The Role of the VirB/VirD4/Bep System in *Bartonella henselae*-triggered Vascular Proliferation

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Florine Scheidegger
aus Basel

Basel, 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Christoph Dehio
Prof. Cécile Arrieumerlou

Basel, den 09.12.2008

Prof. Dr. Eberhard Parlow
(Dekan)

for my family

The most exciting phrase to hear in science, the one that heralds new discoveries, is not “Eureka!” (I've found it!), but “That's funny...”

Isaac Asimov

Statement to my Thesis

My thesis is written in a cumulative format. It consists of a synopsis about a topic related to my work (the role of bacterial infection in tumourigenesis), followed by chapters presenting a published research article, a submitted manuscript, a manuscript in preparation with a short summary and unpublished results obtained during my work. Finally, I resume the major findings of my thesis, draw conclusions and discuss some aspects and open questions of this work.

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Synopsis

The Role of Bacterial Pathogens in Tumour Formation

General introduction

Francis Peyton Rous' discovery early in the last century that an infectious agent, later identified as a virus, can cause tumours arising from the connective tissue in chicken established the link between infection and tumour (Rous, 1911). Today, experimental and epidemiological evidence propose a variety of infectious agents to be one of the main causes of tumours worldwide – accounting for estimated 17.8% (1.9 million cases) of the incidence of tumours in 2002 (Parkin, 2006). This fact was acknowledge by this years Nobel prize in medicine given to H. zur Hausen for his discovery of human papilloma viruses causing cervical cancer (Nobelprize.org, 2008). Part of the reason for the interest in investigating the infectious origins of tumours lies in the realization that a tumour connected to infection is preventable (Kuper *et al.*, 2000). Amongst the major infectious agents classified as carcinogenic by the IARC (International Agency for Research on Cancer) *Helicobacter pylori* is so far the only one of bacterial origin, the others being viruses and parasites. Nonetheless, a number of bacteria are thought to be associated with tumour formation (Vogelmann and Amieva, 2007; Mager, 2006). The awareness that the infectious agent may persist silently for many years and actually cause tumours in only a few of the chronically infected individuals and that especially bacteria often are not sufficient to induce tumour on their own, that chronic inflammation might be an important underlying process and independent alterations in oncogenic signalling pathways might be needed, illustrates the complexity between infection and tumourigenesis and emphasizes the central question of "cause and effect". Understanding pathogen-host interactions is thereby a key component to unravel bacteria-associated tumour formation.

The first part of this synopsis introduces the basic and relevant mechanisms of tumour formation. The second part gives examples of bacteria associated with tumourigenesis.

Mechanisms of tumour formation

Definition

Originally, “tumour” described any abnormal swelling, lump or mass. Today however, it is used as a synonym for neoplasm – the medical term for a pathological lesion characterized by the progressive or uncontrolled proliferation of cells. A neoplasm can be benign or malignant. In contrast to malignant tumours (also called cancer), benign tumours usually grow slowly and remain localized – meaning that they do not grow in an unlimited, aggressive manner and especially do not invade surrounding tissues and do not metastasize (McGraw-Hill, 2004). Yet not all tumours classified as benign are harmless to the host. Benign tumours may give rise to cancers via additional genetic changes in a subpopulation of the tumour cells or secondary difficulties may occur as a result of mechanical pressure.

Steps of tumour formation

The genetic model of oncogene activation, loss of tumour suppressor genes and genomic instability for colorectal tumour development (Vogelstein *et al.*, 1988) provided the framework for studies addressing the independent multiple steps involved in the transition of a normal cell towards an overt and complex disease via tumour initiation, promotion and progression (illustrated in figure 1).

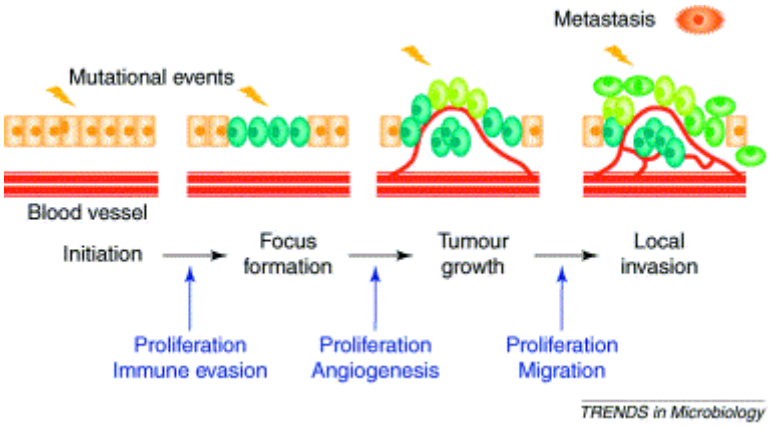

Figure 1 Tumour initiation occurs when a cell is freed from growth restraints, usually irreversibly by mutation. Promoting agents, mutagenic and non-mutagenic, provide an anti-apoptotic and proliferative state enabling the mutated cell to build up a colony. Further progression and growth of tumour needs the capacity to induce blood-vessel formation and leads to an increase in malignant features such as invasion and metastases (Lax and Thomas, 2002).

The so-called hallmarks of cancer include the acquisition of self-sufficient signals for growth, the capacity to extend proliferation, resistance to growth-inhibiting signals, the ability to evade cell death signals (apoptosis), the potential for tissue invasion and metastasis, and the power to induce blood-vessel formation (angiogenesis) (Hanahan and Weinberg, 2000). Whereas some of these traits are the properties of the tumour cells themselves explained by mutations affecting cell cycle, proliferation, programmed cell-death and genome integrity, others depend on communication between tumour cells and their environment. Although having provided a wealth of information about processes inside cells, the reductionist focus on tumour as renegade cell-based disease is heavily discussed. Alternatively tumour is viewed as a complex tissue in which tumour cells interact with and subvert normal cells – as a developmental process gone awry (Sonnenschein and Soto, 2008; Hanahan and Weinberg, 2000) (figure 2).

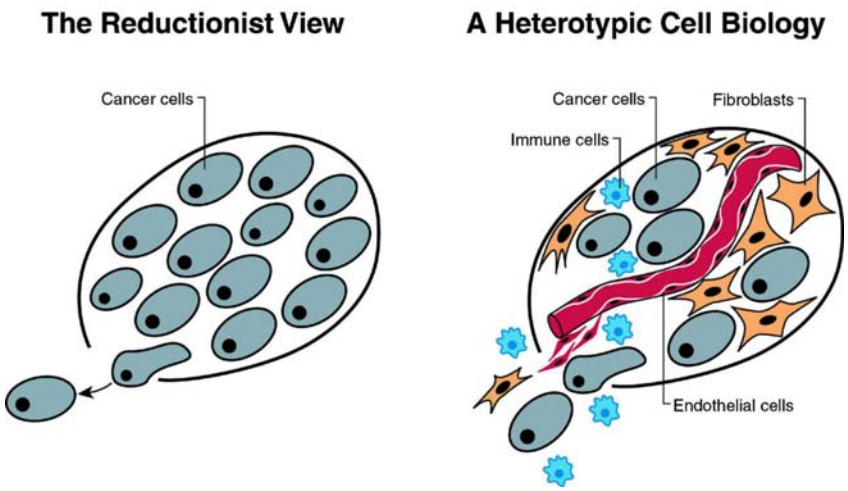

Figure 2 Left side: Cell-centred reductionist concept of tumour formation. Right side: tumour as a complex tissue. The interactions between the genetically altered tumour cells and the supporting normal cells will prove to be critical to understand tumour pathogenesis (Hanahan and Weinberg, 2000).

Tumour angiogenesis

Like normal tissues, tumours require an adequate supply of oxygen, metabolites and an effective way to remove waste products (Papetti and Herman, 2002). This obligates virtually all cells in a tissue to reside within 100 to 200 µm of a capillary blood vessel – the diffusion limit for oxygen. Hence, angiogenesis – the growth of new capillaries out of existing blood vessels – is central to the growth of

tumours to overcome size limitation and is one of the discrete steps of tumour progression (see figure 1) (Bergers and Benjamin, 2003). Angiogenesis itself is a highly orchestrate and tightly regulated process. A balance of pro- and anti-angiogenic signals usually keeps the vasculature in a quiescent state (figure 3). An up-regulation of pro-angiogenic factors (such as vascular endothelial growth factor, VEGF), or a down-regulation of anti-angiogenic factors (such as angiostatin) may trigger the “angiogenic switch” – the initiation of angiogenesis.

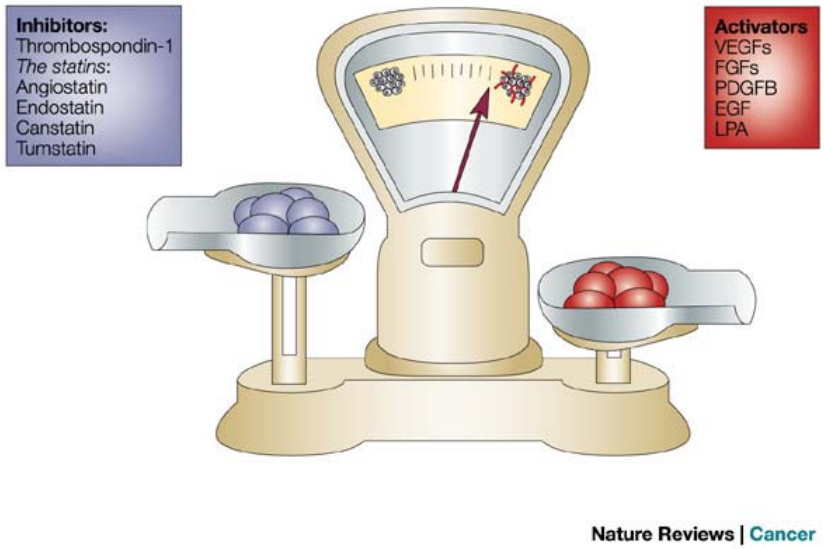

Figure 3 Angiogenesis is orchestrated by a variety of activators and inhibitors — only a few of which are listed above. In general, the levels of activators and inhibitors dictate whether an endothelial cell will be in a quiescent or an angiogenic state. It is believed that changes in the angiogenic balance mediate the angiogenic switch (Bergers and Benjamin, 2003).

Various signals that trigger the angiogenic switch have been discovered. These include metabolic stress (low pO_2, low pH, low level of sugar), mechanical stress (pressure generated by proliferating cells), immune/inflammatory response (cells from the immune system that have infiltrated the tissue), and genetic mutations (activation of oncogenes or deletion of tumour suppressor genes controlling the production of angiogenesis regulators) – demonstrating a complex interplay of environmental and genetic mechanisms influencing tumour angiogenesis. Pro- and anti-angiogenic stimuli might come from tumour cells themselves, endothelial cells (ECs), stromal cells, blood and extracellular matrix (Carmeliet and Jain, 2000).

In contrast to normal physiological angiogenesis (such as for example angiogenesis in injured tissue) where new vessel rapidly mature and become stable, tumours are described as “wounds that never heal” having lost the proper balance between positive and negative control (Dvorak, 1986). Tumour blood vessels are different in their architecture than their normal counterparts – they are less organized, irregularly shaped, dilated, have altered interactions with pericytes, and are often leaky. Whereas in normal tissue vessel density is dynamically controlled by the metabolic needs of nutrients and oxygen, tumour vasculature fails to become quiescent and enables constant growth of new tumour blood vessels (Bergers and Benjamin, 2003).

Chronic inflammation and tumourigenesis

Links between tumour and inflammation were first made on the basis of observations that tumours often arose at sites of chronic inflammation and that inflammatory cells are present in tumour environment (Balkwill and Mantovani, 2001). Several other lines of evidences summarized in table 1 are consistent with the view that inflammation plays an important role in malignant progression.

Table 1 The links between cancer and inflammation (Balkwill *et al.*, 2005).

• Chronic inflammation increases risk of cancer, and many cancers arise at sites of chronic inflammation
• The immune cells that mediate chronic inflammation are found in cancers and promote tumor growth in cell transfer experiments
• The chemical mediators that regulate inflammation are produced by cancers
• Deletion or inhibition of inflammatory mediators inhibits development of experimental cancers
• Genetic variations in inflammatory genes alter susceptibility to and severity of cancer
• Long-term use of nonsteroidal anti-inflammatory agents reduces risk of some cancers

Acute inflammation is a rapid and self-limiting process: chemical mediators are induced in a tightly regulated sequence, and immune cells move in and out of the affected area, destroying infectious agents, repairing damaged tissue, and initiating a specific and long-term response to the pathogen. However, acute inflammation does not always resolve. In chronic conditions initiating factors persist or mechanisms

required for resolving the inflammatory response fail. This can lead to abnormalities and ultimately pathogenesis (Balkwill *et al.*, 2005). As mentioned before tumours can bee viewed as “wounds that never heal” (Dvorak, 1986).

Chronic inflammation is thought to be implicated in all stages of tumourigenesis, i.e. initiation, promotion and progression. The interplay of both the extrinsic pathway (driven by inflammatory conditions within the microenvironment surrounding a pre-tumourous cell) and the intrinsic pathway (driven by genetic alterations in the pre-tumourous cell that cause inflammation) is required for inflammation-associated tumourigenesis (Balkwill and Mantovani, 2001). Persistent inflammation leads to tissue damage – nitric oxide and reactive oxygen species from inflammatory cells may induce DNA damage, which increase the risk for malignant transformation of a cell. In addition various factors such as cytokine, chemokines and growth factors from inflammatory cells (table 2) as well as produced by tumour cells themselves alter crucial biological processes responsible to maintain normal cellular homeostasis and genomic stability, increasing the risk of tumour development (figure 4) (Kundu and Surh, 2008; Yoshimura, 2006).

Table 2 Macrophage products with potential to influence tumourigenesis (Yoshimura, 2006).

Biological effect	Factor s and molecules
Growth and survival	bFGF, EGF, HGF, PDGF, IL-6, TNF, polyamine, PGE2, WNT7b
Angiogenesis	VEGF, MMP-9, IL-1, IL-8, uPA, CXCL-1,CXCL-8,HIF-1α, HIF-2α, PGE2
Tissue invasion	Chemokines, PGE2, MMP-9, uPA, TIMP, plasmin
DNA damage	ROS, NO
Suppressors of immune responses	IL-10, TGF-β, IDO, arginase, PGE2, ROS, NO
Activation of immune responses	IL-12, TNF, CD80, CD86, MHC, CD40, chemokines, BAFF

IL, interleukin; MMP, matrix metalloproteinases; NO, nitric oxide; ROS, reactive oxygen species; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

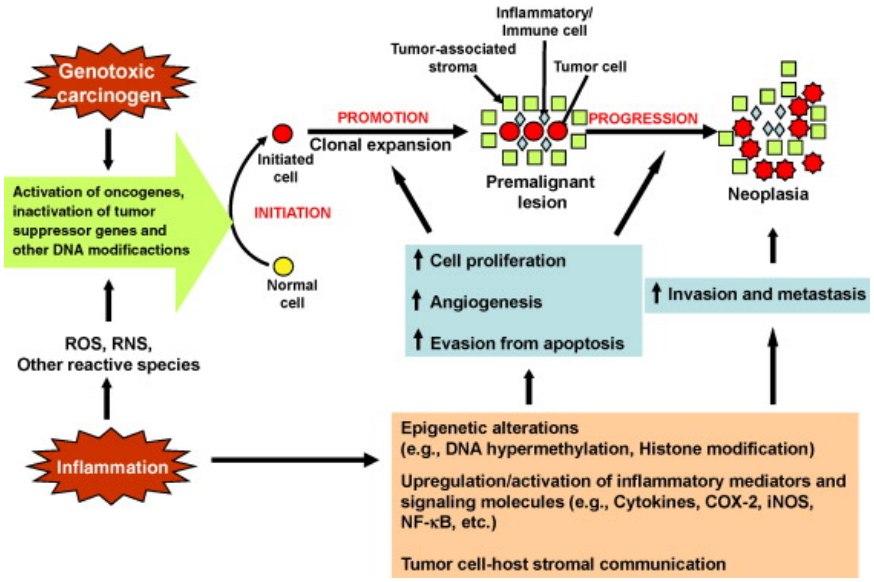

Figure 4 Inflammation is implicated in multi-step tumourigenesis. ROS/RNS or other reactive species derived from inflammatory stress can attack DNA and cause mutations in oncogenes/tumour suppressor genes or other genetic alterations. This will lead to initiation of tumour formation. Inflammation also contributes to tumour promotion and progression by stimulating the proliferation of initiated or premalignant cells, enhancing angiogenesis and metastasis, and inhibiting cell apoptosis (Kundu and Surh, 2008).

The role of bacterial infection in tumourigenesis

Bacterial infection and tumour

A number of epidemiological evidences link bacterial infections to tumour, but causality and the underlying molecular mechanism are far from clear. Studies in animal models support a causative role for several bacterial pathogens in tumour formation, but the relevance of experimental animal results needs to be transmitted to humans. Many bacteria and their products show properties that could modulate tumourigenesis, but there is no known link to human tumour. A general perception in all those studies aiming to unravel the role of bacterial infection in tumourigenesis is the awareness that bacteria often are not sufficient to induce tumour on their own, that the process might be accompanied by chronic inflammation and modulation of host immune response, depend on susceptibility factors on the host side, and need independent alterations in oncogenic signalling pathways (Vogelmann and Amieva, 2007; Mager, 2006). Bacteria would thus promote tumour formation by disrupting interactions between cells involved in maintaining tissue organization, repair, and local homeostasis – altering microenvironments and relaxing restraints controlled by tissue organization. Hence, the knowledge about bacteria-host interactions is crucial to the understanding of the role of bacterial infection in tumour development.

The paradigm of increased host inflammatory response

The Gram-negative bacterium ***Helicobacter pylori*** (*Hp*) colonises the stomachs of more than half of the world's population from childhood – making it one of the most successful bacterial pathogens (Suerbaum and Michetti, 2002). The majority of those infected will carry and transmit *Hp* without any symptoms of disease (Blaser, 1997). However, *Hp* infections are also responsible for a heavy toll of morbidity and mortality as a consequence of ulcer disease, lymphoma of the mucosa-associated lymphoid tissue (MALT) and, the most dangerous complication of *Hp* infection, gastric adenocarcinoma (Peek and Blaser, 2002; Sepulveda and Graham, 2002). The IARC classification of *H. pylori* infection as class I carcinogen itself was entirely based on epidemiological evidence (IARC, 1994). Meanwhile pathogenesis of *Hp* has been addressed by infection studies using animal models, analysis of cellular signalling mechanism perturbed by *Hp* infection, and the elucidation of possible roles of *Hp* gene products (Kusters *et al.*, 2006). *Hp* isolates

are surprisingly diverse in their genome sequence and their virulence. Increased pathogenicity has been linked, although not exclusively, to the presence of the *cag* pathogenicity island (PAI), encoding a type IV secretion system (T4SS), and the thereby translocated **CagA** protein. CagA can activate a number of signal transduction pathways that resemble signalling by growth factor receptors. CagA is also involved in binding and perturbing the function of epithelial junctions, resulting in aberrations in tight junction function, cell polarity and cellular differentiation (Fig. 5) (Amieva and El-Omar, 2008; Backert and Selbach, 2008).

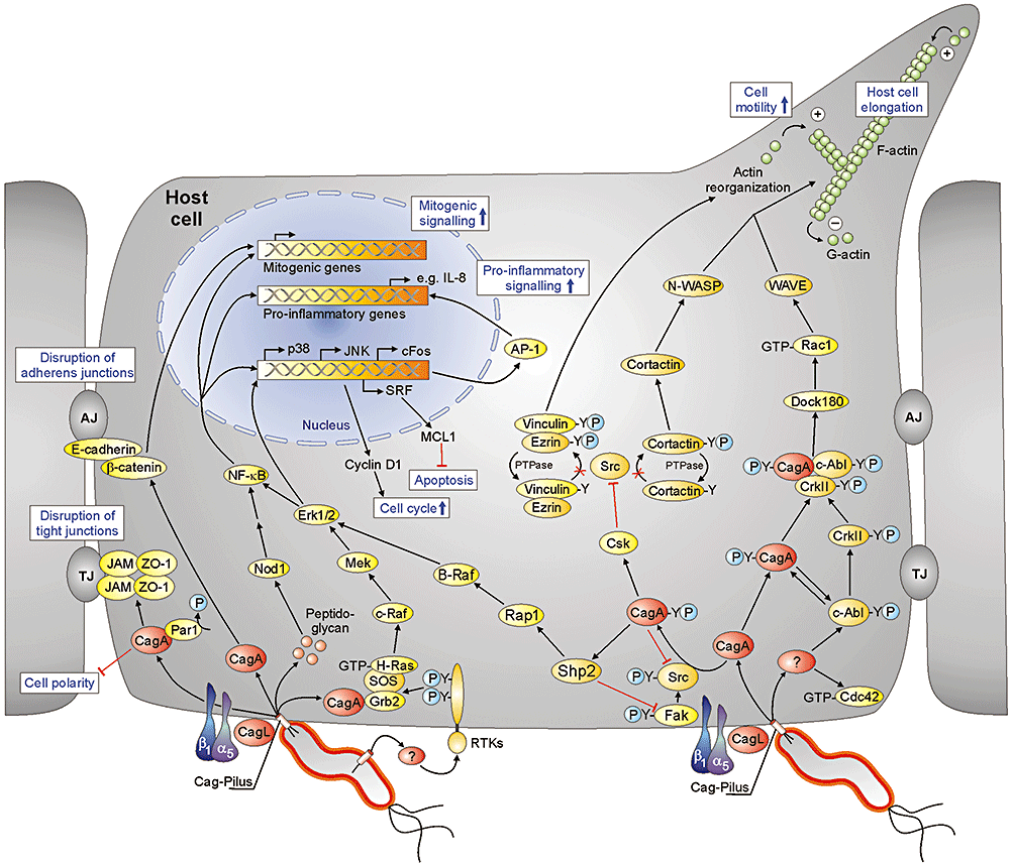

Figure 5 Model for the role of Hp T4SS effectors CagL, peptidoglycan and CagA in host cell interaction and signal transduction. T4SS effectors alter different cellular processes in gastric epithelial cells as illustrated. Bacterial effector molecules are shown in red, host signalling molecules in yellow and actin filaments in green. CagA is injected into the membrane of infected gastric epithelial cells and modulates various signalling cascades associated with cell proliferation, motility, actin cytoskeletal rearrangements, disruption of tight junctions, pro-inflammatory responses and suppression of apoptosis, as shown. Receptor tyrosine kinases, ERK and small Rho GTPases Rac1 and Cdc42 can also be activated by a yet unknown T4SS protein. In addition, translocated peptidoglycan appears to activate the intracellular receptor Nod1 which activates NF-κB in a T4SS-dependent manner (Backert and Selbach, 2008).

Another amongst the numerous *Hp* virulence factors is the vacuolating toxin **VacA**, demonstrating multiple actions contributing to *Hp* colonization of the stomach (Cover and Blanke, 2005). *Hp* virulence factors elicit both pro-inflammatory and immunosuppressive effects in the gastric mucosa resulting in a robust but specific form of chronic inflammation, ineffective in clearing the infection (Amieva and El-Omar, 2008). The resulting continuous production of reactive oxygen species can give rise to DNA damage, thus inducing the multiple mutations thought to be required for initiation of tumourigenesis (Kundu and Surh, 2008). In summary, *Hp* expresses several proteins that might independently influence tumour development and cancer risk would be the summation of the polymorphic nature *Hp* virulence factors, environmental exposures, as well as host genetic factors affecting differentially host inflammatory responses and epithelial-cell physiology (Amieva and El-Omar, 2008; Peek and Blaser, 2002).

Streptococcus bovis (*Sb*), a member of the gastrointestinal tract flora of humans, is considered as a lower grade pathogen frequently involved in bacteraemia and endocarditis. Yet, *Sb* infection is often associated to colorectal tumours (Klein *et al.*, 1979). Moreover, it was reported that faecal carriage of *Sb* was increased in patients with colon carcinoma (Klein *et al.*, 1977). Taken together these data suggest a correlation between *Sb* infection and the development of colorectal cancer. Indications for an active role of *Sb* in tumourigenesis are given by studies done with rats pre-treated with a chemical carcinogen (azoxymethane, AOM). Injections of *Sb* or bacterial wall extracted antigens (WEA) promoted the progression of early AOM-induced preneoplastic lesions in the colonic mucosa, enhanced expression of proliferation markers and increased production of the chemokine interleukin (IL)-8 (Ellmerich *et al.*, 2000). Similarly as for *Hp*, the synthesis of pro-inflammatory proteins (e.g. IL-8, prostaglandin E2, cyclooxygenase-2) in human colon carcinoma cells (Caco-2) and in the rat colonic mucosa point to a role of oxygen radicals in colon tumourigenesis induced by chronic infection with *Sb* (Biarc *et al.*, 2004). Thus, *Sb* could contribute to tumour development in a chronic infection / inflammation-dependent manner where bacterial components in addition interfere with cell function. Presently though, the available data do not prove a causal relationship and it remains to be answered whether the increased presence of *Sb* in colonic cancers results from the preferential bacterial colonization of these cancers and their precursors or whether *Sb* as such initiates tumourigenesis (zur Hausen, 2006).

The gram-negative obligate intracellular ***Chlamydia (Chlamydophila) pneumoniae*** (*Cp*) are a common cause of acute respiratory infections, several chronic lung diseases, and proposed to be associated to an elevated risk of lung cancer (Laurila *et al.*, 1997). Similar as proposed for *Hp*, *Cp* could increase the risk through mediators of inflammation. Furthermore studies showed that *Cp* can inhibit host cell apoptosis (Airenne *et al.*, 2002) and triggers the type of activation required for the induction of secreted macrophage-derived mediators of angiogenesis, such as VEGF (Carratelli *et al.*, 2007). It is also proposed that *Cp* acts synergistically with smoking to increase the risk of cancer. Smoking impairs lung immunity which may allow *Cp* to more easily colonize the lungs and then initiate a chronic inflammatory response (Littman *et al.*, 2005). The current evidences linking *Cp* and lung cancer are based on serology and direct antigen detection and are somewhat controversial and at the end – although maybe truly suggesting an association – it remains uncertain whether *Cp* infection increases the risk of lung cancer, or whether lung cancer favours a *Cp* infection. Indeed, a recent publication negates that *Cp* infection is an important cause of lung cancer, in particular among non-smokers (Smith *et al.*, 2008). Likewise, studies done to address the role of *Chlamydia trachomatis* in cervical cancer are discussed (Al-Daraji and Smith, 2008).

Proliferative infections

Inflammation is not always a feature of bacterial infections associated with proliferative lesions. The obligate intracellular bacterium ***Lawsonia intracellularis*** (*Li*) was identified as the agent triggering proliferative intestinal lesions termed proliferative enteropathy (PE) in pigs and other animals (Smith and Lawson, 2001). *Li* infects the undifferentiated crypt cells of the epithelium of the intestinal tract and promotes their proliferation without affecting the normal uninfected epithelium (McOrist *et al.*, 1996). Cells infected with *Li* continue to divide and migrate to populate the epithelium and thus colonise the epithelium with bacteria. In addition, dividing cells better promote the growth of bacteria than non-dividing cells (Lawson *et al.*, 1993). Remarkably, the stimulatory effect of bacteria does no persist once the lesion becomes fully developed (McOrist *et al.*, 1996). Furthermore, despite showing a fully developed proliferative lesion the inflammatory response is very limited (Rowland and Rowntree, 1972). Hence, persistent infection, facilitated by the absence of cellular inflammation and limited immune response, enables the bacteria

to promote a cellular niche for its replication and spread. The bacterial factors involved in induction of cell proliferation and modulation of the immune response remain to be discovered (Smith and Lawson, 2001).

Similar lesions are observed in mice infected with the non-invasive enteric pathogen ***Citrobacter rodentium*** (*Cr*), which causes a disease called transmissible murine colonic hyperplasia (TMCH) characterised by epithelial proliferation and variable limited inflammation (Barthold *et al.*, 1978). *Cr*-infection increases the susceptibility to the carcinogenic effect of a chemical (DMH). Hyperplasia induced by *Cr* reduces the latency period for early neoplastic lesions (Barthold and Jonas, 1977). *Cr* uses the same molecular mechanisms of type III secretion system (T3SS) as enteropathogenic (EPEC) or enterohemorrhagic *E. coli* (EHEC) to colonise the epithelial cells of the gut. A pathogenicity island termed locus of enterocyte effacement (LEE) encodes the gene needed to induce attaching and effacing (A/E) lesions where the brush-border microvilli are destroyed and bacteria intimately attach to the host epithelial cells (Garmendia *et al.*, 2005). The ubiquitous transcription factor NF-κB, controlling the expression of genes involved in immune responses, apoptosis, and cell cycle, is activated in TMCH. Nevertheless, blocking NF-κB activity does not alter *Cr*-induced hyperproliferation (Wang *et al.*, 2006), implying other mechanisms to explain the mitogenic stimulus. The mitochondrial associated protein (**Map**) is amongst the LEE-encoded effector proteins. Not essential for A/E lesion formation *in vitro*, Map has been reported to be targeted to host mitochondria, where it interferes with membrane potential. Disruption of mitochondrial structure and function by *Cr* is proposed to drive the infected cells into impaired cellular metabolism. Reduction in host cell energy might be beneficial for *Cr* by limiting the ability of the cells to combat the infection (Ma *et al.*, 2006). Furthermore, Δ*map* mutants induced a low level of hyperplasia while efficiently colonizing the colon at late stage of the infection suggesting that Map thus could modulate host cell responses influencing proliferation (Mundy *et al.*, 2004).

Bacterial effectors with tumourigenic potential

Bacteria express a range of proteins that interact with host cell in a precise manner. Bacterial products could directly damage DNA by enzymatic attack, indirectly by provoking an inflammatory cell response producing free radicals, or even by affecting DNA repair mechanisms. Interference with signal pathway resulting in

the disruption of the normal balance of growth, cell division and apoptosis might encourage tumour promotion. Metastatic potential and tumour progression could be facilitated by uncoupling of the anchorage-dependent growth (table 3). An underlying characteristic that is likely to be important for most aspects of tumour causation is chronic exposure to a stimulus (Lax, 2005).

Table 3 Potential tumourigenic properties of bacterial toxins (Lax, 2005).

Bacterium	Toxin	Activity	Carcinogenic potential
Helicobacter pylori	CagA	Binds to SHP2 and c-Met	Cell scattering
Helicobacter pylori	*cag* PAI	?	Inflammation
Helicobacter pylori	VacA	Vacuolation, ?	Apoptosis, inflammation
Citrobacter rodentium	MAP	?	Proliferation
Escherichia coli	Cif	?	Cell cycle
Escherichia coli	CNF1	Modifies Rho, Rac, Cdc42	Inflammation, cell cycle
Escherichia coli	CDT	DNase	DNA damage, cell cycle
Campylobacter spp.	CDT	DNase	DNA damage, cell cycle
Shigella dysenteriae	CDT	DNase	DNA damage, cell cycle
Haemophilus ducreyi	CDT	DNase	DNA damage, cell cycle
Actinobacillus actinomycetemcomitans	CDT	DNase	DNA damage, cell cycle
Helicobacter hepaticus	CDT	DNase	DNA damage, cell cycle
Salmonella enterica	CdtB	DNase	DNA damage, cell cycle
Pasteurella multocida	PMT	Modifies G_q?	Proliferation
Bacteroides fragilis	BFT	Cleaves E-cadherin	Proliferation

The so far best characterized example of a bacterial protein with tumourigenic potential is the mitogenic toxin **PMT** produced by some strains of ***Pasteurella multocida***. PMT has been shown to modulate the activity of the $G_{q/11}$ family of heterotrimeric G proteins as well as the small GTPase Rho resulting in the stimulation of a number of signalling proteins involved in tumourigenesis, such as RhoA, Src, focal adhesion kinase (FAK), epidermal growth factor receptor (EGFR), and extracellular signal-regulated kinase ERK1/2 (Oswald *et al.*, 2005). Similarly, the Rho family of small GTPase is activated by cytotoxic necrotizing factor (**CNF**), expressed by many human ***Escherichia coli*** isolates (Landraud *et al.*, 2004). Although inducing quiescent cells to enter the cell cycle and undergo DNA synthesis, CNF interferes via activation of Rho GTPase with normal cytokinesis – thus cell cycle is perturbed and multinucleated cells are generated (Horiguchi *et al.*, 1993). There are also evidences that CNF inhibits apoptosis by increasing the expression of the Bcl-2 family proteins (Fiorentini *et al.*, 1998). Cycle inhibiting factor (**Cif**) translocated by the LEE type III secretion system of **EHEC** and **EPEC** *E. coli* strains is another

bacterial factor inhibiting cell cycle transition, yet independent of Rho activation but linked to inactivation of the cyclin-dependent kinase Cdc2 (Marches *et al.*, 2003). Another pathway to disturb cell cycle is used by the ***Bacterioides fragilis*** toxin (**BFT**). BFT is a metalloproteinase cleaving E-cadherin, an important protein for cell-cell interaction, thus releasing β-catenin which migrates to the nucleus and mediates the transcritptional activation of *c-myc* resulting in cell proliferation (Wu *et al.*, 2003). In contrary to the proteins mentioned before, cytolethal distending toxin (**CDT**) directly damages DNA. The CtdB subunit is a DNase harbouring a nuclear localization signal and catalyzing double-stranded DNA breaks that induce repair mechanisms to cause cell cycle arrest (Hassane *et al.*, 2003). CDT is produced by a broad range of bacteria including ***E. coli***, ***Shigella dysenteriae***, ***Campylobacter spp.***, ***Salmonella typhi***, ***Haemopilus ducreyi***, ***Hp*** species, and ***Actinobacillus actinomycetemcomitans*** (Oswald et al., 2005).

In summary, it is tempting to speculate that the activity of cyclomodulins such as CDT could also act as predisposing factors for the initiation of tumour. Likewise, cyclomodulins such as Cif or CNF inducing DNA replication in absence of cell division could promote genetic abnormalities. In general, alterations in cell growth – the ratio of apoptosis to proliferation and differentiation – are important mechanisms driving the clinical manifestation of bacterial infection (Oswald *et al.*, 2005).

Bartonella species and tumour-like vasoproliferative lesions

Verruga peruana (VP) and Bacillary angiomatosis / peliosis (BA/BP) lesions illustrate the remarkable capacity of three among 21 *Bartonella* species. to trigger vasoproliferative processes resembling tumour angiogenesis (Chian *et al.*, 2002). Bartonellae are gram-negative α-proteobacterial pathogens infecting in a rather specific manner their mammalian hosts (Dehio, 2004). Bacteria are transmitted via blood-sucking arthropods or by direct contact. The hallmark of *Bartonella*-infection is a long-lasting intraerythrocytic bacteraemia in their reservoir host(s). Whereas ECs seem to be targeted in both reservoir and incidental host (Dehio, 2005).

The human specific ***Bartonella bacilliformis*** (*Bb*) causes Carrion's disease – a biphasic disease endemic in the highlands of Peru, where the ecological conditions meet the needs of its transmission vector the sandfly *Lutzomia verrucarum* (Spach and Koehler, 1998). The initial acute phase, Oroya fever, is characterized by sever – even fatal if not treated – haemolytic anaemia (Caceres-Rios *et al.*, 1995). Thus, in

contrary to the other *Bartonella*-infections were infected erythrocytes keep circulating in the bloodstream for their remaining lifespan (Schulein *et al.*, 2001), *Bb* induces lysis of red blood cells. The subsequent chronic phase, VP, presents as vascular tumour-like lesions showing varying degrees of inflammation, compact proliferation of ECs and the formation of Rocha-Lima's inclusions, EC colonization with *Bb* (Arias-Stella et al., 1986). The mechanism by which an angiogenic response is accomplished remains elusive. The bacteria could synthesize some diffusible product that, in analogy with known angiogenic factors, could stimulate ECs to participate in angiogenesis (Garcia *et al.*, 1990). The bacterial chaperon GroEL has been proposed to be involved in the observed *Bb* mitogenic activity (Minnick *et al.*, 2003). Alternatively or in addition, host angiogenic factors could be produced as a consequence of either the infection of ECs or an immune response against the bacteria, or the infected cells themselves may have angiogenic potential. VP has been proposed to be the result of *Bb*-induced autocrine production of angiopoietin-2 (Ang-2), with paracrine contribution of VEGF secreted outside of the infected endothelium (Cerimele *et al.*, 2003).

Bartonella quintana (*Bq*) is another human specific pathogen. *Bq* is transmitted by human body lice and causes a spectrum of clinical diseases such as cycling fever (trench fever), endocarditis, chronic lymphadenopathy, and the already mentioned BA lesions (Maurin and Raoult, 1996). BA lesions are histologically very similar to VP associated with infection by *Bb*. Little is known about the mechanisms of *Bq* pathogenicity related to vasoproliferation. *Bq* replicates within ECs in *Bartonella*-containing vacuoles (BCVs) (Brouqui and Raoult, 1996). While early during infection apoptosis is induced by the activation of Apaf-1 and caspase-8, at later times the apoptotic process is inhibited by an overexpression of the anti-apoptotic *bcl-2*. Bcl-2 activation could partially explain survival and proliferation of ECs (Liberto *et al.*, 2003). The triggered peak of apoptosis was further associated with induction of p38 MAPK and SAPK/JNK, followed by next to Bcl-2 induction also a mitotic activation via the cyclin-dependent kinase Cdc2 (Liberto *et al.*, 2004). Variably expressed outer membrane proteins (Vomps) belong to a class of trimeric autotransporter adhesins (TAA) (Linke *et al.*, 2006; Zhang *et al.*, 2004). Vomp expression has been shown to correlate with the ability of *Bq* to induce paracrine production of VEGF in infected THP-1 macrophages and HeLa cells (Schulte *et al.*, 2006).

The zoonotic pathogen ***Bartonella henselae*** (*Bh*), the object of research in this work, causes asymptomatic long-lasting intraerythrocytic bacteraemia in its natural feline reservoir host and is responsible for a number of clinical outcomes during infection of the human incidental host. Disease manifestations range from self-limiting but long-lasting swelling of lymph nodes (cat-scratch-disease, CSD) in immunocompetent patients to the aforementioned tumour-like vasoproliferative lesions on the skin or inner organs known as BA/BP especially in patients with a compromised immune system, such as AIDS patients (Fig. 6) (Florin *et al.*, 2008).

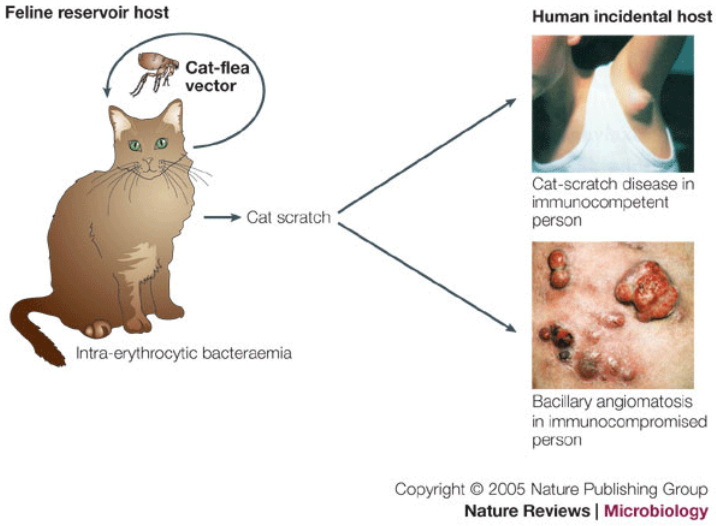

Figure 6 *Bh* causes a sub-clinical intraerythrocytic bacteraemia in its reservoir host, the cat. Through the bite of an infected cat flea or through direct contact (cat scratch), the pathogen is transmitted from cat to cat or from cat to human, respectively. Depending on the immune status of the human host, *Bh* can lead to different clinical manifestations, including CSD (swelling of lymph nodes with fever) in immunocompetent individuals, or BA/BP in immunocompromised patients (Dehio, 2005).

The fact that antibiotic treatment leads to regression of those lesions (Koehler and Tappero, 1993) indicate a causative and active role of *Bh* triggering these vascular proliferations. In addition, the close association of *Bh* with proliferating ECs in BA/BP (Chian *et al.*, 2002) prompted to speculate that *Bh* could subvert EC functions. Indeed, striking changes have been described for human umbilical vein endothelial cells (HUVECs) upon infection with *Bh in vitro* – namely (i) uptake of individual bacteria in so-called BCVs (Kyme *et al.*, 2005), (ii) massive cytoskeletal actin rearrangements leading to a unique structure termed invasome (Dehio *et al.*, 1997), (iii) a NF-κB-dependent pro-inflammatory response (Fuhrmann *et al.*, 2001), (iv) the inhibition of apoptosis (Kirby and Nekorchuk, 2002), and a direct mitogenic

stimulation of ECs (Conley *et al.*, 1994). With the exception of BCVs, in part the NF-κB-dependent pro-inflammatory response, and the direct mitogenic stimulus these activities dependent on the VirB/VirD4 T4SS of *Bh* and its translocated *Bartonella* effector proteins (BepA-BepG) (Fig. 7) (Schulein *et al.*, 2005; Schmid *et al.*, 2004). Intriguingly, the VirB/VirD4 T4SS even counterbalances the potent mitogenic stimulus (Schmid *et al.*, 2004). T4SSs are membrane-associated transporter complexes ancestrally related to conjugation systems used by a number of bacteria to deliver proteins or protein-DNA complexes into a wide range of target cells (Christie *et al.*, 2005). Common to all Beps is a modular structure harbouring a bipartite translocation signal at the C-terminus composed of a BID (Bep intracellular delivery) domain of about 140 amino acids and an unconserved positively charged tail. In their N-terminus the Beps contain next to additional BID domains (BepE-BepG) other domains such as FIC (filamentation induced by cAMP, BepA-BepC) or tandem-repeated peptide motifs resembling eukaryotic tyrosine-phosphorylation sites (BepD-F) (Backert and Selbach, 2005; Schulein *et al.*, 2005).

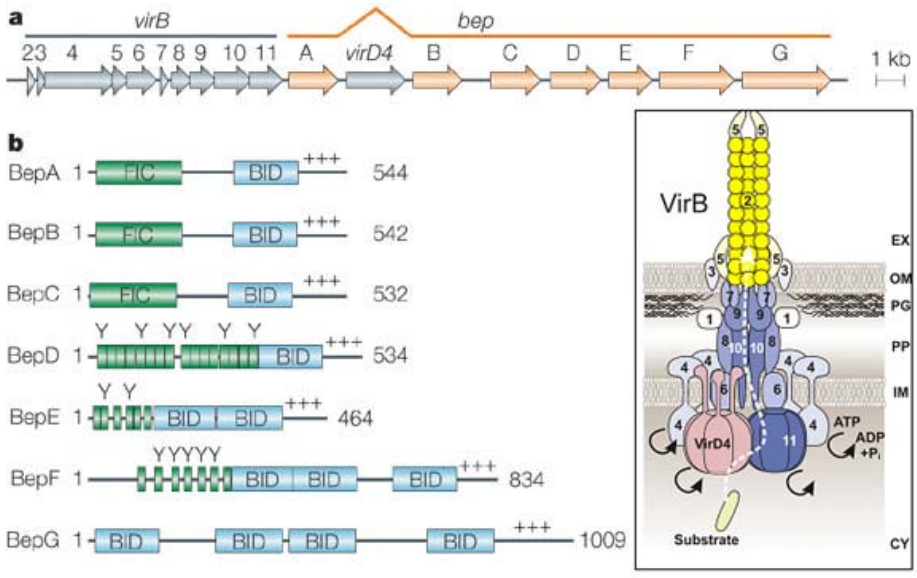

Figure 7 a) The genetic structure of the *virB/virD4/bep* pathogenicity island. b) Model of the VirB/VirD4 T4SS machinery and modulary domain structure of BepA-G. Green boxes represent tyrosine-containing sequence repeats resembling tyrosine-phosphorylation motifs (indicated by Y) or FIC (filamentation induced by cAMP) domain and blue the BID (Bep intracellular delivery) domain (Dehio, 2005; Schroder and Dehio, 2005).

Experimental infection of rats with *B. tribocorum* (*Bt*), a close relative of *Bh*, further emphasizes the importance of the VirB/VirD4 T4SS – non-polar inframe deletions of structural component such as *virB4* or *virD4* result in the complete loss of the ability of the bacteria to cause bacteraemia (Schulein and Dehio, 2002). Additional data indicating a relevance for *Bh*-EC interaction are the expression of the *Bh virB*-operon induced upon EC contact (Schmiederer *et al.*, 2001) and the transcriptional profiling of HUVECs upon infection with *Bh* revealing the regulation of a number of genes controlling innate immune responses, cell cycle, and vascular remodelling (Dehio *et al.*, 2005).

The molecular and cellular mechanisms targeted by the Beps are subject of current research. BepA has been shown to be targeted to the host cell plasma membrane and to inhibit apoptosis in HUVECs via a rise in the cytosolic concentration of the second messenger cAMP (see chapter 1) (Schmid *et al.*, 2006). BepD is tyrosine-phosphorylated upon translocation in ECs (Schulein *et al.*, 2005). The c-Src tyrosine kinase (Csk) binds to a tyrosine-phosphorylation motif, which is very similar to the Csk-binding site in VE-cadherin, in the N-terminus of BepD. Also the protein tyrosine phosphatase SHP2 binds to BepD in a tyrosine-phosphorylation dependent manner (Guye, 2006). Thus BepD is hypothesised to provide docking sites for cell signalling proteins once translocated into the host cell (Pulliainen and Dehio, 2008). BepG is able to promote actin rearrangements required for invasome-mediated invasion. This process seems independent of the small GTPase RhoA but relying on Rac1 and Cdc42 involving the recruitment of the Arp2/3 complex (Rhomberg *et al.*, 2008). Despite those striking VirB/Bep-dependent changes in ECs, the causal role and importance of the *Bh* VirB/VirD4/Bep system in BA/BP remains elusive, in part due to the lack of a suitable animal model or a sophisticated *in vitro* model (addressed in chapter 2).

The vasoproliferative lesions are typically infiltrated by polymorphonuclear neutrophils (PMN) and monocytes indicating an inflammation (Chian *et al.*, 2002). Activation of a pro-inflammatory response might favour the recruitment of monocytes, which upon infection with *Bh* are activated to release pro-angiogenic factors such as VEGF, which could promote EC proliferation in a paracrine manner (Resto-Ruiz *et al.*, 2002; Kempf *et al.*, 2001). Thereby another *Bh* virulence factor, the nonfimbrial adhesin BadA (*Bartonella* adhesin A) a homologue of the *Bq* Vomps (Linke *et al.*, 2006), is thought to be an essential factor eliciting the secretion of VEGF by

activating the transcription factor hypoxia-inducible factor (HIF)-1 (Riess *et al.*, 2004). NF-κB, a major factor controlling inflammation, has been shown to be activated both dependent on the VirB/VirD4 T4SS (Schmid *et al.*, 2004) and generally upon *Bh*-infection (Fuhrmann *et al.*, 2001). The role of the VirB/VirD4/Bep system in triggering VEGF production upon infection has not been addressed so far (see chapter 4), neither was the effect of exogenous VEGF, and thus the contribution of the paracrine loop to BA/BP, on *Bh*-infected ECs assessed (see chapter 3). In summary, the current model for *Bh*-triggered vascular tumour formation proposes a direct activation of ECs and stimulation of proliferation, inhibition of apoptosis, and the activation of a paracrine loop of pro-angiogenic factors such as VEGF released from infected monocytic cells (Fig. 8).

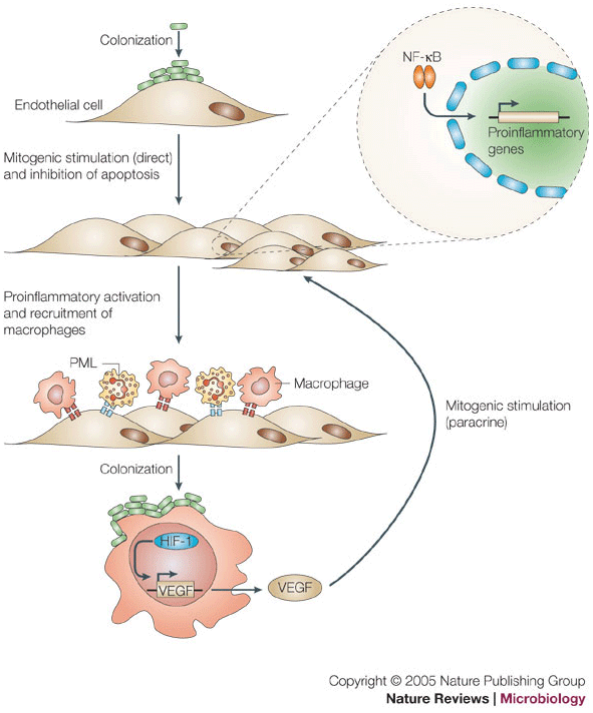

Figure 8 Infection of ECs with *Bh* results in the direct stimulation of EC proliferation and inhibition of apoptosis. Bacteria also trigger a nuclear factor NF-κB-dependent pro-inflammatory response leading to the recruitment of monocytes. Bacterial colonization of macrophages results in hypoxic conditions that lead to activation of HIF-1 and the subsequent upregulation of VEGF expression. The release of VEGF by macrophages results in paracrine stimulation of EC proliferation (Dehio, 2005).

As mentioned in the “Mechanisms of tumour formation” part, the development of a tumour is a multistep process that can take years before clinical manifestations can be observed. Especially infectious agents might be cleared long before the obvious onset of disease. Benign proliferative tumour lesions triggered by pathogenic bacteria like *Bartonella* spp., *L. intracellularis* or *C. rodentium* occur much faster and thus are experimentally more assessable. Besides providing insights into the pathogenesis of *Bh*, the study of bacteria-host cell interactions might be useful to explore general mechanisms involved in processes such a pathological angiogenesis – a hallmark of tumour growth and of cardiovascular and inflammatory diseases. In addition the VirB/VirD4/Bep system itself provides an elegant though complex tool to elucidate cellular functions.

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Aim of the Thesis

Aim of the Thesis

Started in November 2004, the primary aim of this thesis was to elucidate the molecular and cellular mechanisms involved in *Bartonella henselae* (*Bh*)-triggered vasoproliferation – to asses the angiogenic properties of *Bh* with a focus on the role of the VirB/VirD4/Bep system. Following a participation in the characterization of the VirB/VirD4-translocated effector BepA protecting endothelial cells (ECs) from apoptosis, I adapted and used a three-dimensional *in vitro* sprouting angiogenesis assay of collagen-embedded EC spheroids to elucidate pro- and anti-angiogenic effects of *Bh*. In addition to directly assessing the impact of the VirB/VirD4/Bep system on a paracrine loop model of *Bh*-triggered vascular endothelial growth factor (VEGF), I also investigated the effect of exogenous VEGF on *Bh*-infected ECs.

Chapter 1

A Translocated Bacterial Protein Protects Vascular Endothelial Cells from Apoptosis

M.C. Schmid, F. Scheidegger, M. Dehio, N. Balmelle-Devaux, R. Schulein, P. Guye, C.S. Chennakesava, B. Biedermann, C. Dehio

PLoS Pathog. 2006 Nov;2(11):e115

The capacity of *Bartonella henselae* (*Bh*) to inhibit apoptosis during endothelial cell (EC) infection is dependent on a functional VirB/VirD4 type IV secretion system (T4SS) and at least one of the T4SS-translocated *Bartonella* effector proteins (Beps). In this study, BepA was identified to mediate the inhibition of apoptosis in human umbilical vein endothelial cells (HUVECs).

A nonpolar deletion of *bepA* resulted in complete loss of the anti-apoptotic effect, which could be restored by complementation *in trans*. Furthermore, the expression of BepA in the effector-less ∆*bepA*-G mutant restored the anti-apoptotic activity to wild-type level. Using caspase-3/-7 assays we tested the ability of BepA homologues of different *Bartonella* species to inhibit apoptosis. Only BepA homologues from *Bartonella* species known to induce angioproliferative tumour formation were able to protect ECs from apoptosis. The calmodulin-dependent adenylate cyclase (*cya*)-reporter assay was used to demonstrate translocation of the two putative effector proteins BepA from *Bh* and *B. quintana* (*Bq*) into HUVECs. Translocation of BepA into the host cell was confined to the C-terminus and dependent on a functional VirB/VirD4 T4SS.

We constructed different GFP-BepA fusion proteins to delineate the functional domain of BepA required to inhibit apoptosis. These fusion proteins were ectopically expressed in HUVECs and the apoptotic cell population was determined. A 140 aa domain of BepA was sufficient to inhibit apoptosis in HUVECs. This domain corresponds to the conserved BID domain of BepA, which is part of the bipartite translocation signal. To investigate the subcellular localization of BepA in the host cell different GFP-BepA fusion constructs were expressed in HUVECs and samples were analyzed by immunocytochemistry as well as by subcellular fractionation experiments. BepA was localized to the cytoplasmic membrane of the host cell.

Interestingly, we observed that only anti-apoptotic GFP-BepA fusion constructs associated to the membrane.

To elucidate the mechanism of inhibition of apoptosis by BepA, microarray data obtained for the transcriptome of HUVECs infected with wild-type versus the *ΔvirB4* mutant were studied. These revealed a VirB-dependent upregulation of NF-κB and the cAMP/CREM/CREB regulon in infected endothelial cells. The NF-κB pathway did not show any specific activation by anti-apoptotic homologues of BepA. However, upregulation of cAMP-regulated gene expression and increased intracellular cAMP level correlated with the ability of BepA to inhibit apoptosis. Artificially elevated cAMP levels protected ECs from apoptosis, thus BepA might mediate anti-apoptosis of ECs by increasing 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 (CTL) mediated cell death.

These results indicate that BepA, a novel T4SS effector protein, could contribute indirectly to vasoproliferative growth by enhancing cell survival.

Statement of the own participation

In addition to addressing the experimental part of the paper revision, I planed and performed subcellular fractionation experiments to asses the localization of BepA in the cell (Fig. 3D, p. C-8), the caspase-3/-7 assays testing the effect of artificially elevated intracellular cAMP on HUVECs (Fig. 6, p. C-11) as well as further developed the FACS assay used to analyze the capacity of BepA to inhibit CTL mediated cell death (Fig. 7, p. C-12). Microarray data and the experimental setup for real time PCR were provided by Dr. M. Dehio. The other data showed in the manuscript were provided by Dr. M. Schmid.

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PLoS PATHOGENS

A Translocated Bacterial Protein Protects Vascular Endothelial Cells from Apoptosis

Michael C. Schmid[1], Florine Scheidegger[1], Michaela Dehio[1], Nadège Balmelle-Devaux[1], Ralf Schulein[1], Patrick Guye[1], Cuddapah S. Chennakesava[2], Barbara Biedermann[2], Christoph Dehio[1]*

1 Division of Molecular Microbiology, Biozentrum, University of Basel, Basel, Switzerland, **2** Department of Research, University Hospital, Basel, Switzerland

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* and *B. quintana* to cause vascular tumor formation in immunocompromised 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 ECs allowed mapping of the anti-apoptotic activity of BepA to the Bep intracellular delivery 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 *B. henselae* and *B. quintana* and correlated with (i) protein localization to the host cell plasma membrane, (ii) elevated levels of intracellular cyclic adenosine monophosphate (cAMP), and (iii) increased expression of cAMP-responsive genes. The pharmacological elevation of cAMP levels protected ECs from apoptosis, indicating that BepA mediates anti-apoptosis by heightening cAMP levels by a plasma membrane–associated mechanism. Finally, we demonstrate that BepA mediates protection of ECs against apoptosis triggered by cytotoxic T lymphocytes, suggesting a physiological context in which the anti-apoptotic activity of BepA contributes to tumor formation in the chronically infected vascular endothelium.

Citation: Schmid MC, Scheidegger F, Dehio M, Balmelle-Devaux N, Schulein R, et al. (2006) A translocated bacterial protein protects vascular endothelial cells from apoptosis. PLoS Pathog 2(11): e115. doi:10.1371/journal.ppat.0020115

Introduction

Bacterial pathogens have developed various strategies to subvert host cell functions to their benefit. In particular, intracellular bacteria have 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 a nuclear factor kappa B (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 meningitidis* [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 *N. 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 endothelial cells (ECs), 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 *Bartonella henselae (Bh), B. quintana (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

Editor: Pascale Cossart, Institut Pasteur, France

Received January 17, 2006; **Accepted** September 19, 2006; **Published** November 24, 2006

Abbreviations: aa, amino acids; AC, adenylate cyclase; *Bh, Bartonella henselae;* BID, Bep intracellular delivery; *Bq, Bartonella quintana; Bt, Bartonella tribocorum;* Cya, calmodulin-dependent adenylate cyclase; cAMP, cyclic adenosine monophosphate; CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; CRAfT, Cre-recombinase assay for translocation; EC, endothelial cell; FIC, filamentation induced by cyclic adenosine monophosphate; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cell; IBMX, 3-isobityl-1-methylxanthine; IL-8, interleukin 8; IPTG, isopropyl β-d-thiogalactosidase; MHC, major histocompatibility complex; MOI, multiplicity of infection; NFκB, nuclear factor kappa B; PI, propidium iodide; SD, standard deviation; T4S, type IV secretion; WGA, wheat germ agglutinin

* To whom correspondence should be addressed. E-mail: christoph.dehio@unibas.ch

Synopsis

The capacity of infected host cells to die by apoptosis (programmed cell death) is critical for controlling pathogen replication and survival. Bacterial pathogens have thus developed strategies to inhibit host cell apoptosis, allowing them to preserve their cellular habitat during chronic infection. For instance, the capacity of the facultative intracellular pathogen *Bartonella henselae* to trigger tumor formation as a consequence of chronic infection of the human vasculature is linked to the inhibition of endothelial cell apoptosis. This study describes the identification and functional characterization of the anti-apoptotic bacterial effector protein BepA of *B. henselae,* which is shown to be sufficient to inhibit endothelial cell apoptosis, i.e., as triggered by activated cytotoxic T lymphocytes. Upon translocation into endothelial cells via a bacterial type IV secretion system, BepA localizes to the plasma membrane, where it triggers the production of second messenger cyclic adenosine monophosphate in quantities effective for blocking apoptosis. Strikingly, the capacity of BepA to mediate membrane localization, cyclic adenosine monophosphate production, and the resulting inhibition of apoptosis is confined to a conserved domain that originally evolved in bacteria as a signal for type IV secretion. This study thus highlights the convergent evolution of an anti-apoptotic effector protein of purely bacterial origin.

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 type IV secretion (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 that encodes next to the *virB/virD4* locus seven putative T4S substrates, the *Bartonella*-translocated effector proteins *Bh* BepA–G [13]. Deletion of the pathogenicity island regions encoding *bepA–G* abolishes 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, called the Bep intracellular delivery (BID) 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 (*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 *B. tribocorum (Bt)* [16]. Remarkably, all BepA homologs encode in their N-terminal part a conserved FIC (filamentation induced by cyclic adenosine monophosphate [cAMP]) 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 identify 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 ECs. 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 ECs. Finally, we demonstrate that BepA can inhibit cytotoxic T lymphocyte (CTL)–mediated apoptosis of ECs.

Results

Bh BepA Mediates Anti-Apoptosis in ECs

The anti-apoptotic activity of *Bh* on human umbilical vein endothelial cells (HUVECs) 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 *bepA–G* mutant and assayed for anti-apoptotic activity. To this end, HUVECs were infected with the different *Bh* strains and apoptosis was induced by exposure to actinomycin D. Morphological examination (Figure 1A), measuring of caspase-3/-7 activity (Figure 1B), and flow cytometric analysis of Annexin V– and propidium iodide (PI)–stained cells (Figure 1C) were used to monitor apoptosis. Δ*bepA–G* did not display anti-apoptotic activity as reported previously [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 actinomycin D–triggered apoptosis (unpublished data). Consistently, strain Δ*bepA* carrying an in-frame deletion of *bepA* did not protect HUVECs from apoptosis, whereas complementation of this mutant with p*Bh* BepA restored the anti-apoptotic activity (Figure 1). To test for functionality of the VirB/VirD4 system in the Δ*bepA* 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 Δ*bepA* strain (unpublished data).

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

Bh BepA Is a VirB/VirD4 Substrate Translocated into ECs

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 (unpublished data). Here, we adapted the calmodulin-dependent adenylate cyclase (Cya) reporter assay [19,20] as an alternative to test for translocation of *Bh* BepA into HUVECs. 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 determine the stability of fusion proteins by immunoblot analysis (Figure 2A and 2C). As positive control, the T4S signal of *Bh* BepD (*Bh* $BepD_{352–534}$) [13] was fused to the FLAG-Cya reporter

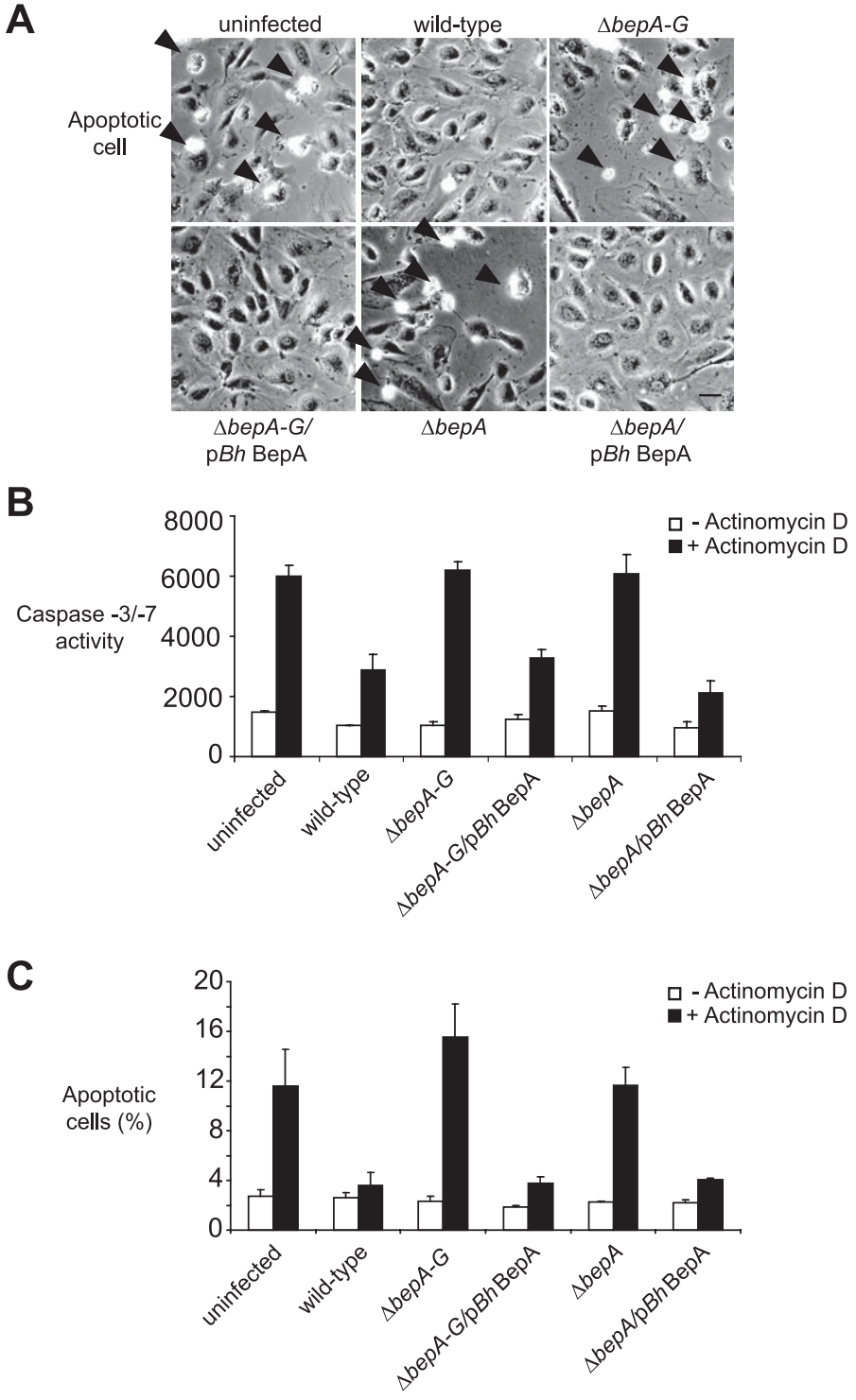


Figure 1. *Bh* BepA Meditates Inhibition of Apoptosis in ECs

(A and B) HUVECs 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 actinomycin D. Then, 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 (A) indicate apoptotic cells displaying membrane blebbing.

(C) Twenty-four hours after induction of apoptosis, the loss of membrane asymmetry was quantified by flow cytometric analysis of AlexaFluor488 Annexin V– and PI-stained cells, allowing us to quantify the rate of apoptotic cells (Annexin V–positive and PI-negative). Mean and standard deviation (SD) are shown for one representative out of three independent replica experiments. All strains were tested a minimum of three times in triplicate samples.

doi:10.1371/journal.ppat.0020115.g001

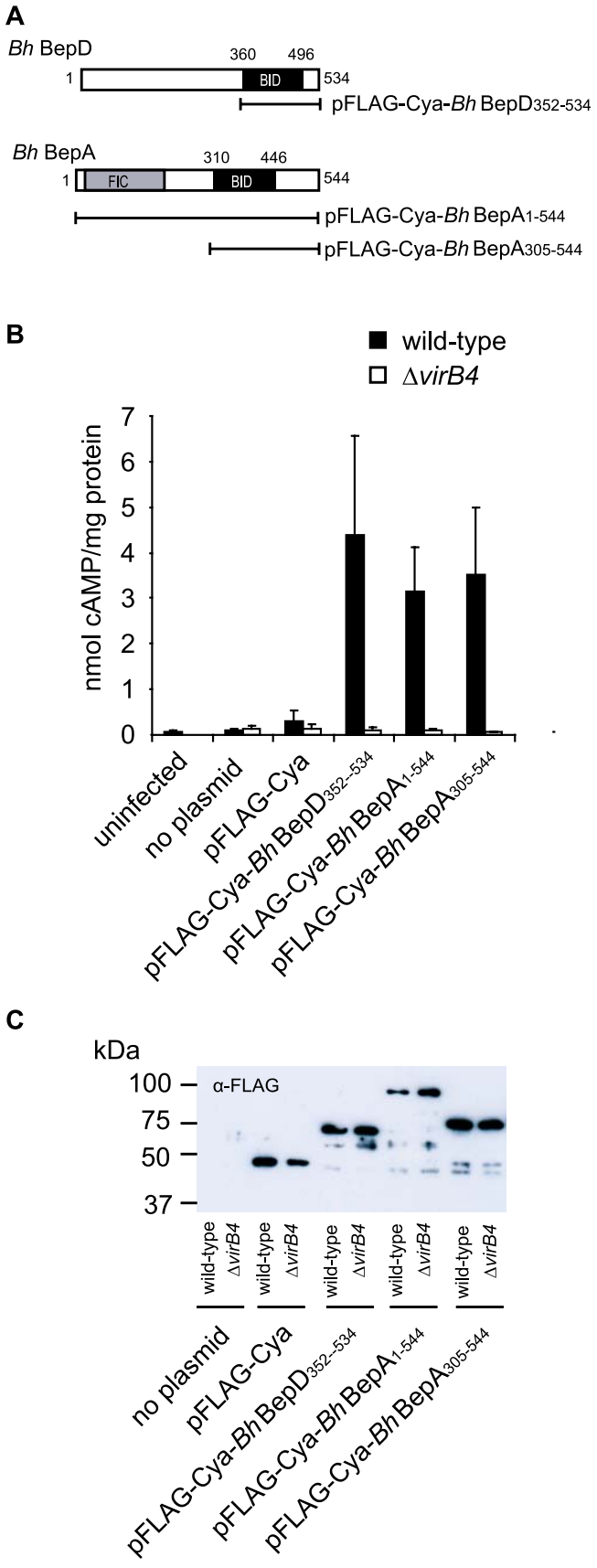


Figure 2. *Bh* BepA Is a Genuine VirB/VirD4 T4S Substrate That Is Translocated into ECs

(A) The bars indicate the parts of *Bh* BepA or *Bh* BepD that were fused to Cya. These reporter fusions were used to monitor translocation via the VirB/VirD4 system. All constructs contain an N-terminal FLAG epitope for immunological detection of the encoded fusion protein.

(B) Quantification of the amount of intracellular cAMP in HUVECs infected for 20 h with the indicated bacterial strains (MOI = 300). Isogenic strains with a functional (wild-type) or non-functional (Δ*virB4*) VirB/VirD4 T4S system 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.

doi:10.1371/journal.ppat.0020115.g002

(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. HUVECs infected with wild-type-expressing FLAG-Cya did not display any significant increase of the cAMP level compared with 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 HUVECs (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 functional 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 HUVECs is sufficient to mediate anti-apoptosis. N-terminal green fluorescent protein (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). Then, 24 h after transfection of HUVECs with the different GFP fusion constructs, cells were either exposed to actinomycin D (apoptosis induction) or left untreated (non-induced control) for another 12 h. Then, cells were stained with Annexin V and PI, and the proportion of apoptotic cells among the GFP-positive cell population was quantified by flow cytometry.

HUVECs transfected with pGFP and treated with actinomycin D displayed an apoptotic rate of 13% (Figure 3B). 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 that of 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 amino acids 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 amino acids [aa]) to GFP (pGFP–*Bh* $BepA_{305–446}$) was sufficient to inhibit apoptosis, whereas fusion of the positively charged C-terminal tail plus only part of the BID domain (pGFP–*Bh* $BepA_{403–544}$) did not result in anti-apoptosis (Figure 3B). Rather, expression of the latter construct had a pro-apoptotic effect, as 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 ECs 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 host cells, the generated GFP fusions (Figure 3A) were ectopically expressed in HEK293T cells for 24 h, followed by immunocytochemical staining for the cell surface with Texas Red–conjugated wheat germ agglutinin (WGA). 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 an *xz*-plane (indicated in Figure 3C by dashed lines). Ectopically expressed GFP localized to the cytoplasm of HEK293T cells, 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 that did not confer protection localized primarily to the cytoplasm.

Fractionation of post-nuclear extracts of the transfected HEK293T cells by ultracentrifugation was used as an independent biochemical assay for analyzing the membrane or cytosolic localization of ectopically expressed GFP-BepA fusions. The obtained data (Figure 3D) were in good agreement with the microscopic analysis presented in Figure 3C, except that construct pGFP–Bh $BepA_{403–544}$ displayed both cytosolic and membrane localization.

Taken together, these data suggest that association with 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. *Bq* was previously shown to cause vascular tumor formation, whereas *Bt* was never associated with vascular lesions [9]. Consistent with these observations, only *Bh* and *Bq,* but not *Bt* wild-type, were able to block actinomycin D–triggered caspase-3/-7 activation in a T4S-dependent manner (Figure 4A). In the genome sequence of *Bq,* the *bepA* gene is annotated as a pseudogene [21]. Closer inspection of the sequence revealed that because of an internal stop codon and a downstream-located start codon in frame, this *bepA* locus is split into two open reading frames *(Bq bepA1* and *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 with *Bh* BepA revealed high similarity (59% and 63%, respectively). We cloned *Bt bepA* and *Bq bepA2* into expression plasmids, which were introduced into *ΔbepA–G* (Figure 4B). 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 4B and unpublished data). HUVECs were infected for 24 h with the different isogenic strains, and subsequently apoptosis was induced by actinomycin D. Of the tested BepA homologs, only *Bh* BepA and *Bq* BepA2 inhibited actinomycin D–triggered caspase-3/-7 activation (Figure 4C). These findings revealed that *Bq bepA2* encodes an anti-apoptotic effector. To assign *Bq* BepA2 as a novel T4S substrate, we translocated a FLAG-Cya-BepA2 fusion from *Bh* into HUVECs by the Cya reporter assay (unpublished data). 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 that composes a functional T4S signal [13].

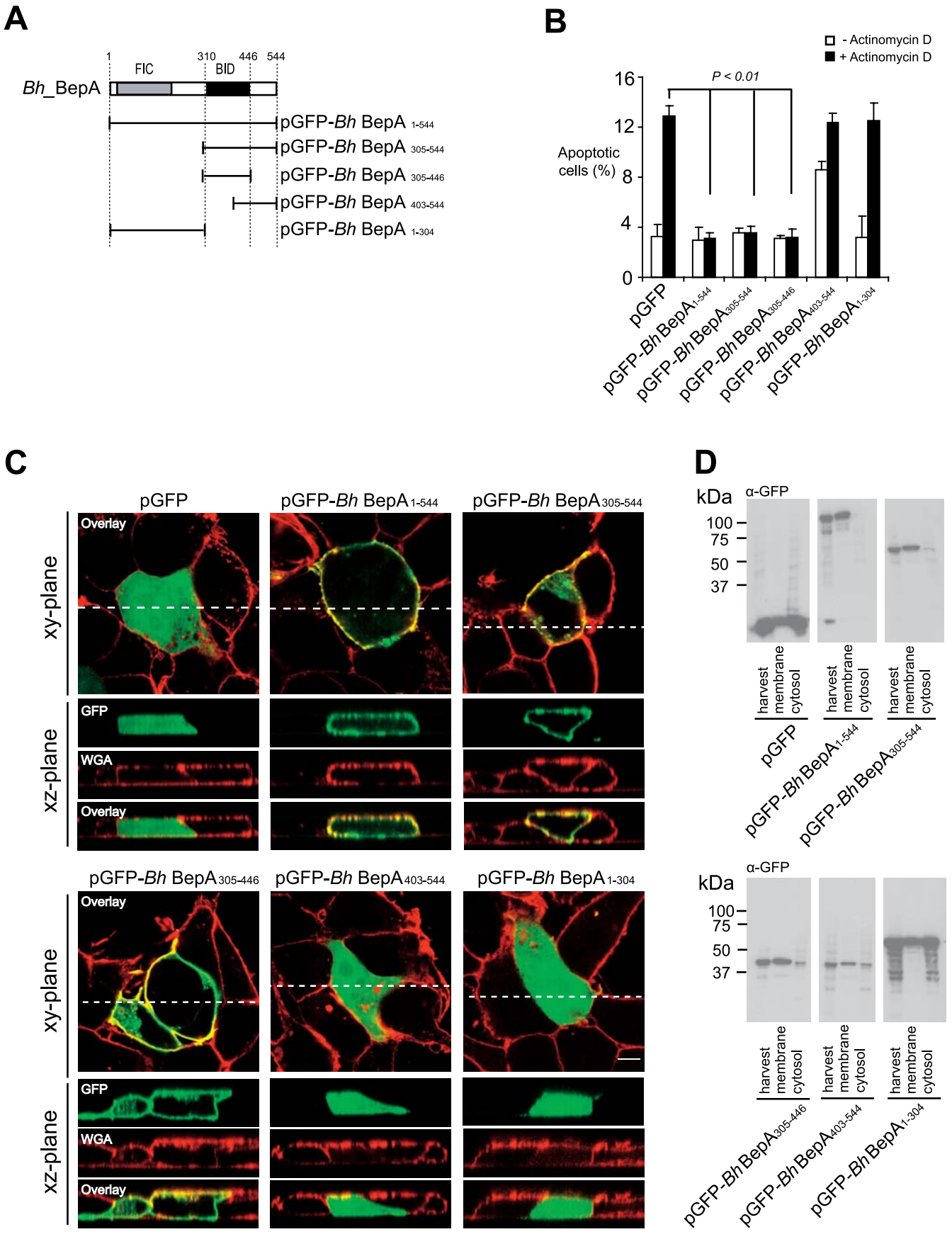
Inhibition of Apoptosis Correlates with an Increased Intracellular cAMP Level

To identify potential survival pathways mediated by anti-apoptotic BepA, we analyzed Affymetrix GeneChip data obtained for the transcriptome of HUVECs infected with *Bh* wild-type versus the Δ*virB4* 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 HUVECs. Both signaling pathways have been described to mediate, among other functions, 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 HUVECs by measuring the release of interleukin 8 (IL-8). *Bh* wild-type triggered an increased IL-8 release compared with Δ*bepA–G* or the uninfected control. In contrast, none of the BepA homologs expressed in Δ*bepA–G* induced increased IL-8 secretion (Figure 5A). This finding indicates that the survival mechanism triggered by anti-apoptotic BepA homologs is independent from the activation of the NFκB pathway triggered by wild-type bacteria. 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 significant manner (Figure 5B). By quantifying intracellular cAMP upon infection of HUVECs 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 ECs against Apoptosis

Since 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 (ACs) generating cAMP, and phosphodiesterases degrading cAMP [27,28]. To trigger a physiological rise of the cAMP level in ECs, we activated ACs with forskolin and in parallel inhibited cAMP degradation by adding the phosphodiesterase-inhibiting drug 3-isobityl-1-methylxanthine (IBMX) [29,30]. HUVECs were infected with the substrate-free Δ*bepA–G* strain and with the BepA-expressing strain Δ*bepA–G*/p*Bh* BepA in the absence and presence of forskolin/IBMX. Apoptosis was induced by exposure to actinomycin D and monitored by measuring caspase-3/-7 activity. By adding forskolin/IBMX we were able


A
1
310
446
544
FIC
BID
Bh_BepA
pGFP-Bh BepA 1-544
pGFP-Bh BepA 305-544
pGFP-Bh BepA 305-446
pGFP-Bh BepA 403-544
pGFP-Bh BepA 1-304
B
- Actinomycin D
+ Actinomycin D
P < 0.01
Apoptotic cells (%)
16
12
8
4
0
pGFP
pGFP-BhBepA1-544
pGFP-BhBepA305-544
pGFP-BhBepA305-446
pGFP-BhBepA403-544
pGFP-BhBepA1-304
C
pGFP
pGFP-Bh BepA1-544
pGFP-Bh BepA305-544
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pGFP-Bh BepA403-544
pGFP-Bh BepA1-304
xy-plane
xz-plane
Overlay
GFP
WGA
D
kDa
α-GFP
100
75
50
37
harvest
membrane
cytosol
pGFP
pGFP-BhBepA1-544
pGFP-BhBepA305-544
pGFP-BhBepA305-446
pGFP-BhBepA403-544
pGFP-BhBepA1-304

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 HUVECs for 24 h, followed by 12 h incubation in the presence or absence of actinomycin D as indicated. The loss of membrane asymmetry in transfected cells (GFP-positive) was then quantified by flow cytometric analysis of APC-Annexin V– and PI-stained cells, allowing us to quantify the rate of apoptotic cells (Annexin V–positive and PI-negative). 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 24 h in HEK293T cells. Cells were immunochemically stained to label the cell surface with Texas Red–conjugated WGA. Confocal pictures were taken for GFP (green channel) and WGA (red channel) in the *xy*-plane (upper image, overlay both channels, bar = 10 μm), and also in the *xz*-plane at the dashed stroke line (lower images, single channels and overlay channels).

(D) Fractionation of GFP–*Bh* BepA fusion proteins into membrane and cytosolic fractions by ultracentrifugation of post-nuclear extracts harvested from transfected HEK293T cells.

doi:10.1371/journal.ppat.0020115.g003

to reduce caspase-3/-7 activity in uninfected HUVECs and cells infected with the Δ*bepA–G* strain to a level similar to that found in cells infected with Δ*bepA*–G/p*Bh* BepA. Interestingly, the addition of forskolin/IBMX did not have an additive protection effect in Δ*bepA–G*/p*Bh* BepA–infected HUVECs (Figure 6A). The same results were obtained by adding the cell-permeable cAMP analog dibutyryl cAMP to the culture medium (Figure 6B). From these data we conclude that a moderately increased intracellular cAMP level, as triggered by *Bh* BepA, is sufficient to protect ECs against apoptosis.

The Role of *Bh* BepA in Evading CTL-Mediated Apoptosis

During colonization of the human endothelium, intracellular *Bh* are hidden from antibody- and complement-mediated immune responses [9]. However, the infected ECs may be killed by CTLs [31]. To study a putative role of *Bh* BepA in protecting ECs from CTL-mediated apoptosis, we used a major histocompatibility complex (MHC) class I–restricted CTL clone that kills HLA-A2-positive HUVECs upon extracellular loading with a Y-chromosome-encoded, male-specific minor histocompatibility Ag peptide with the sequence FIDSYICQV (SMCY peptide) [32]. To specifically measure the rate of EC apoptosis without interference by apoptotic CTLs, we pre-labeled the EC population with the fluorogenic dye CSFE. Pre-labeled HUVECs were left uninfected (control), or infected with wild-type, Δ*bepA–G*, or Δ*bepA–G*/p*Bh* BepA. Then, 24 h after infection, HUVECs were either pre-loaded for 30 min with the CTL-stimulating SMCY peptide (10^{-5} M) or not pre-loaded. After washing, CTLs were added in an effector-to-target cell ratio of 5:1. At different time points (0 h, 2.5 h, and 6 h), Annexin V and PI staining was performed to quantify the amount of apoptotic HUVECs (CSFE-positive, Annexin V–positive, and PI-negative) (Figure 7A and 7B). SMCY-treated HUVECs, which were either uninfected or pre-infected with the BepA-deficient Δ*bepA*–G mutant strain, displayed a time-dependent increase in the apoptotic population in comparison with respective control cells without pre-loading of the CTL-activating SMCY peptide. In sharp contrast, SMCY-treated HUVECs pre-infected with wild-type or the BepA-expressing strain Δ*bepA–G*/p*Bh* BepA did not display any significant increase of apoptosis over control cells without pre-loading with the SMCY peptide. These data indicate that BepA is capable of protecting ECs against the MHC class I–restricted apoptotic activity of CTLs. Consistently, we noticed that BepA-expressing bacteria, as well as the ectopic expression of a GFP–*Bh* BepA fusion protein alone, protected ECs against cell death triggered by a different CTL clone, which was activated by phytohemagglutinin in an MHC class I–independent manner (unpublished data). Together, these data indicate that translocated *Bh* BepA can effectively protect the chronically infected vascular endothelium 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 their 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 need 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, remain 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 ECs [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,* whereas expression of *Bh bepA* in *trans* restored the activity to wild-type level. *Bh* BepA was previously described as a putative substrate of the T4S system VirB/VirD4 [13]; we used the Cya reporter assay to demonstrate that *Bh* BepA is indeed translocated into ECs in a T4S-dependent manner. *Bh* BepA is thus a genuine T4S effector that inhibits apoptosis upon translocation into HUVECs. It is worth noting 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 readout by CRAfT [13], suggesting that the negative readout 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 GFP–*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,

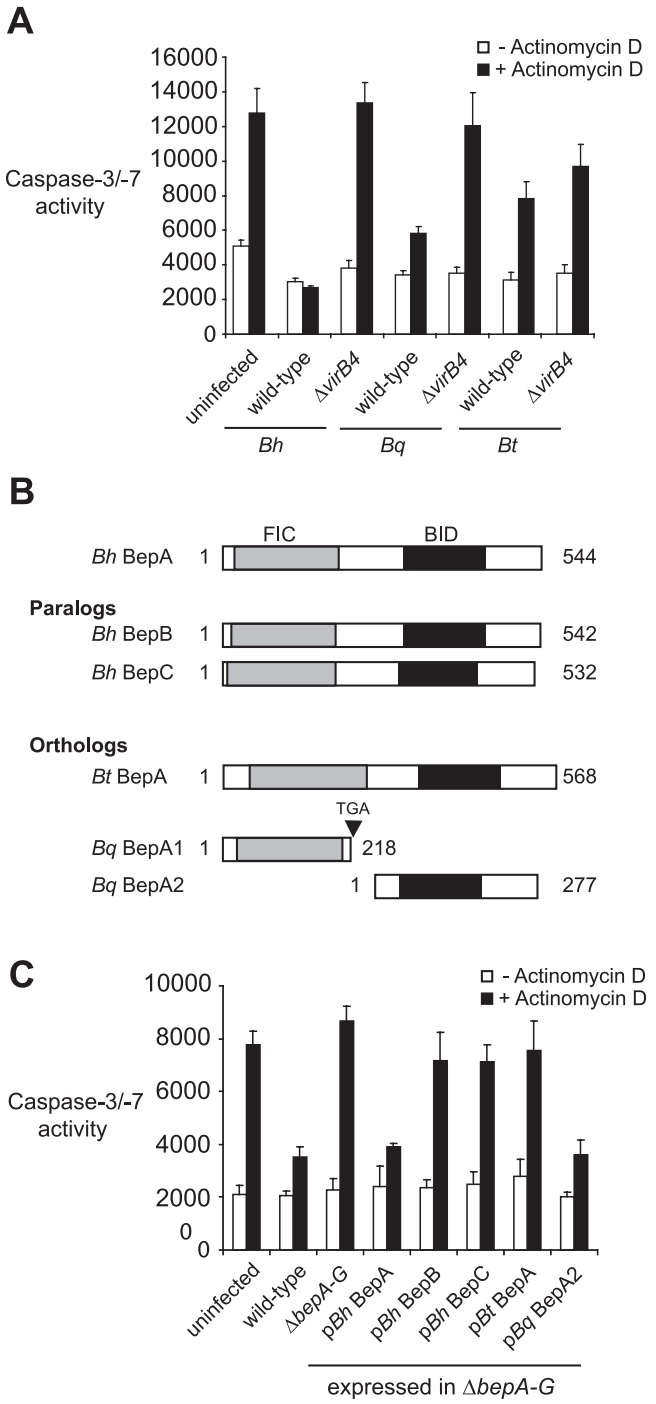


Figure 4. Comparison of the Anti-Apoptotic Activities of BepA Homologs

(A) Anti-apoptotic activities of wild-type and isogenic Δ*virB4* mutant strains of *Bh* in comparison with *Bq* and *Bt*.

(B) Domain structure of *Bh* BepA, its paralogs *Bh* BepB and *Bh* BepC, and the orthologs *Bt* BepA and *Bq* BepA1/*Bq* BepA2. These homologs contain conserved FIC and BID domains in their N-terminal and C-terminal regions, respectively, except for *Bq*, where the orthologous locus is split between these domains into two separate open reading frames by an internal stop codon.

(C) Anti-apoptotic activity of BepA homologs. HUVECs were infected with the indicated *Bh* strains for 24 h, followed by apoptotic induction with actinomycin D 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.

doi:10.1371/journal.ppat.0020115.g004

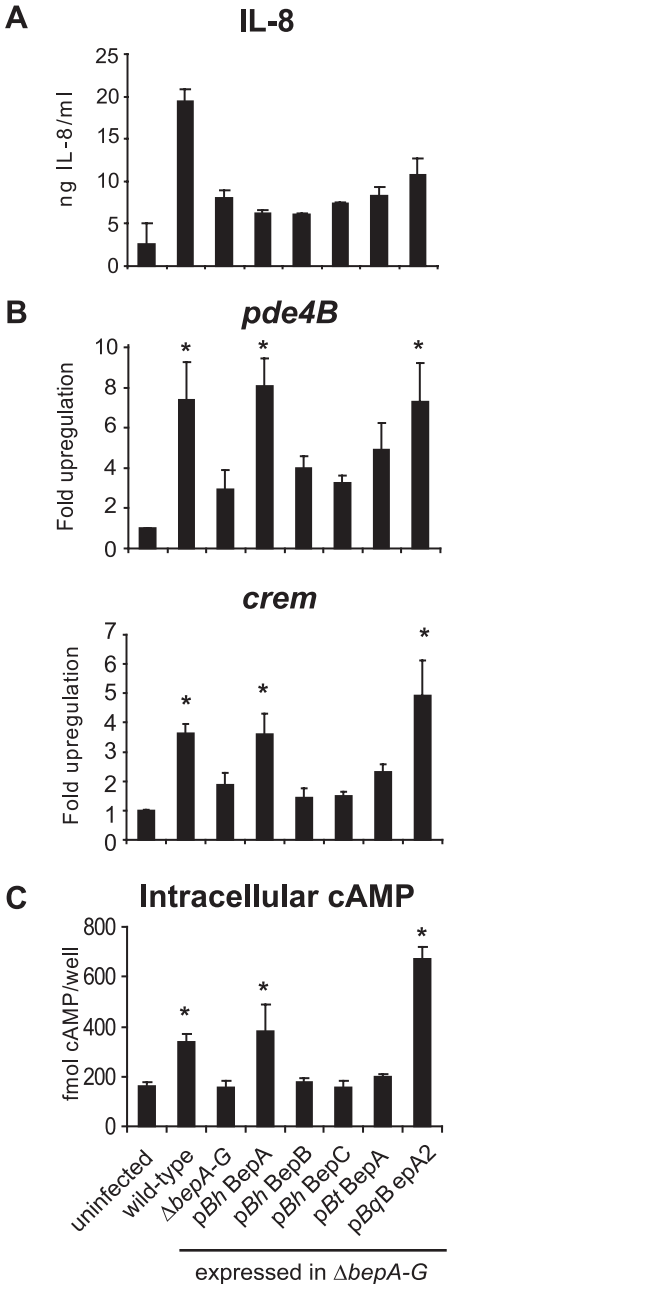


Figure 5. Anti-Apoptotic BepA Homologs Mediate an Increase in Intracellular cAMP and an Upregulation of cAMP Response Genes

HUVECs were infected with the indicated *Bh* strains. The means and SD of one out of three independent replica experiments performed in triplicate samples are presented.

(A) IL-8 was determined in culture supernatants after infection for 54 h with 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 MOI = 300.

(C) Intracellular cAMP levels were determined after infection for 30 h with MOI = 150.

In (B) and (C), samples marked with an asterisk ($p < 0.05$) differ statistically significantly from Δ*bepA–G* using an unpaired Student's *t*-test.

doi:10.1371/journal.ppat.0020115.g005

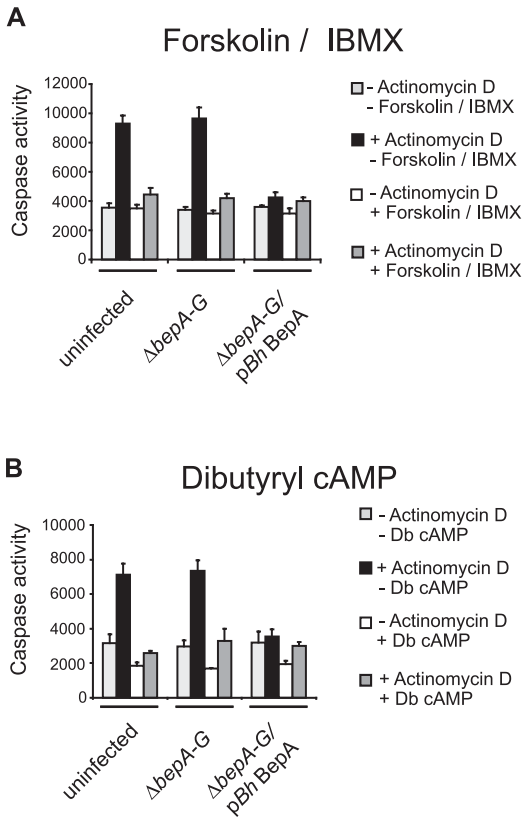


Figure 6. A Pharmacologically Increased cAMP Level in ECs Mimics the Anti-Apoptotic Effect of *Bh* BepA

HUVECs 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 (1 mM). If indicated, apoptosis was then induced with actinomycin D. Caspase-3/-7 activities were determined 9 h later. All strains were tested in triplicates a minimum of three times.

doi:10.1371/journal.ppat.0020115.g006

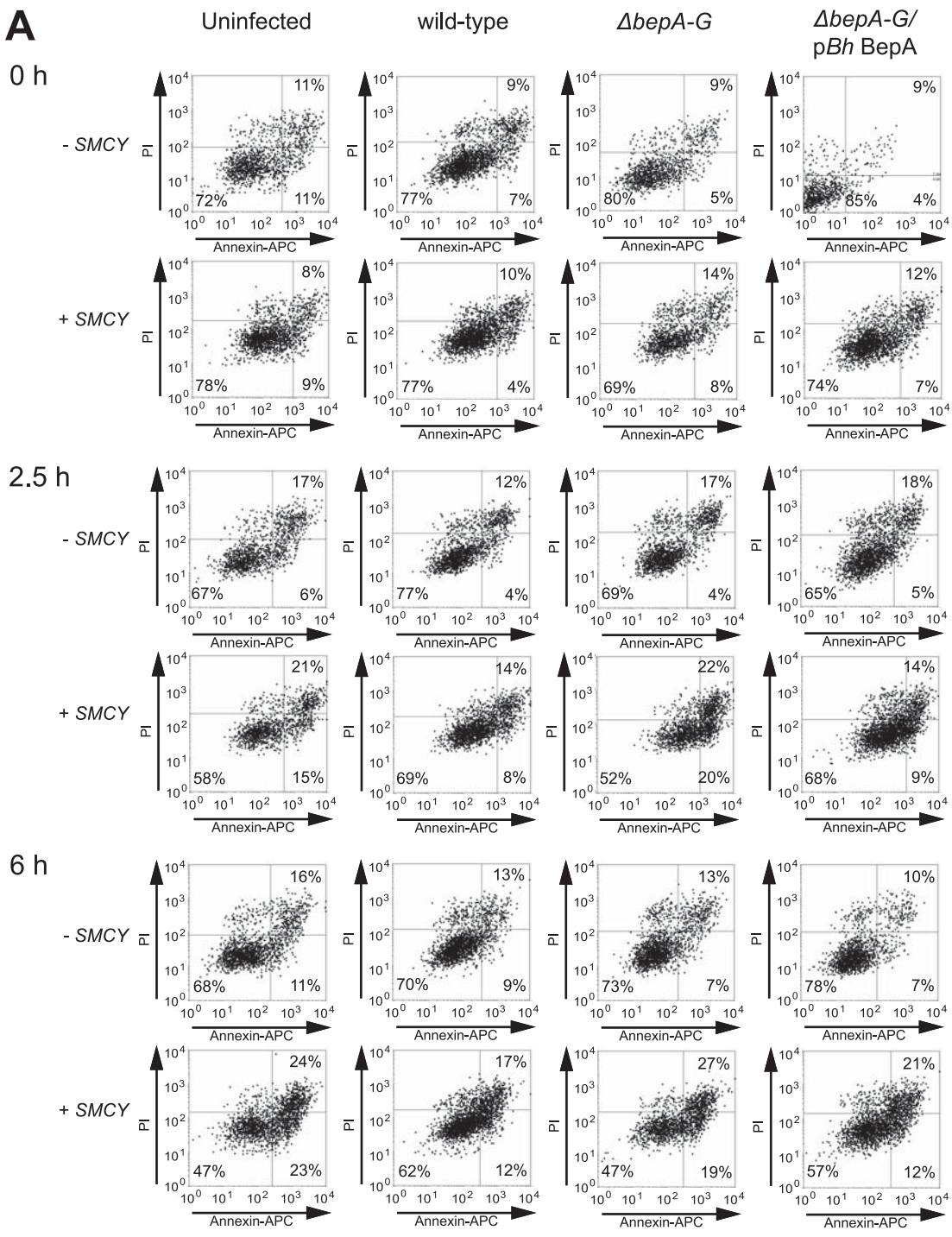
and that this anti-apoptotic effector localizes to the plasma membrane. Moreover, ectopic expression of various parts of *Bh* BepA fused to GFP revealed a strict correlation of the capacity to mediate anti-apoptosis with localization to the plasma membrane (as opposed to the primarily cytoplasmic localization 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 of the cellular pathway by which *Bh* BepA mediates anti-apoptosis in ECs, we first tested whether an NFκB-dependent survival pathway is involved. The anti-apoptotic activity of *R. rickettsii* in ECs 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 signaling 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 HUVECs correlates with elevation of the intracellular cAMP level and results 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 protein kinase A, extracellular signal regulated, mitogen-activated protein kinase, or guanine nucleotide exchange factor 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: efforts to block putatively involved signaling molecules (i.e., protein kinase A) failed because of 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 has been reported to fully protect ECs against apoptosis [45]. Consistent with this, we observed that increased cAMP levels caused by the combined action of the AC-activating drug forskolin and the phosphodiesterase-inhibiting drug IBMX resulted in a complete suppression of actinomycin D–induced apoptosis in HUVECs. Forskolin/IBMX-treated cells displayed an increase in the expression of cAMP-regulated genes similar to that observed upon infection with anti-apoptotic *Bh* strains. cAMP is produced in eukaryotic cells by the family of membrane-anchored ACs. ACs are activated by heterotrimeric G proteins that are regulated by G protein–coupled receptors [46]. Since the anti-apoptotic activity of *Bh* BepA in HUVECs is strictly associated with the plasma membrane localization of this effector, concomitant with an increase in the cAMP level, we propose that *Bh* BepA may trigger anti-apoptosis by interacting either with ACs 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 was 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 been associated with anti-apoptosis. This indicates that subsequent to the expansion of this paralogous protein family by gene duplication, the *Bh* BepA BID domain acquired, in addition to its crucial function as a signal for 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 ECs.

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


A
Uninfected
wild-type
ΔbepA-G
ΔbepA-G/ pBh BepA
0 h
- SMCY
+ SMCY
2.5 h
- SMCY
+ SMCY
6 h
- SMCY
+ SMCY
PI
Annexin-APC
11%
72%
11%
9%
77%
7%
9%
80%
5%
9%
85%
4%
8%
78%
9%
10%
77%
4%
14%
69%
8%
12%
74%
7%
17%
67%
6%
12%
77%
4%
17%
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4%
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69%
8%
22%
52%
20%
14%
68%
9%
16%
68%
11%
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73%
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10%
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12%
27%
47%
19%
21%
57%
12%

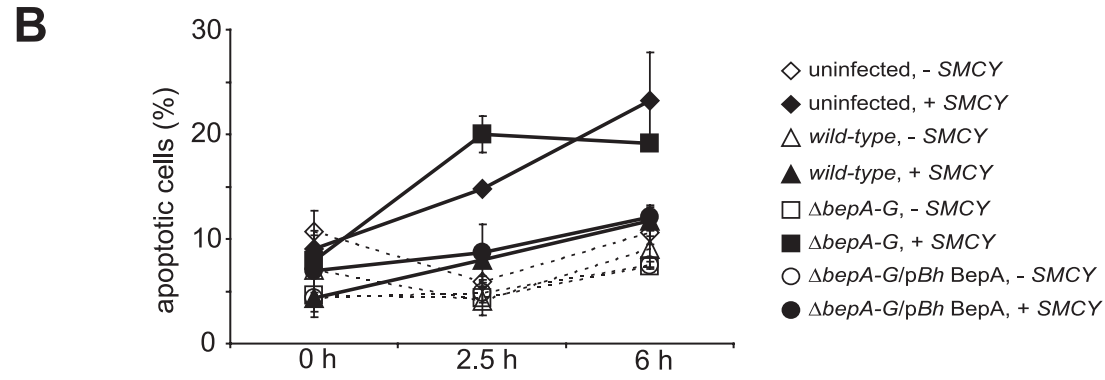
B
apoptotic cells (%)
30
20
10
0
0 h
2.5 h
6 h
uninfected, - SMCY
uninfected, + SMCY
wild-type, - SMCY
wild-type, + SMCY
ΔbepA-G, - SMCY
ΔbepA-G, + SMCY
ΔbepA-G/pBh BepA, - SMCY
ΔbepA-G/pBh BepA, + SMCY

Figure 7. *Bh* BepA Protects ECs against CTL-Mediated Cell Death

Confluent HUVEC monolayers fluorescently labeled with the cell-tracking dye CFSE were infected with the indicated bacterial strains for 24 h or left uninfected (control). Then cells were incubated in the presence (+ SMCY) or absence (− SMCY) of SMCY peptides, followed by washing. CTLs were added in an effector-to-target cell ratio of 5:1 for the indicated period, and after washing and Annexin V– and PI-staining, the apoptotic cell population of HUVECs was determined by flow cytometry analysis (CFSE-positive, Annexin V–positive, and PI-negative).

(A) Dot plots are shown for representative samples.

(B) Summary graph representing HUVEC apoptosis during CTL co-culture. The mean and SD of one of two independent experiments performed in duplicates is shown.

doi:10.1371/journal.ppat.0020115.g007

important for the effector function of *Bh* BepB and *Bh* BepC, our results demonstrate 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* split this locus into two separate open reading frames *(Bq bepA1* and *Bq bepA2). Bq* BepA1 has the FIC domain but lacks a T4S signal, and thus cannot be translocated into ECs. In contrast, *Bq* BepA2 consists only of the T4S signal, which we have shown mediates both T4S-dependent translocation and anti-apoptosis in ECs. It thus appears plausible that the functional diversification of BepA into an anti-apoptotic effector occurred in the common ancestor of the closely related species *Bh* and *Bq* [21], and that following speciation the acquisition of additional 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* does 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 HUVECs with human CTLs. CTLs execute cell-mediated immunity, an immune mechanism probably involved in the elimination of *Bh* infection [9,12,47]. Cell-mediated immunity 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, CTLs 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 that is activated in an MHC class I–restricted manner by extracellular loading of HLA-A2-positive HUVECs with an HLA-A2-specific peptide (SMCY). Such activated CTLs kill their target cells in a perforin-dependent manner [32], primarily by triggering granzyme-dependent apoptosis [50,51]. *Bh* BepA–expressing strains indeed inhibited CTL-triggered EC apoptosis, indicating that the biological function of *Bh* BepA expression in vivo might be to protect the integrity of its colonized cellular niche. The *Bh* BepA–mediated resistance of ECs to CTL-dependent cell death points towards an important role of BepA in escaping cell-mediated immunity and thus in protecting the integrity of the chronically infected vasculature, which is a prerequisite for vascular proliferation. The establishment of an animal model for *Bartonella*-triggered vasoproliferation now appears an urgent need for studying 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 of the evolution of new pathogenic traits in bacteria. The delineation of their anti-apoptotic activity to the conserved BID domain, and their proposed role in maintaining the cellular habitat by mediating anti-apoptosis via specific interaction with the plasma membrane–associated, cAMP-generating signaling cascade of ECs, 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. *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 d. Strain RSE247, a spontaneous streptomycin-resistant strain of *Bh* 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). *Escherichia coli* strains were cultivated in Luria-Bertani liquid medium, or after addition of agar on plates, 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.

DNA manipulation. Plasmids used in this study are listed in Table 1. Primers are listed in Table 2.

Plasmids for in-frame deletions and complementation of deletion mutants were constructed as follows: 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. Plasmid pRS63, used for generating a Δ*virB4* in-frame deletion mutant in *Bq,* was constructed as follows. The BamHI insert of pRS14 [16] was replaced by a 1,318-bp BamHI fragment of the *Bq virB* locus containing a 2,112-bp in-frame deletion in *virB4*. This fragment was constructed by megaprime PCR from two PCR products. Product 1 (0.75 kb) was amplified with primers prRS226 and prRS227 and contained the first 115 bp of the *virB4* gene and upstream sequences. Product 2 (0.55 kb) was amplified with primers prRS228 and prRS229 and contained the last 120 bp of the *virB4* gene and downstream sequences. Megapriming and PCR amplification with primers prRS226 and prRS229 were performed as described [16].

Plasmids for complementation of *ΔbepA* mutant and *ΔbepA–G* mutant were constructed as follows: 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 and *Bt,* using oligonucleotide primers prPG92/93, prPG95/129, prPG97/130, or pMS27/28, and 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. Then, 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

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain or Plasmid	Name	Genotype or Relevant Characteristics	Reference or Source
***Bh* strains**	ATCC 49882^T	Houston-1, isolated from a bacteremic HIV patient	[57]
	MSE150	Δ*bepA–G* mutant of RSE247	[13]
	MSE154	Δ*bepA* mutant of RSE247	This work
	MSE156	MSE150 containing pPG101	This work
	MSE159	MSE150 containing pMS007	This work
	MSE162	MSE150 containing pRS51	[13]
	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	[13]
	RSE242	ΔvirB4 mutant of RSE247	[12]
	RSE247	Spontaneous Sm^r strain of ATCC 49882^T, serving as wild-type	[12]
	RSE308	RSE247 containing pRS51	[13]
***Bq* strains**	JK-31	Isolated from a bacteremic patient with bacillary angiomatosis	[58]
	RSE356	Spontaneous Sm^r strain of JK-31, serving as wild-type	This work
	RSE569	*ΔvirB4* mutant of RSE356	This work
	VR 358		[58]
***Bt* strains**	IBS 506^T	Isolated from a brown rat *(Rattus norvegicus)* (CIP 105476^T)	[16]
	RSE149	Spontaneous Sm^r strain of IBS 506T, serving as wild-type	[16]
	RSE173	*ΔvirB4* mutant of RSE149	[16]
***E. coli* strains**	β2150	*F′ lacZDM15 lacI^q traD36 proA+B+ thrB1004 pro thi strA hsdS lacZΔM15* Δ*dapA*::erm (Erm^R) pir	[16]
	NovaBlue	*endA1 hsdR17*(r K12–m K12+) *supE44 thi-1 recA1 gyrA96 relA1 lac*[*F′ proA+B+ lacI^{q}ZΔM15*::Tn*10* (Tc^R)]	Novagen, Madison
Plasmids	pPG100	*E. coli– Bartonella* spp. shuttle vector, encoding a short FLAG epitope	[13]
	pPG101	Derivative of pPG100, encoding FLAG::*Bh* BepA	This work
	pRS40	Cre vector encoding NLS::Cre	[13]
	pRS48	Cre vector containing NLS::Cre::*Bh* BepA (aa 305–544)	This work
	pRS49	Cre vector encoding NLS::Cre::*Bh* BepB (aa 303–542)	[13]
	pRS50	Cre vector encoding NLS::Cre::*Bh* BepC (aa 292–532)	[13]
	pRS51	Cre vector encoding NLS::Cre::*Bh* BepD (aa 352–534)	[13]
	pRS55	Cre vector encoding NLS::Cre::*Bt* BepA (aa 326–568)	This work
	pMS005	Mutagenesis vector for generating a *ΔbepA* in-frame deletion in *Bh*	[13]
	pMS006	Derivative of pPG100, encoding FLAG::*Bh* BepB	This work
	pMS007	Derivative of pPG100, encoding FLAG::*Bh* BepC	This work
	pMS013	Cre vector containing NLS::Cre::*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	[19]
	pMS400	pMS100 containing a 1.23-kb PCR fragment carrying the *cya* of *Bordetella pertussis* (amplified from pMS111-Cornelis)	This work
	pMS401	Cya vector encoding *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

doi:10.1371/journal.ppat.0020115.t001

Table 2. 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	CGGGAATTCCATATGCAAAAGGGAATATTTCACT	NdeI
prMS028	CGGGAATTCCATATGTTAGCTGGCTATAGCGAGTA	NdeI
prMS037	ACGCGTCGACCTCCAAAGGCAAAAGCAAAAACG	SalI
prMS038	TCCCCCCGGGTTAGCTAGCCATGGCAAGC	XmaI
prMS068	TCCCCCCGGGATGCCAAAGGCAAAAGCAAAAA	XmaI
prMS069	CTAGTCTAGATTAGCTAGCCATGGCAAGC	XbaI
prMS074	TCCCCCCGGGAAGCATGTTGCACTCCTCA	XmaI
prMS075	CTAGTCTAGATTATTCGGCTTTTGGAGCTGTAA	XbaI
prMS076	TCCCCCCGGG GAGCTTGAAAACACGCTCAT	XmaI
prMS077	CTAGTCTAGATTAGCTGGCAATAGCAAGCG	XbaI
prMS078	GGAATTCCATATGCAGCAATCGCATCAGGCTG	NdeI
prMS083	TCCCCCCGGGGAATTAAAAAAAACACTCATCCC	XmaI
prMS084	CTAGTCTAGATTAACTTGGCATAGGGACCTCTT	XbaI
prMS090	TCCCCCCGGGTTACGAGGCCTGGCGTTCCACTGCGCCCA	XmaI/StuI
prMS102	ACGCGTCGACCGATGAGCCATATGAAAAATATCAAA	SalI
prMS103	TCCCCCCGGGTTAGCAAACTATTTTAGCTTGC	XmaI
prMS104	AGCTTTGTTTAAACGATGAGCCATATGAAAAATATCAAA	PmeI
prPG92	GGAATTCCATATGCCAAAGGCAAAAGCAAAAA	NdeI
prPG93	GGAATTCCATATGTTAGCTAGCCATGGCAAGC	NdeI
prPG095	GGAATTCCATATGTTAGCTGGCAATAGCAAGCG	NdeI
prPG097	GGAATTCCATATGTTAGTTGGTAAGAGCCCTTG	NdeI
prPG129	GGAATTCCATATGCCAAAAGCAAAAGCAAAAAA	NdeI
prPG130	GGAATTCCATATGTTAGAGCATAATTATCTGTA	NdeI
prRS167	TCCCCCCGGGTTAGCTAGCCATGGCAAGC	XmaI
prRS180	ATGGTGTCGAAAGAATTAAAAAAAACACTCATCC	SalI
prRS187	ATGGTGTCGACAAGAGTTAAAAAATATTCTCATTC	SalI
prRS188	TCCCCCCGGGTTAGCTGGCTATAGCGAGTA	XmaI

[a]Restriction endonuclease cleavage sites are underlined.
doi:10.1371/journal.ppat.0020115.t002

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-kb fragment was shuttled into the SalI/XmaI sites of pMS100. Using oligonucleotide primers 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 amino acids of a given Bep protein fused to the N-terminal FLAG: pMS100-A (shuttled from pRS48, aa 305–544 of *Bh* BepA), pMS100-B (shuttled from pRS49, aa 303–542 of *Bh* BepB), pMS100-C (shuttled from pRS50, aa 292–532), and pMS100-D (shuttled from pRS51, aa 352–534 of *Bh* BepD).

Plasmids for expression of Cya-Bep fusion proteins were constructed as follows: To construct pMS400, the *cya* 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 were constructed as follows: To construct eGFP-Bep fusion proteins (see Figure 4A), pWAY21 (Molecular Motion Lab, http://momotion.cn-s.montana.edu), a CMV-driven EGFP for C-terminal fusion, was used as basic vector. The plasmids resulting from this step and the oligonucleotide primers used (with incorporated XmaI/XbaI sites used for cloning to the corresponding sites of pWAY21) are given below, as well as the range of amino acids of a given Bep protein fused to eGFP: pMS21 (primers prMS68/69, aa 1–544 of *Bh* BepA), pMS22 (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), and 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-Aldrich, http://www.sigmaaldrich.com).

Generation of deletion mutants. pRS63 was used to generate the *ΔvirB4* mutant RSE569 in the RSE356 background as previously described for *Bt* [16]. The spontaneous streptomycin-resistant mutant RSE356 was obtained by selection of *B. quintana* JK-31 on 100 mg/l streptomycin.

Cell lines and cell culture. HUVECs were isolated as described [18]. HUVECs 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. HUVECs (passage 3–7) were plated as described before [12]. Unless stated differently, cells were infected with a multiplicity of infection (MOI) of 300 bacteria per cell [10] in M199/10% FCS/500 μM IPTG and incubated for the indicated time. If specified, 100 nM actinomycin D (Sigma-Aldrich) was added to trigger apoptosis as described before [10,12].

HUVEC transfection. HUVECs were transfected using Amaxa nucleofection technology (Amaxa, http://www.amaxa.com) following the manufacturer's guidelines for HUVEC transfection. After transfection, cells were seeded into 24-well plates or six-well plates.

HEK293T cell transfection. Subconfluent (2.5 million cells) HEK293T cells in 8-cm cell culture dishes were transfected with a total of 2.5 μg DNA following the protocol "Calcium phosphate–mediated transfection of eukaryotic cells with plasmid DNAs" [52]. After 12 h, the cell culture medium was replaced and the cells kept in culture for an additional 24 h before fixation for immunocytochemical staining or harvesting for subcellular localization of the transfected constructs.

Subcellular fractionation. Subcellular fractionation was performed according to standard protocols [53,54]. Briefly, confluent monolayers were washed three times with cold homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 250 mM sucrose [pH 7.4]), and cells were harvested by scraping and homogenized with a syringe and a 22-gauge 1/4 inch needle. The post-nuclear supernatant was obtained by centrifugation at 240*g* (15 min). Membranes were separated from cytosol by ultracentrifugation of the post-nuclear supernatant at 100,000*g* (30 min).

Caspase activity assay. The infection of HUVECs and the determination of caspase-3/-7 activity (MOI = 300) were carried out as described [12].

***cya* assay.** cAMP was assayed after 20 h contact between bacteria (MOI = 300) and HUVECs in 24-well plates. HUVECs were washed one time in pre-warmed PBS and lysed in denaturizing conditions as described previously [19]. cAMP was assayed by an EIA system (Biotrak; Amersham, http://www.amershambiosciences.com). Total cell proteins were assayed by the method of Bradford [55] (Bradford Reagent, Sigma-Aldrich).

CRAfT. The infection of the stably transfected Ea.hy926/pRS56-c#B1 cell line and the quantification of GFP-positive cells (percent positive events) by flow cytometry were carried out as described [13].

Immunoblot analysis. To monitor the steady-state level of Cya fusion proteins, bacteria were grown on IPTG-containing medium for 2 d. Cells were harvested and processed as described previously [13], except that anti-FLAG M2 (Sigma-Aldrich) was used as the primary antibody.

The stability of the different GFP fusion proteins used for ectopic expression in HUVECs was monitored after transient transfection of HEK293T cells. Cells were transfected by FuGENE 6 (Roche, http://www.roche.com) following the manufacturer's instructions. After 24 h expression, cells were washed twice with PBS and harvested in 100 μl of sample buffer. Samples were then further processed as described before [13] and probed with anti-GFP (Roche) antibody.

Annexin V assay. Twenty-four hours after infection or transfection of HUVECs, apoptosis was induced for 12–24 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 PI (1 ug/ml) and Annexin V AlexaFluor488 (Molecular Probes, http://probes.invitrogen.com) or Annexin V APC (Alexis, http://www.axxora.com). The total apoptotic population was determined by analyzing Annexin V–positive and PI-negative/positive cells with a FACSCalibur flow cytometer (BD Biosciences, http://www.bdbiosciences.com). Prior to determining the apoptotic population, transfected cells were gated by their positive GFP signal.

Transfection and immunocytochemistry. HEK293T cells were transfected for 30 h with different GFP-Bep fusion constructs, fixed with 3.7% paraformaldehyde, and immunocytochemically stained with Texas Red–labeled WGA (Invitrogen, http://www.invitrogen.com). Specimens were analyzed by confocal microscopy as described [12].

Determination of IL-8 secretion. The infection (MOI = 300) of HUVECs and the determination of IL-8 secretion by IL-8 DuoSet ELISA kit (R&D Systems, http://www.rndsystems.com) were performed as described before [12].

Determination of intracellular cAMP level. After infection (MOI = 150) of HUVECs 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 manufacturer.

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 [56] using primers listed in Table 2.

Effects of exogenous cAMP and cAMP-elevating agents. HUVECs were infected in the absence or presence of either forskolin (Sigma-Aldrich) and 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/-7 activities were monitored as described above.

CTL assay upon infection. Confluent HLA-A2-positive HUVEC (a gift from B. C. Biedermann, Department of Research, University Hospital Basel, Basel, Switzerland) monolayers were labeled with 5 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes), in M199 without FCS according to the manufacturer's protocol. Cells were then infected with indicated *Bh* strains (MOI = 300) or left uninfected in M199/10% FCS/500 μM IPTG for 24 h. The HLA-A2-restricted CTL clones specific to the Y-chromosome-encoded, male-specific minor histocompatibility peptide antigen SMCY were generated as described previously [32]. CTLs were washed by centrifugation and were added in an effector-to-target cell ratio of 5:1 in replacing the infection medium with CTL assay medium (M199, 2% FCS, 500 μM IPTG). Plates were centrifuged to bring the cells in contact. To quantify the surviving and dead cell rate, we used Annexin V and PI staining and flow cytometry (described above). Prior to determining the surviving and dead cell populations, CFSE-positive cells were gated by their positive GFP signal.

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for orthologous sequences to *Bh* BepA are *Bq* (NC 005955) and *Bt* (CAD37389).

Acknowledgments

We are grateful to M. Quebatte for excellent technical assistance in performing real-time PCR. We also thank Dr. Henri Saenz and Dr. Gunnar Schröder for critical reading of the manuscript. The Bruderholzspital Basel is acknowledged for providing human umbilical cords.

Author contributions. CD conceived and designed the experiments. MCS, FS, MD, and NBD performed the experiments. MCS, FS, MD, and CD analyzed the data. RS, PG, CSC, and BB contributed reagents/materials/analysis tools. MCS and CD wrote the paper.

Funding. This work was supported by grant 3100–06177700 from the Swiss National Science Foundation and grant 55005501 from the International Research Scholar program of the Howard Hughes Medical Institute, as well as a student fellowship from the Misrock Foundation in Basel.

Competing interests. The authors have declared that no competing interests exist.

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Chapter 2

Distinct Activities of Bartonella henselae Type IV Secretion Effector Proteins Modulate Capillary-like Sprout Formation

F. Scheidegger, Y. Ellner, P. Guye, T.A. Rhomberg, H. Weber, H.G. Augustin, C. Dehio

Submitted to Cellular Microbiology

Bartonella-triggered vascular proliferations – termed bacillary angiomatosis and peliosis in case of *B. henselae* (*Bh*)-infection – are reminiscent of tumour angiogenesis, the pathological process of formation of new capillaries out of pre-existing vessels. Those bacteria-induced vasoproliferative lesions are benign and depend on the continuous presence of bacteria, which apparently produce factors that maintain the angiogenic process. In this study we have addressed the angiogenic potential of *Bh* by employing a three-dimensional *in vitro* sprouting angiogenesis assay of collagen-embedded human umbilical vein endothelial cell (HUVEC) spheroids, which allows the quantitative assessment of both pro- as well as anti-angiogenic factors.

Whether non-infected or infected with *Bh* wild-type respectively different *Bh* mutants, HUVECs formed three-dimensional spheroids of similar size under culture condition that allow cellular aggregation. Noteworthy, pre-infection of HUVECs with wild-type bacteria resulted in a slightly less regular and compact morphology of spheroids. Embedded in collagen gel uninfected spheroids showed a low spontaneous sprouting, but capillary-like outgrowths could be readily induced by VEGF. Spheroids from HUVECs pre-infected with *Bh* wild-type showed an increased sprouting activity, albeit sprout morphology was distinct from the sprouts induced by VEGF. Formation of these sprouts was in part dependent on a functional VirB/VirD4 T4SS. Importantly, we observed that three out of the seven known translocated *Bartonella* effector proteins (Beps) had distinct and in part opposing activities in this assay.

BepA – inhibiting apoptosis in HUVECs (chapter 1) – showed a marked pro-angiogenic activity, promoting sprouts similar to those induced by VEGF. Paralleling what was previously described for the anti-apoptotic activity, an N-terminally truncated version of BepA harbouring only the BID-domain and the C-terminal tail

sequence was sufficient to promote sprouting. Likewise, the ectopic expression of a GFP-BepA fusion protein alone was sufficient to trigger sprout formation, indicating that the same protein domains and effector mechanisms could be involved in anti-apoptosis and sprout-induction. BepD – phosphorylated on specific tyrosines upon translocation into endothelial cells (ECs) – had a moderate stimulating effect. Those phosphorylated tyrosines may serve as specific docking sites for host cell signalling proteins and the resulting signalling complex could modulate angiogenesis. In contrast, BepG – involved in cytoskeletal rearrangement – showed a potent interference with sprout formation. BepG might block sprouting angiogenesis by altering the actin cytoskeleton and therefore the protrusive force needed for outgrowth and EC migration.

Taken together, we provide in this study a novel *in vitro* model of *Bh*-triggered sprout formation and an initial characterization of the distinct activities of VirB/VirD4-translocated Bep effectors modulating sprouting angiogenesis and contributing to the regulation of the *Bh* angiogenic activity in the course of chronic infection of the human vasculature.

Statement of the own participation

The data reported in this manuscript were generated by me. I am grateful to Y. Ellner and C. Mistl for technical help and to O. Siedentopf and Dr. H. Weber for introducing me to the spheroid model and supply with protocols and recombinant VEGF. Some of the constructs and strains used were generated by Drs. P. Guye and T. Rhomberg.

Distinct Activities of *Bartonella henselae* Type IV Secretion Effector Proteins Modulate Capillary-like Sprout Formation

F. Scheidegger[1], Y. Ellner[1], P. Guye[1], T.A. Rhomberg[1], H. Weber[2,3], H.G. Augustin[2,4,5], C. Dehio[1,*]

[1]Biozentrum, University of Basel, CH-4056 Basel, Switzerland; [2]Tumor Biology Center, University of Freiburg, D-79106 Freiburg, Germany; [3]Present address: ProQinase GmbH, D-79108 Freiburg, Germany; [4]Joint Research Division Vascular Biology of the Medical Faculty Mannheim (CBTM), University of Heidelberg, D-68167 Mannheim, Germany, and [5]German Cancer Research Center (DKFZ), D-69120 Heidelberg, Germany.

*For correspondence:

Prof. Christoph Dehio, Ph.D.

Biozentrum, University of Basel

Klingelbergstrasse 70

CH-4056 Basel, Switzerland

E-Mail: christoph.dehio@unibas.ch

Tel. (+41) 61 267 2140

Fax (+41) 61 267 2118

Running title: *Bartonella* modulates sprouting of endothelial spheroid

Abstract

The zoonotic pathogen *Bartonella henselae* (*Bh*) can lead to vasoproliferative tumour lesions in the skin and inner organs known as bacillary angiomatosis and bacillary peliosis. The knowledge on the molecular and cellular mechanisms involved in this pathogen-triggered angiogenic process is confined by the lack of a suitable animal model and a physiologically relevant cell culture model of angiogenesis. Here we employed a three-dimensional *in vitro* angiogenesis assay of collagen gel-embedded endothelial cell (EC) spheroids to study the angiogenic properties of *Bh*. Spheroids generated from *Bh*-infected ECs displayed a high capacity to form sprouts, which represent capillary-like projections into the collagen gel. The VirB/VirD4 type IV secretion system (T4SS) and a subset of its translocated *Bartonella* effector proteins (Beps) were found to profoundly modulate this *Bh*-induced sprouting activity. BepA, known to protect ECs from apoptosis, strongly promoted sprout formation. In contrast, BepG, triggering cytoskeletal rearrangements, potently inhibited sprouting. Hence, the here established *in vitro* model of *Bartonella*-induced angiogenesis revealed distinct and opposing activities of T4SS effector proteins, which together with a VirB/VirD4-independent effect may control the angiogenic activity of *Bh* during chronic infection of the vasculature.

Key words: type IV secretion, effector protein, *Bartonella henselae*, endothelial cell spheroid, pathological angiogenesis

Introduction

Unique within the bacterial kingdom, three members of the gram-negative α-proteobacterial genus *Bartonella* have the capacity to cause vasoproliferative tumour lesions in humans. The human-specific pathogen *Bartonella bacilliformis* (*Bb*) causes vasoproliferative skin lesions known as verruga peruana (VP). Especially in immuno-compromised patients, infections with the human-specific pathogen *Bartonella quintana* (*Bq*) and the feline-specific zoonotic pathogen *Bartonella henselae* (*Bh*) lead to vasoproliferative lesions in the skin that are termed bacillary angiomatosis (BA). *Bh* also causes similar lesions in inner organs, which are known as bacillary peliosis (BP) (Dehio, 2005; Dehio, 2003). BA manifests as reddish-brown papules on the skin - an accumulation of immature blood vessels with misshapen endothelial cells (ECs), a mixed inflammatory infiltrate, and numerous bacteria associated with proliferating ECs (Chian *et al.*, 2002). BP is characterized by vascular proliferation in liver and spleen, which results in the formation of blood-filled cysts. Similar to BA, bacteria are found in association with proliferating ECs (Tappero *et al.*, 1993).

These bacteria-triggered vascular proliferations are reminiscent of tumour angiogenesis, the pathological process of the formation of new capillaries out of pre-existing vessels. Angiogenesis is a highly orchestrated multistep process involving EC activation, the degradation of extracellular matrix (ECM) and basement membranes, EC proliferation, EC migration and invasion into the surrounding matrix, and finally the formation of tubular structures to build immature vessels later stabilized by tight interactions with smooth muscle cells and pericytes (Carmeliet, 2003). Other than normal vessels, the vessels triggered by tumour angiogenesis are not properly matured and will regress upon deprival of the pro-angiogenic signals released by the tumour (e.g. overproduction of vascular endothelial growth factor,

VEGF). Likewise, *Bartonella*-triggered vessels do not mature properly, and eradication of the bacteria colonizing these newly formed vessels by antibiotic treatment leads to vessel regression (Koehler and Tappero, 1993). Thus, the *Bartonella*-induced vascular proliferations are benign and depend on the continuous presence of bacteria (Dehio, 2004), which apparently produce angiogenic factors that maintain the angiogenic process.

Most of the current knowledge on *Bartonella*-EC interaction is derived from two-dimensional cell culture infection assays with *Bh.* The VirB/VirD4 type IV secretion system (T4SS) and the thereby translocated *Bartonella* effector proteins (Beps) of *Bh* mediate most of the cellular phenotypes associated with infection of human umbilical vein endothelial cells (HUVECs), such as (i) cytoskeletal actin rearrangements leading to the internalization of a large bacterial aggregate by a unique structure called the invasome, (ii) NFκB-dependent pro-inflammatory activation, and (iii) inhibition of apoptosis (Schmid *et al.*, 2004). The capacity of *Bh* to prevent EC apoptosis is dependent on the effector BepA and could contribute indirectly to vasoproliferative growth by enhancing cell survival (Schmid *et al.*, 2006; Kirby and Nekorchuk, 2002). Another prominent phenotype of *Bh* infection of ECs is a direct mitogenic stimulation, which is VirB/VirD4/Bep-independent and is even counterbalanced by the VirB/VirD4 system at a high multiplicity of infection (MOI) (Schmid *et al.*, 2004). The activation of a pro-inflammatory response in ECs, which occurs both in a VirB/VirD4-dependent (Schmid et al., 2004) and -independent manner (Fuhrmann *et al.*, 2001), may lead to the recruitment of monocytes, which upon activation by *Bh*-infection release pro-angiogenic factors, such as VEGF, that promote EC proliferation in a paracrine manner (Resto-Ruiz *et al.*, 2002; Kempf *et al.*, 2001). The non-fimbrial adhesin BadA (*Bartonella* adhesin A) is considered to trigger the secretion of VEGF by activating hypoxia-inducible factor (HIF)-1 (Riess *et al.*,

2004). In summary, the current model for *Bartonella*-triggered vascular tumour formation proposes for *Bh*-infected ECs a direct stimulation of proliferation, inhibition of apoptosis, and the activation of a paracrine loop of pro-angiogenic factors such as VEGF released from infected monocytic cells (Dehio, 2005).

A number of rodent animal models of *Bartonella*-infection have been used to study different aspects of the *Bartonella* infection cycle and the general immune response to infection (Resto-Ruiz *et al.*, 2003; Schulein and Dehio, 2002; Arvand *et al.*, 2001; Koesling *et al.*, 2001; Schulein *et al.*, 2001). With the exception of one report (Velho *et al.*, 2002) no publication indicated the appearance of vasoproliferative lesions in *Bartonella*-infected animals. Due to the absence of a suitable animal model, appropriate *in vitro* models of angiogenesis may represent an alternative to study the molecular basis of *Bartonella*-triggered vasoproliferation. Three-dimensional cell culture model offer an option to the complexity of live tissue experiments, providing a defined setup where physiological cell-cell and cell-ECM interactions are better mimicked than in conventional two-dimensional cultures (Pampaloni *et al.*, 2007). *Bh* was shown to be able to promote survival of EC cords in type I collagen matrix as well as matrix invasion, survival and tubular differentiation of single embedded cells (Kirby, 2004). The *in vitro* cord formation assay measures the ability of cells to form a web-like network of interconnected cells (cord) and is believed to model a late morphogenic step of new vessel formation (Goodwin, 2007), whereas formation of capillary networks originating from single gel-embedded cells might rather represent a model to study the process of vasculogenesis – the *de novo* formation of a vessel (Davis *et al.*, 2002). Neither of these models does really reflect sprouting angiogenesis originating from the confluent endothelium of the pre-existing vessel. Systems aiming to mimic a vessel *in vitro* are based on focal delivery of aggregates of EC from which sprouting can occur (Korff and Augustin, 1999). Single

non-adherent EC will undergo apoptosis and will not respond to survival factors. In contrast, spheroidal aggregation stabilizes the ECs and renders them responsive. Within 24 h of spheroid formation, the cells of the spheroidal aggregate spontaneously establish a surface monolayer of elongated cells and a core of unorganized cells. Embedded in matrices such as type I collagen and stimulated with growth factors, e.g. VEGF, those spheroids display radial sprouting. Cross sections of collagen gels showed numerous capillary-like structures forming a true lumen. Beyond a critical spheroid density, capillary sprouts from neighbouring spheroids will even form a complex anatomizing capillary-like network (Korff and Augustin, 1999).

Here we adapted this three-dimensional *in vitro* angiogenesis assay of collagen gel-embedded HUVEC spheroids to study the angiogenic properties of *Bh* involved in pathogen-triggered vasoproliferation. *Bh* wild-type was found to activate HUVEC spheroids and to trigger radial outgrowths. Different VirB/VirD4 T4SS effectors of *Bh* individually expressed *in trans* in the effector-less mutant Δ*bepA-G* had distinct and opposing effects on spheroid sprouting, suggesting that these effectors play a modulatory role contributing to the regulation of the *Bh* angiogenic activity in the course of human infection.

Results

The *Bartonella henselae* VirB/VirD4/Bep system modulates *in vitro* angiogenesis of collagen gel-embedded HUVEC spheroids

To establish an *in vitro* assay for studying the angiogenic activity of *Bh* we adapted the in-gel based three-dimensional *in vitro* angiogenesis model described by Korff and Augustin (1998). A defined number of HUVECs (400 cells) cultured as a hanging drop in methocel-containing EGM medium for 24 h were found to form a spheroid of similar size, regardless whether cells were not infected or pre-infected for 24 h with a multiplicity of infection (MOI) of 300 bacteria per cell. Noteworthy, compared to the typical regular spheroid morphology formed by HUVEC which were uninfected or pre-infected by the isogenic deletion mutants Δ*virB4* (deficient for a functional T4SS (Schmid et al., 2004)) or Δ*bepA-G* (lacking the entire set of translocated effector proteins (Schulein et al., 2005)), HUVECs pre-infected with wild-type bacteria (a spontaneous streptomycin-resistant variant of the well-characterized clinical isolate and typing strain ATCC49882^T (Schmid *et al.*, 2004)) resulted in a slightly less regular and compact morphology. This is illustrated in Fig. 1A by bright field imaging or maximum intensity z-projections of confocal microscopy stacks of spheroids stained with the EC marker CD31.

To study the ability of *Bh* to manipulate sprouting, these spheroids were embedded in collagen gels and the outgrowth of sprouts representing capillary-like structures was evaluated after 24 h. Quantification of *in vitro* angiogenesis was assessed by measuring the cumulative sprout length (CSL) of individual spheroids (Fig 1B, determined as the mean +/- SD of 10 spheroids per experimental group). Spheroids of uninfected HUVECs embedded in collagen-gel showed a very low level of spontaneous sprouting (Fig. 1B, C), but were readily responsive to exogenous addition of VEGF (25 ng/ml) resulting in an approximately five-fold increased CSL

(Fig. 1B, G). Compared to uninfected control spheroids, we observed a higher sprouting activity of spheroids formed from HUVECs pre-infected with *Bh* wild-type (Fig. 1B, D), displaying an increase in CSL comparable to the stimulation by VEGF. Compared to wild-type, the sprouting activity was reduced about two-fold in spheroids of HUVECs pre-infected with the isogenic deletion mutants ∆*virB4* (Fig. 1B, E) or ∆*bepA-G* (Fig. 1B, F). Hence, *Bh* wild-type showed a clear VirB/VirD4- and Bep-dependent angiogenic activity on top of a basal level of VirB/VirD4/Bep-independent sprout formation. Similar results were obtained when bacteria were added at the stage of spheroid-formation (data not shown). In contrast, the addition of bacteria onto the collagen gel immediately after embedding of spheroids had no stimulatory effect on sprouting, probably as the non-motile *Bh* can not sufficiently interact with the spheroids embedded in the collagen matrix (data not shown). Strikingly, spheroids of wild-type infected HUVECs displayed similar, but distinct sprout morphology in comparison to those induced by VEGF (Fig. 2). *Bh* wild-type bacteria clearly stimulated invasion of ECs into the gel, but the thickness of the capillary sprouts seemed reduced and appeared less regular. Moreover, less cell nuclei migrated into the collagen gel and some of the outgrowths rather had the appearance of cell protrusions than migratory cells.

Pro- and anti-angiogenic activities of *Bartonella* effector proteins (Beps)

To investigate the role of translocated Bep effectors in VirB/VirD4-mediated angiogenesis, we performed assays using isogenic strains of the effector-less mutant ∆*bepA-G* expressing individual *bep* genes (*bepA* to *bepG*) on a plasmid *in trans*. Spheroids were generated and embedded into collagen gels. 24 h later sprouting was quantified by measuring the CSL (Fig. 3A). Expression of BepA, which is involved in protection of HUVECs from apoptosis (Schmid *et al.*, 2006), showed a

strong sprout-promoting activity (Fig. 3A, F), with a three-fold increase in CSL compared to the Δ*bepA-G* mutant and a two-fold increase compared to wild-type. We also observed a moderate increase of sprouting for the Δ*bepA-G* mutant expressing *bepD in trans*, with a 1.5-fold increased CSL in comparison to the Δ*bepA-G* mutant (Fig. 3A, I). BepD is known to become tyrosine-phosphorylated upon translocation into host cells (Schulein *et al.*, 2005) and possibly interferes with host cell signalling processes (Backert and Selbach, 2005). The Δ*bepA-G* mutant expressing *bepG in trans* displayed a marked reduction in CSL of about six-fold in comparison to the Δ*bepA-G* mutant and almost nine-fold compared to wild-type (Fig. 3A, L). BepG is involved in actin rearrangements leading to invasome-formation (Rhomberg *et al.*, 2008). No statistically significant differences in CSL were observed for the Δ*bepA-G* mutant strains expressing *in trans* either *bepB*, *bepC*, *bepE*, or *bepF* (Fig.3A, G, H, J, K).

Confirmative evidence for the data obtained with Δ*bepA-G* mutant strains expressing individual Bep effectors *in trans* was obtained by analysing a set of isogenic mutants carrying in-frame deletions of genes encoding individual Bep effectors (Fig. 4; ΔbepA, Δ*bepC*, Δ*bepD*, Δ*bepE*, Δ*bepF*, and Δ*bepG*). Compared to wild-type, the Δ*bepA* mutant showed a three-fold reduced CSL (Fig. 4A, F). We measured a slight decrease of 1.3-fold in CSL for the Δ*bepD* mutant (Fig.4A, H), whereas the Δ*bepG* stimulated a statistically significant 1.3-fold increase of the CSL in comparison to wild-type (Fig. 4A, J). Loss of *bepG* though did not completely unlock CSL to levels triggered by BepA, possibly indicating that additional factors might be involved in inhibition of sprouting. Deletion of *bepC* (Fig. 4A, G), *bepE* (data not shown), or *bepF* (Fig. 4A, I) had no significant effect on CSL compared to wild-type. In conclusion, BepA and BepD showed distinct pro- and BepG a marked

anti-angiogenic activity in the established three-dimensional *in vitro* angiogenesis assay.

Most of the single gene deletion mutants (i.e. Δ*bepA*, Δ*bepD*, Δ*bepE*, and Δ*bepF*, bright field images Fig, 4F, H and I) also stimulated the altered sprout morphology as illustrated for wild-type (Fig. 2). In contrast, the sprouts stimulated by Δ*bepC* and Δ*bepG* deletion mutants had the typical sprout appearance as previously described for VEGF stimulation of uninfected spheroids (bright field images Fig. 4G and J). Sprouts induced by the expression of individual Bep effectors in the Δ*bepA-G* mutant background showed as well a morphology comparable to those promoted by VEGF (Fig.2, illustrated for BepA, bright field images Fig. 3F-L). The slightly altered sprout morphology triggered by wild-type bacteria thus appears to depend on the translocation of multiple Bep effectors, including at least BepC and BepG.

The C-terminal part of BepA is sufficient to promote sprouting

BepA is sufficient to mediate protection of ECs against apoptosis. Moreover, deletion of its N-terminal Fic domain (a domain of unknown molecular function) demonstrated that the BID domain and the proximal positively charged C-terminal tail, which together constitute a functional signal for effector translocation (Schulein *et al.*, 2005), are enough for the full anti-apoptotic activity. In accordance with this finding also the *Bq* ortholog BepA2, which due to an internal stop codon lacks a FIC domain, is inhibiting EC apoptosis (Schmid *et al.*, 2006). To elucidate whether the prominent sprout-promoting activity of BepA is also independent of the Fic domain we compared the ability of full-length BepA, a truncated version harbouring only the BID domain plus positively charged C-terminal tail (tBepA encoded by plasmid pt*bepA*), and the *Bq* ortholog BepA2 (encoded by plasmid *Bq* p*bepA2*) to promote sprouting of spheroids. BepC and a truncated version (tBepC encoded by plasmid

pt*bepC*) were used as negative controls. Spheroids formed form HUVECs infected with the ∆*bepA-G* mutant complemented *in trans* with the indicated plasmids were embedded in collagen and sprout formation was quantified after 24 h (Fig. 5A). Similar to full-length BepA, the truncated version of BepA, as well as the *Bq* ortholog BepA2 resulted in an increased CSL, whereas infection with ∆*bepA-G* mutant strains expressing either full-length BepC or truncated BepC *in trans* had no effect on sprouting.

Ectopic expression of full-length BepA fused to GFP was previously shown to be sufficient to protect ECs from apoptosis (Schmid *et al.*, 2006). To test whether BepA alone is also enough to promote sprouting in the established three-dimensional angiogenesis assay in the absence of any additional bacterial factor, we transfected HUVECs prior to spheroid formation for 24 h with plasmids encoding GFP (*gfp*) or a GFP-BepA fusion (*gfp-bepA*), or left cells untransfected for this period. Spheroids formed by those cell populations were embedded in collagen gels and *in vitro* angiogenesis was quantified 24 h later by measuring the CSL (Fig. 5B). Ectopic expression of the GFP-BepA fusion (Fig. 5B, F) lead to a statistically significant increase of CSL of about two-fold compared to spheroids formed from either GFP-expressing or untransfected HUVECs (Fig. 5B, C, E). Conclusively, paralleling what was previously described for the anti-apoptotic activity (Schmid et al., 2006) the BID-domain plus C-terminal tail of BepA was sufficient to promote sprouting.

BepG interferes with sprout-formation from spheroids

Invasome-mediated entry of *Bh* into ECs (Dehio et al., 1997) depends on the VirB/VirD4 T4SS (Schmid et al., 2004) and thereby translocated effector(s) (Schulein et al., 2005). A ∆*bepA-G* mutant expressing BepG was recently found to promote the actin cytoskeletal rearrangements characteristic for the invasome structure, whereas

no other single Bep elicits a similar actin remodelling (Rhomberg et al., 2008). To test the impact of this F-actin modulating effector on spheroid sprouting, we infected HUVECs with BepG-expressing strains of either the Δ*bepA-G* mutant background (not expressing BepA) or the Δ*bepB-G* mutant background (expressing BepA). As negative control we used the Δ*bepA-G* and Δ*bepB-G* mutant backgrounds expressing BepC, which alone does not interfere with spheroid sprouting. Spheroids formed from such infected ECs were embedded in collagen and *in vitro* angiogenesis was quantified after 24 h (Fig. 6A). Overexpression of BepG in the Δ*bepA-G* mutant lead to a marked reduction of CSL in comparison to the Δ*bepA-G* mutant (Fig. 6A, G), while BepC did not alter the basal VirB/VirD4-independent sprouting activity (Fig. 6A, F). Similarly, when BepG was over-expressed in the Δ*bepB-G* background, this effector reduced the BepA-induced increase in CSL more than three-fold (Fig. 6A, J), whereas BepC had no influence (Fig. 6A, I). Thus, BepG antagonizes the BepA-triggered sprouting activity, but also generally interfered with VirB/VirD4-independent sprouting in the in-gel based three-dimensional *in vitro* angiogenesis model.

Discussion

BA, BP, and VP lesions illustrate the remarkable capacity of the three *Bartonella* species *Bh*, *Bq*, and *Bb*, to trigger vasoproliferative processes resembling tumour angiogenesis. The molecular and cellular bases of these pathogen-triggered vascular proliferations, as well as their significance for establishing chronic infection of the vasculature are poorly understood. The underlying process of sprouting angiogenesis – the growth of new capillary vessels out of pre-existing ones – involves a sequence of highly orchestrated morphogenic events. In the healthy adult homeostatic mechanisms typically keep numerous positive and negative regulators of angiogenesis in balance in order to maintain a quiescence state of the vasculature. However, changes in the physiological state (e.g. in the microenvironment of tumours), that either result in an up-regulation of pro-angiogenic factors (such as VEGF) or a down-regulation of anti-angiogenic factors (such as angiostatin) may trigger angiogenesis (Carmeliet, 2003).

In this study we have addressed the angiogenic potential of *Bh* by employing a three-dimensional *in vitro* sprouting angiogenesis assay of collagen-embedded HUVEC spheroids, which allows the quantitative assessment of both pro- as well as anti-angiogenic factors (Korff and Augustin, 1999). We could show that *Bh* triggers an angiogenic response, which is manifested by a cumulative sprout length in a similar range as induced by the potent angiogenic factor VEGF. Formation of these sprouts was in part dependent on a functional VirB/VirD4 T4SS. Importantly, we observed that three out of the seven known VirB/VirD4-translocated Bep effectors had distinct and in part opposing activities in this assay. BepA showed a marked and BepD a rather moderate pro-angiogenic effect, while BepG displayed a prominent anti-angiogenic activity.

BepA is known to inhibit apoptosis in HUVECs via a rise in the cytosolic concentration of the second messenger cyclic AMP (cAMP) (Schmid et al., 2006). As reported for the cAMP-dependent anti-apoptotic activity, an N-terminally truncated version of BepA harbouring only the BID-domain and the C-terminal tail sequence was sufficient to promote sprouting. Likewise, the ectopic expression of a GFP-BepA fusion protein alone was sufficient to trigger sprout formation, indicating that the same protein domains and effector mechanisms could be involved in anti-apoptosis and sprout-induction. The potent angiogenic response triggered by VEGF is known to depend on its distinct activities on ECs – not only as a mitogen but also as a survival factor that inhibits apoptosis (Ferrara *et al.*, 2003). The strong angiogenic activity of BepA demonstrated in the employed three-dimension *in vitro* angiogenesis assay could therefore depend to a large extent on its cAMP-mediated anti-apoptotic activity, boosting the VirB/VirD4-independent mitogenic activation of ECs. Remarkably, overexpression of *Bh* BepA or the BepA2 homologue of *Bq* in an otherwise Bep-deficient genetic background of *Bh* was found to display a pro-angiogenic activity even superior to that of VEGF. Likewise, *Bartonella* exceeded the effect of VEGF in the *in vitro* cord formation and 3-D collagen assay (Kirby, 2004).

BepD displayed a significant, although much weaker pro-angiogenic activity than BepA. Upon translocation in ECs, this effector becomes phosphorylated on specific tyrosine residues (Schulein et al., 2005) that may serve as specific docking sites for host cell signalling proteins bearing phosphotyrosine binding (PTB) or Src-homology 2 (SH2) domains (Backert and Selbach, 2005). This signalling complex could then modulate angiogenesis. Interestingly, the effectors BepE and BepF that bear similar tyrosine-phosphorylation motifs than BepD did not significantly affect sprout formation, which indicates the specificity of the pro-angiogenic signaling events triggered by BepD.

BepG was the only effector that demonstrated a marked anti-angiogenic activity. BepG was previously shown to be able to promote actin rearrangements required for invasome-mediated invasion, a specific mode of *Bh* entry into ECs (Dehio *et al.*, 1997). This F-actin dependent BepG-triggered sequence of events appears to inhibit the uptake of individual bacteria via conventional endocytosis (Rhomberg et al., 2008). Notably, invasome formation is associated with a reduction in EC motility (Dehio et al., 1997), which may represent a link of the BepG-dependent processes of invasome-medicated invasion and the inhibition of *in vitro* angiogenesis as reported here. It is unknown how BepG interferes with endocytosis and migration in ECs, however, these activities may relate to the co-localization of BepG with F-actin (Rhomberg et al., 2008). BepG might thus block sprouting angiogenesis by altering the actin cytoskeleton and therefore the protrusive force needed for outgrowth and EC migration.

Further to the pro- and anti-angiogenic activities of individual Bep effectors, we observed that the interaction of several effectors can result in modified sprout morphology. The altered sprouting phenotype seemed to require multiple Bep proteins, including BepG, which by itself inhibits sprout formation, and BepC, which by itself does not affect sprout formation, and probably in addition at least one sprout-inducing effector, such as BepA. The atypical representation of capillaries observed in BA lesions (Chian *et al.*, 2002) may represent the clinical correlate of the altered sprouts induced by *Bh* wild-type in the employed *in vitro* angiogenesis assay.

It is unclear whether *Bartonella*-induced vasoproliferation represents a cause or rather a consequence. Bacterial factors may directly target the angiogenic switch in order to trigger proliferation as part of a dedicated infection strategy that expands the specific host cell habitat (Kempf et al., 2002). However, vasoproliferation may as well represent a “biological accident” induced by the complex physiological changes

resulting from chronic vascular colonization. It is likely that a yet unidentified immunological parameter on the host side influences the different clinical manifestations of *Bartonella*-infection, as BA and BP lesions triggered by *Bh* or *Bq* infections are almost exclusively observed in patients with a severe immuno-suppression, although chronic infections by these pathogens also occur in immuno-competent individuals. *Bb* on the contrary regularly causes VP lesions in immuno-competent humans (Chian *et al.*, 2002). Based on these clinical observations it is tempting to speculate that all three vasoproliferative *Bartonella* species encode vascular colonization factors that, either directly or indirectly, contribute to a potent pro-angiogenic response, while *Bh* and *Bq* may in addition encode anti-angiogenic factors to compensate this pro-angiogenic activity, at least in individuals with an intact immune response. The Bep effector proteins translocated by the VirB/VirD4 T4SS of *Bh* and *Bq* represent strong candidates for such modulators of the angiogenic response. First, the VirB/VirD4/Bep system was horizontally acquired and evolved as host adaptation system in the “modern” *Bartonella* lineage comprising *Bh* and *Bq*, while it is absent from the ancestral lineage solely represented by *Bb* (Saenz et al., 2007). Second, the VirB/VirD4 system and its translocated effector can suppress a VirB/VirD4-independent strong proliferative response of ECs (Schmid et al., 2006, Schulein et al., 2005). Third, the here observed prominent but adverse effects of BepA and BepG on spheroid-sprouting point towards a modulatory role of these effectors on angiogenesis.

Taken together, we provide in this study a novel model and an initial characterization of the distinct activities of VirB/VirD4-translocated Bep effectors of *Bh* in modulating sprouting angiogenesis. The adapted collagen gel-embedded HUVEC spheroid model represents a validated quantitative *in vitro* assay of sprouting angiogenesis that in further studies will allow elucidating the molecular basis of the

described pro- and anti-angiogenic activities of BepA, BepD and BepG. Noteworthy, a recent report demonstrated that HUVEC spheroids as used here for *in vitro* sprouting angiogenesis can also be used *in vivo* (Alajati *et al.*, 2008). To this end, HUVEC spheroids embedded in a mixture of matrigel and fibrin are subcutaneously injected into severe combined immunodeficiency (SCID) mice. In the presence of pro-angiogenic factors (i.e. a mixture of VEGF and FGF-2) the grafted HUVEC spheroids were shown to build a complex three-dimensional capillary network that even connected to the mouse vasculature and displayed blood flow. Using this novel murine xenotransplantation model with HUVEC spheroids pre-infected with *Bh* as described here may allow in future studies to establish the badly needed animal model to study *Bartonella*-triggered angiogenesis *in vivo*.

Experimental procedure

Reagents

Recombinant human vascular endothelial growth factor-A165 (VEGF) was produced in Sf9 insect cells (Tumor Biology Centre, University of Freiburg). Antibiotics and Carboxymethylcellulose (methocel, 4000 centipoises) were from Sigma (http://www.sigmaaldrich.com). Collagen type I was isolated from rat tail tendons (Augustin, 2004). The monoclonal mouse anti-human CD31 antibody (clone JC70A) was purchased from DAKO (http://www.dako.com). Monoclonal mouse anti-human VE-cadherin (clone 55-7H1) was obtained from BD Pharmingen (http://www.bdbiosciences.com). Secondary AlexaFluor 488-conjugated goat anti-mouse antibody was from Molecular Probes (http://www.invitrogen.com). TRITC-phalloidin from Sigma (http://www.sigmaaldrich.com) was used to stain F-actin and DAPI from Roche (https://www.roche-applied-science.com) to stain double-stranded DNA.

Bacterial strains and Growth Conditions

The bacterial strains used are listed in Table 1. *Bartonella* spp. were grown on Columbia agar (CBA) plates containing 5% defibrinated sheep blood in a humified atmosphere at 35°C and 5% CO_2 for 2-3 days. Strain RSE247, a spontaneous streptomycin-resistant strain of ATCC 49882^T (Schmid *et al.*, 2004) served as wild-type in this study. 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, http://www.applichem.de) when indicated.

Construction of In-frame Deletions

In-frame deletion mutants of *B. henselae* RSE247 were generated by a two-step gene replacement procedure as described (Schulein & Dehio, 2002, Schmid et al., 2004). The basic mutagenesis vector pTR1000 was described before (Schmid *et al.*, 2004). All mutagenesis plasmids harbour a cassette with the flanking regions of the in-frame deletion in the gene(s) of interest. This cassette was generated from two PCR fragments amplified from chromosomal DNA of RSE247 as template, either by megaprime PCR or by conventional cloning.

Construction of the in-frame mutant Δ*bepA* has been described previously (Schmid *et al.*, 2004).

pTR1069 used for generating a Δ*bepC* in-frame mutant was constructed as follows. Oligonucleotide primers prTR082 and prTR083 amplified fragment 1 (846 bp*)* and prTR084 and prTR085 amplified fragment 2 (753 bp, containing 256 bp of 3` end of *bepD*). Both fragments were combined by megaprime PCR with oligonucleotide primers prTR082 and prTR085, resulting in a fragment of 1.59 kb carrying an in-frame deletion of 1.58 kb in *bepC*. By using flanking *BamHI* sites, the fragment was inserted into the corresponding site of pTR1000, yielding pTR1069. The use of pTR1069 for gene replacement in RSE247 resulted in the Δ*bepC* mutant TRB288.

pPG161 used for generating a Δ*bepD* in-frame mutant was constructed as follows. Oligonucleotide primers prTR041 and prTR051 amplified fragment 1 (853 bp, containing 321 bp of 5` end of *bepC)* and prTR042 and prTR044 amplified fragment 2 (860 bp, containing 473 bp of 3` end of *bepE*). Both fragments were combined by megaprime PCR with oligonucleotide primers prTR041 and prTR044, resulting in a fragment of 1.67 kb carrying an in-frame deletion of 1.54 kb in *bepD*. By using flanking *BamHI* sites, the fragment was inserted into the corresponding site of

pTR1000, yielding pPG161. The use of pPG161 for gene replacement in RSE247 resulted in the Δ*bepD* mutant PGC08.

pPG162 used for generating a Δ*bepE* in-frame mutant was constructed as follows. Oligonucleotide primers prPG134 and prPG135 amplified fragment 1 (898 bp, containing 497 bp of 5` end of *bepD)* and prPG136 and pr137 amplified fragment 2 (906 bp, containing 522 bp of 3` end of *bepF*). Both fragments were combined by megaprime PCR with oligonucleotide primers prPG134 and prPG137, resulting in a fragment of 1.75 kb carrying an in-frame deletion of 1.33 kb in *bepE*. By using flanking *BamHI* sites, the fragment was inserted into the corresponding site of pTR1000, yielding pPG162. The use of pPG162 for gene replacement in RSE247 resulted in the Δ*bepE* mutant PGD20.

pTR1075 used for generating a Δ*bepF* in-frame mutant was constructed as follows. Oligonucleotide primers prTR053 and prTR054 amplified fragment 1 (736 bp, containing 356 bp of 5` end of *bepE)* and prTR055 and prTR056 amplified fragment 2 (665 bp, containing 345 bp of 3` end of *bepG*). Both fragments were combined by megaprime PCR with oligonucleotide primers prTR053 and prTR056, resulting in a fragment of 1.38 kb carrying an in-frame deletion of 2.45 kb in b*epF*. By using flanking *Xba*I sites, the fragment was inserted into the corresponding site of pTR1000, yielding pTR1075. The use of pTR1075 for gene replacement in RSE247 resulted in the Δ*bepF* mutant TRB222.

pTR1078 used for generating a Δ*bepG* in-frame mutant was constructed as follows. Oligonucleotide primers prTR057 and prTR058 amplified fragment 1 (713 kb, containing 339 bp of 5` end of *bepF)* and prTR059 and prTR060 amplified fragment 2 (625 kb). Both fragments were combined by megaprime PCR with oligonucleotide primers prTR057 and prTR060, resulting in a fragment of 1.26 kb carrying an in-frame deletion of 2.93 kb in *bepG*. By using flanking *Xba*I sites, the fragment was

inserted into the corresponding site of pTR1000, yielding pTR1078. The use of pTR1078 for gene replacement in RSE247 resulted in the Δ*bepG* mutant TRB223.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated as described before (Dehio *et al.*, 1997) and cultured in EGM medium (http://www.promocell.com) in a humified atmosphere at 37°C and 5% CO_2.

Infection Assay

HUVEC (passage 4-7) were plated in gelatine-coated well plates at $6*10^4$/ml using EGM. The next day cells were washed twice with M199 with Earls salts (M199, Gibco, http://www.invitrogen.com) supplemented with 10% fetal calf serum (FCS, http://www.invitrogen.com) and unless stated differently infected with a multiplicity of infection (MOI) of 300 bacteria per cell in M199/10% FCS/500 µM IPTG and incubated for 24 hours.

HUVEC Transfection

HUVECs were transfected using Amaxa nucleofection technology (Amaxa, http://www.amaxa.com) following the manufacturer's guidelines for HUVEC transfection. After transfection, cells were seeded into gelatine-coated six-well plates.

Generation of endothelial cell (EC) spheroids

EC spheroids of defined cell number were generated as described previously (Korff and Augustin, 1998) with some modifications. In brief, uninfected respectively infected EC monolayers were trypsinized and suspended in EGM containing 20% methocel (Weber *et al.*, 2008). Spheroids were generated by pipetting 400 EC (in 25

µl) on quadratic Greiner Petri dishes (http://www.huberlaborworld.ch) and grown overnight as hanging drops in a humified atmosphere at 35°C and 5% CO_2. Under these conditions, all suspended cells contribute to the formation of a single spheroid of defined cell number per drop.

Spheroid whole-mount staining

Staining of spheroids was performed in 2 ml Eppendorf tubes as done previously (Scharpfenecker *et al.*, 2005). Spheroids were harvested and fixed for 1 hour in PFA-fixans (0.02mM $CaCl_2$, 1.36 % w/v saccharose, 4% PFA in PBS). The fixative was removed and spheroids were washed three times with 1 ml PBS. Permeabilization was done with 150 µl 0.1% Triton X-100 in PBS for 10 minutes. Spheroids were washed three times with 1 ml PBS and blocked with 300 µl 3% FCS in PBS. Spheroids were then incubated for 1 hour with a mouse anti-human-CD31 antibody (1:100) diluted in 150 µl blocking solution. After three washing steps with 1 ml PBS, spheroids were then exposed to AlexaFluor 488-conjugated goat anti-mouse antibody (1:200) and DAPI (1:1000, 1mg/ml stock) in 150 µl blocking solution. After 1 hour, spheroids were washed tree times with 1 ml PBS and supernatants were removed. Then, one drop of mowiol (http://www.sigmaaldrich.com) was added and spheroids were transferred to glass slides and sealed with coverslips. Short pieces of human hair or parafilm were used as spacers in order to not destroy the 3D structure of the spheroids. Z-stacks were acquired using a spinning disc confocal microscope (Olympus IX81, Andor Revolution XD) and analyzed using the ImageJ software (http://rsb.info.nih.gov/ij/).

Spheroid-based *in vitro* angiogenesis assay

The *in vitro* angiogenesis assay was performed as described (Weber *et al.*, 2008). The generated spheroids were harvested and suspended in the methocel solution containing 20% FCS. Subsequently, the ice-cold collagen solution (rat tail type I collagen in 0.1% acidic acid) was neutralized by adding 10% 10-fold Medium 199 and approx. 10% 0.2 N NaOH to adjust the pH to 7.4. EC spheroid/ methocel solution was mixed 1:1 with the neutralised collagen solution and 1 ml of the mixed solution containing approximately 50 EC spheroids were pipetted into individual wells of a pre-warmed 24 well plate to allow polymerisation in the incubator in a humified atmosphere at 35 °C, 5% CO_2. After 30 min VEGF was added to an end-concentration of 25 ng/ml by pipetting 100 µl of a 10-fold concentrated working dilution on top of the polymerised gel. Plates were incubated in a humified atmosphere at 35 °C, 5% CO_2 for 24 h and fixed by adding 3.7% paraformaldehyde. Pictures were taken with a Leica DM IRBE inverted microscope using a MicroMAX camera (Princeton Instruments with MetaMorph software) using the 20x objective. I*n vitro* angiogenesis was digitally quantitated by measuring the cumulative length of the sprouts (CSL) that had grown out of each spheroid using the MetaMorph software analyzing 10 spheroids per experimental group and experiment.

In-gel staining of sprouting spheroids

HUVEC monolayers were infected with indicated *Bh* strains (MOI 300) or left uninfected in M199/10% FCS/500 µM IPTG for 24 h. As for the spheroid angiogenesis assay EC spheroids were generated, harvested, suspended in methocel 20% FCS and mixed 1:1 with neutralized collagen. 2 ml of the mix was distributed over the wells of an 8 well µ-slide (http://www.ibidi.de) and was allowed to polymerize. 50 µl of a 10-fold concentrated working dilution of VEGF was pipetted on

top of the polymerised gel. The µ-slides were incubated in a humified atmosphere at 35 °C, 5% CO_2 for 24 h and fixed by adding 3.7% paraformaldehyde. Gels were washed tree times with 150 µl PBS and blocked with 200 µl PBS 5% bovine serum albumin (BSA) for 2 hours before being incubated with DAPI and TRITC-phalloidin in 100 µl PBS-BSA overnight at 4°C. Gels were washed tree times with PBS and analyzed by confocal spinning disc microscopy (Olympus IX81, Andor Revolution XD). 3D reconstruction of in-gel sprouting spheroids was done using image stacks and the ImageJ software.

Acknowledgments

We are grateful to Arto Pulliainen for critically reading of the manuscript. We thank C. Mistl and O. Siedentopf for technical assistance. The work was supported by grant 3100A0-109925/1 from the Swiss National Science Foundation (SNF) to C.D., grant 55005501 from the Howard Hughes Medical Institute (HHMI) to C.D., and a PhD fellowship from the Misrock Foundation to F.S. We acknowledge the donation of human umbilical cords from the University Women's Hospital Basel.

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Table 1 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	(Regnery *et al.*, 1992)
RSE247	Spontaneous Sm^{r} strain of ATCC 49882 ^{T}, serving as wild-type	(Schmid *et al.*, 2004)
RSE242	*ΔvirB4* mutant of RSE247	(Schmid *et al.*, 2004)
MSE150	*ΔbepA-G* mutant of RSE247	(Schulein *et al.*, 2005)
TRB106	*ΔbepB-G* mutant of RSE247	(Schulein *et al.*, 2005)
MSE156	MSE150 containing pPG101 (Δ*bepA-G*/p*bepA*)	(Schmid *et al.*, 2006)
MSE167	MSE150 containing pMS006 (Δ*bepA-G*/p*bepB*)	(Rhomberg *et al.*, 2008)
MSE159	MSE150 containing pMS007 (Δ*bepA-G*/p*bepC*)	(Rhomberg *et al.*, 2008)
PGD03	MSE150 containing pPG104 (Δ*bepA-G*/p*bepD*)	(Rhomberg *et al.*, 2008)
PGD10	MSE150 containing pPG105 (Δ*bepA-G*/p*bepE*)	(Rhomberg *et al.*, 2008)
TRB171	MSE150 containing pPG106 (Δ*bepA-G*/p*bepF*)	(Rhomberg *et al.*, 2008)
TRB169	MSE150 containing pPG107 (Δ*bepA-G*/p*bepG*)	(Rhomberg *et al.*, 2008)
MSE269	MSE150 containing pMS106 (Δ*bepA-G*/p*bepA2*)	(Schmid *et al.*, 2006)
MSE218	MSE150 containing pMS100-A (Δ*bepA-G*/pt*bepA*)	(Schmid *et al.*, 2006)
MSE228	MSE150 containing pMS100-C (Δ*bepA-G*/pt*bepC*)	(Schmid *et al.*, 2006)
MSE154	*ΔbepA* mutant of RSE247	(Schmid *et al.*, 2006)
TRB288	*ΔbepC* mutant of RSE247	This work
PGC80	*ΔbepD* mutant of RSE247	This work
PGD20	*ΔbepE* mutant of RSE247	This work
TRB222	*ΔbepF* mutant of RSE247	This work
TRB223	*ΔbepG* mutant of RSE247	This work

Plasmids

pPG100	E.coli – Bartonella spp. Shuttle vector, encoding a short FLAG epitope	(Schulein *et al.*, 2005)
pPG101	Derivative of pPG100, encoding FLAG::*Bh*_BepA	(Schmid *et al.*, 2006)
pMS006	Derivative of pPG100, encoding FLAG::*Bh*_BepB	(Schmid *et al.*, 2006)
pMS007	Derivative of pPG100, encoding FLAG::*Bh*_BepC	(Schmid *et al.*, 2006)
pPG104	Derivative of pPG100, encoding FLAG::*Bh*_BepD	(Schulein *et al.*, 2005)
pPG105	Derivative of pPG100, encoding FLAG::*Bh*_BepE	(Rhomberg *et al.*, 2008)
pPG106	Derivative of pPG100, encoding FLAG::*Bh*_BepF	(Rhomberg *et al.*, 2008)
pPG107	Derivative of pPG100, encoding FLAG::*Bh*_BepG	(Rhomberg *et al.*, 2008)
pMS100	Inserton of a multiple cloning site into pPG100	(Schmid *et al.*, 2006)
pMS106	Derivate of pMS100, encoding FLAG::*Bq*_BepA2	(Schmid *et al.*, 2006)
pMS100-A	Derivate of pMS100, encoding FLAG::*Bh*_BepA$_{305-542}$	(Schmid *et al.*, 2006)
pMS100-C	Derivate of pMS100, encoding FLAG::*Bh*_BepC$_{292-542}$	(Schmid *et al.*, 2006)
pWAY21	Plasmid for ectopic expression of N-terminal eGFP fusion proteins	Molecular Motion, Montana Lab
pMS21	Derivate of pWAY21, encoding GFP::*Bh*_BepA	(Schmid *et al.*, 2006)
pTR1000	Basic mutagenesis vector to generate in-frame mutants of RSE247	(Schulein *et al.*, 2005)
pTR1069	Derivate of pTR1000 to generate a Δ*bepC* in-frame mutant in *Bh*	This work
pPG161	Derivate of pTR1000 to generate a Δ*bepD* in-frame mutant in *Bh*	This work
pPG162	Derivate of pTR1000 to generate a Δ*bepE* in-frame mutant in *Bh*	This work
pTR1075	Derivate of pTR1000 to generate a Δ*bepF* in-frame mutant in *Bh*	This work
pTR1078	Derivate of pTR1000 to generate a Δ*bepG* in-frame mutant in *Bh*	This work

Table 2 Oligonucleotide primers used in this study.

Name	Sequence [a]	Restriction site
prPG134:	CGGGATCCCGAAAGATGAAGTTACTTCAAAGC	*BamHI*
prPG135:	GTTTAGATGGCGAAAGCTATTGTTTCTGTGGAA	
	TGAGTTGTCG	
prPG136:	CGACAACTCATTCCACAGAAACAATAGCTTTCG	
	CCATCTAAAC	
prPG137:	CGGGATCCGTGTAGCTCCTTGTCTTTTGC	*BamHI*
prTR041:	GCGGATCCGCACACTCTGTGGAAAAACC	*BamHI*
prTR042:	CATTCCTTTTTGATTTGTGGGAGTCGGAGGGGATGGTCG	
prTR044:	CGGGATCCGCTTGGACCAATGGATTGT	*BamHI*
prTR051:	CGACCATCCCCTCCGACTCCCACAAATCAAAAAGGAATG	
prTR053	GCTCTAGAGGGGCATAATGTATGTGGC	*XbaI*
prTR054	GCCAGCACCATTTTTTGTGGAGATGTGGAAGAGGATGG	
prTR055	CACAAAAAATGGTGCTGGC	
prTR056	GCTCTAGAGGCGTTACACAGGGAAGAG	*XbaI*
prTR057	GCTCTAGAGCTCCGCTTGCTGGCAAG	*XbaI*
prTR058	GGTCGAACTATCTGTTCTACAGGTGTTGAAGGGGCTGG	
pTR059	GTAGAACAGATAGTTCGACC	
prTR060	GCTCTAGAGTTACGAGTCTGTCCAGCC	*XbaI*
prTR082	CGCGGATCCGGGCGCTCATTTTACCTCC	*BamHI*
prTR083	CGTGTTTAGTTGGTAAGAGCCTCTAACATATATTTTCTCCTTG	
prTR084	GCTCTTACCAACTAAACACG	
prTR085	CGCGGATCCCGGCACGTCGCCCATTC	*BamHI*

[a] Restriction endonuclease cleavage sites are underlined

Figure 1: *Bartonella henselae* VirB/VirD4/Bep system modulates sprouting angiogenesis of collagen gel-embedded EC spheroids

(A) Human umbilical vein endothelial cells (HUVEC) where infected with a MOI of 300 with the indicated strains 24 h before a defined number of cells was suspended in culture medium containing methylcellulose and grown overnight as hanging drops. Spheroids were whole-mount stained with a mouse anti-human CD31-specific monoclonal antibody and detected with an anti-mouse secondary antibody conjugated to AlexaFluor488. Bright field image and maximum intensity z-projections of stacks acquired by confocal spinning disc microscopy are shown. (B-G) Spheroids out of uninfected HUVECs or HUVECs infected with the indicated strains were embedded in collagen and control uninfected spheroids were stimulated with VEGF (25 ng/ml). (B) Quantification of *in vitro* angiogenesis of collagen embedded spheroids after 24 h by calculating the cumulative length of sprouts from individual spheroids. Shown are means / SD of ten spheroids per experimental group. Differences between experimental groups were analyzed by Student's t-test. * $p < 0.05$, ** $p < 0.01$ were considered statistically significant. (C-G) Bright field images of one-day-old collagen embedded HUVEC spheroids. Shown are representative images demonstrating strong capillary-like sprouting of spheroids upon stimulation with VEGF (G), as well as VirB/D4 T4SS-dependent sprouting of spheroids out of *Bh* wild-type-infected HUVECs (D). The bar represents 100 µm.

Figure 2: *Bh* wild-type induces sprout outgrowth with a distinct morphology

Spheroids from uninfected HUVECs or HUVECs infected with the indicated strains were embedded in collagen and control was stimulated with VEGF (25 ng/ml). Collagen gels were incubated with the DNA stain DAPI as well as TRITC-phalloidin binding to actin. Stacks were acquired by confocal microscopy. Shown are maximum intensity z-projections. DAPI staining demonstrated that nuclei migrate into the collagen gel, TRITC and DIC showed that especially outgrowths induced by *Bh* wild-type display some loss of sprout integrity.

Figure 3: Modulation of spheroid sprout formation by *Bartonella henselae* mutant strains translocating individual Bep effectors

Spheroids out of uninfected HUVECs or HUVECs infected with *Bh wt* or the effector-less mutant Δ*bepA-G* trans-complemented with single *bep* genes were embedded in collagen and control was stimulated with VEGF (25 ng/ml). (A) Quantification of *in vitro* angiogenesis of collagen embedded spheroids after 24 h. The cumulative length of sprouts of individual spheroids was calculated. Shown are means / SD of ten spheroids per experimental group. Differences between experimental groups were analyzed by Student's t-test. * $p < 0.05$, ** $p < 0.01$ were considered statistically significant. (B-L) Corresponding representative bright field images of one-day-old gel-embedded spheroids. BepA involved in anti-apoptosis promoted strongly sprout formation (F). BepG, involved in cytoskeletal rearrangement, showed a potent interference with sprout formation (L). BepD, which is tyrosine phosphorylated by eukaryotic kinases, exhibited a positive modulatory effect on sprout formation (I).

Figure 4: Modulation of spheroid sprout formation by *Bartonella henselae* mutant strains deficient for the translocation of individual Bep effectors

Spheroids out of uninfected HUVECs or HUVECs infected with *Bh wt*, the effector-less mutant Δ*bepA-G*, or the indicated in-frame deletion mutants of individual bep genes were embedded in collagen and control was stimulated with VEGF (25 ng/ml). (A) Quantification of *in vitro* angiogenesis of collagen embedded spheroids after 24 h. The cumulative length of sprouts of individual spheroids was calculated. Shown are means / SD of ten spheroids per experimental group. Differences between experimental groups were analyzed by Student's t-test. * $p < 0.05$, ** $p < 0.01$ were considered statistically significant. (B-J) Corresponding representative bright field images of one-day-old gel-embedded spheroids. Loss of BepA (F) reduced sprouting activity of *Bh* wt to Δ*bepA-G* mutant basal sprouting level (E). A small reduction in sprouting was observed if BepD is missing (H). Knock-out of *bepG* enhanced induction of spheroid sprouting upon infection (J).

Figure 5: Ectopic expression of BepA promotes sprouting and BepA BID-domain plus C-tail is sufficient to promote sprouting of infected HUVEC

Spheroids out of uninfected respectively untransfected HUVECs, HUVECs infected with the indicated strains, or transfected with the indicated constructs were embedded in collagen and control was stimulated with VEGF (25 ng/ml). (A, B) Quantification of *in vitro* angiogenesis of collagen embedded spheroids after 24 h. The cumulative length of sprouts of individual spheroids was calculated. Shown are means / SD of ten spheroids per experimental group. Differences between experimental groups were analyzed by Student's t-test. * $p < 0.05$, ** $p < 0.01$ were considered statistically significant. (A) N-terminally truncated BepA (tBepA) harbouring BID-domain plus C-tail (sufficient for anti-apoptosis) is enough to promote

sprouting of spheroids. Moreover the *B. quintana* homolog BepA2, encoded by the down-stream ORF of the split *bepA* locus containing only the BID-domain and C-tail, also strongly promoted sprouting. (C-F) Representative bright field images of one-day-old gel-embedded spheroids for the ectopic expression of GFP-BepA fusion. HUVEC transfected with gfp-BepA (pIMS21, F) show increased capillary-like sprouting compared to untransfected cells or cells transfected with control gfp (pWay21, E).

Figure 6: BepG is interfering with sprouting of spheroids

Spheroids out of uninfected HUVECs or HUVECs infected with the indicated strains were embedded in collagen and control was stimulated with VEGF (25 ng/ml). (A) Quantification of *in vitro* angiogenesis of collagen embedded spheroids after 24 h. The cumulative length of sprouts of individual spheroids was calculated. Shown are means / SD of ten spheroids per experimental group. Differences between experimental groups were analyzed by Student's t-test. * $p < 0.05$, ** $p < 0.01$ were considered statistically significant. (B-J) Corresponding representative bright field images of one-day-old gel-embedded spheroids. The overexpression of BepG in the Δ*bepA-G* mutant background which promotes invasome formation reduces baseline sprouting (G); moreover BepG overexpression in the Δ*bepB-G* mutant background reduces BepA-induced sprouting (J).

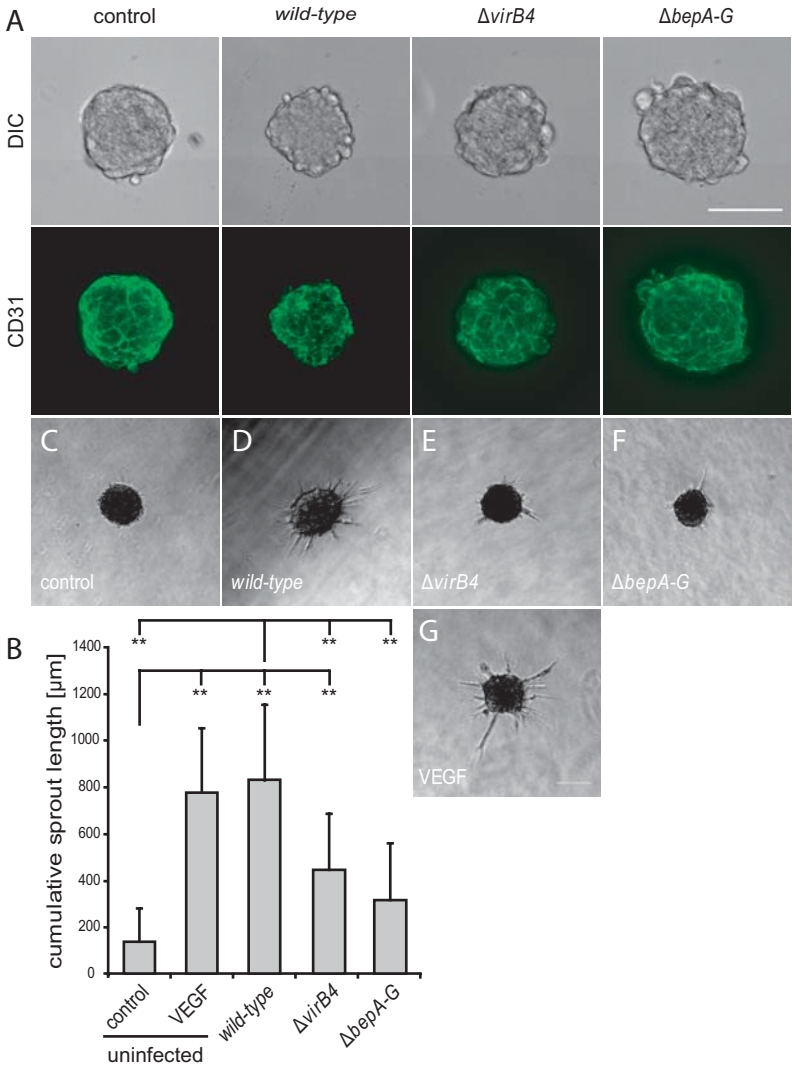

A
control
wild-type
ΔvirB4
ΔbepA-G
DIC
CD31
C
D
E
F
control
wild-type
ΔvirB4
ΔbepA-G
B
G
VEGF
cumulative sprout length [µm]
1400
1200
1000
800
600
400
200
0
**
**
**
**
**
control
VEGF
wild-type
ΔvirB4
ΔbepA-G
uninfected

Figure 1

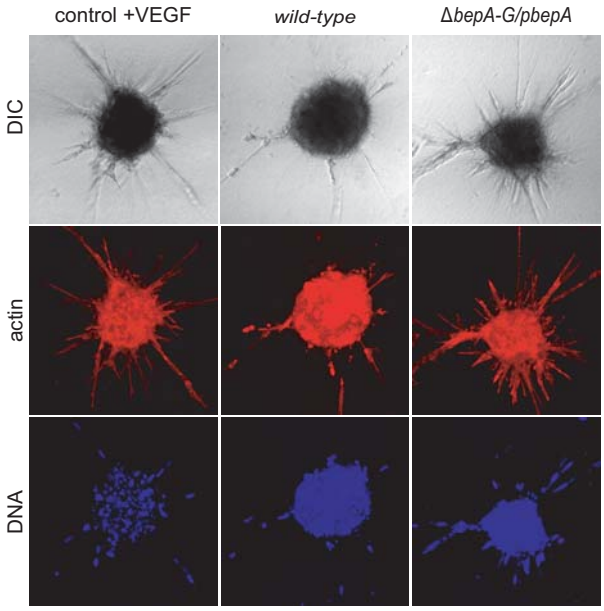


Figure 2

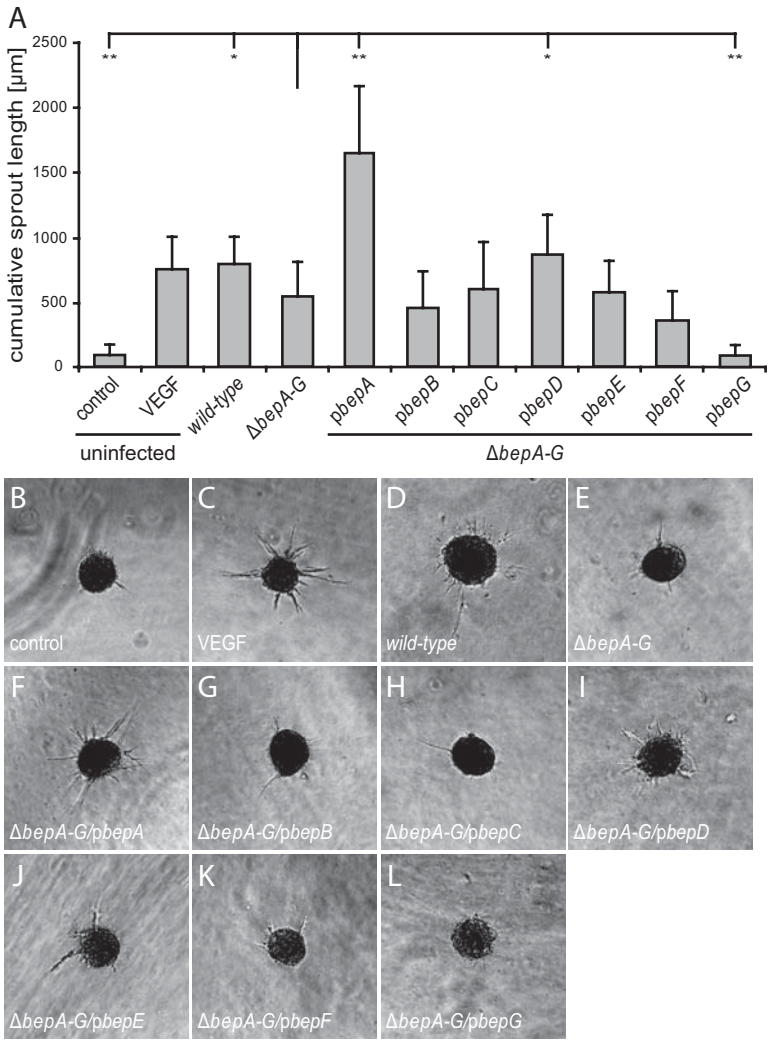
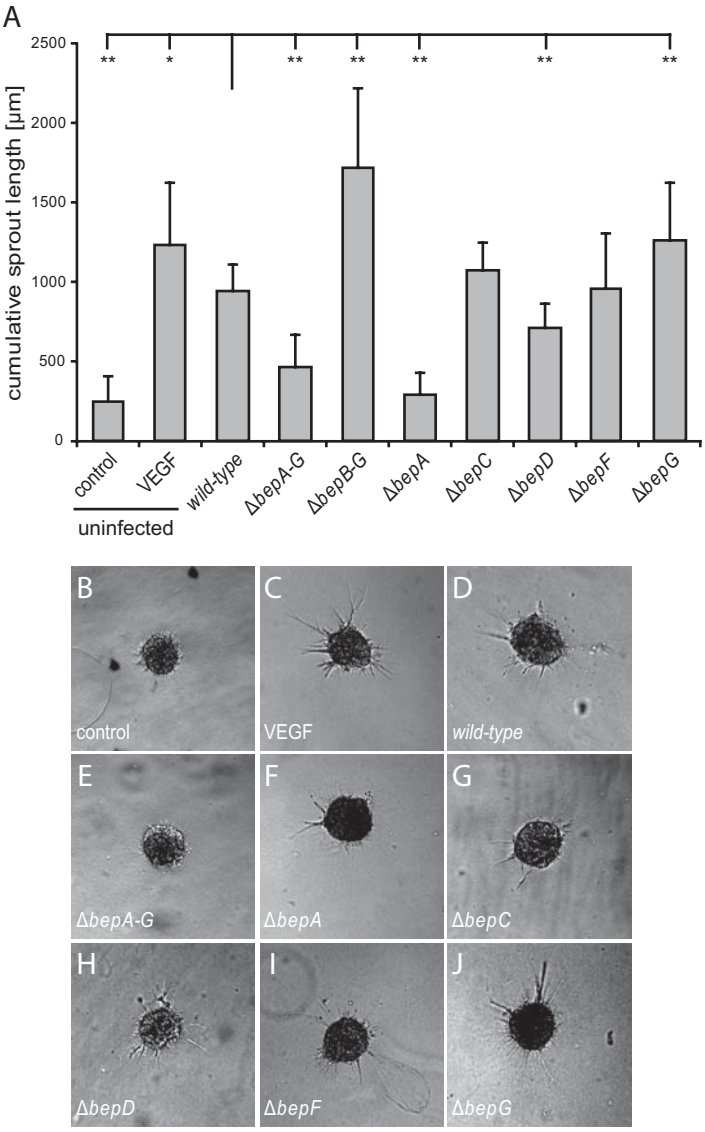

A
cumulative sprout length [µm]
2500
2000
1500
1000
500
0
**
*
**
*
**
control
VEGF
wild-type
ΔbepA-G
pbepA
pbepB
pbepC
pbepD
pbepE
pbepF
pbepG
uninfected
ΔbepA-G
B
control
C
VEGF
D
wild-type
E
ΔbepA-G
F
ΔbepA-G/pbepA
G
ΔbepA-G/pbepB
H
ΔbepA-G/pbepC
I
ΔbepA-G/pbepD
J
ΔbepA-G/pbepE
K
ΔbepA-G/pbepF
L
ΔbepA-G/pbepG

Figure 3


A
cumulative sprout length [µm]
2500
2000
1500
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500
0
**
*
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**
control
VEGF
wild-type
ΔbepA-G
ΔbepB-G
ΔbepA
ΔbepC
ΔbepD
ΔbepF
ΔbepG
uninfected
B
control
C
VEGF
D
wild-type
E
ΔbepA-G
F
ΔbepA
G
ΔbepC
H
ΔbepD
I
ΔbepF
J
ΔbepG

Figure 4

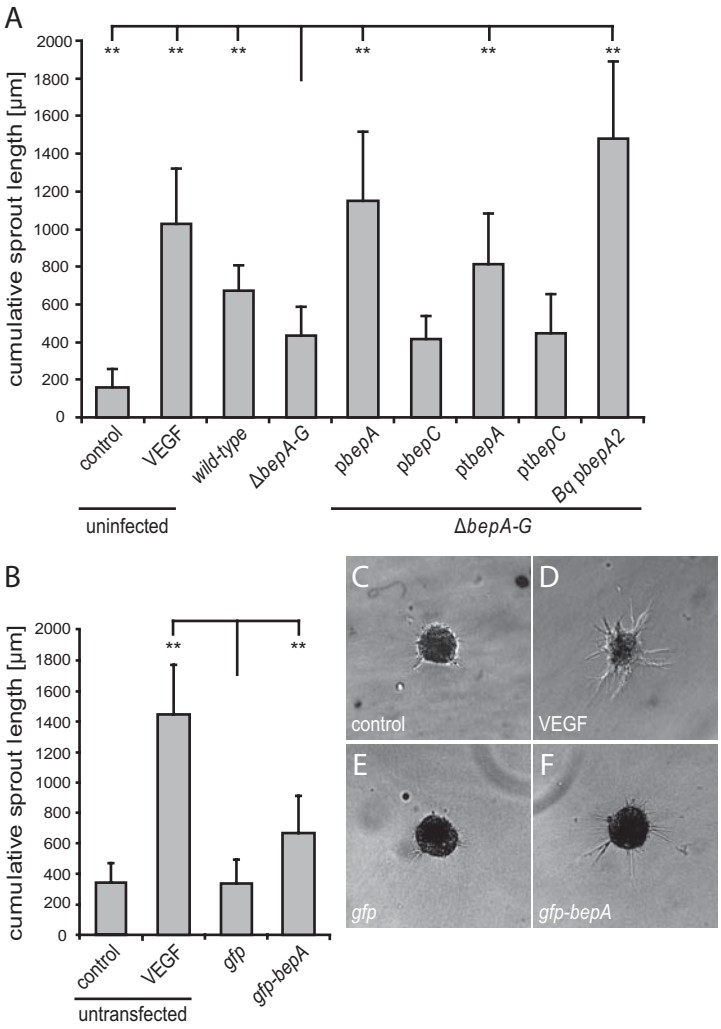
A
cumulative sprout length [µm]
2000
1800
1600
1400
1200
1000
800
600
400
200
0
**
control
VEGF
wild-type
ΔbepA-G
pbepA
pbepC
ptbepA
ptbepC
Bq pbepA2
uninfected
ΔbepA-G
B
cumulative sprout length [µm]
control
VEGF
gfp
gfp-bepA
untransfected
C
control
D
VEGF
E
gfp
F
gfp-bepA

Figure 5

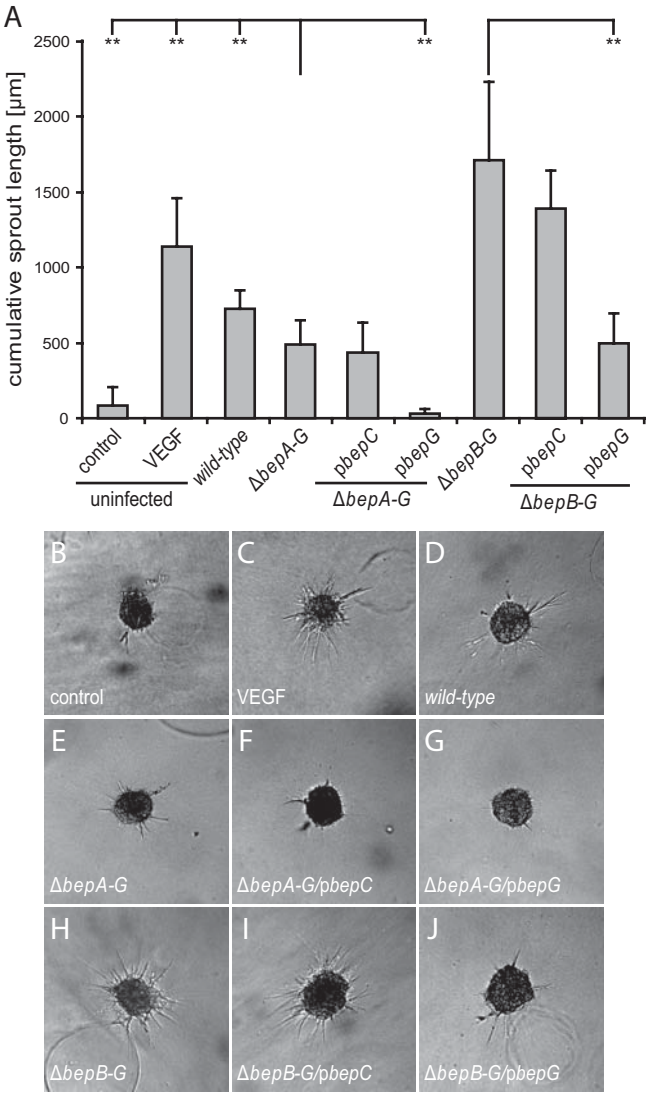
A
cumulative sprout length [µm]
2500
2000
1500
1000
500
0
**
**
**
**
**
control
VEGF
wild-type
ΔbepA-G
pbepC
pbepG
ΔbepB-G
pbepC
pbepG
uninfected
ΔbepA-G
ΔbepB-G
B
control
C
VEGF
D
wild-type
E
ΔbepA-G
F
ΔbepA-G/pbepC
G
ΔbepA-G/pbepG
H
ΔbepB-G
I
ΔbepB-G/pbepC
J
ΔbepB-G/pbepG

Figure 6

Chapter 3

The *Bartonella henselae* VirB/VirD4/Bep System Interferes with VEGF-induced ERK1/2-mediated Endothelial cell proliferation

F. Scheidegger and C. Dehio

In preparation

The close association of *Bartonella henselae* (*Bh*) with proliferating endothelial cells (ECs) in bacillary angiomatosis / peliosis (BA/BP) and the fact that antibiotic treatment leads to regression of those lesions indicate an active role of *Bh* triggering these vascular proliferations. In addition to a direct stimulation of proliferation and inhibition of apoptosis in ECs the activation of a paracrine loop of vascular endothelial growth factor (VEGF), an essential regulator of physiological as well as pathological angiogenic processes, is thought to be involved in *Bh*-induced vascular tumour formation. We addressed in this study the effect of exogenous VEGF on *Bh*-infected ECs.

Assessing the biological activity of VEGF in assays such as proliferation, wounding assay and capillary-like sprout formation revealed an intriguing interference of the VirB/VirD4/Bep system with responsiveness of infected ECs to stimulation with this potent growth factor. Sprouting, proliferation, and migration could be readily induced by VEGF if human umbilical vein endothelial cells (HUVECs) were uninfected or infected with the Δ*bepA-G* mutant. Infection with *Bh* wild-type interfered with EC responsiveness to VEGF.

Analysis of the VEGF receptor 2 (VEGFR2) pathways further showed that *Bh* wild-type inhibited phosphorylation of tyrosine 1175 (Tyr1175) in the C-terminus of VEGFR2. Tyr1175 is one of the critical docking sites for adaptor proteins mediating VEGFR2 downstream signalling. Phospholipase Cγ1 (PLCγ1) is amongst the proteins binding to Tyr1175. The membrane phospholipid phophatidylinositol (4,5)-bisphosphate (PIP_2) is hydrolysed by PLCγ1 giving rise to the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG is the activator of PKC, mediating the activation of the mitogen-activated protein kinase ERK1/2 which is the major mediator of VEGF-induced cell proliferation. IP_3 generation results in increase of intracellular concentration of calcium $[Ca^{2+}]_i$. Consistent with a lack of Tyr1175 phosphorylation upon stimulation of *Bh* wild-type-infected ECs with VEGF,

we observed no PLCγ1 recruitment and phosphorylation and no calcium flux and ERK1/2 phosphorylation. First experiments done with other stimuli than VEGF, such as ATP or thrombin triggering GPCR-coupled calcium flux, indicate that *Bh* interference is at least specific for receptor tyrosine kinases.

The data presented in this work challenge the proposed VEGF-driven paracrine loop in case of an active VirB/VirD4/Bep system. Furthermore, together with the fact that the VirB/VirD/Bep system masks a potent VirB-independent mitogenic activity and that BepG potently interferes with sprouting (chapter 2), this interference of the VirB/VirD4/Bep system with the signalling of one of the most important regulator of angiogenesis speaks against the VirB T4SS as major pro-angiogenic factor in *Bh* pathogenesis. Thus, the VirB/VirD4/Bep system more likely represents a host adaptation system, a modulator needed to balance the angiogenic activity of *Bh* during chronic infection of the human vasculature.

Statement of the own participation

The data reported in this manuscript were generated by me. I am grateful to C. Mistl for technical help and to R. Jayachandran and P. Müller for help setting up the calcium assays.

The *Bartonella henselae* VirB/VirD4/Bep System Interferes with VEGF-induced ERK1/2-mediated Endothelial cell proliferation

F. Scheidegger and C. Dehio*

Biozentrum, University of Basel, CH-4056 Basel, Switzerland

*For correspondence:

Prof. Christoph Dehio, Ph.D.

Biozentrum

University of Basel

Klingelbergstrasse 70

CH-4056 Basel, Switzerland

E-Mail: christoph.dehio@unibas.ch

Tel. (+41) 61 267 2140

Fax (+41) 61 267 2118

Running title: *Bartonella henselae* interferes with VEGF-induced cell proliferation

Word count (including legends and references)**:** 6922

Abstract

The zoonotic gram-negative pathogen *B. henselae* (*Bh*) causes amongst various clinical manifestations tumour-like vascular proliferations. A direct stimulation of endothelial cell (EC) proliferation, inhibition of apoptosis in ECs, and the activation of a paracrine loop of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) are thought to be involved. VEGF is an essential regulator of angiogenesis under physiological as well as pathological conditions. Here we report that *Bh* actually interferes with responsiveness of ECs to VEGF. Proliferation or wound closure could not be stimulated by VEGF in cells infected with *Bh*, neither could VEGF induce capillary-like sprouting of spheroids from *Bh*-infected ECs. Tyrosine-phosphorylation on position 1175 of the VEGF receptor 2 (VEGFR2) was inhibited; consequently we observed a lack of PLCγ1 activation and interference with VEGF-triggered cytosolic calcium flux. Thus, *Bh* constrained the signalling cascade leading to the activation of the mitogen-activated protein kinase ERK1/2 upon stimulation of VEGFR2 with its ligand. This interference was dependent on a functional VirB/VirD4 type IV secretion system and its effectors, the *Bartonella*-effector proteins (Beps). The presented data emphasize the role of the VirB/VirD4/Bep system as an important factor balancing the angiogenic potential of *Bh* during chronic infection of the vasculature.

Introduction

Rarely showing any symptoms in the feline reservoir host *Bartonella henselae* (*Bh*) is responsible for a number of clinical outcomes during infection of the human incidental host. Clinical manifestations range from self-limiting but long-lasting swelling of lymph nodes (cat-scratch-disease, CSD) in immunocompetent patients to tumour-like vasoproliferative lesions on the skin or inner organs known as bacillary angiomatosis or peliosis (BA/BP) especially in patients with a compromised immune system (Florin *et al.*, 2008). The close association of *Bh* with proliferating endothelial cells (ECs) in BA/BP (Chian *et al.*, 2002) and the fact that antibiotic treatment leads to regression of those lesions (Koehler and Tappero, 1993) indicate an active role of *Bh* triggering these vascular proliferations. *Bh*-triggered vasculoproliferative lesions resemble tumour angiogenesis – the pathological process of the formation of new capillaries out of pre-existing blood vessels. Foremost among the several different growth factors and their associated receptor tyrosine kinases (RTKs), the vascular endothelial growth factor (VEGF) family and VEGF receptors (VEGFRs) are essential regulators of angiogenesis under physiological as well as pathological conditions (Takahashi and Shibuya, 2005).

The secretion of VEGF from *Bh*-infected macrophages, recruited to the site of infection as a result of a pro-inflammatory response in ECs (Schmid *et al.*, 2004; Fuhrmann *et al.*, 2001), or from infected epithelial cells surrounding the blood vessel is proposed to contribute to BA/BP by promoting EC proliferation in a paracrine manner (Resto-Ruiz *et al.*, 2002; Kempf *et al.*, 2001). *Bh* also demonstrates a potent direct mitogenic stimulation of ECs (Conley *et al.*, 1994). Furthermore, the inhibition of EC apoptosis upon *Bh*-infection might play indirectly a role in vasoproliferation by increasing cell survival (Kirby and Nekorchuk, 2002). Another characteristic of infection of human umbilical vein endothelial cells (HUVECs) with *Bh* are massive

cytoskeletal actin rearrangements leading to a unique structure termed invasome (Dehio *et al.*, 1997). Almost all these activities depend on the VirB/VirD4 type IV secretion system (T4SS) of *Bh* and the thereby translocated *Bartonella* effector proteins (BepA-BepG) (Schulein *et al.*, 2005; Schmid *et al.*, 2004). The NFκ-B-dependent pro-inflammatory is to some extend T4SS-independent (Fuhrmann *et al.*, 2001), whereas the direct mitogenic stimulus is completely lying outside of the VirB/VirD4 T4SS. Intriguingly, this potent direct stimulation of EC proliferation is even counterbalanced by the VirB/VirD4/Bep system (Schmid *et al.*, 2004). The role of the VirB/VirD4 T4SS and the Beps in the VEGF-based paracrine loop is unknown. A recently established three-dimensional spheroid sprouting assay allowed the assessment of the complex angiogenic properties of *Bh* in an elaborate *in vitro* model and revealed distinct but also adverse activities of the Beps modulating capillary-like sprout formation (Scheidegger *et al.*, 2008).

Observations done during the establishment of the spheroid-based *in vitro* model prompted us to study the effect of exogenous VEGF on *Bh*-infected ECs. Spheroids formed from HUVECs pre-infected with *Bh* wild-type showed sprouting which could not be further stimulated by VEGF. VEGF-stimulated proliferation as well as migration was suppressed in *Bh* wild-type-infected ECs. Immunoblot analyses demonstrate that the *Bh* VirB/VirD4/Bep system altered VEGFR2 signalling, more precisely inhibited the signalling cascade leading to the activation of the mitogen-activated protein kinase ERK1/2 by interfering with tyrosine-phosphorylation of a specific site of the VEGFR2. The presented data emphasize the role of the VirB/VirD4/Bep system, which displays both pro- as well as anti-angiogenic characteristics, as an important factor balancing the angiogenic potential of *Bh* in the course of human vascular infection.

Results

The VirB/VirD4/Bep system of *Bartonella henselae* inhibits VEGF-induced spheroid sprouting

The recently established three-dimensional spheroid-based *in vitro* model of *Bartonella*-triggered angiogenesis (Scheidegger *et al.*, 2008) was used as an elaborate assay for VEGF activity. Therefore spheroids from uninfected HUVECs respectively HUVECs infected with a multiplicity of infection (MOI) of 300 with *Bh* wild-type or the isogenic effector-less mutant Δ*bepA-G* were embedded in collagen and subsequently either stimulated with VEGF (25 ng/ml) or left untreated. After 24 h *in vitro* angiogenesis was quantified by calculating the cumulative sprout length (CSL) of individual spheroids (Fig 1A, determined as the mean +/- SD of 10 spheroids per experimental group). To quantify the effect of VEGF we calculated a VEGF sprout factor by dividing CSL of spheroids stimulated with VEGF by CSL of spheroids without VEGF (Fig 1B, shown are means +/- SD of the indicated number of experiments). As previously observed spheroids formed from HUVECs pre-infected with *Bh* wild-type displayed an increase in CSL in comparison to uninfected control spheroids. Compared to wild-type, the sprouting activity was reduced in spheroids of HUVECs pre-infected with the Δ*bepA-G* mutant. Surprisingly, spheroids made from HUVECs previously infected with *Bh* wild-type were not responsive to VEGF as the CSL with or without VEGF were comparable (Fig. 1A, C), leading to a VEGF sprout factor of 1 (Fig. 1B). In contrast, sprouting could be readily induced about six- respectively 2.5-fold by VEGF for uninfected spheroids or for spheroids from HUVECs pre-infected with the mutant lacking the VirB/VirD4-translocated effectors BepA-BepG (Fig. 1A, B, C).

The VirB/VirD4/Bep system of *Bartonella henselae* interferes with VEGF-induced proliferation and wound closure

Further to the spheroid-based three-dimensional *in vitro* angiogenesis assay quantifying sprout formation, two classical *in vitro* assays were used to asses the effect of exogenous VEGF on ECs infected with *Bh*. To determine the effect of *Bh*-infection on VEGF-stimulated proliferation, cell numbers were compared after 4 days in presence or absence of VEGF (25 ng/ml) of uninfected cells and HUVECs either infected with wild-type or the effector-less Δ*bepA-G* mutant (Fig. 2A, determined as the mean +/- SD of 10 images per well). Cells were seeded in 96-well plates and infected with a MOI of 50. Cell numbers were determined by staining nuclei with DAPI, automated image acquisition and automated cell counting using the CellProfiler software (Carpenter *et al.*, 2006). As reported previously the Δ*bepA-G* mutant as such strongly promoted cell proliferation and this T4SS-independent stimulus was only slightly affected in *Bh* wild-type infection at the low MOI used in this assay. Addition of VEGF stimulated cell proliferation about four-fold for uninfected HUVECs. Proliferation of HUVECs infected with the Δ*bepA-G* mutant could be further increased 1.5-fold by exogenous VEGF. In contrary, VEGF had no effect on wild-type-infected cells.

To asses the effect of infection and exogenous VEGF on cell migration cells were seeded in 6-well plates and infected for 24 h with a MOI of 150 with the indicated strains. The confluent cell layers were wounded by scratching with a pipette tip, stimulated with VEGF (25 ng/ml) and followed by microscopy (Fig. 2B). Stimulation of uninfected HUVECs with VEGF led to complete closure of the wound after two days. Similarly if not faster, HUVECs pre-infected with the Δ*bepA-G* mutant and stimulated with VEGF readily moved to fill the damaged area. Pre-infection with *Bh* wild-type led to a prominent interference with migration, which could not be

rescued by exogenous VEGF. Interestingly, wild-type infected HUVECs did project towards the centre of the wound, thus they were activated to migrate, but the rear back of those cells seemed to stick to the surface, indicating that HUVECs infected with *Bh* wild-type might have difficulties to detach from the substrate.

The VirB/VirD4/Bep system alters VEGF receptor 2 Tyr1175 signalling

VEGFR2 signalling was assessed in a series of immunoblots using antibodies against a number of key components of the VEGR2 signalling cascade (Fig. 3). For this, HUVECs were infected for 24 h with a MOI of 300 with the indicated strains. Subsequently, cells were either stimulated with VEGF (25 ng/ml) for 5 minutes or left untreated and total cell lysates were harvested. In HUVECs infected with *Bh* wild-type and stimulated with VEGF general tyrosine-phosphorylation of VEGFR2 was slightly reduced, albeit showing similar levels of total VEGFR2. This effect was VirB/Bep-dependent. Tyrosine 1175 (Tyr1175) in the C-terminus of VEGFR2 has been shown to be one of the important docking sites for downstream signalling proteins and to have a critical role in the regulation of angiogenesis (Holmes *et al.*, 2007; Sakurai *et al.*, 2005). *Bh* interfered in a VirB/Bep-dependent manner with phosphorylation on this position. Phospholipase Cγ1 (PLCγ1) is amongst the proteins binding to phosphorylated Tyr1175. This interaction promotes phosphorylation and subsequent increase in the catalytic activity of PLCγ1, which further mediates the activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase-1/2 (ERK1/2) (Takahashi *et al.*, 2001). Consistent with the lack of Tyr1775-phosphorylation in HUVECs infected with wild-type and stimulated with VEGF, less PLCγ1 was recruited and the enzyme did not get phosphorylated. Moreover, we observed a VirB/Bep-dependent reduced phosphorylation of the ERK1/2 kinase with no difference in total ERK1/2. The phosphorylation of p38 MAPK

was generally increased upon infection. We saw no differences, also not upon stimulation with VEGF, for the phosphorylation of the serine/threonine kinase AKT/PKB, and the phosphorylation of the activating Tyr418 or inhibitory Tyr529 of Src. The adaptor protein Shb has also been reported to bind to phosphorylated Tyr1175 (Holmqvist *et al.*, 2004). Shb is able to bind a number of proteins, amongst them the focal-adhesion kinase (FAK) (Holmqvist *et al.*, 2003), an important factor for cellular attachment and cellular migration (Parsons, 2003). In *Bh* wild-type-infected ECs phosphorylation of FAK following stimulation with VEGF might be impaired.

The VirB/VirD4/Bep system blocks calcium flux upon stimulation with VEGF

ERK-phosphorylation involves a protein kinase C (PKC)-dependent pathway starting from the activation of PLCγ1. PLCγ1 hydrolyses the membrane phospholipid phophatidylinositol (4,5)-bisphosphate (PIP_2) to generate the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 generation further results in increase of intracellular concentration of calcium $[Ca^{2+}]_i$ through opening of IP_3-operated calcium-release channels (IP_3R) of the endoplasmatic reticulum (ER), whereas DAG is the activator of a number of PKC isoforms (Holmes *et al.*, 2007). HUVECs infected for 24 h with different MOI of *Bh* wild-type were loaded with the fluorescent Ca^{2+} indicator Fluo-4 NW to further asses the nature of *Bh* interference along the ERK1/2 signalling cascade. Baseline fluorescence was measured before cells were stimulated with VEGF (25 ng/ml). Application of VEGF to HUVECs induced a rapid increase in $[Ca^{2+}]_i$, followed by a sustained plateau phase clearly above baseline (Fig. 4A, control). With increasing MOI the initial calcium peak was diminished to baseline level (Fig. 4A). A MOI of 150 cut the calcium peak by half. Treatment with Ionomycin (Fig. 4 A, B) or Thapsigargin (data not shown) showed that calcium stores are not emptied upon infection.

ECs either infected with the Δ*virB4* mutant deficient for translocation via the VirB/VirD4 T4SS or the Δ*bepA-G* mutant lacking BepA to BepG behaved as uninfected ECs (Fig. 4B). Furthermore, heat-killed *Bartonella* or bacteria lacking *batR*, the two-component-regulator of *virB/virD4/bep* expression, did not affect VEGF-induced increase in $[Ca^{2+}]_i$ (data not shown). Thus, the MOI-correlating interference with calcium flux was dependent on viable bacteria with a functional VirB/VirD4 T4SS and at least one of the effectors.

To test the specificity of *Bh*-interference with calcium flux we assessed $[Ca^{2+}]_i$ in HUVECs after stimulation with Thrombin (1 U/ml) or ATP (100 µM). Both stimuli lead to accumulation of IP_3 and subsequent increase of $[Ca^{2+}]_i$ by the activation of PLCβ connected to G protein-coupled receptors (GPCRs) (Rozengurt, 2007; McLaughlin *et al.*, 2005; Communi *et al.*, 2000). *Bh* wild-type neither blocked Thrombin- nor ATP-induced calcium flux (Fig. 4C). Hence, the results obtained so far indicate that *Bh* can interfere with calcium flux, that follow the activation of PLCγ coupled to RTKs.

Loss of *bep* genes had effects in both directions (Fig. 5). Infections with a mutant lacking *bepD-F*, harbouring tyrosine phosphorylation motifs, as well as a mutant having a single deletion in *bepE* led to a more potent suppression of the VEGF-triggered calcium flux. Surprisingly, infection with the Δ*bepA* mutant, BepA being the effector responsible for inhibition of apoptosis (Schmid *et al.*, 2006), resulted in VEGF-induced calcium flux comparable to the one triggered in cells infected with the Δ*bepA-G* mutant. Cells infected with the a mutant lacking BepG, the effector able to promote actin rearrangements required for invasome-mediated invasion (Rhomberg *et al.*, 2008) , showed already half of the interference with calcium flux. When infected with a double-knockout of *bepC* and *bepG*, a mutant not

able to make invasomes (Rhomberg, T. unpublished observation), the cells showed unhindered VEGF-stimulated calcium flux.

Discussion

Major phenotypic changes have been described for *Bh*-infected ECs *in vitro.* Whereas some depend on the VirB/VirD4/Bep system others are VirB/VirD4/Bep-independent (Scheidegger *et al.*, 2008; Schulein *et al.*, 2005; Schmid *et al.*, 2004). They are thought to play a role in the remarkable capacity of *Bh* to trigger BA and BP which are vasoproliferative processes resembling tumour angiogenesis. In addition to a direct stimulation of proliferation and inhibition of apoptosis in ECs the activation of a paracrine loop of pro-angiogenic factors such as VEGF is thought to be involved in *Bartonella*-triggered vascular tumour formation (Dehio, 2005). VEGF is known as an essential regulator of physiological as well as pathological angiogenic processes (Takahashi and Shibuya, 2005). Based on observations done with a recently established spheroid-based *in vitro* model of *Bh*-triggered angiogenesis (Scheidegger *et al.*, 2008) we addressed in this study the effect of exogenous VEGF on *Bh*-infected ECs.

Surprisingly, the VirB/VirD4/Bep system interfered with EC responsiveness to stimulation with the potent EC specific growth factor VEGF. If HUVECs were pre-infected with *Bh* wild-type neither sprouting, nor proliferation, nor migration could be induced by VEGF. *Bh* wild-type inhibited phosphorylation of Tyr1175, one of the critical docking site for different adaptor proteins mediating VEGFR2 downstream signalling (Holmes *et al.*, 2007). PLCγ1 is one of those proteins. PLCγ1 feeds into the signalling cascade leading to the activation of the mitogen-activated protein kinase ERK1/2. ERK1/2 has a pivotal role in VEGF-stimulated EC proliferation (Takahashi *et al.*, 2001). PLCγ1-recruitment and phosphorylation upon VEGF-stimulation was inhibited by *Bh* in a VirB/Bep-dependent manner. Consequently, we observed also a lack of downstream calcium release from the ER. The importance of PLCγ1 for VEGF function is highlighted by the fact that *PLCγ1* deficient zebrafish embryos fail

to respond to exogenous VEGF and that overall the mutant phenotype of these embryos resembles the ones of mutants lacking VEGF function (Lawson *et al.*, 2003).

Another adaptor protein binding to Tyr1175 is Shb, which can bind FAK (Holmqvist *et al.*, 2004; Holmqvist *et al.*, 2003). FAK is involved in focal-adhesion turnover during cellular migration (Parsons, 2003). In ECs infected with *Bh* wild-type FAK-phosphorylation upon stimulation with VEGF appeared to be reduced, which could explain the defects in migration observed during the scratch wound assay even in the presence of VEGF. Small interfering RNA (siRNA)-mediated gene silencing of Shb results in an inhibition of not only VEGF-induced cytoskeletal reorganisation and migration but also activation of phosphoinositide 3-kinase (PI3K) (Holmqvist *et al.*, 2003). Signalling pathways downstream of PI3K affect cell growth, cell survival, and cell movement. Amongst the downstream targets of PI3K the protein serine-threonine kinases AKT (also called protein kinase B, PKB) is of particular interest (Cantley, 2002). AKT mediates VEGF-induced cell survival (Gerber *et al.*, 1998) and regulates NO (Fulton *et al.*, 1999). The fact that serum induces AKT-activation (Andjelkovic *et al.*, 1996) might explain why in this setup we did not observe any changes in AKT-phosphorylation, neither upon stimulation with VEGF nor influenced by infection. *Bh* requires serum for efficient translocation of the Bep effector proteins via the VirB/VirD4 T4SS (Quebatte, M. unpublished observation).

Other signalling molecules have been implicated in VEGF-induced migration, such as the sequential activation of CDC42, p38 MAPK and phosphorylation of heat-shock protein-27 (HSP27) following the phosphorylation of Tyr1214 of VEGFR2 (Lamalice *et al.*, 2004). The activation p38 MAPK seems to have various and somewhat adverse effects on processes associated with angiogenesis, such as being pro-migratory but restraining cell proliferation (McMullen *et al.*, 2005). ECs

infected with *Bh* generally showed an increase in p38-phosphorylation. The p38 MAPK is strongly activated by environmental stresses and inflammatory cytokines, and less by serum and growth factors (Cuenda and Rousseau, 2007). Hence, the effect we wanted to address upon stimulation with VEGF could have been masked as even in uninfected cells we could not see a difference between stimulated and unstimulated ECs. Nevertheless, *Bh*-triggered activation of p38 might play a role in pathogenesis as proposed for *B. quintana* (*Bq*) and *B. bacilliformis* (*Bb*) (Liberto *et al.*, 2004; Verma and Ihler, 2002).

Experiments done with other stimuli than VEGF, such as ATP or thrombin triggering GPCR-coupled calcium flux, indicate that *Bh* interference might be specific for RTKs. The observation that the *Bh* Δ*bepA-G* mutant lacking the seven known effectors behaved as uninfected cells, strongly suggests a role for the Beps in the observed interference of *Bh* with responsiveness towards VEGF. An initial analysis of individual in-frame mutants in calcium flux assays points to an interplay of the anti-apoptotic BepA and BepC / BepG involved actin rearrangements. It remains to be investigated how the VirB/VirD4/Bep system interferes with VEGFR2 signalling, whether for example Beps directly interact with signalling components of the VEGF signalling cascades or whether they would indirectly interfere by sequestering key factors. Other open questions need to be addressed, such as if VEGFR2 kinase activity itself is changed upon *Bh*-infection, whether other tyrosine-phosphorylation sites known to bind adaptor proteins are influenced, and if signalling kinetics are altered, and last but not least the translation of these *in vitro* observations into *in vivo* relevance. Another important fact to consider is that the experiments addressing the role of *Bh* in the paracrine loop model and attributing a crucial role to the non-fimbrial adhesin BadA (*Bartonella* adhesin A) in the induction of a pro-angiogenic cell response (Riess *et al.*, 2004; Kempf *et al.*, 2001), were carried out with the *Bh*

Marseille strain (Drancourt *et al.*, 1996). Whereas here, a spontaneous streptomycin-resistant variant of the Houston-1 clinical isolate and typing strain ATCC49882^T served as wild-type (Schmid *et al.*, 2004; Regnery *et al.*, 1992). Although the length of the gene varies significantly, *badA* seems to be present in all *Bh* strains tested so far. Single-base deletions or insertions and recombinations are proposed to affect BadA expression, resulting in the coexistence of BadA-positive and BadA-negative substrains within one *Bh* type strain (Riess *et al.*, 2007). BadA and its relevance have not been tackled so far in the strain used in this study.

In summary, we demonstrated an intriguing effect of the VirB/VirD4/Bep system, antagonizing a major signalling cascade of VEGFR2 following its activation by VEGF. Observations done on the level of phosphorylation to assess activation of proteins were reflected in assays such as proliferation, migration and sprout formation assessing the biological function of VEGF. Together with the fact that the VirB/VirD/Bep system masks a potent T4SS-independent mitogenic activity (Schmid *et al.*, 2004) and that BepG potently interferes with sprouting (Scheidegger *et al.*, 2008), this strong interference of the VirB/VirD4/Bep system with the signalling of one of the most important regulator of angiogenesis speaks against the VirB/VirD4 T4SS as major pro-angiogenic factor in *Bh* pathogenesis, at least for cells infected with *Bh*. Along this line, the human-specific *Bb* strongly promotes vascular proliferation (Garcia *et al.*, 1990) even though it has no VirB/VirD4 T4SS (Saenz *et al.*, 2007). Thus, the VirB/VirD4/Bep system more likely is a true host adaptation system, a modulator needed for the regulation of the angiogenic activity of *Bh* during infection – antagonizing potent mitogenic stimuli but inducing minor perturbation in cell proliferation and cell death tolerated by the host but necessary in the long run for chronic vascular infection.

Experimental procedures

Reagents

Vascular endothelial growth factor-A_{165} (VEGF) was a generous gift from Dr. H. Weber, Tumor Biology Centre, University of Freiburg. Antibiotics, ATP and Carboxymethylcellulose (methocel, 4000 centipoises) were from Sigma (http://www.sigmaaldrich.com). Collagen type I was isolated from rat tail tendons (Augustin, 2004). Thrombin and Ionomycin were purchased from Calbiochem (http://www.emdbiosciences.com). The mouse anti-phosphotyrosine antibody (clone 4G10) and mouse anti-actin (clone C4) were purchased from Millipore (http://www.millipore.com). Rabbit anti-VEGFR2 (clone 55B11), rabbit anti-phospho VEGFR2 (Tyr1175, clone 19A10), mouse anti-p44/42 (ERK1/2) MAP Kinase, mouse anti-phospho p44/42 (Thr202/Tyr204, clone E10), rabbit anti-phospho p38 (Thr180/Tyr182, clone12F8), rabbit anti-phospho AKT (Thr308, clone 244F9), rabbit anti-PLCγ1, and rabbit anti-phospho PLCγ1 (Tyr783) antibodies were purchased from Cell Signalling Technology (http://www.cellsignal.com). The mouse anti-FAK (clone 77) was obtained from BD Pharmingen (http://www.bdbiosciences.com), rabbit anti-phospho FAK (Tyr397) from abcam (http://www.abcam.com). Rabbit anti-phospho Src (Tyr418 and Tyr529) antibodies were from Biosource (http://www.biosource.com). Secondary HRP-conjugated antibodies were obtained from Amersham (http://www.amersham.com).

Bacterial strains and Growth Conditions

The bacterial strains used are listed in Table 1. *Bartonella* spp. were grown on Columbia agar (CBA) plates containing 5% defibrinated sheep blood in a humified atmosphere at 35°C and 5% CO_2 for 2-3 days. Strain RSE247, a spontaneous streptomycin-resistant strain of ATCC 49882^T (Schmid *et al.*, 2004) served as wild-

type in this study. 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, http://www.applichem.de) if needed.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated as described before (Dehio *et al.*, 1997) and cultured in EGM medium (http://www.promocell.com) in a humified atmosphere at 37°C and 5% CO_2.

Infection Assay

HUVECs (passage 4-7) were plated in gelatine-coated well plates using EGM. The next day cells were washed twice with M199 with Earls salts (M199, Gibco, http://www.invitrogen.com) supplemented with 10% fetal calf serum (FCS, http://www.invitrogen.com) and infected with the indicated multiplicity of infection (MOI) bacteria per cell in M199/10% FCS/500 µM IPTG and incubated for 24 hours.

Generation of endothelial cell spheroids

EC spheroids of defined cell number were generated as described previously (Korff and Augustin, 1998) with some modifications. In brief, uninfected respectively infected HUVEC monolayers were trypsinized and suspended in EGM containing 20% methocel (Weber *et al.*, 2008). Spheroids were generated by pipetting 400 ECs (in 25 µl) on quadratic Greiner Petri dishes (http://www.huberlaborworld.ch) and grown overnight as hanging drops in a humified atmosphere at 35°C and 5% CO_2. Under these conditions, all suspended cells contribute to the formation of a single spheroid of defined cell number per drop.

Spheroid-based *in vitro* angiogenesis assay

The *in vitro* angiogenesis assay was performed as described (Weber *et al.*, 2008). The generated spheroids were harvested and suspended in the methocel solution containing 20% FCS. Subsequently, the ice-cold collagen solution (rat tail type I collagen in 0.1% acidic acid) was neutralized by adding 10% 10-fold Medium 199 and approx. 10% 0.2 N NaOH to adjust the pH to 7.4. EC spheroid/ methocel solution was mixed 1:1 with the neutralised collagen solution and 1 ml of the mixed solution containing approximately 50 EC spheroids were pipetted into individual wells of a pre-warmed 24 well plate to allow polymerisation in the incubator in a humified atmosphere at 35°C, 5% CO_2. After 30 min VEGF was added to an end-concentration of 25 ng/ml by pipetting 100 µl of a 10-fold concentrated working dilution on top of the polymerised gel. Plates were incubated in a humified atmosphere at 35°C, 5% CO_2 for 24 h and fixed by adding 3.7% paraformaldehyde. Pictures were taken with a Leica DM IRBE inverted microscope using a MicroMAX camera (Princeton Instruments with MetaMorph software) using the 20x objective. I*n vitro* angiogenesis was digitally quantitated by measuring the cumulative length of the sprouts (CSL) that had grown out of each spheroid using the MetaMorph software analyzing 10 spheroids per experimental group and experiment.

Proliferation assay

1000 HUVECs were seeded in 96-well plates and infected with a MOI of 50 in the presence or absence of VEGF (25 ng/ml). After 4 days cells were fixed with 3.7% paraformaldehyde and exposed to DAPI (1:1000, 1mg/ml stock). Plates were imaged with an automated fluorescence microscope (IXM, MDC Molecular Devices) and assessed quantitatively by using CellProfiler (Carpenter *et al.*, 2006). Results are given as average cell number per image, analysing ten images per well.

Scratch wound assay

$1.2*10^5$ HUVECs were seeded in 6-well plates and infected with a MOI of 150. Small areas of the confluent monolayers were disrupted by scratching three straight lines with a p200 pipette tip. Debris were removed by washing the cells once, stimulated with VEGF (25 ng/ml) and followed by microscopy with a 10x objective. To obtain the same fields during the image acquisition we used a Leica DM-IRBE microscope with automated staging.

Immunoprecipitation and Immunoblotting

$6*10^5$ HUVECs were seeded in 10-cm dishes and infected with a MOI of 300 with the indicated strains for 24 hours and then stimulated with 25 ng/ml VEGF for 5 min. Proteins from total cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybond-C, http://www.amersham.com), and examined using the indicated antibodies (1:1000). The secondary horseradish peroxidase (HRP)-conjugated antibody (1:2000) was visualized by enhanced chemiluminescence (http://www.perkinelmer.com). If indicated membranes were treated with strip-buffer (0.2 M Tris-HCl ph 6.8, 2% w/v SDS, 0.7% v/v mercaptoethanol) and reprobed.

Ca^{2+} mobilization measurements

5000 HUVECs were seeded per well of a 96-well plate and unless stated differently infected with a MOI of 300 in M199/10% FCS for 24 hours. Media was removed and cells were loaded with the fluorescent Ca^{2+} indicator Fluo-4 NW (http://www.invitrogen.com) for 45 min in the incubator in the dark. Following the manufacturers protocol baseline fluorescence was measured for two minutes before cells were stimulated with VEGF (25 ng/ml) or Thrombin (1 U/ml) respectively ATP

(100 μM) and fluorescence was recorded for another seven minutes before Ionomycin (0.5 μM) was added if indicated.

Acknowledgments

We are grateful to Arto Pulliainen for critically reading of the manuscript. We thank C. Mistl, Dr. R. Jayachandran, and P. Müller for technical assistance. The work was supported by grant 3100A0-109925/1 from the Swiss National Science Foundation (SNF) to C.D., grant 55005501 from the Howard Hughes Medical Institute (HHMI) to C.D., and a PhD fellowship from the Misrock Foundation to F.S. We acknowledge the donation of human umbilical cords from the University Women's Hospital Basel.

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Table 1 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	(Regnery *et al.*, 1992)
RSE247	Spontaneous Sm^r strain of ATCC 49882 ^{T}, serving as wild-type	(Schmid *et al.*, 2004)
RSE242	*ΔvirB4* mutant of RSE247	(Schmid *et al.*, 2004)
MSE150	*ΔbepA-G* mutant of RSE247	(Schulein *et al.*, 2005)
MSE154	*ΔbepA* mutant of RSE247	(Schmid *et al.*, 2006)
TRB288	*ΔbepC* mutant of RSE247	(Rhomberg *et al.*, 2008)
PGC80	*ΔbepD* mutant of RSE247	(Rhomberg *et al.*, 2008)
PGD20	*ΔbepE* mutant of RSE247	(Rhomberg *et al.*, 2008)
TRB222	*ΔbepF* mutant of RSE247	(Rhomberg *et al.*, 2008)
TRB223	*ΔbepG* mutant of RSE247	(Rhomberg *et al.*, 2008)
Plasmids		
pTR1000	Basic mutagenesis vector to generate in-frame mutants of RSE247	(Schmid *et al.*, 2004)
pTR1069	Derivate of pTR1000 to generate a Δ*bepC* in-frame mutant in *Bh*	(Scheidegger *et al.*, 2008)
pPG161	Derivate of pTR1000 to generate a Δ*bepD* in-frame mutant in *Bh*	(Scheidegger *et al.*, 2008)
pPG162	Derivate of pTR1000 to generate a Δ*bepE* in-frame mutant in *Bh*	(Scheidegger *et al.*, 2008)
pTR1075	Derivate of pTR1000 to generate a Δ*bepF* in-frame mutant in *Bh*	(Scheidegger *et al.*, 2008)
pTR1078	Derivate of pTR1000 to generate a Δ*bepG* in-frame mutant in *Bh*	(Scheidegger *et al.*, 2008)

Figure 1: *Bh* VirB/VirD4/Bep system interferes with VEGF-induced spheroid sprouting

Spheroids made from uninfected HUVECs respectively HUVECs infected with the indicated strains were embedded in collagen and either stimulated or not with VEGF (25 ng/ml). (A) Quantification of *in vitro* angiogenesis of collagen embedded spheroids after 24 h by calculating the cumulative length of sprouts originating from an individual spheroid (CSL). Shown are means +/- SD of ten spheroids per experimental group. Differences between experimental groups were analyzed by Student's t-test. $p < 0.05$ was considered statistically significant. (B) VEGF sprout factor was calculated by dividing CSL stimulated by VEGF by unstimulated CSL. Shown are means +/- SD of the indicated number of experiments (C) Bright field images of one-day-old collagen embedded HUVEC spheroids. Shown are representative images demonstrating strong capillary-like sprouting of spheroids upon stimulation with VEGF for control spheroids as well as spheroids from Δ*bepA-G*-infected HUVECs, but interference with responsiveness to VEGF by infection with *Bh* wild-type.

Figure 2: *Bh* VirB/VirD4/Bep system interferes with VEGF-induced proliferation and wound closure

(A) Cell numbers were determined after 4 days of infection in presence and absence of VEGF (25 ng/ml). Proliferation was stimulated by infection, especially with the Δ*bepA-G* mutant. Whereas proliferation could be further increased in uninfected cells or HUVECs infected with the Δ*bepA-G* mutant, *Bh* wild-type completely blocked the effect of exogenous VEGF. Shown are average numbers and SD of ten acquired images (B) Confluent cell layers, uninfected or pre-infected with the indicated strains for 24 h, were wounded by scratching three straight lines with a pipette tip, stimulated

with VEGF (25 ng/ml) and followed by microscopy. Stimulation of uninfected HUVECs with VEGF led to complete closure of the wound after two days. Pre-infection with *Bh* wild-type severely interfered with cell migration even in the presence of VEGF, whereas HUVECs pre-infected with the Δ*bepA-G* mutant readily moved to fill the damaged area. Shown are representative bright field images.

Figure 3: Changes in VEGF receptor 2 Tyr1175 signalling upon infection

HUVECs were infected with the indicated strains or left uninfected for 24 h before being subjected to VEGF (25 ng/ml) for 5 min. Total cell extracts were separated by SDS-PAGE and immunoblots were performed with the indicated antibodies. General tyrosine-phosphorylation of VEGFR2 upon stimulation with VEGF was reduced in HUVECs infected with *Bh* wild-type. *Bh* wild-type interfered with Tyr1175 phosphorylation of VEGFR2, consequently PLCγ1 was less recruited and thus not activated as shown by decreased phosphorylation of PLCγ1 and weaker signal for total PLCγ1. VEGF-stimulated downstream Erk1/2- and less pronounced FAK-phosphorylation were affected. These observations were dependent on a functional VirB/VirD4 T4SS and at least one of the Beps. Phosphorylation of p38 MAP Kinase was generally slightly increased upon infection. No differences were observed for phosphorylation of AKT/PKB and Src. For total PLCγ1, phospho-SRC (Tyr529), and total FAK imunnoblots for phospho-PLCγ1, phospho-SRC (Tyr418), and phospho-FAK respectively were stripped and reprobed.

Figure 4: VirB/Bep-dependent interference with receptor tyrosine kinase specific calcium flux upon stimulation with VEGF

Uninfected HUVECs respectively. HUVECs infected with the indicated strains were loaded with the calcium indicator Fluo4. Baseline fluorescence was measured for two minutes, then cells were stimulated with VEGF (25 ng/ml) or Thrombin (1 U/ml) respectively ATP (100 µM) and fluorescence was recorded for another seven minutes before Ionomycin (0.5 µM) was added if indicated. (A) *Bh* wild-type interferes with calcium flux initiated by VEGF in a MOI-dependent manner. (B) Interference is VirB/Bep-dependent. (C) GPCR (Thrombin or ATP)-triggered calcium flux is not influenced by *Bh*.

Figure 5: Role of Beps in interference with calcium flux upon stimulation with VEGF

Uninfected HUVECs respectively HUVECs infected with *Bh* wild-type or the indicated inframe-deletion mutants were loaded with the calcium indicator Fluo4. Baseline fluorescence was measured for two minutes, then cells were stimulated with VEGF (25 ng/ml) and fluorescence was recorded for another seven minutes. Double-deletion of *bepC* and *bepG* lead to loss of interference, surprisingly also the knockout of *bepA*.

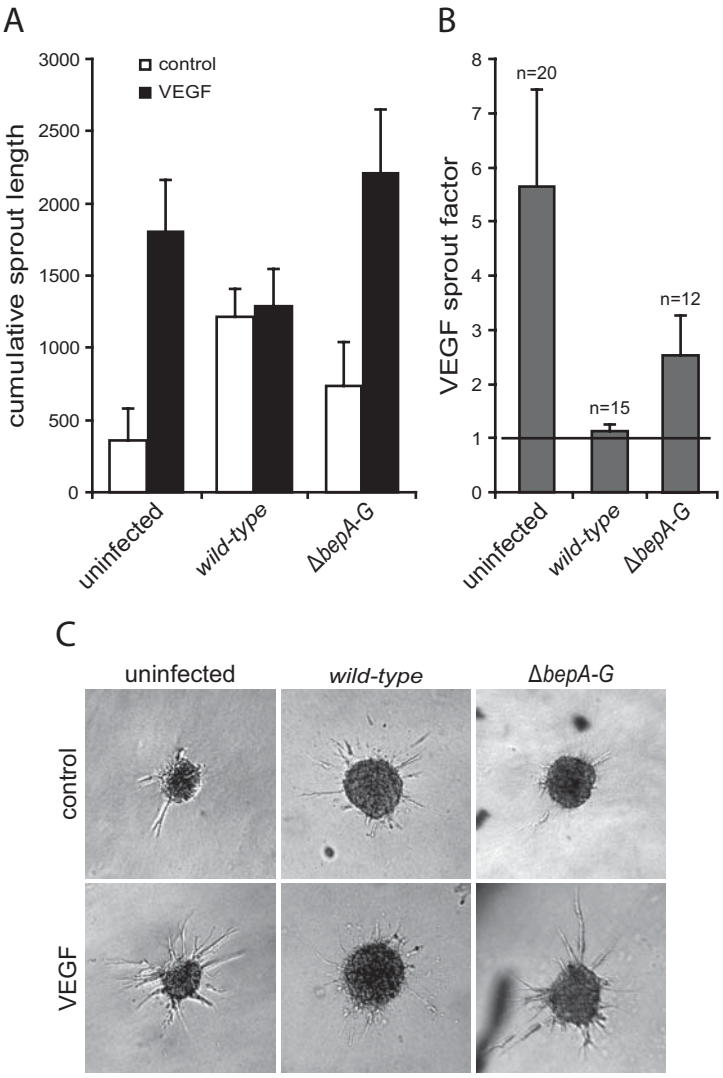
A
B
C
control
VEGF
cumulative sprout length
3000
2500
2000
1500
1000
500
0
uninfected
wild-type
ΔbepA-G
VEGF sprout factor
8
7
6
5
4
3
2
1
0
n=20
n=15
n=12
uninfected
wild-type
ΔbepA-G
control
VEGF

Figure 1

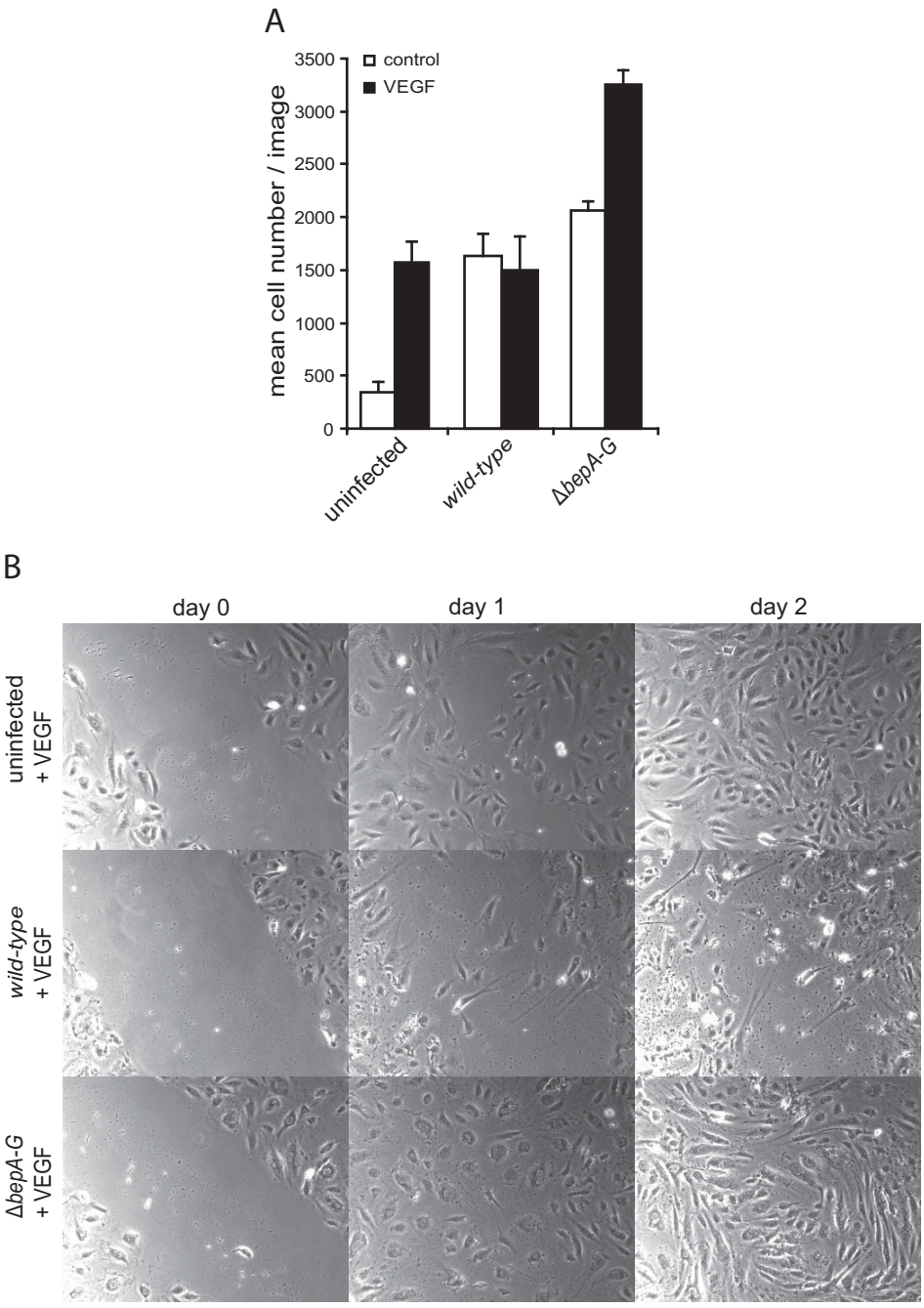
A
control
VEGF
mean cell number / image
3500
3000
2500
2000
1500
1000
500
0
uninfected
wild-type
ΔbepA-G
B
day 0
day 1
day 2
uninfected + VEGF
wild-type + VEGF
ΔbepA-G + VEGF

Figure 2

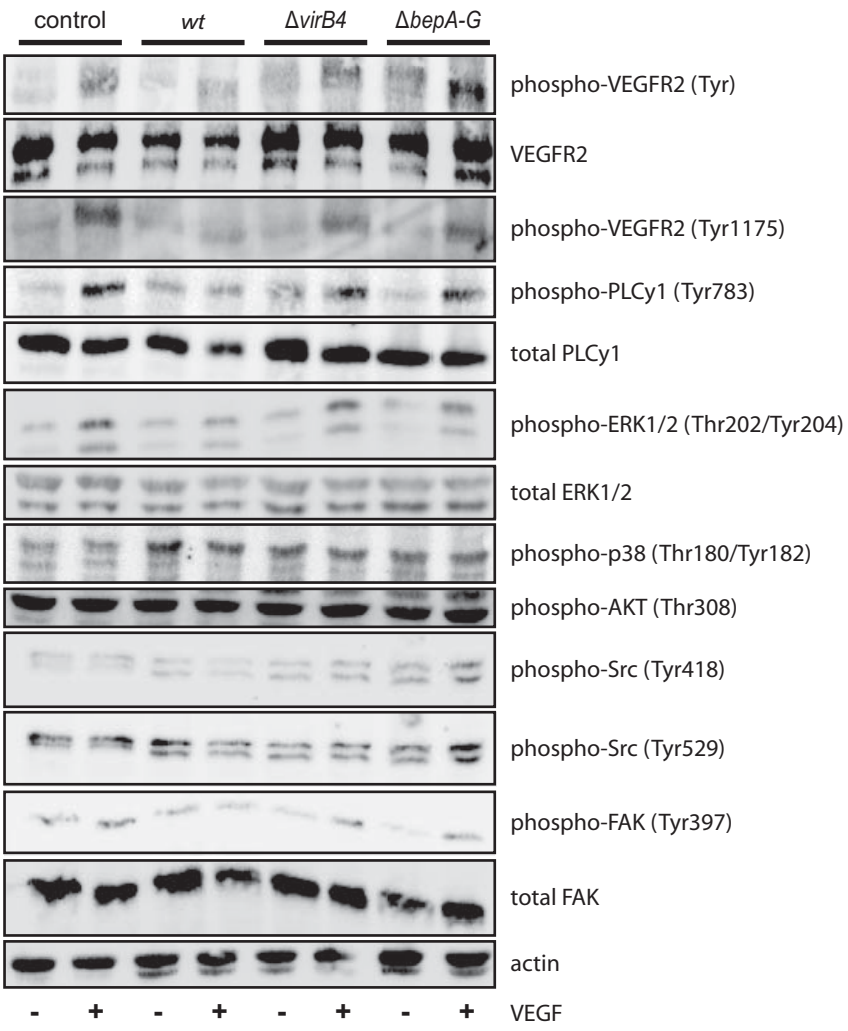
control
wt
ΔvirB4
ΔbepA-G
phospho-VEGFR2 (Tyr)
VEGFR2
phospho-VEGFR2 (Tyr1175)
phospho-PLCy1 (Tyr783)
total PLCy1
phospho-ERK1/2 (Thr202/Tyr204)
total ERK1/2
phospho-p38 (Thr180/Tyr182)
phospho-AKT (Thr308)
phospho-Src (Tyr418)
phospho-Src (Tyr529)
phospho-FAK (Tyr397)
total FAK
actin
- + - + - + - +
VEGF

Figure 3

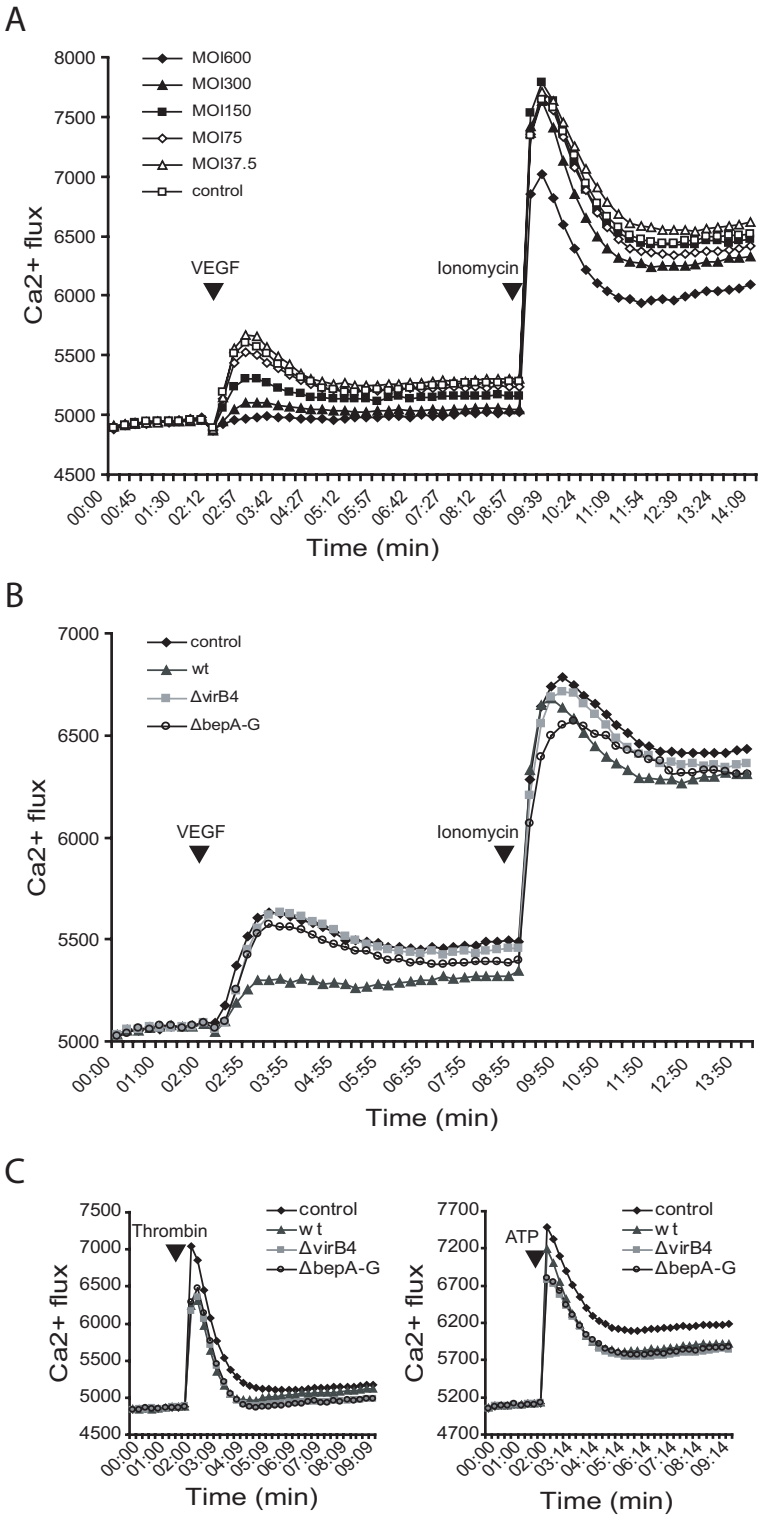
A
MOI600
MOI300
MOI150
MOI75
MOI37.5
control
VEGF
Ionomycin
Ca2+ flux
Time (min)
B
control
wt
ΔvirB4
ΔbepA-G
VEGF
Ionomycin
Ca2+ flux
Time (min)
C
Thrombin
control
wt
ΔvirB4
ΔbepA-G
Ca2+ flux
Time (min)
ATP
control
wt
ΔvirB4
ΔbepA-G
Ca2+ flux
Time (min)

Figure 4

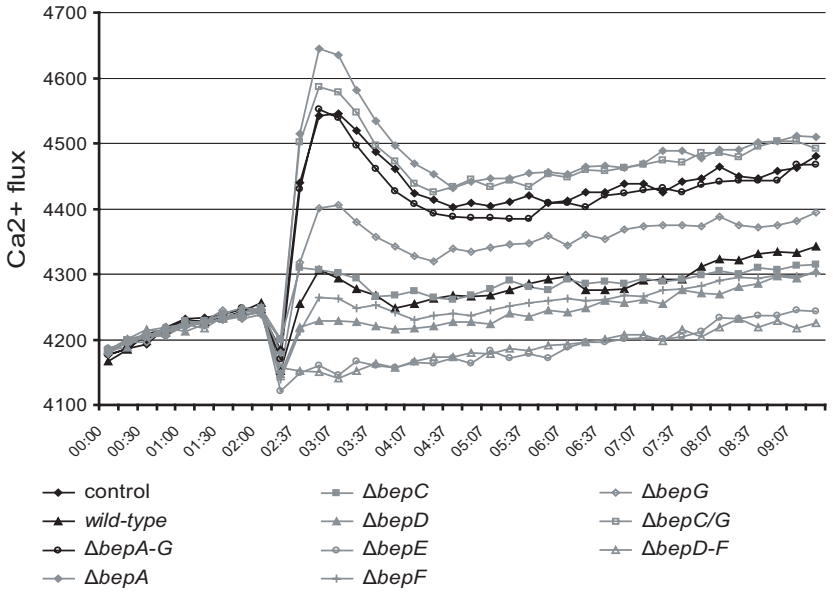
4700
4600
4500
4400
4300
4200
4100
Ca2+ flux
00:00
00:30
01:00
01:30
02:00
02:37
03:07
03:37
04:07
04:37
05:07
05:37
06:07
06:37
07:07
07:37
08:07
08:37
09:07
control
wild-type
ΔbepA-G
ΔbepA
ΔbepC
ΔbepD
ΔbepE
ΔbepF
ΔbepG
ΔbepC/G
ΔbepD-F

Figure 5

Chapter 4

The Role of the VirB/VirD4/Bep System in the Paracrine Loop Model of *Bartonella henselae*-triggered VEGF Production (unpublished)

The vascular endothelial growth factor (VEGF) is an essential regulator of angiogenesis under physiological as well as pathological conditions (Takahashi and Shibuya, 2005). Evidence for a role of VEGF in *Bartonella henselae* (*Bh*)-induced vasoproliferation came from co-culture experiments done with Ea.hy926 cells (a fusion of human umbilical vein endothelial cells, HUVECs, with the lung carcinoma cell line A549), HeLa cells (an epithelial cervical cancer cell line), or THP-1 cells (a human acute monocytic leukemia cell line) where it was shown that upon infection with *Bh* those cells secrete biologically active VEGF. Either taken as a model for monocytes recruited to the site of infection or cells of epidermal origin surrounding blood vessels, those cells would provide a paracrine source of VEGF stimulating endothelial cell (EC) proliferation and thus contributing to bacillary angiomatosis and peliosis (BA/BP) (Resto-Ruiz *et al.*, 2002; Kempf *et al.*, 2001).

Results

VirB/Bep-induced increase in VEGF secretion upon infection of HeLa cells with *Bartonella henselae*

To address whether the VirB/VirD4/Bep system affects VEGF production, I measured VEGF levels by ELISA in culture supernatants of HeLa and Ea.hy926 cells infected with a MOI of 300 with different *Bh* mutant strains. Stimulation with 25 ng/ml PMA (phorbol-12-myristate-13-acetate) served as control. As previously observed HeLa cells produced large amounts of VEGF upon exposure to *Bh* (Fig. 1A). Infection with *Bh* wild-type even exceeded the effect of PMA. The deletion of *virB4*, leading to a non-functional T4SS, reduced VEGF secretion, which could be restored by *in trans*-complentation of *virB4* on a plasmid. Comparable to the Δ*virB4* mutant a Δ*bepA-G* mutant, lacking the known effector proteins, triggered less VEGF, indicating that at least one of the Beps is responsible for the induction of increased VEGF production. Similarly also Ea.hy926 were stimulated to secrete VEGF in a VirB/Bep-dependent manner, although showing in general low levels (Fig. 1B). Dead bacteria were not able to induce VEGF production (data not shown).

Initial experiments with THP-1 cells showed an increased VEGF production upon infection in general for monocytes (Fig. 1C) and indicated a slight T4SS-dependent effect on top of a VirB-independent action in PMA-differentiated THP-1 macrophages (Fig. 1D). Noteworthy are the already high levels of VEGF in uninfected cells.

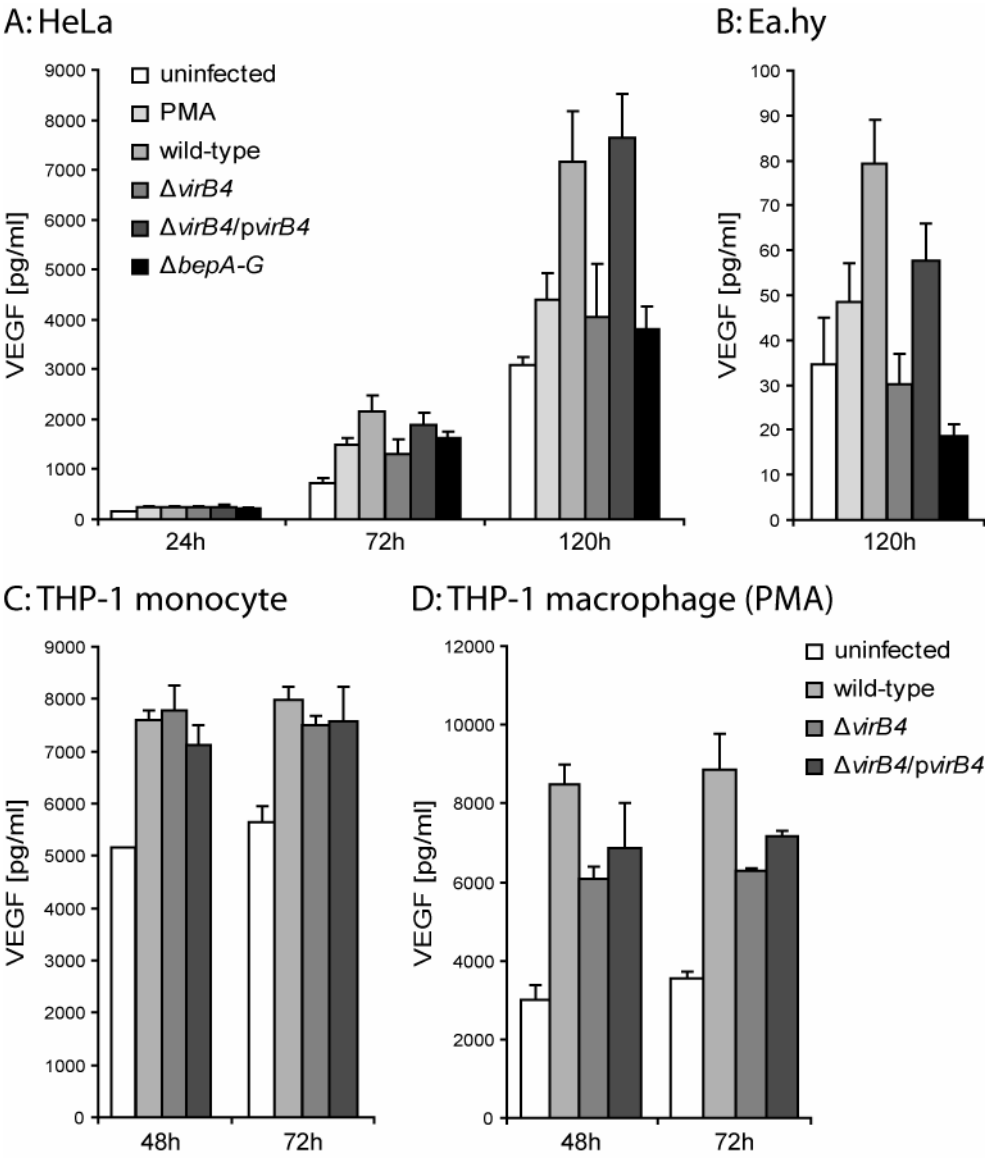

Figure 1 VEGF production of (A) HeLa cells, (B) Ea.hy926 cells, (C) THP-1 monocytes, and (D) THP-1 macrophages (differentiated with 100ng/ml PMA, phorbol-12-myristate-13-acetate) infected with a MOI of 300 with different *Bh* mutant strains. Control cells were uninfected or stimulated with PMA (25 ng/ml). Determination of VEGF by ELISA in cell culture supernatants after 24, 48, 72, and 120 hours respectively. Shown are mean / SD of triplicates.

The VirB/VirD4 T4SS effector BepD promotes VEGF production in HeLa cells

I performed ELISA measurements of supernatants from infections with the Δ*bepB-G* mutant *trans*-complemented with single *bep* genes and compared resulting levels to those triggered by wild-type or the effector-less Δ*bepA-G* mutant to further elucidate which of the Beps are involved in triggering VEGF. I observed in this experiment an already strong VirB/Bep-independent level of VEGF production although still increased about 1.8-fold for wild-type infection. Infection with the Δ*bepB-G* mutant still expressing *bepA* showed a 1.3-fold increased stimulation of VEGF secretion in comparison to the Δ*bepA-G* mutant. On top of this, BepD further augmented VEGF production about 1.7-fold (2.2-fold if compared to the Δ*bepA-G* mutant) exceeding the amount of VEGF triggered by the wild-type as such (Fig. 2).

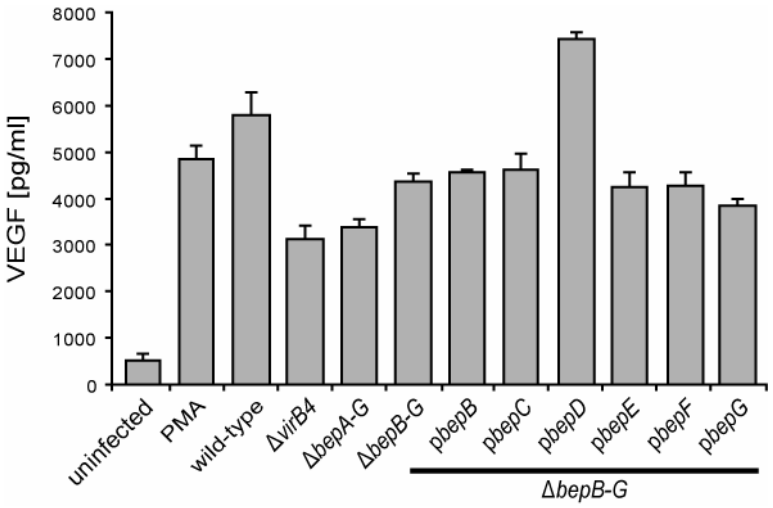

Figure 2 VEGF production of HeLa cells infected with a MOI of 300 with *Bh* wild-type, the effector-less Δ*bepA-G* and the Δ*bepB-G* mutant *trans*-complemented with single *bep* genes. Control cells were uninfected or stimulated with PMA (25 ng/ml). Determination of VEGF by ELISA in cell culture supernatants after 120 hours. Shown are mean / SD of triplicates.

BepD carries the classical bipartite translocation signal in its C-terminus, composed of the BID (Bep intracellular delivery) domain and an unconserved positively charged tail. The N-terminus harbours several minor repeats condensed in two major repeats with tyrosine-phosphorylation motifs, such as EPLYA and ETIYA. Indeed, BepD becomes tyrosine-phosphorylated by host cell-derived tyrosine kinases upon translocation into ECs (Schulein *et al.*, 2005) and thereby possibly provides docking sites for cell signalling proteins (Backert and Selbach, 2005). To trigger VEGF production in HeLa cells both major repeats seemed to be needed (data not

shown). The importance of tyrosine-phosphorylation in eukaryotic signalling pathways as reversible switches (Hunter, 1995) makes it an attractive target for pathogens. By changing signalling cascades with tyrosine-phosphorylated proteins or adapters, virtually any function of the host cell can be accessed and subverted. Further studies could provide insights into how BepD is able to interfere with signalling pathways to alter host cell functions such as secretion of VEGF.

Acidification of cell culture supernatant upon VirB/VirD4 T4SS activity

During the ELISA experiments I noticed dramatic changes in the colour of the HeLa cell culture supernatant. Cell media often contain phenol red (PR, phenolsulfonephtalein) as a pH indicator to assure near-neutral pH needed for cell growth. Under normal conditions these growth media will have a pink-red colour. In cases were cells are overgrown by bacteria or waste products accumulate, the pH will gradually decrease turning the solution to orange and then yellow. Bacteria can stimulate the metabolism of the cultured cells but also can be themselves metabolically very active. The supernatant of HeLa cells stimulated with PMA or infected with the Δ*virB4* or the Δ*bepA-G* mutant was slightly shifted to a pale pink colour. For cells infected with *Bh* actually translocating effectors the colour of the cell culture supernatant turned to orange, even more yellowish for the Δ*bepB-G* mutant overexpressing BepD *in trans* on a plasmid (Fig. 3).

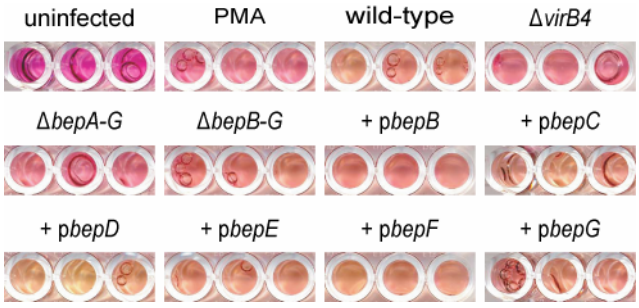

Figure 3 Colour changes of HeLa cell culture media after 120 h. Uninfected cells compared to PMA-stimulated cells or cells infected with a MOI of 300 with the indicated *Bh* strains.

The conversion of the pink form of PR to the orange form can be followed by measuring absorbance at 440 nm (acidic form of PR, yellow) and 560 nm (basic form of PR, red) respectively (Wang *et al.*, 2003). Indeed, measurements of absorbance indicating decrease of the basic and increase of the acidic form of PR paralleled the

visual observations, though did not show a significant difference for Δ*bepB-G* mutant overexpressing BepD (Fig. 4A).

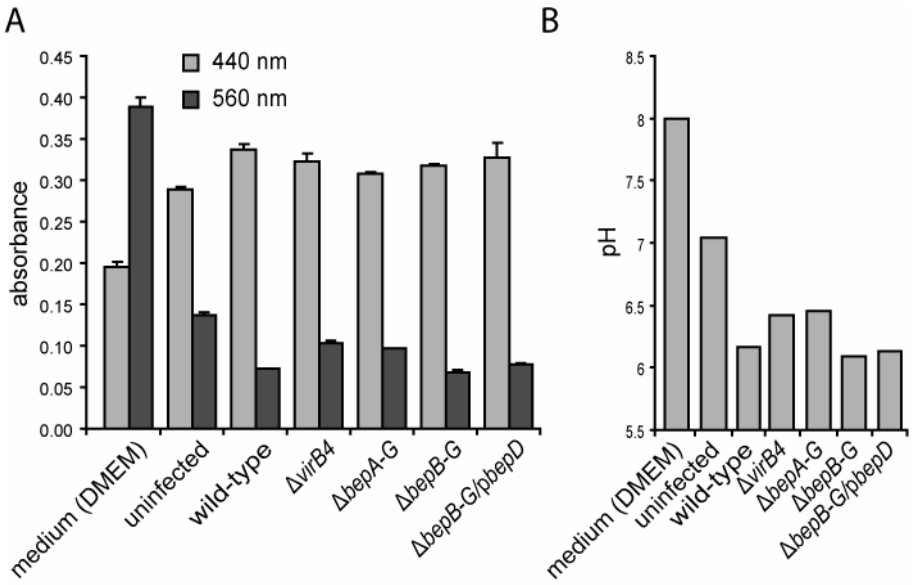

Figure 4 (A) Measurement of absorbance to follow the acidic form (440 nm, yellow) and basic form (560 nm, red) of phenol red respectively of HeLa cell culture supernatants. Shown are mean / SD of duplicates. (B) Corresponding measurement of pH.

A shift in colour depending on PR has also been reported for horseradish peroxide-mediated oxidation of PR by hydrogen peroxide (Pick and Keisari, 1980). Therefore I measured the pH of the HeLa cell culture supernatants (Fig. 4B). VirB/VirD4 T4SS translocation activity correlated with a decrease of pH, indicating that the colour changes observed are probably based on changes in pH. Interestingly, acidic extracellular pH has been shown to be able to induce VEGF mRNA expression and protein production (Shi *et al.*, 2001). Thus, the increase in VEGF secretion observed upon infection with the Δ*bepB-G* mutant background could be explained by decrease in pH associated with infection with *Bh* harbouring an active VirB T4SS translocating effector proteins.

Dulbecco's modified essential medium (DMEM), used in the aforementioned experiments, is a nutrient-rich growth media commonly used in mammalian tissue culture experiments usually buffered by addition of sodium bicarbonate. HEPES is a zwitterionic organic chemical buffering agent. Compared to bicarbonate buffers it is better at maintaining physiological pH despite changes in carbon dioxide. However, addition of HEPES to DMEM generally lowered absorbance measured at 560 nm but

did not prevent the shift in colour of the cell culture media upon infection (Fig. 5A) nor influenced the pattern of VEGF production (Fig. 5B).

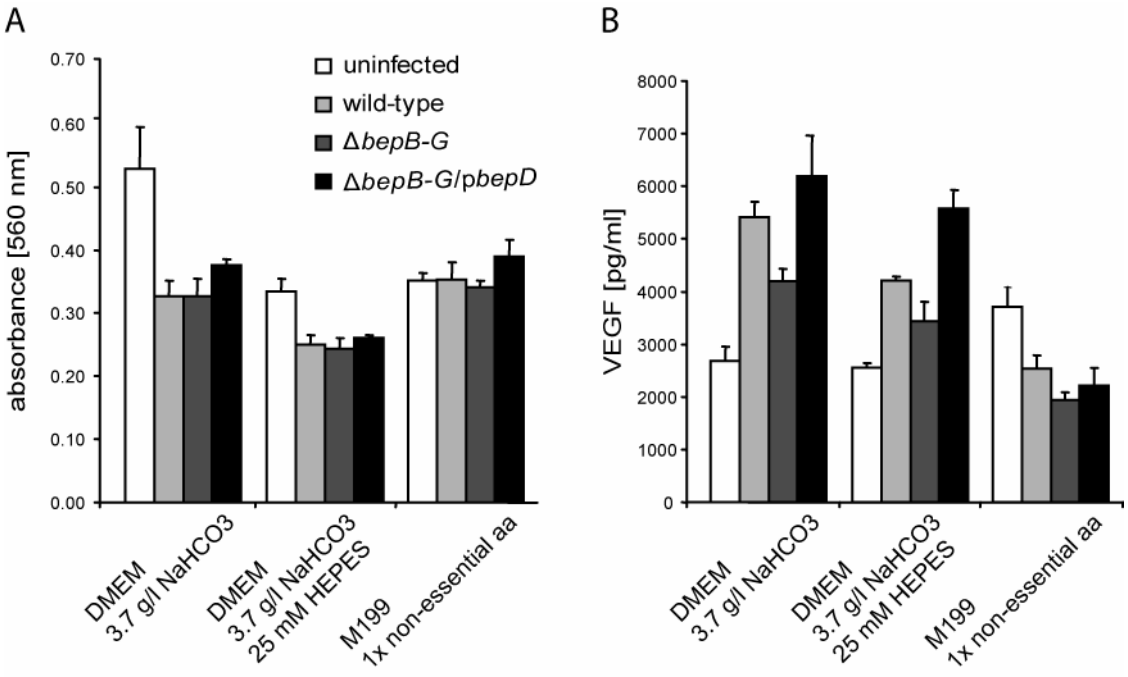

Figure 5 (A) Measurement of absorbance to follow the basic form (560 nm, red) of phenol red of HeLa cell culture supernatants. Shown are mean / SD of duplicates. (B) Corresponding measurement of VEGF production. Shown are mean / SD of triplicates.

Observations done by M. Quebatte in the lab revealed a narrow window of optimal pH for the expression of the *virB*- and *bep*-operons which is best reached performing the infections using the cell culture media M199 buffered with HEPES. I did not observe a shift in colour when using M199 supplemented with non-essential amino acids (Fig. 5A) and rather a *Bh*-dependent reduction than induction of VEGF production (Fig. 5B). HeLa cells severely suffered during prolonged infections performed in M199 regardless of the *Bh* strain used (data not shown), indicating that M199 might not cover the metabolic demands of HeLa cells during infection. This possibly explains the observed general reduction in VEGF secretion in *Bh*-infected cells. As for the source responsible for acidification of cell culture supernatant, a plausible explanation would be an increase in cell metabolism, especially glycolysis. A glycolytic phenotype has been proposed to arise as an adaptation to local hypoxia, resulting from limited oxygen, and altered glucose metabolism is considered a common property of tumours. Glycolysis under low oxygen settings requires first the

conversion of glucose to pyruvate and then to the waste product lactic acid. Thus, constitutive and persistence glylcolysis results in acidification of the local environment (Gatenby and Gillies, 2004). Proposed by Kempf *et al.* (Kempf *et al.*, 2005) inhibition of apoptosis and replication of intracellular bacteria resulting in high oxygen consumption and reduced ATP levels in the host cell would lead to the stabilization of the transcription factor hypoxia-inducible factor-1α (HIF1α). VEGF itself, glucose transporters, and glycolytic enzymes are amongst the various downstream targets of HIF1α (Kaelin, 2008).

Conclusions

In summary it remains unclear how *Bh* triggers VEGF production in HeLa cells and whether VEGF production and the pH shift in cell culture media I observed are connected “cause and effect” or parallel events. Infection with *Bh* could provide an acidic extracellular milieu inducing VEGF by for example increasing the metabolic need of their host cell or directly affecting the cell culture media by bacterial growth and VirB T4SS translocation activity. Alternatively, as already proposed, *Bh* might induce a hypoxia-like cell response stabilising HIF1α leading to a cell metabolism shifted toward glycolysis and stimulation of growth factors such as VEGF involved in angiogenesis. Especially the prominent action of BepD cannot be explained only by changes in pH or oxygen. Albeit I noticed a shift in cell culture supernatant going even more towards yellow for the overexpression of BepD in the Δ*bepB-G* background – BepD-triggered VEGF production neither correlated with additional shift in PR absorbance nor pH in comparison to the Δ*bepB-G* mutant, speaking for an additional mechanism probably involving tyrosine-phosphorylation and interference with host cell signalling.

An important fact to consider are the different *Bh* genotypes such as *Bh* Marseille (Drancourt *et al.*, 1996) and *Bh* Houston-1 (Regnery *et al.*, 1992) used to address the role of *Bh* in the paracrine loop model. Studies attributing a crucial role to the non-fimbrial adhesin BadA in the induction of a pro-angiogenic cell response were done with the *Bh* Marseille strain (Riess *et al.*, 2004), whereas I used as wild-type a spontaneous streptomycin-resistant variant of the Houston-1 clinical isolate and typing strain ATCC49882^T (Schmid *et al.*, 2004), previously used in our lab to characterize the VirB/VirD4/Bep system. For this strain, BadA expression is unknown so far. Whereas the original ATCC49882^T strain expresses BadA, a lab variant thereof lacks BadA. Loss of BadA expression, by single-base deletions or insertions and recombinations, upon extensive passaging on agar plates *in vitro* without any ecological pressure are proposed to affect BadA expression – resulting in the coexistence of BadA-positive and BadA-negative substrains within one *Bh* type strain (Riess *et al.*, 2007). However, I observed a prominent VirB/Bep-dependent induction of VEGF production upon infection with our *Bh* Houston-1 variant. Initial CRAFT experiments done to test effector translocation (Schulein *et al.*, 2005) indicate that the *Bh* Marseille does not show an efficient VirB T4SS translocation activity, especially not in the presence of BadA (Pulliainen, A., Teoh, J., unpublished

observation). Hence, possibly two independent mechanisms are able to elicit the observed induction of VEGF production in cells used as a model for a paracrine source upon infection with *Bh*. It is thus important to address both the presence of BadA and the activity of the VirB/VirD4/Bep system to understand *Bh*-host cell interaction.

Experimental procedures

Bacterial strains and Growth Conditions

The bacterial strains used are listed in Table 1 of chapter 2 (p. D-31). *Bartonella* spp. were grown on Columbia agar (CBA) plates containing 5% defibrinated sheep blood in a humified atmosphere at 35°C and 5% CO_2 for 2-3 days. Strain RSE247, a spontaneous streptomycin-resistant strain of ATCC 49882^T (Schmid *et al.*, 2004) served as wild-type in this study. 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, http://www.applichem.de) if needed.

Cell Culture

HeLa and Ea.hy926 cells were grown in Dulbecco's Modified Eagle Media (DMEM, Gibco, http://www.invitrogen.com) with 10% fetal calf serum (FCS, http://www.invitrogen.com). The human acute monocytic leukemia THP-1 cell line was cultured in RPMI 1640 medium (http://www.sigmaaldrich.com) with 10% FCS. Cells were grown in a humified atmosphere at 37°C and 5% CO_2.

Infection Assay

HeLa and Ea.hy926 cells were plated in 24-well plates at 10^5/ml using DMEM with 10% FCS. The next day cells were washed and unless stated differently infected with a multiplicity of infection (MOI) of 300 bacteria per cell in DMEM/10% FCS/500 µM IPTG and sedimented onto cultured cells by centrifugation for 5 minutes at 1800 rpm. Cells were incubated for the indicated time. For some assay M199 with Earls salts (M199, Gibco, http://www.invitrogen.com) and 10% FCS was used. The medium was supplemented with non-essential amino acids or 25 mM HEPES where indicated. THP-1 monocytes were directly seeded in 24-well plates at $7*10^5$/ml and infected with a MOI of 300 in RPMI/10% FCS/500 µM IPTG. Bacteria-cell contact was facilitated by centrifugation for 5 minutes at 1800 rpm. Alternatively, THP-1 cells were differentiated into macrophages by overnight treatment with 100 ng/ml phorbol-12-myristate-13-acetate (PMA, http://www.sigmaaldrich.com). Media was changed the next day and cells left for two days before there were infected as described for THP-1 monocytes.

Detection of VEGF in cell culture supernatants

Stimulation of VEGF secretion by PMA (25 ng/ml) served as a positive control. At the indicated timepoints cell culture supernatants were collected, centrifuged for 3 minutes at 12k rpm at 4°C and stored at -20°C. VEGF concentration in culture medium was measured using a human $VEGF_{165}$-ELISA kit (http://www.rndsystems.com) following the manufacturer's instructions.

Measurement of absorbance of cell culture supernatants

To measure the absorbance at 560 nm and 440 nm respectively, 100 µl of cell culture supernatants were transferred to a 96-well plate and assed with a SpectraMax M2 microplate reader (http://www.moleculardevices.com).

Determination of pH of cell culture supernatants

300 µl of cell culture supernatants were transferred to 1.4 ml U-tubes (http://www.micronic.com) and pH was measured with a high-precision pH meter (827 pH lab, http://www.metrohm.com).

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Summary

Summary

Bartonella henselae (*Bh*) rarely shows any symptoms in its feline reservoir host but is responsible for a number of clinical outcomes during infection of the human incidental host. *Bh* shares with *B. quintana* (*Bq*) and *B. bacilliformis* (*Bb*) the remarkable capacity to induce tumour-like vascular proliferations. The study of *Bartonella*-host cell interactions might not only provide insides into the pathogenesis of *Bh* but might also serve as a model to investigate general mechanisms involved pathological angiogenesis, which is a hallmark of tumour growth. The *Bh* VirB/VirD4 type IV secretion system (T4SS) and the thereby translocated *Bartonella*-effector proteins (Beps) have been shown to be responsible for the subversion of a number of endothelial cell (EC) functions upon infection. This thesis aimed to elucidate the role of the VirB/VirD4/Bep system in *Bh*-triggered angioproliferation.

The first chapter characterizes the *Bh* VirB T4SS translocated protein BepA as an effector mediating the inhibition of apoptosis in human umbilical vein endothelial cells (HUVECs). Either overexpressed in the effector-less Δ*bepA*-G mutant or ectopically expressed, BepA was sufficient to promote the anti-apoptotic activity. Delineation of BepA revealed that this activity is confined to the BID (Bep intracellular delivery) domain of BepA. Interestingly, only the homologue BepA2 (corresponding to the BID domain plus C-terminus of BepA) from *Bq* inhibited apoptosis as well. In contrast to *Bh* and *Bq*, *B. tribocorum* is not associated to vascular proliferation and its BepA-homologue did not show any anti-apoptotic activity. Upon translocation into the host cell BepA was targeted to the membrane. BepA-mediated anti-apoptosis correlated with an increase in cAMP and increased expression of cAMP-responsive genes, pointing to a mechanism involving the regulation of this second messenger. BepA not only inhibited chemically ActD-induced but also CTL-triggered apoptosis. Thus, BepA has the potential to play a role in vasoproliferation in an indirect way by enhancing cell survival.

Chapter 2 presents a spheroid-based three-dimensional *in vitro* sprouting assay established to address the angiogenic potential of *Bh*. Compared to spheroids from uninfected HUVECs, spheroids from HUVECs pre-infected with *Bh* wild-type showed an increased sprouting activity, albeit sprout morphology was distinct from

the sprouts induced by vascular endothelial growth factor (VEGF). Formation of those sprouts was in part VirB/Bep-dependent. Overexpressed BepA in the Δ*bepA*-G mutant strongly promoted sprout formation. Delineation of the domain required for this stimulation indicates parallels to the anti-apoptotic activity. BepD showed a moderate sprout-promoting effect. In contrary, BepG, involved in cytoskeletal rearrangement, displayed a potent interference with sprout formation. This novel *in vitro* model of *Bh*-triggered sprouting angiogenesis revealed distinct activities of the Beps in modulating sprout formation and contributing to the regulation of the *Bh* angiogenic activity in the course of the chronic vascular infection.

In chapter 3 the effect of exogenous VEGF on *Bh*-infected ECs is addressed. VEGF is thought to be involved in *Bh*-induced vascular tumour formation by promoting EC proliferation in a paracrine manner. Assessing the biological activity of VEGF in assays such as proliferation, wound assay and capillary-like sprout formation revealed an intriguing interference of the VirB/VirD4/Bep system with responsiveness of infected ECs to stimulation with VEGF. Analysis of the VEGF receptor 2 pathways showed that *Bh* inhibited phosphorylation of tyrosine 1175. In *Bh*-infected ECs stimulated with VEGF, PLCγ1 was less recruited and phosphorylated and consequently downstream calcium flux and ERK1/2 activation were blocked. These data rather challenge the idea of a VEGF-driven paracrine loop in the presence of an active VirB/VirD4/Bep system and emphasize the modulatory role of the VirB T4SS balancing the angiogenic activity of *Bh*.

The last chapter reports the role of the VirB/VirD4/Bep system in the paracrine-loop model of VEGF production. HeLa cells produced VEGF upon exposure to *Bh* in a VirB/Bep-dependent manner. BepD elicited a strong stimulation of VEGF secretion in HeLa cells when overexpressed in the Δ*bepB*-G mutant. A shift in colour of the cell culture supernatant, depending on the cell culture media used, was associated to VirB T4SS translocation activity but not particularly to VEGF production. The shift probably is due to acidification of the supernatant. Hence, in addition to the previously reported BadA also the VirB/VirD4/Bep system, especially BepD, independently is able to trigger the production of an essential regulator of angiogenesis.

Concluding remarks

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The anti-apoptotic effector BepA

The protection of ECs against apoptosis by *Bh* can be mediated by a single effector, BepA (chapter 1) (Schmid *et al.*, 2006).

Knowledge of the cellular binding partners and interactions of BepA is a requirement for understanding the association of BepA, cAMP, and anti-apoptosis. Ongoing work approaches this issue by mass spectrometry-based proteomics (Aebersold and Mann, 2003). Classical co-immunoprecipitation and co-localization studies as well as functional approaches such as small interfering RNA (siRNA) knockdown experiments and ectopic expression of variants can be used to validate potential hits. An image-based siRNA screen combined with assays involving inhibitors, antibodies, drugs, or molecular probes might further give insights into the host cell signalling pathways targeted by BepA to mediated anti-apoptosis.

Manipulation of EC apoptosis has been proposed to accompany as well the mitogenic stimulation observed for *Bq* (Liberto *et al.*, 2004; Kirby and Nekorchuk, 2002). The human specific *Bq* is thought to have evolved from *Bh* by reductive evolution (Alsmark *et al.*, 2004) and indeed showed an inhibition of apoptosis depending on the VirB/VirD4 T4SS and the *Bq* BepA homologue (chapter 1) (Schmid *et al.*, 2006). Interestingly, *Bartonella* species not associated to angioproliferative lesions such as *B. vinsonii* or *B. elizabethae* did not inhibit cell death (Kirby and Nekorchuk, 2002). Likewise, neither *B. tribocorum* nor its BepA did show any anti-apoptotic activity (chapter 1) (Schmid *et al.*, 2006). In contrast to *Bh* and *Bq*, *Bb* lacks a corresponding VirB/VirD4/Bep system (Saenz *et al.*, 2007). GroEL has been proposed to affect EC apoptosis during co-culture of cells with *Bb* (Smitherman and Minnick, 2005), but so far there are no data showing if *Bb* would influence ECs challenged with for example chemically ActD-induced apoptosis.

Another fact that needs to be taken into account is the prominent activation of an NF-κB-dependent pro-inflammatory phenotype in ECs upon infection with *Bh*, which is even though in part depending on the VirB/VirD4 T4SS not associated to BepA (Schmid *et al.*, 2006; Schmid *et al.*, 2004). NF-κB has been shown to have a role in the regulation of the apoptotic program (Karin and Lin, 2002) indicating a possible additional mechanism how *Bh* could manipulate EC cell death.

BepA was able to promote capillary-like sprout formation in a spheroid-based three-dimensional *in vitro* model of sprouting angiogenesis. Deletion of *bepA* significantly reduced sprouting induced by *Bh*, which indicates a prominent role for BepA in *Bh*-triggered sprouting angiogenesis. An initial delineation indicates parallels to the anti-apoptotic activity of BepA (chapter 2) (Scheidegger *et al.*, 2008). Further research is needed to elucidate whether indeed the ability of BepA to induce sprouting is directly connected to inhibition of cell death and whether cAMP for example is involved. Knowing the cellular interaction partners of BepA might here as well give insights into the pathways manipulated by BepA. Comparing sprouting induced by ectopic expression of BepA to sprout formation of spheroids from HUVECs pre-infected with the Δ*bepA-G* mutant overexpressing BepA, might point to synergistic effects of inhibition of apoptosis and VirB/VirD4 T4SS-independent mitogenic stimulation. Similarly, VEGF is known to act on ECs not only as a mitogen but also as a survival factor (Ferrara *et al.*, 2003).

Surprisingly, deletion of *bepA* was sufficient to unlock the antagonizing effect on VEGF-induced calcium flux otherwise observed for *Bh* wild-type (chapter 3). It remains to be shown whether in ECs infected with a Δ*bepA* mutant and stimulated with VEGF the phosphorylation of VEGFR2 Tyr11775, PLCγ1 recruitment and activation, and downstream ERK1/2-phosphorylation follow the control pattern observed for uninfected ECs. The double knockout of *bepC* and *bepG* behaved as the Δ*bepA* mutant in the calcium assay. Thus, the presence alone of BepA is not responsible for the interference with VEGF signalling. How BepA presumably together with BepC and BepG is able to manipulate EC responsiveness to VEGF and whether this is a direct action or the indirect result of manipulation of EC function by the Beps, remains to be discovered.

The recurrent involvement of BepA puts a strong emphasis on this VirB/VirD4 T4SS effector. The specificity of the observations connected to BepA, notably to the BID domain of BepA, is astonishing. BepB and BepC are paralogues of BepA neither showing any anti-apoptotic effect nor affecting sprouting (chapter 1 and 2) (Scheidegger *et al.*, 2008; Schmid *et al.*, 2006). Moreover, the BID domain is part of the bipartite translocation signal found in all Beps and as well in the conjugative relaxases of plasmid-borne bacterial conjugation systems (Schulein *et al.*, 2005). Structural information might help to solve the uniqueness lying in the BID domain of BepA. In the end yet, the relevance and actual contribution of the anti-apoptotic and

possible other activities of BepA to the process of vascular tumor formation remains a central question and calls for an appropriate animal model to study *Bartonella*-triggered vasoproliferation *in vivo*. However, BepA clearly belongs to those bacterial effectors displaying a tumourigenic potential.

A three-dimensional *in vitro* model for sprouting angiogenesis

The established spheroid-based *in vitro* model of sprouting angiogenesis revealed pronounced yet opposing effects of individual Beps on sprout formation (chapter 2) (Scheidegger *et al.*, 2008).

Each *in vitro* assay has its benefits and limitations. Moving from cell monolayers to three-dimensional cultures is motivated by the need to work with cellular models that mimic the functions of living tissues, but where complexity is reduced to a defined setup and standardized experimental conditions can be applied (Pampaloni *et al.*, 2007). Issues to consider using such a three-dimensional assay are the need of advanced imaging techniques to visualize moderately thick specimens and complicated biochemical analysis due to the lower cell density and the additional steps required to separate the cells from the matrix. Spinning-disk confocal laser microscopy (CLSM), used in this work to image spheroids, offers a fast image acquisition and represents the commercially available state-of-the-art imaging method, but like other CLSM suffers from a limited penetration depth, marked difference in lateral and axial resolution, and sample bleaching. Selective plane illumination microscopy (SPIM), where the sample is not illuminated by a light volume but a light sheet, has the potential to overcome the limitations of CLSM (Huisken *et al.*, 2004). Considering the matrix itself, most of the currently available ECM gels are extracted from animals or cultured cells and hence quality control is difficult. Components between batches may vary and affect the reliability and reproducibility of the assay (Pampaloni *et al.*, 2007).

The collagen gel-embedded HUVEC spheroid model adapted during this work represents a validated quantitative and robust *in vitro* assay of sprouting angiogenesis (Korff and Augustin, 1999). Amongst the advantages over other three-dimensional assays such as cord formation or formation of capillary networks originating from single gel-embedded cells, the spheroid-based assay better reflects the angiogenic process originating from the confluent endothelium of the pre-existing vessel still allowing the assessment of both pro- as well as anti-angiogenic factors.

Furthermore, the assay is assessable by treatment of gel-embedded spheroids with soluble compounds such as inhibitors, activators or antibodies and ECs can be manipulated before the formation of spheroids by for example siRNA knockdown or ectopic expression of genes of interest. This provides the possibility to elucidate the molecular basis of the observed angiogenic activities of *Bh*. The fact that spheroids as used for *in vitro* sprouting angiogenesis can also be used *in vivo* (Alajati *et al.*, 2008) offers an attractive opportunity to refine the spheroid-based model of *Bh*-triggered sprouting towards the establishment of the badly needed animal model.

Interference with VEGF signalling

The presence of an active VirB/VirD4/Bep system intriguingly inhibited EC responsiveness to VEGF (chapter 3). Yet, VEGF production was increased in a VirB/Bep-dependent manner in epithelial HeLa cells (chapter 4).

Time-course experiments are needed to evaluate the effects of *Bh*-infection on the phosphorylation kinetics of VEGFR2 and downstream signalling components in ECs to validate the so far observed phosphorylation pattern. Additional readouts such as the measurements of PIP_2, the precursor for the second messengers DAG and IP_3 (Berridge and Irvine, 1984), nitric oxide (NO) required for VEGF-induced vascular permeability (Fukumura *et al.*, 2001; Busse and Mulsch, 1990) or prostacyclin (PGI_2) acting together with NO on vascular smooth muscle cells (VSMCs) to promote vasodilation and increased blood flow production (Li *et al.*, 2002) might further give insights into the VEGFR2 Tyr1175 downstream cascade manipulated by *Bh*. Careful analysis of VEGFR2 expression would strengthen the fact that the observed interference is due to direct influence on signalling and not to reduced presence of the receptor. Deciphering how the Beps interfere with VEGF-signalling is a challenge, which likely requires the understanding of the interplay of BepA, BepC and BepG.

The cellular and molecular mechanisms underlying VirB/Bep-induced production of VEGF remain unclear. Albeit a wealth of information on fundamental process that occurs in human cells, both normal and abnormal, was gained using the cancer-derived HeLa cells as a model system, there is no common stock of HeLa cells but these cells show genetic and phenotypic differences as a result of genetic drift and being subjected to different conditions in various laboratories (Masters, 2002). Thus, a major concern regarding the approach taken to propose the paracrine

loop is the use of those cells as model for an epithelial cell surrounding a blood vessel or even a monocytic cell. Experiments using THP-1 or MonoMac6 cells as model for the macrophages present in bacillary angiomatosis (BA) (Arrese Estrada and Pierard, 1992) are more convincing. Macrophages are indeed capable to produce potent angiogenic factors upon activation (Sunderkotter *et al.*, 1994). SMCs involved in the stabilization of the blood vessels would represent cells in close contact to ECs (Armulik *et al.*, 2005) and could possibly be a target for paracrine activity on ECs. Reports about an interaction of *Bartonella* with SMCs are restricted to the observation that SMCs do not proliferate upon co-culture with *Bartonella* (Maeno *et al.*, 1999; Garcia *et al.*, 1990).

In summary, whereas experiments done with HeLa cells indicate an involvement of the VirB/VirD4 T4SS in the paracrine-loop model, the relevance of this VEGF-based model of paracrine support of EC proliferation itself, in presence of an active VirB/VirD4 T4SS, is questioned. Thus, the careful choice of a model cell line for *in vitro* experiments is part of the crucial parameters influencing the interpretation of the role of the VirB/VirD4/Bep system in *Bh*-triggered angioproliferation. Hence it would be crucial to know with which cell type *Bh* actually interacts *in vivo*.

Bartonella henselae, the VirB/VirD4/Bep system and tumour-like vasoproliferative lesions

The knowledge about the involvement of the VirB/VirD4 T4SS in *Bh*-host cell interaction gathered using different model systems revealed both pro- as well as angiogenic traits lying in the VirB/VirD4/Bep system. Many of those effects have been addressed individually to reduce complexity. Often, although observing a clear phenotype for the Δ*bepA-G* mutant lacking the known effectors, it remains unclear which Beps actually are involved. Hence, the timing of effector translocation, the quantities of translocated Beps and the composition of the effector set injected into a given cell type is an open question possibly determining whether pro- or anti-angiogenic activities of the VirB/VirD4 T4SS would prevail.

Nevertheless, interference of the VirB/VirD4/Bep system with both potent intrinsic (direct mitogenic stimulus) and extrinsic (paracrine-loop) pro-angiogenic *Bh* factors strongly argues against its function as a major element driving BA. Consistently, the human-specific *Bb* strongly promotes very similar vascular proliferative lesions (Garcia *et al.*, 1990) although it lacks the VirB/VirD4 T4SS

(Saenz *et al.*, 2007). The VirB/VirD4 T4SS has recently emerged as a potent host adaptation factor and is proposed to be a highly conserved molecular platform equipped with a set of different effectors to modulate the cellular habitats of the corresponding reservoir hosts (Dehio, 2008). In this respect, vasoproliferation associated to *Bh* in the human incidental host may as well represent a "biological accident" induced by the complex physiological changes resulting from chronic vascular colonization and a maladjusted action of the bacteria on cell signalling. Speculatively, the VirB/VirD4/Bep system therefore could have an important role as a balancing factor actually preventing drastic effect on its host such as BA but promoting subtle changes needed for a chronic infection. Again an appropriate animal model would be required to asses this hypothesis experimentally.

The causative role of *Bh* in vascular proliferation is clear. The involvement of the VirB/VirD4 T4SS though is ambiguous. It appears that the VirB/VirD4/Bep system represents a modulator controlling the angiogenic potential of *Bh* by affecting several EC functions connected to angiogenesis. *Bh* could indeed contribute to an anti-apoptotic, proliferative state prone to tumour initiation, promotion, and progression by perturbing cell signalling processes (Lax and Thomas, 2002). But the VirB/VirD4 T4SS also interferes with strong pro-angiogenic traits of *Bh*. Thus, the VirB/VirD4/Bep system clearly influences *Bh*-induced vasoproliferation, yet its causative role remains open for future research. A better understanding of the VirB/VirD4/Bep system during infection of the reservoir host might clarify as well the role of the VirB/VirD4 T4SS in pathogenesis during interaction with the incidental host. Furthermore, this could also give some indication whether induction of vascular proliferations indeed represent a dedicated infection strategy to expand the specific host cell habitat or whether it is a result of unbalanced interaction of *Bh* with its host. Research aiming at the understanding of mechanisms, such as the VirB/VirD4/Bep system, involved in the control of the balance between host and pathogen will help to answer the questions of how and to what extend bacteria are involved in tumourigenesis. Yet one needs to keep in mind that the molecular mechanisms underlying bacteria-induced tumour formation remain a challenge governed by the right mixture of multiple factors including bacterial properties, host factors, and environmental triggers.

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Acknowledgements

Acknowledgements

This work was carried out in the group of Prof. Christoph Dehio in the Focal Area of Infection Biology at the Biozentrum of the University of Basel.

I would like to thank Christoph Dehio for giving me the opportunity to expand my first steps in science into a fascinating journey that has been my PhD. I appreciated his support and guidance, analytical criticism and suggestions as well as the freedom given to explore an intriguing aspect of bacterial pathogen-host cell interaction.

I also want to thank Prof. Cécile Arrieumerlou and Prof. Jean Pieters for joining my PhD committee and accompanying me with helpful comments and discussion.

Many thanks to present and past members of the Dehio lab for all the help, discussions and chitchats and a great working atmosphere – Michaela Dehio, Phillip Engel, Marco Faustmann, Jérémie Gay-Fraret, Arnaud Goepfert, Patrick Guye, Barbara Hauert, Sonja Huser, Marius Liesch, Rusudan Okujava, Thomas Rhomberg, Henri Saenz, Hermine Schein, Michael Schmid, Gunnar Schröder, Ralf Schülein, Isabella Toller, David Tropel, and Matthias Truttmann.

For the critical and careful reading of my paper manuscripts I am very grateful to Arto Pulliainen.

I thank Nadège Devaux, Yvonne Ellner, Claudia Mistl, and Maxime Québatte for support as well as for taking care of the daily lab organization contributing to an excellent working environment.

I would like to acknowledge the valuable support by the many people of the technical and administrative staff of the 4th floor. Thank you very much for all those things running in the back of the every day business of science.

I enjoyed the fruitful scientific interactions with Abdullah Alajati, Oliver Siedentopf, and Holger Weber from the Tumor Biology Center of the University of Freiburg to establish the spheroid-model. Thanks as well to Rajesh Jayachandran and Philipp Müller from the Pieters' lab for helping me setting up the calcium assays.

Schliesslich ein grosses Danke an meine Eltern Véronique und Claude, an meine Schwestern Noémy, Camille und Moïra, an sonstige Verwandten und Freunde. Danke Dir Krystof für so Vieles. Ihr seid mein Rückhalt – die Basis für meine Abenteuer! Merci für euer Vertrauen, Unterstützung, Motivation, Aufmunterung und eure Neugier.

Curriculum vitae

Last Name Scheidegger
First Name Florine
Date of Birth 29.01.1980
Nationality Swiss

Adress Office
Klingelbergstrasse 50/70
CH-4056 Basel
+41 61 267 21 39
florine.scheidegger@stud.unibas.ch

Adress Private
Leuengasse 28
CH-4057 Basel
+41 61 354 63 68

Education and working experience

2004 – 2008	Ph.D. thesis Focal Area of Infection Biology, Biozentrum, University of Basel CH Supervisor, Prof. Dr. Christoph Dehio Committee Members, Prof. Dr. Cécile Arrieumerlou and Prof. Dr. Jean Pieters "The Role of the VirB/VirD4/Bep System in *Bartonella henselae*-triggered Vascular Proliferation"
2003 – 2004	Graduate Studies, awarded M.Sc. Division of Molecular Microbiology, Biozentrum, University of Basel, CH Supervisor, Prof. Dr. Christoph Dehio "Functional characterization of the type IV secterion substrates BepD1 and BepD2 of *Bartonella tribocorum*"
2000 – 2003	Undergraduate Studies in Molecular Biology University of Basel, CH
1996 – 1999	Matura Type C (university-entrance diploma) at the Gymnasium Oberwil, Oberwil BL, CH

Teaching experience

2004 – 2008	Assistant and tutor for undergraduate students in practical courses "Microbiology", once per year

Publications

Schmid MC, Scheidegger F, Dehio M, Balmelle-Devaux N, Schulein R, Guye P, Chennakesava CS, Biedermann B, Dehio C. (2006) A translocated bacterial protein protects vascular endothelial cells from apoptosis. PLoS Pathog. 2 (11): e115

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F. Scheidegger and C. Dehio. The *Bartonella henselae* VirB/VirD4/Bep System Interferes with VEGF-induced ERK1/2-mediated Endothelial cell proliferation. In preparation.