Cellular Vacuolation and Mitochondrial Cytochrome c Release Are Independent Outcomes of Helicobacter pylori Vacuolating Cytotoxin Activity That Are Each Dependent on Membrane Channel Formation*

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Helicobacter pylori vacuolating toxin (VacA) is a secreted toxin that is reported to produce multiple effects on mammalian cells. In this study, we explored the relationship between VacA-induced cellular vacuolation and VacA-induced cytochrome c release from mitochondria. Within intoxicated cells, vacuolation precedes cytochrome c release and occurs at lower VacA concentrations, indicating that cellular vacuolation is not a downstream consequence of cytochrome c release. Conversely, bafilomycin A1 blocks VacA-induced vacuolation but not VacA-induced cytochrome c release, which indicates that cytochrome c release is not a downstream consequence of cellular vacuolation. Acid activation of purified VacA is required for entry of VacA into cells, and correspondingly, acid activation of the toxin is required for both vacuolation and cytochrome c release, which suggests that VacA must enter cells to produce these two effects. Single amino acid substitutions (P9A and G14A) that ablate vacuolating activity and membrane channel-forming activity render VacA unable to induce cytochrome c release. Channel blockers known to inhibit cellular vacuolation and VacA membrane channel activity also inhibit cytochrome c release. These data indicate that cellular vacuolation and mitochondrial cytochrome c release are two independent outcomes of VacA intoxication and that both effects are dependent on the formation of anion-selective membrane channels.

Persistent infection of the human gastric mucosa with the Gram-negative pathogen Helicobacter pylori causes chronic inflammation and is a strong risk factor for development of peptic ulcer disease or gastric cancer (1–9). Pathogenic strains of H. pylori secrete a vacuolating cytotoxin (VacA),1 that induces the formation of large intracellular vacuoles within gastric epithelial cells both in vitro and in vivo, presumably by causing a defect in vesicular trafficking (10–12). Oral administration of purified VacA to mice results in degeneration of the gastric mucosa and inflammatory cell recruitment (13, 14). In addition, VacA was recently reported to be important for colonization of H. pylori in a mouse model (15). Whereas increasing evidence indicates that VacA is an important virulence factor in H. pylori associated peptic ulcer disease, the exact role that VacA plays in H. pylori colonization and pathogenesis has not yet been elucidated.

VacA has recently been implicated as an H. pylori factor capable of inducing apoptosis both in vitro and in vivo (16–19). Apoptosis within the gastric mucosa is strongly associated with H. pylori infection (20–23), and H. pylori has been shown to induce apoptosis in murine and gerbil models of infection (24–28). Although multiple H. pylori factors have been linked to the initiation of apoptosis (29–32), VacA was recently demonstrated to be sufficient to induce apoptosis in gastric epithelial cells (33). Intracellular expression of VacA in transiently transfected HeLa cells or the application of purified toxin to HeLa monolayers has been reported to induce the release of cytochrome c from the intermembrane space of mitochondria, which suggests that VacA-induced apoptosis may occur via a mitochondria-dependent pathway (16). However, neither the mechanism of VacA-mediated cytochrome c release nor the relationship of this cellular activity to the more thoroughly characterized cell-vacuolating activity of VacA has been investigated.

To explore the relationship between VacA-mediated cellular vacuolation and cytochrome c release, we conducted single cell analyses using a stably transfected HeLa cell line expressing a genetically derived fusion protein comprising cytochrome c and green fluorescence protein (CcGFP-HeLa), which is localized to the mitochondria (34). The CcGFP-HeLa cell line has emerged as a powerful system for studying fundamental mechanisms of mitochondrial perturbations leading to cytochrome c release (34–38). Collectively, our data indicate that cellular vacuolation and cytochrome c release are two independent cellular effects induced by VacA and that both are dependent on VacA-associated intracellular channel activity.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium, fetal bovine calf serum, phosphate-buffered saline (PBS), penicillin-streptomycin, cell dissociation buffer, and trypsin-EDTA were purchased from Invitrogen. H. pylori strain J1 was used in all experiments. Antimicrobial agents were purchased from Sigma.

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5-nitro-2-(3-phenylpropylamino)benzoic acid; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.
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60190 (ATCC 49503) was received from the American Type Culture Collection (Manassas, VA). Bacterial culture media were obtained from Difco, Vancomycin, bafloxicin A1, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), DIDS, anti-rabbit immunoglobulin G-alkaline phosphatase conjugate, Phalloidin-TRITC, and most common laboratory chemicals and reagents were obtained from Sigma. 25- and 75-cm² plastic flasks, 8-well chamber slides, and 96-well plates were obtained from Corning (Cambridge, MA). Centricon 100 centrifugal microconcentrators and filtration units were from Millipore (Bedford, MA). Purification matrices were from Amersham Biosciences. Tyramide signal amplification kit and anti-green fluorescent protein-Alexa Fluor 488 conjugate were from Molecular Probes (Eugene, OR).

Cell Culture—CcGFP-HeLa cells were a gift from Dr. D. B. Green (34). HeLa cells and CcGFP-HeLa cells were grown in minimal essential medium supplemented with 2 mM glutamine, 200 mg of penicillin/ml, 100 µg of streptomycin sulfate/ml, 100 µg of G418 sulfate/ml (CcGFP-HeLa only), and 10% fetal bovine calf serum and maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO₂.

H. pylori Growth and VacA Purification—H. pylori 60190 and 60190 isogenic mutant strains were cultivated and purified as described previously (39). H. pylori was grown in culture flasks on a rotary platform shaker for 18 h at 37 °C under a 5% CO₂ atmosphere in bisulfite- and sulfite-free brucella broth (10 g of tryptone/-liter, 10 g of Bacto-proteose peptone/liter, 5 g of NaCl/liter, 2 g of yeast extract/liter, 1 g of dextrose/liter) supplemented with either 2 g of β-cyclodextrin/liter or 5 g of activated charcoal/liter. H. pylori cultures were harvested by centrifugation at a relative centrifugal force of 7,500 by using a Sorvall RC2-B centrifuge at 4 °C. VacA was initially fractionated and concentrated by ammonium sulfate (50%) precipitation. The ammonium sulfate-saturated pellet was resuspended and dialyzed into PBS, pH 7.2. Concentrated VacA was further purified by fast protein liquid chromatography by using a Superose 6 preparative grade resin in a HR16/50 column previously equilibrated at 4 °C in PBS, pH 7.2. The fractions were concentrated using Centricon 100 centrifugal microconcentrators, tested for vacuolating activity, and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. For all of the assays requiring acid activation, 0.2 (v/v) 300 mM HCl was added to VacA preparations and incubated for 30 min at 37 °C and then neutralized with the same volume of 300 mM NaOH. As a control, PBS was “acid-activated” using the same protocol.

Vacuolation Assay—Relative vacuolation was quantified based on the uptake of the dye Neutral Red within mammalian cells as described previously (40). The experiments were performed in 96-well plates, and Neutral Red uptake was determined using a Dynatech MBR5000 microtiter plate reader to measure the absorbance at 530 nm (minus the absorbance at 410 nm). Unless otherwise specified, all of the assays were performed in the presence of 5 mM NH₄Cl including controls (41).

Cytochrome c Release Quantification—Confocal images were collected using a Zeiss LSM 310 confocal microscope (Thornwood, NY) with an Omnichrome external Ar/Kr ion laser (Melles Griot, Irvine, CA). CcGFP-HeLa cells were seeded at 5 × 10⁴ cells/ml and 400 µM well on 8-well microscopy slides and grown overnight. At the indicated intoxication times, cells were fixed, permeabilized, and stained with 4% paraformaldehyde or stained as described above. When necessary for signal enhancement, cells were permeabilized with 0.2% Triton X-100 for 10 min, washed three times with PBS, and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 h followed by 60-min incubation with 5 µg of anti-green fluorescent protein-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR). Images were collected with a lubricator attenuated at 50% using a ×40 or 63 objective at ×2–4 zoom, and frame was averaged four times. Release of cytochrome c-GFP was determined by field count. Data are expressed as the percentage of cells demonstrating cytochrome c release. All of the data represent the average (or a representative image) of at least 20 cells from the same culture.

VacA Internalization Assay—HeLa cells were seeded on 8-well Lab-Tek chamber slides at 5 × 10⁴ cells/ml and 400 µM well and were grown overnight under 5% CO₂. Acid-activated or unactivated VacA was applied for 18 h. Cells were then washed three times with PBS, fixed, and permeabilized as indicated above. At room temperature, the monolayers were washed three times with PBS and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 h followed by 1 h of incubation with 1:5000 dilution of rabbit polyclonal anti-VacA. The slides were then washed three times with PBS and incubated with 1:100 anti-rabbit immunoglobulin peroxidase conjugate in blocking buffer for 30 min at room temperature. For detection of VacA, we used the tyramide signal amplification kit with Alexa Fluor 488 according to the manufacturer’s specifications. Cells were then incubated with 1 µg of phallolidin-TRITC/ml for 20 min, washed twice with PBS, and imaged by confocal microscopy as indicated above.

RESULTS

VacA Induces Cytochrome c Release into the Cytosol of Intact Cells—A previous study (16) reported that intracellular expression of VacA or application of purified toxin to HeLa cells resulted in the release of cytochrome c from mitochondria based on the detection of cytochrome c in the soluble fraction of lysates prepared from VacA-intoxicated cells. However, cellular fractionation can potentially induce mechanical disruption of mitochondria (34). To study effects of VacA on cytochrome c release in a system that does not require cellular fractionation, we performed single cell analysis of HeLa cells with a stably integrated gene expressing cytochrome c fused to green fluorescence protein (34, 37, 38). An analysis of untreated individual cells within monolayers by fluorescence microscopy revealed punctate green fluorescence in the cytosol of each cell, which is the expected pattern when cytochrome c-GFP is localized to the mitochondria (Fig. 1, panel A). In contrast, incubation of monolayers with the apoptosis-inducing agent actinomycin D (1 µM) yielded cells demonstrating diffuse green fluorescence throughout the cell including the nucleus (Fig. 1, panel B), which is the pattern expected following release of cytochrome c-GFP from the mitochondria (34).

To test whether VacA induces cytochrome c release within intact cells, monolayers of CrGFP-HeLa cells were incubated with purified acid-activated VacA (750 nm, calculated using 88-kDa VacA molecular mass (42)) for 18 h. In contrast to the untreated cells, virtually all of the VacA-treated cells within the monolayer demonstrated diffuse green fluorescence, indicating that cytochrome c-GFP was dispersed throughout the cells (Fig. 1, panel C). Because the cells were extensively vacuolated (Fig. 1, panel F), which is a well documented characteristic of VacA-intoxicated cells (11, 43), the diffuse green fluorescence was most readily evident in the nucleus. These results indicate that VacA induces cytochrome c release from mitochondria within living intact cells. This finding confirms and extends the previous observation that expression of VacA in the cytosol of transfected cells or application of purified toxin results in the release of cytochrome c from mitochondria (16). In addition, the use of mammalian cells stably transfected with cytochrome c-GFP provides a convenient and powerful approach to determine the percentage of cells within a monolayer that have undergone cytochrome c release.
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**Cellular Vacuolation and Cytochrome c Release as Function of VacA Concentration**—To determine the relationship between VacA-induced cellular vacuolation and VacA-induced cytochrome c release, we investigated the extent of vacuolation and cytochrome c release as a function of VacA concentration. Monolayers of CcGFP-HeLa cells were incubated with a range of different concentrations of purified acid-activated VacA (1–1000 nM). After 18 h, the monolayers were analyzed for both vacuolation and cytochrome c release. These studies revealed very different dose-response profiles for vacuolation and cytochrome c release (Fig. 2). Whereas vacuolation was detectable at 10 nM VacA and maximum vacuolation of monolayers occurred at 100 nM toxin (44), cytochrome c release was detected only with VacA concentrations >200 nM and maximum release of cytochrome c required VacA concentrations of 500 nM. The dose-response curves for vacuolation and cytochrome c release did not change when monolayers incubated with VacA were analyzed after extended periods (48 or 72 h) (data not shown), suggesting that changes in the cell because of VacA intoxication occur prior to 18 h.

**Temporal Relationship between VacA-mediated Vacuolation and Cytochrome c Release**—To determine the temporal relationship between cellular vacuolation and cytochrome c release, we monitored the time course of cellular vacuolation and cytochrome c release in cells intoxicated with VacA. Acid-activated VacA (750 nM) was incubated with monolayers of CcGFP-HeLa cells, and both vacuolation and cytochrome c release were monitored over 18 h as described under “Experimental Procedures.”

These experiments revealed that cellular vacuolation and cytochrome c release occur by distinct temporal programs within intoxicated cells (Fig. 3). Vacuolation was evident within the monolayer as early as 1 h after the addition of toxin and progressively increased until approximately a 4-h time point when Neutral Red uptake reached a maximum value. In contrast, no cytochrome c release could be detected within the monolayer at the 4-h time point, but by 5 h, the vast majority of cells within any given field demonstrated cytochrome c release. Thus, vacuolation was detected in intoxicated cells at relatively early time points compared with the release of cytochrome c.

**Inhibition of VacA-induced Cellular Vacuolation Does Not Block VacA-mediated Cytochrome c Release**—To further explore the relationship between cellular vacuolation and cytochrome c release, we tested the effect of a known inhibitor of VacA-induced vacuolation on cytochrome c release. Cellular vacuolation induced by VacA is dependent on the action of vacuolar-type ATPase proton pumps (45). Vacuolation in VacA-intoxicated cells is blocked by bafilomycin A1, which targets vacuolar-type ATPase proton pumps (46, 47). To determine whether VacA-mediated vacuolation is essential for cytochrome c release in VacA-intoxicated cells, CcGFP-HeLa cells were preincubated with bafilomycin A1 (40 nM) for 1 h prior to application of acid-activated VacA (750 nM). As expected, there was no detectable vacuolation in bafilomycin A1-treated monolayers after 18 h, whereas control monolayers not exposed to bafilomycin A1 were fully vacuolated (data not shown). In contrast, essentially all of the cells within monolayers treated with bafilomycin A1 demonstrated cytochrome c release (Fig. 4, panel B), indicating that even in cells in which vacuolation had been blocked by bafilomycin A1, VacA was able to induce cytochrome c release.

**Activation of VacA Is Important for Toxin-mediated Mitochondrial Cytochrome c Release**—There is substantial evidence that VacA associates with and enters mammalian cells to elicit an intracellular activity resulting in vacuolation (44, 47–51). Notably, the entry of VacA into target cells is facilitated by activation, which consists of briefly exposing the toxin to acidic pH (44). Activation of VacA results in a significant increase in cellular vacuolation (39, 44, 52–54), which suggests that internalization is an important step in the mechanism of VacA-mediated cellular vacuolation. Because vacuolation experiments are routinely performed with activated VacA, we next tested whether activation of VacA is important for toxin-mediated cytochrome c release. Both activated and unactivated VacA were incubated with CcGFP-HeLa cells for 18 h. Using confocal microscopy, we confirmed that unactivated VacA enters cells much less efficiently than activated VacA (Fig. 5, panel B). In contrast to experiments conducted with activated VacA (Fig. 5, panel D), cytochrome c release was not detected in monolayers incubated with VacA (up to 1 μM) that had not been previously activated, even after 48 h (Fig. 5, panel E). Whereas previous experiments with transfected cells indicated that expressing VacA directly within the cytosol of target cells is sufficient to induce cytochrome c release (16), these data now suggest that VacA entry into cells is essential for cytochrome c release.
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**Figure 4.** The effects of blocking VacA-mediated cellular vacuolation on cytochrome c release. Monolayers of CcGFP-HeLa cells incubated at 37 °C in a humidified atmosphere under 5% CO₂ with PBS (panel A) or acid-activated VacA (750 nM, panel B). 1 h prior to PBS or VacA application, bafilomycin A1 was applied to the cells and maintained at 40 nM throughout. After 18-h intoxication, the cells were analyzed by confocal microscopy for cytochrome c release.

**Figure 5.** Acid-activation of VacA is required for internalization of VacA. Monolayers of HeLa cells (panels A–C) or CcGFP-HeLa cells (panels D–F) were incubated at 37 °C in a humidified atmosphere under 5% CO₂ with acid-activated VacA (750 nM, panels A and D), unactivated VacA (750 nM, panels B and E), or PBS (panels C and F). After 18 h, the cells were fixed and processed as described under "Experimental Procedures" and analyzed by confocal microscopy. In panels A–C, the cells were visualized with phalloidin-TRITC stain (red) and indirect immunofluorescence (green) for VacA.

VacA-mediated Cytochrome c Release Is Sensitive to Channel Inhibitors—VacA-mediated cellular vacuolation was earlier demonstrated to be sensitive to channel inhibitors that had been shown to block VacA membrane channel activity (55, 56), which suggests that membrane channels formed by VacA may be important for vacuole biogenesis. To explore whether membrane channels formed by VacA may also be involved in cytochrome c release, we tested the effects of pretreating CcGFP-HeLa cells with channel inhibitors known to block both VacA-mediated channel activity and cellular vacuolation. CcGFP-HeLa cells were pretreated for 1 h with DIDS (400 μM) or NPPB (200 μM) prior to incubation with VacA (750 nM) at 37 °C. These experiments revealed that both DIDS and NPPB inhibited vacuole formation and cytochrome c release to approximately the same extent (Fig. 6).

**Mutant Forms of VacA Deficient in Ion-conducting Channel Activity Are Unable to Induce Cytochrome c Release**—To further explore the potential correlation between VacA channel activity and toxin-induced cytochrome c release, we tested mutant forms of VacA attenuated in channel-forming activity for the ability to induce cytochrome c release in CcGFP-HeLa cells. We previously demonstrated that substituting alanine for either Pro-9 or Gly-14 completely ablated VacA-mediated intracellular vacuolation activity (57, 58). Pro-9 and Gly-14 were the first single amino acid substitutions identified that fully eliminated the vacuolating activity of the toxin and suggested that the VacA amino terminus is especially sensitive to mutation. Recently, both VacA-P9A and VacA-G14A were demonstrated to be deficient in VacA channel activity, further supporting a functional link between the membrane channel activity of the toxin and cellular vacuolation (57, 58). To probe whether these single point mutations also affect VacA-mediated cytochrome c release, CcGFP-HeLa cells were incubated at 37 °C with purified VacA-G14A and VacA-P9A (1 μM) produced from isogenic mutant strains. After 18 h, neither VacA-G14A nor VacA-P9A induced detectable cytochrome c release (Fig. 7, panels C and D). Confocal microscopy confirmed that both of these VacA mutant forms entered cells in a manner similar to wild-type toxin (data not shown). Unpurified culture filtrates from wild-type H. pylori strain 60190 induced cytochrome c release, whereas culture filtrates from H. pylori isogenic mutants strains producing either VacA-G14A or VacA-P9A, whose concentrations were normalized based on Western blot analysis, did not induce cytochrome c release (data not shown). This provides strong evidence that VacA alone is the factor released by H. pylori that is responsible for this cellular activity.

**VacAΔ-(6–27) Is Dominant Negative for Toxin-mediated Cytochrome c Release**—A mutant form of VacA with residues 6–27 deleted (VacAΔ-(6–27)) was earlier shown to be unable to induce cellular vacuolation (59). Characterization of VacAΔ-(6–27) revealed that this mutant form of the toxin was similar to wild-type toxin in stability, oligomer formation, lipid association, cell binding, and cellular uptake. However, closer examination revealed that VacAΔ-(6–27) was defective in the capacity to form anion-selective membrane channels (59, 60). To determine whether VacAΔ-(6–27) induces cytochrome c release, we incubated CcGFP-HeLa cells with either VacAΔ-(6–27) alone or mixtures of VacAΔ-(6–27) and wild-type toxin. Examination of monolayers after 18 h revealed no detectable cytochrome c release in cells incubated with VacAΔ-(6–27) (Fig. 8). Confocal microscopy confirmed that VacAΔ-(6–27) entered cells in a manner similar to wild-type toxin (data not shown).

When added to cells together with wild-type VacA, VacAΔ-(6–27) inhibits the vacuolating activity of wild-type VacA in a dominant negative manner, presumably because of the formation of mixed hetero-oligomers (59, 60). Notably, mixed hetero-oligomers comprised of wild-type VacA and VacAΔ-(6–27) were also deficient in channel formation (59, 60). We tested whether VacAΔ-(6–27) also could inhibit the capacity of wild-type VacA to induce cytochrome c release. When different molar ratios of wild-type VacA and VacAΔ-(6–27) were mixed and added to cells, detectable cytochrome c release was not observed in monolayers until greater than a 5-fold excess of wild-type toxin was used (Fig. 8). These data suggest that VacAΔ-(6–27) inhibits VacA-mediated cytochrome c release in a dominant negative manner, similar to the inhibition of vacuolation activity, and further supports the model that VacA-induced release of cytochrome c is dependent on the formation of membrane channels. Although VacA-P9A and VacA-G14A were also deficient in channel activity (58), these mutant forms were not capable of inhibiting wild-type toxin for either vacuolation or cytochrome c release activity in a dominant-negative fashion (data not shown), suggesting fundamental differences between the effects of these mutations and the perturbation resulting from the deletion of residues 6–27.

**Discussion**

Vacuolation and cytochrome c release are two seemingly independent, albeit very different, cellular outcomes of VacA intoxication. In the current study, we sought to understand whether common biochemical mechanisms underpin these two activities. Our data indicate for the first time that VacA channel-forming activity is important for cytochrome c release. Specifically, we have demonstrated that three distinct VacA mu-
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In vacuolation-deficient forms of VacA with single amino acid substitutions are unable to induce cytochrome c release. A–H, monolayers of CcGFP-HeLa cells incubated at 37 °C in a humidified atmosphere under 5% CO2 with PBS (panels A and F), acid-activated VacA (panels B, C, E, and G), and F or 400 μM DIDS (panels C, D, G, and H) was applied to cells and maintained at these concentrations after VacA was applied. After 8 h, the cells were analyzed by laser-scanning confocal microscopy (LSM, panels A–D) or stained with Neutral Red and imaged using light microscopy (panels E–H).

Fig. 6. VacA induction of mitochondrial cytochrome c release is sensitive to channel inhibitors. A–H, monolayers of CcGFP-HeLa cells incubated at 37 °C in a humidified atmosphere under 5% CO2 with acid-activated VacA (750 nM) (panels B, D, F, and H) or PBS (panels A, C, E, and G), 1 h prior to VacA application, 200 μM NPPB (panels A, B, E, and F) or 400 μM DIDS (panels C, D, G, and H) was applied to cells and maintained at those concentrations after VacA was applied. After 8 h, the cells were analyzed by laser-scanning confocal microscopy (LSM, panels A–D) or stained with Neutral Red and imaged using light microscopy (panels E–H).

Fig. 7. Inhibition of VacA-induced cytochrome c release by a dominant negative mutant form of VacA. Monolayers of CcGFP-HeLa cells incubated at 37 °C in a humidified atmosphere under 5% CO2 with PBS, acid-activated VacA, and VacA-D-Δ-(6–27) (750 nM for each protein) or mixtures of VacA and VacA-D-Δ-(6–27) acid-activated together at the indicated molar ratios are shown. After 18 h, the monolayers were analyzed for cytochrome c release as described under “Experimental Procedures.” Data represent the average of at least 20 cells over three independent experiments. *, p < 0.05 compared with samples treated with PBS.

Fig. 8. Inhibition of VacA-induced cytochrome c release by a dominant negative mutant form of VacA. Monolayers of CcGFP-HeLa cells incubated at 37 °C in a humidified atmosphere under 5% CO2 with acid-activated VacA, and VacA-D-Δ-(6–27) (750 nM for each protein) or mixtures of VacA and VacA-D-Δ-(6–27) acid-activated together at the indicated molar ratios are shown. After 18 h, the monolayers were analyzed for cytochrome c release as described under “Experimental Procedures.” Data represent the average of at least 20 cells over three independent experiments. *, p < 0.05 compared with samples treated with PBS.

Although our data indicate that cytochrome c release and vacuolation are each dependent on the formation of membrane channels by VacA, these two phenotypes represent independent outcomes of VacA intoxication. We initially hypothesized that VacA-induced cytochrome c release could lead to vacuolation as a downstream cellular stress response. Not only does the release of cytochrome c from mitochondria promote the formation of the apoptosis as a key step during apoptosis, it is also potentially devastating to the cell from a bioenergetics standpoint (63). However, this scenario is unlikely because we have demonstrated that VacA induces vacuolation at lower toxin concentrations than those required to induce cytochrome c release. Furthermore, at high VacA concentrations, vacuole biogenesis occurs at a time point significantly earlier than cytochrome c release. These results support the conclusion that cellular vacuolation is not a downstream consequence of VacA-mediated cytochrome c release.

Alternatively, the enormous commitment of cellular energy and resources to vacuole biogenesis and maintenance could ultimately result in apoptotic cellular death via a mitochondria-dependent pathway. In this case, cytochrome c release would be expected to occur only after the appearance of cellular vacuoles. However, this scenario is also unlikely because we have demonstrated cytochrome c release in the absence of cellular vacuolation through the application of bafilomycin A1. Moreover, the VacA dose-response curve for cytochrome c release did not change when vacuolation was blocked in this manner (data not shown). These results suggest that cytochrome c release is not a downstream consequence of vacuolation induced by VacA. Collectively, these results support a model in which both cellular vacuolation and cytochrome c release are dependent on VacA channel activity but neither cellular outcome is a direct result of the other.

It is not currently clear how VacA intoxication results in these two different cellular phenotypes. VacA entry into target cells is essential for both vacuolation and cytochrome c release, and therefore, we presume that VacA may form membrane channels in an intracellular location. Consistent with this model of intracellular channel formation, we recently demon-
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strated that VacA monomers associate inside of intoxicated cells and that assembly into a higher ordered structure is essential for intracellular vacuolating activity (61, 62). Conceivably, VacA could form ion-conducting channels at a single location within cells with the cellular outcome dependent on the concentration of toxin. For example, our data indicate that cytochrome c release requires a higher concentration of VacA than that for vacuolation, suggesting that higher levels of VacA channel activity may be required for cytochrome c release than for vacuolation. Alternatively, VacA may elaborate channel activity at multiple different sites within an intoxicated cell. Notably, VacA has been reported by different investigators to be localized to the cytosol (49), vacuoles (50), and the mitochondria (16). Conceivably, a single VacA activity, such as the formation of ion-conducting channels, may stimulate different cellular consequences depending only on localization of VacA within the cell. For example, VacA channels within membrane-bound vesicles could result in vacuole formation, whereas VacA channels at the mitochondria could result in cytochrome c release. Because vacuolation occurs well before cytochrome c release, we also cannot rule out at this time the possibility that a VacA binding site related to vacuolation becomes saturated before the toxin can bind to a second site related to the release of cytochrome c. Future research will require correlating intracellular localization of VacA to the different activities of the toxin.

In conclusion, our data support a model in which VacA enters cells and produces at least two independent cellular effects (vacuolation and cytochrome c release), which are each dependent on the formation of anion-selective membrane channels. The finding that VacA can produce two very different concentration-dependent outcomes supports the intriguing hypothesis that the in vivo function of VacA could depend upon, at least in part, controlling toxin levels at different times of infection within the stomach. Future research will be required to fully explore the temporal aspects versus spatial aspects of VacA elaboration by H. pylori and the relationship of the different VacA cellular activities to H. pylori-mediated pathogenesis.

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