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Functional properties of *Helicobacter pylori* VacA toxin m1 and m2 variants

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Abstract

Helicobacter pylori colonizes the gastric mucosa and secretes a pore-forming toxin (VacA). Two main types of VacA, m1 and m2, can be distinguished by phylogenetic analysis. Type m1 forms of VacA have been extensively studied but there has been relatively little study of m2 forms. In this study, we generated *H. pylori* strains producing chimeric proteins in which VacA m1 segments of a parental strain were replaced by corresponding m2 sequences. In comparison to the parental m1 VacA protein, a chimeric protein (designated m2/m1) containing m2 sequences in the N-terminal portion of the m-region was less potent in causing vacuolation of HeLa cells, AGS gastric cells or AZ-521 duodenal cells and had reduced capacity to cause membrane depolarization or death of AZ-521 cells. Consistent with the observed differences in activity, the chimeric m2 VacA protein bound to cells at reduced levels compared to the parental m1 protein. The presence of two strain-specific insertions or deletions within or adjacent to the m-region did not influence toxin activity. Experiments with human gastric organoids grown as monolayers indicated that m1 and m2/m1 forms of VacA had similar cell-vacuolating activities. Interestingly, both forms of VacA bound preferentially to the basolateral surface of organoid monolayers and caused increased cell vacuolation when interacting with the basolateral surface compared to the apical surface. These data provide an improved understanding of functional correlates of sequence variation in the VacA m-region.

Introduction

Helicobacter pylori is highly adapted for colonization of the human stomach, and persistent colonization with these bacteria is a risk factor for development of gastric cancer and peptic ulcer disease (1, 2). *H. pylori* secretes several proteins that contribute to the pathogenesis of these diseases. One such protein, VacA toxin, is secreted through a type V (autotransporter) secretion pathway and released into the extracellular space as a soluble protein (3-12). CagA, an oncogenic effector protein, is secreted and translocated into gastric cells by a type IV secretion system (13-17). Numerous other proteins are released into the extracellular space, either by bacterial autolysis or through more selective processes that remain poorly understood (18-22).

The *vacA* gene encodes a 140 kDa protein, which undergoes proteolytic cleavage of an amino terminal signal sequence and a C-terminal β-barrel domain to yield a secreted 88 kDa protein (23-25). The secreted 88 kDa VacA protein is comprised of an N-terminal p33 domain and a C-terminal p55 domain (25, 26). Crystallographic and electron microscopic studies indicate that both domains have predominantly a β-helical structure [[ref]]. Both the p55 and p33 domains contribute to the process by which VacA binds to host cells [[ref Torres JBC 2005]]. The p33 domain contains an amino-terminal segment with multiple GXXXG motifs, which is required for membrane channel formation [[ref Vinion-Dubiel JBC 1999, McClain JBC 2003 and others]].

Although *vacA* is present in all *H. pylori* strains, there is a high level of sequence diversity among *vacA* alleles. Three main regions of sequence diversity have been designated: a 5' region encoding the signal sequence and the amino-terminus of the 88 kDa protein (s-region), intermediate region (i-region), and mid-region (m-region) (27-29) (Figure 1). Multiple combinations of these regions (for example, s1i1m1, s1i1m2, s2i2m2) can arise through natural

transformation and homologous recombination. Sequence variation in the s-region is the main determinant of VacA activity (27, 30, 31), but sequence variation in other regions also influences toxin activity (28, 32, 33). Epidemiological studies have shown that *H. pylori* strains containing specific *vacA* allelic types (for example, s1i1m1) are associated with an increased risk of gastric cancer and peptic ulcer disease, compared to strains that contain other *vacA* allelic types (e.g., s2i2m2) (27-29, 34-37).

Two main families of *vacA* alleles, m1 and m2, are recognized based on diversity in the region encoding the p55 domain (27, 38). Both m1 and m2 *vacA* allelic types are common throughout the world, and subfamilies of m1 or m2 *vacA* alleles are present in specific geographic regions (39). A prototypical m1 VacA protein and a prototypical m2 VacA protein exhibit 55% amino acid sequence diversity within a ~281 amino acid segment of the p55 domain (38). The exact boundaries of the VacA m-region have not been precisely defined. Phylogenetic analysis indicates that there is a relatively strong selective pressure for preservation of m1 and m2 *vacA* alleles, such that chimeric alleles arising through recombination (containing both m1 and m2 elements) are uncommon (38). Based on this observation, it has been proposed that m1 and m2 forms of VacA have distinct activities that each confer a selective advantage compared to m1-m2 chimeric forms of the protein (38). (40-43). Thus far, structural analyses have only been undertaken for s1i1m1 forms of VacA, but other forms presumably have a similar overall β-helical structure. When comparing m1 and m2 forms of VacA, the sites of amino acid polymorphisms are predicted to be clustered and surface-exposed (38, 40).

Most studies of VacA activity have analyzed s1i1m1 forms of the toxin. Type s1i1m1 VacA can cause a wide range of cellular alterations in cultured epithelial cells, including stimulation of intracellular vacuole formation, membrane depolarization, mitochondrial alterations, altered cell signaling, autophagy, disruption of cell-cell junctions, and potentially cell death (1, 6, 8, 10, 36,

44). VacA also can cause alterations in immune cells, including inhibition of the activation and proliferation of T-cells and B-cells and altered signal transduction in macrophages (6, 7, 11, 45, 46). Most of the reported VacA-induced cellular alterations are attributed to its capacity to form membrane channels in host cells, and therefore, VacA is classified as a pore-forming toxin (47-49).

Previous studies of m2 VacA proteins have used mostly non-gastric cell lines (such as HeLa or RK-13 cells), which are of uncertain relevance to *H. pylori* biology in the human stomach. Early studies reported that m2 VacA proteins have a reduced capacity to cause vacuolation of HeLa cells, compared to m1 VacA proteins (33, 50). Conversely, several studies reported that m1 and m2 VacA have similar capacity to vacuolate RK-13 cells (rabbit kidney origin), leading to the hypothesis that m1 and m2 proteins differ in cell type specificity (33, 50). Two studies suggested that m1 VacA exhibits increased binding to HeLa cells compared to m2 VacA proteins (33, 51). Notably, there have been several limitations in previous comparative studies of m1 and m2 VacA proteins. Some studies compared m1 and m2 VacA proteins produced by wild-type *H. pylori* strains, which have variations outside of the m-region of VacA (33, 50). Due to the difficulty of purifying the m2 forms of VacA, most studies have used concentrated broth culture supernatants or water extracts of intact bacteria as sources of m2 VacA (33, 50, 52). The presence of numerous bacterial proteins in these preparations potentially can influence cellular responses and complicate the interpretations, and the standardization of broth culture supernatants or water extracts to ensure equivalent VacA concentrations has been challenging. For example, some studies did analyses of relative VacA concentrations using polyclonal antibodies against m1 and m2 VacA that potentially differ in affinities (33). Finally, most comparative studies of m1 and m2 VacA proteins have provided few insights into the mechanisms or structural features underlying observed differences in activity.

In this current study, we sought to investigate the activity of m2 forms of VacA on gastric epithelial cells and elucidate the mechanistic basis for any observed differences in activity between m1 and m2 forms. We describe the generation of *H. pylori* strains producing chimeric VacA proteins in which segments of the p55 domain (m1 in the parental strain) were replaced by corresponding m2 sequences. We then report the results of comparative experiments in which these proteins were added to cultured human gastric epithelial cells or human gastric organoids grown as monolayers. We show that sequence variation in the N-terminal portion of the VacA p55 domain influences toxin activity and VacA binding to transformed cell lines, thereby influencing toxin potency. Importantly, both m1 and m2 forms of the toxin are capable of causing alterations in all of the cell lines tested, as well as human gastric organoids. These data provide an improved understanding of functional correlates of sequence variation in the VacA m-region.

Results

Replacement of m1 VacA sequences with m2 sequences. To undertake comparative analyses of type m1 and type m2 forms of VacA, we generated *H. pylori* strains in which segments of the *vacA* m-region in strain 60190 (which harbors a type s1/i1/m1 *vacA* allele) were replaced with corresponding segments of *vacA* from strain Tx30a (type s2/i2/m2 *vacA*) (Table 1, Figure 1A). Each of the chimeric *vacA* genes was also engineered to encode a Strep-tag II at a conserved region near the carboxy terminal end of the secreted VacA protein, which allows purification of the secreted proteins.

One of the strains (RRC4) was designed so that the *vacA* region encoding nearly the entire p55 domain was altered (corresponding to a change from m1 to m2; designated m2 in Figure 1). We also engineered *H. pylori* strains in which smaller regions of the m-region were altered. In one

strain (RRC3) the 5' portion of the m-region was changed from m1 to m2 (designated m2m1 in Fig. 1), and in the other strain (RRC2) the 3' portion of the m-region was changed from m1 to m2 (designated m1m2 in Figure 1). Figure 1B shows the cryo-EM structure of a hexamer formed by wild-type VacA from strain 60190 and depicts regions that were replaced with VacA sequences from strain Tx30a (41, 42). Each of these chimeric VacA proteins was secreted into the extracellular space, and the secreted proteins were successfully purified by use of the Strep-tag II. SDS-PAGE and Coomassie blue staining showed that all the secreted proteins were similar in size (about 88 kDa) (Figure 2A). To determine if the VacA variants retained the ability to oligomerize, we utilized negative stain electron microscopy (EM). All of the m2 VacA chimeras assembled into flower-shaped oligomeric structures (Figure 2B), similar to m1 VacA. Collectively, these experiments suggested that the replacement of various segments of the parental m1 sequence with corresponding m2 sequences did not have detrimental effects on protein folding.

Cell-vacuolating effects of VacA. When added to cultured epithelial cells, s1m1 VacA causes multiple cellular alterations, including cell vacuolation (3, 6-8, 36). To compare the vacuolating activity of the m1/m2, m2/m1 and m2 forms of VacA with that of the m1 form, we tested the effects of the proteins on HeLa cells (which are commonly used for assessing VacA activity) and AGS gastric epithelial cells. Vacuolation was quantified using a neutral red uptake assay, as described in Methods. The m1 VacA form and the m1/m2 form had similar activities, and these activities were significantly greater than those of the m2/m1 or m2 forms in both HeLa cell and AGS cell assays (Figure 3). These results provided evidence that sequence variation in the N-terminal portion of the p55 domain influences toxin potency, and suggest that the key functional differences between m1 and m2 VacA proteins are due to sequences differences in the amino-proximal region of the m-region. Because we consistently obtained relatively low yields of

purified m2 protein compared to other forms of VacA, we used the m2/m1 VacA protein for subsequent experiments.

Effects of VacA proteins on AZ-521 cells. AZ-521 cells, a duodenal cell line, are highly susceptible to cell death in response to s1m1 forms of VacA (53), but the effects of m2 forms of VacA on these cells have not been studied in detail. Therefore, we tested the effects of the chimeric VacA proteins on this cell line. Type m1 and m1/m2 types of VacA caused similar levels of vacuolation in AZ-521 cells, and these VacA forms induced significantly greater vacuolation compared to the m2/m1 form (Figure 4A). Similar to the results of the vacuolation studies, the m1 and m1/m2 VacA types each caused a significant reduction in cellular ATP levels compared to the m2/m1 form (Figure 4B). The reduction in ATP levels reflect altered metabolic homeostasis and have also been linked to cell death in VacA-treated AZ-521 cells (53).

When added to AZ521 cells, type s1m1 VacA causes depolarization of the plasma membrane potential (54). To compare the depolarizing activities of the different forms of VacA, we conducted cell depolarization assays, as described in the Methods. A reduced fluorescent signal was detected for cells incubated with m2/m1 VacA compared to the m1 and m1/m2 types (Figure 4C), indicating that the m2/m1 form caused less cell depolarization than the m1 or m1/m2 forms.

Analysis of a strain-specific insertions or deletions in the m-region. The results shown in Figure 3 suggest that differences in the activity of m1 and m2 forms of VacA are mainly due to sequence differences in the N-terminal portion of the p55 domain. Two of the most striking differences in this region are a ~23-amino acid segment at the junction of the p33 and p55 domains (denoted as the d-region in some publications) (55, 56), typically present in m1 but not

m2 VacA proteins, and a ~25-amino acid segment within the p55 domain of m2 but not m1 VacA proteins. To determine if these regions are important determinants of VacA activity, we generated strains (RRC5 and RRC6) engineered to produce VacA proteins in which these segments were individually altered (Figure 1). We also generated a strain (RRC7) producing a VacA protein in which both regions were altered. All of these mutants secreted VacA, similar to the parental strain (data not shown). When tested for activity in vacuolation and cell death assays, each of these mutants exhibited activity that was not significantly different compared to that of the parental m1 VacA protein (Supplemental Fig. 1).

VacA cellular binding, internalization, and localization. We next compared the ability of the different forms of VacA to interact with host cells. AGS or HeLa cells were incubated with fluorescently labeled VacA proteins at 4°C (to assess VacA binding to cells) or 37°C (to assess VacA internalization into cells), and the cells were then visualized by fluorescence microscopy. These studies revealed that the m1 form of VacA binds to both HeLa and AGS cells at higher levels compared to the m2/m1 form (Figure 5). The m1 form was also internalized at higher levels by HeLa and AGS cells compared to the m2/m1 form (Figure 6).

To determine if the different activities of m1 and m2/m1 VacA proteins might be attributable to differences in intracellular localization, we labeled m1 and m2/m1 forms of VacA with different fluorophores (Alexa 488 or 594) and added the proteins to cells, either consecutively or simultaneously. The VacA-treated cells were then incubated at 37°C for 4 h. Confocal microscopy revealed substantial co-localization of the two VacA types (Figure 7). Collectively, these results suggest that reduced binding of the m2/m1 VacA form to cells compared to the m1 type correlates with reduced internalization of the m2/m1 toxin. However, once m2/m1 VacA is internalized, it localizes in the same intracellular compartments as the m1 VacA type.

VacA interactions with human gastric organoids. We also assessed VacA interactions with polarized monolayers derived from human gastric organoids, grown on transwell filters. In initial experiments, we tested organoid cell lines derived from gastric tissue of three different human donors and added VacA proteins to either the apical (top) or basolateral (bottom) compartment of the chambers. Both m1 and m2/m1 VacA proteins caused cell vacuolation (assessed by neutral red uptake assay) when added to the basolateral compartment but not the apical compartment (Figure 8). There were no significant differences in vacuolating activity when comparing m1 and m2/m1 VacA (Figure 8). We then selected one of the organoid cell lines for further studies and analyzed VacA binding to the cells. Fluorescently labeled VacA proteins were added to either the apical or basolateral compartment, the cells were incubated at 4°C, and then were visualized using fluorescence microscopy. Higher levels of VacA binding were detected when the proteins were added to the basolateral compartment than when added to the apical compartment (Figure 9). Similar to the results observed in experiments with cultured transformed cells, the levels of m1 binding to the gastric organoids were significantly higher than levels of m2/m1 binding (Figure 9).

Discussion

In the current study, we analyzed the functional correlates of sequence diversity (corresponding to m1 and m2 variations) in the VacA p55 domain. We found that a chimeric m2/m1 VacA protein was less potent than the parental m1 VacA protein in causing vacuolation of multiple transformed cell lines. The m1 form of the toxin also induced greater cellular depolarization and cell death in AZ521 cells, a duodenal cell line, compared to the m2/m1 toxin. The region responsible for the observed difference in potency was mapped to the N-terminal portion of the p55 domain.

Previous studies compared the activity of different forms of VacA in the context of non-gastric cells (HeLa cells and RK-13 cells) and reported that m1 and m2 forms of VacA had similar vacuolating activity when tested on RK-13 cells, whereas the m1 form was more active than the m2 form when tested on HeLa cells (33, 50, 51). Therefore, it was proposed that m2 forms of VacA have a cell type-specific activity. The current study focuses on gastric or duodenal epithelial cells, which are presumably more biologically relevant than non-gastric cell types.

Nearly all previous studies of m2 VacA proteins analyzed *H. pylori* broth culture supernatants or *H. pylori* extracts containing VacA, instead of purified m2 VacA protein (33, 50, 52). One likely reason for the rare use of purified m2 VacA proteins in previous studies relates to difficulty in purifying sufficient quantities of the m2 protein. Most strains producing s2m2 forms of VacA secrete relatively low levels of VacA compared to strains producing s1m1 forms of VacA; this difference has been attributed to different levels of *vacA* transcription (57). In the current study, we manipulated a parental strain (containing an s1m1 *vacA* allele and secreting high levels of VacA) to yield strains containing modified forms of *vacA* (harboring segments of m2 *vacA*), which allowed the *vacA* promoter region to remain unchanged in all the strains tested. The construction of these strains and use of a strep tag for purification allowed us to purify sufficient quantities of most of the chimeric VacA proteins to allow analyses of VacA activity, but we consistently purified lower amounts of VacA from the strain in which the entire m1 region was replaced with an m2 sequence than from the parental strain. Although the reasons for this phenomenon are not entirely clear, we hypothesize that reduced stability of this chimeric protein and/or reduced levels of secretion are potential reasons for the low purification yields. Because of this limitation and our observations that the m2/m1 chimera had vacuolating activity similar to that of the protein with a complete m2 sequence, most experiments utilized the m2/m1 VacA form.,

Our current studies revealed that the m2/m1 VacA chimera exhibits decreased binding to the plasma membrane of AGS gastric epithelial cells, compared to the parental m1 protein. The reduced level of binding presumably accounts for the reduced level of m2/m1 VacA protein internalized into host cells, as well as the observed reduction in activity. Several putative cell-surface receptors for m1 VacA on epithelial cells have been reported, including receptor protein tyrosine phosphatase alpha and beta (RPTPα and RPTPβ) (58-61), lipoprotein receptor-related protein-1 (LPR1) (62), epidermal growth factor receptor (EGFR) (63), sphingomyelin (64, 65), heparan sulfate, glycosphingolipids and phospholipids (6, 8, 35, 36). The relative importance of these individual receptors in mediating VacA binding to cells or VacA internalization into host cells remains unclear (52, 66). In future studies it will be important to investigate further the binding of m1 and m2 VacA proteins to specific receptors and evaluate if differential binding to specific receptors accounts for the observed differences in toxin potency.

In addition to analyzing VacA interactions with transformed cultured cell lines, we analyzed VacA interactions with human gastric organoids grown as monolayers, which potentially replicate in vivo conditions more closely than cultured transformed cell lines. Unexpectedly, we found that VacA bound at higher levels to the basolateral surface of the monolayers compared to the apical surface. Similarly, VacA caused increased cell vacuolation following interaction with the basolateral surface compared to the apical surface. Preferential binding of VacA to the basolateral surface could potentially reflect a higher concentration of VacA receptors on this surface compared to the apical surface. Alternatively, mucus overlying the apical surface might impede VacA binding. Similar to the patterns of binding observed with cultured transformed cells, we detected a difference in binding of m1 and m2/m1 forms of VacA to the organoids.

H. pylori strains containing m2 *vacA* alleles are prevalent throughout the world. Such strains can potentially cause gastric cancer or peptic ulcer disease, particularly if the strains contain the

cag pathogenicity island and s1/i1/m2 forms of *vacA* *[[ref]],* and we speculate that both m1 and m2 forms of VacA confer fitness advantages to *H. pylori* compared to strains that do not produce VacA [[ref]]. The current study provides further understanding of the capacities of such proteins to cause alterations in gastric epithelial cells.

Methods

H. pylori culture methods. H. pylori strains were cultured on Trypticase soy agar plates containing 5% sheep blood at 37°C in room air supplemented with 5% CO_2. Liquid cultures were grown in Brucella broth containing 1x cholesterol (Gibco).

Generation of H. pylori mutant strains. Plasmids containing chimeric *vacA* sequences, derived in part from *H. pylori* strain 60190 (type m1 *vacA;* Genbank accession number Q48245.1) and in part from *H. pylori* strain Tx30a (type m2 *vacA;* Genbank accession number Q48253.1), and encoding a strep tag (strep-tag II) at a position corresponding to amino acid 808 in *vacA* from strain 60190 (Str_{808}), were designed as shown in Figure 1 and were synthesized by Genscript. The VacA chimeric proteins analyzed in this study do not represent naturally occurring proteins, but were engineered to facilitate analysis of VacA regions of interest. A previously described counter-selection approach (43) was used to construct the mutant strains. *H. pylori* strain 60190 Δ*rdxA* containing a *cat::rdxA* cassette replacing amino acids 420 to amino acid 820 in *vacA* (AflII and NheI restriction sites) (VM218) was transformed with the plasmids described above. Metronidazole-resistant transformants were isolated and expanded and genomic DNA was isolated. Introduction of the desired mutations was confirmed by PCR and sequencing the PCR-amplified regions of interest. Mutant strains are described in Table 1.

VacA purification. H. pylori strains were grown in broth culture for two days, and bacteria were removed by centrifugation at 7,500 x *g* for 15 min. Ammonium sulfate was added to the supernatant, resulting in a 50% saturated solution of ammonium sulfate. Precipitated proteins were pelleted by centrifugation at 7,500 x *g* for 15 min and resuspended in phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.02% sodium azide. Strep-tagged VacA proteins were incubated with Strep-Tactin resin (IBA) in a gravity column. The resin and associated proteins were washed with wash buffer (50 mM Tris, 150 mM NaCl [pH 8.0]), and subsequently VacA was eluted with elution buffer (50 mM Tris, 150 mM NaCl, and 5 mM D-desthiobiotin [pH 8.0]) (43).

Negative stain electron microscopy. Samples were prepared for electron microscopy (EM) analysis by diluting the samples to 40 µg/mL in 20 mM of HEPES buffer and applying about 5 µL to a glow-discharged copper grid covered with carbon-coated collodion film. After washing grids in 4 drops of water and staining in uranyl formate (0.75 %), samples were imaged by electron microscopy (Morgagni) at a magnification of 28,000x (67).

Cell culture methodology. HeLa cells (human cervix origin) and AZ-521 cells (human duodenal origin) were grown in minimal essential medium (modified Eagle medium containing Earle's salts and L-glutamine) supplemented with 10% FBS and nonessential amino acid solution 100x (Sigma), and AGS cells (human gastric origin) were grown in RPMI 1640 medium (containing L-glutamine and 25 mM Hepes) supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C.

Cell vacuolation and neutral red assay. Wild-type (WT) and mutant VacA proteins were tested for vacuolating activity using a neutral red uptake assay (68). Cells were seeded in tissue culture-treated flat bottom 96-well plates at a density of 1-2.5 x 10^4 cells per well and incubated overnight at 37°C in room air supplemented with 5% CO_2. Oligomeric purified VacA was acid-

activated with 200 mM HCl until a pH of 3.0 was reached, and the acid-activated VacA then was added to adherent cells in media supplemented with 5 mM ammonium chloride. After incubation for 4 h or 24 h at 37°C in room air supplemented with 5% CO_2, vacuolation was quantified by removing media and adding neutral red dye (68). Once the dye was taken up by vacuolated cells, the cells were washed three times with 0.9% saline. Acid alcohol (97% ethanol and 3% HCl) was added to release the dye and OD_{540} was determined using a plate reader.

VacA-induced cell death. Cell death was assessed using the ATPlite Luminescence Assay (Perkin Elmer) (53). AZ-521 cells were grown in minimal essential medium (modified Eagle medium containing Earle's salts and L-glutamine) supplemented with 10% FBS and MEM non-essential amino acid solution in a 5% CO_2 atmosphere at 37°C. Cells were plated on black-walled tissue culture-treated plates with a clear bottom (Corning). Acid-activated VacA was added to 1-2.5 x10^4 cells per well as described above for neutral red assays. After incubation for 24 h, the assay was completed following the instructions provided by the manufacturer.

Assessing VacA binding to cells and internalization using fluorescent microscopy. HeLa or AGS cells were plated onto glass coverslips in wells of a 24-well plate at a density of 7 x 10^4 cells/ml and incubated overnight. VacA proteins were labeled with Alexa 488 (Molecular Probes), which reacts with primary amines (43, 69). m1 and m2/m1 proteins contain similar numbers of primary amines in the region that was experimentally changed from m1 to m2 (14 lysines in the m1 VacA form and 15 in the m2/m1 VacA form). The average degree of labeling (moles dye/mole protein) for m1 VacA was 7.6 $\pm$ 3.6 moles dye/mole protein, and the average degree of labeling for m2/m1 VacA was 6.6 $\pm$ 4.2 moles dye/mole protein (analysis of 11 batches of labeled m1 and 11 batches of m2/m1 labeled toxin), calculated as described in Alexa Fluor 488 Microscale Protein Labeling Kit product information sheet. To assess VacA binding to cells, 5 µg/mL (~60 nM) of each fluorescently labeled acid-activated VacA variant was added to cells for 1 h and

incubated at 4°C. When assessing VacA internalization, 5 μg/mL of each labeled VacA variant was acid-activated, added to cells and cells were incubated for 5 minutes at 37°C. The VacA-containing media was removed and fresh media with 5 mM of ammonium chloride was added, and cells then were incubated at 37°C for 4 h. The cells were washed once with PBS and fixed with 4% paraformaldehyde (PFA). Cell membranes were stained with Wheat Germ Agglutinin Alexa Fluor 594 Conjugate (WGA) (Molecular Probes) and nuclei were stained with DAPI (Invitrogen) according to the manufacturer's instructions. All cells were imaged using confocal microscopy (LSM710) and analyzing using ImageJ. VacA co-trafficking studies were performed by fluorescently labeling two different VacA preparations with Alexa 488 or Alexa 594. The Pearson correlation coefficient (determined with ImageJ) was used to quantify colocalization of m1 and m2/m1 VacA or colocalization of m1 VacA with DAPI.

Cell depolarization. AZ521 cells were detached with trypsin/EDTA then incubated with 1,5-Bis (5-oxo-3-propylisoxazol-4-yl) pentamethine oxonol (oxonol VI) (Millipore Sigma) (final concentration 2.5 μM) for 15 min at 37 °C (54). A cell suspension (2 ml) was placed in a stirred quartz cuvette at 37 °C in a PerkinElmer Life Sciences LS50B fluorimeter. After stabilization of the fluorescence signal (excitation 585 nm, slit 10 nm; emission, 645 nm, slit 5 nm), acid-activated VacA (final concentration 20 μg/ml) or an acidified buffer control was added to the cells. The fluorescent signal was monitored for about 3.5 minutes after addition of VacA.

VacA interactions with cultured human organoids. De-identified donor gastric tissue specimens of corpus origin were obtained from sleeve gastrectomy surgeries and gastric glands were isolated as described previously, using IRB-approved protocols (70, 71). Gastric spheroid cultures were established following a 1 h incubation with 0.5 U/mL of collagenase in a 37 °C water bath at 200 rpm, samples were vortexed to release the glands, and centrifuged at 4 °C for

5 min. After resuspending the pellet in cold DPBS (Hyclone GE Healthcare Life Sciences) and vortexing for 30 s, glands were removed from the supernatant fraction, transferred to a 50 mL tube, pelleted and transferred to Matrigel (Corning, Bedford, MA, USA). The Matrigel-suspended glands were added to prewarmed 24-well plates and overlaid with conditioned L-WRN media composed of Advance DMEM/F12 (Gibco) and supplemented with 10 mM HEPES, 10 μM Y-27632 and 10 μM SB431542 (Tocris). Cells were maintained as described in (72).

To generate two-dimensional monolayer cultures, gastric spheroids from three different human donors were dissociated using trypsin and mechanical dissociation. Cells were then added to transwells (Thermo Fisher) previously coated with type I collagen, overlaid with L-WRNA conditioned medium containing penicillin and streptomycin, and cultured until confluent. Fluorescently labeled acid-activated VacA was added to either the apical or basolateral side as described above. Cells were washed with PBS and fixed as indicated above, and transwells were cut out using a scalpel and mounted using Prolong gold antifade (Invitrogen). VacA binding was assessed using confocal microscopy as described above. To quantify VacA intensity, z-stacks were merged and maximum intensity was analyzed in ImageJ. Integrated density values were divided by the number of nuclei in the field.

VacA-induced vacuolation was quantified using a neutral red uptake assay similar to that used for transformed cell lines (described above) but modified for compatibility with transwells. Acid-activated VacA proteins were added to either the apical or basolateral compartments in medium containing 5 mM ammonium chloride and cells were incubated for 24 h at 37°C in room air supplemented with 5% CO_2. Vacuolation was then quantified by removing media and adding neutral red dye to both the apical and basolateral compartments for 20 minutes. Cell surfaces on both sides of the transwell then were washed three times with 0.9% saline. Acid alcohol was

added to the apical compartment to release the dye, 75 µL of dye-cell mixture was transferred to a 96 well plate, and OD_{540} was determined.

Statistical analysis. Statistical significance of vacuolation and cell death data were determined by two-way ANOVA. Microscopy data were analyzed using ANOVA and Dunnett's or Dunn's multiple test corrections for parametric and non-parametric analyses, respectively. Neutral red data to assess vacuolation of epithelial monolayers derived from human gastric organoids were analyzed by two-way ANOVA with Bonferroni multiple corrections. The Mann Whitney test was used to evaluate the significance of differences in VacA binding to cells derived from gastric organoids.

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Table 1. *H. pylori* strains analyzed in this study

***H. pylori* strain name**	**Description of strain or VacA protein**	**VacA type**
VM218	Strain 60190 with *cat::rdxA* cassette replacing VacA amino acids 420-820	*vacA* null mutant
60190-Str_{808}	Wild-type VacA secreted from *H. pylori* strain 60190 engineered to produce VacA containing a Strep-tag II introduced at amino acid 808 (43)	m1
RRC2	60190-Str_{808} containing VacA amino acid sequences from *H. pylori* strain Tx30a replacing amino acids 625-804	m1/m2
RRC3	60190-Str_{808} containing VacA amino acid sequences from *H. pylori* strain Tx30a replacing amino acids 315-623	m2/m1
RRC4	60190-Str_{808} containing VacA amino acid sequences from *H. pylori* strain Tx30a replacing amino acids 315-804	m2
RRC5	60190-Str_{808} containing VacA amino acid sequences from *H. pylori* strain Tx30a replacing amino acids 299-347, which includes a 23 amino acid deletion	299-347 m2
RRC6	60190-Str_{808} containing VacA amino acid sequences from *H. pylori* strain Tx30a replacing amino acids 462-485, which includes a 25 amino acid insertion	462-485 m2
RRC7	60190-Str_{808} containing the changes described for RRC5 plus the changes described for RRC6	double mutant

Figure legends

Figure 1. Schematic depiction of VacA proteins analyzed in this study. *vacA* segments from *H. pylori* strain Tx30a (which contains type m2 VacA) were introduced in place of corresponding *vacA* segments in strain 60190. Strain designations are explained in Table 1. (A) In the schematic, the p33 domain of VacA is depicted in black, the p55 domain containing native m1 VacA sequences is blue, and regions containing m2 VacA sequences from strain Tx30a are red. Amino acid numbers of swapped regions are listed below each schematic (based on numbering of VacA from strain 60190). Asterisks indicate position of a 23-amino acid region present in VacA from strain 60190 but absent in VacA from strain Tx30a. Caret symbols indicate position of a 25-amino acid region present in VacA from strain Tx30a but absent in VacA from strain 60190. (B) The cryo-EM structure of a hexamer formed by VacA from wild-type 60190 (type m1 VacA) is shown (PDB 6NYF). Regions in which m1 sequences were replaced with m2 sequences are shown in red. Blue indicates regions in which VacA sequences of the parental strain are unchanged.

Figure 2. Properties of VacA chimeras in which regions in VacA from strain 60190 (type m1) were replaced with corresponding regions of VacA from strain Tx30a (type m2 VacA). (A) VacA proteins were purified from *H. pylori* culture supernatants and analyzed by SDS-PAGE and Coomassie blue staining. (B) Negative stain electron microscopy revealed that all of the VacA proteins assemble into water-soluble oligomers.

Figure 3. Cell-vacuolating activity of VacA proteins. Purified VacA proteins were acid-activated, serially diluted and added to (A) HeLa or (B) AGS cells in the presence of 5 mM ammonium chloride for 24 h. Neutral red uptake assays were performed to assess cell vacuolation (quantified by measuring optical density at 540 nm). Data from two or three independent

experiments were normalized to results for the m1 VacA control. *, $p < 0.05$ for m2/m1 or m2 VacA proteins compared to the m1 VacA type.

Figure 4. Effects of VacA proteins on AZ-521 cells. (A) AZ-521 cells were incubated with the indicated purified acid-activated VacA proteins for 4 h in the presence of 5 mM ammonium chloride. Cell vacuolation was quantified by neutral red uptake assay. Data were normalized to results for the m1 VacA control. *, $p < 0.05$ for m2/m1 VacA compared to m1 VacA. (B) AZ-521 cells were incubated with VacA proteins for 24 h and ATP levels were analyzed using the ATPlite reagent. Data from three independent experiments were normalized to results for control cells incubated with medium only. *, $p < 0.05$ for m2/m1 compared to m1 VacA. (C) AZ-521 cells were loaded with oxonol VI (a probe used to monitor membrane potential). Following stabilization of the fluorescent signal for 2.5 minutes, acid-activated VacA proteins (20 μg/ml) or acidified PBS was added to cells. The fluorescent signal was monitored for about 3.5 minutes after addition of VacA. m2/m1 VacA has reduced ability to alter membrane potential compared to m1 VacA. The graph shows representative data from three independent experiments.

Figure 5. m2/m1 VacA exhibits reduced binding to AGS cells compared to the m1 form. (A) AGS cells were treated with 5 μg/mL of acid-activated fluorescently labeled VacA (green) for 1 h at 4°C to evaluate toxin binding to cells. Cells were stained with fluorescently labeled WGA (magenta), a cell membrane marker, and DAPI (blue) for nuclear staining, and then were imaged by confocal microscopy. Cells were outlined in ImageJ and the VacA fluorescence intensity was quantified. VacA intensity was then normalized to signals in control cells treated with PBS. Experiments with HeLa cells yielded similar results (images not shown). The m1 form of toxin binds to HeLa (B) and AGS cells (C) at higher levels compared to m2/m1 VacA. Data points represent results from three (HeLa) or four (AGS) independent experiments (analysis of ≥10 cells per condition per experiment). ***, $p< 0.001$

Figure 6. m2/m1 VacA exhibits reduced internalization into AGS cells compared to the m1 form. HeLa and AGS cells were treated with 5 μg/mL of fluorescently labeled acid-activated VacA (green) for 5 minutes. The medium overlying cells was replaced with fresh medium containing 5 mM ammonium chloride, and cells then were incubated for 4 h at 37°C. Cells were stained with fluorescently labeled WGA (magenta), a cell membrane marker, and DAPI (blue) for nuclear staining, and then were imaged by confocal microscopy. Cells were outlined in ImageJ and the VacA fluorescence intensity was quantified. VacA intensity was then normalized to signals in control cells treated with PBS. (A) Images for experiments with AGS cells are shown. Experiments with HeLa cells yielded similar results (images not shown). The m1 form of toxin is internalized by HeLa (B) and AGS cells (C) at higher levels compared to m2/m1 VacA. Data points represent results from three (HeLa) or four (AGS) independent experiments (analysis of ≥15 cells per condition per experiment). **, $p<0.01$ and ***, $p< 0.001$

Figure 7. m1 and m2/m1 VacA co-localize within intracellular compartments. (A) m1 and m2/m1 VacA types were labeled with different fluorophores (m1, green; m2/m1, magenta) and added either simultaneously ("mix") or sequentially ("m1, m2/m1" or "m2/m1, m1") to AGS cells. In the latter case, cells were treated with 5 μg/mL of the first fluorescently labeled acid-activated VacA protein for 5 minutes, washed, and then treated with the second labeled protein for 5 minutes. The medium overlying cells was replaced with fresh medium containing 5 mM ammonium chloride, and cells then were incubated for 4 h at 37°C. Cells then were imaged by confocal microscopy. Co-localization of m1 and m2/m1 toxin is indicated by a white color in the merged images (right panels). (B) The Pearson correlation coefficient was used to quantify the degree of colocalization between m1 VacA and m2/m1 VacA. (C) The same images were also analyzed to assess the degree of colocalization between the m1 toxin and DAPI.

Figure 8. Vacuolation of epithelial monolayers derived from human gastric organoids. Acid-activated VacA proteins were added at the indicated concentrations to either the apical or basolateral compartments of transwells containing epithelial monolayers derived from human gastric organoids. Epithelial monolayers derived from three different human donors were tested. Cells were incubated for 24 h at 37°C, and VacA-induced vacuolation was quantified by a neutral red dye uptake assay as described in Methods. *, $p<0.05$ and ****, $p<0.0001$

Figure 9. Binding of m1 and m2/m1 VacA to epithelial monolayers derived from human gastric organoids. Fluorescently labeled acid-activated VacA proteins (5 μg/mL) were added to the apical or basolateral compartments of transwells containing human gastric epithelial cells for 1 h at 4°C to assess toxin binding (green). Nuclei were stained with DAPI (blue), and cells were imaged by confocal microscopy. NT, not treated (no VacA added). Bottom right panel: Cell-associated VacA was quantified as described in Methods. A, apical; B, basolateral. Each data point represents results from 4 fields per condition per experiment (two independent experiments). B-m1 vs. B-m2/m1 $p = 0.0379$; A-m1 vs A-m2/m1 $p = 0.003$; B-m1 vs A-m1 $p = 0.007$; B-m2/m1 vs A-m2/m1 $p = 0.0002$

Supplemental Fig. 1. Strain-specific VacA insertions or deletions are not determinants of toxin activity. VacA proteins in which the segment at the p33-p55 junction is deleted (299-347 m2, produced by *H. pylori* strain RRC5), a ~ 25 segment is inserted into the p55 domain (462-485 m2, produced by *H. pylori* strain RRC6), or both alterations (double mutant, produced by *H. pylori* strain RRC7) are depicted in Figure 1. VacA preparations were acid-activated and added (final concentration 5 μg/ml) to (A) HeLa or (B) AGS cells for 24 h or (C) AZ-521 cells for 4 h in the presence of 5 mM ammonium chloride. Neutral red uptake was measured as described in Methods. Data from three to seven independent experiments were normalized to results for the m1 VacA control. (D) To analyze cell death, AZ-521 cells were incubated with the indicated

concentrations of VacA for 24 h and luminescence was measured after the addition of the ATPlite reagent. Data from three independent experiments were normalized to results for control cells incubated with medium only. There were no significant differences between m1 VacA and VacA mutants in analyses of cell vacuolation or cell death (two-way ANOVA).