# Chapter 4
# *Helicobacter pylori* VacA Exhibits Pleiotropic Actions in Host Cells

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**Abstract** *Helicobacter pylori* vacuolating cytotoxin (VacA) is a major virulence factor, with pleiotropic actions on target cells including induction of vacuole formation, mitochondrial dysfunction leading to apoptosis, modulation of signal transduction pathways associated with autophagy, inhibition of T cell proliferation, and production of inflammatory cytokines. Numerous epidemiological studies have indicated that the allelic diversity within four variable regions of the *vacA* gene might be associated with cell type-specific binding as well as specific clinical outcomes in *H. pylori* infection. VacA binds to receptors such as receptor protein tyrosine phosphatases (RPTPα and RPTPβ), low-density lipoprotein receptor-related protein-1 (LRP1), fibronectin, CD18, and sphingomyelin to facilitate its action, suggesting the involvement of these receptors in the pathogenesis of *H. pylori* infection. RPTPβ contributes to ulceration in gastric epithelial cells and LRP1 is involved in the induction of autophagy. Interestingly, it has been suggested that CagA is degraded by VacA-induced autophagy and that the interaction between these two molecules is associated with the pathogenesis of gastric diseases. Therefore, better understanding of the mechanism of VacA toxicity may provide valuable information regarding appropriate medical care for gastroduodenal diseases caused by *H. pylori* infection.

**Keywords** *Helicobacter pylori* • VacA • Vacuolation • Autophagy

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## 4.1 Introduction

*Helicobacter pylori* is a Gram-negative bacterium that causes gastroduodenal diseases including chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric lymphoma. A number of virulence factors are involved in the pathogenesis of *H. pylori* infection. During colonization of the gastric epithelium, *H. pylori* secretes a potent protein toxin, vacuolating cytotoxin, termed VacA, which is considered to be an important virulence factor associated with peptic ulcer disease, indicating that VacA is critical to the pathogenicity of *H. pylori* [1–4]. Although vacuolation, a hallmark of VacA function (Fig. 4.1), is readily observed in VacA-intoxicated cells in vitro, its function in vivo in *H. pylori* infection is unclear.

In 1988, Leunk et al. reported that the broth culture filtrates of *H. pylori* caused the generation of large vacuoles in the cytoplasm of cultured mammalian cells [5]. After Cover et al. purified an 87-kDa VacA from culture supernatant [6], the entire nucleotide sequence of the *vacA* gene and its deduced amino acid sequence were determined, revealing the presence of a protoxin of about 140 kDa that lacked homology to any other protein [7]. Vacuolating activity was enhanced by acidic (<pH 5.5) or alkaline (>pH 9.5) conditions due to conformational changes in the protein [8, 9]. Further, vacuolating activity was also potentiated by weak bases, including ammonium chloride and nicotine [10]. After binding to the cell surface receptors, VacA internalizes into epithelial cells by endocytosis and then induces the formation of vacuoles, which are derived from late endosomes. Maturation of vacuoles involves several host molecules including a small GTPase, Rab7, and vacuolar-type ATPase (V-ATPase) proton pump. Rab7 is essential for the efficient

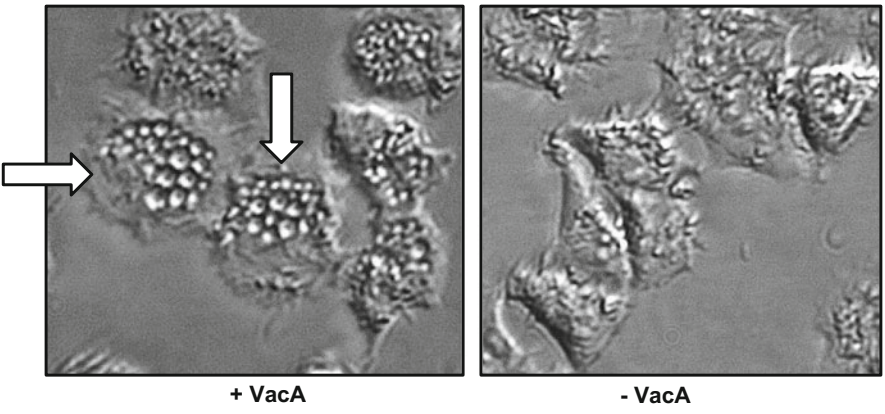


**Fig. 4.1** Vacuolation in the cytoplasm of mammalian cells. Purified VacA (*left*) or vehicle (*right*) was added, and cultured mammalian cells were incubated at 37 °C, 5 % $CO_2$ for several hours. Many vacuoles (*arrows*) were observed in the cytoplasm of cells incubated in the presence of VacA, while vehicle did not cause vacuolation. Thus, VacA is the responsible protein for vacuole formation

membrane flow from early to late endosome, and V-ATPase is involved in osmotic swelling of the endosomal compartment, resulting in generation of VacA-dependent vacuolation [11–13].

VacA also causes mitochondrial dysfunction, leading eventually to apoptotic cell death through cytochrome *c* release and caspase activation [14–16]. Furthermore, VacA also modulates membrane potential and signal transduction pathways associated with immune responses, induction of autophagy, and inhibition of T cell proliferation [17–21]. There is some evidence of an antagonistic relationship between VacA and cytotoxin-associated gene A (CagA), another important *H. pylori* virulence factor [22–24], suggesting that both toxins contribute to the development of gastric disorders in *H. pylori* infection. In this review, we will discuss the multiple functions of VacA on target host cells and also discuss the latest research on VacA function.

## 4.2 Protein Structure and Domains of VacA

All isolated strains of *H. pylori* contain the *vacA* gene encoding a 140-kDa precursor protein, which has three major domains, i.e., signal sequence, mature toxin, and autotransporter domains. Proteolytic cleavage of the signal sequence and autotransporter domain from a VacA protoxin results in a mature VacA toxin of about 90-kDa molecular mass, which is secreted into the extracellular space by a type V secretion system (Fig. 4.2a) [1, 6, 7, 25]. Secreted mature VacA forms an oligomeric structure, and the assembled VacA has an expected molecular mass of around 1000 kDa under neutral pH or non-denaturing conditions [6]. Upon imaging analysis by electron microscopy, VacA oligomer is composed of six or seven VacA monomers that form a flower-shaped structure with a central ring. VacA oligomerization leads to the assembly of a double-layered form of a single VacA oligomer (consisting of 12 or 14 VacA monomers), rather than the single-layered oligomer [26–29]. VacA oligomers observed in the lipid bilayer of cell or mitochondrial membrane seem to contribute to anion-selective membrane channel formation [26, 30]. The mature VacA is further cleaved into two fragments by proteolytic digestion and consists of 33-kDa N-terminal (termed p33) and 55-kDa C-terminal fragments (termed p55) [1, 27, 31]. It has been proposed that in the three-dimensional structures of VacA oligomers, p55 represents the peripheral arms and p33, the central core of the complexes [29].

Although many studies about the cellular functions of both p33 and p55 have been performed, their functions are still debated. Several lines of evidence have indicated that p55 is involved in binding to host cells, whereas p33 plays a role in the formation of anion channels in the lipid membrane and is targeted to the inner mitochondrial membrane, leading to the loss of membrane potential [14]. In addition, it has been reported that p33 is crucial in pore formation in lipid bilayers [32]. The N-terminal of p33 is predicted to contain a hydrophobic region, which is

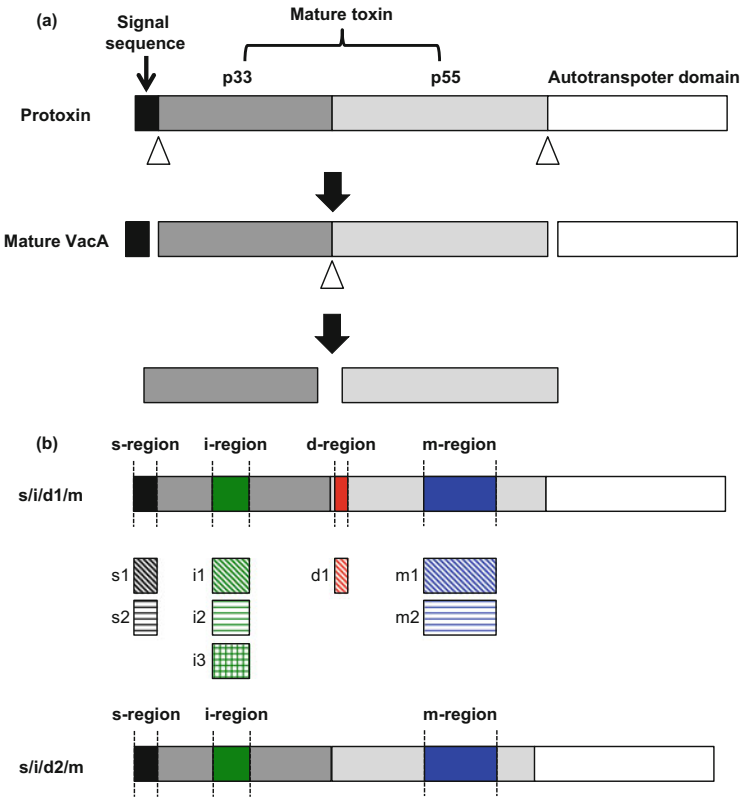


**Fig. 4.2** *vacA* gene allelic and protein structures. (**a**) Precursor VacA is synthesized in *H. pylori* with three functional domains (signal sequence, mature toxin, and autotransporter domain). Mature VacA protein, which lacks two fragments (signal sequence and autotransporter domain), is generated by proteolytic digestion and is then secreted by a type V secretion system. The p33 and p55 fragments might be generated from the mature form of VacA. *Arrowheads* indicate cleavage sites. (**b**) The *vacA* gene as four variable regions: s-region (*black*, s1 and s2), i-region (*green*, i1, i2, and i3), d-region (*red*, d1), and m-region (*blue*, m1 and m2). The *vacA* gene allele d2 is missing the d1 region (*lower figure*)

required for not only channel formation but also vacuolating activity and trafficking to the inner mitochondrial membrane [33, 34]. Furthermore, the proline residue at position 9 and glycine residue at position 14 in p33 play key roles in these activities [35]. In addition, GXXXG motif at residues 14–18 in p33 is also important for channel formation [34]. However, mutant VacA lacking a part of the hydrophobic region retained channel activity in planar lipid bilayers [32]. Other studies have shown that p33 and p55 are involved in cell binding and channel formation [36, 37]. Thus, further studies to define the molecular basis underlying p33 and p55 activities are required.

## 4.3 Gene Structure of *vacA*

### *4.3.1 Polymorphism of the* vacA *Gene*

Although all *H. pylori* strains isolated from patients possess a *vacA* gene, only approximately 50 % of the isolates show vacuolating activity because of allelic diversity within the *vacA* gene [2, 6]. Nucleotide sequence analyses of the *vacA* gene have revealed that polymorphisms exist in four variable regions (Fig. 4.2b) [38]. The most characterized allelic diversities are observed within the signal sequence region (s-region) and the mid-region (m-region). The s-region is located at the 5′ end of the *vacA* gene and includes the signal sequence of VacA (designated s1 and s2). Interestingly, epidemiological studies have shown that strains possessing the s1 type of *vacA* are linked to the presence of the *cagA* gene [39, 40]. The s1-type allele is further classified into three subtypes s1a, s1b, and s1c, respectively [41]. Strains possessing the s2 type of *vacA* allele fail to induce vacuolation in cultured mammalian cells [2]. A hydrophilic segment consisting of 12-amino acids in the s2 type, which the s1 type lacks, suppresses VacA-induced vacuolating activity without any effect on its production [42–44]. Two m-regions (classified as m1 and m2) are located within p55; m2 is further divided into two subtypes, m2a and m2b [2, 41]. The entire m-region composed of m1 and m2 alleles is required for vacuolating activity [45, 46]. However, there is a clear difference in cell specificity between m1 and m2 [47], e.g., m2-type VacA, in contrast to m1-type VacA does not induce vacuole formation in HeLa cells irrespective of acid or alkaline activation [48]. De Guzman et al. have indicated that m2-type VacA does not bind to receptor protein tyrosine phosphatase (RPTP) α, one of the VacA-binding receptors, on membranes of HeLa cells, suggesting that posttranslational modifications of RPTPα of HeLa cells may determine sensitivity to m2 VacA [48]. Combinations of *vacA* alleles (s1/m1, s1/m2, and s2/m2) are commonly found in clinically isolated strains of *H. pylori*, but s2/m1 type of *vacA* gene is rare [2, 49].

Two other variable regions in the *vacA* gene have been identified. The intermediate region (i-region) has been identified as a third polymorphism region and is located between s- and m-regions (noted as i1, i2, and i3). Rhead et al. has indicated that the s1/m1 allele strains were predominantly i1 type, whereas all s2/m2 allele strains were i2 type and s1/m2 allele strains were variable in the i-region [50]. In addition, there is some evidence that the i-region affects human T cell functions, e.g., i2 type had a diminished capacity to inhibit the activation of nuclear factor of activated T cells (NFAT) and bound to Jurkat cells less avidly than did i1 type [51]. The fourth polymorphism is observed in the deletion region (d-region), which is located between i- and m-regions (noted as d1 and d2) [52]. It has been demonstrated that there is no deletion in the d1 genotype of the *vacA* gene, but d2 genotype contains a deleted region. However, the effects of the d-regions are still unclear.

### 4.3.2 *Association Between* vacA *Gene Alleles and Clinical Outcomes*

Many epidemiological studies indicate that a clear functional association between *vacA* gene alleles and clinical outcome of gastroduodenal diseases has not been found. Generally, s1/m1 *vacA* is a more toxic genotype than are s1/m2 and s2 types of the *vacA* alleles [44]. The s1/m1 *vacA* allele is predominantly isolated from patients, suggesting that strains possessing the s1/m1 *vacA* allele are more likely to be associated with gastroduodenal disease than other *vacA* alleles, whereas strains possessing s2 type of *vacA* are rarely associated with disease. The s1 or s1/m1 *vacA* allele is associated with duodenal ulceration and gastric cancer in the United States and Western Europe. On the other hand, most of the clinical isolates possess the s1/m1 *vacA* allele in East Asia including Japan and South Korea, where there is a high incidence of gastric cancer [2, 39, 53–56]. In addition, it has been proposed that i1 genotype is associated with gastric cancer and gastric ulcer in Italian populations, and therefore, the i-region might be a good indicator of the carcinogenic ability of *H. pylori* strains, while any combinations of three variable regions (s-, m-, and i-regions) in the *vacA* gene were not disease determinants in East Asian and Southeast Asian countries [50, 57, 58]. From these observations, relationships between *vacA* genotypes and specific clinical outcomes may be different in different geographic areas.

## 4.4 VacA Receptors on Target Cells

It is important to identify specific bacterial toxin receptors on the cell surface. These receptors play a critical role in bacterial toxin binding and entry into cells, followed by intoxication. Thus, interaction between toxin and receptors induce unique signal transduction pathways, leading to effects (e.g., morphological changes, cell damage) on the target cell.

The C-terminal domain of VacA is responsible for binding to cell surface receptors [14, 47]. We identified toxin-binding cultured cell compartments by immunoprecipitation analysis with purified VacA and biotin-labeled cultured cell lysate. In VacA-sensitive cells, we found that biotinylated cell surface proteins of 140 kDa (p140) and 250 kDa (p250) were precipitated with VacA but not inactivated VacA [59]. On the other hand, Seto and colleagues reported that the EGF receptor might be involved in VacA endocytosis, leading to induction of vacuolating activity [60]. Since these VacA-binding proteins, p140 and p250, were modified by *N*-linked sugar, we purified these proteins using lectin agarose. By LC-MS/MS analysis, RPTPα and RPTPβ were identified as VacA-binding proteins [9, 61].

De Bernard et al. reported that phorbol 12-myristate 13-acetate (PMA) induces differentiation of HL-60 cells into macrophage-like cells and induced the

susceptibility to VacA [62]. It was found that expression of RPTPβ mRNA and protein was significantly induced during differentiation of HL-60 cells by PMA [63]. Consistently, knockdown of RPTPβ by antisense oligonucleotide resulted in reduction of VacA-induced vacuolation in parallel with suppression of RPTPβ expression [63]. In addition, it is known that acid or alkaline treatment induced conformational changes in VacA, which increased binding, internalization, and cytotoxicity [9, 64]. The activated VacA showed increased binding to RPTPβ, suggesting that conformational changes of VacA promote its interaction with cell surface RPTPβ [9]. Fujikawa et al. demonstrated that oral administration of VacA to wild-type mice, but not RPTPβ knockout mice, resulted in gastric ulcers, indicating that RPTPβ is essential for intoxication by VacA in gastric tissue [65]. These results suggest that VacA binding to RPTPβ has functional effects.

With G401 cells, a human kidney tumor cell line that lacks RPTPβ but forms vacuoles in the presence of VacA, we also identified p140 as RPTPα. The finding that silencing of RPTPα gene by antisense oligonucleotide in G401 cells inhibited VacA binding and induction of vacuolation supports the hypothesis that RPTPα mediates VacA intoxication [61].

Other surface factors appear to interact with VacA and serve as cell surface receptors. By surface plasmon resonance-based biosensor studies, it has been proven that VacA binds to heparin sulfate, a component of the extracellular matrix [66]. Hennig et al. demonstrated that VacA binds to fibronectin of HeLa cells, resulting in inhibition of HeLa cell adhesion, suggesting that VacA affects cytoskeleton organization and cell adhesion via interaction with fibronectin [67]. Further, Gupta et al. also reported that VacA-induced vacuolation in HeLa cells is reduced in the presence of sphingomyelinase [68]. Sewald et al. have reported that β2-integrin subunit CD18 plays an important role in VacA uptake into human T lymphocytes [69]. Through this mechanism, VacA has immunosuppressive effects on cells by inhibiting cell growth and interleukin-2 (IL-2) secretion [69]. They also demonstrated that VacA endocytosis is PKC-dependent and clathrin-independent in primary T cells [70]. Recently, we identified a new VacA receptor, low-density lipoprotein receptor-related protein-1 (LRP1), which is essential for VacA-induced autophagy and apoptosis [19].

## 4.5 VacA Uptake Pathway

After binding to its receptors on target cells, VacA is internalized and found in endocytic vesicles. To enter into epithelial cells, VacA associates with lipid rafts [71, 72] and then internalizes via the Cdc42-dependent pinocytic pathway without requirement for dynamin 2, ADP-ribosylation factor 6, or RhoA GTPase. In this process, VacA was associated with detergent-resistant membranes [73]. Gupta et al. also reported that sphingomyelin is important in VacA uptake and intracellular translocation [74]. Furthermore, there is some evidence that VacA is endocytosed via a GPI-anchored protein-enriched early endosomal compartment (GEEC)-

dependent pathway. Filamentous actin is involved in VacA translocation from GEECs to late endosomes [75]. In primary T lymphocytes, VacA endocytosis is regulated by PKC-, Cdc42- and Rac1-dependent pathways [70].

## 4.6 Biological Activities of VacA

### *4.6.1 Vacuolation and Autophagy*

The vacuoles induced by interfering with intracellular membrane fusion in VacA-intoxicated cells contain markers of a pre-lysosomal compartment, including Rab7, Lgp110, and LAMP1 [76–78]. Further, various host factors, including dynamin, Rac1 and PIKfyve, are involved in VacA-induced vacuole formation [79–82]. The facts that VacA-induced vacuolation is suppressed by a V-ATPase inhibitor, bafilomycin A1, and V-ATPase colocalized with Rab7 in VacA-induced vacuoles suggest that a pH gradient generated through the activity of V-ATPase proton pump is required for this process (Fig. 4.3a) [83, 84].

Recent studies have reported that VacA induces autophagy, which is different from the large vacuoles formed in VacA-intoxicated cells [85–87]. VacA-induced autophagosomes and autophagolysosomes are dependent on functions of Atg family proteins (e.g., Atg5, Atg12, Atg16L1), with LC3-positive vesicles observed by confocal microscopy [85]. The internalized VacA partially colocalized with LC3 and LRP1 but not mitochondria. Additionally, the channel activity of VacA was also required for autophagosome formation [19, 85]. These findings indicate that VacA associates with autophagosomes and autophagolysosomes. Tsugawa et al. recently demonstrated that CagA is degraded by VacA-induced autophagy, which is regulated by a reactive oxygen species (ROS)-Akt-p53 signaling pathway (Fig. 4.3b) [24]. Interestingly, they found that CD44v9-expressing gastric cancer stem-like cells accumulated CagA by inhibition of VacA-induced autophagy through a mechanism involving cellular increase of glutathione due to stabilization of cystine transporter. A recent study showed that *H. pylori* have a strategy to avoid clearance by autophagy, i.e., *H. pylori* infection increased miR30BA, which directly downregulates autophagy regulatory proteins, Atg12 and Beclin1 [88]. Thus, *H. pylori* use several mechanisms to escape host autophagy, and then the persistent pathogen can produced various virulence factors, which trigger the pathological processes involved in diseases.

### *4.6.2 Apoptosis*

VacA also induces apoptosis of various cells [11, 89]. It has been suggested that p33 N-terminal fragment of VacA has a unique sequence, which translocates into

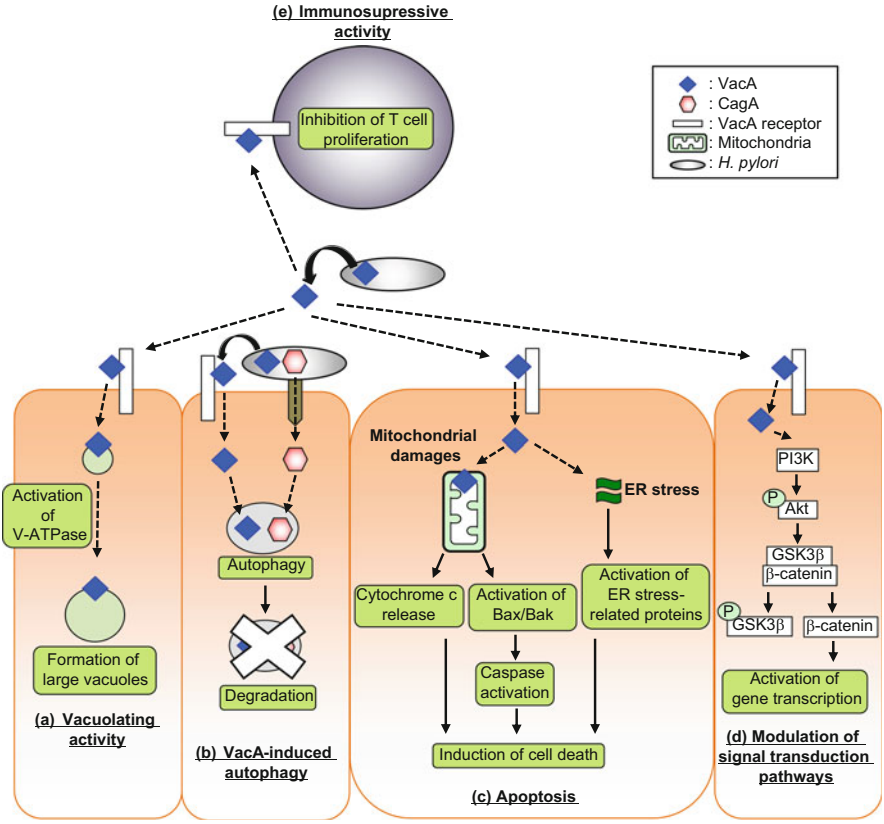


**Fig. 4.3** Pleiotropic functions of VacA. During *H. pylori* infection, VacA is internalized into target host cells via its receptors. (**a**) VacA generates the formation of large vacuoles in the cytoplasm by activation of V-ATPase. (**b**) After translocation of CagA into gastric epithelial cells, CagA is degraded by VacA-induced autophagy. (**c**) VacA localizes to the mitochondria and modulates the mitochondrial functions leading to cytochrome *c* release or caspase activation, resulting in apoptosis. In addition, ER stress is also involved in VacA-induced apoptosis. (**d**) VacA modulates signal transduction pathways, e.g., VacA activates PI3K/Akt signaling pathway, resulting in phosphorylation of GSK3β, associated with induction of β-catenin release from the GSK3β/β-catenin complex. Resulting β-catenin can translocate from cytoplasm to nucleus to induce expression of genes such as cyclin D1. (**e**) VacA binds to CD18 and modulates signal transduction pathways, resulting in inhibition of T cell proliferation

mitochondria to facilitate intoxication [90]. Apoptosis induced by VacA may proceed by two mechanisms (Fig. 4.3c). After internalization into cytosol, VacA directly moves to mitochondria, followed by modulation of mitochondrial membrane permeability by its channel activity, leading to cytochrome *c* release and apoptosis [14, 91–93]. Another pathway involves the proapoptotic Bcl-2 family protein in VacA-induced apoptosis, i.e., VacA causes caspase activation and PARP cleavage, subsequent to activation of Bax/Bak on the mitochondria [16, 94, 95]. Calore et al. have demonstrated that VacA-induced apoptosis requires Bax/

Bak-dependent juxtaposition of endosomes and mitochondria and accumulation of VacA and Bax in mitochondria [96]. These findings suggest that movements of both VacA and Bax/Bak into mitochondria are important in the apoptotic pathway. Matsumoto et al. reported that VacA downregulates STAT3 expression, followed by reduction of the amounts of Bcl-2 and Bcl-xL, which are anti-apoptotic proteins [97]. Jain et al. reported that VacA-induced activation of dynamin-related protein 1 (Drp1), a regulator of mitochondrial fission, is critical to apoptosis and that activation of Drp1-dependent mitochondrial fission requires channel activity of VacA, but not Bax activation, suggesting that cross talk between Drp1 and Bax may be unidirectional in VacA-treated cells [98].

Recently, it was demonstrated that VacA activates PERK, an endoplasmic reticulum (ER) stress sensor protein, and stimulates phosphorylation of eIF2α, followed by induction of C/EBP homologous protein (CHOP), a key protein of ER stress-induced apoptosis [99], suggesting that ER stress is also involved in VacA-induced apoptosis in AZ-521 and dendritic cells. More recently, Radin et al. have reported that connexin 43 (Cx43), a major component of the gap junction, in AZ-521 cells contributes to VacA-induced cell death [100].

On the other hand, in eosinophils, VacA induced apoptosis via p38 mitogen-activated protein kinase (MAPK) activation, leading to Bax translocation and cytochrome *c* release, although VacA promotes inhibitor of apoptosis protein (c-IAP)-2 expression at an early step in the process [94].

### *4.6.3 Immunosuppressive Activity of VacA*

VacA might contribute to chronic infection of *H. pylori* in the stomach by preventing protective immunity [51, 94, 101]. Molinari et al. have reported that VacA inhibits the Ii-dependent process of antigen presentation through MHC class II [102]. Other groups also indicated that VacA could not only interfere with T cell activation by preventing NFAT activity, resulting in suppression of IL-2 expression, but also activate MAPK kinase (MKK) 3/6 and Vav/Rac1, leading to massive actin reorganization in T cells (Fig. 4.3e) [103, 104]. In addition, Kim et al. reported that VacA treatment inhibits LPS-induced dendritic cell maturation, suggesting that VacA negatively regulates dendritic cell maturation through the restoration of E2F1 [105].

ROS also plays important roles in host defense [106]. In macrophages, VacA inhibits ROS production by interfering with expression of integrin-linked kinase (ILK), followed by suppression of endothelial nitric oxygen synthase (eNOS), resulting in increased survival of VacA-positive *H. pylori* [107].

### *4.6.4 Cell Signal Transduction by VacA*

VacA has diverse roles in signal transduction including proliferation, apoptosis, and host defense [18, 108]. Nakayama et al. reported that in AZ-521 cells, VacA activates p38 MAPK/activating transcription factor 2 (ATF-2) signaling pathway, which requires interaction with an unknown GPI-anchored membrane protein in lipid rafts [72]. Furthermore, VacA induced phosphoinositide-3-kinase activation, leading to phosphorylation of Akt and glycogen synthase kinase-3β (GSK3β). This signal transduction pathway interfered with β-catenin translocation from cytoplasm to nucleus by inhibiting β-catenin release from GSK3β/β-catenin complex (Fig. 4.3d) [109]. Another study showed that VacA treatment activates prostaglandin E2 production through induction of cyclooxygenase 2 (COX-2) expression via the p38 MAPK/ATF-2 pathway, leading to stimulation of cis-acting replication element (CRE) site in the COX-2 promoter [110].

In T cells and monocytic cell line U937, VacA enhanced NF-κB activation [111, 112]. In U937 cells, VacA induced IL-8 production via activation of p38 MAPK through intracellular $Ca^{2+}$ release, leading to stimulation of IL-8 promoter activation by binding of transcription factors, ATF-2, CRE-binding protein (CREB), and NF-κB [112]. These results indicate that *H. pylori* VacA is a pleiotropic toxin, able to affect various signaling transduction pathways and disrupt cell homeostasis.

## 4.7 Conclusion

VacA has pleiotropic actions in vitro and is considered to be one of the most important virulence factors of *H. pylori*. In addition to the findings related to VacA toxicity, it is interesting to note that the biological activities of VacA and the prevalence of some types of gastroduodenal disease caused by *H. pylori* infection are strongly associated with specific *vacA* gene alleles, suggesting that the *vacA* gene allele is a putative genetic biomarker of *H. pylori*-induced gastric diseases. Although many studies have demonstrated more clearly the importance of VacA in *H. pylori* infection, current investigations are focused on understanding the mechanisms by which VacA causes the pathogenesis of gastroduodenal diseases. Therefore, research on VacA may lead to a better understanding of its role in the development of gastric disorders and modulation of host immune defense in *H. pylori* infection. Thus, further studies may provide new insights into the role of VacA as a factor that promotes gastric diseases.

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