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Review

Vacuolating cytotoxin A (VacA) – A multi-talented pore-forming toxin from *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori is associated with severe and chronic diseases of the stomach and duodenum such as peptic ulcer, non-cardial adenocarcinoma and gastric lymphoma, making *Helicobacter pylori* the only bacterial pathogen which is known to cause cancer. The worldwide rate of incidence for these diseases is extremely high and it is estimated that about half of the world's population is infected with *H. pylori*. Among the bacterial virulence factors is the vacuolating cytotoxin A (VacA), which represents an important determinant of pathogenicity. Intensive characterization of VacA over the past years has provided insight into an ample variety of mechanisms contributing to host-pathogen interactions. The toxin is considered as an important target for ongoing research for several reasons: i) VacA displays unique features and structural properties and its mechanism of action is unrelated to any other known bacterial toxin; ii) the toxin is involved in disease progress and colonization by *H. pylori* of the stomach; iii) VacA is a potential and promising candidate for the inclusion as antigen in a vaccine directed against *H. pylori* and iv) the *vacA* gene is characterized by a high allelic diversity, and allelic variants contribute differently to the pathogenicity of *H. pylori*. Despite the accumulation of substantial data related to VacA over the past years, several aspects of VacA-related activity have been characterized only to a limited extent. The biologically most significant effect of VacA activity on host cells is the formation of membrane pores and the induction of vacuole formation.

This review discusses recent findings and advances on structure-function relations of the *H. pylori* VacA toxin, in particular with a view to membrane channel formation, oligomerization, receptor binding and apoptosis.

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1. Introduction

Given the enormous impact on human health, *Helicobacter pylori* is now among the best characterized bacterial human pathogens and intensive research efforts are devoted to the analysis of the molecular pathobiology of this organism. *Helicobacter pylori*, a Gram-negative, spiral-shaped, microaerophilic organism is able to persistently colonize the human stomach for decades and causes serious chronic diseases such as dyspepsia, gastric atrophy, peptic ulcer disease, gastric adenocarcinoma and gastric cell lymphoma (Cover and Blaser, 2009; Montecucco and Rappuoli, 2001; Suerbaum and Michetti, 2002). Striking differences are associated with gastric cancer prevalence in different geographical locations. Gastric cancer is the third leading type of cancer worldwide and it is the fifth most common cancer in Europe (Ferlay et al., 2010). The risk of developing gastric cancer is strongly correlated to the prevalence of *H. pylori*–associated atrophic gastritis (Wen and Moss, 2009). *H. pylori* is now considered as the most common bacterial infectious agent of relevance to human health (Atherton, 2006; Ferlay et al., 2010) and it is obvious that a pathogen with this impact on human health became a priority target for the extensive analysis of its molecular physiology. The clinical presentation of infections with *H. pylori* is determined by a complex interaction of multiple factors such as strain diversity, host genetic predisposition, environmental factors and nutrition (Wen and Moss, 2009). *H. pylori* is contagious and most likely transmitted *via* oral-oral, fecal-oral, and gastro-oral (mediated by a reflux of gastric juice) routes (Boehnke et al., 2015; Brown, 2000; El-Sharouny et al., 2015; Khalifa et al., 2010; Krueger et al., 2015; Rakhmanin and German, 2014; Santiago et al., 2015; Tirodimos et al., 2014; Yokota et al., 2015). Epidemiological data related to disease prevalence have to be interpreted with caution as a large proportion of infected individuals (approx. 80%) can remain symptomless over long periods of time. Moreover, diagnostic procedures appropriate for *H. pylori* infections may be limited or not available at all in some developing countries (Akguc et al., 2014; Batts et al., 2013; Shrestha et al., 2014; Taylor and Blaser, 1991; Vilaichone et al., 2014). Nevertheless, current information on the incidence of *H. pylori*-infections suggests a high burden to public health care, especially in developing countries where acquisition of the disease appears to occur at higher rates than in developed countries (Aziz et al., 2015; Weaver, 1995; Archampong et al., 2015). In addition, infections during the childhood seem to be more frequent in developing countries where up to 50% of children (5 years of age) and 90% of adults are infected (Khalifa et al., 2010). Marked geographic differences in *H. pylori* prevalence were previously attributed to differential acquisition rates during early childhood and developing countries display higher incidences of *H. pylori* infections during childhood (<10 years of age) than developed countries (Pounder and Ng, 1995).

The vacuolating cytotoxin VacA represents an important determinant of pathogenicity with highly complex interactions between *H. pylori* and the epithelial cells of the gastric mucosa which have now become a paradigm for host–pathogen association. Virulence factors are generally defined as molecules produced by pathogens that contribute to colonization, attachment to host cells, evasion of the host immune response and consumption of nutrients. Among the major *H. pylori* virulence factors are flagellin, urease, catalase, mucinase, lipase, neutrophil activating protein (NAP), outer membrane proteins (OMP), lipopolysaccharides, cytotoxin cytotoxin-associated gene pathogenicity island (cagPAI) and the vacuolating cytotoxin VacA (Essawi et al., 2013; Rieder et al., 2005). Some actions of the VacA toxin are apparently antagonized by opposing effects mediated through CagA (Argent et al., 2008). Highly pathogenic ("*type I*") strains of *H. pylori* display a constant association of VacA and CagA and it was speculated that CagA-VacA interaction would be required to promote long-term colonization of the stomach by ameliorating the detrimental effects of the bacterial virulence factor (Oldani et al., 2009). The cagPAI covers approximately 40 kb and comprises 30 genes including a type IV bacterial secretion system utilized by the CagA protein, which contributes to a pro-inflammatory response. However, recent studies have shown that CagA plays only a minor role, if any, during the release of pro-inflammatory cytokines such as interleukin-8 (Fischer et al., 2001; Kusters et al., 2006).

Regulatory effects exerted by CagA depend on tyrosine phosphorylation by host kinases. Accumulation of unphosphorylated CagA triggers an anti-apoptotic mechanism at the mitochondria without affecting the intracellular trafficking of the toxin, whereas phosphorylated CagA apparently prevents VacA to reach its intracellular target compartments (Oldani et al., 2009). Although it seems plausible that the proinflammatory and anti-apoptotic effects of CagA are contributors to the development of peptic ulcer and gastric cancer, the progress of gastroduodenal diseases is probably attributable to a multitude of bacterial virulence factors as well as various host factors (de Bernard and Josenhans, 2014). It is interesting to note that the cagPAI pathogenicity island is not present in all strains of *H. pylori* and humans infected with cagPAI-negative strains apparently remain symptomless (Ferreira et al., 2014).

While there is a number of excellent reviews published recently on host-pathogen interactions of *H. pylori* (Backert and Tegtmeyer, 2010; Bornschein and Malfertheiner, 2014; Cid et al., 2013; Yamaoka and Graham, 2014), this review will focus mainly on structure-mechanism relations of the VacA toxin from *H. pylori*. For a comprehensive overview, the reader is directed to previous reviews summarizing the history of key findings obtained for VacA (Boquet and Ricci, 2012; Isomoto et al., 2010; Palframan et al., 2012). We apologize to authors whose work we have failed to cite owing to space constraints.

2. VacA – structure

The VacA cytotoxin represents a multifunctional protein of about 860 amino acid residues which displays structural, mechanistic and functional features unrelated to other known bacterial toxins (Cover and Blanke, 2005). The *vacA* gene is present as sole chromosomal copy in all strains investigated to date and can vary in length from 3.86 to 3.94 kbp. The gene sequences encoding VacA show considerable variations and some strains were identified which ostensibly express a functionally inactive form of the toxin (Cover et al., 1994). The toxin can interact with a variety of mammalian cells and thereby exerts pleiotropic effects which include **i**) the formation of pores in the membrane of the target cell leading to leakage of ions and small molecule nutrients (Czajkowsky et al., 2005); **ii**) the generation of large intracellular

vacuoles derived from membranous compartments of the endocytic pathway (de Bernard et al., 1997); **iii**) the induction of apoptosis by interaction with mitochondrial membranes (Cho et al., 2003) and **iv**) a modulation of the host immune response by interference with signal transduction pathways involved in regulation of T-cell proliferation (Gebert et al., 2003). The fact that multiple effects of VacA on gastric epithelial cells finally lead to the establishment of a persistent immune response and concomitant inflammation was established in a huge number of scattered observations extensively reviewed elsewhere (Isomoto et al., 2010).

The *vacA* gene from *H. pylori* model strain 60190 encodes a ~140 kDa protoxin of 1287 amino acid residues which is proteolytically processed to yield the mature ~90 kDa toxin containing 821 residues (Cover and Blaser, 1992; Papini et al., 2001). In a first cleavage reaction, the N-terminal 3 kDa signal sequence of 33 residues is removed from the protoxin during transport into the periplasmic space. Secretion of the toxin into the extracellular medium is mediated by a type Va secretion system. Secreted VacA is cleaved into the p88 mature protein and the 10.5 kDa passenger domain (p10). The 88 kDa mature toxin can be further proteolytically processed by yet unidentified protease(s) into 2 domains with distinct functions: a N-terminal fragment of 33 kDa (residues 33–311) exhibits pore-forming activity involved in vacuole formation and a C-terminal fragment of 55 kDa (residues 312–821) plays a role in receptor-binding to target cells and also for the assembly of VacA toxin molecules into oligomeric structures required for pore formation (Sewald et al., 2008a). While cleavage into the p33 and p55 subunits was observed *in vitro*, no cleavage *in vivo* was detected thus suggesting that cleavage is not a strict prerequisite for toxin activity (Backert and Tegtmeyer, 2010).

The overall structure of VacA displays similarities to intracellularly acting bacterial exotoxins of the A-B type such as anthrax-, diphtheria- and cholera toxins. B-components of A-B toxins typically bind to specific receptors on the cell surface, whereas A-components carry enzymatic functions which modify specific intracellular target molecules leading to a perturbation of cellular metabolism. In an attempt to identify a suspected “A-component” for VacA, N- and C-terminal truncations of VacA were constructed and assayed for induction of vacuolation in transfected HeLa cells (Ye et al., 1999). These investigations revealed that an N-terminal portion of 422 residues within VacA is sufficient to mediate the formation of vacuoles upon intracellular expression. No vacuolating activity was observed with N-terminal truncations exceeding 17 residues, thus indicating the importance of the N-terminal region for vacuolation. The N-terminal 311 residues (p33) and the C-terminal fragment (p70) were unable to induce vacuolation; however, co-transfection of separate plasmids expressing both p33 and p70 resulted in vacuole formation. It was proposed recently that VacA represents the prototype of a new class of monofunctional A-B toxins in which the enzymatic A-subunit is replaced by a pore-forming moiety (Boquet and Ricci, 2012). As membrane pore formation accounts for almost all significant cellular effects of VacA which are known to date, this seems to represent an attractive working hypothesis under which the numerous and diverse effects of VacA could become integrated into a refined mechanistic model of VacA action.

Mixtures of the p33 and p55 domains display enhanced binding to plasma membranes and detectable cytotoxic activity when compared to the individual domain fragments (Torres et al., 2005). Sequential addition of p55 followed by p33 resulted in internalization whereas the reverse order was ineffective in cell vacuolation, thereby suggesting that binding of p55 to the cell surface is needed prior to an internalization of the p33 domain and that co-localization of the 2 subunits is indispensable for pore formation (Torres et al., 2004). Both the p33 and p55 subunits are first imported into mitochondria and subsequently insert into the mitochondrial inner membrane eventually generating an ion channel. This localization to the organelle does not require proteolysis of p88, however, is dependent on both time and the electrochemical gradient across the inner mitochondrial membrane and requires the presence of multiple targeting signal sequences at the N- and C-terminus of the p55 subunit (Foo et al., 2010). The N-terminal sequence of the VacA protein displays no significant homology to other known membrane-inserting bacterial toxins, but resembles internal sequences of ion channel and transporter proteins (Ito et al., 1998) (see Fig. 1).

3. Oligomerization and receptor binding

The crystal structure of the VacA p55 domain has been determined at a resolution of 2.4 Å and consists of a right-handed β-helix (residues 355–735) and a small C-terminal globular domain (residues 736–811) (Fig. 2) (Gangwer et al., 2007). The p55 structure has two main parts: residues 355–735 within p55 represent a right-handed parallel β-helix and a small globular domain at the C –terminus (residues 736–811) exhibits mixed α/β secondary structure elements. This structure is consistent with the elution of p55 as a dimer from gel filtration columns. A comparison of 92 VacA sequences identified two novel conserved regions located at the N- and C-terminal moieties of p55. The C-terminal region contains a disulfide bridge formed by two conserved cysteine residues in strands β37 and β40. It was proposed that this surface-exposed structure might constitute a potential receptor-binding site; however, targeted mutagenesis of these cysteine residues had no effect on vacuolating activity in HeLa cells but reduced the amounts of secreted VacA, thereby suggesting a role in bacterial toxin secretion rather than receptor-binding (Letley, 2006). Experimental data on vacuole formation in cellular assay systems are supportive of a role of the N-terminal region, in particular strands β3, 6 and 9, for toxin oligomerization. The crystal structure of the p55 domain also provides a functional explanation for the N-terminal part (residues 351–360) in mediating an interaction between 3 loops of two p55 units in the dodecameric form of the VacA oligomer. The p55 domain alone is unable to function in internalization in the absence of the p33 domain as the latter is required to extend the β-helix fold of the p55 domain and thereby promoting oligomerization (Gangwer et al., 2007). The model was also supported by biochemical analysis that showed VacA residues 1–422 could induce vacuolation in cultured cells when expressed intracellularly, whereas p33, p55, and VacA residues 1–394 cannot (Ye and Blanke, 2002).

This model of VacA oligomerization by contacts between a large portion of p33 and the N-terminal region of p55 is also corroborated by yeast two-hybrid experiments in which residues 312–478 of p55 were observed to interact with the p33 domain (Torres et al., 2004).

Cell internalization of VacA was demonstrated to proceed by a clathrin-independent pathway of endocytosis which requires the small GTPases Rac1 and Cdc42 (Gauthier et al., 2005; Ricci et al., 2000). Different receptors have been identified which are involved in VacA binding to the cell surface and intoxication by uptake of the toxin. VacA binds to 2 high-affinity receptors identified by co-immunoprecipitation as receptor-like protein tyrosine phosphatases, RPTPα and RPTPβ, on the surface of AZ-521 gastric epithelial cells and G-401 kidney cells (Yahiro et al., 2003, 2004). A function for RPTPβ as VacA receptor was confirmed by a drastic decrease of VacA-induced vacuolation in RPTPβ-knockout HL60 leukaemic cells (Padilla et al., 2000). The human kidney tumor cell line G401 lacks RPTPβ but responds to VacA by utilizing RPTPα (previously designated as ‘p140’) as receptor for VacA (Padilla et al.,

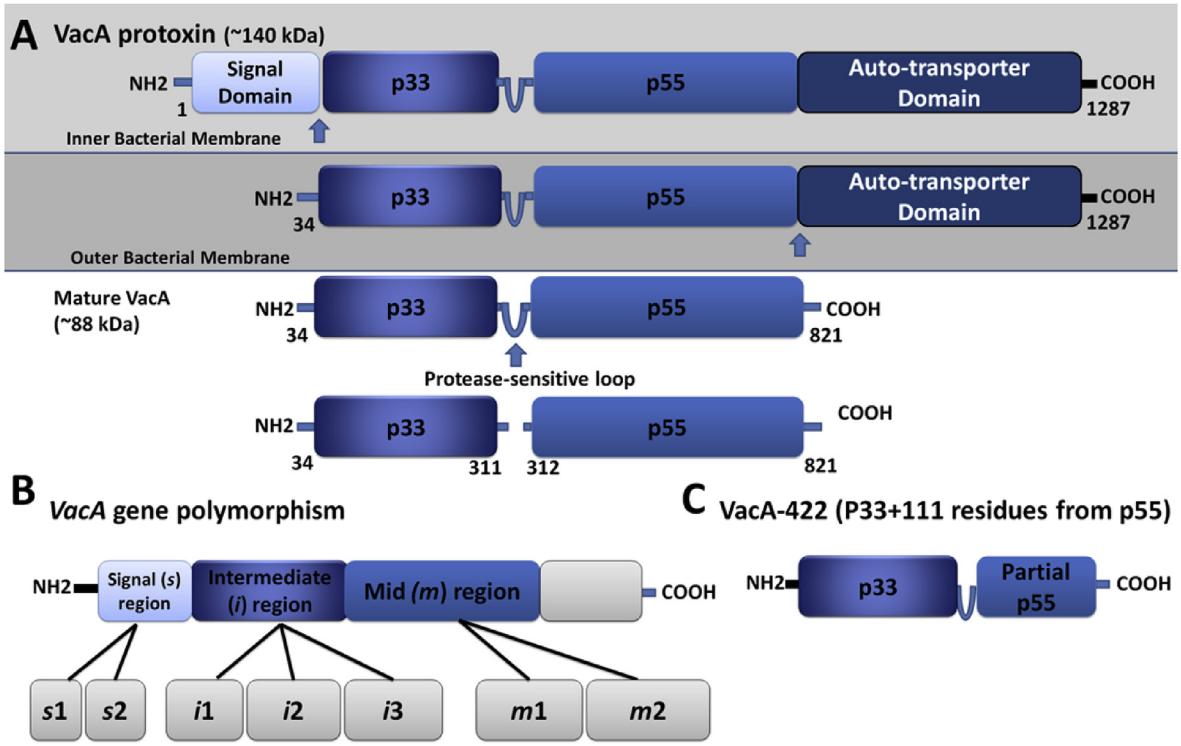

Fig. 1. Architecture of the *vacA* gene from *H. pylori*. (**A**) VacA is produced as protoxin of approximately 140 kDa. The signal sequence is cleaved upon translocation in the periplasm and the C-terminal autotransporter domain is cleaved during transportation across the outer membrane. The mature 88 kDa protoxin can be further cleaved by yet unidentified protease(s) into an N-terminal domain (amino acids 33–311) designated as p33, and a C-terminal p55 domain (residues 312–821). The p33 domain is involved in membrane insertion and the generation of ion channels whereas the p55 domain has a role in receptor binding and oligomerization. Proteolytic cleavage of p88 into the p33 and p55 subunits is not mandatory for toxin action. (**B**) Allelic diversity and polymorphisms within the *vacA* gene. The *vacA* gene contains three highly variable polymorphic regions which are the signal sequence region (*s*1, *s*2), the intermediate region (*i*1, *i*2, *i*3) and the mid region (*m*1, *m*2). Strains of *H. pylori* with the s1m1 phenotype are more frequently associated with severe disease symptoms than strains carrying other combinations of these alleles. (**C**) The minimal N-terminal structure (422 residues) of VacA necessary for vacuolation consists of the p33 domain and 111 additional residues of the p55 domain (Ye et al., 1999).

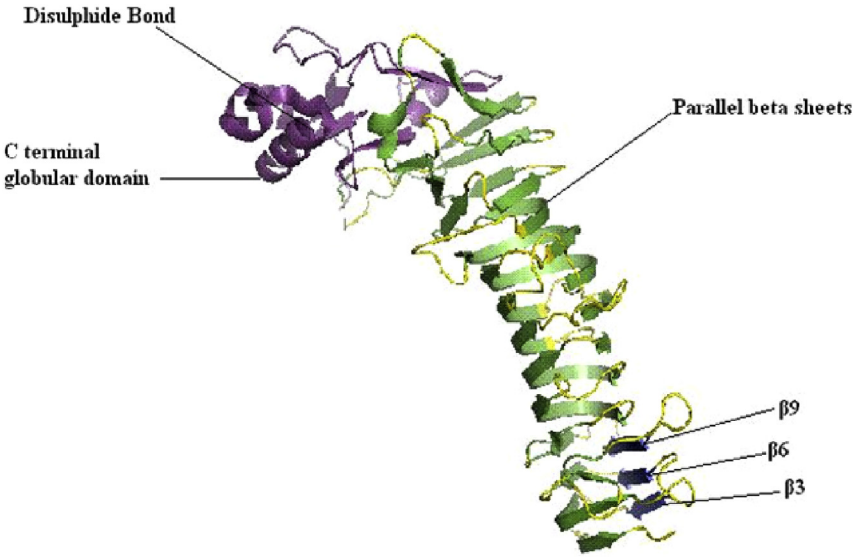

Fig. 2. Crystallographic structure of VacA p55 domain. The p55 domain is composed mainly of beta sheets (green), connected by loops of varying size and shapes (yellow). The C-terminus of the p55 domain contains both alpha helices and beta sheets (violet) and a disulphide bond (red) is present in the globular domain (Gangwer et al., 2007). β-sheets which are supposedly involved in toxin oligomerization are indicated.

2000). A specific receptor for VacA on T-cells was identified as β2 integrin subunit of LFA-1 (lymphocyte function-associated antigen 1, CD18) (Sewald et al., 2008), thereby supporting the notion that VacA uses different receptors for the intoxication of epithelial cells or modulation of cells of the immune system. VacA–induced cell vacuolization is reduced upon treatment of cells with PI-PLC

(phosphatidylinositol-specific phospholipase C) which specifically cleaves proteins anchored to the plasma membrane by a glycosylphosphatidyl-inositol (GPI)–anchor, a finding indicating localization of VacA in the GPI-anchored-protein-enriched early endosomal compartment (GEEC). Glycosylation at specific residues in a sequence of 5 amino acids is essential for VacA-binding to RPTPβ suggesting that an interaction of VacA with defined sugar residues is necessary for internalization (Ricci et al., 2000). VacA initially binds to RPTPβ localized in non-lipid raft microdomains followed by concentration of the VacA–receptor complex as result of its relocation into lipid raft structures (Nakayama et al., 2006). Sphingomeyelin, a main constituent of lipid rafts, was reported to function as a receptor for VacA in epithelial cells, thus implying that a VacA receptor could permanently reside in lipid raft structures (Gupta et al., 2010). RPTPβ was later hypothesized to trigger a signaling pathway by phosphorylation of Git-1 (synonymous to Cat-1), a multi-domain protein which controls vesicle trafficking, cell adhesion and cytoskeletal organization (Fujikawa et al., 2003). Deletion of approximately 100 amino acid residues at the C-terminus of p55 or antiserum against p55 resulted in decreased binding to cells (Garner and Cover, 1996; Wang and Wang, 2000). Specific residues in VacA as well as in RPTPs α and β which mediate specific toxin-receptor interactions have not been identified to date.

VacA appears to have activity on a large number of different cell types such HeLa cells, gastric tumor cells (AGS, AZ-521), kidney cells (G-401) and immune cells such as T-cells and macrophages. Work in our laboratory has shown that VacA toxin of the s1/m2 allelic type purified from a clinical isolate exhibited significant apoptosis on epithelial T84 and MDCK cells, whereby the observed activity was markedly higher on MDCK cells (Junaid et al., 2014). The toxin can seemingly interact with the plasma membrane of certain target cells without internalization but is able to induce there a pro-inflammatory response resulting in cytokine release (Isomoto et al., 2010). Oligomerization of VacA and clustering in detergent-resistant membrane domains was shown to be a prerequisite for endocytosis by a clathrin-independent pathway which involves cellular components such as the small GTPase Cdc42 and the cytoskeleton protein actin (Reyrat et al., 1999).

4. Membrane insertion, vacuolation and apoptosis

VacA is able to insert into lipid membranes and form low-conductance pores which display moderate selectivity for anions over cations (Iwamoto et al., 1999). The mechanism of VacA insertion into biological membranes and the structural requirements for pore formation are not well understood at present and no 3-dimensional structural model based on crystallographic data for the p33 pore forming domain is available. However, it is well established that the ultimate result of ion channel formation mediated by VacA is a depolarization of the membrane potential, mitochondrial fragmentation, formation of reactive oxygen species and apoptosis (Rassow and Meinecke, 2012).

Molecular modeling of the VacA membrane pore predicted a structure formed by 6 α-helices within the N-terminal part of p33 that was reminiscent of the mechano-sensitive channel S protein of *Escherichia coli* (Kim et al., 2004). Secondary structure prediction with the p33 domain suggests that α-helical elements presumably capable of channel formation are located between residues 1 to 71 and that β-strands are predicted to begin at residue 87 and extend into a region between residues 120–288 predicted to represent a β-helix. The assembly of 6 VacA molecules is conducive to the generation of anion-selective channel in planar lipid bilayers which can be inhibited by non-specific chloride channel blockers such as 5-nitro-2-(3-phenyl)-propylamino-benzoic acid (NPPB) (Tombola et al., 1999). Binding of VacA to lipids and association with detergent-resistant membrane domains (lipid rafts) is enhanced upon acid activation of the water-soluble oligomeric toxin molecules. Acid-initiated activation of the insertion process is essentially supported by data which have shown that disassembly of VacA oligomers at pH < 5 results in increased membrane insertion in artificial liposomes and plasma membranes (Molinari et al., 1998).

Our current knowledge on oligomerization of VacA monomers and the native pore-forming conformation of VacA bound to biological membranes is relatively limited since available experimental data seem to reflect mainly preferred conformations in solution. Low resolution of imaging techniques and in particular the lack of structural information for the p33 domain had left a gap in our understanding of the pore-forming mechanism of VacA and its significance for *H. pylori* pathogenesis. The structure of VacA oligomers has been investigated by cryo-electron microscopy at a resolution of 19 Å, atomic force microscopy and electrophysiological methods and the results indicated that VacA is able to undergo major conformational changes, accompanied by changes in its state of oligomerization, under different natural and experimental conditions (Adrian et al., 2002; Czajkowsky et al., 1999; Iwamoto et al., 1999). A variety of oligomeric forms has been observed with different imaging techniques and VacA monomers have been shown by deep-etch electron microscopy to assemble into hexameric and heptameric oligomers of 'flower-like' shape as well as double-layered structures in which 12 90-kDa subunits are interlocked in 2 six-membered arrays (El-Bez et al., 2005).

A recent approach by Chambers et al. employing single-particle electron microscopy determined three-dimensional structures of VacA oligomeric conformations at ~15 Å resolution (Chambers et al., 2013). In addition to the analysis of intramolecular interactions that support oligomerization, an examination of VacA mutants differing from wild-type (WT) VacA in oligomeric structural features revealed that VacAΔ6–27, a mutant that fails to form membrane channels, lacks an organized p33 central core. It is interesting to note that the p33 central core structure of VacA s2/m1 allelic type (deficient in cell-vacuolating activity) and VacAΔ301-328 (which retains vacuolating activity) is similar to those of wild-type VacA oligomers. From the structural analysis of the numerous VacA oligomer conformations, a model was proposed where soluble VacA first oligomerizes into hexamers or heptamers and then these single layers interact to form double-layer structures. It is yet an unresolved question whether the large dodecameric forms of VacA are formed during intracellular transport in endosomes or whether substantial conformational rearrangements precede insertion into endosomal and/or mitochondrial membranes.

Earlier studies on VacA insertion into mammalian mitochondria demonstrated the formation of membrane channels by the star-shaped p33/p55 co-complex leading to a decrease in the mitochondrial transmembrane electrochemical potential, release of cytochrome c and ultimately execution of apoptosis (Willhite and Blanke, 2004; Yamasaki et al., 2006).

There is evidence that a unique hydrophobic sequence at the N-terminus of the p33 domain (residues 6–27) is essential for membrane channel formation (McClain et al., 2003). This sequence contains a predicted hydrophobic region with 3 tandem GXXXG motifs (G14, 18, 22, 26) typical for trans-membrane dimerization sequences. Targeted mutagenesis including residues G14 and G18 has been demonstrated to abrogate vacuolating activity and the ability to form channels in planar lipid bilayers. Several protease-protected regions were identified at residues 40–66, 111–169, 205–266, 548–574 and 723–767, which were proposed to represent sites that interact with the membrane (Wang et al., 2000). It is not likely, however, that all of these regions participate in pore

formation as some of them are located in the p55 domain.

Vacuolation is a unique property of the VacA toxin and involves the participation of a number of intracellular protein components. While at present there is no evidence for a direct physical interaction of VacA with the small GTP-binding protein Rab7, dominant-negative mutations of Rab7 were shown to effectively prevent vacuolation in HeLa cells (Papini et al., 1997). Vacuole formation was dependent on the presence of a functionally active Rab7 protein, whereas Rab5 and Rab9 mutations were only partially inhibitory or ineffective. These effects indicated that homotypic fusion between late endosomes and the membrane flow in the endocytic pathway are necessary for vacuolation.

VacA-induced vacuolization is also inhibited in dominant-negative mutants of syntaxin 7, an integral membrane protein which cooperates with vesicle-associated membrane protein 7 (VAMP7) in lysosome-endosome fusion (Suzuki et al., 2003). The process of vacuolation also requires activity of the vesicular ATPase (V-ATPase) which compensates the influx of Cl^--ions through the VacA pore by increased pumping of protons into the vesicle; and dynamin, a high molecular weight GTP-binding protein which functions as mechano-chemical enzyme in vesicle formation (Suzuki et al., 2001). The docking factor CD2-associated protein (CD2AP) bridges F-actin structures with VacA-containing early endosomes and is required to transfer VacA from early endosomal compartments to late endosomes (Gauthier et al., 2007). In light of these data, VacA-induced vacuolation is basically the result of substantial alterations in intracellular vesicle metabolism and endocytic membrane traffic. In the context of VacA-induced pore-formation, it is important to realize that the toxin can seemingly interact with different cellular sites for membrane channel formation such as the cytoplasmic membrane, the membranes of the late endocytic compartments and the mitochondrial membrane. It is not entirely clear at present whether pore formation at different cellular target sites proceeds by a uniform mechanism of membrane insertion, oligomerization and channel formation or whether different mechanisms underlie these processes.

The molecular mechanism responsible for VacA-induced apoptosis and especially delivery of the toxin from endosomes to mitochondria is currently debated as one of the most challenging problems in VacA research. In particular, it is unclear whether both the p33 and p55 subunits are required for pore-formation at the inner mitochondrial membrane and the precise structural determinants and requirements for toxin internalization, intracellular transport and finally execution of membrane insertion leading to cell death are largely uncharacterized.

Earlier studies have demonstrated that VacA is able to induce the formation of mitochondrial apoptotic channels, the activation of proapoptotic multidomain Bcl-2 family member proteins Bax/Bak and the release of caspases (Yamasaki et al., 2006). The p33 subunit was shown to act as a pore-forming protein at the inner mitochondrial membrane and was able to trigger apoptosis independently of the p55 domain (Domanska et al., 2010). A recent study has proposed that the 32 hydrophobic residues at the N-terminus of p33 could represent a mitochondrion targeting sequence which promotes the transfer of VacA to the IMM by using the TOM translocase of the outer membrane (Galmiche and Rassow, 2010). While the N-terminal 36 residues of p33 were found to be essential and sufficient for mitochondrial targeting, it was demonstrated that these residues were dispensable for toxin assembly, oligomerization and channel formation in planar lipid bilayer experiments. It is noteworthy that the N-terminal sequence of p33 is not only required for mitochondrial import but also for the entry of the holotoxin into target cells. In contrast, Foo et al. proposed the presence of putative mitochondrion targeting sequences within p55 which could direct VacA to the IMM independent of the N-terminal p33 sequence (Foo et al., 2010). However, the question remains to be solved as of whether cleavage of the toxin and the presence of both domains is a strict requirement for the induction of apoptosis.

A straightforward model for VacA-induced mitochondrial apoptosis is now challenged by observations raising the possibility that mechanisms independent from pore-formation could operate during apoptosis and that VacA exerts effects on the activation of proapoptotic proteins as well as on mitochondrial morphology. By employing a cell-based analysis utilizing mouse embryonic fibroblasts (MEF), it was recently demonstrated that VacA induces an endosome-mitochondria juxtaposition preceding the retrieval of active Bax on mitochondria (Calore et al., 2010). This translocation of Bax leads to the co-segmentation of endosomal and mitochondrial membranes and is strictly dependent on the presence of the pore-forming p33 domain. Although no evidence has been obtained to suggest a direct interaction between VacA and Bax or Bak, the possibility exists that some interaction between these proteins occurs within the membrane. Bax and Bak double-knockout MEF cells are completely resistant to VacA-induced apoptosis.

VacA has been shown to induce activation of the dynamin-related protein 1 (Drp1) and subsequent mitochondrial fission (Jain et al., 2011). The GTPase-activity of Drp1 is required to activate Bax/Bak and subsequent mitochondrial fragmentation and permeabilization of the outer membrane.

Toxin-dependent mitochondrial fragmentation requires VacA channel activity; however, data obtained from morphological inspection experiments suggested that activation of Bax was not strictly required for VacA-induced mitochondrial fission mediated by Drp-1. It should be noted that the cellular site at which toxin channel activity is required for fragmentation has not been identified and the possibility exists that VacA-mediated mitochondrial fragmentation arises independently of the toxin localization to mitochondria.

It remains an open question as of whether VacA is delivered to the mitochondria by a release *via* VacA channels or by vacuole swelling and destruction (Palframan et al., 2012). Based on an observed release of LDH and the proinflammatory protein HBMG1 in VacA-treated AGS cells, a recent study has suggested the possibility of a necrotic mechanism operating in VacA induced cell death (Radin et al., 2011).

5. Allelic diversity

Strains of *H. pylori* can demonstrate considerable variations in their production of VacA cytotoxin activity due to extensive polymorphisms in *vacA* gene structure. Clinical isolates of *H. pylori* were reported that fail to express a functionally VacA protein due to internal duplications, deletions, 1 bp insertions or nonsense mutations (Ito et al., 1998). A section of the *vacA* gene which exhibits maximum sequence diversity corresponds to approximately 800 bp in the middle of the gene within the p55 domain and was designated 'm-region' (middle) (Atherton et al., 1995). Various m sequences have been grouped into 2 distinct families of alleles, *m*1 and *m*2, which roughly correspond to 281 amino acid residues spanning the region from D455 to V735 in the VacA sequence of *H. pylori* model strain 60,190 (Ye et al., 1999).

A second region of significant sequence diversity is located at the signal sequence of the protoxin and the N-terminus of the mature toxin, referred to as 's'—region (van Doorn et al., 1998). Two main allelic families designated *s*1 and *s*2 (with subtypes *s*1a, *s*1b and *s*1c) were recognized and these allelic subtypes were previously linked to restrictions in the geographical distribution of *H. pylori* strains (Rudi et al., 1998). All combinations of the s and m allelic types (*s*1/*m*1, *s*2/*m*1, *s*1/*m*2, *s*2/*m*2) can arise in nature as

result of recombination and have been isolated, however, *s*2/*m*1 forms are exceptionally rare.

Later it became evident that both the *m* and *s* distinct alleles correlate with the severity of gastroduodenal diseases in *H. pylori* – infected patients and the biological activity of the VacA toxin (Han et al., 1998; Rudi et al., 1998). Extensive studies have provided evidence that strains containing the *s*1 *vacA* alleles are more frequently associated with peptic ulceration than strains containing the *s*2 allele and individuals infected with *s*1 – carrying strains are at higher risk for developing gastric carcinoma (Nogueira et al., 2001). Similarly, strains harboring the *m*1 allele are more significantly associated with gastric carcinomas than strains with the *m*2 marker. Strains carrying the *m*1 allele show a higher correlation with atrophic gastritis and intestinal metaplasia. It is noteworthy that strains containing the *s*1/*m*1 allelic form of the *vacA* gene encode a variant of VacA which exhibits high levels of toxin activity *in vitro*. In Japan, an unusually high incidence of gastric adenocarcinoma correlates with the presence of strains that almost exclusively carry the *s*1/*m*1 allele. For reasons which are not completely understood at present, the phylogenetic separation between allelic forms of *vacA* genes is maintained in nature despite the ability of *H. pylori* to perform extensive recombination (Atherton et al., 1999).

The m region also determines specificity of the VacA toxin for certain cell types whereby the *m*1 type is cytotoxic to HeLa cells and the *m*2 toxin induces vacuolation in gastric and non-gastric cell types such as rabbit kidney cells (RK13), but is not cytotoxic to HeLa cells (Pagliaccia et al., 1998). These findings would indicate that the m region is critically involved in cell type-specific receptor-mediated interactions of the VacA toxin. The cell type-specific binding region of the *m*1 form of VacA was mapped to residues 460–496. The *m*2 type contains an insertion of 23 residues at position 475 consisting of an imperfect repeat of the upstream sequence (Ji et al., 2000).

The structural basis for the significant phenotypic differences between *s*1 and *s*2 forms of VacA is less clear. It was proposed that export of the VacA toxin across the cytoplasmic membrane into the periplasmic space is less efficient in *s*2 strains when compared to *s*1 strains (Atherton et al., 1999). The signal sequence of *s*1 strains is strongly hydrophobic whereas the signal sequence of the *s*2 type contains a short, predominantly hydrophilic extension of 12 amino acid residues. These differences may account for variations in VacA protein activity between *s*1 and *s*2 alleles. It is conceivable that the high potency of *s*1/*m*1 forms of VacA is not only attributable to the structural properties of the encoded VacA but also to the quantity of product formation as the *s*1 form reportedly is secreted in higher amounts than the *s*2 variant (Forsyth et al., 1998). These differences may be caused by an elevated level of transcription of the *vacA* gene in *s*1/*m*1 strains of *H. pylori*.

Recently, the intermediate '*i*'–region was identified by a survey within the Iranian population as a third polymorphic site within the *vacA* sequence (Rhead et al., 2007). The *i*–region is located within the p33 domain and 3 types, *i*1, *i*2 and *i*3, were commonly found in clinical isolates of *H. pylori* (Bridge and Merrell, 2013). The *i*–region type is linked to the *s* and m phenotypes whereby *s*1/*m*1 strains contain only the *i*1 region and *s*2/*m*2 strains contain the *i*2 region. For reasons which are unknown at present, naturally occurring *s*1/*m*2 strains can possess either one *i*1 or one *i*2 sequence. Vacuolation assays demonstrated that vacuolating activity of *s*1/*m*2 was dependent on the presence of the *i*-region, thereby suggesting a pivotal role for VacA activity. How the *i*-region functions in the context of VacA activity needs to be elucidated in future studies.

6. Conclusions

Currently the VacA cytotoxin of *H. pylori* is intensively investigated for a number of reasons. The structural and mechanistic properties of VacA are unrelated to any other known bacterial toxin. The VacA toxin is an important virulence factor for the bacterial colonization of the stomach and the development of gastroduodenal diseases. The gene encoding VacA is characterized by a high degree of allelic diversity and variants of the toxin have been linked to different risks of diseases caused by *H. pylori*. Moreover, the VacA toxin appears to be a promising candidate antigen for inclusion in a vaccine against *H. pylori*. There remain still many questions to be answered about structure-function relations within the VacA toxin and its mechanism of action, however, the multitude of factors involved and allelic variation among *H. pylori* strains complicates the analysis of possible molecular interactions. Considerable controversy exists for the question of intracellular trafficking, mitochondrial delivery and induction of apoptosis by the toxin. The 3-dimensional structure of the putative pore-forming domain p33 has to be resolved and determinants and structural requirements for membrane insertion and pore-forming activity need to be identified. Moreover, specific interactions of the VacA toxin with potential receptor(s) are largely uncharacterized to date. These further studies may not only contribute to improved predictive diagnostic procedures but ultimately lead to the discovery of novel antimicrobial inhibitors against infections with *H. pylori* which target receptor-binding, oligomerization or membrane channel formation of the VacA toxin.

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Ethical statement

This submission is a review article. No ethical issues are involved.

Conflict of interest

The authors confirm that there are no conflicts of interest in relation to this submission.

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