Bh* and *Bq* and correlated with (i) protein localization to the host cell plasma membrane, (ii) elevated levels of intracellular cAMP, and (iii) increased expression of cAMP-responsive genes. The pharmacological elevation of cAMP levels protected EC from apoptosis, indicating that BepA mediates anti-apoptosis by heightening cAMP levels by a plasma membrane-associated mechanism. Finally, we demonstrate that BepA mediates a marked protection of EC against cell death triggered by CTL, suggesting a physiological context in which the anti-apoptotic activity of BepA contributes to tumor formation in the chronically infected vascular endothelium.

Word count: 200

Introduction

Bacterial pathogens have developed various strategies to subvert host cell functions to their benefit. Especially intracellular bacteria adapted mechanisms to modulate the apoptotic pathway of their host cells (1). The resulting induction or inhibition of apoptosis is often crucial for a successful infection of the host. Pathogen-induced apoptosis can serve to eliminate key immune cells or to evade other host defenses (1). Several bacteria elicit an inflammatory process by inducing a specific form of apoptotic cell death, which at the place of infection leads to the disruption of tissue barriers and thus may secure efficient microbial spread in the host (2, 3). In contrast, inhibition of apoptosis may be essential for intracellular pathogens to establish chronic infection. Pathogen-triggered anti-apoptosis of infected host cells facilitates a slow microbial replication process and enables persistence in the infected host. For example, the obligate intracellular pathogen *Chlamydia pneumoniae* degrades by an unknown effector mechanism pro-apoptotic BH-3 host cell proteins (4). *Rickettsia rickettsii* protects invaded host cells from apoptosis by activating an NFκB-dependent survival pathway (5). For many human pathogens, the activation and inhibition of the apoptotic machinery of the infected host cell thus has a central role during the infection process (1).

The bacterial effectors known to modulate apoptosis are mostly pro-apoptotic. The only described anti-apoptotic effector is the outer membrane protein PorB of *Neisseria meningitis* (6). PorB has been shown to interact with mitochondria, where it is thought to block apoptosis by preventing mitochondrial depolarization and cytochrome C release. Interestingly, opposite effects were reported for the PorB ortholog of *Neisseria gonorrhoea*, which provokes a pro-apoptotic effect upon interaction with mitochondria (7, 8).

Bartonellae are facultative intracellular pathogens associated with the formation of vasoproliferative tumors in humans (e.g. bacillary angiomatosis, bacillary peliosis, and verruga peruana) (9). These vascular lesions consist of an increased number of EC, which are colonized by extracellular and intracellular bacteria. Although at least nine *Bartonella* species

are known to infect humans, vascular proliferation is mainly caused by the species *Bh, Bq, and B. bacilliformis* (9). Upon EC infection *in vitro*, *Bh* and *Bq* are able to stimulate proliferation and inhibition of apoptosis (9-11). The anti-apoptotic activity of bartonellae is considered to contribute synergistically to an unrelated mitogenic activity and results in the formation of vascular tumors (10, 11).

In *Bh,* inhibition of apoptosis is dependent on a functional VirB/VirD4 system (12, 13). This T4S system is a major virulence determinant for *Bartonella*-EC interaction (12-14). T4S systems are versatile transporters ancestrally related to bacterial conjugation machines. Different human pathogens have recruited such conjugation machineries to inject macromolecular effectors across the bacterial and host cell membranes directly into the host cell cytosol, where they alter various cellular processes (15). *Bh* carries a pathogenicity island (PAI) that encodes next to the *virB/virD4* locus seven putative T4S substrates, the *Bartonella*-translocated effector proteins *Bh*_BepA-G (13). Deletion of the PAI-regions encoding *bepA-G* abolished the anti-apoptotic activity and all other VirB/VirD4-dependent EC phenotypes (13). However, the contribution of individual *Bh*_Beps to anti-apoptosis and the other cellular phenotypes is unknown. All seven *Bh*_Beps carry in their C-terminus at least one conserved region, defined as BID (<u>B</u>ep <u>i</u>ntracellular <u>d</u>elivery) domain (13). The positively-charged C-terminal tail sequence together with the proximal BID domain constitute a bipartite signal for T4S. Based on a Cre-Recombinase Assay for Translocation (CRAfT), this T4S signal was shown to be functional for VirB/VirD4-dependent transfer of four different *Bh*_Beps (i.e. *Bh*_BepB, *Bh*_BepC, *Bh*_BepD, and *Bh*_BepF) (13). Evidence for translocation of *Bh*_BepA, *Bh*_BepE, and *Bh*_BepG is still missing.

*Bh*_BepA, *Bh*_BepB and *Bh*_BepC are paralogous proteins. A *Bh*_BepA ortholog is found in the animal pathogen *Bartonella tribocorum (Bt)* (16). Remarkably, all BepA homologs encode in their N-terminal part a conserved FIC (<u>f</u>ilamentation <u>i</u>nduced by <u>c</u>AMP)

domain, which was proposed to be involved in bacterial cell division (17), while the putative effector function within human cells is unknown.

In this paper, we identified BepA as anti-apoptotic effector of the vasoproliferative bartonellae *Bh* and *Bq*. We show that this effector is translocated by the VirB/VirD4 system into EC. Further, we show that the anti-apoptotic activity is confined to the BID domain of *Bh*_BepA, which also mediates localization to the EC plasma membrane. We demonstrate that *Bh*_BepA provokes an elevation of intracellular cAMP and the upregulation of cAMP-responsive genes, suggesting that plasma membrane-associated *Bh*_BepA triggers cAMP production and signaling, resulting in the abrogation of apoptotic processes. Accordingly, we were able to mimic the anti-apoptotic effect of BepA by artificially increasing the intracellular cAMP level in EC. Finally, we demonstrate that expression of BepA during endothelial cell infection results in resistance against CTL-mediated cell death.

Results

*Bh*_BepA mediates anti-apoptosis in EC.

The anti-apoptotic activity of *Bh* on human umbilical vein endothelial cells (HUVEC) requires a functional VirB/VirD4 system and at least one of the putatively secreted effectors *Bh*_BepA-G (12, 13). To identify the effector(s) required for inhibition of apoptosis, we expressed them individually in the substrate-free *Bh_ΔbepA-G* mutant and assayed for anti-apoptotic activity. To this end, HUVEC were infected with the different *Bh* strains and apoptosis was induced by exposure to actinomycin D (actD). Morphological examination (Figure 1A) and measuring of caspase-3/-7 activity (Figure 1B) was used to monitor apoptosis. *Bh_ΔbepA-G* did not display anti-apoptotic activity (13). Strikingly, the expression of *Bh*_BepA (by plasmid p*Bh*_BepA) restored the anti-apoptotic activity to wild-type level (Figure 1), whereas no other *Bh*_Bep interfered with actD-triggered apoptosis (data not shown). Consistently, strain *Bh_ΔbepA* carrying an in-frame deletion of *bepA* did not protect HUVEC from apoptosis, whereas complementation of this mutant with p*Bh*_BepA restored the anti-apoptotic activity. To test for functionality of the VirB/VirD4 system in the *Bh_ΔbepA* mutant we monitored the T4S-dependent process of invasome formation (12, 18), and furthermore quantified the translocation efficiency of a reporter substrate (encoded by pRS51) using CRAfT (13). Both assays confirmed that the VirB/VirD4 T4S system is functional in the *Bh_ΔbepA* strain (data not shown).

Taken together, these results provide evidence that *Bh* requires the putative T4S substrate *Bh*_BepA to protect HUVEC from apoptosis.

*Bh*_BepA is a VirB/VirD4 substrate translocated into EC.

CRAfT was used to demonstrate functionality of most of the C-terminal bipartite T4S signals of *Bh*_Beps (13), while this assay was negative for the putative T4S substrate *Bh*_BepA (data not shown). Here, we adapted the calmodulin-dependent adenylate cyclase (*cya*) reporter

assay (19, 20) as alternative to test for translocation of Bh_BepA into HUVEC. Translocation of a Cya-reporter fusion is demonstrated by the increase in the intracellular cAMP level. An N-terminal FLAG-tag epitope was fused to Cya to allow determining the stability of fusion proteins by immunoblot analysis (Figure 2A,C). As positive control, the T4S signal of Bh_BepD (Bh_BepD$_{352-534}$) (13) was fused to the FLAG-Cya reporter (pFLAG-Cya-Bh_BepD$_{352-534}$). To test for translocation of Bh_BepA, either full-length protein (Bh_BepA$_{1-544}$), or the C-terminal domain harboring the putative T4S signal (Bh_BepA$_{305-544}$) was fused to FLAG-Cya. These reporter constructs were expressed either in Bh wild-type or in the $ΔvirB4$ mutant lacking a functional VirB/VirD4 system. HUVEC infected with wild-type expressing FLAG-Cya did not display any significant increase of the cAMP level as compared to wild-type without the pFLAG-Cya plasmid. In contrast, FLAG-Cya fused to either Bh_BepD$_{352-534,}$ Bh_BepA$_{1-544,}$ or Bh_BepA$_{305-544}$ resulted in an approximately 10-fold increase of intracellular cAMP levels in HUVEC (Figure 2B). This effect was dependent on a functional VirB/VirD4 system. In summary, we show that BepA is a VirB/VirD4 substrate that harbors a C-terminal T4S signal.

The BID domain of Bh_BepA is sufficient to inhibit apoptosis.

Next we determined whether ectopic expression of Bh_BepA in HUVEC is sufficient to mediate anti-apoptosis. N-terminal GFP-fusions to full-length Bh_BepA or fragments thereof were constructed in an appropriate eukaryotic expression vector. As control we included the expression vector pGFP encoding just GFP (Figure 3A). Twenty-four hours after transfection of HUVEC with the different GFP-fusion constructs, cells were either exposed to actD (apoptosis induction) or left untreated (non-induced control) for another 12 h. Then, cells were stained with AnnexinV and propidium iodide (PI) and the proportion of apoptotic cells (AnnexinV positive, PI negative) among the GFP-positive cell population was quantified by flow cytometry.

HUVEC transfected with pGFP and treated with actD displayed an apoptotic rate of 13%. Fusion of full-length Bh_BepA to the C-terminus of GFP (pGFP-Bh_BepA$_{1-544}$) reduced the apoptotic population almost 4-fold to a level similar to the non-induced control. The same anti-apoptotic activity was observed when only the C-terminal bipartite T4S signal of Bh_BepA was fused, thus missing the first 304 N-terminal aa encoding the FIC domain (pGFP-Bh_BepA$_{305-544}$). In contrast, fusion of the FIC domain (pGFP-Bh_BepA$_{1-304}$) did not result in inhibition of apoptosis. Further analysis showed that fusion of the BID domain (142 aa) to GFP (pGFP-Bh_BepA$_{305-446}$) was sufficient to inhibit apoptosis, whereas fusion of the positively charged C-tail plus only part of the BID domain (pGFP-Bh_BepA$_{403-544}$) did not result in anti-apoptosis (Figure 4B). Expression of the latter construct rather had a pro-apoptotic effect, indicated by the increased apoptotic cell population in the untreated sample. Taken together, these data demonstrate that ectopic expression of the BID domain as part of the bipartite T4S signal of Bh_BepA in EC is sufficient to mediate protection against apoptosis.

Anti-apoptotic Bh_BepA constructs associate with the plasma membrane.

To test for the subcellular localization of Bh_BepA in EC, the generated GFP fusions (Figure 3A) were ectopically expressed in HUVEC for 30 h, followed by immunocytochemical staining for the surface marker α_v-integrin. Samples were analyzed by confocal microscopy by taking images in the xy-plane. To better distinguish between cytoplasmic and membrane-associated localization, we also captured images in a xz-plane at sections with increased cytoplasmic volume close to the nucleus (indicated in Figure 3C by dashed lines). Ectopically expressed GFP localized to the cytoplasm of HUVEC, whereas the GFP-Bh_BepA$_{1-544}$ fusion localized to the plasma membrane (Figure 3C). Interestingly, all fusion proteins with anti-apoptotic activity localized to the plasma membrane, while those

which did not confer protection localized to the cytoplasm. These findings indicate that association to the plasma membrane is critical for the anti-apoptotic activity of *Bh*_BepA.

Only BepA orthologs from vasoproliferative *Bartonella* species display anti-apoptotic activity.

To assess whether anti-apoptosis is a general feature of BepA homologs, we tested the orthologs encoded by other *Bartonella* species for this activity. In the public databases, orthologous sequences to *Bh*_BepA are available for *Bq* (NC_005955) and *Bt* (CAD37389). *Bq* was previously shown to cause vascular tumor formation, while *Bt* was never associated with vascular lesions (9). In the genome sequence of *Bq* the *bepA* gene is annotated as a pseudogene (21). Closer inspection of the sequence revealed that due to an internal stop codon and a downstream located start codon in frame this *bepA* locus is split into two ORFs (*Bq_bepA1, Bq_bepA2*). *Bq_bepA1* encodes a FIC domain, and *Bq_bepA2* encodes a BID domain and the positively charged C-terminal tail, the latter representing a putative T4S signal. Comparison of the amino acid sequences of *Bq*_BepA1 and *Bq*_BepA2 to *Bh*_BepA revealed high similarity (59% and 63%, respectively). We cloned *Bt_bepA* and *Bq_bepA2* into expression plasmids, which were introduced into *Bh_ΔbepA-G* (Figure 4A). As controls we included the paralogs *Bh_bepB* and *Bh_bepC*, which in the initial screening of *Bh*_Beps were found to lack anti-apoptotic activity (Figure 4A and data not shown). HUVEC were infected for 24 h with the different isogenic strains, and subsequently apoptosis was induced by actD. Of the tested BepA homologs, only *Bh*_BepA and *Bq*_BepA2 inhibited actD-triggered caspase-3/-7 activation (Figure 4B). These findings revealed that *Bq_bepA2* encodes an anti-apoptotic effector. To assign *Bq*_BepA2 as a novel T4S substrate, we demonstrated translocation of a FLAG-Cya-BepA2 fusion from *Bh* into HUVEC by the Cya-reporter assay (data not shown). These results show that the anti-apoptotic activity of BepA is conserved among two human pathogens with vasoproliferative capacity (*Bh* and *Bq*), but not in *Bt* which

has not been associated with vasoproliferation. Moreover, these data confirm the localization of the anti-apoptotic activity to the C-terminal region of BepA composing a functional T4S signal (13).

Inhibition of apoptosis correlates with an increased intracellular cAMP level.

To identify potential survival pathways mediated by anti-apoptotic BepA, we analysed Affymetrix GeneChip data obtained for the transcriptome of HUVEC infected with *Bh* wild-type versus the *ΔvirB4* mutant impaired in T4S (M. Dehio, M. Schmid, M. Quebatte and C. Dehio, unpublished data). These data revealed a T4S-dependent upregulation of the NFκB and cAMP-dependent CREM/CREB regulons in HUVEC. Both signaling pathways were described to mediate, among other functions, the protection against apoptosis (22-24).

We tested whether these pathways were activated by anti-apoptotic BepA homologs. We monitored activation of the NFκB pathway in infected HUVEC by measuring the release of IL-8. *Bh* wild-type triggered an increased IL-8 release compared to *Bh_ΔbepA-G* or the uninfected control. In contrast, none of the BepA homologs expressed in *Bh_ΔbepA-G* induced increased IL-8 secretion (Figure 5A). This finding indicates that activation of the NFκB pathway by wild-type is independent of the survival mechanism triggered by anti-apoptotic BepA homologs. Activation of the cAMP-dependent CREM/CREB pathway was assayed by quantitative real-time PCR of two cAMP-inducible genes, namely *pde4B* and *crem* (25, 26). Only strains translocating anti-apoptotic BepA homologs were found to induce the expression of these genes in a statistically significantly manner (Figure 5B). By quantifying intracellular cAMP upon infection of HUVEC by the various isogenic *Bh* strains, we further demonstrated that anti-apoptotic BepA homologs significantly increase the intracellular cAMP level (Figure 5C).

A rise in intracellular cAMP results in protection of EC against apoptosis.

As translocation of anti-apoptotic BepA homologs resulted in a rise of the intracellular cAMP level, we further studied the role of cAMP in anti-apoptosis. The intracellular cAMP level is regulated by adenylate cyclases (AC) generating cAMP, and phosphodiesterases (PDE) degrading cAMP (27, 28). To trigger a physiological rise of the cAMP level in EC, we activated AC with forskolin and in parallel inhibited cAMP degradation by adding the PDE-inhibiting drug 3-isobityl-1-methylxanthine (IBMX) (29, 30). HUVEC were infected with the substrate-free *Bh_ΔbepA-G* strain and with the BepA-expressing strain *Bh_ΔbepA-G/pBh_*BepA in the absence and presence of forskolin/IBMX. Apoptosis was induced by exposure to actD and monitored by measuring caspase-3/-7 activity. By adding forskolin/IBMX we were able to reduce caspase-3/-7 activity in uninfected HUVEC and cells infected with the *Bh_ΔbepA-G* strain to a similar level as found in cells infected with *Bh_ΔbepA-G/pBh_*BepA. Interestingly, the addition of forskolin/IBMX did not have an additive protection-effect in *Bh_ΔbepA-G/pBh_*BepA infected HUVEC (Fig. 6A). The same results were obtained by adding the cell-permeable cAMP analog dibutyryl cAMP to the culture medium (Fig. 6B). From these data we conclude, that a moderately increased intracellular cAMP level, as triggered by *Bh_*BepA, is sufficient to protect EC against apoptosis.

The role of *Bh_*BepA in evading CTL-mediated cell death.

During colonization of the human endothelium, intracellular *Bh* are hidden from antibody and complement-mediated immune responses (9). However, the infected EC may be killed by CTL (31). To study the role of *Bh_*BepA in this context, we used PHA-activated CTL for triggering apoptosis of *Bh* infected EC (32). To specifically measure the rate of EC apoptosis without interference by apoptotic CTL, we pre-labeled the EC population with the fluorogenic dye CSFE. Pre-labeled HUVEC were infected with *Bh_ΔbepA-G* or with

*Bh_ΔbepA-G/pBh_*BepA. Twenty-four hours after infection, CTL were added in an E/T cell ratio of 13:1. At different time points morphological examination and AnnexinV/PI staining was performed to quantify the amount of surviving (AnnexinV negative, PI negative) and early-apoptotic or dead HUVEC (AnnexinV positive, PI negative and positive) (Fig. 7A/B).

Starting with a confluent monolayer with adherent CTL, considerable differences in remaining HUVEC numbers were observed during the course of CTL exposure in the various conditions tested (Fig. 7A). In the early time points (2 h and 6 h), uninfected HUVEC were more susceptible to CTL-mediated cell death than pre-infected cells. Importantly, in pre-infected HUVEC the translocation of *Bh_*BepA provoked a marked increase in resistance to CTL-mediated cell death. After 6 h CTL-exposure the monolayer pre-infected with *Bh_ΔbepA-G* was heavily disrupted and remaining HUVEC were found in patches. In *Bh_ΔbepA-G/pBh_*BepA pre-infected HUVEC the grade of disruption of the monolayer was strongly diminished. This *Bh_*BepA-dependent increase in EC resistance to CTL-mediated cell death was even more pronounced during prolonged CTL exposure time. After 48 h exposure to CTL, the persistence of a HUVEC monolayer was strictly dependent on the presence of *Bh_*BepA.

The same results were obtained by quantifying the surviving and dead HUVEC populations by AnnexinV/PI staining. Uninfected HUVEC revealed a rapid decrease of the surviving cell population in the presence of CTL (Fig. 7B), as indicated by 7% surviving cells and 80% dead cells after 6 h exposure to CTL. The percentage of surviving HUVEC was dependent on the presence of bacteria, however, during all stages of incubation with CTL, the presence of *Bh_*BepA caused increased HUVEC survival and strongly reduced the dead cell population compared to the substrate-free *Bh_ΔbepA-G* strain. After 48 h of HUVEC exposure to CTL, *Bh_*BepA caused the persistence of 30% of HUVEC, while survival of uninfected or *Bh_ΔbepA-G* infected HUVEC declined to 1% and 5%, respectively (Fig. 7C).

These data indicate that translocated *Bh*_BepA effectively protects the chronically infected vascular endothelium against CTL-mediated cell death.

Ectopic expression of *Bh*_BepA reduces CTL-mediated cell death.

To test whether *Bh*_BepA alone is sufficient to mediate increased resistance to CTL-triggered EC death, we ectopically expressed the anti-apoptotic pGFP-*Bh*_BepA$_{1-544}$ construct in HUVEC (Fig.3A). pGFP served as a negative control. Twenty-four h after transfection, CTL were added to the HUVEC monolayer in an E/T cell ratio of 4:1 or 13:1. Upon 22 h of incubation, bright field pictures were taken, followed by AnnexinV/PI staining to measure the surviving rate of GFP-positive cells by flow cytometry. While in the absence of CTL the monolayer of pGFP and pGFP-*Bh*_BepA$_{1-544}$ transfected cells stayed intact (Fig. 8A), the addition of CTL decreased the surviving cell population in an E/T cell-dependent manner. This CTL-mediated cell death was significantly reduced by expression of pGFP-*Bh*_BepA$_{1-544}$ for both E/T cell ratios tested.

From these data, we conclude that ectopic expression of *Bh*_BepA in HUVEC is sufficient to provoke increased resistance against CTL-mediated cell death.

Discussion

The modulation of host cell apoptosis is a recurrent theme in bacterial pathogenesis (1).
Research in this area focused initially on the pro-apoptotic mechanisms triggered by
pathogens that typically cause acute infections (e.g. *Shigella*, *Salmonella,* and *Yersinia*
species). Some pro-apoptotic effectors and the targeted cellular pathways have been studied in
molecular detail (33, 34). More recently, pathogen-triggered anti-apoptosis was recognized as
an important virulence trait of bacteria that predominately cause chronic infection (e.g.
Bartonella, *Brucella*, *Chlamydia*, *Helicobacter*, *Mycobacterium*, and *Rickettsia* species) and
thus require to protect their cellular habitats by suppressing host-triggered apoptosis (35).
However, the bacterial effectors and to a large extent also the cellular pathways involved in
mediating pathogen-induced anti-apoptosis remained poorly defined. In the case of *Bh* and
Bq, the formation of vascular tumors in immunocompromised patients was shown to be linked
to the inhibition of apoptosis of infected EC (10). In this report we identified the anti-
apoptotic factor of these vasoproliferative bartonellae. Deletion of the *Bh_bepA* gene resulted
in the complete loss of the anti-apoptotic activity of *Bh,* while expression of *Bh_bepA in trans*
restored the activity to wild-type level. *Bh*_BepA was previously described as putative
substrate of the T4S system VirB/VirD4 (13). In this report, we used the Cya-reporter assay to
demonstrate that *Bh*_BepA is indeed translocated into EC in a T4S-dependent manner.
*Bh*_BepA is thus a genuine T4S effector that inhibits apoptosis upon translocation into
HUVEC. It is worth to note, that we have been unable to show translocation of *Bh*_BepA by
CRAfT, an assay that we previously used to demonstrate translocation of several other
*Bh*_Beps (13). Nuclear import of the Cre-reporter protein fusion is a prerequisite for a positive
read-out by CRAfT (13), suggesting that the negative read-out obtained for *Bh*_BepA fusions
could result from protein recruitment to an intracellular localization that interferes with
nuclear import. Indeed, ectopic expression of full-length *Bh*_BepA fused to GFP revealed a
prominent localization of the fusion protein to the plasma membrane, in contrast to the

cytosolic localization of GFP alone. Unlike ectopically expressed GFP, the GPF-*Bh*_BepA

fusion also conferred protection against EC apoptosis. These data demonstrate that *Bh*_BepA

is not only required, but also sufficient for inhibiting EC apoptosis, and that this anti-apoptotic

effector localizes to the plasma membrane. Moreover, ectopic expression of various parts of

*Bh*_BepA fused to GFP revealed a strict correlation of the capacity to mediate anti-apoptosis

with localization to the plasma membrane (opposed to the cytoplasmic localization of fusions

that did not cause anti-apoptosis). This finding indicates that plasma membrane targeting may

be important for the anti-apoptotic activity of *Bh*_BepA.

In search for the cellular pathway by which *Bh*_BepA mediates anti-apoptosis in EC,

we first tested whether an NFκB-dependent survival pathway is involved. The anti-apoptotic

activity of *Rickettsia rickettsii* in EC was reported to depend on NFκB activation (5), and we

have previously shown that *Bh* activates NFκB in a VirB/VirD4 and Bep-dependent manner

(13). Here, we demonstrated that *Bh*_BepA alone does not trigger the NFκB-dependent

secretion of IL-8, even so it fully protects against apoptosis. Thus, NFκB does not seem

critical for *Bh*_BepA-mediated anti-apoptosis. Affymetrix genechip experiments indicated

that *Bh* also triggers a cAMP-signalling pathway in a T4S-dependent manner (M. Dehio, M.

Schmid, M. Quebatte and C. Dehio, unpublished data). Here, we have shown that the anti-

apoptotic activity of *Bh*_BepA in HUVEC correlated with the elevation of the intracellular

cAMP level and resulted in the upregulation of cAMP-stimulated gene expression. Recently,

it emerged that the regulation of apoptosis is an important facet of cAMP signal transduction

(30, 36-38). Moderately elevated cAMP levels were reported to protect several cell types

against apoptosis, while the survival mechanisms differed from cell type to cell type and were

considered to require the activation of PKA, ERK, MAPK, or a GEF-signaling pathways,

which subsequently resulted in the expression of anti-apoptotic genes (23, 36, 37, 39-44). In

our system, the specific downstream signaling pathway mediating cAMP-dependent anti-

apoptotis remains elusive, because efforts to block putatively involved signaling molecules

(i.e. PKA) failed due to apparent cytotoxic effects of effective inhibitor concentrations during the extended time-frame of the apoptosis assay.

Importantly, a physiological rise in the intracellular cAMP level was reported to fully protect EC against apoptosis (45). Consistently, we observed that increased cAMP levels as a result of the combined action of the AC-activating drug forskolin and the PDE-inhibiting drug IBMX resulted in a complete suppression of actD-induced apoptosis in HUVEC (Fig. 6). Forskolin/IBMX-treated cells displayed a similar increase in the expression of cAMP-regulated genes as observed upon infection with anti-apoptotic *Bh* strains. Cyclic AMP is produced in eukaryotic cells by the family of membrane anchored AC. AC are activated by heterotrimeric G proteins that are regulated by G protein-coupled receptors (46). Since the anti-apoptotic activity of *Bh*_BepA in HUVEC is strictly associated with the plasma membrane localization of this effector (Fig. 3), concomitant with an increase in the cAMP level (Fig. 5), we propose that *Bh*_BepA may trigger anti-apoptosis by interacting either with AC directly, or with plasma membrane-associated heterotrimeric G proteins or G protein-coupled receptors that regulate AC activity.

Ectopic expression of GFP-fusions with different parts of *Bh*_BepA confined the anti-apoptotic activity to a region of 142 aa, which corresponds to the BID domain. This conserved domain was previously shown to be present in a least one copy in all Beps of *Bh* and *Bq* (13, 14, 21). The BID domain plus the C-terminal positively charged tail sequence of the Beps were shown to constitute a bipartite translocation signal for T4S (13). Interestingly, in contrast to the BID domain of *Bh*_BepA, the conserved BID domains of the paralogs *Bh*_BepB and *Bh*_BepC have not be associated with anti-apoptosis. This indicates that subsequent to the expansion of this paralogous protein family by gene duplication, the BID domain of *Bh*_BepA acquired in addition to its crucial function in mediating T4S the capacity to mediate anti-apoptosis. How this rather short domain may mediate these unrelated activities is presently unknown. It may be speculated that the BID domain represents a basic fold that mediates a

protein-protein interaction with the T4S machinery that is crucial for protein translocation, and that in *Bh*_BepA this basic fold is adapted to also mediate specific interaction with the plasma membrane-associated cAMP-generating signaling cascade of EC. Each of the paralogs *Bh*_BepA, *Bh*_BepB and *Bh*_BepC carries in the N-terminal region one copy of the FIC domain. While this conserved domain of unknown function might be important for the effector function of *Bh*_BepB and *Bh*_BepC, our results demonstrated that the FIC domain is dispensable for the anti-apoptotic activity of *Bh*_BepA. Interestingly, an internal stop codon and downstream located start codon within the *bepA* ortholog of *Bq* is splitting this locus into two separate ORFs (*Bq_bepA1* and *Bq_bepA2*). *Bq*_BepA1 consists of the FIC domain but lacks a T4S signal, and thus can not be translocated into EC. In contrast, *Bq*_BepA2 consists only of the T4S signal, which we have shown to mediate both T4S-dependent translocation, as well as anti-apoptosis in EC. It thus appears plausible that the functional diversification of BepA into an anti-apoptotic effector occurred in the common ancestor of these closely species *Bh* and *Bq* (21), and that following speciation the acquisition of mutations resulted in the elimination of the dispensable N-terminal FIC domain in *Bq*.

Interestingly, unlike *Bh*_BepA and *Bq*_BepA2, the BepA ortholog of *Bt* did not mediate any anti-apoptotic activity, nor any measurable activation of the cAMP pathway. This observation is in agreement with the previously reported finding that among the bartonellae the anti-apoptotic activity is limited to the vasoproliferative species *Bh* and *Bq* (10).

To asses if the here identified anti-apoptotic activity of Bh_*BepA* might be able to protect the cellular habitat of *Bh* during the infection process, we used a co-culture system of HUVEC with human CTL. CTL execute cell-mediated immunity (CMI), a physiological mechanism involved in the elimination of *Bh* infection (9, 12, 47). CMI particularly serves as a defense mechanism against microbes that survive and replicate inside infected host cells. Upon recognition of MHC class I displaying microbial peptides, CTL are activated to kill their target cells by the release of perforin and granzymes (31, 48). Perforin forms pores in the

target cell membrane and assists the delivery of pan-caspase activating granzymes into the cytoplasm of the target cell (44, 49, 50). In our study, we used a CTL clone, which is pharmacologically activated by PHA and kills HUVEC in a perforin-dependent manner, primarily by triggering granzyme-dependent apoptosis (50, 51). In this experimental context, the release of cytotoxic effector proteins by this CTL clone is independent of the MHC class I-restricted presentation of microbial peptides (32). Hence, we can exclude differences in peptide-presentation and CTL activation between different *Bh* stains. *Bh*_BepA mediated increased resistance to CTL-dependent cell death, indicating that the biological function of *Bh*_BepA expression *in vivo* might be to protect the integrity of its colonized cellular niche. Interestingly, *Bh*_BepA could not prevent EC death completely in this CTL co-culture system, suggesting that *Bh*_BepA might inhibit the apoptotic pathway mediated by granzymes, while apoptosis-independent killing mechanisms such as perforin-mediated lyses are unaffected.

Via the ectopic expression of *Bh*_BepA in HUVEC we further showed a protective effect of this bacterial effector against CTL-mediated cell death in the absence of bacterial infection (Fig. 8). These data confirm the cytoprotective effect of *Bh*_BepA observed during infection assays and allowed us to exclude any interference of *Bh*_BepA with CTL-activity by effector-translocation into CTL (Fig. 8B).

The *Bh*_BepA-mediated resistance of EC to CTL-dependent cell death points towards an important role of BepA in escaping CMI and thus in protecting the integrity of the chronically infected vasculature, which represents a prerequisite for vascular proliferation. The establishment of an animal model for *Bartonella*-triggered vasoproliferation now appears an urgent need to study the precise contribution of BepA-mediated anti-apoptosis in the process of vascular tumor formation.

In summary, the anti-apoptotic T4S effectors *Bh*_BepA and *Bq*_BepA2 characterized in this study represent striking examples for the evolution of new pathogenic traits in bacteria. The delineation of their anti-apoptotic activity to the conserved BID domain and its proposed

role to maintain the cellular habitat by mediating anti-apoptosis via specific interaction with the plasma membrane-associated cAMP-generating signaling cascade of EC should pave the way for future studies to elucidate the molecular and structural basis of *Bartonella*-mediated anti-apoptosis in the vascular endothelium and the role of this pathological process in vasoproliferative tumor growth.

Materials and methods

Bacterial strains and Growth Conditions. The bacterial strains used in this study are listed in Table 1 of the Online Supplemental Material. *Bartonella* spp. were grown on Columbia agar plates containing 5% defibrinated sheep blood (CBA plates) at 35°C and 5% CO_2 for 2-4 days. Strain RSE247, a spontaneous streptomycin-resistant strain of ATCC 49882^T (12) served as wild-type in this study. When indicated, media were supplemented with 30 µg/ml kanamycin, 100 µg/ml streptomycin, 12.5 µg/ml gentamicin, and/or 500 µM isopropyl β-D-thiogalactosidase (IPTG). *E. coli* strains were cultivated in Luria-Bertani liquid medium (LB) or after addition of agar on plates (LA) at 37°C overnight. When indicated, media were supplemented with 50 µg/ml kanamycin, 200 µg/ml ampicillin, 25 µg/ml gentamicin, 500 µM IPTG, and/or 1 mM diaminopimelic acid (DAP).

DNA-Manipulation

Plasmids used in this study are listed in Table 1 of the Online Supplemental Material. Primers are listed in Supplementary Material Table 2.

Construction of In-Frame Deletions and Complementation of the Deletion Mutants. pMS5 used for creating the Δ*bepA* mutant was already described before (13). The use of pMS5 for gene-replacement in RSE247 resulted in the Δ*bepA* mutant MSE154.

Plasmids for complementation of Bh_ΔbepA mutant and Bh_ΔbepA-G mutant. For constructing vectors expressing N-terminal FLAG-tagged Bep proteins, the plasmid pPG100 was used. The fragments were generated by PCR-amplification from chromosomal DNA of RSE247 (*Bh*) and *Bt*, respectively, using oligonucleotide primers prPG92/93, prPG95/129, prPG97/130, or pMS27/28 were inserted by the flanking *NdeI*-site into the corresponding site of pPG100, resulting in pPG101 (encoding *Bh*_BepA), pMS006 (encoding *Bh*_BepB), pMS007 (encoding *Bh*_BepC), and pMS011 (encoding *Bt*_BepA).

To construct plasmid pMS100, carrying a multiple cloning site, prGS01 and prGS02 were annealed together and the resulting 45 bp fragment was inserted into pPG100 using the *NdeI*-site. Using oligonucleotide primers pMS102 and prMS103, a fragment of 0.85 kb was amplified using chromosomal DNA of *Bq* as template. Using *SalI/XmaI*-sites this fragment was inserted into the corresponding site of pMS100, giving rise to pMS106.

Plasmid pMS105 was constructed as follows. Using flanking *SalI/XmaI*- sites, the 1.65 kb PCR-fragment generated by prMS37/38 (chromosomal DNA of RSE247 serves as a template) was inserted into the corresponding sites in pRS40, yielding pMS13. Using flanking *SalI/XmaI*- sites the 1.65 fragment was shuttled into pMS100 using the corresponding sites.

Using oligonucleotide primer prRS167/180 and prRS187/188, fragments of 0.73 kb size were amplified using chromosomal DNA of RSE247 and of *Bt* respectively as template. Using flanking *SalI/XmaI*-sites, the fragments were inserted into the corresponding site of pRS40, giving rise to pRS48 and RS55, respectively.

C-terminal sequences of the different Bep-proteins were shuttled into pMS100 using the flanking SalI/XmaI-sites. The plasmids resulting from this step are given below, as well as the range of aa of a given Bep protein fused to the N-terminal FLAG: pMS100-A (shuttled from pRS48, aa 305-544 of BepA), pMS100-B (shuttled from pRS49, aa 303-542 of BepB), pMS100-C (shuttled from pRS50, aa 292-532), and pMS100-D (shuttled from pRS51, aa 352-534 of BepD).

Plasmid for expression of Cya-Bep-fusion proteins. To construct pMS400, the calmodulin-dependent adenylate cyclase of pMS111 was PCR-amplified with oligonucleotide primers prMS78/90 introducing a start and a stop codon to the resulting *cya* fragment. Using flanking *NdeI/XmaI*-sites, the 1.23 kb fragment was inserted in the corresponding sites of pMS100. Plasmid pMS401 was derived by insertion of a 1.2 kb *NdeI/StuI*-fragment of pMS400, which includes the *cya*-gene without stop codon, into the corresponding sites of pMS100-A. The *NdeI/StuI*-fragment of pMS400 was further inserted in the corresponding site of pMS100-D

and pMS105, giving rise to pMS404 and pMS405, respectively. To construct pMS406, *Bq*_BepA2 was PCR-amplified from chromosomal DNA of *Bq* with oligonucleotide primers prMS103/104. Using flanking *PmeI/XmaI*-sites, the 0.86 kb fragment was inserted in the *StuI/XmaI*-sites of pMS400.

Plasmids for ectopic expression of GFP-BepA-fusion proteins. To construct eGFP-Bep fusion proteins (see Figure 4A), pWAY21 (Molecular Motion Montana Labs), a CMV driven EGFP for C-terminal fusion, was used as basic vector. The plasmids resulting from this step and the used oligonucleotide primers (with incorporated *XmaI/XbaI*-sites used for cloning to the corresponding sites of pWAY21) are given below, as well as the range of aa of a given Bep protein fused to eGFP: pMS21 (primers prMS68/69, aa 1-544 of *Bh*_BepA); prMS22 (primer prMS76/77, aa 302-542 of *Bh*_BepB); pMS23 (primers prMS83/69, aa 305-544 of *Bh*_BepA); pMS24 (primers prMS83/84, aa 305-446 of *Bh*_BepA); pMS25 (primers prMS74/69, aa 403-544 of *Bh*_BepA); pMS26 (primers prMS68/75, aa 1-304 of *Bh*_BepA); pMS27 (primers prMS105/106, aa 1-277 of *Bq*_BepA2).

The integrity of all constructs was confirmed by sequence analysis and western blotting using anti FLAG M2 antibodies (SIGMA, Saint Louis, USA).

Cell Lines and Cell Culture. Human umbilical vein endothelial cells (HUVEC) were isolated as described (18). HUVEC and the human embryonic kidney cell line HEK293T were cultured as described before (12). The stable transfected endothelial cell line Ea.hy296/pRS56-c#B1 was cultured as reported (13).

Infection Assay. HUVEC (passage 3-7) plated as described before (12). Unless stated differently, cells were infected with a multiplicity of infection (MOI) 300 bacteria per cell (10) in M199/10% FCS/500 μM IPTG and incubated for the indicated time. If specified, 100 nM actD (Sigma Aldrich) was added to trigger apoptosis as described before (10, 12).

Transfection Assay. Huvec were transfected using Amaxa nucleofection technology^{TM} (Amaxa, Koeln, Germany) following the manufactures guidelines for HUVEC transfection. After transfection cells were seeded onto cover-slips into 24-well plates or 6 well plates for subsequent immunocytochemistry or AnnexinV assay, respectively.

Caspase Activity Assay. The infection of HUVEC and the determination of caspase-3/-7 activity (MOI=300) were carried out as described (12).

Calmodulin Dependent Adenylate Cyclase Assay. cAMP was assayed after 20h contact between bacteria (MOI=300) and HUVEC in 24-well plates. HUVEC were washed one time in pre-warmed PBS and lysed in denaturizing conditions as described previously (19). cAMP was assayed by a EIA system (Biotrak, Amersham). Total cell proteins were assayed by the method of Bradford (52)(Bradford Reagent, SIGMA).

Cre-Recombinase Reporter Assay for Translocation (CRAfT). The infection of the stable transfected Ea.hy926/pRS56-c#B1cell line and the quantification of GFP positive cells (positive events in %) by flow cytometry was carried out as described (13).

Immunoblot Analysis. To monitor the steady-state level of Cya-fusion proteins, bacteria were grown on isopropyl β-D-thiogalactosidase-containing medium for 2 days. Cells were harvested and proceed as described previously (13), excepting as primary antibody anti FLAG M2 (Sigma, Saint Louis, USA) was used.

The stability of the different GFP-fusion protein used for ectopic expression in HUVEC was monitored after transient transfection of HEK293T cells. Cells were transfected by Fugene6 (Roche, Switzerland) following the manufactures instructions. After 24 h

expression cells were washed twice with PBS and harvested in 100ul sample buffer. Samples were then further proceed as described before (13) and probed with anti-GFP (Roche, Switzerland) antibody.

AnnexinV Assay. 24 h after transfection of HUVEC apoptosis was induced for 12 h. Cells were then collected by mild trypsinization and briefly centrifuged together with the culture supernatant. The cell pellet was washed, resuspended, and stained with AnnexinV APC (Alexis, Switzerland). Propidium iodide (PI, 1ug/ml) was added to counterstain necrotic cells and sample were then analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Prior to determine the apoptotic population, transfected cells were gated by their positive GFP signal.

Transfection and Immunocytochemistry. HUVEC were transfected for 30 h with different GFP-Bep fusion constructs, fixed with 3.7% paraformaldehyde, and immunocytochemically stained against α_v-integrin (Merck). Specimens were analyzed by confocal microscopy as described (12).

Determination of IL-8 Secretion. The infection (MOI=300) of HUVEC and the determination of IL-8 secretions by IL-8 DuoSet ELISA kit (R and D Systems, Minneapolis, MN) was performed as described before (12).

Determination of Intracellular cAMP Level. After infection (MOI=150) of HUVEC for 30 h in 24-well plates, cells were washed with pre-warmed PBS and lysed. Intracellular cAMP level was determined by the EIA system (Biotrak, Amersham) as described by the manufacture.

Real time PCR. To quantify the activation of the cAMP pathway, total cellular RNA was isolated at 54 h after infection as described above. RNA manipulation and real time PCR was performed as previously described (53) using primers listed in Table II.

Effects of Exogenous cAMP and cAMP-elevating Agents. HUVEC were infected in the absence or presence of either forskolin (Sigma-Aldrich) and 3-isobityl-1-methylxanthine (IBMX) (Sigma-Aldrich) or dibutyryl cAMP (Sigma-Aldrich) for 24 h with the indicated *Bh* strains (MOI = 300) or left uninfected (control). Apoptosis was induced and caspase-3 and -7 activities were monitored as described above.

CTL Assay upon Infection. Confluent HUVEC monolayers were labeled with 5uM carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes) in M199 without FCS according to the manufactures protocol. Cells were then infected with indicated *Bh* strains (MOI=300) or left uninfected in M199/ 10% FCS/ 500uM IPTG during 24 h. CTL clones were generated as described previously (51). CTL were washed twice by centrifugation and were added in a ratio of E/T cell of 13:1 in replacing the infection medium with CTL-assay medium (M199, 2%FCS, 5mM HEPES, 2mM Glutamine, 500μM IPTG). Plates were centrifuged to bring the cells in contact followed by adding phytohemagglutinin PHA (Sigma Aldrich) in a final concentration of 1μg/ml . Cells were washed with prewarmed PBS (excepted time point 0h) prior transmission light pictures were taken at indicated time points by a Leica DM IL microscope equipped with a Leica DFC 280 camera and Leica Image Manager 50 software. To quantify the surviving and dead cell rate, we used AnnexinV/PI staining and flow cytometry (described above). Prior to determine the surviving and dead cell populations, CFSE-positive cells were gated by their positive GFP signal.

CTL Assay upon Transfection. HUVEC were transfected for 24 h with indicated GFP-fusion constructs. CTL were added in an E/T cell ratio of 4:1 and 13:1. Cells were brought in contact by centrifugation and PHA was added (1µg/ml). After 22 h incubation, pictures were taken and surviving cells were quantified by AnnexinV/PI staining as described above. Prior to determine the surviving cell populations using flow cytometry, GFP-positive cells were gated by their positive GFP signal.

Acknowledgments

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Figure legends

Figure 1. *Bh*_BepA meditates inhibition of apoptosis in EC.

(A, B) HUVEC were infected for 24 h with the indicated *Bh* strains (MOI=300) or left uninfected for this period (control). If not indicated differently, apoptosis was then triggered by the addition of actD. 12 h later **(A)** morphological changes were visualized by recording phase contrast images (bar = 40 μm), and **(B)** caspase-3/-7 activities were determined with a specific fluorogenic peptide substrate. Arrowheads indicate apoptotic cells displaying membrane blebbing. All strains were tested a minimum of three times in triplicate samples.

Figure 2. *Bh*_BepA is a genuine VirB/VirD4 T4S substrate that is translocated into EC.

(**A**) The bars indicate the parts of *Bh*_BepA or *Bh*_BepD, which were fused to calmodulin-dependent adenylate cyclase (*cya*). These reporter fusions were used to monitor translocation via the VirB/VirD4 system. All construct contain an N-terminal FLAG epitope for immunological detection of the encoded fusion protein. (**B**) Quantification of the amount of intracellular cAMP in HUVEC infected for 20 h with the indicated bacterial strains (MOI=300). Isogenic strains with a functional (wild-type) or non-functional (*ΔvirB4*) VirB/VirD4 T4S systems were used to express the different *cya*-reporter constructs. Mean and SD are shown for one representative out of three independent replica experiments. (**C**) Steady-state FLAG-Cya fusion protein levels of the indicated *Bh* strains grown on IPTG-containing medium.

Figure 3. Delineation and subcellular localization of the region of *Bh*_BepA required for inhibition of apoptosis.

(**A**) Schematic presentation of N-terminal GFP-fusions to parts of *Bh*_BepA. (**B**) Determination of apoptosis following ectopic expression of the constructs illustrated in (A). GFP-BepA fusion proteins were ectopically expressed in HUVEC for 24 h, followed by 12 h incubation in the presence or absence of actD as indicated. The loss of membrane asymmetry in transfected cells (GFP-positive) was then quantified by flow cytometric analysis of APC-AnnexinV and PI stained cells, allowing to distinguish apoptotic (AnnexinV positive, PI negative) and necrotic cells (AnnexinV positive, PI positive). The means and SD of three independent replica experiments are shown. The *P* values were determined by using an unpaired Student's t-test. (**C**) The GFP-*Bh*_BepA fusion proteins illustrated in (A) were ectopically expressed for 30 h in HUVEC. Cells were immunochemically stained for the cell surface marker α_v-integrin. Confocal pictures were taken for GFP (green channel) and α_v-integrin (red channel) in the xy-plane (upper image, overlay both channels, bar = 10 µm), and at the dashed stroke line in the xy-plane also in the xz-plane (lower images, single channels and overlay channels)

Figure 4. Comparison of the anti-apoptotic activities of BepA homologs. (**A**) Domain structure of *Bh*_BepA, its paralogs *Bh*_BepB and *Bh*_BepC, and the orthologs *Bt*_BepA and *Bq*_BepA1/2. These homologs contain conserved FIC and BID domains in their N-terminal and C-terminal regions, respectively, except for the ortholog *Bq*_BepA, which due to an internal stop codon is split between these domains into two separate ORFs. (**B**) Anti-apoptotic activity of BepA homologs. HUVEC were infected with the indicated *Bh* strains for 24 h, followed by apoptotic induction with actD for 12 h. Caspase-3/-7 activities were then determined with a specific fluorogenic peptide substrate. Mean and SD are illustrated for one representative out of three independent experiments.

Figure 5. Anti-apoptotic BepA homologs mediate an increase in intracellular cAMP and an upregulation of cAMP response genes. HUVEC were infected with the indicated *Bh* strains. **(A)** IL-8 was determined in culture supernatants after infection for 54 h with an MOI=300. (**B**) Expression of the cAMP-responsive genes *pde4B* and *crem* was determined by quantitative real-time PCR after infection for 54 h with an MOI=300. (**C**) Intracellular cAMP levels were determined after infection for 30 h with a MOI=150. The means and SD of three independent replica experiments are presented. **(B, C)** Samples marked with an asterisk ($P<0.05$) differ statistically significantly from *Bh_ΔbepA-G* by using an unpaired Student's t-test.

Figure 6. A pharmacologically increased cAMP level in EC mimics the anti-apoptotic effect of *Bh*_BepA. HUVEC were infected for 24 h with the indicated *Bh* strains or left uninfected (control) in the absence or presence of either **(A)** forskolin (1 μM) and IBMX (10 μM) or **(B)** dibutyryl cAMP (Db cAMP, 1 mM). If indicated, apoptosis was then induced with actD. Caspase-3/-7 activities were determined 9 h later. All strains were tested in triplicates for a minimum of three times.

Figure 7. *Bh*_BepA protects EC against CTL-mediated cell death. Confluent HUVEC monolayers were infected with the indicated bacterial strains for 24 h or left uninfected (control). (**A**) CTL were added in a E/T cell ratio of 13:1 for the indicated period and after washing the disruption of the HUVEC monolayer was monitored by phase contrast transmission light microscopy (size bar = 50 μm). (**B**) Dotplots of surviving and dead cell populations at indicated time points upon AnnexinV/PI staining and flow cytometry analysis. Percentage (mean) of surviving and dead cells (dashed boxes) are presented. (**C**) Summary graph representing HUVEC cell survival during CTL co-culture as quantified by AnnexinV/PI staining. The mean and SD of one of three independent experiment performed in triplicates is shown.

Figure 8. Ectopic expression of *Bh*_BepA in EC mediates cytoprotection against CTL-mediated cell death. HUVEC expressing GFP or GFP-*Bh*_BepA$_{1-544}$ were incubated with CTL for 22 h at different E/T cell ratios, or left untreated (control). (**A**) Cells were washed and phase contrast microscopic images were taken (size bar = 50 μm). (**B**) Quantification of the surviving cell proportion by AnnexinV/PI staining and flow cytometry analysis. *P* values were determined by using an unpaired Student's *t*-test. The mean and SD of one out of two independent assays performed in duplicates is shown.

A

Uninfected   Wild-type   *Bh_ΔbepA-G*

Apoptotic cell

Bh_ΔbepA-G/ p*Bh*_BepA   *Bh_ΔbepA*   *Bh_ΔbepA/* p*Bh*_BepA

B

Caspase -3/-7 activity

□ - Actinomycin D
■ + Actinomycin D

8000
6000
4000
2000
0

Uninfected
Wild-type
Bh_ΔbepA-G
Bh_ΔbepA-G/pBh_BepA
Bh_ΔbepA
Bh_ΔbepA/pBh_BepA

Figure 1

A

*Bh*_BepD

360   496

1   [BID   ] 532

[——— pFLAG-Cya-*Bh*_BepD352-532

*Bh*_BepA

310   446

1   [FIC] [BID] 544

[——————————] pFLAG-Cya-*Bh*_BepA1-544

[————————] pFLAG-Cya-*Bh*_BepA305-544

B

■ Wild-type
□ Δ*virB4*

nmol cAMP/mg protein

Uninfected
No plasmid
pFLAG-Cya
pFLAG-Cya-*Bh*_BepD352–534
pFLAG-Cya-*Bh*_BepA1-544
pFLAG-Cya-*Bh*_BepA305-544

C

kDa

α-FLAG

100 —
75 —
50 —
37 —

Wild-type Δ*virB4*
Wild-type Δ*virB4*
Wild-type Δ*virB4*
Wild-type Δ*virB4*
Wild-type Δ*virB4*

No plasmid
pFLAG-Cya
pFLAG-Cya-*Bh*_BepD352–534
pFLAG-Cya-*Bh*_BepA1-544
pFLAG-Cya-*Bh*_BepA305-544

Figure 2

Figure 3

Figure 4

A IL-8

B PDE4

CREM

C Intracellular cAMP

expressed in *Bh_ΔbepA-G*

Figure 5

A

Forskolin / IBMX

Caspase activity

- Actinomycin D
  - Forskolin / IBMX
+ Actinomycin D
  - Forskolin / IBMX
- Actinomycin D
  + Forskolin / IBMX
+ Actinomycin D
  + Forskolin / IBMX

Uninfected   Bh_ΔbepA-G   Bh_ΔbepA-G/
                            pBh_BepA

B

Dibutyryl cAMP

Caspase activity

- Actinomycin D
  - Db cAMP
+ Actinomycin D
  - Db cAMP
- Actinomycin D
  + Db cAMP
+ Actinomycin D
  + Db cAMP

Uninfected   Bh_ΔbepA-G   Bh_ΔbepA-G/
                            pBh_BepA
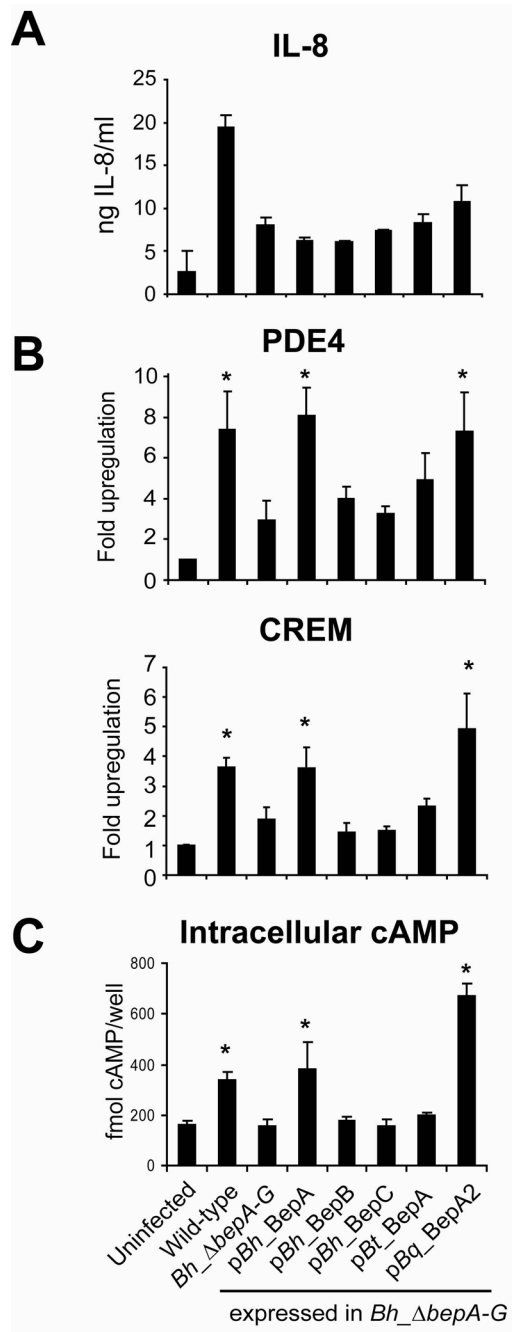
Figure 6

Figure 7

Figure 8

Online Supplemental Material

Table I: Bacterial strains and plasmids used in this study

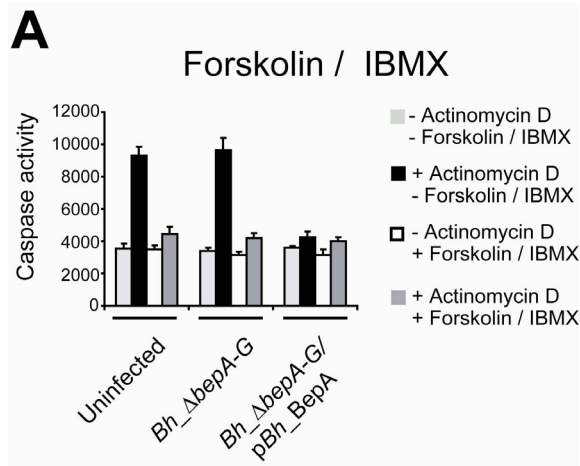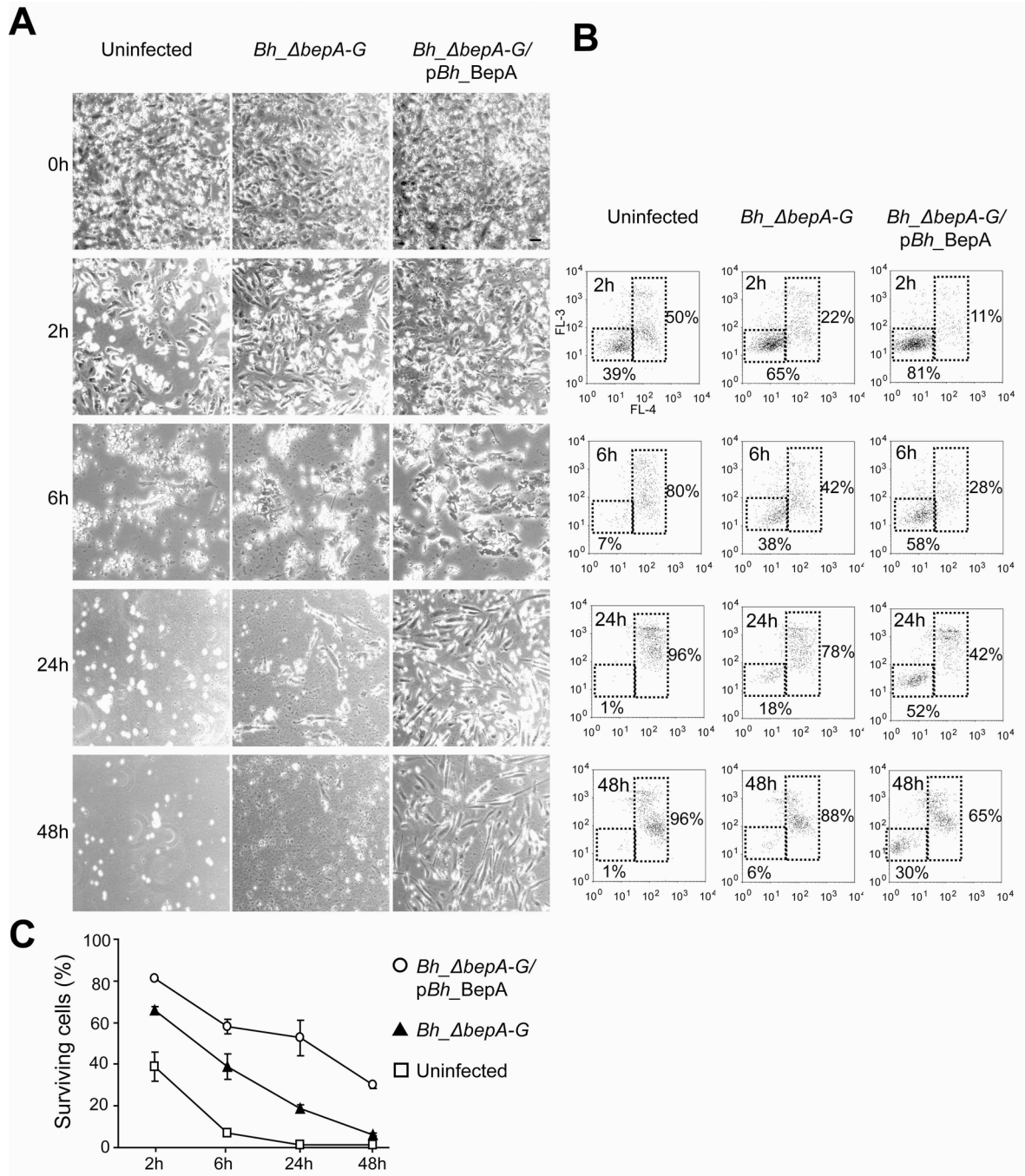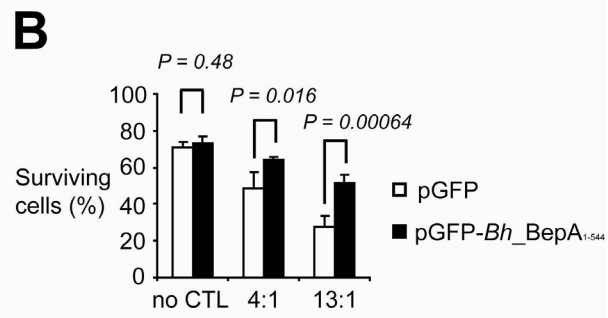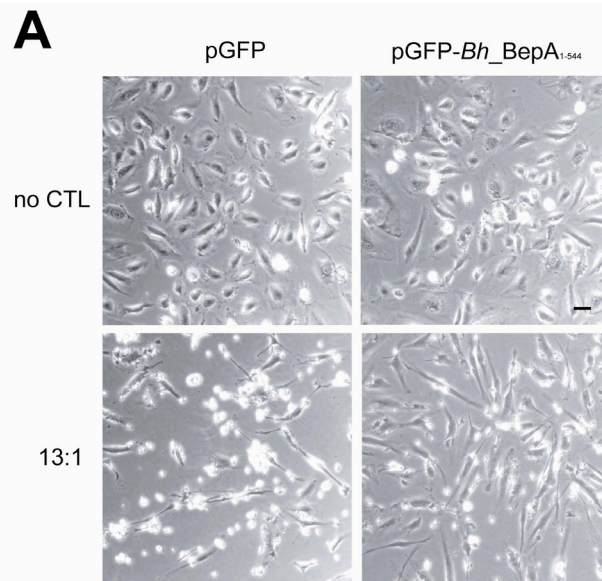| Strain or plasmid	Genotype or relevant characteristics	Reference or source
***B. henselae* strains**		
ATCC 49882 [T]	'Houston-1', isolated from a bacteremic HIV-patient	(1)
MSE150	*ΔbepA-G* mutant of RSE247	(2)
MSE154	*ΔbepA* mutant of RSE247	This work
MSE156	MSE150 containing pPG101	This work
MSE159	MSE150 containing pMS007	Ths work
MSE162	MSE150 containing pRS51	(2)
MSE164	MSE154 containing pRS51	This work
MSE166	MSE154 containing pPG101	This work
MSE167	MSE150 containing pMS006	This work
MSE175	MSE150 containing pMS011	This work
MSE218	MSE150 containing pMS100-A	This work
MSE220	MSE150 containing pMS100-D	This work
MSE226	MSE150 containing pMS100-B	This work
MSE228	MSE150 containing pMS100-C	This work
MSE240	MSE150 containing pMS102	This work
MSE242	MSE150 containing pMS104	This work
MSE249	RSE247 containing pMS400	This work
MSE251	RSE247 containing pMS401	This work
MSE253	RSE247 containing pMS404	This work
MSE255	RSE242 containing pMS400	This work
MSE257	RSE242 containing pMS401	This work
MSE259	RSE242 containing pMS404	This work
MSE269	MSE150 containing pMS106	This work
MSE273	RSE247 containing pMS406	This work
MSE275	RSE242 containing pMS406	This work

MSE277	RSE247 containing pMS405	This work
MSE279	RSE242 containing pMS405	This work
RSE232	RSE242 containing pRS51	(2)
RSE242	*ΔvirB4* mutant of RSE247	(3)
RSE247	Spontaneous Sm^r strain of ATCC 49882^T, serving as wild-type	(3)
RSE308	RSE247 containing pRS51	(2)
***B. quintana* strain**		
VR 358		(4)
***B. tribocorum* strain**		
IBS 506^T		(5)
***E. coli* strains**		
β2150	*F' lacZDM15 lacI^q traD36 proA+B+ thrB1004 pro thi strA hsdS lacZΔM15 ΔdapA::erm* (Erm^R) *pir*	(6)
NovaBlue	*endA1 hsdR17*(r K12–m K12+) *supE44 thi-1 recA1 gyrA96 relA1 lac*[*F' proA+B+ lacI^{q}ZΔM15*::T*n10* (Tc^R)]	Novagen, Madison
Plasmids		
pPG100	*E. coli- Bartonella* spp. shuttle vector, encoding a short FLAG epitope	(2)
pPG101	Derivative of pPG100, encoding FLAG::*Bh*_BepA	This work
pRS40	Cre-vector encoding NLS::Cre	(2)
pRS48	Cre-vector containing NLS::Cre::*Bh*_BepA (aa 305-544)	This work
pRS49	Cre-vector encoding NLS::Cre:: *Bh*_BepB (aa 303-542)	(2)
pRS50	Cre-vector encoding NLS::Cre:: *Bh*_BepC (aa 292-532)	(2)
pRS51	Cre-vector encoding NLS::Cre:: *Bh*_BepD (aa 352-534)	(2)
pRS55	Cre-vector encoding NLS::Cre:: *Bt*_BepA (aa 326-568)	This work
pMS005	Mutagenesis vector for generating a *ΔbepA* in-frame deletion in *B. henselae*	(2)
pMS006	Derivative of pPG100, encoding FLAG::*Bh*_BepB	This work
pMS007	Derivative of pPG100, encoding FLAG::*Bh*_BepC	This work

pMS013	Cre vector containing::*Bh*_BepA (aa 1-544)	This work
pMS011	Derivate of pPG100, encoding FLAG::*Bt*_BepA	This work
pMS100	Insertion of a multiple cloning site into pPG100	This work
pMS106	Derivate of pMS100, encoding FLAG::*Bq*_BepA2	This work
pMS100-A	Derivate of pMS100, encoding FLAG::*Bh*_BepA (aa 305-542)	This work
pMS100-B	Derivate of pMS100, encoding FLAG::*Bh*_BepB (aa 303-542)	This work
pMS100-C	Derivate of pMS100, encoding FLAG::*Bh*_BepC (aa 292-542)	This work
pMS100-D	Derivate of pMS100, encoding FLAG::*Bh*_BepD (aa 352-534)	This work
pMS105	Insertion of *Bh*_BepA into pMS100	This work
pMS111	containing the hybrid *yopE-cyaA* gene	(7)
pMS400	pMS100 containing a 1.23 kb PCR fragment carrying the cya of *Bordetella pertussis* (amplified from pMS111-Cornelis)	This work
pMS401	Cya vector encodning *Bh*_BepA (aa 305-542)	This work
pMS404	Cya vector encoding *Bh*_BepD (aa 252-534)	This work
pMS405	Cya vector encoding *Bh*_BepA	This work
pMS406	Cya vector encoding *Bq*_BepA2	This work

Table II: Oligonucleotide primers used in this study

Name	Sequence [a]	Restriction site
prCREMfw	ATCGCCCGGAAGTTTGC	
prCREMrv	CAGCTCTCGTTTGCGTGTTG	
prGAPDHfw	GAAGGTGAAGGTCGGAGTC	
prGAPDHrv	GAAGATGGT GATGGGATTTC	
prGS001	TATGAAGGCCTCGAGTCGACCGCGGCCGCATCGATGACCCGGG (NdeI/StuI/XhoI/SalI/SacII/NotI/ClaI/XmaI)	
prGS002	TACCCGGGTCATCGATGCGGCCGCGGTCGACTCGAGGCCTTCA (XmaI/ClaI/NotI/SacII/SalI/XhoI/StuI/NdeI)	
prMS027	CGGGAATTC<u>CATATG</u>CAAAAGGGAATATTTCACT	NdeI
prMS028	CGGGAATTC<u>CATATG</u>TTAGCTGGCTATAGCGAGTA	NdeI
prMS037	ACGC<u>GTCGAC</u>CTCCAAAGGCAAAAGCAAAAACG	SalI
prMS038	TCCC<u>CCCGGG</u>TTAGCTAGCCATGGCAAGC	XmaI
prMS068	TCCC<u>CCCGGG</u>ATGCCAAAGGCAAAAGCAAAAA	XmaI
prMS069	CTAG<u>TCTAGA</u>TTAGCTAGCCATGGCAAGC	XbaI
prMS074	TCCC<u>CCCGGG</u>AAGCATGTTGCACTCCTCA	XmaI
prMS075	CTAG<u>TCTAGA</u>TTATTCGGCTTTTGGAGCTGTAA	XbaI
prMS076	TCCC<u>CCCGGG</u> GAGCTTGAAAACACGCTCAT	XmaI
prMS077	CTAG<u>TCTAGA</u>TTAGCTGGCAATAGCAAGCG	XbaI
prMS078	GGAATTC<u>CATATG</u>CAGCAATCGCATCAGGCTG	NdeI
prMS083	TCCC<u>CCCGGG</u>GAATTAAAAAAAACACTCATCCC	XmaI
prMS084	CTAG<u>TCTAGA</u>TTAACTTGGCATAGGGACCTCTT	XbaI
prMS090	TCCC<u>CCCGGG</u>TTACG<u>AGGCCT</u>GGCGTTCCACTGCGCCCA	XmaI/StuI
prMS102	ACGC<u>GTCGAC</u>CGATGAGCCATATGAAAAATATCAAA	SalI
prMS103	TCCC<u>CCCGGG</u>TTAGCAAACTATTTTAGCTTGC	XmaI
prMS104	AGCTTT<u>GTTTAAAC</u>GATGAGCCATATGAAAAATATCAAA	PmeI
prPG92	GGAATTC<u>CATATG</u>CCAAAGGCAAAAGCAAAAA	NdeI
prPG93	GGAATTC<u>CATATG</u>TTAGCTAGCCATGGCAAGC	NdeI
prPG095	GGAATTC<u>CATATG</u>TTAGCTGGCAATAGCAAGCG	NdeI

prPG097	GGAATTC<u>CATATG</u>TTAGTTGGTAAGAGCCCTTG	NdeI
prPG129	GGAATTC<u>CATATG</u>CCAAAAGCAAAAGCAAAAAA	NdeI
prPG130	GGAATTC<u>CATATG</u>TTAGAGCATAATTATCTGTA	NdeI
prRS167	TCCC<u>CCCGGG</u>TTAGCTAGCCATGGCAAGC	XmaI
prRS180	ATGG<u>TGTCGA</u>AAGAATTAAAAAAAACACTCATCC	SalI
prRS187	ATGG<u>TGTCGA</u>CAAGAGTTAAAAAATATTCTCATTC	SalI
prRS188	TCCC<u>CCCGGG</u>TTAGCTGGCTATAGCGAGTA	XmaI

[a] Restriction endonuclease cleavage sites are underlined

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Concluding remarks

Bartonella-endothelial cell interaction

Bartonella species are vasculotropic bacterial pathogens responsible for clinical manifestations including trench fever, Carrion's disease, cat-scratch disease, and bacillary angiomatosis-peliosis. The endothelium is proposed to be the primary niche for bartonellae upon transmission via an arthropod vector (Dehio, 2001). *Bartonella* are able to adhere to and invade endothelial cells (EC). In humans, the colonization of the endothelium by the three *Bartonella* species *B. henselae, B. quintana, and B. bacilliformis*, is associated with vascular tumor formation (Dehio, 2004). The property of bartonellae to stimulate the proliferation of human EC is unique among bacterial pathogens. EC are lining the inside of our blood vessels and are in close interaction with smooth muscle cells and pericytes. The blood vessel is finally embedded in an extracellular matrix composed of different rigid protein structures. This tight interaction of all components provides the required stability for the blood vessel (Folkman, 1995). The process of forming new vessels out of existing ones is called angiogenesis. The vasculature is usually quiescent in the adult, and endothelial cells are among the longest-lived cells outside the nervous system. The few adult tissues that do require ongoing "normal" angiogenesis include the female reproduction organs, organs that undergo physiological growth, and injured tissue (Hanahan and Folkman, 1996). Induction of "pathological" angiogenesis is often observed during tumor formation. To contribute to an angiogenic process, the normally quiescent EC require a specific stimulus to proliferate.

Considering their localization, EC play an important role during an inflammatory response (Johnson-Leger and Imhof, 2003). By expressing different adhesion molecules and chemokines, they control the transmigration of immune cells to the site of infection. The first published report of my PhD thesis is related to this pro-inflammatory activation of EC: "***B. henselae* induces NFkB-dependent upregulation of adhesion molecules in cultured endothelial cells: possible roles of outer membrane proteins as pathogenic factors**" (Chapter 1, p. B-1). In this work, we provide evidence, that *B. henselae*-EC interaction results in an induction of a pro-inflammatory response of the host cell. Upon *B. henselae* infection the normally quiescent EC started to express several genes involved in a pro-inflammatory process. This included the expression of the adhesion molecules

E-selectin and ICAM-1, which are known to mediate the adherence, rolling, and transmigration of leukocytes *in vivo.* The pro-inflammatory response was regulated by the activation of the NF-κB pathway by *B. henselae.* To biochemically characterize the bacterial stimulus mediating this pro-inflammatory response, *B. henselae* was subfractionated. We were able to restrict the observed pro-inflammatory response to the outer membrane protein fraction of *B. henselae.* Further, we excluded the involvement of bacterial lipopolysaccharide (LPS) in the observed inflammation. LPS, also called endotoxin, is a potent inducer of a local and systemic inflammation. By binding to toll-like receptor 4 (TLR4), LPS induces a strong pro-inflammatory response in EC (Faure et al., 2001; Miller et al., 2005). In our study, the activation of EC by *B. henselae* was determined to be of proteinaceous origin, independent of the LPS, because adding the common LPS inhibitor polymyxin B to the outer membrane fraction did not show any effect. Our findings were finally confirmed by the recently solved LPS structure of *B. henselae* by Zähringer and co-workers (Zahringer et al., 2004). They gave evidence that the endotoxic activity of purified LPS from *B. henselae* is 10^3- to 10^4-times lower compared to enterobacterial LPS. These results strongly support our previous conclusions, that indeed bacterial proteins rather than LPS mediate the pro-inflammatory response of the endotoxin-sensitive EC. In summary, our study provides evidence that the pro-inflammatory activation of EC by *B. henselae* is mediated by bacterial proteins. The low endotoxic activity of *B. henselae* and the specific induction of a pro-inflammatory response by bacterial proteins might indicate an evolved characteristic feature of a highly adapted vasculotropic pathogen.

Role of the VirB type IV secretion system in subversion of EC function

Different pathogenic bacteria have adapted conjugation systems to translocate effector proteins into the cytosol of host cells. During bacterial infection, these injection machineries, also called type IV secretion systems (T4SS), represent major virulence factors (Cascales and Christie, 2003). *B. henselae* harbors two T4SS, the Trw and the VirB T4SS, classified by their evolutionary origin. In the study "**The VirB T4SS of *B. henselae* mediates invasion, pro-inflammatory activation, and anti-apoptotic protection of endothelial cells**" (Chapter 2, p. C-1), we were driven by the interest to reveal the role of the VirB T4SS during endothelial cell interaction. Different aspects of the subversion of EC through *Bartonella* infection were already

described in literature, like (i) the massive rearrangement of the actin cytoskeleton, resulting in the formation of the so-called invasome (Dehio et al., 1997), (ii) the induction of a pro-inflammatory response (Fuhrmann et al., 2001), (iii) the protection against apoptosis (Kirby and Nekorchuk, 2002), and (iv) a mitogenic activation (Maeno et al., 1999). To specify the contribution of the VirB T4SS during EC-interaction, we first abrogated the functioning of the VirB T4SS of *B. henselae* by a nonpolar deletion of the *virB4* gene. Then, we established several cell-assays to dissect the different influences of the VirB T4SS during EC infection. Using wild-type bacteria, a *ΔvirB4* inframe mutant, and a *trans*-complemented *ΔvirB4* mutant, we were able to dissect and classify the *B. henselae*-EC interactions. The majority of the analyzed phenotypes were dependent on a functional VirB T4SS. Beside the mitogenic activity of *B. henselae*, all known characteristics were found to be mediated by the VirB T4SS. Other bacterial pathogens are also known to use their T4SS for subversion of host cell function. For example the translocation of CagA via the VirB-related Cag T4SS of the gastric pathogen *Helicobacter pylori* induces elongation and spreading of host cells (Segal et al., 1999). However, such a variety of T4SS-dependent phenotypes as observed upon *B. henselae* infection was never described for any other T4SS. As inactivation of the translocation machinery resulted in the loss of a wide array of phenotypes, we suggested that several yet unknown T4SS substrates could be translocated into the host cell, where they could act in an individual or synergistic manner.

Taken together, by determining VirB T4SS-dependent cellular processes in EC, we revealed various aspects of bacteria-host cell interaction which may play a crucial role during pathogenesis. During this study, we further established cell-biology assays which facilitate our future *Bartonella* research to monitor the differential behavior of genetically modified bacteria during EC infection.

Show me the type IV substrates

The subversion of EC function by the VirB T4SS described in chapter 2 was proposed to be mediated by yet unidentified translocated effector proteins. Our report "**A bipartite signal mediates the transfer of T4SS substrates of *B. henselae* into human cells**" (Chapter 3, p. D-1) describes the discovery of these putative VirB T4SS effector proteins in *B. henselae*. By sequencing downstream of the *virB* locus, the genes encoding the type IV secretion coupling protein VirD4 and seven putative

Bartonella effector proteins (Bep) were identified. The nonpolar deletion of the genes encoding these putative Beps abolished the ability of *B. henselae* to trigger host cell phenotypes, which were previously described to be mediated by the VirB T4SS (Chapter 2, p. C-1). These findings confirmed our assumption that the observed subversion of host cell function is mediated by putative effector proteins. A so-called Bep intracellular delivery (BID) domain, conserved in all Beps, was further characterized by a Cre-recombinase reporter assay for translocation (CRAFT). We identified a VirB/VirD4-dependent bipartite translocation signal, composed of the conserved BID domain and a short positively charged tail sequence. Interestingly, the VirB/VirD4 T4SS of *B. henselae* shares high homology to bacterial conjugation systems, which mediate the transfer of DNA into recipient cells. We could provide evidence, that a bipartite translocation signal of a relaxase of the conjugation machinery AvhB/TraG of *Agrobacterium tumefaciens* still mediates translocation by the VirB/VirD4 T4SS of *B. henselae*. This allows the speculation that the VirB/VirD4 T4SS could also have retained the capacity of transferring DNA.

To summarize, the identification of translocated effector proteins was an exciting step towards a molecular understanding of host cell subversion by *B. henselae*. Next, we were ambitious to further investigate the here identified bacterial effectors to characterize their individual functions in host cell subversion.

The anti-apoptotic factor

One of the VirB/VirD4-dependent processes described in chapter 2 and 3 is the protection of EC from apoptotic cell death. This anti-apoptotic activity of *B. henselae* is proposed to contribute to the mitogenic activity resulting in enhanced vasculoproliferative tumor formation (Kirby, 2004; Kirby and Nekorchuk, 2002). In the report "**The BID domain of the translocated effector protein BepA of *B. henselae* is sufficient to protect endothelial cells from apoptosis**" (Chapter 4, p. E-1), we identified BepA as the anti-apoptotic factor of *B. henselae*. A nonpolar deletion of *bepA* resulted in the loss of the anti-apoptotic activity of *B. henselae*. Because translocation of BepA could not been shown by the CRAFT assay (Chapter 3, p. D-1), we adapted a calmodulin-dependent adenylate cylclase (*cya*) reporter assay to demonstrate BepA-translocation by the VirB/VirD4 T4SS. This defined BepA as a novel VirB/VirD4 T4SS substrate and as the first *Bartonella* effector protein with an assigned function in host cells. Moreover, it represents the first identified secreted

substrate of a type III or type IV secretion system known to mediate an anti-apoptotic activity (Synopsis, p. A-1). Strikingly, the anti-apoptotic activity of BepA was found exclusively among *Bartonella* species associated with bacillary angiomatosis. BepA of the human pathogen *B. quintana* showed even a more pronounced anti-apoptotic effect than the orthologue of the zoonotic species *B. henselae.* Genome comparison of these two pathogens showed that *B. quintana,* which is highly adapted to humans, evolved from *B. henselae* by reductive evolution (Alsmark et al., 2004). The increased efficiency of BepA of *B. quintana* to protect EC from apoptotic cells death might indicate the importance of the anti-apoptotic activity of *Bartonella* during human infection. We have to consider that the formation of angioproliferative lesions, including new vessel sprouting, is a tightly controlled mechanism, in which several mechanisms are acting in a orchestral order (Bergers and Benjamin, 2003). Additionally, stimulation of EC proliferation is not sufficient to trigger vascular tube formation in a three-dimensional matrix (Kirby, 2004). The central question is to define the contribution of the anti-apoptotic activity of BepA to the process of vascular tumor formation induced by *B. henselae*. There is an emerging need for an appropriate animal model to study bacillary angiomatosis *in vivo* and to unravel the putative synergistic effects of the mitogenic and anti-apoptotic activity of *B. henselae* during the formation of vasculoproliferative lesions. Beside the putative involvement of BepA during tumor formation, the here identified anti-apoptotic bacterial factor might be useful for biotechnical applications, or might represent a valuable tool to specifically manipulate EC.

To summarize, my PhD thesis project aimed at characterizing *Bartonella*-EC interactions. The core work of this thesis consists of two major findings. First, the role of the VirB T4SS in mediating most of the EC changes observed during bacterial infection, like invasion, pro-inflammatory activation, and anti-apoptotic protection of EC. Second, the discovery of BepA as a novel VirB/VirD4 T4SS substrate, protecting EC from apoptosis.

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Education

University of Basel

Since Jan. 2001   PhD-student under the supervision of Prof. Christoph Dehio,

Division of Molecular Microbiology

Topic: "Subversion of host cell functions by the

human pathogen *Bartonella henselae"*

University of Bern, studies in Biology since 1996

Nov. 2000   Diploma in Biology, General Microbiology

1999-2000   Diploma work at the Institute for Immunology and Allergology,

of the University of Bern.

Topic: "Gene expression profiling of tumor-derived

endothelial cells"

1995-1996   Language and working residence in New Zealand

Staatliches Seminar Bern, Lerbermatt 1990-1995

1995   Teaching-Patent

Working experience

Jul. – Oct. 1997   Anatomical Institute, Department of Cell Biology, University of Bern,

Topic: "Intussuceptive Angiogenesis"

Jul. – Oct. 1998   Burnham Institute, Department of Apoptosis, San Diego, CA

Topic: "BAG-family proteins and Bcl-xL-binding proteins"

Presentations / Awards

Oct. 2003   Poster at the Biozentrum Symposium in Basel, Switzerland: "A type IV secretion system of the bacterial pathogen *Bartonella* mediates anti-apoptotic protection of infected endothelial cells." (M.C. Schmid, R. Schulein, M. Dehio, P. Guye, T. Rhomberg, I. Carena, C. Dehio)

Oct. 2003   Poster at the European Cell Death Organization (ECDO) conference in Ghent, Belgium: "A type IV secretion system of the bacterial pathogen *Bartonella* mediates anti-apoptotic protection of infected endothelial cells." (M.C. Schmid, R. Schulein, M. Dehio, P. Guye, T. Rhomberg, I. Carena, C. Dehio)

Oct. 2004   Poster-prize at the Biozentrum Symposium in Basel, Switzerland: "A novel bacterial protein inhibiting apoptosis after injection into the host cell." (M.C. Schmid, M. Dehio, N. Devaux, C. Dehio)

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