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Helicobacter pylori Vacuolating Cytotoxin, VacA, Is Responsible for Gastric Ulceration

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Pathogenic strains of *Helicobacter pylori* produce a potent exotoxin, VacA, which causes progressive vacuolation as well as gastric injury. Most *H. pylori* strains secrete VacA into the extracellular space. After exposure of VacA to acidic or basic pH, re-oligomerized VacA (mainly 6 monomeric units) at neutral pH is more toxic. Although the mechanisms have not been defined, VacA induces multiple effects on epithelial and lymphatic cells, i.e., vacuolation with alterations of endo-lysosomal function, anion-selective channel formation, mitochondrial damage, and the inhibition of primary human CD4$^+$ cell proliferation. VacA binds to two types of receptor-like protein tyrosine phosphatases (RPTP), RPTPα and RPTPβ, on the surface of target cells. Oral administration of VacA to wild-type mice, but not to RPTPβ KO mice, results in gastric ulcers, suggesting that RPTPβ is essential for intoxication of gastric tissue by VacA. As the potential roles of VacA as a ligand for RPTPα and RPTPβ are only poor understood, further studies are needed to determine the importance of VacA in the pathogenisis of disease due to *H. pylori* infection.

Key words: gastric ulcer, *Helicobacter pylori*, RPTP (receptor-like protein tyrosine phosphatase), VacA (vacuolating cytotoxin), vacuolation.

The role of VacA in *Helicobacter pylori* infection

Helicobacter pylori, a Gram-negative bacterium that colonises the gastric mucosa of humans, plays a major role in gastric disease (*1–3*). Although more than half of the world human population is chronically infected with *H. pylori*, most people have no symptoms. Colonization with *H. pylori*, however, induces gastric mucosal inflammation, and is a risk factor for the development of chronic gastritis and peptic ulcers, and gastric cancer. Many *H. pylori* strains isolated from patients contain the *cagA* gene (cytotoxin-associated gene A) and produce the vacuolating cytotoxin, VacA. Additional *H. pylori* products, including urease, the neutrophil-activating protein NapA, adhesins, heat-shock protein, and lipopolysaccharide appear to be involved in virulence (*1–3*). Recent molecular and cellular studies of VacA action have shown that it is a major virulence factor that is involved in the pathogenesis of inflammation in *H. pylori*–induced gastritis and ulceration (*4–6*).

Multiple structures of VacA

The *vacA* gene encodes a ~139-kDa protoxin (including the amino-terminal signal peptide). The signal peptide and a ~50-kDa carboxy-terminal fragment are proteolytically cleaved during VacA secretion by *H. pylori* (*7*, *8*). Under denaturing conditions, the mature toxin has a molecular mass of 87–95 kDa, whereas native VacA in *H. pylori* culture medium exists as a large oligomeric complex with a mass of approximately 1,000 kDa (*9*, *10*). The VacA oligomer is thought to be composed of two rings, each comprising 6–7 VacA monomers, and forming a flower-like structure. Exposure of VacA to acid or alkaline pH increases the vacuolating activity by disassembly of the oligomer into monomers (*11*, *12*). When VacA is exposed to acidic conditions, it exhibits increased activity for planar lipid bilayers and forms membrane-associated hexamers (*11*). These hexamers act as anion-selective, voltage-dependent channels (*13*, *14*). The channel-forming activity is inhibited by deletion or mutation of the amino-terminal portion of VacA (see below). It is believed that the VacA channel induces toxicity in susceptible cells by inducing changes in the osmotic balance across some membranes (*e.g.*, plasma, vacuolar, and mitochondrial membranes) (Fig. 1).

VacA has amino-terminal 34–37 kDa (p37) and carboxy-terminal 58 kDa (p58) domains (*11*, *15*) with an exposed protease-sensitive loop between these fragments (*10*) (Fig. 1). It is believed that the p37 domain has vacuolating activity whereas the p58 domain is responsible for VacA binding to the cell (*16*, *17*). Although VacA has structural similarity to AB-type toxins, the cleavage of the mature toxin to the p37 and p58 fragments does not correlate with vacuolating activity (*9*). The interaction between the p37 and p58 fragments has been detected by yeast two-hybrid techniques (*18*). Mutant proteins with internal deletions in the p37 fragment are unable to interact with p58 fragments. In addition, studies using *H. pylori* strains containing these same deletion mutations in the *vacA* gene have revealed that secreted mutant VacA proteins fail to assemble into large oligomeric complexes (*18*, *19*). These data suggest that the

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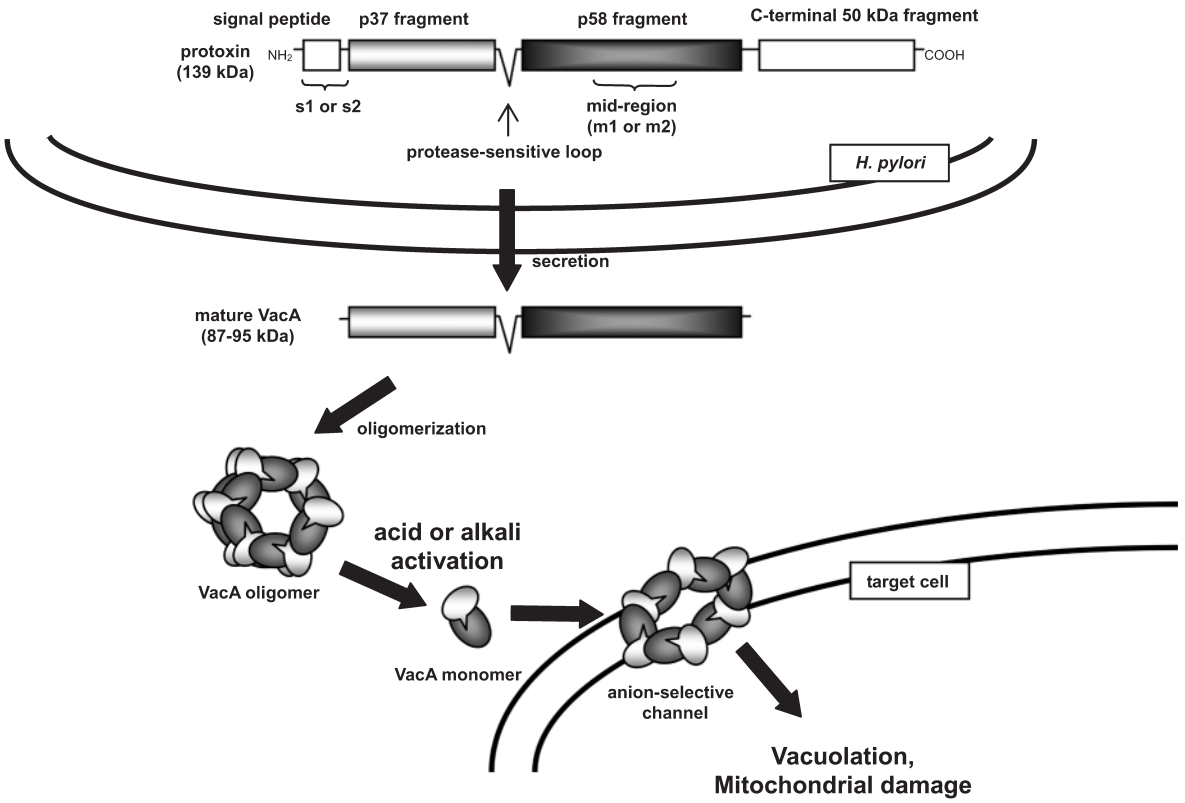

Fig. 1. **Schematic view of VacA structure, processing and action.** The *vacA* gene product (protoxin) consists of five fragments, *i.e.*, signal peptide, p37 fragment, protease-sensitive loop, p58 fragment and C-terminal 50-kDa fragment. There is a structurally characteristic mid-region (m1/m2) in the p58 fragment, and an additional characteristic region (s1/s2) near the amino terminal end of the protoxin, including the signal peptide and amino terminal portion of the p37 fragment. The signal peptide and C-terminal 50-kDa fragment are cleaved during the secretion of the toxin. Secreted, mature VacA assembles into a large flower-like oligomer in the extracellular space. The oligomer is disassembled by exposure to acidic or alkaline conditions. The VacA monomer associates with plasma membranes of target cells in a receptor-mediated manner, and forms an anion-selective channel.

interaction between the p37 and p58 domains is important to VacA toxicity.

The p58 domain has a structurally characteristic region, termed the mid-region, which is classified into two families (m1 and m2) based on primary amino acid sequence. In addition, two families (s1 and s2) are distinguished by primary structure near the amino-terminal end of the protoxin (*20*) (Fig. 1). All possible combinations of these regions (s1/m1, s1/m2, s2/m1 and s2/m2) have been reported in clinical isolates of *H. pylori*, although s2/m1 alleles are rare (*20*, *21*). In studies of the clinical relevance of *vacA* genotypes, the s1 type *vacA* alleles were found to be associated with the presence of peptic ulcers (*20*). It has also been reported that s1 type *vacA* gene products are secreted at higher levels than s2 type *vacA* products. Other studies, which examined the relationship between the quantity of VacA and gastric ulcers using a very sensitive method, bead-ELISA, revealed that the amount of VacA secreted by *H. pylori* correlates with the presence of gastric ulcers (*22*). In an *in vitro* study of vacuolating activity on HeLa cells, the culture supernatants of the strains encoding the s1/m1 *vacA* gene had the greatest vacuolating activity, the strains encoding the s1/m2 *vacA* gene had intermediate vacuolating activity, and the strains encoding the s2/m2 *vacA* gene had no detectable cytotoxic activity (*20*). One possible cause of these differences in cytotoxicity is the different cell type specificities of the mature toxins. The m1 and m2 types of VacA (termed m1VacA and m2VacA), which are mainly produced by strains containing the s1/m1 *vacA* and s1/m2 *vacA* genes, respectively, have different cell type specificities in cytotoxicity assays. Whereas m1VacA is cytotoxic to HeLa cells, m2VacA is able to induce vacuolization in primary cultured human gastric cells and in non-gastric cell lines such as RK-13, but has no cytotoxic activity on HeLa cells (*23*). These preferences in cell types are caused by different binding abilities of the two types of VacA to the cells. These differences suggest that some receptor-mediated interactions exist between cell surface and VacA that are important in determining cytotoxicity.

The p37 fragment has three tandem GxxxG motifs in an amino-terminal hydrophobic region (*24*) that is essential for membrane channel formation by VacA. Alanine replacement mutants for glycine residues in the GxxxG motifs (Gly-14 and Gly-18) diminish VacA oligomerization, vacuolation activity and anion-selective membrane channel forming activity in planar lipid bilayers. In addition, the proline residue in the amino-terminal hydrophobic region (Pro-9) is essential for anion-selective membrane channel formation as determined by alanine replacement. The structural modeling study supports these experimental data using mutants (*25*). This study

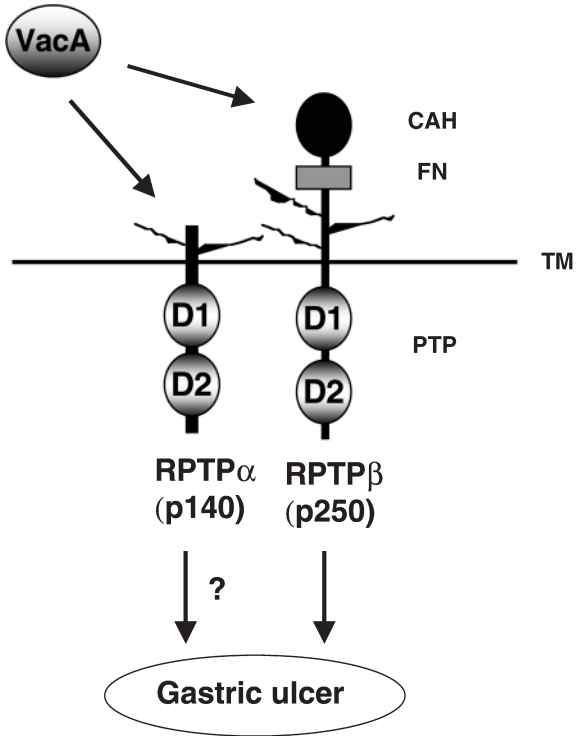

Fig. 2. **VacA interacts with two receptor-like protein tyrosine phosphatases, RPTPα and RPTPβ.** Both RPTPα and RPTPβ are single transmembrane-spanning receptors that contain tyrosine phosphatase (PTP) domains (D1, D2) in their intracellular region. RPTPβ contains a carbonic anhydrase domain (CAH) and fibronectin type III repeat domain (FN). These RPTPs are glycoproteins. RPTPβ-mediated gastric ulceration by VacA might involve signal transduction processed in gastric cells, based on the activities of RPTPα and RPTPβ. The role of RPTPα signaling in the pathogenesis of gastric ulcers remains unknown.

indicated that the amino-terminal regions of p37 fragments form a homooligomeric transmembrane helical (VacA-TM helix) structure that resembles the structure of an anion selective channel of *E. coli,* termed the mechanosensitive channel of small conductance, MscS (*26*). By comparison of VacA with the MscS structure, it has been found that Gly-14 and Gly-18 are essential for the packing of the VacA-TM helix structure. The structural modeling study also suggested that alanine and valine residues, which exist in three tandem GxxxG motifs, are essential for packing in the VacA-TM helix structure, and suggested that threonine, isoleucine and lysine residues, which exist in or in the neighborhood of three tandem GxxxG motifs, are essential for VacA channel function as a low-conductance anion channel.

Receptors on target cells

VacA binding to mammalian cell surface receptor proteins is the first step in toxin action on gastric epithelial cells. Most mammalian cells are susceptible to vacuolation induced by m1/VacA, suggesting that the m1/VacA receptor is a ubiquitous cell surface molecule. By immuno precipitation analysis of biotinylated cell surface proteins with VacA and anti-VacA antibody, a 140-kDa protein (p140) was detected when VacA-susceptible cells such as AZ-521, AGS, and COS-7 cells were examined, but not with HL-60 cells, which are nonsusceptible cells (*27*). A higher molecular mass VacA receptor, a 250 kDa protein (p250), was detected only in the case of AZ-521 cells, gastric epithelial–derived cells (*27*). The p250 protein, purified by peanut agglutinin affinity and Superose 6 chromatography, was identified following isolation and sequencing as a receptor-like protein tyrosine phosphatase β (RPTPβ) (*12*). Chemical agents that promote the differentiation of HL-60 cells into macrophage- and monocyte-like cells, but not granulocyte-like cells, enhance VacA sensitivity by increasing the expression of the cell surface protein, RPTPβ (*28*). Suppression of RPTPβ expression with antisense oligonucleotides inhibits VacA sensitivity of PMA-stimulated HL-60 cells, and transfection of the RPTPβ gene into BHK-21 cells results in the induction of VacA sensitivity. These data support the hypothesis that RPTPβ is a VacA receptor (*28*). G401 cells, a human kidney tumor cell line, lack RPTPβ but respond to VacA, and p140 has been identified as a major receptor protein for VacA. Internal amino acid sequences of p140, which was purified by PNA-affinity chromatography from G401 cells, are identical to those of RPTPα (*29*). The overexpression of V5-tagged RPTPα cDNA confers VacA binding to COS-7 cells. Treatment of G401 cells with RPTPα antisense oligonucleotide before exposure to VacA, inhibits vacuolation. These data suggested that RPTPα acts as a receptor for VacA in G401 cells. VacA is the first extracellular ligand to be discovered for RPTPα. Thus, our previous studies showed that VacA binds on the surface of target cells to two types of receptor-like protein tyrosine phosphatases (RPTP), RPTPα (*12*) and RPTPβ (*29*) (Fig. 2). As the potential roles of VacA as a ligand for RPTPα and RPTPβ are only poorly understood, further studies are needed to determine the importance of VacA-receptor complexes to intracellular signaling.

Cytotoxicity of VacA

Cellular vacuolation is a unique function of VacA that was originally found by Leunk et al. in 1988 (*30*). The membranes of these vacuoles contain the small GTP-binding protein Rab7, late endosome and lysosomal markers, and the membrane protein Lgp110 (*31*). Accordingly, VacA might disrupt normal membrane trafficking at or near the level of late endosomes. Vacuolation depends not only on VacA, but also on the presence of permeant weak bases in the extracellular medium (*32*). The microinjection of VacA or the transfection of plasmids containing the VacA gene into HeLa cells results in the formation of intracellular vacuoles (*33*), providing evidence that VacA introduced into the cytosol acts on an intracellular target, potential targets include the vacuolar ATPase, Rab7, and Rac1 (*34*, *35*). It has been well established that VacA-induced vacuolation requires V-ATPase activity, and that its inhibitor, bafilomysin A1, reduces VacA-induced vacuolation in mammalian cells (*36*). Rab7 may be important for supporting membrane deposition and homotypic fusion between late endosomes, and Rab7 may control cytoskeletal elements affecting membrane traffic. Recently, we reported that dynamin, a high molecular weight GTP-binding protein functioning as a mechanochemical enzyme in vesicle formation, is involved in VacA-induced vacuolation (*37*). In addition, transient transfection of the dominant-negative

mutant syntaxin 7 was also found to inhibit VacA-induced vacuolation (*38*). Syntaxin 7 is an integral membrane protein present on both late endosomes and lysosomes and functions in their heterotypic fusion as part of the SNARE complex in association with other SNARE proteins. The observation that the expression of both syntaxin 7 mRNA and protein in AGS cells is enhanced by exposure to VacA strongly suggests that syntaxin 7 is involved in VacA-induced vacuolation (*38*). These results suggest that VacA-induced vacuolation is a result of a toxin-induced alteration of intracellular vesicle trafficking.

VacA induces alterations in endosomal function, resulting in degenerative vacuolation (*30*). Mitochondrial damage (*39*, *40*) and apoptosis (*41–43*) appear to be vacuolation-independent effects of VacA. Other cytotoxic actions of VacA, such as the inhibition of the invariant chain (Ii)–dependent pathway of antigen presentation by MHC class II (*44*), suppression of nuclear translocation of nuclear factors of activated T cells, NFAT (*45*, *46*), and ATF-2 activation in a gastric cell line (*47*), have been reported, although the mechanisms have not been defined.

It is believed that VacA forms anion-selective channels in endosomal membranes through its interaction with lipids (*48*, *49*). As with other bacterial toxins such as hemolysin, VacA is also able to form channels in biological membranes. The assembly of six molecules of VacA forms an anion-selective channel in lipid bilayers, and its channel activity is inhibited by typical chloride channel blockers. A nonspecific chloride channel blocker, NPPB, can inhibit the channel activity of VacA, resulting in a reduction of vacuolation and cytochrome *c* release caused by VacA (*45*). However, p38 activation in VacA-treated cells is independent of the chloride channel activity of VacA (*45*).

VacA is endocytosed by cells via a clathrin-independent pathway (*50*). Treatment of VacA-sensitive cells with PI-PLC, which specifically removes proteins anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor, reduces and delays VacA-induced cell vacuolation (*50*). Although VacA does not bind to GPI-anchored proteins (*51*), these proteins may be important for VacA-induced vacuolation and channel formation. Similar to GPI-anchored proteins, VacA associates with lipid rafts, as was shown by examining immunoblot analyses of sucrose density gradient centrifugation. Treatment of cells with methyl-β-cyclodextrin (a cholesterol-depleting agent) do not inhibit the VacA-induced depolarization of the plasma membrane, but interfere with the internalization and intracellular localization of VacA, and inhibit the capacity of the toxin to induce cell vacuolation (*52*). These results suggest that the association of the toxin with lipid rafts is important for VacA activity.

Yeast two-hybrid analysis revealed an interaction between VacA and a 54-kDa protein (VIP54) in HeLa cells, cosistent with an intracellular target for VacA action (*53*) and the hypothesis that VacA affects various cellular functions through association with intracellular molecules. Both full-length VacA and its p37 domain genes transfected into Hep-2 cells localize to the mitochondrial matrix, whereas the p58 domain remains in the cytosol (*41*). In that report, VacA expressed in the cytosol was found to localize to the mitochondoria and resulted in apoptosis, which was associated with the release of cytochrome *c*, activation of caspase 3, and the cleavage of poly(ADP-ribose)polymerase (*41*). Although the extracellular addition of VacA also induced cytochrome *c* release, no data regarding VacA-induced caspase 3 activation were not reported (*41*). These data indicate that VacA or its p37 is released into the cytosol, and that the target organelle might be mitochondria.

Recently, it was reported that VacA has immunosuppressive effects, with direct action on T cells rather than antigen-presenting cells (*45*, *46*). VacA inhibits Jurkat T cells as well as human peripheral blood lymphocytes. Studies in Jurkat T cells indicate that VacA blocks activation of the nuclear factor of activated T cells (NFAT), a key transcriptional factor required for T cell activation. VacA partially mimics the activity of the immunosuppressive drug FK506 by possibly inducing immune suppression. More recently, Sundrud et al. reported that VacA inhibits the proliferation of primary human $CD4^+$ T cells and demonstrated that this inhibitory effect on proliferation is not attributable to VacA effects on NFAT activation of IL-2 expression (*54*). In addition, VacA suppresses IL-2-induced cell cycle progression without affecting IL-2–dependent survival (*54*). In these studies, the VacA-mediated inhibition of primary T cell proliferation was found to depend on VacA-channel activity.

VacA-induced gastric damage in mice through RPTPβ function

RPTPβ, a chondroitin sulfate proteoglycan with an extracellular region containing a carbonic anhydrase-like domain and a single FNIII domain, has been shown to play an important role in cell migration, differentiation, synaptogenesis, synaptic function, and myelination in the central nervous system. RPTPβ was originally found to be highly expressed in brain. For RPTPβ to have functional importance in mediating VacA toxicity, RPTPβ needs to be expressed in gastric tissues. Virtually all glands in the gastric corpus of wild-type mouse contain detectable RPTPβ, and expression is localized to the glandular basal region (*55*).

In mice, orally administered VacA causes degeneration of the gastric mucosa and acute inflammation, followed by gastric ulcer disease (*56*). Using this mouse model system, RPTPβ KO mice were subjected to VacA administration. Indeed, oral administration of VacA to wild-type mice, but not to RPTPβ KO mice, resulted in gastric ulcers, suggesting that RPTPβ is essential for the intoxication of gastric tissue by VacA (*55*). However, VacA induces the same amount of vacuolation in epithelial gastric cells of wild-type mouse and RPTPβ KO mice. Since RPTPα is expressed ubiquitously in many tissues, including stomach, VacA-induced vacuolation in RPTPβ KO mice may result from VacA acting through RPTPα. A variety of extracellular ligands of RPTPβ have been identified including PTN (pleiotoropin), N-CAM, Ng-CAM, tenascin, contactin, and midkine (*57*). Among these RPTPβ ligands, PTN and midkine are secreted proteins that exert their effects through the inhibition of RPTPβ phosphatase activity. Interestingly, PTN also induces gastric ulcers in wild-type mice, but not in RPTPβ KO mice, and PTN causes no cellular vacuolation as is seen with VacA. RPTPβ-mediated gastric ulcer might be

important for signal transduction in gastric cells, but not vacuolation (Fig. 2). Signal transduction involving another VacA receptor, RPTPα, may also contribute to receptor-mediated gastric ulcesr. Studies of the effects of VacA in RPTPα KO mice may yield important clues for understanding cellular information by VacA (Fig. 2).

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