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## Pleiotropic Actions of *Helicobacter pylori* Vacuolating Cytotoxin, VacA

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*Helicobacter pylori* produces a vacuolating cytotoxin, VacA, and most virulent *H. pylori* strains secrete VacA. VacA binds to two types of receptor-like protein tyrosine phosphatase (RPTP), RPTP $\alpha$  and RPTP $\beta$ , on the surface of host cells. VacA bound to RPTP $\beta$ , relocates and concentrates in lipid rafts in the plasma membrane. VacA causes vacuolization, membrane anion-selective channel and pore formation, and disruption of endosomal and lysosomal activity in host cells. Secreted VacA is processed into p33 and p55 fragments. The p55 domain not only plays a role in binding to target cells but also in the formation of oligomeric structures and anionic membrane channels. Oral administration of VacA to wild-type mice, but not to RPTP $\beta$  knockout mice, resulted in gastric ulcers, in agreement with the clinical effect of VacA. VacA with s1/m1 allele has more potent cytotoxic activity in relation to peptic ulcer disease and appears to be associated with human gastric cancer. VacA activates pro-apoptotic Bcl-2 family proteins, and induces apoptosis via a mitochondria-dependent pathway. VacA can disrupt other signal transduction pathways; VacA activates p38 MAPK, enhancing production of IL-8 and PGE<sub>2</sub>, and PI3K/Akt, suppressing GSK-3 $\beta$  activity. VacA has immunomodulatory actions on T cells and other immune cells, possibly contributing to the chronic infection seen with this organism. *H. pylori* virulence factors including VacA and CagA, which is encoded by *cytotoxin-associated gene A*, along with host genetic and environmental factors, constitute a complex network to regulate chronic gastric injury and inflammation, which is involved in a multistep process leading to gastric carcinogenesis.

**Keywords:** *Helicobacter pylori*/vacuolating toxin/VacA

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Persistent *Helicobacter pylori* (*H. pylori*) infection of the gastric epithelium is world wide and one of the most common infections. *H. pylori* is accepted as a major cause of gastroduodenal diseases including peptic ulcer disease, gastric lymphoma, and gastric adenocarcinoma. *H. pylori* virulence, host genetic and environmental factors constitute a complex network, leading to persistent immune and inflammatory responses, which are instrumental in a multi-step process resulting in gastric cancer (Blaser and Atherton 2004).

The role of *H. pylori* in gastroduodenal diseases is firmly established. Pathology results from the intricate, ongoing interplay between environmental, bacterial, and host factors (Blaser and Atherton 2004). Strain-to-strain genetic variability in bacterial virulence factors not only affects the ability of the organism to colonize and cause disease but also affects inflammation and gastric acid output.

During the interactions with the host, *H. pylori* is able to adapt by mutations and DNA rearrangements, generating novel genotypes. On the host side, variations in the immune response to the chronic presence of *H. pylori* directly impact *H. pylori*-associated gastroduodenal diseases and affect gastric acid output and thereby, the density and location of the bacteria (Blaser and Atherton 2004; Kusters et al. 2006). Many of these *H. pylori*-host interactions have similarities with the interactions between the gut flora and the gastrointestinal tract and may serve as a paradigm for the interactions between bacteria and their hosts. A number of bacterial virulence factors have been identified, including flagellin, urease, catalase, neutrophil-activating protein, adhesion, heat shock proteins, outer membrane proteins,  $\gamma$ -glutamyl-transpeptidase, lipopolysaccharide, cytotoxin-associated gene pathogenicity island (*cagPAI*) and vacuolating cytotoxin (VacA) (Fig. 1, Blaser and Atherton 2004; Kusters et

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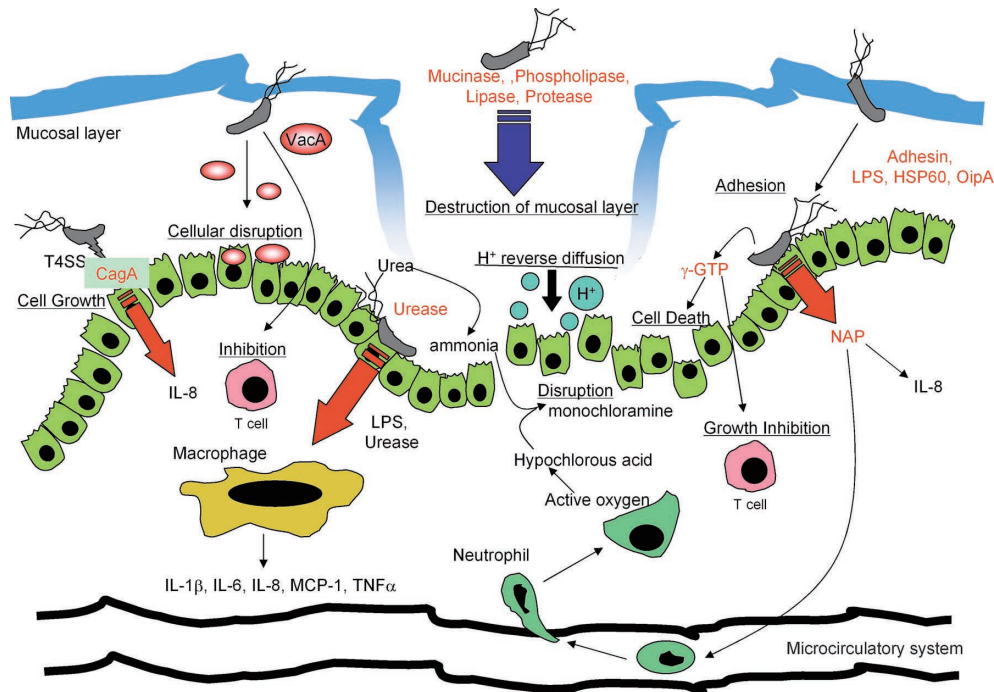


Fig. 1. *Helicobacter pylori* products appear to be involved in virulence. There are bacterial virulence factors such as adhesins (AlpA/B, BabA, HopQ and SabA), urease, catalase, neutrophil-activating protein (NAP), adhesin, heat shock proteins (HSP60), outer membrane proteins (OipA), glutamyltranspeptidase ( $\gamma$ -GTP), lipopolysaccharide (LPS), cytotoxin-associated gene pathogenicity island (*cagPAI*) and vacuolating cytotoxin (*VacA*). The *cagPAI*-encoded proteins serve as building blocks of a type IV secretion apparatus, which forms a syringe-like structure capable of penetrating gastric epithelial cells and facilitating the translocation of CagA. CagA is encoded by *cagA* gene is a marker for the presence of the 40 kb-long genomic *cagPAI* and contributes to the inflammatory response by initiating a signal transduction cascade, resulting in interleukin (IL) 8 production. LPS can also induce enhanced production of various proinflammatory cytokines. NAP plays a role as a chemoattractant for neutrophils. *VacA* causes cellular disorder and inhibition of T-cell activation along with  $\gamma$ -GTP. Urease exerts strong activity to generate ammonia from urea, then producing monochloramine, a harmful bioactive molecule against the epithelium. Additional enzyme products including mucinase, phospholipase, lipase and protease damage mucus layer covering the gastric epithelium.

al. 2006). Host enzymes including nitric oxide synthase (iNOS) and cyclooxygenase may perturb the balance between gastric epithelial cell apoptosis and proliferation, resulting in ulcer formation and gastric cancer. The complete genome sequence of many *H. pylori* strains, isolated from patients with different clinical outcomes has provided useful information to identify new virulence genes by genomic, proteomic, and mutagenetic approaches. Host and bacterial factors may allow *H. pylori* to persist for decades and invoke an intense inflammatory response, leading eventually to damage of host cells.

About three decades of intense research into *H. pylori* virulence factors, in particular CagA and VacA, have revealed many aspects of the relationships between this bacterium, the gastric mucosal surface, and the pathogenesis of disease (Miehike et al. 2001; Cover and Blanke 2005; Kusters et al. 2006; Hatakeyama 2008; Sewald et al. 2008a, Wen and Moss 2009). CagA, encoded by the *cagA* gene, is a marker for the presence of the 40-kb-long genomic Pathogenicity Island, PAI (Covacci et al. 1993) and contributes to the inflammatory response by initiating a signal transduction cascade, resulting in interleukin-8 production. VacA not only causes vacuolization, which is the most

prominent feature and the basis for its name (Fig. 2), but also exerts pleiotropic actions on gastric epithelial cells and other cell types (Kusters et al. 2006). VacA is an important virulence factor in the pathogenesis of peptic ulceration and gastric cancer. VacA can induce multiple cellular activities, including cell vacuolation, membrane channel formation, disruption of endosomal/lysosomal function, apoptosis, and immunomodulation. VacA protein influences cellular processes via different routes, thus assisting in chronic colonization of the gastric mucosa by *H. pylori* (Fig. 3). (1) VacA secreted by type V-secretion system of *H. pylori* may either (2) bind to a cell membrane receptor and initiate a proinflammatory response, (3) be taken up directly by the cell, traffic to the mitochondria, and induce apoptosis, (4) be taken up by endocytosis and induce vacuolization, (5) form a membrane channel, resulting in leakage of nutrients to the extracellular space, or (6) pass through the tight junctions and inhibit T-cell activation and proliferation. Although VacA is not essential for *in vitro* growth of *H. pylori*, it was reported to significantly contribute to murine gastric colonization by *H. pylori* (Salama et al. 2001).

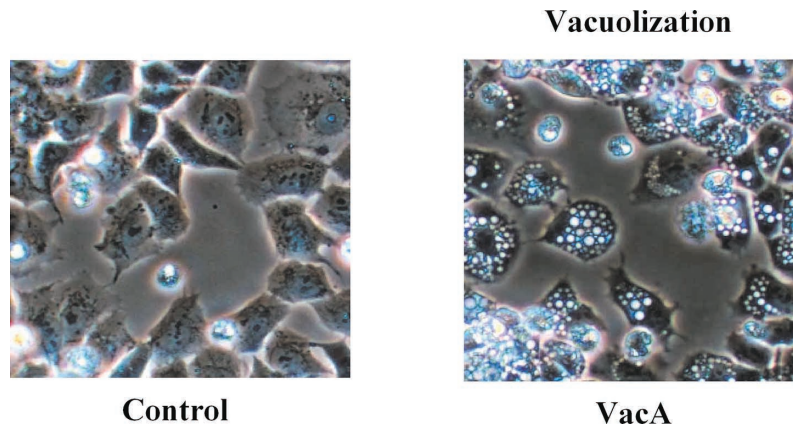


Fig. 2. VacA induces massive vacuolization in gastric epithelial cells (AZ-521, a human gastric cancer cell line).

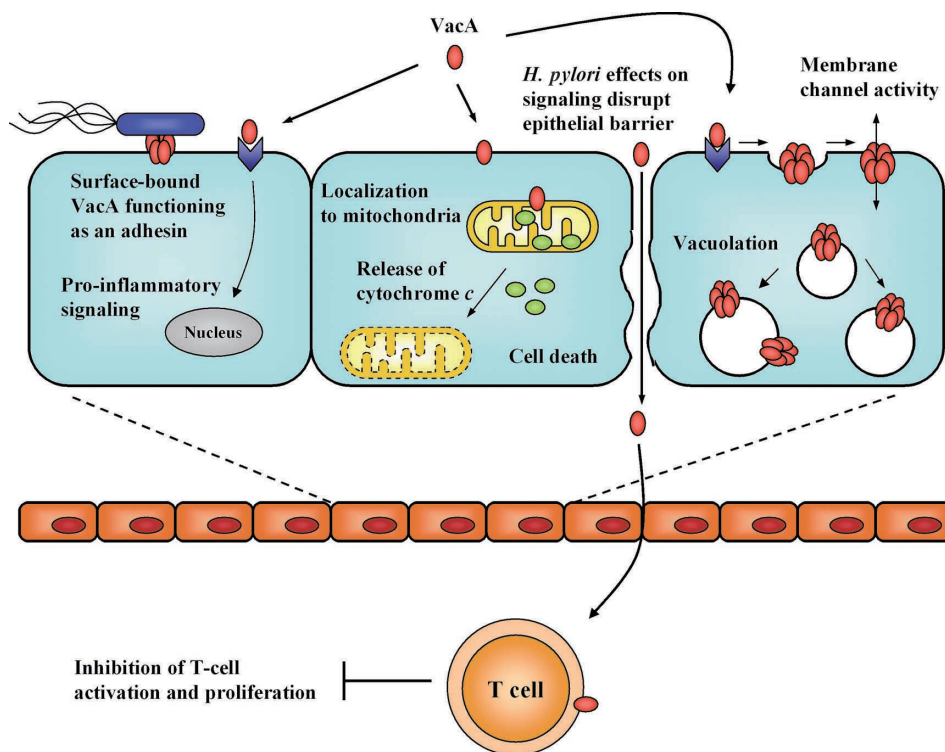


Fig. 3. VacA has pleiotropic effects on human cells including vacuolization, membrane channel formation, disruption of endosomal/lysosomal function, apoptosis, and immunomodulation. Secreted VacA may bind to a cell membrane receptor and initiate a proinflammatory response, traffic to the mitochondria and induce apoptosis, be taken up by endocytosis and induce vacuolization, form a membrane channel, disturb epithelial barrier, or pass through tight junctions and inhibit T-cell activation and proliferation.

### Structure and function of VacA

Unlike the *cagPAI*, the *vacA* gene is present in all strains. The *vacA* gene encodes 140-kDa protoxin (Papini et al. 2001; Cover and Blanke 2005; Sewald et al. 2008a). Proteolytic processing during secretion via the type V-secretion system produces a mature 90 kDa toxin that forms flower-shaped dodecameric aggregates, which are disassembled by acid-activation to increase toxicity (Cover et al. 1997). Secreted VacA can be further processed into an N-terminal fragment of 33 kDa (p33) and a C-terminal frag-

ment of 55 kDa (p55), but cleavage does not seem to be necessary for VacA activity (Papini et al. 2001; Cover and Blanke 2005; Sewald et al. 2008a). The p33 domain exhibits pore-forming activity necessary for vacuole formation (McClain et al. 2003), whereas the p55 domain is responsible for target cell binding (Reyrat et al. 1999). Recently, Ivie et al. (2008) showed that the N-terminal of p55 is important for the formation of VacA oligomeric structures and is essential for VacA-induced vacuolation, for host cell membrane depolarization, and for formation of anionic membrane channels. In addition, Gangwer et al. (2007)

solved the crystal structure of p55, which consists of a parallel  $\beta$ -helix with a carboxy-terminal globular domain, indicating how p55 monomers may assemble into oligomers capable of membrane pore formation.

VacA forms anion-selective channels in endosomal membranes through its interaction with lipids (Tombola et al. 1999; Czajkowsky et al. 1999). The assembly of six molecules of VacA forms an anion-selective channel in lipid bilayers, and its channel activity is inhibited by chloride channel blockers. A nonspecific chloride channel blocker, NPPB, can inhibit the channel activity of VacA, resulting in a reduction of vacuolation.

*H. pylori* strains vary considerably in production of cytotoxin activity and this is primarily due to variations in *vacA* gene structure. The variable regions are located near the 5'-end, s1 or s2, and in the mid-region, m1 or m2. 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 (Atherton et al. 1995; van Doorn et al. 1999; Cover and Blanke 2005). 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 (Atherton et al. 1995). It has also been reported that s1 type *vacA* gene products are secreted at higher levels than s2 type *vacA* products (Sewald et al. 2008a). 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 (Shirasaka et al. 2002). Again, *H. pylori* strains that possess a type s1/m1 *vacA* allele are associated with an increased risk of gastric cancer and enhanced gastric epithelial cell injury (Atherton et al. 1997; Peek and Crabtree 2006) compared with *vacA* s2/m2 strains. The relationship between s1/m1 alleles and gastric cancer is consistent with the distribution of *vacA* genotypes throughout the world. In regions where the background prevalence rate of distal gastric cancer is high, such as Colombia and Japan, most *H. pylori* strains contain type s1/m1 alleles (van Doorn et al. 1999), whereas the converse is true in regions of the world with low rates of non-cardia gastric adenocarcinoma (Peek and Crabtree 2006). VacA exerts effects on epithelial cells that may lower the threshold for carcinogenesis. The *vacA* intermediate (i)-region, which encodes part of the p33 domain of VacA, displays sequence variation at the nucleotide level (Rhead et al. 2007). i1-type strains, but not i2-type strain, were associated with gastric carcinoma in an Iranian population (Rhead et al. 2007; Hussein et al. 2008), and were an independent predictor of peptic ulcer disease in an Italian population (Basso et al. 2008).

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 (Atherton et al.

1995). One possible cause of these differences in cytotoxicity is the different cell type specificities of the mature toxins. The 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 (Pagliaccia et al. 1998). 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 with VacA that are important in determining cytotoxicity.

### VacA receptor binding

As is known for many toxins, binding of VacA to a receptor on the target cell surface is a crucial step for toxicity. Different potential VacA receptors have been described for different types of cells (Sewald et al. 2008a). Among these, receptor-like protein tyrosine phosphatases RPTP $\alpha$  and RPTP $\beta$  have been described as receptors for epithelial cells (Yahiro et al. 1999, 2003). On the other hand, lymphocyte function-associated antigen-1 (LFA-1) was identified as a specific VacA receptor on T-cells (Sewald et al. 2008b) but not epithelial cells. Thus, VacA uses different receptors for intoxication or modulation of epithelial or immune cells.

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. Following isolation and sequencing, the p250 protein was identified as RPTP $\beta$  (Yahiro et al. 1999). 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 RPTP $\beta$  (Padilla et al. 2000). G401 cells, a human kidney tumor cell line, lack RPTP $\beta$  but respond to VacA, and p140 has been identified as another receptor protein for VacA. p140 was shown to be RPTP $\alpha$  (Yahiro et al. 2003), which was responsible for VacA-induced vacuolation in AGS cells (Tegtmeyer et al. 2009). Glycosylation at specific residues was essential for VacA binding to RPTP $\beta$  (Yahiro et al. 2004), supporting the idea that an interaction of VacA with defined sugar residues is necessary for its function (Roche et al. 2007). Cell type-specific binding has been attributed to differences in the m1 and m2 alleles, with m1 VacA binding and inducing vacuoles in HeLa cells, whereas m2 VacA vacuolates rabbit kidney (RK13) and primary epithelial cells, but not HeLa cells (Pagliaccia et al. 1998; Ji et al. 2000). This cell type-specific binding region of m1 VacA was mapped to amino acids 460–496. m2 VacA has an insertion of 23 residues at amino acid 475, consisting of an imperfect repeat of the upstream sequence. It is unclear whether this region is directly involved in receptor binding because RPTP $\alpha$  and RPTP $\beta$  are both recognized by m2 VacA (de Guzman et al. 2005).



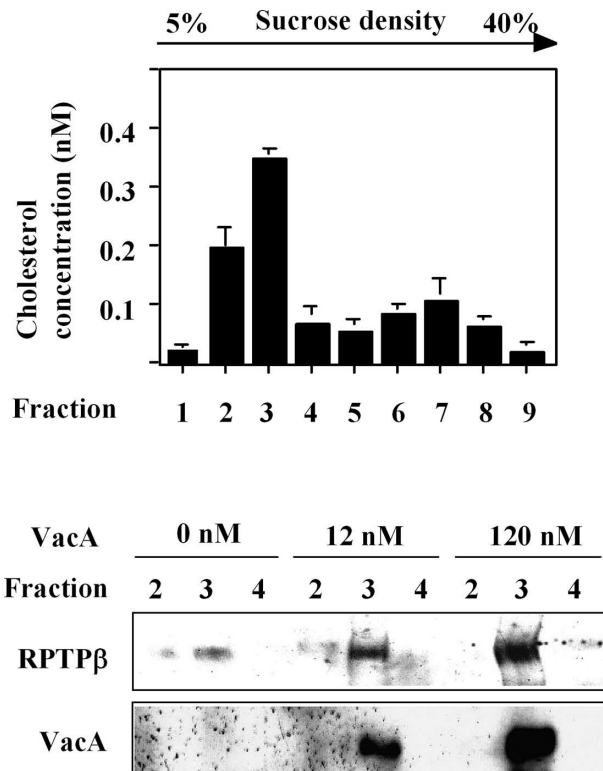


Fig. 4. Translocation of VacA bound to receptor-like protein tyrosine phosphatase (RPTP) $\beta$  to lipid rafts. The binding of VacA to RPTP $\beta$  in non-raft domains of the plasma membrane leads to relocation and concentration of the toxin-receptor complex in lipid rafts. Lipid rafts were isolated by ultracentrifugation in a sucrose density gradient and were enriched in cholesterol (Fraction 3). VacA incubated with cells appeared in the lipid raft fraction in a dose-dependent fashion. In parallel with VacA translocation to lipid rafts, RPTP $\beta$  also accumulated in lipid rafts from non-lipid raft microdomain (Nakayama et al. 2006).

Recently, Gangwer et al. (2007) identified two conserved regions located at the N-terminal part of p55 as well as at the C-terminal region. The C-terminal region includes a disulfide bridge pocket formed by two conserved cysteine residues. This pocket is located at the outward surface of VacA oligomer and serves as a receptor-binding site.

Following binding of monomeric VacA to a specific receptor, oligomerization leads to clustering of VacA in detergent-resistant membrane domains and subsequent endocytosis. We have demonstrated that binding of VacA to RPTP $\beta$  in non-raft domains of the plasma membrane leads to relocation and concentration of the toxin-receptor complex into lipid rafts (Fig. 4) (Nakayama et al. 2006). The oligomerization of VacA is essential for endocytosis (Reyrat et al. 1999). Internalization of VacA then takes place at lipid raft structures and is described as a clathrin-independent pathway of endocytosis that requires cellular components such as the small GTPase Cdc42 and the actin cytoskeleton (Ricci et al. 2000). Treatment of VacA-sensitive cells with PI-PLC (phosphatidylinositol-specific phospholipase C),

which specifically removes proteins anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor, reduces and delays VacA-induced cell vacuolization (Ricci et al. 2000), suggesting involvement of GPI-anchored proteins in VacA-induced vacuolization.

RPTP $\beta$ , 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 (Deuel et al. 2002). RPTP $\beta$  was originally found to be highly expressed in brain. Virtually all glands in the gastric corpus of wild-type mouse contain detectable RPTP $\beta$ , and expression is localized to the glandular basal region (Fujikawa et al. 2003). In mice, orally administered VacA causes degeneration of the gastric mucosa and acute inflammation, followed by gastric ulcer disease (Telford et al. 1994). Using this mouse model system, RPTP $\beta$  KO mice were subjected to VacA administration. Indeed, oral administration of VacA to wild-type mice, but not to RPTP $\beta$  KO mice, resulted in gastric ulcers, suggesting that RPTP $\beta$  is essential for the intoxication of gastric tissue by VacA (Fujikawa et al. 2003).

#### Cellular vacuolization by VacA

Cellular vacuolization is a unique function of VacA (Fig. 2). The membranes of these vacuoles contain the small GTP-binding protein Rab7, late endosome and lysosomal markers, and the membrane protein Lgp110 (Molinari et al. 1997). Accordingly, VacA might disrupt normal membrane trafficking at or near the level of late endosomes (Montecucco and de Bernard 2003). Vacuolation depends not only on VacA, but also on the presence of permeant weak bases in the extracellular medium (Cover and Blaser 1992). Microinjection of VacA or the transfection of plasmids containing the *vacA* gene into HeLa cells resulted in the formation of intracellular vacuoles (de Bernard et al. 1997), providing evidence that VacA introduced into the cytosol acts on an intracellular target; potential targets include the vacuolar ATPase (V-ATPase), Rab7, and Rac1 (Papini et al. 1997; Hotchin et al. 2000). It has been well established that VacA-induced vacuolation requires V-ATPase activity, and that its inhibitor, bafilomycin A1, reduced VacA-induced vacuolation in mammalian cells (Suzuki et al. 2001; Genisset et al. 2007). Rab7 may be important for supporting membrane deposition and homotypic fusion between late endosomes. Dynamin, a high molecular weight GTP-binding protein that functions as a mechanochemical enzyme in vesicle formation, is involved in VacA-induced vacuolation (Cover et al. 1993). In addition, transient transfection of the dominant-negative mutant syntaxin 7 was also found to inhibit VacA-induced vacuolation (Suzuki et al. 2003). 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 (soluble N-ethylmaleimide-sensitive-factor attach-

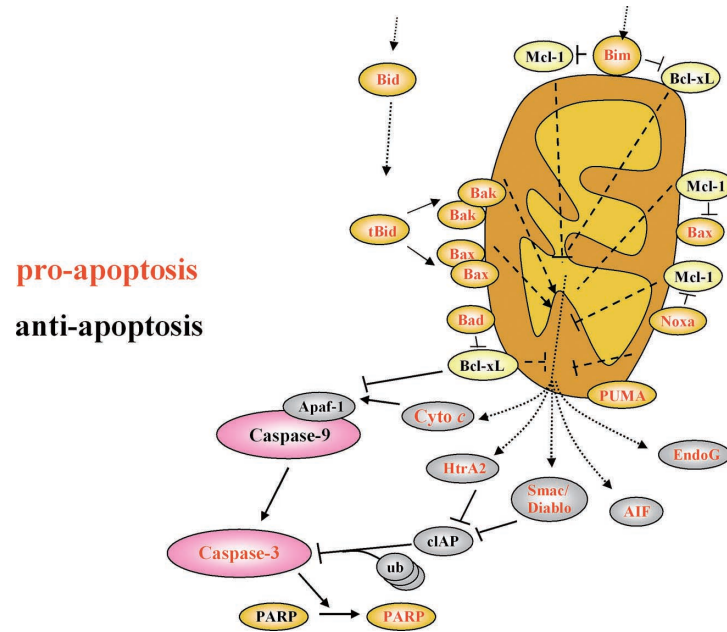


Fig. 5. Regulation of mitochondria-associated apoptosis by Bcl-2 family members. The Bcl-2 proteins are classified into subgroups, according to their function (anti- or pro-apoptotic). Pro-apoptotic Bcl-2 members, Bax and Bak are pivotal regulators of cytochrome c release, leading to activation of caspase 3, cleavage of PARP (poly(ADP-ribose)polymerase) and apoptosis execution. The major members of anti-apoptotic Bcl-2 proteins are Bcl-2, Bcl-X<sub>L</sub>, and myeloid cell leukemia 1 (Mcl-1).

ment protein receptor) complex in association with other SNARE proteins (Südhof and Rothman 2009). In addition, expression of syntaxin 7 mRNA and protein in AGS cells is enhanced by exposure to VacA, indicating the involvement of syntaxin 7 in VacA-induced vacuolation (Suzuki et al. 2003). At the final steps of vacuole formation by VacA, vesicle associated membrane protein 7 (VAMP7) is a partner of syntaxin 7 in the process of lysosome–endosome fusion (Mashima et al. 2008). These results suggest that VacA-induced vacuolization is a result of a toxin-induced alteration of intracellular vesicle trafficking. Interestingly, it was demonstrated that CD2-associated protein (CD2AP), an adaptor protein implicated in intracellular trafficking, which regulates filamentous actin (F-actin) structures, was required to transfer VacA from early to late endosomes (Gauthier et al. 2007) after VacA was internalized by a pinocytic mechanism that involves F-actin and Cdc42 but was independent of clathrin, dynamin, and ARF6 GTPase (Gauthier et al. 2005). They also suggested that sorting of VacA in these compartments requires dynamic F-actin structures on early endosomes, which is characterized as being enriched with GPI-anchored proteins (Ricci et al. 2000; Gauthier et al. 2005).

#### VacA-induced apoptotic cell death

Several lines of evidence have documented that VacA induces apoptotic cell death via a mitochondria-dependent pathway in gastric epithelial cells (Kimura et al. 1999; Galmiche et al. 2000; Yamasaki et al. 2006). It is known that VacA reduces the membrane potential of mitochondria,

resulting in cytochrome c release. 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 (Galmiche et al. 2000). VacA accumulated into the mitochondria inner membrane, resulting in apoptotic cell death (Galmiche et al. 2000). On the other hand, in our experiments using immunostaining and confocal microscopy, most VacA was localized to vacuoles, marked by Rab7-GFP, rather than mitochondria (Yamasaki et al. 2006). These results suggest that VacA may not directly induce cytochrome c release from mitochondria, and instead, suggest that apoptosis-related factors such as proapoptotic Bcl-2 family proteins may be involved (Fig. 5). Bax and Bak are pivotal regulators of cytochrome c release (Mérino and Bouillet 2009). We performed immunostaining and confocal microscopy with conformation-specific antibodies against Bax and Bak, which identify their active forms. After stimulation with VacA, active forms of pro-apoptotic Bax as well as Bak appeared (Yamasaki et al. 2006) and accumulated on mitochondria. The time course of Bax activation in response to VacA paralleled that of cytochrome c release. In line with this, activation of caspase 3 and cleavage of PARP (poly(ADP-ribose)polymerase) were confirmed. Thus, our data have shown that in response to VacA, Bax and Bak activation cause cytochrome c release from mitochondria and apoptotic cellular death. Again, the pro-apoptotic Bcl-2 protein-mediated apoptosis execution was vacuolation-independent (Yamasaki et al. 2006).

More recently, we documented the relationship among

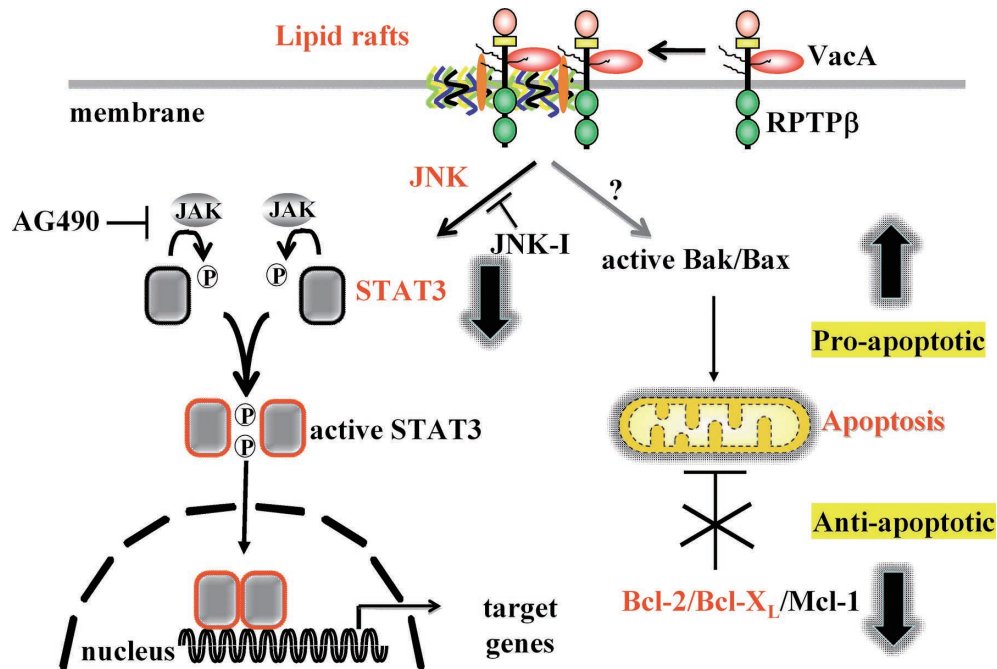


Fig. 6. Hypothetical mechanisms of VacA-induced apoptosis. By binding to receptor, VacA induces STAT (Signal Transduction and Activator of Transcription) 3 down-regulation, possibly via JNK activation. Reduction in STAT3 leads to pro-apoptotic Bcl-2 family proteins, in particular Bcl-2 and Bcl-X<sub>L</sub>. Bcl-2 and Bcl-X<sub>L</sub> down-regulation resulted in the acceleration of apoptosis. Alternatively, VacA intoxication induces Bax/Bak activation. These pro-apoptotic Bcl-2 family proteins accumulate in mitochondria and cause cytochrome c release.

VacA, apoptosis and anti-apoptotic Bcl-2 proteins (in submission). The major members of anti-apoptotic Bcl-2 proteins are Bcl-2, Bcl-X<sub>L</sub> and myeloid cell leukemia 1 (Mcl-1) (Mérino and Bouillet 2009). JAK (Janus kinase)-STAT3 (Signal Transduction and Activator of Transcription) signaling pathway regulates each family member of the Bcl-2 sub-family proteins. STAT3 is phosphorylated by JAKs, then dimerizes and translocates to the nucleus. Active STAT3 dimers bind to the responsible element of each gene promoter, and up-regulate target gene expression (Fig. 6) (Isomoto 2009). By immunoblot analysis, cellular STAT3 expression of the AZ521 cells was substantially reduced by VacA in a dose-dependent manner. In parallel with this, Bcl-2 and Bcl-xL expression was substantially reduced. Down-regulation of STAT3 and Bcl-2 family proteins was independent of VacA-induced vacuolation. Instead, treatment with a JNK inhibitor restored VacA-induced STAT3 inhibition to the basal level, suggesting that STAT3 inhibition in response to VacA may be dependent on JNK activity. The proposed mechanisms of VacA-induced apoptosis in gastric epithelial cells are outlined in Fig. 6.

*H. pylori* induces apoptosis of infected macrophages (Menaker et al. 2004). RAW 264.7 cells infected with a virulent *H. pylori* strain 60190, which possesses the *cagPAI* and *vacA*, displayed morphological features characteristic of an apoptotic cell. Immunoblot analysis showed that bacterial infection induced a time-dependent increase of pro-caspase 8 and disappearance of full-length Bid in macrophages compared with the uninfected cells. Isogenic

mutants of 60190 deficient in either *cagA* or *vacA* induced significantly less apoptosis than the parent strain. These results suggest that *H. pylori* can also induce apoptosis of macrophages in association with alterations in the mitochondrial pathway. Elimination of this key immunomodulatory cell may represent a mechanism employed by the bacterium to evade host immune responses.

#### Other disruptions of cellular signal transduction by VacA

Nakayama et al. (2004) showed VacA-induced activation of the p38 MAPK (mitogen-activated protein kinase)/ATF-2 (activating transcription factor 2)-mediated signal transduction pathway, independent of cellular vacuolization, in gastric epithelium-derived cell line, AZ-521. Interestingly, in AZ-521 cells, VacA enhanced PGE<sub>2</sub> production through induction of cyclooxygenase-2 expression via the p38 MAPK/ATF-2 cascade (Hisatsune et al. 2007). In contrast, Tuo et al. (2009) demonstrated that VacA inhibited PGE<sub>2</sub>-stimulated duodenal mucosal bicarbonate secretion by stimulating the release of mucosal histamine, suggesting that the inhibitory effect of VacA on bicarbonate secretion may impair duodenal mucosal defense against acid injury, eventually leading to ulcer development. On the other hand, VacA stimulated calpain-mediated proteolysis of ezrin in parietal cells, thereby leading to disruption of apical membrane-cytoskeletal interactions and inhibition of gastric acid secretion, modeling the hypochlorhydric state of patients infected with *H. pylori* (Wang et al. 2008).

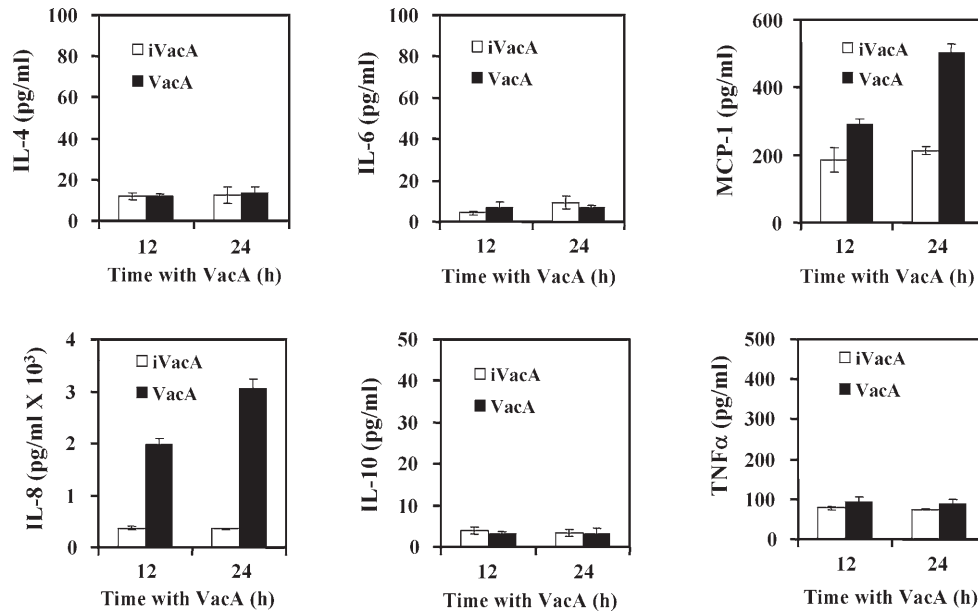


Fig. 7. VacA enhances production and secretion of IL-8. Stimulation of U937 cells, a human monocyte-derived cell line, with VacA (120 nM), increased IL-8 release into cell supernatants. IL-8, followed by monocyte chemoattractant protein 1 (MCP-1) were the most abundantly released cytokines from U937 cells stimulated with active VacA, but not inactivated VacA (Hisatsune et al. 2008).

With regard to a potential role for VacA in inflammation, VacA was shown to induce bone marrow-derived mast cells to produce proinflammatory cytokines, tumor necrosis factor  $\alpha$ , macrophage inflammatory protein 1 $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, IL-10, and IL-13 (Supajatura et al. 2002). Recently, Hisatsune et al. (2008) have shown that VacA enhanced production and secretion of IL-8, an important mediator in the immunopathogenesis of chronic gastritis. Upon stimulation with VacA, increased IL-8 release into the supernatant was observed in some cultured cell types, such as A549 cells, a lung cancer cell line, and monocyte-derived U937 cells. Among various cytokines, IL-8, followed by monocyte chemoattractant protein 1 were found most abundantly released from U937 cells when stimulated with active VacA, but not inactivated VacA (Fig. 7). In U937 cells, VacA directly increased IL-8 production by activation of the p38 MAPK via intracellular Ca<sup>2+</sup> release, leading to activation of the transcription factors, ATF-2 and CREB (cyclic AMP response element-binding protein). In addition, the enhanced production of IL-8 by VacA may be attributable in part to NF (nuclear factor)- $\kappa$ B signaling.

VacA can block activation of NFAT, a key transcription factor required for T cell activation (Boncristiano et al. 2003; Gebert et al. 2003). Moreover, Sundrud et al. (2004) reported that VacA inhibited the proliferation of primary human CD4<sup>+</sup> T cells and demonstrated that this inhibitory effect on proliferation is not attributable to VacA effects on NFAT activation of IL-2 expression. In addition, VacA suppressed IL-2-induced cell cycle progression without affecting IL-2-dependent survival (Sundrud et al. 2004). Furthermore, Torres et al. (2007) reported that VacA inhibited the proliferation of CD8<sup>+</sup> T cells and B cells. VacA

inhibited both T-cell-induced and PMA/anti-IgM-induced proliferation of purified B cells. They proposed that the immunomodulatory actions of VacA on T and B lymphocytes, the major effectors of the adaptive immune response, may contribute to the ability of *H. pylori* to establish a persistent infection in the human gastric mucosa. Interestingly, recent evidence indicated that VacA efficiently enters activated, migrating primary human T lymphocytes by binding to the  $\beta$ 2-integrin receptor subunit (CD18) and exploiting the recycling of LFA-1 (Sewald et al. 2008b). LFA-1-deficient Jurkat T cells were resistant to vacuolation and IL-2 modulation, and genetic complementation restored sensitivity to VacA. VacA targeted human, but not murine, CD18 for cell entry, consistent with the species-specific adaptation of *H. pylori*. Before interaction with CD18, VacA needed to bind to a thus far unknown, probably GPI-anchored, membrane-associated protein (factor X) to be recruited together with factor X to the uropod of the trailing edge.

It has become evident that glycogen synthase kinase-3 $\beta$ , GSK3 $\beta$ , is a key enzyme in Wnt/ $\beta$ -catenin signaling and a crucial regulatory component of many cellular pathways, including apoptosis, cell cycle, cell polarity, migration, and gene expression. This multitasking by GSK3 $\beta$  is achieved by its participation in signaling pathways involving the phosphoinositide 3 kinase (PI3K)/ protein kinase B (Akt) cascade. Several studies showed that *H. pylori* activated the PI3K-Akt signaling pathway in epithelial cells (Sokolova et al. 2008; Nakayama et al. 2009; Nagy et al. 2009; Tabassam et al. 2009). Sokolova et al. (2008) demonstrated that *H. pylori* induced inhibition of  $\beta$ -catenin phosphorylation and ubiquitin-dependent degradation, leading to upregulation of



T cell factor/lymphoid enhancer-binding factor (Tcf/Lef)-dependent transcription of cyclin D1 by suppression of GSK3 $\beta$  activity. In addition, GSK3 $\beta$  inhibition through PI3K-Akt activation by *H. pylori* resulted in NF- $\kappa$ B activation and IL-8 production (Tabassam et al. 2009). It was also found that incubation of AZ-521 cells with VacA resulted in phosphorylation of Akt and GSK3 $\beta$  through a PI3K-dependent pathway (Nakayama et al. 2009). Following phosphorylation and inhibition of GSK3 $\beta$ ,  $\beta$ -catenin was released from a GSK3 $\beta$ / $\beta$ -catenin complex, with subsequent nuclear translocation. This VacA-mediated activation of the PI3K/Akt signaling pathway did not require VacA internalization and was independent of vacuolation. In addition, VacA transactivated the  $\beta$ -catenin-dependent cyclin D1 promoter. These data support the hypothesis that Wnt-dependent signaling might play a role in the pathogenesis of VacA-dependent gastric damage, including the development of gastric cancer.

#### Cellular pathway affected by VacA in the presence of CagA

Recent study has shown that CagA activated a transcription factor, NFAT (nuclear factor of activated T-cells) in T lymphocytes, while VacA inhibited NFAT (Yokoyama et al. 2005): CagA and VacA are likely to exert opposite cellular effects in some aspects, and hence are related to a variety of *H. pylori*-dependent diseases. VacA inhibited activation of epidermal growth factor receptor (EGFR) and HER2/Neu, and subsequently Erk1/2, which are important for cell scattering and elongation (Tegtmeyer et al. 2009). Using human gastric epithelial cells in culture, transfected with cDNA encoding for either the wild-type 38 kDa C-terminal signaling domain of CagA or its non-tyrosine-phosphorylatable mutant form, Oldani et al. (2009) found that, depending on tyrosine-phosphorylation by host kinases, CagA inhibited VacA-induced apoptosis by two complementary mechanisms. Tyrosine-phosphorylated CagA prevented pinocytosed VacA from reaching its intracellular compartments. Unphosphorylated CagA triggered an anti-apoptotic activity blocking VacA-induced apoptosis at the mitochondrial level without affecting the intracellular trafficking of VacA. Assays of the level of apoptosis of gastric epithelial cells infected with wild-type CagA+/VacA+ *H. pylori* or isogenic mutants lacking of either CagA or VacA, showed that CagA antagonized VacA-induced apoptosis. These results, showing that VacA can inhibit some CagA-induced responses on epithelial cells, are consistent with previous findings suggesting that VacA and CagA have opposing effects (Argent et al. 2008).

#### Vaccine

Despite efforts over 25 years, an effective vaccine against *H. pylori* has not been developed. The intramuscular vaccine formulation consisting of VacA, CagA and HP-NAP plus aluminium hydroxide adjuvant represents a very promising candidate for the prevention of *H. pylori*

infection (Malfertheiner et al. 2008). A successful vaccine against *H. pylori* will be useful for preventing infection in 50% of the world's population and have a significant impact on global health.

#### Conclusions

*H. pylori* produces a potent exotoxin, VacA, which causes progressive vacuolization and gastric injury. VacA induces multiple effects on gastric epithelial and other cells: vacuolization with alterations of endo-lysosomal function, anion-selective channel formation, mitochondrial-dependent apoptosis, disruption of signal transduction pathways and inhibition of T-cell proliferation and activation. VacA binds to RPTPs, which is an essential step for intoxication. VacA causes gastric mucosal injury, gastric ulcer, and gastric cancer. As described above, however, many bacterial factors, besides VacA, including outer membrane proteins, NAP, and CagA in *cagPAI* contribute to the pathogenicity of *H. pylori*. The multitude of these proteins and allelic variation makes it extremely difficult to test the contribution of each individual factor. Much effort has been put into identifying the mechanisms associated with *H. pylori*-associated gastroduodenal diseases including peptic ulcer and gastric adenocarcinoma.

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