Proteins injected by the bacterial pathogen *Bartonella* subvert eukaryotic cell signaling

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for my family

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1. Introduction

Understanding the mechanisms of pathogenesis means having a glimpse into the hundreds of millions of years of common evolution between the various organisms existing on this planet. While most prominent pathogens elicit acute diseases, which we understandably feel and fear most, they are probably only short-lived sparks in the evolutionary timeline. They represent extreme situations, not well reflecting the massively parallel evolutionary optimization processes discreetly accompanying us in a hidden network of viruses, prions, catalytic RNAs, bacteria, plants and animals. As we discover and describe more and more highly adapted organisms being intimately involved and entangled in our lifestyles, behaviors and our own evolutionary success, the classical, restrained definition of species might well be outlived. Elucidating the molecular mechanisms these highly adapted organisms use to associate themselves to others will have a deep impact on our understanding of biology.

1.1 Signaling by means of phosphotyrosines

One of the most important means of controlling, regulating and computing communication in a complex system is to use reversible switches. This permits a much greater complexity without increasing the actual number or types of objects involved. Such a molecular switch, regulating many functions in and between the proteins it is present, is the phosphorylation of tyrosine residues. Proteins catalyzing the addition of a phosphate group to tyrosines are known as tyrosine kinases, the ones removing it as tyrosine phosphatases (1). In the late 1970's, viral oncogenes such as v-Src, v-Abl and v-Fbs were discovered whose products had an intrinsic activity in phosphorylating tyrosine residues in proteins (2-5). Their precursors and cellular counterparts (c-Src, c-Abl, etc…), as well some surface receptors binding growth factors were also shown to contain such an activity (6, 7). Hoping to understand more about the cell signaling and the oncogenic mechanisms used by these proteins, a very fruitful quest was started to unravel the underlying machinery of these covalent modifications. The knowledge accumulated the last 35 years concerning this modification and its manifold effects in the cell highlights its immense importance in the regulation of eukaryotic cells.

1.1.1 The Src Family Kinases and Csk

Tyrosine kinases are present either in the form of receptors anchored into the membrane with an intracellular kinase activity, the receptor tyrosine kinases (RTK), or as soluble forms, being facultatively anchored of tethered to the membrane or to signaling complexes (8, 9). The Src Family Kinases (SFKs) got their name for its best studied and prominent member, c-Src, and encompasses eight proteins in mammals (c-Src, Lyn, Yes, Fyn, Lck, Hck, Fgr, and Blk). They all contain an N-terminal fatty acylation site, a unique domain, where they are most divergent between the different members of the SFKs, a polyproline binding SH3 domain, an SH2 domain, a kinase domain and a C-terminal tail.

SFKs are potent activators of signaling pathways as illustrated by their prominent role as oncogenes and need therefore to be tightly regulated. While phosphatases undo the tyrosine-phosphorylation caused by the kinases, a more economical way is to regulate the activity of the SFKs themselves. Most of the work in unriddling the regulation of the SFKs has been achieved by studying Src. The transforming v-Src has enhanced kinase activity in comparison to c-Src. Intriguingly, v-Src lacking Y527 was shown to be mainly tyrosine-phosphorylated on Y416, whereas c-Src is mainly tyrosine-phosphorylated on Y527 (10). This led to the notion that Y416 activates, and Y527 inhibits the kinase activity of c-Src (11). Y416 was later identified as an autophosphorylation site and Y527 as being phosphorylated by the C-terminal Src kinase (Csk) (12). Further studies showed the SH2 domain of c-Src having a low affinity for Y527, which, when phosphorylated, forms a loop with the domain and inhibits the kinase activity. The solved structure of c-Src and Hck completed the picture (13, 14). In its inactive form, the SH2 domain of Src interacts with its C-terminal phosphotyrosine-527, while the SH3 domain binds to a short polyproline motif between the kinase and the SH2 domain. These two intracellular loops close the protein an render it inactive.

Csk is a potent inhibitor of the SFKs. It is ubiquitously expressed, notably in hematopoietic cells. The modular structure of Csk is very similar to the one of the SFKs. The main differences are the lack of the N-terminal unique sequence including the acylation site, and a lack of both regulatory tyrosines present in the

SFKs. This raised some fundamental questions concerning the regulation of its activity. The lack of an acylation site at the N-terminus suggests that Csk is mainly present in the cytoplasm, and must somehow be recruited to the membrane-associated SFKs to exert its function. This relocalization was shown to be mediated by proteins interacting with the SH2 or SH3 domains of Csk, as for example the Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains (Cbp/PAG). Cbp/PAG is associated to lipid rafts in the membrane (15, 16) and is phosphorylated on Y314 by activated SFKs. Following phosphorylation of this residue, Csk is recruited to the membrane to bind Cbp/PAG on Y314, by virtue of its SH2 domain. Additionally, the binding of its SH2 domain fully activates Csk, enabling it to down regulate the activity of the SFKs present at the membrane by phosphorylating their C-terminal inhibitory tyrosine. This straightforward feedback loop is thought to enable the cell to keep the amount of activated SFKs under control.

1.1.2 The SH2 domain

The SH2 domain can be considered as *the* prototypical domain for modular cell signaling. It was initially discovered in the retroviral protein tyrosine kinase v-Fps as non-catalytical domain which altered the localization and the kinase activity of this protein (17). Subsequently, a similarly localized sequence of about 100 amino acids was discovered in the tyrosine kinases Src and Abl, and termed SH2 (for *Src Homology domain 2*), with SH1 being already used to delineate the kinase domain (18). This suggested (i) that the kinase has a modular organisation (ii) that target specificity is not mediated by the kinase domain alone.

The idea of a modular organization for many proteins involved in cell signaling was further supported by the discovery of v-Crk, and its cellular counterpart c-Crk. This protein contains an SH2 domain and additionally a then newly described SH3 domain (19), which was later shown to bind to polyprolines (20). V-Crk enhances tyrosine-phosphorylation of cellular proteins without encoding a kinase domain. This finding supported the possibility of Crk recruiting kinases to form a complex encompassing multiple proteins.

The association of SH2 domain-containing proteins such as the phospholipase C (PLCγ) to activated and therefore tyrosine-phosphorylated RTKs finally led to the notion that this domain could bind phosphotyrosines. Single isolated SH2 domains from PLCγ were shown to bind to a variety of tyrosine-phosphorylated proteins, proving that an SH2 domain is sufficient for this binding (21). Finally, studies using phosphopeptides identical to the putative binding sites of SH2 domains in the C-terminus of RTKs demonstrated that these peptides bind to the domain in a tyrosine-phosphorylation dependent manner (22, 23).
Binding analyses *in vitro* and *in vivo* highlighted that the SH2 domain specifically recognizes the phosphorylated tyrosine and the amino acids located to the immediate C-terminus of it. This concept was used in a hallmark screen where a large library containing peptides with a phosphotyrosine and degenerated flanking amino acids was screened with different SH2 domains, discovering the importance of the positions +1 and +3 C-terminally to the phosphotyrosine (24). The relatively modest dissociation constant of 500-1000 nM for the SH2-phosphotyrosine-containing peptide interaction supports its dynamic nature (25). Structural analysis of SH2 domains binding their cognate phosphorylated peptides showed that their common fold are two α-helices surrounding an antiparallel β-sheet with the phosphorylated tyrosine embedded in a positively charged pocket in the β-sheet (26).
An SH2 domain is often capable of associating to multiple phosphotyrosine containing sequences in different proteins and the reverse is also true. A certain phosphopeptide ligand may bind several different SH2 domains with various affinities, enabling cells to build complex and dynamic interaction networks (27). Most proteins contain in addition to the SH2 domain multiple other domains with functions as various as binding (SH3, PTB), enzymatic activity (kinases, phosphatases, GTP-exchange factors), and transcription factors (STAT), supporting the notion of the modularity of cell signaling.

1.1.3 The PTB domain

The phosphotyrosine binding (PTB) domain was first recognized in the Shc scaffold protein by screening expression libraries for proteins binding the intracellular, tyrosine-phosphorylated domain of the EGF-receptor. Remarkably,

although an intraction with Shc could be expected because of its SH2 domain, fragments lacking the SH2 domain were recovered, which indicated that another domain than SH2 in Shc could bind to phosphotyrosines (28, 29). Further work elucidated this domain in Shc to be about 200 amino acids in size, and to associate to the Asn-Pro-X-pTyr motif in activated RTKs. As more proteins containing PTB domains were described, it became clear that this domain was much less strictly defined as the SH2. Surprisingly, a phosphorylated tyrosine is by no means a necessity, as many motifs without it are recognized and bound by PTB domains (30). Structural analysis of seven PTB domains showed their common structure being a pleckstrin-homology superfold (31), which differs from the SH2-fold structure. This suggests separate evolutionary developments between the SH2 and the PTB domains. Although of great importance in cell signaling, the variety of ligands binding in many different ways to the PTB domains are a considerable challenge in studying this domain, and led to the notion that PTB stands for “Promiscuous, Tolerant and Bizarre” (31).

1.1.4 SHP1 and SHP2

The catalysts to remove of a phosphate group from a tyrosine are known as tyrosine phosphatases (1). As with the tyrosine kinases, they come in two flavors. Either as receptors anchored to the membrane, the receptor protein tyrosine phosphatases (RPTP) or as facultatively soluble, non-transmembrane forms, which will be discussed here (32). SHP2, encoded by the gene *PTPN11*, contains two SH2 domains in its N-terminus, followed by a phosphatase domain and a C-terminal tail. In its inhibited state, a loop of the N-terminal SH2 domain binds to the phosphatase domain and prevents it from being active. The more C-terminal SH2 domain is free and can mediate the binding to a first phosphotyrosine-containing motif of an interaction partner. This causes a relocalization and then facilitates the binding of the N-terminal SH2 domain to the second phosphotyrosine motif, thereby freeing and activating the phosphatase domain (33). Deletion of exon 3 of murine SHP2 leads to defects in gastrulation, lymphopoeisis, heart development, and to embryonic lethality (34). Mutations in the *PTPN11* gene are associated in humans with the Noonan and Leopard Syndrome, autosomal-dominant disorders leading to a multitude of developmental defects (35, 36).

While the tyrosine phosphatase function of SHP2 is well defined, the manner in which it influences the signaling is quite complex (34, 37). Dominant-negative phosphatase dead SHP2 inhibits the activation of the Ras-Erk pathway mediating the cellular response for many cytokine receptors, suggesting a positive signaling for SHP2 in these cascades upstream of Ras (38, 39). An explanation for this positive signaling of SHP2 was published in 2004 (40), where Zhang et al. showed that growth factor-evoked SFK activation required SHP2 activity. By dephosphorylating Cbp/PAG, SHP2 inhibits the localization of Csk to the plasma membrane; subsequently, the tyrosine phosphorylation of the SFKs by Csk on their inhibitory tyrosine is reduced. By indirectly influencing the activity of the SFKs in a positive manner, SHP2 might therefore govern the wide variety of pathways that are controlled by the SFKs. SHP2 has also been associated to negative signaling in the JAK-STAT pathway, where data indicate that JAK1 is a

direct substrate of SHP2 (41). These findings lead to the conclusion that SHP2 can act both in an activatory and in an inhibitory way.

SHP1, encoded by *PTPN6*, is predominantly expressed in hematopoietic cells, where it down-regulates signaling of transmembrane receptors as various as cytokine receptors, growth factor receptors with an intrinsic tyrosine-phosphorylation activity, and receptors involved in immune signaling (42). Its crystal structure suggests the same type of regulation as SHP2, with the N-terminal SH2 domain blocking the phosphatase domain in its deactivated state and the more flexible C-terminal SH2 being involved in scanning for potential binding partners. Mice with defects in SHP1 expression, termed the *motheaten* mice, exhibit severe developmental abnormalities in many hemopoeitic lineages and die approximately three weeks after birth (43).

A further prominent role for SHP1 and SHP2 is their association to the inhibitory receptors of the immune system, which is discussed in the next chapter.

1.2 Immune Receptors

The immune system faces the challenge of having to be extremely reactive against pathogens, pathogen-infected and malignant cells, some of which it has never encountered before, and, at the same time, tolerate the many cell types and processes normally occurring in an organism. This balance is achieved by regulation networks influencing positively and negatively the activation of the immune cells.
Whereas the activation and suppression of B-cells, T-cells, natural killer (NK) cells, macrophages, mast cells, neutrophils and dendritic cells (DCs) is mediated by diverse receptors, the intracellular signaling mechanisms are highly conserved.

1.2.1 Stimulatory Immunoreceptors

Most activatory receptors contain in their intracellular domains immunotyrosine-based activating motifs (ITAMs) or recruit co-receptors containing such motifs. These motifs share the consensus Tyr-X-X-(Leu/Ile)-X(6-8)-Tyr-X-X-(Leu/Ile), where X can be any amino acid. ITAMs were discovered in proteins involved in T-cell and B-cell receptor (TCR/BCR) signaling, in some Fc Receptors, and in the activating form of the killer immunoglobulin receptors (KIRs) (44). Upon stimulation of these receptors, for example by the binding of antigens to the TCR, the two tyrosines in the ITAM get phosphorylated by SFKs, which then enables the Syk-family kinases and ZAP-70 to bind to these motifs with their tandem SH2 domains (45, 46). Following their activation, the Syk-family kinases then phosphorylate adapters and downstream effectors such as the novel B-cell linker (Blnk), the linker molecule LAT and the SH2-domain containing linker protein SLP-76. The ensuing signaling cascade activating the cell has been well described, with the induction of PLCγ, protein kinase C (PKC), phosphoinositol-3-kinase (PI3K), the Ras/MAPK pathway and mobilization of intracellular calcium stores (47, 48).

1.2.2 Inhibitory Immunoreceptors

Three major mechanisms are though to contribute to the negative regulation in the immune system. The inhibitory cytokines, as TGFβ and IL-10, inhibit inflammatory responses (49, 50), whereas facultatively inhibitory cytokines, as IL-4, can mediate inhibition or activation, depending on other stimuli (51). Apoptosis controls many steps in the development and responses of immune cells, as defects in the apoptotic pathways leads to lymphoproliferative and autoimmune diseases (52, 53). The third mechanism comprises the inhibitory immune receptors, which attenuate and downregulate activating signals. Knock-out mice for these receptors produce autoantibodies, symptoms similar to systemic lupus erythematosus and arthritis similar to rheumatoid arthritis (54, 55).

The inhibitory receptors share three structural similarities: (i) an extracellular domain containing one or more immunoglobin- or lectin-like repeats, (ii) a transmembrane spanning region, and (iii) an intracellular domain containing between one to four immunotyrosine inhibitory motifs (ITIMs) or immunotyrosine-based switch motifs (ITSMs). The ITIMs were initially discovered in the FcγRII receptor and defined as (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val) (56, 57). The ITSM was later identified in the intracellular domain of the CD150 receptor and defined as (Thr)-X-Tyr-X-X-(Leu/Val) (58-60). In most receptors, ITIMs/ITSMs are tandemly repeated, with a poorly conserved spacer sequence of about 15 to 30 amino acids between the motifs. Upon binding their extracellular ligands, such as MHC-I complex in the case of the inhibitory KIRs, the inhibitory immune receptors are tyrosine-phosphorylation by SFKs on these motifs. Intriguinly, the extracellular ligands of many inhibitory receptors have not yet been identified. Following their engagement, these receptors recruit phosphatases as the inositol phosphatase SHIP, SHP-1 and/or SHP2 to their ITIMs/ITSMs. The ITSMs can additionally bind the adapter proteins SAP and/or EAT-2.

1.2.3 Inhibitory Signaling over ITIMs

The tandem SH2-containing phosphatases SHP1 and SHP2 are predisposed to bind to the tandem ITIMs/ITSMs. Recruited to the inhibitory receptors they dephosphorylate tyrosine-phosphorylated proteins in the activatory cascades (61, 62). While the effects of SHP1 recruitment by inhibitory receptors has been well documented (63), many inhibitory receptors bind SHP1 and SHP2 simultaneously, making it difficult to assess their individual contribution. This is further hampered by the embryonic lethality of SHP2 knock-outs. The ITIM-containing CD31 receptor (PECAM-1) binds SHP1, SHP2 and was shown to negatively regulate the BCR/TCR signaling, as mice deficient in CD31 exhibit hyper responsive B-cells and developed autoimmune diseases (64). While the inhibitory effect of CD31 was also present in normal B-cells which express both SHP1 and SHP2, the inhibition was abrogated in SHP2-deficient cells, indicating a requirement of SHP2 in the inhibitory signaling (65). In addidition, expression of a dominant-negative SHP2 but not a dominant negative SHP1 abolished the inhibition of cytotoxicity in NK cells by the KIR2DL5 receptor (66). These data indicate that although both SHP1 and SHP2 can bind the same motifs and inhibit signaling, their functions are often not redundant.

The initial report involving Csk in the inhibition of the immune signaling came from studying the effects of its overexpression in a T-cell line (67). In these cells, the TCR phosphorylation and the amount of secreted IL-2 were significantly reduced. In contrast, over-expressing Csk caused only minor effects on the phagocytic activity of macrophages, and no effects at all in B-cells (68, 69). Taking into account the knowledge that Csk associates to Cbp/PAG and inhibits SFKs activity, the following model was proposed for T-cells (62); Upon recognition of ligands by the TCR, Cbp/PAG undergoes dephosphorylation by an yet unknown mechanism and Csk leaves the membrane to become cytoplasmic. In doing so, Csk does not repress the SFKs anymore, which become activated and can initiate the activatory and inhibitory signaling by tyrosine-phosphorylating protein residues in the cluster of the TCR signaling complex. With the PEST-domain enriched protein tyrosine phosphatase (PEP) a protein binding the SH3 domain of Csk was identified in an yeast two-hybrid screen (70). PEP is

exclusively expressed in hematopoietic cells, and predominantly (50-80%) constitutively associated to Csk. The association of Csk with PEP opened completely new views on the mechanisms by which it can act. The human homolog of the mouse PEP, the lymphoid phosphatase Lyp (PTPN22), can, associated to Csk, inhibit T-cell activation (71). Mutations in Lyp have been associated with a variety of autoimmune diseases as rheumatoid arthritis (72), systemic lupus erythematosus, Graves disease and susceptibility to type I diabetes (73). The risk variant associated to the susceptibility to type I diabetes encodes a mutation (R620W) in Lyp abrogating the binding of Csk to Lyp, stressing the importance of the association between Csk and tyrosine phosphatases in the regulation of the immune system. Through sequence homologies, a second binding partner to the SH3 domain of Csk was identified, the protein phosphatase PTP-PEST (74), which is ubiquitously expressed and associates in a wide range of cell types with Csk. While PTP-PEST has important roles in signaling in immune cells, it is also involved in controlling the assembly, migration, and cytokinesis of cellular focal adhesions (75) and preventing the activation of the Ras pathway signaling (76).

1.2.4 Inhibitory and Activatory Signaling over ITSMs

While the concept of separate activatory and inhibitory receptors holds true in many cases, an additional layer of complexity is added by the presence of receptors containing ITSMs and having a dual function, *switching* the signaling to activation or inhibition depending on the availability of cytoplasmic adapter proteins.

The SLAM (signaling lymphocitic activation molecules) subfamily of receptors encompasses six members (CD150, CD84, CD229, CD244, NTB-A, CS1), which contain no ITIMs but at least two ITSMs in their cytoplasmic domains. Other receptors integrate both motifs in their intracellular domain, such as PD-1, CD31, CD33, C-CAM (each containing one ITIM-ITSM tandem) and SHPS-1 (two ITIM-ITSM tandems). The ITSM can, as mentioned earlier, bind to SHP1, SHP2, or

SHIP, and, additionally, to at least two adapter proteins, SH2D1A (SAP) and SH2D1B (EAT-2).

SAP is a 15 kDa protein expressed in T- and NK cells (77), and contains only an SH2 domain and a short C-terminal tail. Mutations in the *SH2D1A* gene encoding SAP are associated to the X-linked immunoproliferative disease (XLP), characterized by impaired responses to viral infections and by the presence of B-cell non-Hodgkin's lymphoma (58). Surprisingly, SAP binds the ITSM in SLAM irrespectively of its tyrosine-phosphorylation, with an affinity comparable to the SH2-phosphotyrosine interactions (78), which is only increased about 5-fold by phosphorylating the tyrosine in the ITSM (79). The crystal structure for this small adapter protein revealed a novel mode of ligand binding. Whereas the classical SH2 domain recognizes its cognate peptide trough two surface pockets, the phosphotyrosine and the amino acids C-terminal to it, the unusual SH2 domain of SAP uses three surface pockets, recognizing residues on both sides of the phosphotyrosine (79). Both SHP2 and SAP bind to the same sites in SLAM, supporting the notion of a competition between both proteins. Indeed, it was shown that SAP, which has a higher affinity, blocks the binding of SHP2. In the absence of SAP, SHP2 binds with its tandem SH2 domains two ITSMs. If SAP is expressed, it displaces SHP2, occupies one ITSM, and the other ITSM becomes accessible to proteins like SHIP, which can also bind a single ITSM, although with a lower affinity than SHP2 (60, 78). Such a molecular switch changes the signaling capabilities of a receptor, depending on the cell type it is being expressed. Further studies revealed a recruitment of the Fyn kinase by SAP (80). This recruitment and therefore coupling of Fyn to the SLAM receptor is mediated by a small prominent non-proline based sequence in the SH2 domain of SAP, which binds the SH3 domain of Fyn, increasing its kinase activity markedly (81, 82).

EAT-2 is expressed in NK-, B-cells, macrophages, and DCs (77) and contains the same unusual SH2 domain as SAP, therefore binding to ITSMs. In contrast to SAP, it does not recruit kinases, but contains tyrosine-phoshorylation sites. NK cells from mice deficient for EAT-2 exhibit an increased killing rate and IFNγ secretion, supporting the idea of EAT-2 being a negative regulator of their SLAM receptor activity (83). EAT-2 is thought to recruit SHP1, SHP2 and/or Csk by means of its phosphotyrosines, exerting its inhibitory function by these effectors.

The function of EAT-2 in the context of the DCs is not known, and may give us novel understandings how our immune system coordinates its activities.
The complex cell-type depending outcome of the signaling by a certain inhibitory receptor is exemplarily illustrated by SHPS-1 (MyD-1, BIT, SIRPα), which contains two ITIM-ITSM pairs. When SHPS-1 is binding its ligand CD47 in macrophages, it inhibits their phagocytic activity (84) and at the same time induces their nitric oxide production (85), whereas the same binding in dendritic cells inhibits their maturation (80), and therefore effective antigen presentation. Co-ligation of this inhibitory receptor with FcεRI in mast cells inhibits their IgE-induced production of cytokines (86). In monocytes, ligation of SHPS-1 inhibits the LPS-induced TNFα secretion (87).

1.3 Tyrosine-phosphorylated effectors of bacterial pathogens

The immense importance of tyrosine-phosphorylation in eukaryotic signaling pathways opens the door for abuse by pathogens. By tweaking the signaling cascades with tyrosine-phosphorylated proteins or adapters, virtually any function of the host cell can be accessed and subverted. It is therefore not surprising that tyrosine-phosphorylation itself was first discovered being mediated by the protein of a pathogen (2).
To subvert the eukaryotic signaling by means of tyrosine-phosphorylated effectors proteins, these proteins have in first place to be transported into these cells. Gram-negative bacteria have two membranes, the eukaryotic cells one. Bacteria have therefore elaborated complex machineries to secrete their effectors across these multiple membranes directly into the eukaryotic cell. Of interest here are the Type III secretion system (T3SS) and the Type IV secretion system (T4SS) of gram-negative bacteria. Both apparatuses are used for delivering effectors into host cells, where they are subsequently tyrosine-phosphorylated. The T3SS apparatus is a needle-like structure spanning both membranes of the bacterial cell, and secretes effector proteins as well as components of the apparatus. It shares many similarities with the flagellar hook basal body, suggesting an evolutionary relationship between both (88).
The T4SS is, in contrast to the T3SS, evolutionarily related to conjugation machineries. It is capable of secreting proteins and additionally also DNA into host cells (89).

The first bacterial effector protein reported to be tyrosine-phosphorylated was the translocated intimin receptor (Tir) of enteropathogenic *Escherichia coli* (EPEC) (90). At first mistakenly identified as a host-cell protein being tyrosine-phosphorylated upon infection, this protein was shown to be secreted by the EPEC Type III T3SS. Upon secretion, Tir inserts into the eukaryotic membrane, where it acts as the receptor for the bacterial surface protein intimin, enabling intimate attachment of the bacteria to its host cell. Its intracellular domain gets tyrosine-phosphorylated on Y474 by SFKs (91) and binds the adapter protein Nck

(92). This adapter protein is composed of one SH2 and three SH3 domains and couples Tir to N-WASP, thereby inducing actin polymerization by the Arp2/3 complex (93). This actin reorganization was shown to be crucial for the formation of pedestrals, which are membrane extrusions of the infected cells, on which the bacteria locate.

The mouse pathogen *Citrobacter rodentium* also expresses a Tir protein, which was shown to be essential for virulence in a rodent model to induce actin rearrangements and to be tyrosine-phosphorylated (94). Intriguingly, in contrast to the Tir of EPEC, it mediates the actin rearrangements independently of its tyrosine-phosphorylation. This finding stresses that - in addition to tyrosine-phosphorylation dependent effects - the secreted effectors can also have a tyrosine-phosphorylation independent function.

The obligate intracellular bacterium *Chlamydia trachomatis* is one of the most commonly sexually transmitted human pathogens and the etiologic agent of trachoma and lymphogranuloma venereum. This pathogen secretes the Tarp protein through its T3SS protein into human cells. In the case of HELA cells, Tarp was shown to be tyrosine-phosphorylated upon translocation and to recruit actin (95). While its exact function is not known, it has been hypothesized to play a role in the invasion of the host cells.

The CagA protein of the human pathogen *Helicobacter pylori* is secreted by its T4SS upon attachment into gastric epithelial cells (96). It has a size of 120-145 kDa and contains in its C-terminus a variable number of “EPIYA” motifs, depending on the bacterial strain. These motifs were, based on their similarities, classified into EPIYA-A,-B,-C and -D. While CagA of Western *H. pylori* strains contain the motif combinations A-B-C, A-B-C-C, or A-B-C-C-C, the more virulent East-Asian counterparts express a CagA containing the A-B-D motifs (97, 98). EPIYA-C and -D are the most prominently tyrosine-phosphorylated motifs, whereas EPIYA-A and -B exhibit a reduced phosphorylation. Tyrosine-phosphorylation of these motifs by host SFKs elicits what has been termed the “hummingbird phenotype” (99), which is characterized by increased host cell motility and elongation (100). While SHP2 and Csk have been reported to interact with CagA depending on its tyrosine-phosphorylation (101, 102), PLCγ, Grb2, c-Met do this independently of the phosphorylation state (103, 104). It has been proposed that Csk inhibits the phosphorylation of CagA by suppressing the

activity of the SFKs, therefore enabling an autoregulatory loop to control the amount of phosphorylated CagA (102, 105).

A large number of studies suggest an increased risk for individuals, carrying CagA-positive *H. pylori* strains, to develop gastric cancer (106, 107). Concomitantly, studies reporting oncogenic proliferations associated to missense mutations in SHP2 showed the oncogenic potential of this phosphatase (36). SHP2 binds to the EPIYA-C and -D motifs in CagA. The severity of the gastric carcinoma or atrophic gastritis has been linked to the number of EPIYA-C motifs in Western *H. pylori* strains (98). In the more virulent East-Asian strains, the EPIYA-D motif was shown to have a stronger SHP2 binding and activation capacity than EPIYA-C (108). Even though the host genetic variability and susceptibility may play an important role in the severity of the disease by *H. pylori*, the CagA-SHP2 interaction is a prime candidate being responsible for the oncogenic transformations.

Bartonella and type IV secretion

Bartonellae are gram-negative, facultative intracellular pathogens. They cause long lasting intra-erythrocytic bacteraemia in their mammalian species-specific reservoir hosts, facilitating the transmission by blood sucking arthropods. No intra-erythrocytic bacteraemia is detected in their incidental hosts, whereas they have in both the reservoir and the incidental hosts a tropism for endothelial cells. Currently, over twenty *Bartonella* species infecting a wide range of mammals are known, two having humans as reservoir host (*B. bacilliformis* and *B. quintana*), seven inducing diseases in humans as zoonotic pathogens, and fourteen not known to infect humans as yet (109, 110).

To elucidate the mechanisms of *Bartonella* infection, Schulein et al. developed an *in vivo* infection model for *B. tribocorum,* which has rats as reservoir hosts (111). Intravenously injected wild-type *B. tribocorum* are rapidly cleared from the bloodstream, which remains sterile for at least three days. The primary niche, which allows the bacterial to survive and replicate in these first three days is yet unknown. On day four, the bacteria re-appear in the bloodstream, adhere to and invade erythrocytes, where they replicate for a few rounds without shortening the lifespan of the erythrocyte. Approximately every five days, a new wave of bacteria enters the bloodstream and invades the erythrocytes, sustaining the bacteremia for about ten weeks. This is in accordance with other animal models (112). The affinity of *Bartonella* for endothelial cells *in vitro* and the fact that these cells coat the blood vessels suggests the endothelial cells being the primary niche, where the bacteria hide the first three to four days, and from where they seed into the bloodstream.

B. henselae has cats as reservoir host, and is the etiological agent of cat scratch disease in incidentally infected immunocompetent humans. *B. henselae* has been furthermore associated with symptoms like endocarditis, bacteremia with fever, neuroretinitis, and, in immunocompromised individuals, with bacillary angiomatosis/peliosis (113). Bacillary angiomatosis is a *Bartonella*-triggered tumor-like vasoproliferation of endothelial cells, which in addition to *B. henselae* also *B. quintana* and *B. bacilliformis* can induce (114). Strikingly, *B. henselae* does also mediate anti-apoptosis in endothelial cells, a mechanism which might

support the induction of the bacillary angiomatosis (115). By infecting endothelial cells *in vitro*, it has been shown that *B. henselae* induced massive actin rearrangements in these cells, a structure which has been termed the invasome (116).

Bartonellae contain two T4SS which were shown to be essential for intra-erythrocytic bacteremia in the animal model: the Trw and the VirB/VirD4 systems (117, 118). The Trw system exhibits a high level of sequence identity to the conjugative machinery of the broad-host-range antibiotic-resistance plasmid R388 of *E. coli*. In contrast, the VirB/VirD4 system has its closests relative in the AvhB/TraG conjugation system of the cryptic plasmid pATC58 of *Agrobacterium tumefaciencs,* as we will show this in the first manuscript of this thesis. It is encoded by an operon of ten genes (*virB2-virB11*) and the *virD4* gene closely located downstream. The VirB proteins form a pore complex encompassing both membranes of gram-negative bacteria and potentially the membrane of infected eukaryotic cells. VirD4, also called the coupling protein, mediates the interaction between the VirB pore complex and the substrates being secreted by this apparatus, therefore coupling the secretion apparatus to the secreted proteins. In conjugative T4SS, the exported substrate is a DNA-protein complex, the protein being termed the relaxase. The relaxase cleaves and covalently attaches to one strand of the plasmid DNA, followed by the export of this protein-DNA complex by the plasmid encoded T4SS.

The combined knowledge of Bartonellae using T4SSs to mediate pathogenicity, of other pathogenic bacteria secreting effectors by the means of such systems, and of the peculiar phenotypes *B. henselae* elicits in the endothelial cells, prompted us to look for VirB/VirD4 T4SS secreted effector proteins. Preliminary data indicated that the VirB T4SS of *B. henselae* mediates invasion, proinflammatory activation and antiapoptotic protection of endothelial cells (119), therefore associating phenotypes to the presence of an intact T4SS. The first manuscript in this thesis describes the discovery of seven putative substrates, the *Bartonella* exported proteins (Bep), being exported by the VirB/VirD4 secretion apparatus, the characterization of their modular organization and their secretion-mediating domain. The second manuscript focuses on BepE, where all data acquired so far indicate that this protein mimicks

inhibitory immune receptors. The third chapter in the results part contains the abstracts of two manuscripts, in which the effects of BepA and BepG on the endothelial cells are presented. Last, the fourth chapter contains unpublished, additional findings concerning BepD and BepF.

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2 – Aim of the Thesis

2. Aim of the Thesis

Started in April 2002, the primary aim of this thesis was to investigate the VirB/VirD4 T4SS of *Bartonella henselae* for the presence of secreted substrates and signals mediating this secretion. Following the identification of these substrates, I then focused on the characterization of their functions and interaction partners in the host cell, with an emphasis on the putative tyrosine-phosphorylated effectors.

3 – Results

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

Ralf Schulein, Patrick Guye, Thomas A. Rhomberg, Michael C. Schmid, Gunnar Schröder, Annette C. Vergunst, Ilaria Carena, and Christoph Dehio

PNAS (2005) 102(3), p. 856-861

Members of our group showed earlier that the T4SS of *Bartonella tribocorum* is essential to establish intraerythrocytic infection in an rat-infection model (1), and that the T4SS of *B. henselae* mediates subversion of endothelial cells upon bacterial infection (2). We report here the discovery and characterization of multiple protein substrates, subsequently termed *Bartonella*-translocated effector proteins (Beps), being translocated by the VirB/VirD4 T4SS of *B. henselae* into infected host cells.

Sequencing 23 kb downstream of the *virB* locus of *B. henselae*, we found an open reading frame (ORF) encoding a coupling protein (*virD4*) and a group of seven ORFs encoding proteins with a common C-terminal domain, which was subsequently termed the *Bartonella* intracellular delivery (BID) domain. By constructing a Hidden Markov Model of these BID domains and querying protein databases, we found many similar domains in the relaxases of conjugative plasmids in the α-proteobacteria, as for example in the C-terminus of the TraA relaxase from the AvhB/TraG conjugation system in *A. tumefaciens*. We then showed that this BID domain of TraA was translocated trough the VirB/VirD4 T4SS of *Bartonella henselae*. This finding does not only support the common evolutionary ancestry between the *Bartonella* T4SS and the T4SS of conjugative plasmids, but furthermore opened up the fascinating possibility that the *Bartonella* VirB/VirD4 T4SS is able to export DNA attached to a relaxase into host cells. No similarities were found between the BID domain and proteins being secreted in a T4SS-dependent manner by other pathogens (*H. pylori, L.*

pneumophila, A. tumefaciens) indicating a separate evolutionary ancestry. These data suggest that the VirB/VirD4/Bep system has rather recently evolved from the T4SS of conjugative plasmids.

To demonstrate the translocation of the Beps through the VirB/VirD4 T4SS, we fused a FLAG-tag to the N-terminus of BepD and could show that BepD is translocated into infected endothelial cells in a VirB/VirD4 T4SS-dependent manner, whereupon it localizes to the cytoplasm of these cells and is tyrosine-phosphorylated by host-cell kinases. The development of the Cre-recombinase reporter assay for translocation (CRAFT) made it possible to delineate precisely the translocation domain, which proved to be bipartite. In addition to the BID domain, a short, positively charged C-terminal amino acid sequence was needed for an effective delivery of proteins.

To assess if the previously observed subversion of endothelial cells upon bacterial infection could be associated to the secretion of the Beps, we generated non-polar in-frame deletions of these seven putative T4SS effector proteins (Δ*bepA-G*) and a non-polar deletion of the ORF encoding the coupling protein (Δ*virD4*). These mutants were abrogated in induction of all the described effects on the endothelial cells as (i) anti-apoptotic protection of endothelial cells, (ii) massive cytoskeletal rearrangements, termed the invasome (iii) cytostatic/cytotoxic effects at higher doses, and (iiii) activation of a pro-inflammatory response.

Statement of my own contribution

I sequenced together with Ralf Schülein and Thomas Rhomberg the locus, coordinated and assembled the sequencing runs (Fig. 1). I described the BID domain (Fig. 1), constructed the Hidden Markov Model, and did the Bioinformatics for finding related domains (Fig. 4), which also led to the description of the translocation domain in the TraA relaxase of the AvhB/TraG system (Fig. 3). By the means of bioinformatics analysis and based on the modular structure of the Beps, I assisted in the planning of constructs used to delinate the secretion signal (Fig. 3). I also did the western blot in Fig. 3 for showing the stability of the Cre-BID fusion constructs. Finally, I constructed the FLAG-tagged BepD and did infection experiments to demonstrate protein translocation by two independent lines of evidence, tyrosine phosporylation and localization in the host cell cytoplasm (Fig. 2).

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

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

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

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

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

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

Materials and Methods

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

This paper was submitted directly (Track II) to the PNAS office.

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

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

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

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

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

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

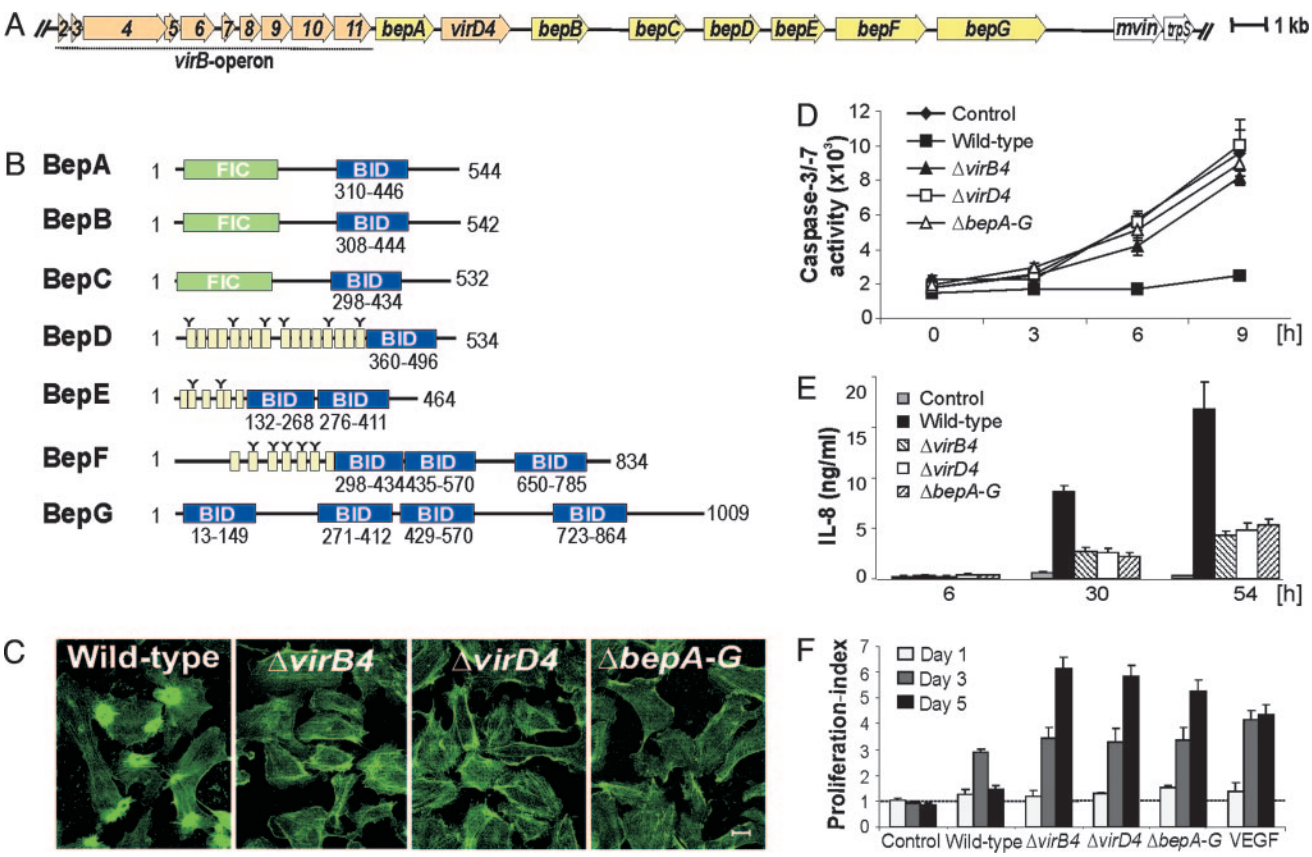

Fig. 1. The Beps mediate VirB/VirD4-dependent invasion, antiapoptotic protection, proinflammatory activation, and control of proliferation of HEC. (*A*) Structure of the *virB/virD4/bep* locus encoding the VirB components (VirB2–VirB11), the T4S coupling protein (VirD4), and seven putative effector proteins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

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

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

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

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

Results

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

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

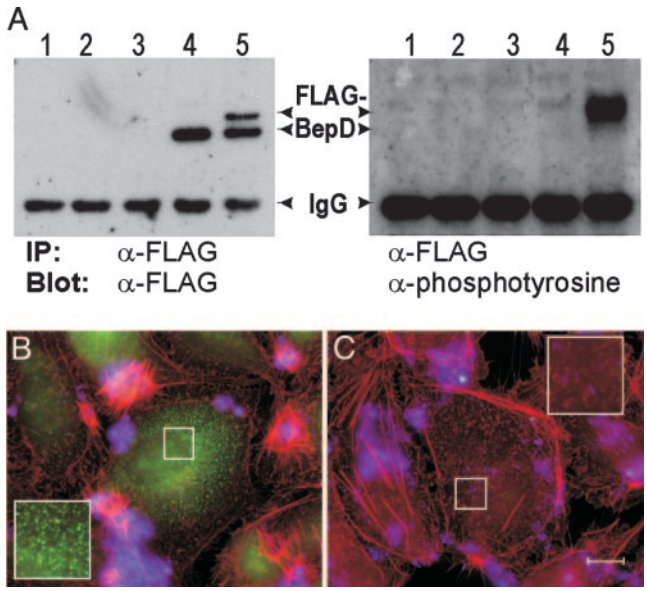

Fig. 2. BepD becomes tyrosine-phosphorylated after VirB4-dependent translocation into HEC. (*A*) VirB4-dependent translocation of BepD into HEC results in tyrosine phosphorylation and a coincident reduction in electrophoretic mobility. Total protein extracts of Ea.hy926 cells uninfected (lane 1) or infected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

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

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

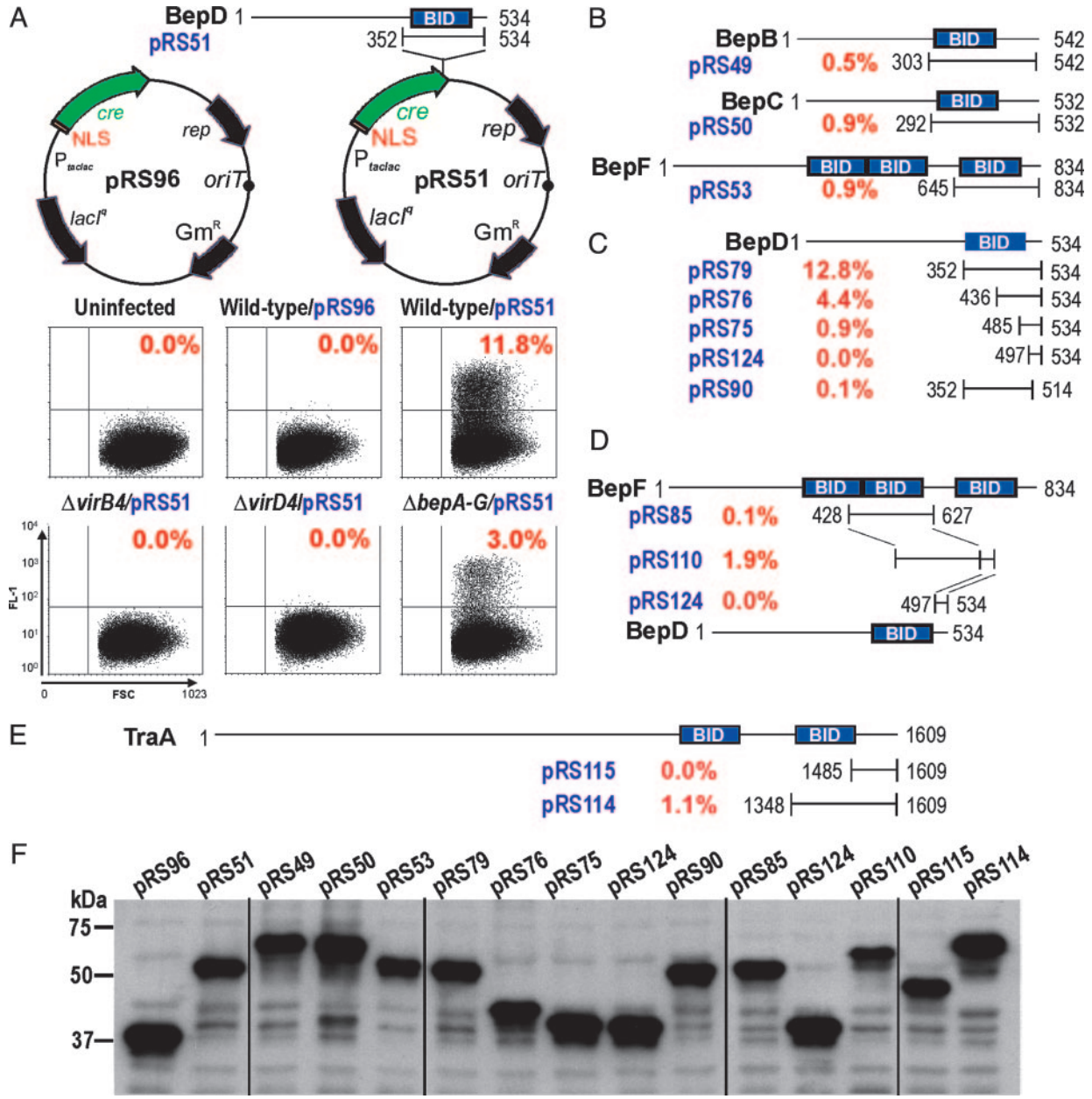

Fig. 3. The C-terminal translocation signal of Beps mediates VirB/VirD4-dependent protein transfer into HEC. Protein transfer was determined by CRAFT. The Cre-tester cell line Ea.hy926/pRS56-c#B1 was infected with the indicated *Bh* strains expressing different NLS–Cre fusion proteins (plasmid names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

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

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

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

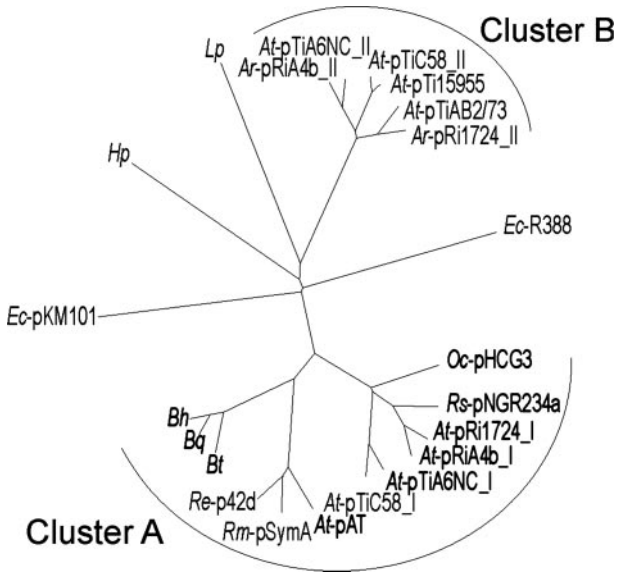

Fig. 4. The coupling proteins (VirD4/TraG) of T4S systems containing a BID domain in their protein substrate(s) form a distinct phylogenetic cluster. This cluster is formed by *Bartonella* VirB/VirD4 systems and α-proteobacterial conjugative DNA transfer systems (cluster A) and does not contain agrobacterial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

Discussion

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

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

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

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

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

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Molecular mimicry of inhibitory immune receptors by the bacterial pathogen Bartonella

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Manuscript in preparation.

The BepE protein of *B. henselae* contains putative tyrosine-phosphorylation sites in its N-terminus, and two C-terminal BID domains thought to mediate the translocation of this protein from the bacteria into the host cells in a T4SS-dependant manner (1).
Here we show that upon translocation, BepE is tyrosine-phosphorylated in its N-terminus, acquires a membrane-proximal localization, and additionally co-localizes with VE-Cadherin at cell-cell contacts in human umbilical endothelial vein cells (HUVECs). Purified BepE is tyrosine-phosphorylated *in vitro* by the c-Src kinase. We describe five putative tyrosine-phosphorylation sites in the N-terminus of this protein, the first similar to a known binding site for the C-terminal c-Src kinase (Csk) in VE-Cadherin, followed by two tandems of immunotyrosine inhibitory and immunotyrosine-based switch motifs (ITIMs, ITSMs). These motifs are widely present in the intracellular domain of inhibitory immune receptors of mammals. By recruiting SHP1, SHP2, SHIP and Csk to these motifs, the inhibitory receptors do inhibit the activation in almost all cells of the immune system. By means of co-immunoprecipitation, we show that both Csk and SHP2 bind BepE in a tyrosine-phosphorylation dependent manner. Performing a systematic exchange of tyrosines to phenylalanines in the N-terminus of BepE, we showed that Csk binds only to the first motif. In contrast, to abrogate all binding of SHP2 to BepE it was necessary to mutate both ITIM-ITSM tandems. The data accumulated so far highly suggest that BepE mimicks inhibitory immune receptors. To show this, we initiated a collaboration with Prof. Gennaro de Libero. This collaboration is ongoing, but due to the time

constraints of my Ph.D. thesis, cannot come to conclusion in this preliminary manuscript.

Statement of own contribution

I did the initial purification of HIS-tagged BepE, and initial *in vitro* and *in vivo* phosphorylation assays, leading to the detection of two phosphorylation sites *in vitro* and one *in vivo*. Hermine Schein further optimized the purification of HIS-tagged BepE, and continued the mapping in collaboration with Paul Jenö's lab (mass spectrometry analysis), which is still ongoing. The plasmid pTR1173 was constructed by Thomas Rhomberg. Matthias Selbach from the laboratory of Matthias Mann investigated the first tyrosine-phosphorylated motif of BepE in the SILAC screen, confirming Csk as an interaction partner for this motif. All other data/constructs reported in this manuscript were generated by me.

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Molecular mimicry of inhibitory immune receptors by the bacterial pathogen Bartonella

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Condensed title: A bacterial protein mimicking immune receptors

Abstract

Inhibitory receptors suppress activatory signals in a wide variety of immune cells by binding and activating phosphatases and kinases, such as SHP2 and the C-terminal c-Src kinase (Csk). Here we show that the VirB/VirD4 Type IV secretion system (T4SS) of the vascular tumor-inducing pathogen *Bartonella henselae* (*Bh*) to secrete a protein, termed BepE, into infected cells, where it localizes to the cell plasma membrane and to cell-cell contacts. Upon secretion, this protein is tyrosine-phosphorylated by host-cell kinases on its immunotyrosine inhibitory motifs (ITIM), immunotyrosine switching motifs (ITSM) and a Csk binding-like motif located in its N-terminus, and binds subsequently Csk and SHP2. We show for the first time a molecular mimicry of such an inhibitory receptor being used by a pathogen to subvert the host cell signaling.

Introduction

Bartonellae are gram-negative, facultative intracellular pathogens, capable of inducing vasoproliferative tumors known as bacillary angiomatosis (1). They can cause long-lasting intra-erythrocytic bacteremia in their mammalian reservoir hosts (2) and subvert multiple endothelial cells functions such as proliferation, apoptosis, inflammation, and cytoskeletal rearrangements (3, 4). Recently, we showed that *Bartonella henselae* (*Bh*) secretes seven proteins, termed BepA-G, into infected host cells through the VirB type IV secretion system (T4SS) (4). The BID domain of these proteins, which is located at their C-termini, mediates their secretion, whereas the N-termini encode putative effector functions. Three of these secreted proteins, BepD-F, contain putative tyrosine-phosphorylation motifs in their N-termini. We showed exemplarily for BepD that this protein is tyrosine-phosphorylated upon secretion into host cells (4).

Tyrosine-phosphorylation plays a key role in many signaling cascades of eukaryotic cells. It is therefore not surprising that multiple bacterial pathogens evolved tyrosine-phosphorylated effector proteins to interfere with and subvert the functions of the host cells they infect (*5*). Most prominent examples are the TIR and Tarp proteins secreted by the Type III secretion system of enteropathogenic *Escherichia coli* (*6*) and *Chlamydia trachomatis* (*7*), respectively, and CagA, which is secreted by the T4SS of *Helicobacter pylori* into gastric epithelial cells (*8*). All three effectors are tyrosine-phosphorylated upon translocation into the host cells by host cell kinases (*5*) and elicit effects as various as enhanced bacterial adherence, invasion, or hypermotility of the host cells.

The immune system of mammals faces a tough dilemma. It has evolved to be exquisitely sensitive in detecting foreign antigens and abnormal or malignant cells, yet, it must synchronously tolerate a huge variety of stimuli associated with the normal processes and cells present in the organism. In this delicate balance, inhibition of the many immune system components is as important as their activation. One of the key components mediating the inhibition are the inhibitory immune receptors (*9*). Almost all cells of the immune system express at least one type of these receptors, most cells many, as in the example of the B cells where

there are at least fourteen distinct inhibitory receptors known to be expressed simultaneously (*10*). Distinctive features of these receptors are an extracellular part containing one or more immunoglobulin- or lectin-like domain, a hydrophobic transmembrane region, and between one to four immunotyrosine inhibitory motifs (ITIMs) on the intracellular side (*11*). The consensus for these ITIMs has been defined as (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), where X denotes any amino acid, and was initially discovered in the FcγRII receptor (*12, 13*). These motifs can, upon tyrosine-phosphorylation by Src Family Kinases (SFK), bind to the SH2 domains of non-receptor phosphatases such as SHIP, SHP1, SHP2, and in some cases Csk (*14*). Bound to SHP1/SHP2 or Csk, the receptors can subsequently dephosphorylate immunotyrosine activation motifs (ITAMs) containing receptors or their tyrosine-phosphorylated substrates, which are for example present in the B-cell or T-cell receptors (BCR / TCR) signaling clusters, or elicit directly signaling events in these cells (*10, 15*). A derivative of the ITIM is the immmunotyrosine switching motif (ITSM) with the consensus (Thr)-X-Tyr-X-X-(Leu/Val). This motif is present in the CD150 subfamily of immune receptors (*16*) and can additionally bind adaptors such as SAP or EAT-2 to signal, depending on the cell type, in a inhibitory or activatory way (*17*). Knowing the effects of these inhibitory receptors, it comes as no surprise that deletions in loci containing these receptors have been associated with autoimmune diseases. Knock-out mice for almost all inhibitory receptors exhibit symptoms of autoimmune diseases, as producing autoantibodies, or symptoms similar to systemic lupus erythematosus and arthritis resembling rheumatoid arthritis (*18*).

Here we characterize the BepE protein of *B. henselae*, which is secreted into infected host cells in a T4S system-dependent manner and which contains two ITIM-ITSM tandems, additionally a motif for binding to the SH2 domain of Csk. We show that upon secretion into the host cells BepE becomes tyrosine-phosphorylated in these motifs by SFKs and subsequently associates with Csk and the tyrosine phosphatase SHP2. Moreover, BepE was found to localize to the plasma membrane and in particular to cell-cell contacts in a phosphorylation-independent manner. Together these data suggest for the bacterial BepE protein molecular mimicry of immunotyrosine inhibitory motifs-containing receptors, to our knowledge the first example of such mimicry by a pathogen.

Results

BepE is tyrosine-phosphorylated upon translocation into the host cell. *Bh* does exhibit a remarkable tropism towards primary human endothelial cells (HUVECs), where it induces a wide range of effects (3). To assess the tyrosine-phosphorylation of BepE upon translocation into HUVECs by the T4SS, we infected these cells with *Bh* strains expressing a FLAG-tagged BepE. Subsequently, we lysed the cells, immunoprecipitated the FLAG-tagged BepE with anti-FLAG agarose and probed the precipitate by western blotting with an anti-phosphotyrosine or an anti-FLAG antibody. BepE was tyrosine-phosphorylated upon translocation into HUVECs as shown in Fig. 1, whereas in a secretion-defective mutant BepE was retained in the bacteria and therefore not accessible to the host cell kinases.

The two C-terminal BID domains of BepE are sufficient for membrane localization and for co-localization with VE-Cadherin in infected HUVECs. HUVECs and HELA cells were infected with *Bh* strains expressing MYC-tagged BepE (aa 1-464) or the MYC-tagged C-terminal part of BepE encompassing the two BID domains (aa 136-464) (Fig. 2a). Using immunofluorescence labeling, the proteins could be detected in association with the plasma membrane of HELA cells (Fig. 2b). The weaker signal in the case of the MYC-tagged C-terminal part of BepE might be due to a folding not optimally exposing the MYC-tag or to reduced stability of the N-terminally truncated protein. Furthermore, both tagged proteins localize to the cell-cell contacts of HUVECs (Fig. 2c) as shown by the overlapping staining for the MYC-tag and the endothelial adherence junction marker VE-Cadherin.

BepE is tyrosine-phosphorylated *in vitro* by the c-Src kinase. It has been shown that c-Src acts as the kinase of other tyrosine-phosphorylated bacterial effectors (*19, 20*). To test whether c-Src also mediates phosphorylation of BepE we expressed and purified HIS-tagged BepE and incubated it together with c-Src and ATP *in vitro*. BepE became tyrosine-phosphorylated as shown in

Fig. 3. In contrast, without c-Src, there was no detectable tyrosine-phosphorylation, excluding the possibility of BepE autosphosphorylation. Noticeably, tyrosine-phosphorylated BepE exhibited a slightly increased apparent mass in the SDS/PAGE gels.

Mapping the phosphorylated tyrosines of BepE by mass spectrometry. *In vitro* phosphorylated and unphosphorylated HIS-BepE was digested by proteases as described in *Materials and Methods* and subjected to mass spectrometry analysis. FLAG-tag containing BepE, co-expressed in HEK293T cells with or without the c-Src kinase was immunoprecipitated and analyzed likewise. Table 1 shows the peptide fragments detected in the *in vitro* tyrosine-phosphorylation mapping screen. Four out of the five putative tyrosine phosphorylation motifs in the N-terminus of BepE could be detected in their unphosphorylated state (peptide fragments encompassing amino acids 24-38, 47-68, 83-99 and 100-122 in BepE). Moreover, three of these motifs were detected as being phosphorylated on their tyrosine in a proportion of 48, 88 and 65 % (peptide fragments 24-38, 47-68, and 100-122). The fact that fragments encompassing the fifth putative tyrosine-phosphorylated motif could not be detected at all, might be due to technical reasons in the mass spectrometry analysis procedure.
The analysis of the *in vivo* tyrosine-phosphorylated motifs in BepE is still ongoing and a first motif (...EPLYATVNK...) could be detected in its unphosphorylated, as well in its tyrosine-phosphorylated form (data not shown).

BepE contains in its N-terminus ITIMs and ITSMs. To gather further indication for a function for these putative tyrosine-phosphorylation motifs, we used the blast tool (http://www.ncbi.nlm.nih.gov/BLAST/) and the ELM database (http://elm.eu.org/) and queried them with short (5-40 amino acids) peptide fragments of BepE. By these means, three distinct classes of motifs could be distinguished in the putative tyrosine-phosphorylated motifs of BepE: ITIMs and ITSMs, both present in subclasses of the IgG-like family of receptors (Fig.

4a), and a sequence ("…EPLYATVNK…") similar to a known binding site for Csk in VE-Cadherin (Fig. 4b) (*21*).

Furthermore, the spacing for ITIM/ITSM tandems binding the two SH2 domains of phosphatases like SHP1 or SHP2 has been published to be in a range between 15 and 30 aa (*22*), which is in accordance to the distance separating the ITIM/ITSM motifs in both tandems of BepE (26 and 22 aa, Fig. 4a). The consensus for the ITIM has been defined as Y/VxYxxV/L and for the ITSM as TxYxxV/L. The CD150 subfamily of receptors were shown to contain ITSMs with additionally a serine or threonine at position -4 in respect to the tyrosine (S/TxTxYxxV/L). This enables these receptors to bind additionally the adaptor proteins SH2D1A or EAT-2 to their ITSMs (*16*). This extended consensus also applies to the two ITSMs of BepE (SETIYTTV and SETLYAEV). The biological importance of the ITIMs/ITSMs as well as of the putative Csk binding site in the genus *Bartonella* is stressed by the high degree of conservation of these motifs between the BepE homologues of *Bartonella henselae (*BepE_Bh, accession-nr. Q6G2A4), *Bartonella quintana* (BepE_Bq, accession-nr. Q6FYV7) and *Bartonella tribocorum* (BepE_Bt, unpublished data, courtesy of H. Saenz) as shown in Fig. 4c. In contrast, the spacer sequence in between these motifs is very divergent, alike the spacer sequences of the various ITIM/ITSM-containing IgG-like receptors (Fig. 4a).

BepE co-immunoprecipitates with Csk and SHP2 in a phosphorylation-dependent manner. To confirm the binding of Csk and SHP2 to BepE, we co-transfected a plasmid expressing FLAG tagged BepE in HEK293T cells together with a plasmid expressing c-Src or the empty vector control. Following immunoprecipitation with the FLAG antibody we probed the precipitate with anti-FLAG, anti-phosphotyrosine, anti-SHP2, and anti-Csk antibodies (Fig. 5). BepE is tyrosine-phosphorylated by the endogenous levels of tyrosine kinases present in the HEK293T cells. This tyrosine-phosphorylation can be dramatically increased by co-transfecting the cells with a vector expressing c-Src. Whereas already weakly tyrosine-phosphorylated BepE binds SHP2 and Csk, enhancing its tyrosine-phosphorylation also massively augments the amounts of SHP2 and Csk which are bound to BepE.

Mapping the binding sites of Csk and SHP2 in BepE by tyrosine to phenylalanine exchanges. By screening a phosphopeptide containing Y32 of BepE ("..EPLYATVNK...") for cellular interaction partners using the SILAC method (*23*), Csk was shown to bind to this peptide in a tyrosine-phosphorylation dependent manner (M. Selbach, P. Guye, M. Mann and C. Dehio, unpublished results). To assess if this is the only binding site of Csk in BepE and to further characterize the binding sites of SHP2, we performed a systematic mutagenesis of the tyrosines in the putative phosphorylation motifs of BepE to phenylalanines. These FLAG-tagged mutants were then co-expressed in HEK293T cells with a vector expressing c-Src or an empty control. The c-Src co-expression was used not to miss any SFK target motifs in BepE that might be weakly tyrosine-phosphorylated by endogenous amounts of the kinases. On the other hand, overexpressing c-Src might mask subtle differential or partial phosphorylation of these motifs, so we also co-expressed BepE with an empty vector control. Following lysis and immunprecipitation for the FLAG tag, we blotted and probed the precipitate for phosphotyrosine, Csk, FLAG tag and SHP2 (Fig. 6).

The BepE mutant in all five putative tyrosine-phosphorylation sites (BepE_FFFFF) did not show any detectable tyrosine-phosphorylation, in contrast to the wild type (BepE_YYYYY), and did neither bind Csk nor SHP2. In the mutant Y37F (BepE_FYYYY) the interaction with Csk is abolished (Fig. 6), demonstrating that Y37 mediates binding of Csk to BepE. Supporting this, exchanging all tyrosines of the ITIMs/ITSMs to phenylalanines and only preserving Y37 (BepE_YFFFF) is sufficient for Csk binding to BepE.

The interaction of SHP2 to BepE is more complex. Whereas the exchange of the tyrosines in all ITIMs/ITSMs (BepE_YFFFF), abolished the interaction between BepE and SHP2, no complete loss of binding was visible by exchanging single or tandem tyrosines. The most striking reduction of SHP2 binding was visible in BepE_YFYYY without co-expression of c-Src, indicating of the first ITIM was the most crucial binding site for one of the SH2 domains of SHP2.

Discussion

ITIM-containing receptors play a crucial role in downregulating the activation of almost all known types of immune cells. Here, we report a protein secreted into infected cells by the pathogen *Bartonella henselae,* for which we have several lines of evidence that it is a molecular mimicry of such a receptor: (i) BepE has a membrane-proximal localization and also localizes to cell-cell contacts of infected cells. (ii) It contains in its N-terminus a Csk-binding motif and two ITIM/ITSM tandems with a spacing matching the one occurring in immune receptors. (iii) BepE is tyrosine-phosphorylated in these motifs by the SFK c-Src *in vitro* and *in vivo*, and binds upon phosphorylation Csk and SHP2. Data of ongoing work also indicate that *Bh* translocates this protein into dendritic cells and monocytes (P. Guye, S. Paoletti, G. de Libero and C. Dehio unpublished results).

BepE is translocated into infected cells by the T4SS of *Bh*, as shown for HELA cells and HUVECs by means of immunofluorescence (Fig. 2). We also show that BepE is only tyrosine-phosphorylated in the infection process when secreted by wild-type *Bh*, as BepE from T4SS-defective *Bh* does not exhibit any tyrosine-phosphorylation. This further supports the notion that BepE is a substrate of the VirB/VirD4 apparatus of *Bh*, as it must be translocated by the T4SS into the host cells for being tyrosine-phosphorylated by host cell kinases. It is important to note that most inhibitory immune receptors are thought to have a very weak or absent basal tyrosine-phosphorylation, which is enhanced upon engagement of the receptors. The constitutive phosphorylation of BepE indicates that this protein might induce signaling in the absence of an external stimulus, as one would expect by an effector mimicking a receptor to subvert the cell signaling. We reported earlier the translocation for some of the Bep proteins using the Cre recombinase reporter assay for translocation (CRAFT) (4). Interestingly, BepE was negative in this screen. This is probably due to its plasma membrane-proximal localization (Fig. 2a), which interferes with the nuclear localization of the Cre-BepE fusion protein needed for the CRAFT readout. The same effect was observed for BepA, which also localizes to the plasma membrane of infected cells and is negative in the CRAFT assay, but positive for translocation in other

readouts, such as the calmodulin-dependent adenylate cyclase (cya)-reporter assay (M. Schmid, Chapter 3.3, this work). Additionally, BepE localizes prominently to the cell-cell contacts of endothelial cells as shown by its co-localization with VE-Cadherin in HUVECs (Fig. 2a). This peculiar localization provided crucial information concerning the putative functions of BepE in endothelial cells, which is currently investigated. Intriguingly, both localizations are not dependent on the tyrosine-phosphorylation of BepE, since the construct lacking the N-terminus localizes like the full-length protein. The weaker signal for this construct in contrast to wild-type BepE (Fig. 2a) might be due to a folding of the fusion protein which does not optimally expose the tag or due to a reduced stability of this truncated protein.

Since it has been reported that the SFKs are responsible for tyrosine-phosphorylation of other bacterial effectors as Tir or CagA, we tested *in vitro* and *in vivo* the effects of c-Src on BepE. While HIS-tag purified BepE is not tyrosine-phosphorylated by incubating it together with ATP, the addition of recombinant active c-Src dramatically induces phosphorylation on tyrosine residues (Fig. 3). This assay rules out an autocatalytic phosphorylation of BepE. To further assess the role of c-Src *in vivo*, we co-transfected HEK293T cells with a vector expressing FLAG-tagged BepE and one expressing c-Src. While the endogenous amounts of SFKs already tyrosine-phosphorylate BepE, the co-expression of c-Src further bolsters this effect (Fig. 5). Strikingly, in both the *in vitro* an *in vivo* kinase assays, tyrosine-phosphorylated BepE undergoes an apparent mass shift towards a higher mass, an effect which has also been reported for other tyrosine-phosphorylated proteins (24).

Bioinformatic analyses of the BepE protein sequence revealed five motifs in the N-terminus as putative tyrosine-phosphorylation sites. These motifs could be further classified as (i) putative Csk-binding sites, similar to a described Csk-binding site in VE-Cadherin (Fig. 4b, one motif), (ii) ITIMs (Fig. 4a, 4c, two motifs), and (iii) ITSMs (Fig. 4a, 4c, two motifs). Furthermore, the spacing between the ITIMs and ITSMs in both tandems matched the consensus for these tandems in inhibitory immune receptors. The comparison of BepE from *Bh*, *Bt* and *Bq* supports the idea of an important biological function, as they are well conserved between the three species in contrast to the surrounding sequences (Fig. 4c).

The ITIMs and ITSMs motifs have been described in inhibitory immune receptors, where they bind upon tyrosine-phosphorylation phosphatases like SHP1, SHP2 and SHIP, or kinases like Csk and thereby suppress the activation of the immune cells.

Mapping of the tyrosine-phosphorylation sites in BepE by mass spectrometry revealed three expected motifs which were tyrosine-phosphorylated in *in vitro* phosphorylated BepE: the Csk-binding site and the two ITIMs. Fragments covering the first ITSM could only be detected in their unphosphorylated state. This finding indicates that c-Src does not phosphorylate this ITSM, but probably another kinase does *in vivo*. We have indeed results indicating Csk being able to tyrosine-phosphorylate BepE *in vivo* (data not shown). As a model, c-Src might first phosphorylate BepE on its Csk-binding site, followed by the binding of Csk to this motif from where it then in turn tyrosine-phosphorylates the ITSM(s). Fragments covering the second ITSM could neither be detected in their tyrosine-phosphorylated, nor in their unphosphorylated state, which is probably due to some technical problems associated to these peptide sequences. Additionally, three more peptides were detected that were tyrosine-phosphorylated by the c-Src kinase *in vitro*, albeit at a significantly lower efficiency (Table 1). A tyrosine to phenylalanine exchange mutant of BepE for the putative Csk-binding site and the two ITIM-ITSM tandems (BepE_FFFFF) showed in co-transfections with c-Src no detectable tyrosine-phosphorylation (Fig. 6), which indicates that the three additionally detected tyrosine-phosphorylated peptides are probably artifacts of this *in vitro* kinase assay. This might be due to the HIS-tag purified BepE exposing residues, which are not accessible *in vivo*, or c-Src kinase and/or kinase buffer conditions not optimally reflecting the *in vivo* conditions. Analysis of *in vivo* tyrosine-phosphorylated BepE revealed one tyrosine-phosphorylated motif ("..EPLYAVNK.."). This analysis is still ongoing for the other motifs.

Having identified tyrosine-phosphorylation motifs also occurring in eukaryotic signaling proteins, we continued by analyzing interactions partners. We showed through co-immunoprecipitation that Csk and SHP2 interact with BepE in a phosphorylation-dependent manner in HEK293T cells (Fig. 5). Additionally, the binding of Csk to a phosphopeptide containing the "EPLYAVNK" sequence was confirmed by the SILAC method in collaboration with the laboratory of Matthias Mann (M. Selbach, P. Guye, C. Dehio, M. Mann, unpublished results). This

powerful novel technique might provide very useful in the retrieval of further interaction partners.

Since BepE contains multiple phosphorylated tyrosines and putative tyrosine-phosphorylation motifs, we systematically exchanged these tyrosines to phenylalanines and studied the effects of these exchanges on the binding of BepE to Csk and SHP2. We expressed the mutant forms of BepE in HEK293T cells by additionally facultatively co-expressing the c-Src kinase, immunoprecipitated it and probed the precipitate for Csk and SHP2 (Fig. 6). Csk binds to the most N-terminal tyrosine-phosphorylation motif of BepE, which does not match the consensus of an ITIM or ITSM pattern, but is similar to the Csk-binding site of VE-Cadherin. The binding of SHP2 is more elusive. Clearly, the binding of SHP2 to BepE can be completely abrogated by mutating the four tyrosines of the two ITIM/ITSM tandems to phenylalanines. Most SHP2 binding is lost by mutating Y64 and Y106, Y64 having a more prominent role in this effect. This might indicate a preference for SHP2 to bind both ITIM motifs with its two SH2 domains, but still having some residual lower affinity for the other ITIM/ITSM combinations. Additionally, we also showed a differential phosphorylation of the motifs *in vitro* by the means of mass spectrometry (Table 1), and *in vivo* by western blotting (Fig. 6), indicating that some motifs like the N-terminal first ITIM are preferentially phosphorylated, and therefore can bind more SHP2. While the binding of only one SH2 domain of SHP2 to BepE may be a possibility, binding of both domains is needed to achieve full phosphatase activity (25). This might of course also be achieved by having two BepE proteins in close contact, with each offering one binding site for an SH2 domain. The complexity of the system is further enhanced by the presence of two ITSMs. These motifs were shown to bind to adapter proteins such as SAP or EAT-2, and could therefore a.) switch the signaling mode from inhibitory to activatory by recruiting further phosphatases or kinases, and/or b.) regulate the accessibility of dual/single SH2-containing phosphatase to the ITIMs/ITSMs (*17*). The binding of these adapters, which are only expressed in hematopoietic cell lines is currently being investigated, and might shed light on how BepE could act as a repressor in one cell type and as an activator in another one.

The sequence similarities, the localization, and the interaction partners of BepE suggest that this protein mimicks inhibitory immune receptors. The subversive

potential of such receptor mimicry in the immune cells is huge. Translocated into NK cells or $CD8^+$ T-cells, it might inhibit the killing of infected cells (26, 27), in $CD4^+$ T-cells it could disturb the activation of B-cells and macrophages (27). In B-cells inhibitory receptors were shown to downmodulate their activation upon ligation of antigens to their BCR (28). In monocytes some of these receptors were shown to suppress the activation of the TLR-2 and TLR-4 pathways, subsequently inhibiting the TNFα secretion (29). Expression of inhibitory receptors on DCs concomitantly with a certain antigen induces tolerance of the immune system to this antigen, by anergizing the respective $CD4^+$ T-cell clones (30). This mechanism could by used by a pathogen to specifically induce tolerance to its antigens in a host, without comprising the immune response to other pathogens.

The binding of SHP2 and Csk by their SH2 domain(s) to BepE also promotes the understanding of BepE's functions in the context of endothelial cells. The co-localization, and the similarity of the Csk-binding site of BepE to VE-Cadherin is currently investigated in our *in vitro* HUVEC models, as this site has been shown in VE-Cadherin to be crucial in mediating the contact-dependent inhibition of cell growth (*21*). The association of Csk over its SH3 domain with the tyrosine phosphatase PEST and their binding as a complex to Paxillin, where they dephosphorylate various proteins building up the focal adhesions, affects the motility of the cells (31, 32). Interfering with this contact inhibition and/or the cell motility by BepE may be one of the factors promoting the bacillary angiomatosis.

A further mode for remodeling the structure of the endothelium *in vivo* is achieved by involving the immune system. Endothelial cells express MHC class I and II complexes and were shown to interact with and present antigens to circulating T-cells. This interaction is bi-directional, as circulating activated T-cells can influence the signaling and behavior of endothelial cells, as for example the formation and remodeling of blood vessels (33). This signaling might be disturbed by a protein mimicking the inhibitory immune receptors and attenuate the activation of the cells involved. The sparse knowledge concerning antigen presentation by endothelial cells, and the mechanisms underlying their interactions with T-cells leaves us regrettably to only speculate about this.

Further characterization of the functions of BepE should give us completely new insights about how highly adapted pathogens survive, subvert and hide in their hosts, possibly even elucidating diseases which were not associated with pathogens up to now.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table S1 of the Supplemental Material. *Bartonella henselae* were grown on Columbia agar plates containing 5% defibrinated sheep blood (CBA plates) at 35°C and 5% CO_2 for 2-4 days. Strain RSE247, a spontaneous streptomycin-resistant strain of ATCC 49882T (34) served as wild-type in this study. When indicated, media were supplemented with 30 µg/ml kanamycin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, and/or 500 µM isopropyl-β-D-thiogalactoside (IPTG). *E. coli* strains were cultivated in Luria-Bertani liquid medium (LB) or on Luria-Bertani agar on plates (LA) at 37°C overnight. When indicated, media were supplemented with 50 µg/ml kanamycin, 200 µg/ml ampicillin, 20 µg/ml gentamicin, 500 µM IPTG, and/or 1 mM diaminopimelic acid (DAP).

Cell lines and culture conditions. HUVECs were isolated as described (34). The embryonic kidney cell line HEK293T and HUVECs were cultured as described (3).

DNA manipulations. Plasmids used in this study are listed in Supplementary Material Table S1, primers are listed in Supplementary Material Table S2.

Plasmid for expression of FLAG-BepE. The plasmid expressing FLAG-BepE was built as follows: Full length *bepE* was amplified with the primer pair prPG101 and prPG101 from chromosomal DNA of RSE247 as template. Using the flanking *Nde*I-sites, the fragment was ligated into previously *Nde*I-cut pPG100.

Plasmid for expression of proteins without an epitope tag. For constructing vector pPG110 expressing native proteins, the oligonucleotide primers prPG90 and prPG91 were used to amplify a 0.4 kb fragment from pRS40 template DNA. Using flanking *Sac*I sites the generated fragment was inserted into the corresponding site of pRS40. Next, a 1.67 kb fragment containing the complete

bepD gene and an N-terminal MYC tag was amplified using oligonucleotide primers prPG141 and prPG145 and chromosomal DNA of RSE247 as template. Using flanking *Nde*I-sites the amplified fragment was ligated into the corresponding site of pPG110, yielding pPG184. The empty MYC tag expression vector, pPG180, was generated by cutting out *bepD* with *Nde*I from pPG184, and religating the vector.

Plasmid for expression of MYC-BepE. By excising full length *bepE* from pPG105 with *Nde*I and inserting it into the respective sites in pPG180, the MYC::BepE expressing vector, pPG185, was constructed. The vector expressing the two C-terminal BID domains of BepE without the N-terminus, pPG172, was constructed by PCR amplifying a fragment of 1 kb from pPG105 with the primers prPG148 and prPG149. After digesting the fragment with *Nde*I, it was inserted in the respective site of pPG180.

Plasmid for expression of HIS-BepE. Full-length *bepE* was amplified using the primer pair prPG83 and prPG84 from chromosomal DNA of RSE247 as template. Following digestion of the fragment with *Xho*I and *Bam*HI, it was ligated into the respective sites of pET15b.

Tyrosine to phenylalanine exchange mutants in BepE. To exchange the putatively phosphorylated tyrosines to phenylalanines in the N-terminus of BepE, we applied megaprime PCR to reamplify the sequence coding for BepE from pPG185 or from the respective mutants as shown in Table S2 of the Supplementary Materials. Briefly, using prPG190 and prPG191 binding on the plasmid pPG185 and two corresponding primers annealing to the site to be mutated, two fragments with a sequence overlap in the mutation site were amplified by PCR. In a second step, these two fragments were joined by megaprime PCR using prPG190 and prPG191, yielding a fragment of 2.1 kb. This fragment was then cut with NdeI, and inserted into the previously NdeI-cut pPG180 vector. The following mutants were generated in a first round using pPG185 as a template: Y37F (BepE_FYYYY), Y64F (BepE_YFYYY), Y91F (BepE_YYFYY), Y106F (BepE_YYYFY), Y129F (BepE_YYYYF) by using prPG192/prPG193, prPG194/prPG195, prPG204/205, prPG206/207, and

prPG196/197 respectively. In a second round, Y64F (BepE_YFYYY) was used as a template to generate Y64F;Y106F (BepE_YFYFY), Y91F (BepE_YYFYY) to generate Y64F;Y91F (BepE_YFFYY), and Y91F;Y129F (BepE_YYFYF), Y129F (BepE_YYYYF) to generate Y106F;Y129F (BepE_YYYFF). Finally, in three sequential mutation rounds, using Y64F;Y106F (BepE_YFYFY), I generated Y64F;Y91F;Y106F (BepE_YFFFY), Y64F;Y91F;Y106F;Y129F (BepE_YFFFF), and Y37F;Y64F;Y91F;Y106F;Y129F (BepE_FFFFF).
The sequences encoding the different BepE mutants were then reamplified by PCR from their respective bacterial shuttle vectors using prTR15 and prTR16, cut with NotI and BamHI, and ligated into the corresponding sites of the pFLAG-CMV2 plasmid, yielding the mutant panel in the eukaryotic expression vector.

Immunocytochemical stainings and immunoprecipitation. HUVEC and HELA were infected with *Bh* strains at a MOI of 150 bacteria per cell for 48 hours. Subsequently, they were fixed in 3.7% paraformaldehyde and stained for F-actin (TRITC-Phalloidin, Sigma, St. Louis, MO/USA), MYC-tagged BepE (mouse anti-MYC, Invitrogen, Carlsbad, CA/USA), VE-Cadherin (rabbit anti-VE-Cadherin, Bender MedSystems, Burlingame, CA/USA), or total bacteria as described (3). Pictures were acquired with a Leica DM-RXE confocal microscope. Immunoprecipitation and Western blotting were performed as described (4).

Transfection of HEK293T cells. 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" (35). After 12 hours, the cell culture media was replaced and the cells kept in culture for additional 24 hours. Harvesting and immunoprecipitation was performed as described (4).

***In vitro* / *in vivo* phosphorylation and mass spectrometry analysis.** Purified HIS-tagged BepE was incubated with recombinant active c-Src kinase (Upstate, Charlottesville, USA) in phosphorylation buffer (33 mM TrisHCl pH 7.2, 40 mM $MgCl_2$, 8 mM $MnCl_2$, 700 μM EGTA, 80 μM sodium vanadate, 500 μM ATP) at 30 °C for 60 minutes.

Transfected FLAG-BepE from HEK293T cells was immunoprecipitated as described (4) and washed five times in wash buffer (50 mM TrisHCl pH 7. 4, 75 mM NaCl) to remove residual detergents. The anti-FLAG agarose bead pellet containing the precipitated proteins was then incubated for 5 minutes on ice in 100 µl low pH buffer (36) (0.1 M Glycine, pH 2.5) to dissociate the FLAG tag from the antibody and centrifuged for 1 minute at 4500 g. For neutralizing the eluated proteins, we immediately removed the supernatant following centrifugation, and added 40 µl neutralization buffer (0.2 M TrisHCl pH 9.0).
The *in vitro* and *in vivo* phosphorylated proteins were then digested with the proteases Trypsin or V8 after they had been dialyzed against 0.1 M NH_4HCO_3. The digests were stopped by adding 1/10th of the volume 10% acetic acid to. The resulting peptides were analysed by capillary liquid chromatography tandem MS (LC-MS/MS) using a Magic C18 100 µm internal diameter, 10 cm HPLC column (Spectronex, Basel, Switzerland) connected on line to a hybrid Finnigan Orbitrap instrument (ThermoFinnigan, San Jose, CA, U.S.A.). A linear gradient from 5 to 75% B [0.1% acetic acid and 80% (v/v) acetonitrile in water] in A (0.1% acetic acid and 2% acetonitrile in water) in 60 min was delivered with a Rheos 2200 HPLC system (Flux, Basel, Switzerland) at 100 µl/min. A precolumn flow splitter reduced the flow to approx. 300 nl/min. 2 µl of the peptides were introduced into the injection loop of the MS. The eluting peptides were ionized by electrospray ionization, detected and the peptide ions were automatically selected and fragmented by collision induced dissociation (MS/MS) in the ion-trap. Individual MS/MS spectra were compared against the protein sequence of BepE using the TurboSequest software. The fragment spectrum of those peptides that had a mass shift of 80 Da were manually inspected to unambiguously assign the phosphorylated tyrosine.

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Table S1: *Bacterial strains and plasmids used in this study*

Strain or plasmid	Genotype or relevant characteristics	Reference
Plasmids		
pRS40	vector for expression of NLS::Cre::MobA fusion proteins	(4)
pPG100	*E.coli-Bartonella* shuttle vector, encoding a short FLAG tag	(4)
pPG110	*E.coli-Bartonella* shuttle vector, for tag-less expression	this study
pPG180	*E.coli-Bartonella* shuttle vector, encoding a short MYC tag	this study
pET15b	vector for expressing HIS-tag tagged proteins in the pET system	Novagen, Madison
pPG125	encoding HIS::BepE, derivative of pET15b	
pPG105	encoding FLAG::BepE	this study
pPG184	encoding MYC::BepD	this study
pPG185	encoding MYC::BepE_WT (wildtype)	this study
pPG185.M2	encoding MYC::BepE_FYYYY	this study
pPG185.M4	encoding MYC::BepE_YFYYY	this study
pPG185.M16	encoding MYC::BepE_YYFYY	this study
pPG185.M20	encoding MYC::BepE_YFFYY	this study
pPG185.M32	encoding MYC::BepE_YYYFY	this study
pPG185.M36	encoding MYC::BepE_YFYFY	this study
pPG185.M52	encoding MYC::BepE_YFFFY	this study
pPG185.M64	encoding MYC::BepE_YYYYF	this study

Table S1 continued

Strain or plasmid	Genotype or relevant characteristics	Reference
pPG185.M80	encoding MYC::BepE_YYFYF	this study
pPG185.M96	encoding MYC::BepE_YYYFF	this study
pPG185.M116	encoding MYC::BepE_YFFFF	this study
pPG185.M118	encoding MYC::BepE_FFFFF	this study
pCMV-FLAG2	eukaryotic expression vector, encoding a short FLAG tag	Eastman Kodak, New Haven
pTR1173	encoding FLAG::BepE_YYYYY (wild-type)	this study
pPG1173.M2	encoding FLAG::BepE_FYYYY	this study
pPG1173.M4	encoding FLAG::BepE_YFYYY	this study
pPG1173.M16	encoding FLAG::BepE_YYFYY	this study
pPG1173.M20	encoding FLAG::BepE_YFFYY	this study
pPG1173.M32	encoding FLAG::BepE_YYYFY	this study
pPG1173.M36	encoding FLAG::BepE_YFYFY	this study
pPG1173.M52	encoding FLAG::BepE_YFFFY	this study
pPG1173.M64	encoding FLAG::BepE_YYYYF	this study
pPG1173.M80	encoding FLAG::BepE_YYFYF	this study
pPG1173.M96	encoding FLAG::BepE_YYYFF	this study
pPG1173.M116	encoding FLAG::BepE_YFFFF	this study
pPG1173.M118	encoding FLAG::BepE_FFFFF	this study

Table S1 continued

Strain or plasmid	Genotype or relevant characteristics	Reference
pCB6	empty eukaryotic expression vector	(37)
pCD211	eukaryotic expressing vector, derivative of pCB6, encoding chicken c-Src	(37)
***E. coli* strains**		
NovaBlue	endA1 hsdR17(r K12–m K12+) supE44 thi-1 recA1 gyrA96 relA1 lac[F' proA+B+ lacI$_q$ZΔM15::Tn10 (Tc^R)]	Novagen, Madison
β2150	F' lacZDM15 lacIq traD36 proA+B+ thrB1004 pro thi strA hsdS lacZΔM15 ΔdapA::erm (Erm^R) pir	(34)
Rosetta (DE3)	F– ompT hsdS (r – m –) gal dcm (DE3) pRARE2 (Cam^R)	Novagen, Madison
***B. henselae* strains**		
RSE242	Δ*virB4* mutant, derivative of RSE247	(3)
RSE247	Spontaneous Sm^R strain of ATCC 49882T, serving as wild-type	(3)
TRB106	Δ*bepB-G* mutant, derivative of RSE247	(4)

Table S1 continued

Strain or plasmid	Genotype or relevant characteristics	Reference
PGB44	RSE247 containing pPG105	this study
PGB45	RSE242 containing pPG105	this study
PGB59	RSE247 containing pPG100	this study
PGB55	RSE242 containing pPG100	this study
PGE76	TRB106 containing pPG180	this study
PGE77	TRB106 containing pPG185	this study
PGH01	TRB106 containing pPG185.M2	this study
PGH02	TRB106 containing pPG180.M4	this study
PGH03	TRB106 containing pPG180 .M16	this study
PGH04	TRB106 containing pPG180 .M20	this study
PGH05	TRB106 containing pPG180 .M32	this study
PGH06	TRB106 containing pPG180 .M36	this study
PGH07	TRB106 containing pPG180 .M52	this study
PGH08	TRB106 containing pPG180 .M64	this study
PGH09	TRB106 containing pPG180 .M80	this study
PGH10	TRB106 containing pPG180 .M96	this study
PGH11	TRB106 containing pPG180 .M116	this study
PGH12	TRB106 containing pPG180 .M118	this study
PGH18	TRB106 containing pPG172	this study

Table S2: *Oligonucleotides used in this study*

Name	Sequence	Restriction sites
prTR15	ATAAGAATGCGGCCGCGATGAAAAGAAATCAACCACCCC	*Not*I
prTR16	CGGGATCCTTAGATGGCGAAAGCTATTGCC	*Bam*HI
prPG83	CCGCTCGAGATGAAAAGAAATCAACCACCC	*Xho*I
prPG84	CGGGATCCTTAGATGGCGAAAGCTATTGC	*Bam*HI
prPG90	CGCGAGCTCTTTAAGAAGGAGATATACATATGGG	*Sac*I, *Nde*I
	GATGCCTGGCAGTTTAT	
prPG91	CGCGAGCTCTAAATCAGAACGCAGAAGCG	*Nde*I
prPG100	GGAATTCCATATGAAAAGAAATCAACCACCC	*Nde*I
prPG101	GGAATTCCATATGTTAGATGGCGAAAGCTATTGC	*Nde*I
prPG141	CGCGCTTATTAATGGAACAAAAACTTATTTCTGAA	*Ase*I, *Nde*I
	GAAGATCTTCATATGAAAAAAAATCGACCATCCCCT	
prPG142	CGCGCTTATTAATCATATGTTACATACCAAAGGCCA	*Ase*I, *Nde*I
	TTCC	
prPG148	GGAATTCCATATGGTCGACTACCACGCCGGATCCAGCAC	*Nde*I, *Sal*I,
	AATACCATCTCTAACAAGAGAG	*Bam*HI
prPG149	GGAATTCCATATGTTAGATGGCGAAAGCTATTGC	*Nde*I
prPG190	GCTCCGCCATCGCCGCT	
prPG191	GGATTTGAACGTTGCGAAGC	
prPG192	CACCGCAACCAGAACCTTTATTTGCAACGGTGAACA	
	AAAGACC	
prPG193	GGTCTTTTGTTCACCGTTGCAAATAAAGGTTCTGGTT	
	GCGGTG	
prPG194	AGCCAACAGGAAGAAGTCGTATTTGCAGCGCTTGAT	
	TTTGAAAATAGATC	

Table S2 continued

Name	Sequence	Restriction sites
prPG195	GATCTATTTTCAAAATCAAGCGCTGCAAATACGACTT CTTCCTGTTGGCT	
prPG196	ATCCGGCAAGCGAAACTTTATTTGCAGAAGTTGCTAT GCAAAGCAC	
prPG197	GTGCTTTGCATAGCAACTTCTGCAAATAAAGTTTCGC TTGCCGGAT	
prPG204	GAATCTAGAAAGCGAAACTATATTTACGACAGTTTCTT CTCAAAG	
prPG205	CTTTGAGAAGAAACTGTCGTAAATATAGTTTCGCTTTC TAGATTC	
prPG206	CAACACAAGCAGAAATACTCTTTGCAGATGTCACTC ACAC	
prPG207	GTGTGAGTGACATCTGCAAAGAGTATTTCTGCTTGT	

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Tables and Figures

Table. 1. Mapping the phosphorylated tyrosines by the means of mass spectrometry. HIS-affinity purified, and *in vitro* phosphorylated BepE was digested with proteases and subjected to mass spectrometry analysis as described in *Materials and Methods*. The sequence of the detected fragments containing the putative phosphorylation sites and their positions in BepE are indicated in the first and second column, the enzyme used for their digestion in the third. The measured masses of the fragments in their unphosphorylated and in their tyrosine-phosphorylated state (mass and pY-mass) are in the fourth and fifth columns. The percentage (pY %) indicates how much of a given fragment was detected to be tyrosine-phosphorylated vs. the total amount (counts) of this fragment. For one fragment, its mass could not be detected in its tyrosine-phosphorylated form (not detectable, n.d.).

Fig. 1. BepE becomes tyrosine phosphorylated after VirB4-dependent translocation into HUVEC. Total protein extracts of HUVECs uninfected (lane 1) or infected with Δ*virB4* (lane 2), wild type (lane 3), Δ*virB4*/pPG105 (lane 4), or wild type/pPG105 (lane 5) were prepared. FLAG-BepE encoded by pPG105 was immunoprecipitated with anti-FLAG antibodies, separated by SDS/PAGE, and immunoblotted with anti-FLAG (*left*) or anti-phosphotyrosine antibodies (*right*).

Fig. 2. BepE localizes to the membrane of HELA cells and to the membrane and cell-cell contacts of infected HUVECs. (A) Full-length MYC tagged BepE (MYC::BepE) and the two MYC tagged C-terminal BID domains (MYC::BIDs). The (+++) indicates the positively charged tail sequence C-terminally to the BID, required for the VirB/VirD4-dependent translocation (B) The two C-terminal BID domains of BepE are sufficient to achieve this localization. HELA cells were infected for 48 h at a MOI of 150 bacteria per cell, fixed, permeabilized, and stained for f-actin (red), MYC-tag (green), and total bacteria (blue). (1) uninfected cells, (2) empty vector control, (3) MYC::BepE, (4) MYC::BIDs. The

scale bar represents 20 μm. (B) HUVECs were infected for 48 h with 150 bacteria per cell, fixed, permeabilized, and stained for VE-Cadherin (red), MYC-tag (green), and f-actin (blue). (1) uninfected cells, (2) empty vector control, (3) MYC::BepE, (4) MYC::BIDs. The scale bar represents 20 μm.

Fig. 3. BepE is tyrosine-phosphorylated *in vitro* by the c-Src kinase. HIS-tag purified BepE was incubated with recombinant purified active c-Src kinase and ATP, separated by SDS/Page stained with Coomassie or immunoblotted with anti-FLAG or anti-phosphotyrosine antibodies.

Fig. 4. BepE contains similar tyrosine-phosphorylation motifs to the ones in eukaryotic inhibitory immune receptors and VE-Cadherin and is conserved in these motifs between different *Bartonella* species. (A) BepE contains two ITIM/ITSM tandems with similar sequence conservation and spacing as in immunotyrosine inhibitory receptors (BepE_Bh: Q6G2A4; LIRB1: Q8NHL6; LAIR-1: Q8NHJ6; CD33: P20138; PD-1: Q15116; NTB-A: Q96DU3; CD244: Q9BZW8; CD31: P16284; SHPS-1: P78324) (B) BepE contains a putative binding site for Csk, as the similarity to the known binding site of Csk in the human VE-Cadherin (accession-nr. P33151) suggests. (C) BepE of *Bartonella henselae*, *Bartonella quintana* and *Bartonella tribocorum* show a high degree of conservation in the putative Csk binding sites and the ITIM/ITSM tandems, suggesting a evolutionary pressure to maintain these motifs. The spacer sequences between the motifs is highly variable, alike the spacer sequences in between the ITIM/ITSM motifs of immunotyrosine inhibitory receptors (B).

Fig. 5. BepE binds Csk and SHP2 in a phosphorylation dependent manner. HEK293T cells were transfected with a vector expressing FLAG-BepE (pTR1173) or the empty vector (pCMV-FLAG2) and with a plasmid expressing c-Src (pCD211) or the respective empty vector (pCB6). Following anti-FLAG immunoprecipitation, separation by SDS/PAGE and western blotting, we probed the membranes with anti-FLAG, anti-phosphotyrosine (pY), anti-Csk and anti-

SHP2 antibodies. Lane 1: pCMV-FLAG2 and pCB6, Lane 2: pTR1173 and pCB6, Lane 3: pCMV-FLAG and pCD211, Lane 4: pTR1173 and pCD211.

Fig. 6. Mapping the binding sites of Csk and SHP2 in BepE. HEK293T cells were transfected with vectors expressing FLAG-tagged tyrosine to phenylalanine mutants of BepE (Table 2 in Supplementary Materials) together with a plasmid expressing c-Src or the empty control vector and probed as described in Fig 5.

peptide	residue (aa)	cutting enzyme	mass (Da)	pY-mass (Da)	pY (%)
QREP**Y**GQSAER	13-23	Trypsin	1304.67	1384.64	17
APSPQPEPL**Y**AT VNK	24-38	Trypsin	1611.84	1691.8	48
ELNKPLSQQE EVV**Y**AALDFENR	47-68	Trypsin	2592.29	2672.26	88
SETI**Y**TTVSS QSTTQAE	83-99	V8	1832.84	n.d.	n.d.
IL**Y**ADVTHTPLH SKHTRRDPASE	100-122	V8	2644.36	2724.33	65
IV**Y**GNSDVLK	165-174	Trypsin	1107.6	1187.57	0.2

Table 1

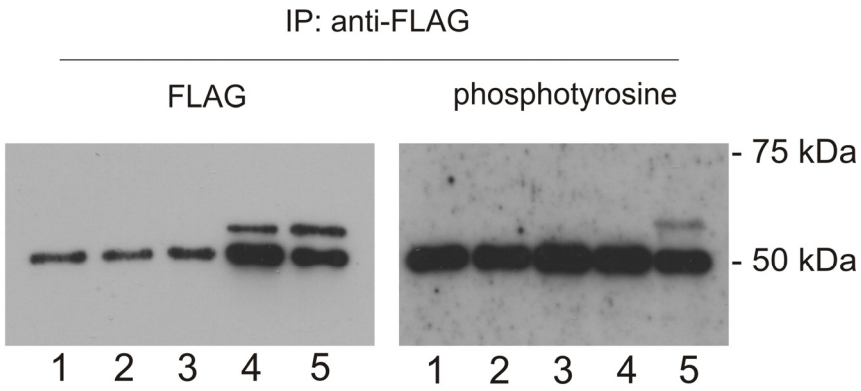
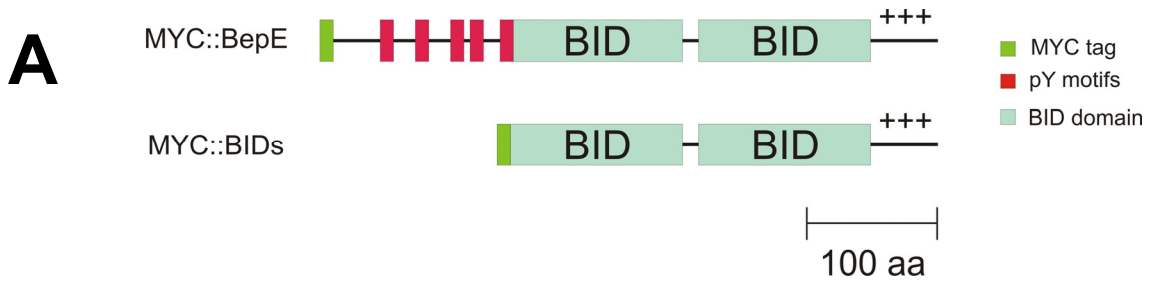


Fig. 1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BID
+++
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+++
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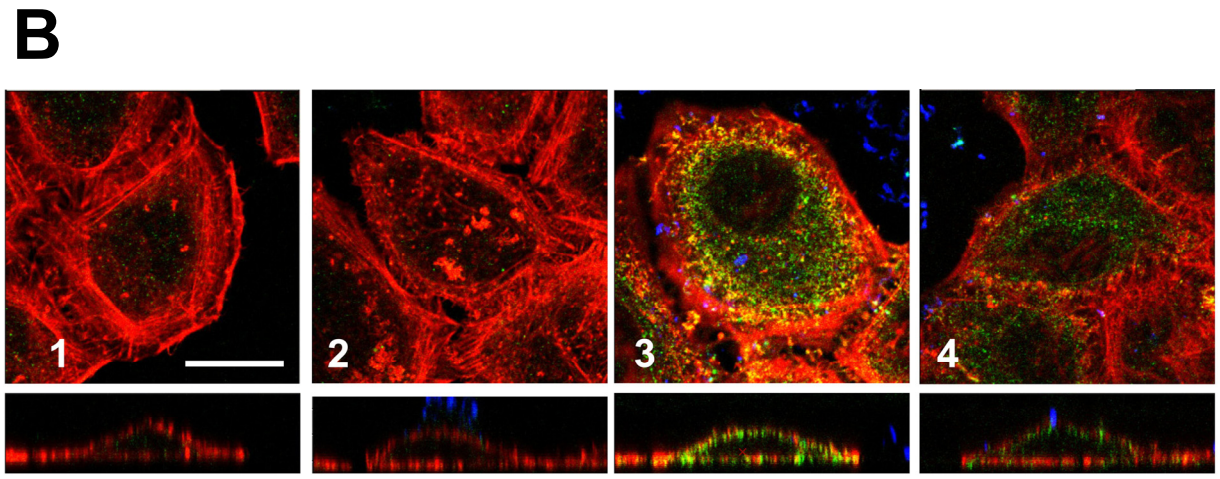
BID domain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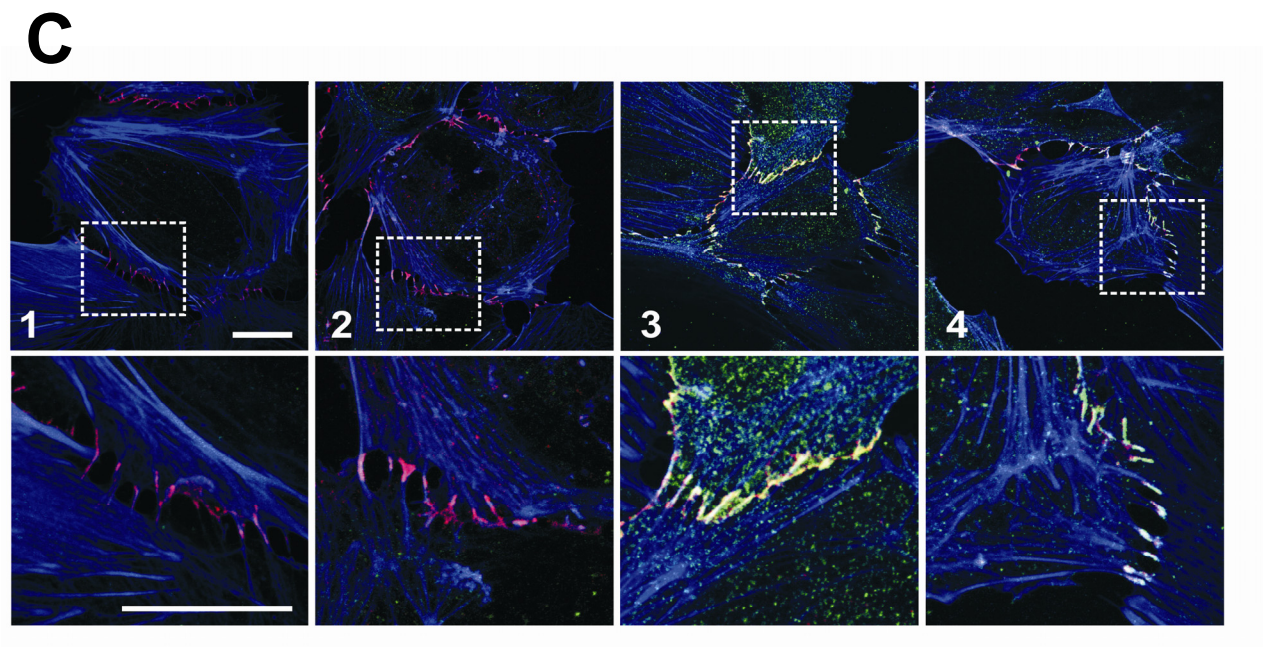
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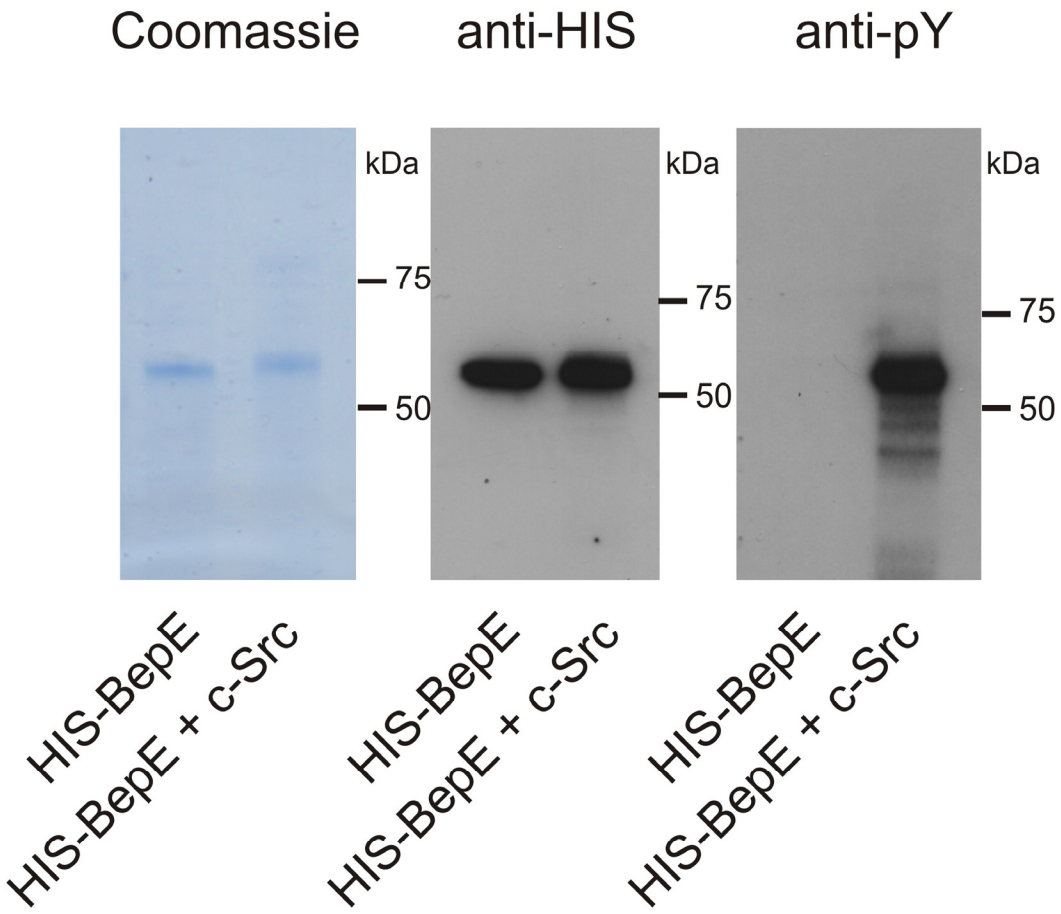
Fig. 2


Coomassie
anti-HIS
anti-pY
kDa
75
50
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75
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kDa
75
50
HIS-BepE
HIS-BepE + c-Src
HIS-BepE
HIS-BepE + c-Src
HIS-BepE
HIS-BepE + c-Src

Fig. 3

A

```
ITIM:  (I/V/L/S)-X-Y-X-X-(L/V)
ITSM:   T-X-Y-X-X-(L/V)

BepE_Bh  (57-96)     SQQEEVVYAALDFENRSHH--PRERYPE---RNLE--SETIYTTVSS
BepE_Bh  (99-134)    TTQAEILYADVTHTPL-HS-----KHTR---RDPA--SETLYAEVAM
NTB-A    (277-313)   SPTNNTVYASVTHSNRETE-----IWTP---REND--TITIYSTINH
CD31     (683-718)   PLNSDVQYTEVQVSSAESH-----K-DL---GKKD--TETVYSEVRK
CD33     (333-363)   EMDEELHYASLNFHGMN--------------PSKD--TSTEYSEVRT
LAIR1    (244-285)   GSSQEVTYAQLDHWALT-QRTARAVSPQST--KPM-AESITYAAVAR
LIRB1    (607-649)   EAPQDVTYAQLHSLTL--RREATEPPPSQEGPSPA--VPSIYATLAI
PD-1     (216-253)   VPVFSVDYGELDFQWR--EK-----TPEPPVPCV--PEQTEYATIVF
SHPS-1   (421-457)   QDTNDITYADLNLPK---GK-----KPAPQAAEPN--NHTEYASIQT
SHPS-1   (462-500)   ASEDTLTYADLDMVHL--NR-----TPKQPAPKPE-PSFSEYASVQV
CD244    (264-302)   PKEFLTIYEDVKDLKTRRNHE-------QEQTFPG-GGSTIYSMIQS
LIRB1    (526-567)   DAQEENLYAAVKHTQPEDGVEMDTRSPH---DEDP--QAVTYAEVKH
CD244    (310-347)   OEPAYTLYSLIO-PSRKSG-------SRKRNHSPSF-NSTIYEVIG-
```

B

```
VE-Cadherin  (672-704)  1  KPPRPALDARPSLYAQVQK-PPRH-   23
BepE_Bh      (27-48)    1  RAPSP--QPEP-LYATVNKRPPRAK   22

Consensus               1  :.* *  :..* *** *:* ***     25
```

C

```
                                                  Csk bdg.-like
BepE_Bh     1  MKRNQPPPPTT-HSTEKQREPYGQSAIRAPSPQPEPLYATVNK--------   42
BepE_Bq     1  MKKNQPSSPSIPRSVEEARRRYEQAPPGSASP-PEPLYATVNKKTSHTAKK   50
BepE_Bt     1  MKKHHP----------------------------HPEPLYAQVNK--------   17

Consensus   1  **:::*      ******* ***                               51

                                                        ITIM
BepE_Bh    43  -----------------RPPRAKDRELNKPLSQQEEVVYAALD--FENRSH   74
BepE_Bq    51  PEQGAPQAPLFAASSPQRPPRLRDRELHKTTIKEEEIVYASLV--HPPHRD   99
BepE_Bt    18  ----------------------QNRGNQRTQNPEEDVLYASVSEVSPLSRG   46

Consensus  52                  ::*  ::.  :*:::**::              102

                          ITSM                          ITIM
BepE_Bh    75  HPRERYPERNLESETIYTTVSSQS-----TTQAE--ILYADVTHTP---LH  115
BepE_Bq   100  H---KHPRKNPDNETLYATVAPQSQAMSPTREKEEGIVYASLVHPP--HTD  145
BepE_Bt    47  R---RPDRKSGGTETDYTEVAPPK------REEE--ILYASVSTSSPLSRG   86

Consensus 103  :   :  .:.  .** *: *:. .     : *   *:**.:  ..       153

                     ITSM
BepE_Bh   116  SKHTRRDPASETLYAEVAMQSTIPSLT  142
BepE_Bq   146  HRHPRKNPDNETLYATVASQNLARQSQ  172
BepE_Bt    87  RHHTQRNEGPETDYTEVSPHQKGRSSS  113

Consensus 154  :*.:::   ** *: *: :.   .     180
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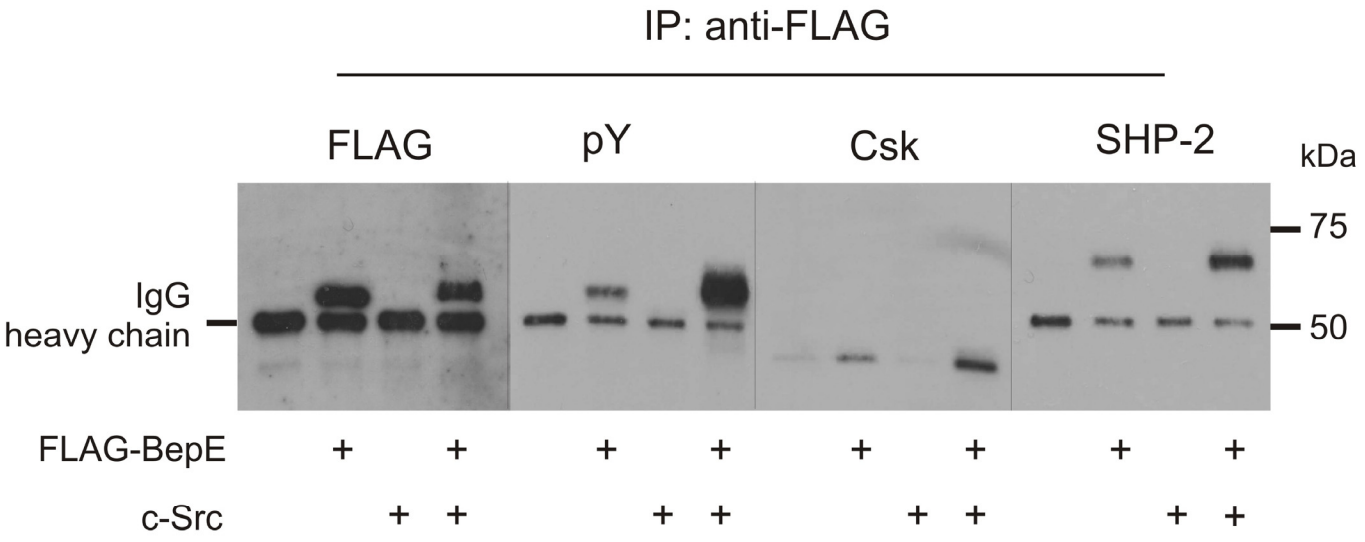
Fig. 4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Csk
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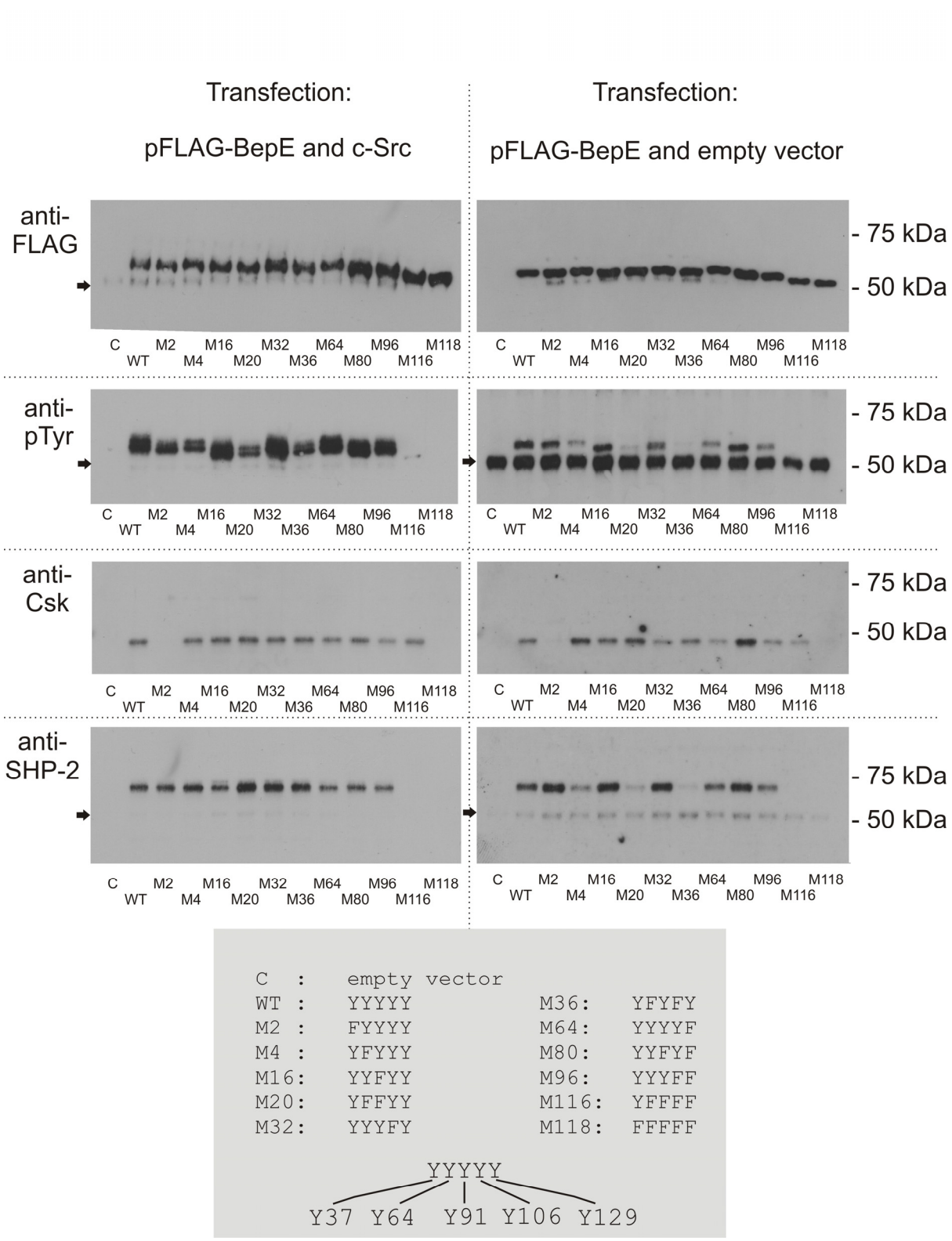
c-Src

Fig. 5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Transfection:
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anti-SHP-2
- 75 kDa
- 50 kDa
C WT M2 M4 M16 M20 M32 M36 M64 M80 M96 M116 M118
C : empty vector
WT : YYYYY
M2 : FYYYY
M4 : YFYYY
M16: YYFYY
M20: YFFYY
M32: YYYFY
M36: YFYFY
M64: YYYYF
M80: YYFYF
M96: YYYFF
M116: YFFFF
M118: FFFFF
YYYYY
Y37 Y64 Y91 Y106 Y129

Fig. 6

Additional manuscripts as co-author

This chapter contains the abstracts of two manuscripts for which I am co-author. In the course of my Ph.D. thesis, my contributions to these studies has been mainly the construction of plasmids and bacterial strains required for the translocation of individual Beps into host cells.

The two manuscripts describe how host cell signaling is subverted by Beps of *Bartonella henselae* other than the phopho-Beps described in the previous and next chapters. Two phenotypes previously associated to the VirB/VirD4 T4SS of *Bartonella henselae*, anti-apoptosis, and cytoskeletal rearrangements, could be directly associated to the effector proteins BepA and BepG, respectively.

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

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Submitted (PLOS Pathogens)

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Abstract

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

Subversion of host cell cytoskeletal function during invasome-mediated uptake of *Bartonella henselae* into human endothelial cells

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In preparation (for Journal of Cell Science)

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Abstract

Uptake of the bacterial pathogen *Bartonella henselae* into human endothelial cells induces re-organization of the host-cell plasma membrane and re-arrangement of the actin cytoskeleton, resulting in the internalization of bacterial aggregates by a unique structure termed invasome. In the present study, we report the identification and characterization of a novel bacterial effector protein mediating the sustained cellular changes leading to invasome formation. Moreover, we show that invasome-mediated uptake requires the action of Rho-family small GTPases Cdc42 and Rac1 but not RhoA and that invasion not only leads to the rearrangement of pre-existing F-actin fibers but as well to localized actin polymerization as suggested by the requirement of Scar1/WAVE adaptor proteins and enrichment of the Arp2/3 complex at sites of invasome-mediated uptake. Finally, we present data indicating that invasome formation may represent a novel mechanism leading to internalization of bacteria but preventing fusion with the endocytic-lysosomal pathway. Taken together, our data suggest that invasome formation is a sophisticated, multi-step process of cellular invasion to establish an intracellular niche in endothelial cells permissive for persistent colonization of the vasculature.

Additional Work (unpublished)

BepD and BepG as antigens in clinical cases of cat-scratch disease

Since the Bep proteins are secreted into infected cells, they are candidates for being prominently exposed by the antigen-presentation machinery of these host cells. To test this hypothesis and the detection of the Beps by serology, I overexpressed some of the FLAG-tagged Beps and probed the total lysate of these cells with serum of patients with clinical symptoms of cat-scratch disease and a secondary anti-human antibody. Since *Bh* does not express the *virB/bep* loci on CBA plates, I could probe for the single Beps expressed with a FLAG-tag on a vector. As shown in Fig. 1, BepD and BepG are recognized by antibodies in the serum of a patient. Further refined, this finding could be used to develop a serology test.

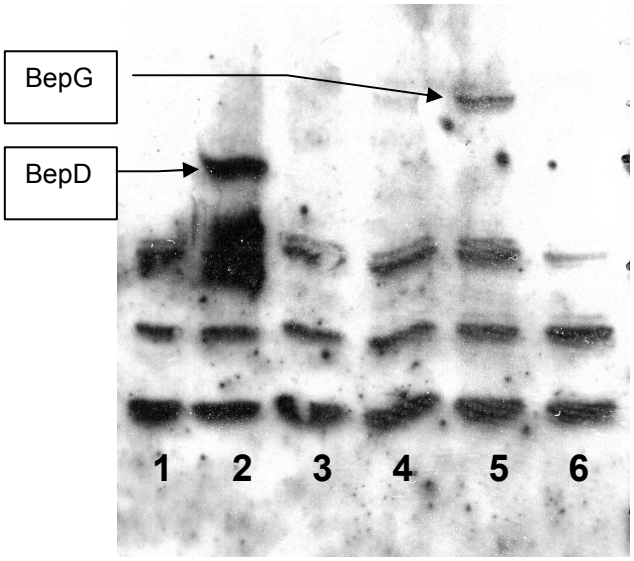

Fig. 1. Total lysates of *Bh* expressing FLAG-tagged 1.) BepA, 2.) BepD, 3.) BepE, 4.) BepF, 5.) BepG, 6.) no Beps (wild-type) probed with serum of a patient with clinical symptoms of cat-scratch disease.

Expression and purification of HIS-tagged BepD

For further studies, I purified BepD by overexpressing the protein in the pET15b system of *E. coli* BL21DE3, lysis by french press, and HIS-affinity purification over a Nickel-agarose column. The protein was subsequently eluted with 250 mM Imidazol, as shown in Fig. 2.

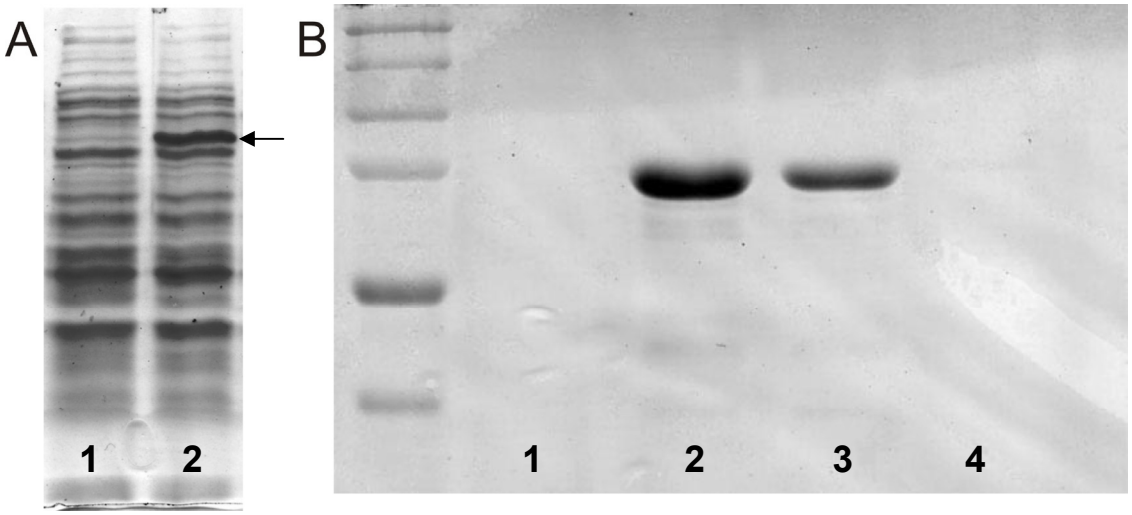

Fig. 2. (A) Total lysate of *E. coli* BL21DE3 containing HIS_BepD, uninduced (lane 1), or induced with 500 μM IPTG (lane 2) for three hours, the arrow marks the size of HIS-tagged BepE. (B) Imidazol-eluted fractions from the Nickel-agarose column, containing HIS-BepD (fractions 2-4).

Mapping tyrosine-phosphorylated sites in BepD

To map the tyrosine-phosphorylation sites of BepD, I performed an *in vitro* phosphorylation reaction as described in the manuscript "Molecular mimicry of inhibitory immune receptors by the bacterial pathogen *Bartonella*". Subsequently, Paul Jenö digested it with trypsin and analyzed the resulting fragments by MALDI-TOF. Two tyrosine-containing peptides phosphorylated by c-Src could be detected in BepD: APSPQAEPLYAQVNKPPR and TPSPQAEPLYAQVNKPPR (Fig. 3).

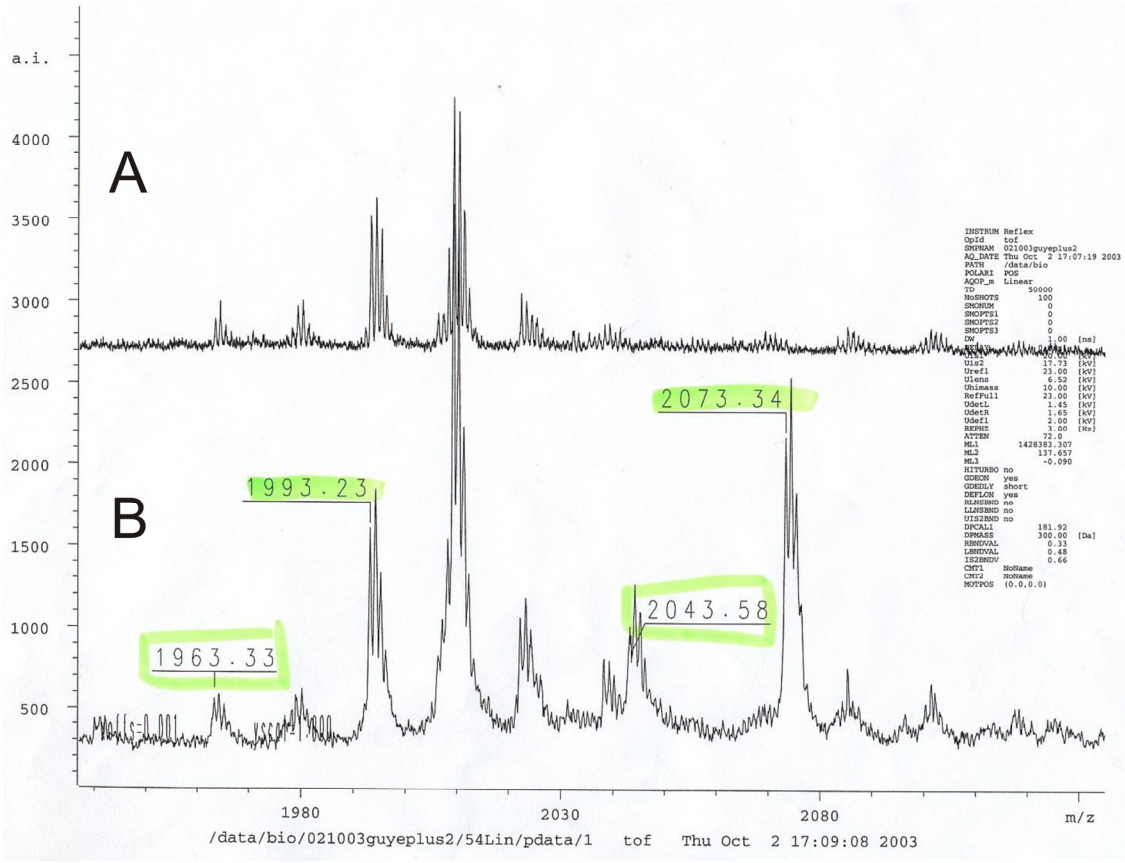

Fig. 3. HIS-tagged BepD incubated in the *in vitro* phosphorylation buffer without (A) or with c-Src kinase. Both fractions were subsequently digested with trypsin and the resulting fragments detected by mass spectrometry. The indicated sizes correspond to the peptides APSPQAEPLYAQVNKPPR and TPSPQAEPLYAQVNKPPR, in their unphosphorylated and tyrosine-phosphorylated state (+ 80 Dalton).

Tyrosine-phosphorylation of BepD results in a relocalization from the Triton X-100 soluble to the Triton X-100 resistant fraction

Tyrosine-phosphorylated BepD exhibited a peculiar property: whenever the FLAG-tagged BepD was tyrosine-phosphorylated by co-expressed c-Src kinase in HEK293T cells, it could not be recovered in 1 % v/v Triton X-100 cell lysates by immunoprecipitation. A glance at the FLAG-tagged BepD content in the total lysate and in the unsoluble pellet of the Triton X-100 lysate showed that BepD became unsoluble in this lysis buffer upon tyrosine-phosphorylation (Fig. 4). A possible explanation for this insolubility is BepD localizing to lipid rafts. Additionally, BepD underwent a pronounced mass shift upon tyrosine-phosphorylation as already shown (1).

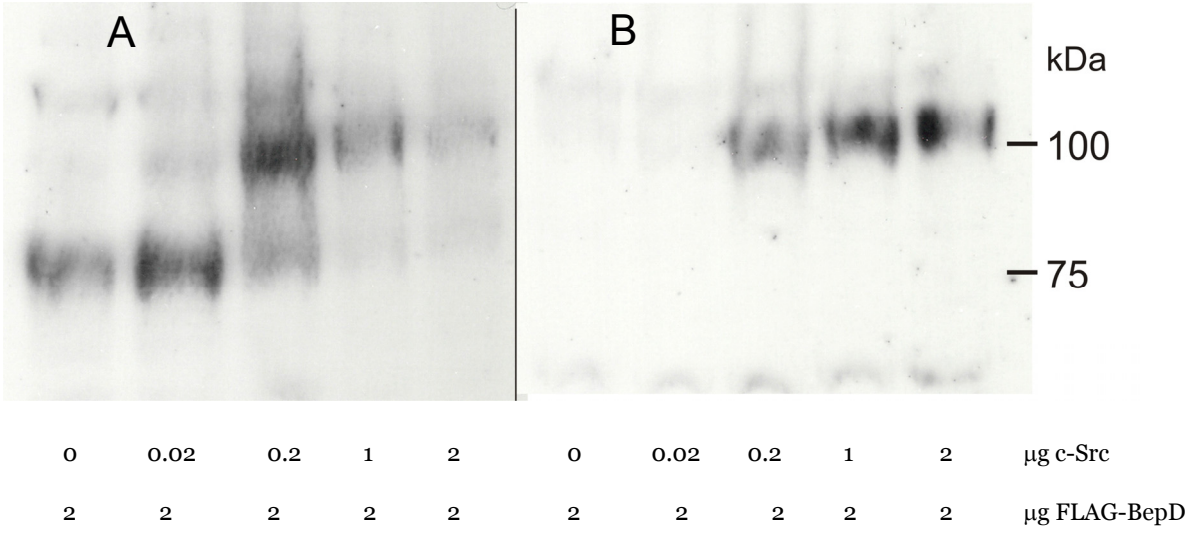

Fig. 4. BepD becomes unsoluble upon tyrosine-phosphorylation in a 1 % Triton X-100 lysate. (A) Total (SDS sample buffer) cell lysate probed for the FLAG-tag (BepD). (B) Unsoluble fraction (pellet) remaining after lysis with 1 % Triton X-100 probed for FLAG-tag (BepD). The amounts of transfected c-Src and FLAG-BepD expressing vectors are given below.

BepD does bind Csk in a tyrosine-phosphorylation dependant manner

BepD contains in its N-terminus a putative tyrosine-phosphorylation motif which is very similar to the one binding Csk in BepE and VE-Cadherin, suggesting that also BepD could have Csk as an interaction partner. To test this, I co-transfected HEK293T cells with a vector expressing MYC-tagged BepE together with one expressing c-Src, lysed the cells, and immunoprecipitated with antibodies against the MYC-tag. The precipitate was then probed with an anti-Csk and a anti-MYC antibody. No IgG heavy chain (from the mouse anti-MYC antibody) is visible in the anti-Csk blot because we used a rabbit/anti-rabbit antibody for the detection there. BepD interacts with Csk in a phosphorylation-dependant manner, as shown in Fig. 5.

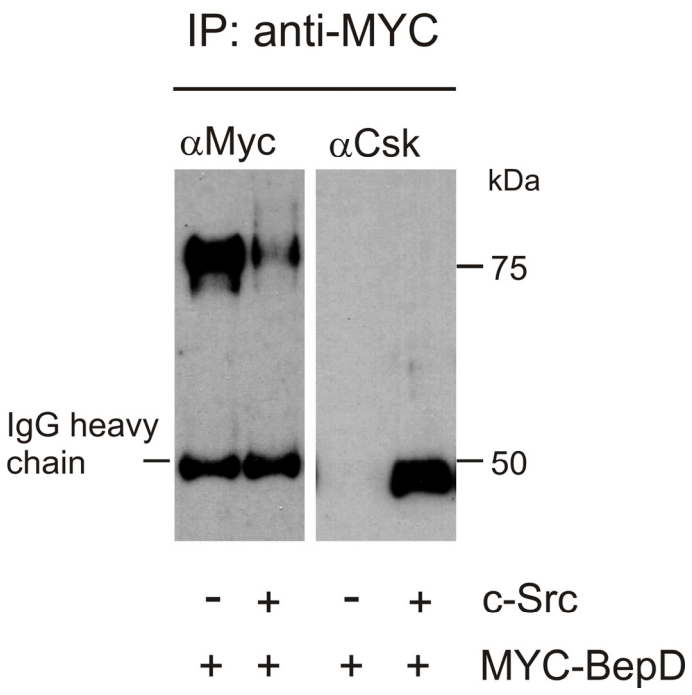

Fig. 5. BepD interacts with Csk in a tyrosine-phosphorylation dependent manner. MYC-BepD and c-Src were co-expressed, and immunoprecipitated with antibodies against the MYC-tag.

BepD binds SHP2 in a tyrosine-phosphorylation dependent manner

As BepD and BepE contain putative tyrosine-phosophorylation motifs predicted by the Scansite software to bind to SH2 domains of SHIP/SHP2 phosphatases, we probed immunoprecipitated FLAG-BepD, FLAG-BepE, FLAG-BepF (kindly provided by T. Rhomberg) with an anti-SHP2 antibody. Clearly, BepD and BepE bound SHP2 in a tyrosine-phosphorylation dependent manner (Fig. 6), and as expected from the bioinformatic analysis, BepF does not. Interestingly, in HEK293T cells, BepF is highly tyrosine-phosphorylated without co-transfection of c-Src, in contrast to BepD and BepE. This may indicate that BepF is tyrosine-phoshorylated by another kinase, present and active in these cells.

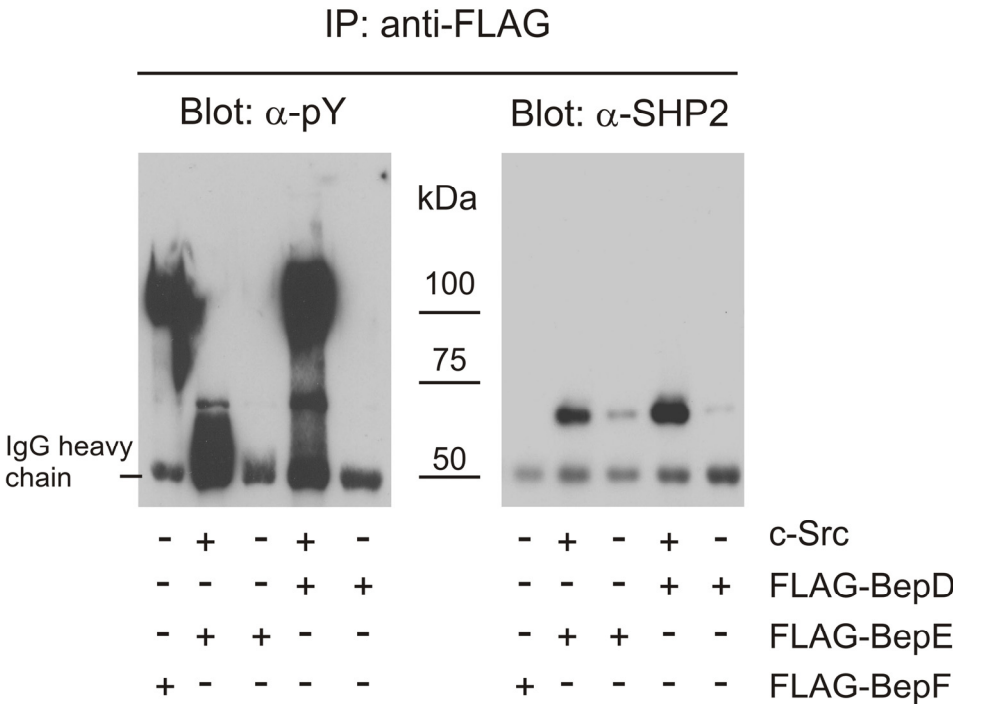

Fig. 6. SHP2 binds to BepD and BepE and not to BepF in a tyrosine-phosphorylation dependent manner. We co-expressed FLAG-BepD, -BepE and BepF facultatively with c-Src, immunoprecipitated with antibodies against the FLAG-tag and probed the precipitate with an anti-SHP2 antibody.

c-Abl is the candidate kinase for BepF

Multiple data indicate that c-Src is not the kinase of BepF. Bioinformatic analysis by using the Scansite algorithm (2) propose c-Abl as the kinase of at least six of the seven putative tyrosine-phosophorylation motifs of BepF (Table 1). Additionally, BepF is highly tyrosine-phosphorylated in HEK293T cells without co-expression of c-Src (Fig. 7). This tyrosine-phosphorylation is not increased by additional expression of c-Src. Moreover, by adding 10 µM of the c-Abl inhibitor Imatinib Mesylate (Gleevec®) to the media of BepF transfected cells, tyrosine-phosphorylation of this protein was reduced by 75% in comparison to cells treated with the vehicle control alone (results not shown). By using the SILAC method, we could show that the adapter protein Crk binds to at least one of these motifs ("PQDSTPLYATPSPQQ", M. Selbach, unpublished results) in an tyrosine-phosphorylation dependent manner.

Tyrosine kinase group (Y_kin)				
Abl Kinase			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
Y149	0.2138	0.002 %	PQDSTPLYATPSPQQ	1.032
Abl Kinase			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
Y213	0.2366	0.002 %	SQDSEPLYATPLPQR	1.032
Abl Kinase			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
Y241	0.2366	0.002 %	NQDSEPLYATPLPQR	1.032
Abl Kinase			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
Y269	0.2366	0.002 %	SQDSEPLYATPLPQR	1.032
Abl Kinase			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
Y185	0.2655	0.003 %	SQDSTPLYATPSPHK	1.032
Abl Kinase			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
Y113	0.2925	0.011 %	SSSQTPLYATPLPQQ	1.032

Table 1. Scansite predicition for motifs in BepF tyrosine-phophorylated by c-Abl. The percentile value of 0.002 signifies that this hit ranks in the best 0.002% in a search space of 430914 motifs, which is an exceptionally good value.

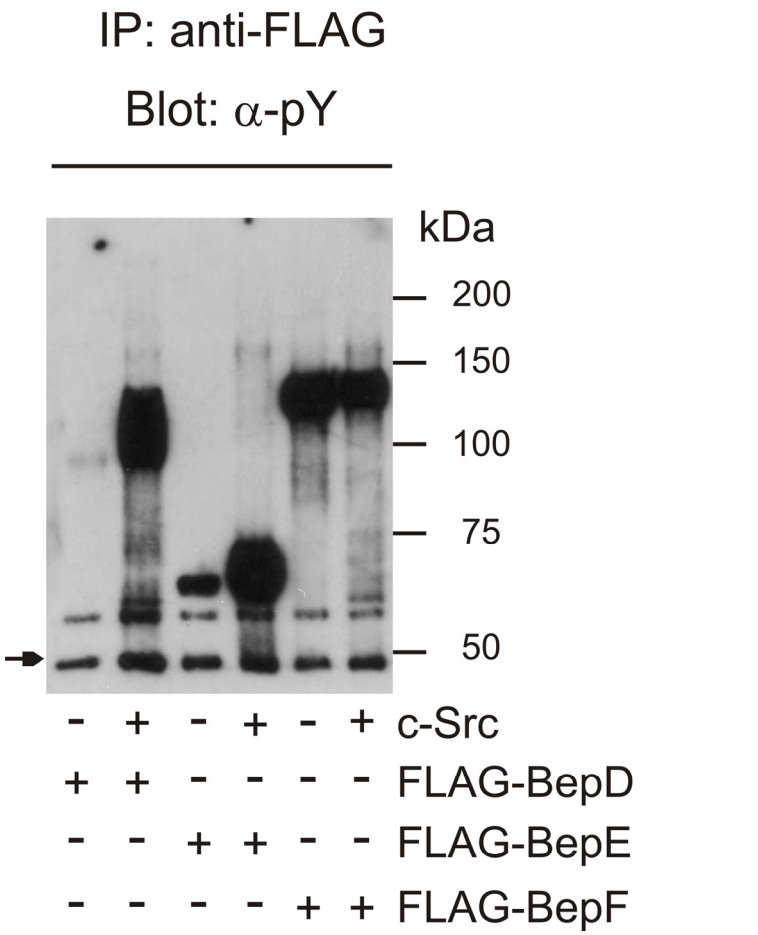

Fig. 7. BepF is strongly tyrosine-phosphorylated by endogenous kinase levels in HEK293T cells. Co-expression of c-Src does not increase this tyrosine-phosphorylation, in contrast to BepD and BepE. The arrow marks the size of the IgG heavy chain.

BepE inhibits host-cell fragmentation

To further assess the role of BepD and BepE in the infection process, I constructed non-polar in-frame mutants in *Bh* (Δ*bepD*, Δ*bepE*, Δ*bepDEF*), while Δ*bepF* was kindly provided by T. Rhomberg. By infecting HUVECs with these strains, a prominent phenotype for the Δ*bepE* and the Δ*bepDEF* mutant became evident, with the effect starting about 20-24 hours post infection, while no phenotype was visible for the Δ*bepD* or Δ*bepF* mutants in comparison to the wild-type bacteria. This assessment was done by time-lapse video microscopy and by immunofluorescence stainings of fixed cells. Whenever BepE was deleted from the wild-type *Bh* strain (Δ*bepE* or Δ*bepDEF)*, the infected HUVECs became highly agitated, deformed, and fragmented multiple times (Fig. 8, Fig. 10). Most of these fragments did not carry a nucleus and got smaller by continuing fragmentation. The fragments erratically roamed on the substrate for a few hours and then came to a halt, possibly because they had used up all their energy. By providing BepE or only the two C-terminal BID domains of BepE *in trans* on a plasmid, the infected HUVECs behaved like infected with wild-type *Bh*, indicating that this effect was indeed mediated specifically by the BID domains of BepE (Fig. 8, Fig. 9) . Intriguingly, the *ΔbepB-G* or the *ΔvirB4* mutant, defective in type IV secretion through the virB apparatus, did not exhibit such a phenotype, suggesting that either BepB, C or G, or another unknown, T4SS mediated effector is responsible for disturbing the cells, which can be counteracted by BepE. This effect was not further pursued, as it is not dependent on the tyrosine-phosphorylated N-terminus of BepE. The reason and mechanisms of this dramatic reaction in the endothelial cells is elusive. So further studies may provide very interesting findings on how cellular motility, focal adhesion turnover and/or cellular integrity is modulated.

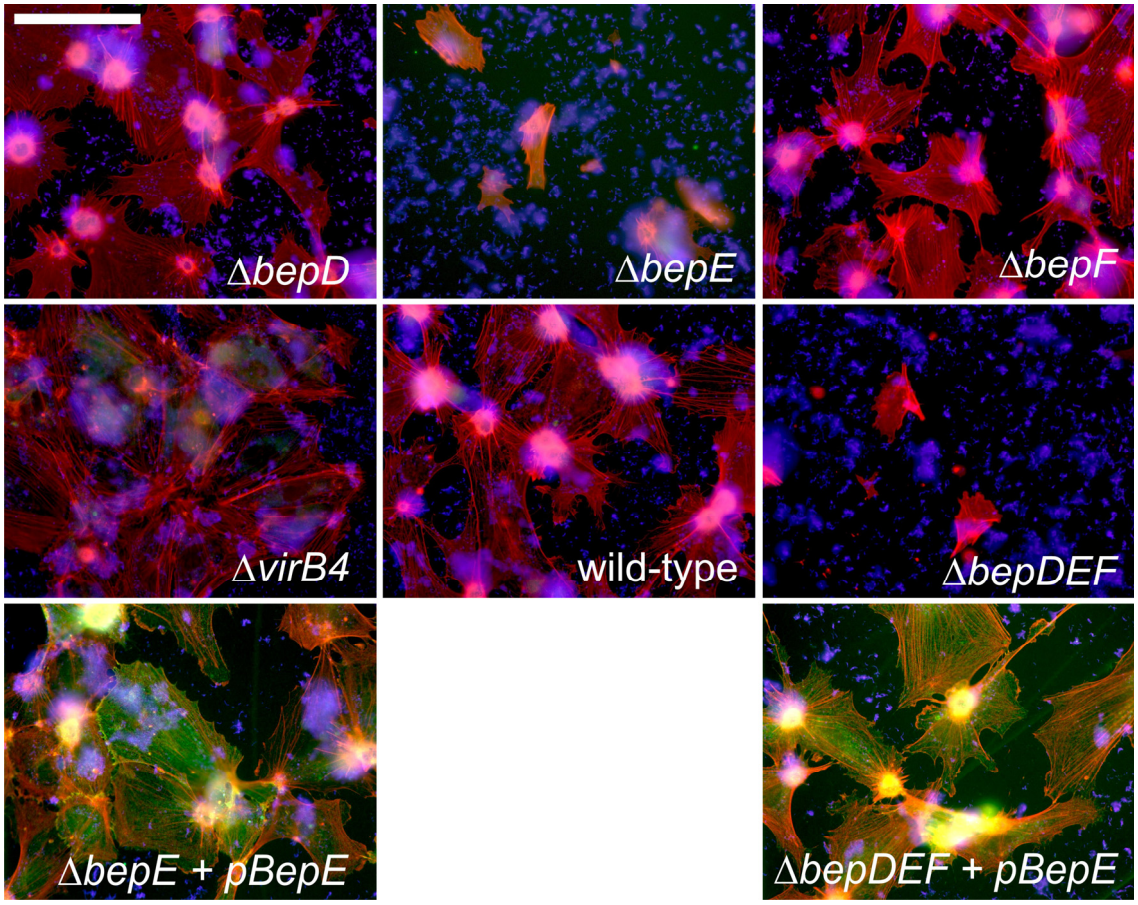

Fig. 8. Lack of BepE in a secretion competent *Bh* strain induces fragmentation of the endothelial cells (24 hours post-infection). This effect is complementable by providing BepE *in trans*. The f-actin is colored in red, the MYC-tag green, and the bacteria in blue.

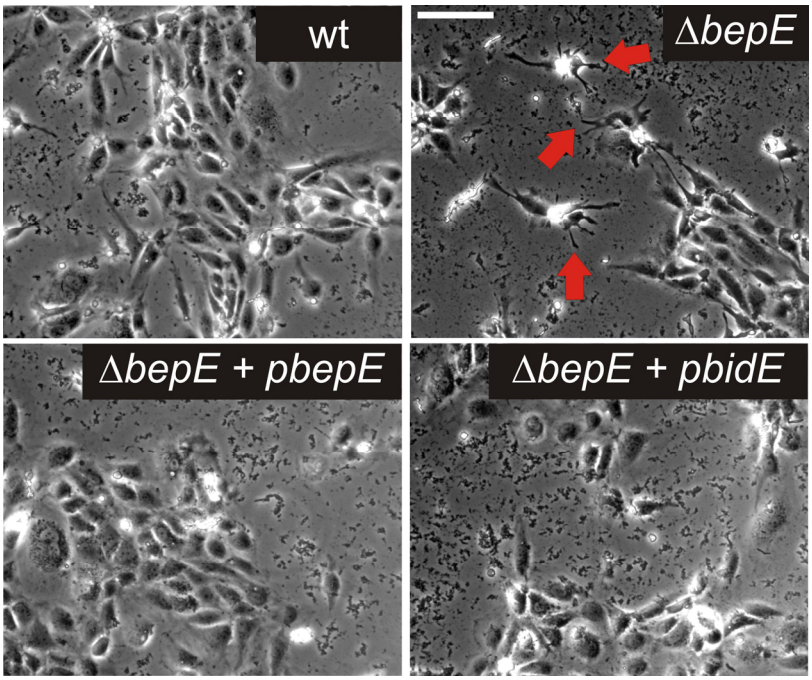

Fig. 9. The two BID domains of BepE are sufficient to inhibit the fragmentation of the endothelial cells, as shown 24 hours post-infection. The red arrows indicate the beginning of fragmentation.

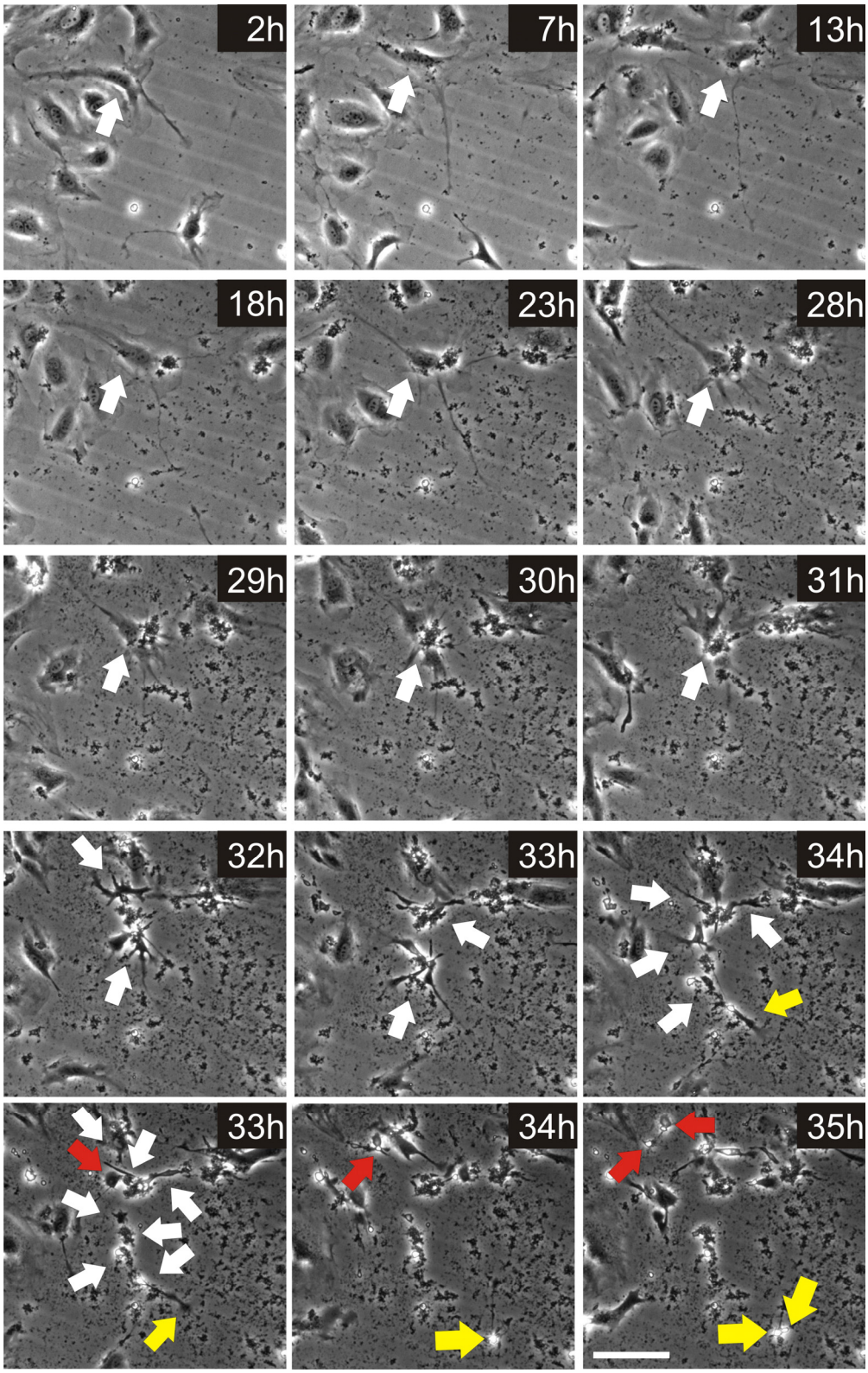

Fig. 10. The fragmention occurs between 24 and 36 hours post-infection. HUVECs were infected with the *Bh* mutant lacking *bepE*, and images were acquired by time-lapse microscopy. The white arrows indicate the beginning of the fragmentation of one cell, the yellow and red arrows further sub-fragmentation. The size bar represents 100 μm.

References

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4 - Summary

4. Summary

We identified in the first manuscript the secreted substrates of the VirB type IV secretion system in *Bh* and first biological effects of this apparatus on endothelial cells. Furthermore, we describe the domain mediating the secretion, which proved to be bipartite. Additionally, we revealed by bioinformatics evolutionarily related translocation domains, which are also secreted by the T4SS of *Bh*, suggesting that this secretion system is able to transfer DNA into host cells.

In the second manuscript we focus on the tyrosine-phosphorylated BepE protein, and show that it contains a binding site for the SH2 domain of Csk and ITIMs-TSMs tandems in its N-terminus, reminiscent of inhibitory immune receptors. BepE is, consistently with this finding, tyrosine-phosphorylated by SFKs in these motifs and subsequently binds to Csk and SHP2. In addition to its localization to the proximity of the membrane, BepE does also co-localize with VE-Cadherin in the cell-cell contacts of HUVECs.

Having contributed to two more publications as a co-author, these two manuscripts are quickly presented by their abstracts in Chapter 3.3, illustrating the advances in elucidating the functions of two other Beps, BepA and BepG, in the infection process.

In the last part, the additional unpublished work, I show that BepD is also tyrosine-phosphorylated by SFKs, whereupon it binds to Csk and SHP2, and furthermore relocalizes to a Triton X-100 insoluble fraction. In contrast, BepF seems to be tyrosine-phosphorylated by another group of kinases, probably from the Abl family. Moverover BepE inhibits by virtue of its double BID domains a dramatic fragmentation of infected cells, as shown by time-lapse microscopy.

5 - Conclusions

5. Conclusions

What are the substrates of the VirB/VirD4 T4SS of *Bartonella henselae*?

Starting point for the first published report ("A bipartite signal mediates the transfer of type IV secreted substrates of *Bartonella henselae* into human cells", Chapter 3.1) in my Ph.D. thesis was the finding that the VirB/VirD4 T4SS of *B. tribocorum* was essential for the pathogen to establish an intra-erythrocytic infection in an animal model (1). Additionally, at that time, unpublished data indicated that many phenotypes which were already known for *B. henselae* infecting HUVECs, as anti-apoptosis (2), cytoskeletal rearrangements (3) and pro-inflammatory activation (4), were dependent on a intact VirB/VirD4 T4SS (5). These findings were suggestive of substrates being translocated through the VirB/VirD4 T4SS of *Bartonella* into the host cells.

Sequencing 23 kb downstream of the *virB* locus of *B. henselae* revealed a coupling protein (*virD4*), and seven genes encoding at least one common C-terminal domain. The proteins encoded by these seven genes were later termed *Bartonella* exported proteins (Beps), the common C-terminal domain they contain *Bartonella* intracellular delivery (BID). By constructing a Hidden Markov Model from these BID domains and querying protein databases, we found similar domains in the C-terminus of relaxases from conjugative plasmids in the α-proteobacteria. We showed exemplarily that the C-terminus of the TraA relaxase from the AvhB/TraG conjugation system in *A. tumefaciens* – one of the top hits in the database search - could still be translocated by the VirB/VirD4 T4SS of *B. henselae*. Both these findings support that the BID domain evolved from conjugative relaxases, in parallel with the T4SS of *B. henselae*. Full-length relaxases bind covalently to plasmid DNA and mediate its transfer through T4SSs. This allows the fascinating speculation that the VirB/VirD4 T4SS of Bartonella could be used to export DNA by those means *in vivo* into host cells.

To demonstrate exemplarily the translocation of the Beps through the VirB/VirD4 T4SS, we fused a FLAG-tag to the N-terminus of BepD and could show that BepD is translocated into infected endothelial cells in a VirB/VirD4 T4SS-dependent manner, whereupon it localizes to the cytoplasm of these cells and is tyrosine-phosphorylated by host-cell kinases. The precise experimental delineation of the domain needed for translocation was made possible by the development of the Cre-recombinase reporter assay for translocation (CRAFT), which showed the translocation domain to be bipartite. In addition to the BID domain, a short, positively charged C-terminal amino acid sequence was needed for an effective delivery of proteins.

Non-polar deletions of all the ORFs encoding the Beps abolished the ability of *Bh* to induce a variety of host-cell phenotypes, suggesting that these proteins elicit biological effects in their eukaryotic target cells. This finding was the fundament for the two Manuscripts presented in Chapter 3.3, "A translocated protein of the vascular-tumor inducing pathogen Bartonella protects human vascular endothelial cells from Apoptosis" and “Subversion of host cell cytoskeletal function during invasome-mediated uptake of Bartonella henselae into human endothelial cells”. In each of these two manuscripts, a major phenotype of the infection of HUVECs by *B. henselae* is shown to depend on a single Bep. While BepA inhibits the apoptosis of endothelial cells, BepG does induce massive cytoskeletal rearrangements. Interestingly, it is the BID domain of BepA which mediates the anti-apoptotic activity in the host cell as well as the localization of this protein to the plasma membrane. This involvement of the BID in localization and function in the host cell holds also true for BepE, as we showed that the two BID domains of this protein are crucial for its localization (Chapter 3.2), and to inhibit the fragmentation of infected endothelial cells (Chapter 3.4).

Many interfaces for interactions over phosphotyrosines

Of the seven Bep proteins, three contain putative tyrosine-phosphorylation motifs in their N-terminus. We showed that both BepD and BepE are tyrosine-phoshorylated by the c-Src kinase. Because of their similar substrate specificities and their variable expression levels depending on the cell type, any member of

the SFK could potentially be the kinase of BepD and BepE *in vivo*. Bioinformatics and preliminary experiments suggest c-Abl as the kinase of BepF. As Crk was shown to bind BepF, and one of the functions of Crk has been described to activate c-Abl, one might speculate that a positive activation loop takes place once BepF has been phosphorylated, which would bind and activate increasing amounts of the c-Abl kinase to BepF through Crk. Whereas c-Abl has been implicated in the invasion process of other pathogens (6), the exact function of BepF in *Bartonella* still remains to be uncovered.

BepD localizes in immunofluorescence stainings to a cytoplasmic vesicular-like compartment. Additionally, it localizes upon its tyrosine-phosphorylation to a Triton X-100 insoluble fraction, and binds SHP2 and Csk. Lipid rafts are prominently associated to Triton X-100 insoluble fractions, and future studies might show BepD interfering with the signaling in these rafts.

I subsequently focused on BepE, for which bioinformatics revealed an intriguing similarity to inhibitory immune receptors of mammals. This resulted in the second manuscript "Molecular mimicry of inhibitory immune receptors by the bacterial pathogen *Bartonella*" (Chapter 3.2). BepE contains two C-terminal BID domains which mediate the localization of this protein to the plasma membrane of HELA cells and to the plasma membrane and cell-cell contacts in endothelial cells, as shown by a co-localizing immunofluorescence staining with VE-Cadherin in HUVECs. This peculiar localization is very intriguing, and might modulate the cell-cell contact strength or the contact inhibition which is crucial in endothelial cells. Recently, a publication demonstrated that VE-Cadherin contains in its intracellular domain a binding site for the SH2 domain of Csk, the binding of Csk to this site being crucial for the contact inhibition of cell growth (7). We identified a very similar motif in the N-terminus of BepE which did also bind Csk. The co-localization and a similar tyrosine-phosphorylation motif binding the same protein makes this finding a good starting point for investigating the functions of BepE in the endothelium.

BepE contains in addition to this Csk-binding motif two immunotyrosine inhibitory motif – immunotyrosine-based switch motif (ITIM/ITSM) tandems. These motifs are commonly found in the intracellular domain of inhibitory immune receptors. *In vitro* phosphorylation with the c-Src kinase and subsequent mapping by mass spectrometry analysis indicated that c-Src

tyrosine-phosphorylates the Csk-binding site and both ITIMs of BepE. No phosphorylation of an ITSM could be detected, which might be due to the fact that another kinase phosphorylates these motifs *in vivo*. Both the inhibitory immune receptors and BepE do contain ITIMs, ITSMs, Csk-binding sites, localize to the plasma membrane, are tyrosine-phosphorylated by Src family kinases and bind SHP2 and Csk. Additionally, BepE is constitutively tyrosine-phosphorylated in HEK293T cells as well as in the primary HUVECs (Chapter 3.2), in contrast to most inhibitory immune receptors, which are thought to only become phosphorylated upon engagement of their extracellular ligands. All these lines of evidence indicate BepE mimicking inhibitory immune receptors.

Csk contains one SH2 domain, SHP2 two. To elucidate the binding sites of these two proteins, and also to be able to use mutants lacking these interactions, we generated a panel of tyrosine-to-phenylalanine exchange mutants in BepE. While Csk binds to one motif (the one with the similarity to the Csk-binding site of VE-Cadherin), SHP2 interacts with motifs in the two ITIM-ITSM tandems. While this interaction study was carried out in HEK293T cells, more potential ITIM/ITSM-binding proteins such as SHP-1 and SHIP, EAT-2 and SAP might increase the complexity of the picture in myeloid and lymphoid cells. The two adapter proteins SAP and EAT-2 have been shown to bind to ITSMs. While some cells such as NK express both adapters, other cells as for example T-cell do only express SAP, and other such as DCs only EAT-2. While SAP recruits Fyn and can lead to an increase in cell activation, EAT-2 recruits phosphatases and Csk, inhibiting the activity. For BepE, this opens the possibility of *switching* its mode of action, depending on the cell type it is translocated into.

Collaborations have been initiated to assess the immunomodulatory potential of BepE and we are currently studying the impact of this protein in our HUVEC models.

To summarize, my Ph.D. thesis aimed at investigating the VirB/VirD4 T4SS of *Bartonella henselae* for the presence of secreted substrates and signals mediating this secretion. Additionally, to describe the functions and interaction partners of these substrates in the host cell, with an emphasis on the putative tyrosine-phosphorylated effectors. . The core findings of this thesis are a.) The discovery

of seven modular substrates secreted by the apparatus b.) Description of the BID domain mediating the secretion of proteins and protein-DNA complexes, c.) Csk and SHP2 being interaction partners for BepD and BepE on the host cell side d.) BepE mimicking inhibitory immune receptors.

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6 - Outlook

6. Outlook

Although we have now some understanding concerning the secretion requirements, the description of which parts of the Beps play a role in secretion, as effectors in cells, or in both, is still incomplete. Deepening this understanding will be of critical importance to further study the role of the VirB/VirD4 secretion apparatus in the pathogenesis of Bartonellae infections.

Describing interaction partners and putative kinases for BepD and F opened the door for future studies, and first exciting results concerning the role of BepD have already been acquired using constructs and mutants described here.

For BepE, sound foundations have been laid out to unravel its functions. Adequate models exist and are currently being used for studying the putative impact of this protein on the endothelium. The assessment of it immunomodulatory potential poses a tremendous challenge, and will not come to completion without an adequate animal model, as the publication history of studying the immune inhibitory receptors shows. Nevertheless, insights on how highly adapted bacteria do modulate our defense lines will be well worth the investments, and may shed light on human diseases previously not connected to pathogens.

7 - Acknowledgements

Acknowledgments

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8 – Curriculum vitae

Curriculum vitae

Name:	Patrick Guye-Vuillème
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Education

Apr. 2002 - Jun. 2006:	Ph.D. work in the lab of Prof. Christoph Dehio at the Biozentrum of the University of Basel. Title: “Proteins injected by the bacterial pathogen Bartonella subvert eukaryotic cell signaling”. Ph.D. committee members: Prof. Christoph Dehio, Prof. Guy Cornelis, Prof. Mike Hall.¨
Sep. 2000 - Mar. 2002:	Diploma thesis in the lab of Prof. Christoph Dehio at the Biozentrum of the University of Basel. Title: “Analysis of the virB-operon

	regulation by promoter fusion to the gene encoding green fluorescent protein".
Jul. 2000 – Jun. 2006:	Tutorial assistant, then part-time employee at the University Computing Center, University of Basel.
Oct. 1997 – Apr. 2002:	Studies of Biology II (molecular biology) at the Biozentrum of the University of Basel.
Jun. 1997:	Matur, type C (university-entrance diploma with specialization in natural sciences) at the Alte Kantonsschule Aarau, Aarau, Switzerland.

Teaching experience:

May 2001 / May 2002 May 2003 / May 2004 May 2005	Tutorage of third-year students: "Blockcourse in Microbiology" at the Biozentrum of the University of Basel, Switzerland
Sep. 2003 – Sep 2004	Tutorage of a diploma student at the Biozentrum of the University of Basel, Switzerland

Other Activities:

1998 – 2005:	Repeated military services, 3 weeks a year, specialization in detection of B-warfare agents (NBC Defense Laboratory, Spiez, Switzerland).
Jul. 1997 – Oct. 1997:	Military service as infantry communications specialist.

Poster Presentations / Conferences:

August 2003: Poster at the 11th European Congress on Biotechnology in Basel, Switzerland: "Intracellular protein delivery in human cells by the VirB/VirD4 type IV secretion system of Bartonella henselae" (R. Schulein, P. Guye, T. Rhomberg, I. Carena, A. Vergunst and C. Dehio).

September 2003: Euresco Conference, biology of type IV secretion process, Giens France, September 12-17, 2003.

Refereed publications:

Schulein, R., P. Guye, et al. (2005). "A bipartite signal mediates the transfer of type IV secretion substrates of Bartonella henselae into human cells." Proc Natl Acad Sci U S A **102**(3): 856-61.

Patent:

Mar. 2003 Schulein, R., Guye, P., Rhomberg, T., Schmid, M., Dehio, M. & Dehio, C. Polypeptides translocated into cells by the VirB/VirD4 type IV secretion system and uses thereof. Patent application no. EP 03004826.8-1222