Intracellular Degradation of *Helicobacter pylori* VacA Toxin as a Determinant of Gastric Epithelial Cell Viability

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ABSTRACT *Helicobacter pylori* VacA is a secreted pore-forming toxin that induces cell vacuolation *in vitro* and contributes to the pathogenesis of gastric cancer and peptic ulcer disease. We observed that purified VacA has relatively little effect on the viability of AGS gastric epithelial cells, but the presence of exogenous weak bases such as ammonium chloride (NH₄Cl) enhances the susceptibility of these cells to VacA-induced vacuolation and cell death. Therefore, we tested the hypothesis that NH₄Cl augments VacA toxicity by altering the intracellular trafficking of VacA or inhibiting intracellular VacA degradation. We observed VacA colocalization with LAMP1- and LC3-positive vesicles in both the presence and absence of NH₄Cl, indicating that NH₄Cl does not alter VacA trafficking to lysosomes or autophagosomes. Conversely, we found that supplemental NH₄Cl significantly increases the intracellular stability of VacA. By conducting experiments using chemical inhibitors, stable ATG5 knockdown cell lines, and ATG16L1 knockout cells (generated using CRISPR/Cas9), we show that VacA degradation is independent of autophagy and proteasome activity but dependent on lysosomal acidification. We conclude that weak bases like ammonia, potentially generated during *H. pylori* infection by urease and other enzymes, enhance VacA toxicity by inhibiting toxin degradation.

KEYWORDS *Helicobacter pylori*, VacA, cell death, cell survival, pore-forming toxins

Helicobacter pylori, a Gram-negative bacterium that colonizes the stomach, is present in over half of the world's population (1). Most *H. pylori*-infected individuals remain asymptomatic, but the presence of *H. pylori* increases the risk of developing peptic ulcer disease and gastric adenocarcinoma (2, 3). One of the important virulence factors implicated in the development of these diseases is vacuolating cytotoxin A (VacA) (4–8).

VacA is secreted from *H. pylori* as 88-kDa monomers which oligomerize to form anion-selective membrane channels (4, 9). VacA monomers are comprised of two domains, an N-terminal p33 domain and a C-terminal p55 domain. A hydrophobic region within the p33 domain is required for formation of membrane channels, and regions within both the p33 and the p55 domains mediate VacA oligomerization and binding to host cells (10–15). VacA can bind the surface of epithelial cells via lipid rafts and is internalized into glycosylphosphatidylinositol-anchored protein (GPI-AP)-

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enriched early endosomal compartments (GEECs) before being trafficked to early and late endosomes (16–21). VacA is reported to cause a wide range of cellular responses, including cell vacuolation, plasma membrane permeabilization, alteration of endosomal and lysosomal function, disruption of mitochondrial function, modulation of autophagy, apoptosis, necrosis, and inhibition of T-cell activation (reviewed in reference 4).

One of the most extensively characterized VacA activities is its ability to induce the formation of large cytoplasmic vacuoles in cultured cells (9, 22). A current model for VacA-induced vacuolation (23, 24) proposes that VacA forms anion-selective channels in late endosomal/lysosomal membranes (10, 25–27), leading to an influx of chloride into endosomes, which stimulates increased proton pumping by the vacuolar ATPase and a subsequent decrease in intraluminal pH (14, 15, 28, 29). Membrane-permeant weak bases that diffuse into the endosome are protonated in the acidic environment and trapped, triggering osmotic swelling that manifests as cell vacuolation (30, 31).

Most cell types are relatively resistant to VacA-induced cell death, which requires exposure of cells to high concentrations of the toxin for long time periods (32–35). One possible explanation is that cells might have mechanisms to protect from VacA-induced toxicity. Indeed, there is growing evidence indicating that cells are able to respond and survive following exposure to several bacterial pore-forming toxins (PFTs), including *Staphylococcus aureus* alpha-toxin (36–38), *Vibrio cholerae* cytolysin (39), *Aeromonas hydrophila* aerolysin (40), *Listeria monocytogenes* listeriolysin O (40), and *Streptococcus pyogenes* streptolysin O (41). Inhibiting cellular repair mechanism(s) enhances the toxicity of these PFTs (36, 38, 39).

Both the formation of VacA-induced vacuoles and VacA-induced cell death are enhanced in the presence of ammonium chloride (NH_4Cl), a weak base (22, 30, 31, 33, 42, 43). Consequently, in experimental studies in which cells are treated with purified VacA, the cell culture medium is often supplemented with NH_4Cl . The presence of weak bases in cell culture medium may mimic the conditions in the stomach during *H. pylori* infection, as *H. pylori* generates ammonia *in vivo* through the actions of urease and other enzymes, such as γ -glutamyl transpeptidase, asparaginase, and glutaminase (44–46). In this study, we investigated the mechanism(s) by which NH_4Cl influences the magnitude of VacA-induced cell death. We report that the presence of supplemental weak bases (such as NH_4Cl) inhibits intracellular VacA degradation while having no detectable effect on VacA intracellular trafficking. Our results indicate that intracellular VacA degradation is independent of autophagy and proteasome activity but dependent on lysosomal acidification. We propose that intracellular degradation of VacA in the lysosome enables host cells to resist VacA-induced vacuolation and cell death and that weak bases enhance VacA activity by inhibiting intracellular degradation of the toxin.

RESULTS

VacA-induced cell death is enhanced in the presence of supplemental NH_4Cl .

As a first step in analyzing VacA-induced cell death, we performed experiments in which cells were treated with multiple successive doses of the toxin, potentially similar to conditions in the stomach where cells continually encounter newly synthesized VacA, in the absence or presence of NH_4Cl . Specifically, we treated AGS gastric epithelial cells once a day for 5 days with VacA (5 $\mu\text{g}/\text{ml}$) in the absence or presence of 5 mM NH_4Cl . Cell vacuolation was detected in the absence of NH_4Cl , but the cells continued to proliferate (Fig. 1A to C). In the presence of NH_4Cl , VacA-induced vacuolation was enhanced (Fig. 1D), and cell proliferation was inhibited (Fig. 1E and F). NH_4Cl alone did not induce significant vacuolation (see Fig. S1 in the supplemental material). After transmitted light images were collected, cellular ATP levels were determined to quantitatively assess cell viability. A small reduction in cell viability was observed in the presence of VacA alone or in the presence of NH_4Cl alone, but a large reduction in cell viability was observed only in the presence of both VacA and NH_4Cl (Fig. 1G). Thus, the reduction in cell viability in the presence of both VacA and NH_4Cl was synergistic, not

FIG 1 Loss of cell viability requires treatment with both VacA and supplemental NH_4Cl . (A to F) AGS cells were treated once a day for 5 days with 5 $\mu g/ml$ of VacA in the absence (–) or presence (+) of 5 mM NH_4Cl . Transmitted-light micrographs were collected after each successive day of VacA intoxication to assess for vacuolation. Scale bar, 50 μm . (G) Quantification of cellular ATP levels from the images shown in panels A to F and in Fig. S1A to F using an ATPlite 1step Luminescence Assay. Values represent luminescence signal normalized to that of the control ($-NH_4Cl$ $-VacA$) cells. (H) AGS cells were treated once a day for 5 days with 20 $\mu g/ml$ of VacA in the absence or presence of 5 mM NH_4Cl . After 5 days, cells were fixed and stained with 5% crystal violet to assess for the presence of cells.

additive. When the experiment was repeated with higher doses of VacA (20 $\mu g/ml$), once again the cells treated with VacA in the absence of NH_4Cl grew to confluence, whereas cells treated with VacA in the presence of NH_4Cl did not (Fig. 1H). Therefore, in the absence of NH_4Cl , repetitive treatment of cells with either low or high concentrations of VacA did not induce substantial cell death. Consistent with previous reports (32, 33), these data indicate that NH_4Cl enhances VacA-induced cell death.

NH_4Cl does not promote VacA trafficking to mitochondria. It has been suggested that VacA can induce cell death by directly trafficking to mitochondria and forming pores in mitochondrial membrane (34, 47–50). Therefore, we tested whether NH_4Cl augments VacA-induced cell death by promoting VacA trafficking to mitochondria. To test this, we used Alexa Fluor 488-labeled VacA (488-VacA) to quantify VacA colocalization with mitochondria over time in the absence and presence of NH_4Cl . We observed that VacA was largely excluded from mitochondria (Fig. 2A), and we did not detect any consistent increase in VacA colocalization with mitochondria over time, regardless of whether cells were treated with VacA in the absence or presence of NH_4Cl (Fig. 2B and C and Table S1). Furthermore, there was no significant increase in VacA colocalization with mitochondria in the presence of NH_4Cl compared to the level of VacA colocalization with mitochondria in the absence of NH_4Cl (Fig. 2D).

We also used live-cell imaging to monitor VacA localization in relation to mitochondria. If VacA localizes to mitochondrial membrane, we expected to observe stable

FIG 2 Treatment with NH_4Cl does not promote VacA trafficking to mitochondria. AGS cells were treated with a pulse of 488-VacA (1 h at 4°C for the 0-min time point; 5 min at 37°C for all other time points), washed, incubated for various lengths of time in the absence or presence of 5 mM NH_4Cl , and then fixed and stained with anti-MTC02 (mitochondria). (A) Representative image of a cell at the 24-h time point in the presence of NH_4Cl . The image is a single, nondeconvolved z-slice. Scale bars, 2 μm (zoom) and 10 μm (all other images). (B to D) The Pearson correlation coefficient was used to quantify colocalization of VacA with MTC02 in the absence (–) and presence (+) of NH_4Cl . (D) Combined data. Open circles indicate absence of NH_4Cl and closed circles indicate presence of NH_4Cl . Each data point represents the Pearson's coefficient of an individual cell measured using ImageJ from a single, nondeconvolved z-slice ($n \geq 10$ cells per condition per experiment from two independent experiments). For panels B to D, error bars indicate standard deviations. For panels B and C, the means for each data set are statistically different as determined by ANOVA ($P \leq 0.0001$). ***, $P \leq 0.001$, by Dunnett's multiple-comparison test. (D) ns (not significant), $P > 0.05$; *, $P = 0.0206$ as determined by an unpaired, two-tailed t test.

colocalization between VacA and mitochondria. However, we did not detect stable colocalization of VacA with mitochondria over time in either the presence (Fig. 3A to F and Movie S1) or absence of NH_4Cl (Fig. S2 and Movie S2). VacA-containing vesicles that appeared to be near or colocalized with mitochondria moved away from mitochondria over time (Fig. 3D to F). In contrast, colocalized movement of VacA with early and late endosomes was routinely observed (Fig. 3G to J and Movie S3). These data indicate that NH_4Cl does not promote VacA trafficking to mitochondria. Additionally, this analysis suggests that either VacA is not targeted to mitochondria or that only a minor subpopulation of VacA traffics to mitochondria, making the interaction difficult to detect.

NH_4Cl inhibits intracellular VacA degradation. While analyzing the fluorescence microscopy images from fixed cells, we noticed that at 24 h, the intracellular level of VacA was significantly higher in cells treated with VacA in the presence of NH_4Cl than in cells treated with VacA in the absence of NH_4Cl (Fig. 4A and B). Ammonium ions accumulate within acidic intracellular organelles, resulting in increased intralysosomal pH, which in turn inhibits the activation of acid proteases required to break down cargo material (51–53). Therefore, we hypothesized that host cells degrade VacA and that NH_4Cl might augment VacA-induced cell death by inhibiting intracellular VacA degradation. To test this hypothesis, we monitored the stability of VacA in AGS cells using Western blot analysis. In the absence of NH_4Cl , VacA was nearly undetectable 4 h after addition of the toxin to cells (Fig. 4C), while in the presence of NH_4Cl , VacA levels remained stable throughout the 8-h time course (Fig. 4D). This difference was not attributable to NH_4Cl -induced alterations in the amount of VacA that binds and is internalized into cells because the levels of VacA associated with cells 0.5 h after addition of the toxin were similar in the absence and presence of NH_4Cl (Fig. 4E and Fig. S3). At a 24-h time point after the addition of VacA to cells, there was a significantly higher level of VacA in the presence of NH_4Cl than in the absence of NH_4Cl (Fig. 4E

FIG 3 Analysis of VacA localization in living cells. (A to C) Live-cell imaging of AGS cells transfected with Mito-RFP. Cells were treated with a pulse of 488-VacA for 5 min at 37°C and then washed and incubated at 37°C in medium supplemented with 5 mM NH₄Cl. Movies were collected at 0.5 h, 4 h, and 24 h post-VacA treatment, as indicated. Panels at left show a single frame of the cell being imaged; at right are sequential images of the regions boxed in the left-hand panels. Time is indicated in seconds relative to the initial frame. Images are single z-slices. Scale bars, 10 μ m (left images) and 2 μ m (right images). (D to F) Line scans of the regions indicated by yellow dashed lines shown in merged panels A to C (right images). The magenta line represents normalized Mito-RFP fluorescence intensity. The green line represents normalized 488-VacA fluorescence intensity. In panels D and F, the initial frame reveals VacA appearing near mitochondria, but subsequent frames reveal VacA moving away from mitochondria. In panel E, the frames reveal VacA initially appearing away from mitochondria, moving near mitochondria, and then moving back away from mitochondria. (G and H) Live-cell imaging of AGS cells transfected with mCh-Rab5a (G) or mCh-Rab7 (H), treated with a pulse of 488-VacA for 1 h at 4°C, and then washed and incubated at 37°C. Movies were collected at 20 min and 45 min posttreatment, as indicated. Panels at left show a single frame of the cell being imaged. At right are sequential images of regions boxed in the left-hand panels. Time is indicated in seconds relative to initial frames. Images are

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FIG 4 NH_4Cl inhibits intracellular VacA degradation. (A) Sum intensity z-section projections of AGS cells treated with a pulse of 488-VacA for 5 min at 37°C and then washed and incubated for 24 h in the absence or presence of 5 mM NH_4Cl . DNA, blue. Intensities for the 488 channel are scaled identically according to a look-up table. Scale bar, 20 μm . (B) Quantification of the images shown in panel A. Error bars indicate standard deviations ($n \geq 50$ cells per condition). ****, $P < 0.0001$ as determined by an unpaired, two-tailed t test. (C and D) Western blots of whole-cell lysates prepared from AGS cells treated for 5 min at 37°C with VacA and then washed and incubated for various lengths of time in the absence (–) or presence (+) of 25 mM NH_4Cl . The blot was probed with antibodies targeting VacA or tubulin (DM1 α); 100- and 58-kDa references are marked on the left. (E) Western blot of whole-cell lysates prepared from AGS cells treated for 1 h at 4°C with VacA and then washed and incubated for 0.5 or 24 h in the absence or presence of 5 mM NH_4Cl . The blot was probed with antibodies targeting VacA and tubulin (DM1 α); 100- and 58-kDa references are marked on the left. (F) Quantification of the blot shown in panel E. Error bars indicate standard deviations ($n = 4$). *, $P = 0.0163$; **, $P = 0.0016$; ****, $P < 0.0001$, as determined by a paired, two-tailed t test. (G) Transmitted-light micrographs of cells used in the experiments shown in panels C and D at each successive time point. Scale bar, 20 μm .

and F). VacA levels detected by Western blot analysis correlated with the presence of cellular vacuolation (Fig. 4G). Similar results were obtained with HeLa cells (Fig. S4). Altogether, these data indicate that cells degrade VacA and that NH_4Cl inhibits VacA degradation.

VacA accumulates in lysosomes and autophagosomes/autolysosomes in both the absence and presence of NH_4Cl . To determine if NH_4Cl altered the intracellular trafficking of VacA to lysosomal or autophagosomal compartments, which are likely sites of intracellular VacA degradation, we evaluated VacA colocalization with lysosomal and autophagosomal markers (LAMP1 and LC3, respectively). VacA colocalized with LAMP1 and LC3 in both the absence and presence of NH_4Cl (Fig. 5). At 4 h and 24 h after addition of VacA, in both the absence and presence of NH_4Cl , there was a statistically significant increase in the colocalization of VacA with LAMP1 compared to what was observed in the 0-min control samples (Fig. 5B and C and Table S1). The same result was

FIG 3 Legend (Continued)

single z-slices. Scale bars, 10 μm (left images) and 2 μm (right images). (I and J) Line scans of the regions indicated by yellow dashed lines shown in merged panels G and H (right images). The magenta line represents normalized mCh-Rab5a (I) or mCh-Rab7 (J) fluorescence intensity, appearing as two peaks due to their membrane localization. The green line represents normalized 488-VacA fluorescence intensity. Frames reveal a stable colocalization of VacA with early (Rab5a) and late (Rab7) endosomes.

FIG 5 VacA accumulates in lysosomes and autophagosomes/autolysosomes in both the absence and presence of NH_4Cl . AGS cells were treated with a pulse of 488-VacA (1 h at 4°C for the 0-min time point; 5 min at 37°C for all other time points), washed, and incubated for various lengths of time in the absence or presence of 5 mM NH_4Cl and then fixed and stained with anti-LAMP1 (lysosomes) or anti-LC3 (autophagosomes/autolysosomes) antibody. (A) Representative images of cells at the 24-h time point in the presence of NH_4Cl . Images are single, nondeconvolved z-slices. Scale bars, 2 μm (zoom images) and 10 μm (all other images). (B to E) The Pearson correlation coefficient was used to quantify colocalization of VacA with LAMP1 and LC3 in the absence (–) and presence (+) of NH_4Cl . Open circles indicate the absence of NH_4Cl , and closed circles indicate the presence of NH_4Cl . Each data point represents the Pearson's coefficient of an individual cell measured using ImageJ from a single, nondeconvolved z-slice. Error bars indicate standard deviations ($n \geq 10$ cells per condition per experiment from two independent experiments). The means for each data set are statistically different ($P \leq 0.0001$) as determined by an ANOVA. **, $P \leq 0.01$; ***, $P \leq 0.001$, as determined by a Dunnett's multiple-comparison test.

observed for VacA colocalization with LC3 (Fig. 5D and E and Table S1). These data indicate that VacA can localize to both lysosomes and autophagosomes/autolysosomes and that the presence of NH_4Cl does not disrupt VacA trafficking to these compartments.

VacA degradation is independent of proteasome activity and autophagy but dependent on lysosomal acidification. Next, we sought to determine the cellular pathway(s) involved in VacA degradation. To test the role of the proteasome, we used the proteasome inhibitor MG132. Treatment of cells with MG132 did not result in increased VacA levels or increased vacuolation compared to the levels observed in control cells (Fig. S5). To test the role of autophagy in VacA degradation, we used both chemical and genetic approaches to inhibit autophagy. First, we treated cells with 3-methyladenine (3-MA), which inhibits type III phosphatidylinositol 3-kinases (PI3K) to block autophagosome formation (54, 55). While we noted that 3-MA alone induced a low level of vacuolation, treatment of cells with VacA in the presence of 3-MA did not result in increased cellular VacA levels or increased vacuolation compared to levels in control cells (Fig. 6A to C). We then analyzed VacA degradation in two stable ATG5 knockdown cell lines (KD1 and KD2) generated using two different ATG5 short hairpin RNA (shRNA) clones. The ATG5 KD cell lines had knockdown efficiencies of 80% for ATG5 KD1 and 88% for ATG5 KD2 (Fig. S6A and B). Inhibition of autophagy by knocking down ATG5 did not result in increased VacA levels or increased vacuolation compared

FIG 6 VacA degradation is independent of autophagy but dependent on lysosome acidification. (A to O) Cells were treated for 1 h at 4°C with VacA and then washed and incubated for 0.5 or 24 h in the absence or presence of respective inhibitors. Cell lysates were collected to assess VacA levels using Western blot analysis. Transmitted-light images were collected at the 24-h time point to assess for vacuolation. (A) Representative Western blot of whole-cell lysates prepared from AGS cells treated with VacA in the absence or presence of 10 mM 3-MA. (B) Quantification of the blot shown in panel A ($n = 3$). Error bars indicate standard deviations. ***, $P = 0.0006$; **, $P = 0.0018$; ns (not significant), $P = 0.4227$, by a paired, two-tailed t test. (C) Transmitted-light micrographs of cells used in the experiment shown in panel A after 24 h of VacA treatment. (D) Representative Western blot of whole-cell lysates prepared from a scrambled control cell line and two AGS ATG5 KD cell lines treated with VacA. (E) Quantification of the blot shown in panel D. Error bars indicate standard deviations ($n = 3$). ***, $P < 0.001$ (paired, two-tailed t test); ns (not significant), $P = 0.9211$ (by ANOVA). (F) Transmitted-light micrographs of cells used in the experiment shown in panel D after 24 h of VacA treatment. (G) Representative Western blot of whole-cell lysates prepared from parental HeLa and HeLa ATG16L1 KO cells treated with VacA. (H) Quantification of the blot shown in panel G. Error bars indicate standard deviations ($n = 3$). ****, $P < 0.0001$; **, $P = 0.0013$; ns, $P = 0.5992$, by a paired, two-tailed t test. (I) Transmitted-light micrographs of cells used in the experiment shown in panel G after 24 h of VacA treatment. (J) Representative Western blot of whole-cell lysates prepared from AGS cells treated with

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to levels in control cells (Fig. 6D to F). We also analyzed VacA degradation in HeLa ATG16L1 knockout (KO) cells generated using CRISPR/Cas9 (Fig. S6C). Inhibition of autophagy by knocking out ATG16L1 did not result in increased VacA levels or increased vacuolation compared to levels in control cells (Fig. 6G to I).

To test the role of the lysosome in VacA degradation, we used chloroquine and bafilomycin A1 to inhibit lysosome acidification. Treatment of cells with chloroquine, a lysosomotropic weak base which, similarly to NH_4Cl , raises intralysosomal pH, resulted in increased VacA levels and increased vacuolation compared to levels in control cells (Fig. 6J to L) (51). Furthermore, treatment of cells with bafilomycin A1, which raises intralysosomal pH by inhibiting the vacuolar H^+ -ATPase, also resulted in increased VacA levels (Fig. 6M and N) (56). Since the vacuolar H^+ -ATPase has previously been shown to be required for osmotic swelling of endosomes in response to VacA (57, 58), bafilomycin A1 inhibited VacA-induced vacuolation despite high intracellular levels of VacA (Fig. 6O). Altogether, these results provide evidence that cells degrade VacA through processes independent of proteasome activity and autophagy but dependent on lysosomal acidification.

DISCUSSION

In this study, we analyzed factors that influence the capacity of *H. pylori* VacA toxin to cause death of gastric epithelial cells. Consistent with previous reports, we show that the presence of supplemental NH_4Cl in cell culture medium enhances VacA-induced cell death (33). Conversely, we show that in the absence of supplemental NH_4Cl , cells can resist VacA-induced vacuolation and cell death by degrading VacA. Our results indicate that VacA degradation is inhibited when cells are treated with NH_4Cl or two other agents that interfere with lysosomal acidification, chloroquine and bafilomycin A1. NH_4Cl is known to enhance VacA-induced vacuolation, a phenomenon that has been attributed to the accumulation of NH_4Cl (a weak base) within endosomal compartments, leading to osmotic swelling (30, 31). In contrast, the role of NH_4Cl in enhancing VacA-induced cell death and mechanisms underlying this phenomenon have not been investigated in any detail. Our work suggests that VacA is degraded in the lysosome and that NH_4Cl enhances VacA-induced cellular damage by inhibiting VacA degradation within lysosomes.

Previous studies have shown that VacA can induce significant vacuolation and cell death in the absence of supplemental NH_4Cl if cells are cocultured with VacA-producing *H. pylori* strains or treated with broth culture filtrate (BCF) from VacA-producing *H. pylori* strains (32, 33, 35, 59). In these experimental systems, ammonia is present at concentrations sufficiently high to influence VacA-induced vacuolation (45). In addition, ammonia-producing *H. pylori* enzymes, such as urease, γ -glutamyl transpeptidase, asparaginase, and glutaminase, are also present (46, 60). Therefore, previous studies using *H. pylori* strains or BCF were not able to assess the effect of VacA on host cells in the absence of ammonia. Experiments described in the current manuscript were performed using purified VacA, which allowed us to specifically evaluate the interactions of VacA with host cells in the presence of ammonia, as well as in the absence of ammonia and ammonia-producing *H. pylori* enzymes. This approach also permitted the concentration of VacA to be experimentally determined and carefully controlled.

Our work reveals that cells can degrade VacA within 4 h after exposure to the toxin.

FIG 6 Legend (Continued)

VacA in the absence or presence of 100 μM chloroquine (CQ). (K) Quantification of the blot shown in panel J. Error bars indicate standard deviations ($n = 4$). ****, $P < 0.0001$; *, $P = 0.0275$; ns, $P = 0.1839$, by a paired, two-tailed t test. (L) Transmitted-light micrographs of cells used in experiment shown in panel J after 24 h of VacA treatment. (M) Representative Western blot of whole-cell lysates prepared from AGS cells treated with VacA in the absence or presence of 10 nM bafilomycin A1 (Baf A1). (N) Quantification of the blot shown in panel M. Error bars indicate standard deviations ($n = 3$). ****, $P < 0.0001$; ns, $P = 0.1532$, paired, two-tailed t test. (O) Transmitted-light micrographs of cells used in the experiment shown in panel M after 24 h of VacA treatment. All blots were probed with antibodies targeting VacA and tubulin (DM1 α); 100- and 58-kDa references are marked on the left. Scale bar, 20 μm .

In contrast, two earlier studies reported that VacA can persist inside cells for substantially longer time periods (61, 62). One study detected VacA by immunoblot analysis in lysates from MKN 28 cells that were treated with *H. pylori* BCF for 16 h and then washed and incubated with medium for an additional 72 h (61). BCF was dialyzed to remove ammonia prior to addition of the BCF to cells, but we presume that urease and/or γ -glutamyl transpeptidase in the BCF would subsequently generate additional ammonia from urea and glutamine, respectively, in the tissue culture medium. Another study reported that VacA persists inside cells for hours with no noticeable degradation (62), but the tissue culture medium was supplemented with 10 mM NH_4Cl in that study.

We propose that the influence of NH_4Cl on VacA degradation is due to the well-established ability of weak bases to raise intralysosomal pH (52). The activity of lysosomal enzymes is known to be dependent on lysosomal pH (53); therefore, it is not possible to discriminate whether the observed effects of NH_4Cl on intracellular VacA levels are specifically due to changes in lysosomal acidification, impaired protease activity, or a combination of these factors. We considered the possibility that NH_4Cl might alter intracellular VacA stability by preventing VacA trafficking to lysosomes. Indeed, weak bases can alter the pH of both endosomes and lysosomes, and regulated acidification is important for endosome maturation (63). However, we show that VacA localizes to both lysosomes and autophagosomes/autolysosomes in both the absence and presence of NH_4Cl . This suggests that NH_4Cl does not inhibit VacA degradation by disrupting the trafficking of VacA to the lysosome.

Recent studies have shown that upon exposure to pore-forming toxins (PFTs), cells can potentially respond, adapt, and survive (36, 40, 64, 65). Degradation of the toxin is one strategy that may be employed by cells to limit the extent of PFT-induced damage (39, 41, 66). Interestingly, a previous study suggested that the stability of intracellular VacA is modulated by autophagy (67). The authors reported that AGS cells treated with a pulse of BCF for 6 h and chased for 24 h degrade VacA over time, concomitant with the disappearance of cell vacuoles, while no degradation of VacA was observed in ATG12 KD cells, and vacuoles remained present (67). In contrast, we show that neither knockdown of ATG5 nor knockout of ATG16L1 has an effect on VacA degradation. One possible explanation for why our results differ from those of the previous study is that cells may have a different response to VacA following a short exposure to the purified toxin (1-h pulse at 4°C in our study) than to extended exposure to BCF from VacA-producing *H. pylori* strains (6-h pulse at 37°C in a previous study) (67). Perhaps the toxin molecules internalized into cells following a short exposure are primarily degraded in the lysosome, whereas cells that internalize high doses of VacA following a long exposure might utilize both lysosomal degradation and autophagy to degrade the toxin.

VacA has been reported to induce cell death via both apoptosis and necrosis (32, 33, 35); conversely, a recent study reported that VacA-induced cellular alterations do not induce apoptosis (68). Proposed mechanisms by which VacA induces cell death include direct pore formation in the mitochondrial membrane (48, 69, 70) or processes independent of mitochondrial targeting (71). In our analysis of VacA localization using live- and fixed-cell imaging, we did not detect a consistent increase in VacA localization to mitochondria over time, and NH_4Cl had no detectable effect on VacA localization to mitochondria. Inability to observe VacA targeting to mitochondria in the current study is consistent with the results of one previous study (71), but this result differs from results of multiple other studies that used fluorescence microscopy to detect mitochondrial localization of VacA (34, 47–50, 70). Other studies have detected interactions of VacA or VacA-containing vesicles with mitochondria using subcellular fractionation, *in vitro* experiments with isolated mitochondria, or immunogold labeling (34, 48, 69, 70, 72). The difference in results reported in the current study compared to those of previous studies may be attributable to the use of a different methodology for detecting VacA localization. Specifically, this is the first time quantitative colocalization measurements have been combined with live-cell imaging to analyze the putative interaction between VacA and mitochondria. In a static fluorescence microscopy image,

FIG 7 Proposed model for the cellular response to VacA intoxication. In a healthy cell, VacA is internalized, trafficked to the lysosome, and degraded. If lysosome activity is inhibited, VacA can accumulate in the lysosome, and cells exhibit vacuolation and eventually cell death.

two objects that are in close proximity can appear colocalized due to resolution limitations. The benefit of live-cell imaging is that it permits the visualization of intracellular dynamics. The use of live-cell imaging allowed us to see that most VacA-containing vesicles that appear to be colocalized with mitochondria at a single time point did not remain colocalized with mitochondria over time. This implies that VacA is sometimes detected in the vicinity of mitochondria but is not stably localized to mitochondria. Consistent with our results, the only other study that used quantitative methods to assess VacA colocalization with mitochondria over time also found that most intracellular VacA is not associated with mitochondria (68). Therefore, the results in the current study suggest that VacA-induced cell death might occur through mechanisms that do not require direct targeting of mitochondria by the toxin. While we cannot rule out the possibility that a minor subpopulation of VacA localizes to mitochondria or that the conditions of our experiments did not permit the targeting of VacA to mitochondria, our results clearly indicate that, in the absence of exogenous NH_4Cl , cells are resistant to VacA-induced cell death.

VacA is known to maintain activity under low-pH conditions and is relatively resistant to proteolytic digestion (73, 74). Therefore, it has been proposed that the toxin might resist degradation in lysosomes (61). The current study provides evidence that cells are capable of degrading intracellular VacA, and the data indicate that intracellular degradation of VacA allows cells to resist VacA-induced cell death. Conversely, if this process is impaired and intracellular levels of VacA accumulate, cells eventually undergo cell death (Fig. 7). We speculate that VacA induces cell death by forming pores at the cell surface and in endosomes/lysosomes to disrupt cellular homeostasis. More specifically, we propose that cells are able to tolerate a low level of VacA-induced homeostatic imbalances but that when intracellular levels of VacA accumulate, these disruptions to cellular homeostasis reach a tipping point and trigger pro-cell-death pathways. In support of this model, a recent study reported alterations in cellular amino acid homeostasis following treatment of cells with VacA (75).

In conclusion, this study shows that the ability of weak bases to enhance VacA activity is due at least in part to an ability of weak bases to inhibit VacA degradation. We presume that the effects of VacA on host cells are determined by the concentrations of toxin to which cells are exposed as well as by the rate at which intracellular VacA is degraded. Individuals infected with *H. pylori* are reported to have elevated levels of ammonia in their stomach due to the activity of *H. pylori* urease and other enzymes (76–78). Therefore, during infection, the concentration of ammonia or other weak bases

in the mucus layer overlying gastric epithelial cells may influence the magnitude of VacA toxicity. Cells in the gastric mucosa directly in contact with *H. pylori*, exposed to high concentrations of VacA and high concentrations of ammonia, would be at the highest risk of undergoing cell death. Conversely, cells localized at a distance from *H. pylori*, exposed to lower concentrations of VacA and lower concentrations of ammonia, would be less likely to undergo VacA-induced cell death; subtle VacA-induced changes in these cells could potentially confer benefits to *H. pylori* *in vivo*. The manipulation of host cell lysosome activity to prevent toxin degradation is presumably not a unique property of *H. pylori*. Many intracellular pathogens are capable of subverting lysosomal function, which potentially alters the degradation of secreted bacterial proteins. In future studies it will be important to investigate whether the manipulation of host cell degradation pathways is a strategy commonly utilized by extracellular bacteria to inhibit toxin degradation.

MATERIALS AND METHODS

Cell culture and stable cell line generation. AGS cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, penicillin, and streptomycin. AGS cells were verified by ATCC to be an exact match of the ATCC human cell line CRL-1739. HeLa Kyoto cells and HeLa ATG16L1 KO and parental cells (Edigene) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin.

A nontargeting scramble control shRNA and two ATG5 shRNAs (Sigma) expressed from the pLKO.1 vector (Addgene) were used to create stable ATG5 KD cell lines using lentiviral transduction. Lentivirus particles containing the shRNA sequences were generated as previously described (79). For lentivirus transduction, AGS cells were grown to 70% confluence, medium was supplemented with 10 μ g/ml Polybrene (Millipore Sigma), and cells were infected with lentivirus for 24 h. After infection, cells were allowed to recover for 24 h. Cells were then cultured in medium supplemented with 4 μ g/ml puromycin to select for stable integration. After selection, AGS scramble and ATG5 KD cell lines were cultured in medium containing 2 μ g/ml puromycin. The cell line created from shRNA clone TRCN0000151963 is referred to as the ATG5 KD1 cell line. The cell line created from shRNA clone TRCN0000330392 is referred to as the ATG5 KD2 cell line.

Purification and Alexa Fluor 488 labeling of VacA. Untagged VacA was purified from broth culture supernatant of *H. pylori* strain 60190 (ATCC 49503), which produces an s1i1m1 form of VacA, as described previously (62, 74). Strep-tagged VacA (with a Strep-tag II at position 808) was purified using Strep-Tactin resin (IBA) as previously described (80). Purified VacA was labeled using an Alexa Fluor 488 Microscale Protein Labeling kit (Thermo Fisher Scientific) or an Alexa Fluor 488 Antibody Labeling kit (Thermo Fisher Scientific) according to the manufacturer's instructions. There are no noticeable differences in activities between untagged, Strep-tagged, or Alexa Fluor 488-labeled VacA proteins (21, 80). Labeled VacA was flash frozen in single-use aliquots and stored at -80°C .

VacA treatment of cells. For all experiments, VacA was acid activated before use by dropwise addition of 200 mM HCl until the pH was reduced to ~ 4.0 (62, 73, 74). Since the physiological concentration of VacA to which cells are exposed *in vivo* is not known, we conducted most experiments using a concentration of VacA (5 μ g/ml or ~ 55 nM) that is sufficiently high to cause detectable cellular alterations but not substantially higher, corresponding to one of the lowest concentrations commonly used for *in vitro* experiments (20, 47, 49, 50, 75). For the cell viability assays shown in Fig. 1, cells were treated with 5 μ g/ml (55 nM) or 20 μ g/ml (222 nM) of VacA once a day for 5 days. For VacA degradation and trafficking studies, cells were treated with a pulse of 5 μ g/ml VacA for either 1 h at 4°C or 5 min at 37°C . In general, the treatment method of 1 h at 4°C was used for experiments involving Western blotting in order to ensure sufficient binding of VacA to cells important for the subsequent detection of protein bands and for experiments involving a 0-min time point in order to ensure that endocytosis was inhibited. Alternatively, for time course experiments requiring addition of VacA to cells in a multiwell plate at multiple different time points, cells were pulsed with VacA for 5 min at 37°C in order to avoid the repeated exposure of cells to 4°C temperatures. Then medium overlying cells, containing unbound VacA, was removed, cells were washed with phosphate-buffered saline (PBS), warm culture medium was added, and cells were incubated at 37°C in the presence of respective inhibitors or mock treatments for the times indicated in the figure legends or figures. Transmitted-light images were acquired with an EVOS FL digital inverted microscope (AMG) to assess for vacuolation.

Cell viability. Cell viability was quantified using an ATPlite 1step Luminescence Assay (Perkin Elmer) according to the manufacturer's instructions. Cells were seeded in 96-well flat-bottom black polystyrene plates (Corning). Luminescence was measured with a Synergy HT microplate reader (BioTek).

Chemical inhibitors. Ammonium chloride (NH_4Cl ; 5 mM or 25 mM final concentrations [Sigma]) and chloroquine (CQ; 100 μ M final concentration [Sigma]) were prepared in culture medium from a water-based stock solution. High levels of NH_4Cl can cause cell death; however, minimal reductions in cell viability are observed in the presence of 5 mM NH_4Cl (81, 82). MG132 (5 μ M final concentration; Calbiochem) and bafilomycin A1 (Baf A1; 10 nM final concentration [Sigma]) were prepared in culture medium from a dimethyl sulfoxide (DMSO)-based stock solution. 3-Methyladenine (3-MA; 10 mM final concentration [Sigma]) was prepared in culture medium from a water-based stock solution made

immediately before each experiment. Control cells were mock treated with either water or DMSO, as appropriate.

Transfections and live-cell imaging. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The following amounts of plasmid DNA were transfected into cells for all experiments: 1 μ g/ml Mito-RFP (where RFP is red fluorescent protein), mCherry-Rab5a, and mCherry-Rab7.

For live-cell imaging of VacA trafficking, transfected cells were seeded on glass-bottom poly-D-lysine-coated dishes (MatTek) ~24 h posttransfection and treated with VacA ~24 h postseeding. Cells were imaged in the presence of CO₂ at 37°C in movie medium (Leibovitz's L-15 medium without phenol red, 10% FCS, penicillin, streptomycin, 7 mM HEPES, pH 7.7) using a 60 $\times$ /1.4 numerical aperture (NA) objective (Olympus) on a DeltaVision Elite imaging system (GE Healthcare) equipped with a Cool SnapHQ2 charge-coupled-device (CCD) camera (Roper). Optical sections were collected at 200-nm intervals and deconvolved in SoftWorx (GE Healthcare). Movies and images were prepared for publication using ImageJ.

Immunostaining and fixed-cell imaging. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences and Alfa Aesar) for 15 min at 37°C. The following primary antibodies were used in this study: anti-MTCO2 at 1:200 (Abcam), anti-LAMP1 at 1:100 (Cell Signaling Technology), and anti-LC3 at 1:200 (Medical and Biological Laboratories). Cells were incubated with primary antibodies for 1 h 45 min. Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen) were used at 1:1,000 for 45 min. DNA was counterstained with 5 μ g/ml Hoechst 33342. Stained cells were mounted with Prolong Gold (Invitrogen) and imaged using a 60 $\times$ /1.4 NA or a 100 $\times$ /1.4 NA objective (Olympus) on the aforementioned DeltaVision Elite system. Either single optical slices or z-sections spaced 200 nm apart were acquired and deconvolved in SoftWorx (GE Healthcare), unless noted otherwise. Images were prepared for publication using ImageJ. To determine the Pearson correlation coefficient (PCC), single z-slice images were collected using the 100 $\times$ /1.4 NA objective, and the PCCs were determined in ImageJ from nondeconvolved images using the intensity correlation analysis plug-in (83). Nuclei were excluded from the colocalization analysis. The number of experimental replicates and the number of cells measured are mentioned in Table S1 in the supplemental material. To quantify fluorescence intensities, z-sections were collected using the 60 $\times$ /1.4 NA objective, and ImageJ was used to create maximum-intensity z-stack projections and to measure the integrated densities of individual cells.

Preparation of total cell lysate. To analyze VacA protein levels in treated cells, trypsinized VacA-treated cells were pelleted, washed with PBS, resuspended in ice-cold NP-40 lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% NP-40) with protease inhibitors, and incubated for 15 min on ice. Total cell lysate was clarified by spinning at maximum speed (16,000 relative centrifugal force [rcf]) for 15 min at 4°C. Extracts were transferred to new microcentrifuge tubes on ice, and protein concentration was determined using a Bradford assay (Bio-Rad). To store samples, protein extracts were mixed with 4 $\times$ lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) with supplemental β -mercaptoethanol (BME) (Sigma), boiled for 5 min, and frozen at -20°C.

Immunoblotting. Equivalent amounts (20 to 60 μ g) of protein extracts were resolved on 4 to 12% Bis-Tris SDS-PAGE gels (Life Technologies) and transferred onto polyvinylidene difluoride (PVDF) (EMD-Millipore) or nitrocellulose membrane (PerkinElmer). Immunoblots were blocked with 50% (vol/vol) Odyssey blocking buffer diluted in PBS or 2% milk diluted in Tris-buffered saline plus Tween 20 (TBST) for 1 h at room temperature (RT) and then probed with primary antibody overnight at 4°C or 1 h at room temperature. The following primary antibodies were used in this work: anti-VacA (19, 22) (1:5,000 or 1:10,000), anti-DM1 α (1:5,000) (Sigma-Aldrich), anti-ubiquitin (Life Sensors), anti-ATG5 (1:1,000) (Abcam), anti-ATG16L1 (1:5,000) (Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000) (Abcam). For fluorescence detection, secondary antibodies conjugated to Alexa Fluor 700 (Invitrogen) or Alexa Fluor 800 (Li-Cor Biosciences) were used at 1:5,000 for 45 min at room temperature, and bound antibodies were detected using an Odyssey CLx imaging system (Li-Cor Biosciences). For chemiluminescence detection, secondary antibodies conjugated to horseradish peroxidase (HRP) (Promega) were used at 1:10,000 for 1 h at room temperature, and bound antibodies were detected by X-ray film. ImageJ was used to quantify signal intensity.

Statistical analysis. GraphPad Prism, version 5, was used to generate all graphs and perform statistical analysis. Differences between two groups were assessed by a *t* test. Differences between multiple groups were assessed by analysis of variance (ANOVA) followed by the Dunnett's multiple-comparison test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00783-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

SUPPLEMENTAL FILE 2, AVI file, 1.2 MB.

SUPPLEMENTAL FILE 3, AVI file, 1.8 MB.

SUPPLEMENTAL FILE 4, AVI file, 0.5 MB.

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N.J.F., K.R., T.L.C., and M.D.O. conceived and designed the experiments. N.J.F. performed the experiments. A.M.C. and S.W.K. provided reagents. N.J.F., K.R., T.L.C., and M.D.O. analyzed the data. N.J.F., T.L.C., and M.D.O. wrote the manuscript. All authors edited and approved the manuscript.

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