Helicobacter pylori vacuolating cytotoxin A (VacA) engages the mitochondrial fission machinery to induce host cell death

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A number of pathogenic bacteria target mitochondria to modulate the host’s apoptotic machinery. Studies here revealed that infection with the human gastric pathogen Helicobacter pylori disrupts the morphological dynamics of mitochondria as a mechanism to induce host cell death. The vacuolating cytotoxin A (VacA) is both essential and sufficient for inducing mitochondrial network fragmentation through the mitochondrial recruitment and activation of dynamin-related protein 1 (Drp1), which is a critical regulator of mitochondrial fission within cells. Inhibition of Drp1-induced mitochondrial fission within VacA-intoxicated cells inhibited the activation of the proapoptotic Bcl-2-associated X (Bax) protein, permeabilization of the mitochondrial outer membrane, and cell death. Our data reveal a heretofore unrecognized strategy by which a pathogenic microbe engages the host’s apoptotic machinery.

Mitochondria preserve cell viability by functioning both as centers for energy production and central regulators of calcium homeostasis, apoptosis, and development. Some pathogenic microbes usurp the mitochondrial apoptotic machinery (1), which, depending on the pathogen and host cell type, can block cell death to preserve a colonization niche or, alternatively, induce cell death to promote escape from an intracellular niche or circumvent immune clearance (2). For the gastric pathogen Helicobacter pylori (Hp), chronic infection is associated with increased apoptosis within the gastric mucosa of humans (3), mice (4), and Mongolian gerbil models (5). Increased apoptosis may alter the gastric environment to promote Hp persistence (6), while at the same time, contribute to gastric disease, including peptic ulcers and gastric adenocarcinoma (7). Earlier studies have indicated that the vaculating cytotoxin A (VacA), an Hp virulence factor that is important for Hp colonization (8) and disease pathogenesis (9), is essential (10) and sufficient (11) for inducing gastric epithelial cell death.

Several studies investigating the mechanism of VacA-induced cell death indicate that VacA is a mitochondrial-targeting toxin. Subsequent to binding plasma membrane sphingomyelin (12, 13), and potentially additional protein components (14, 15), VacA is internalized and induces mitochondrial dysfunction and mitochondrial outer membrane permeabilization (MOMP) (16). Intracellular VacA localizes to mitochondria (16, 17), and isolated mitochondria rapidly import purified VacA beyond the outer membrane (17, 18), resulting in dissipation of the mitochondrial transmembrane potential (\(\Delta \Psi_{m}\)) (19). VacA-dependent MOMP occurs after \(\Delta \Psi_{m}\) dissipation (16) and requires activation of the eukaryotic proapoptotic effector Bel-2-associated X protein (Bax) (19). However, the underlying mechanism by which VacA triggers Bax-dependent MOMP has not been identified.

Here, we report that VacA disrupts the morphological dynamics of mitochondria as a mechanism to induce gastric epithelial cell death. Mitochondria exist in several overall morphologies, which are linked to the health of the cell, and change in a dynamic fashion through frequent and repetitive cycles of fission and fusion that occur in response to cellular energy demands and environmental challenges (20). Deregulation of mitochondrial dynamics has increasingly been linked to the pathologies resulting from inflammatory and neurodegenerative disorders (21) and several cancers (22). However, the extent to which the morphological dynamics of mitochondria may be targeted by pathogenic microbes during host infection, or are associated with the pathophysiology of some infectious diseases, is largely unexplored. Our studies revealed that VacA induces activation of the dynamin-related protein 1 (Drp1), which is a critical regulator of mitochondrial fission within cells. Moreover, inhibition of VacA-mediated Drp1-dependent fission prevented activation of the proapoptotic Bax protein, MOMP, and death of intoxicated cells. Hp disruption of mitochondrial dynamics during infection is a heretofore unrecognized strategy by which a pathogenic microbe engages the host’s apoptotic machinery.

**Results**

**Hp Infection and Mitochondrial Fragmentation.** While studying cellular responses to Hp, we observed Hp-dependent alterations in the structure of cellular mitochondria, similar to those previously reported in studies of Hp-infected human stomach adenocarcinoma (AGS) cells (23). In uninfected AZ-521 gastric epithelial cells, mitochondria were predominantly filamentous networks of interconnected strands (~15–25 μm in length) (Fig. 1A and E). In contrast, at 8 h postinfection with Hp 60190, the mitochondrial network structure was highly fragmented, and individual mitochondria were visible as punctate organelles that were significantly shorter in length (1–2 μm) (Fig. 1B and E). The transition from filamentous to punctate structures was also observed with either Hp 26695 or Hp G27, indicating that Hp-dependent fragmentation of mitochondria is not idiosyncratic to a single strain (Fig. S1A). In addition, Hp-dependent fragmentation was recapitulated across cells lines, as observed in studies using AGS (Fig. S1B) and polarized Madin-Darby canine kidney (MDCK) II cells (Fig. S1 C and D). Fragmentation occurred at multiplicity of infection (MOI) 10, but not at MOI 1 (Fig. S1E), and was time dependent, with progressive fragmentation of the filamentous network observed between 2 and 8 h postinfection (Fig. S1F).

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VacA Is Essential for Hp-Induced Mitochondrial Fragmentation. While characterizing Hp-dependent mitochondrial alterations, we observed fragmentation of the mitochondrial network in AZ-521 cells that had been incubated with culture filtrate prepared from Hp 60190 (Hp culture filtrate, HPCF), but not with HPCF that had been pretreated at 95 °C (Fig. S1G). These results suggested that a proteinaceous factor released by Hp is responsible for mitochondrial fragmentation observed during infection. Consistent with this idea, fragmentation was not detected in cells incubated with heat-killed Hp 60190 (Fig. S1H).

Studies indicating the involvement of an extracellular, heat-labile factor in Hp-mediated mitochondrial network fragmentation prompted us to evaluate a potential role for VacA, which has been previously reported to modulate mitochondrial function (16, 17, 24, 25). In contrast to the extensive fragmentation of mitochondria induced by Hp 60190 (Fig. 1B), an isogenic Hp mutant strain lacking a functional vacA gene [Hp (ΔvacA)] (26) did not induce visible mitochondrial fragmentation within AZ-521 cells (Fig. 1C and E), whereas the complemented strain [Hp (ΔvacA::vacA)] (27) induced robust mitochondrial fragmentation (Fig. 1D and E). Differences in the adherence of Hp 60190, Hp (ΔvacA), or Hp (ΔvacA::vacA) to AZ-521 cells were not observed (Fig. S1J), suggesting that the inability of Hp (ΔvacA) to induce mitochondrial fragmentation was not likely due to attenuated cell association. These results support the idea that VacA is essential for Hp-dependent mitochondrial fragmentation.

VacA Is Sufficient to Induce Mitochondrial Fragmentation. Studies to evaluate the sufficiency of VacA for Hp-dependent mitochondrial fragmentation revealed that VacA, which had been purified from culture filtrates of Hp 60190, induced the transition of mitochondrial networks into significantly shorter punctiform organelles (Fig. 2A), an effect that was abrogated when the toxin was preincubated at 95 °C or with VacA antiserum (Fig. S2A). More convincing, however, were studies conducted with recombinant VacA comprising the toxin’s amino- and carboxy-terminal fragments, called p33 and p55, respectively, that had been expressed separately in Escherichia coli and purified (28). The combined p33–p55 fragments induced robust fragmentation, whereas neither p33 nor p55 alone induced detectable alterations in the mitochondrial network (Fig. 2B). Together, these results indicate that VacA is sufficient to induce mitochondrial fragmentation.
Mitochondria within infected cells is VacA dependent (Fig. 3C). These results suggested a possible involvement of the cellular fission machinery in VacA-dependent mitochondrial network fragmentation.

**Drp1 GTPase Activity is Important for VacA-Dependent Mitochondrial Fragmentation.** A critical step during mitochondrial fission is the assembly of Drp1 into spiral chains at the mitochondrial scission sites (33), which is driven by the intrinsic GTPase activity of Drp1 (34). To evaluate the importance of Drp1 function for VacA-dependent mitochondrial fission, AZ-521 cells were intoxicated with VacA in the absence or presence of the mitochondrial division inhibitor mdivi-1, which specifically blocks Drp1 self-assembly and GTPase activity (35). These studies revealed that mdivi-1 significantly inhibits VacA-dependent mitochondrial fragmentation in AZ-521 cells (Fig. 4A and Fig. S44) and in AGS cells (Fig. S4B). Mitochondrial fragmentation was also significantly inhibited by mdivi-1 in AZ-521 cells infected with Hp 60190 (Fig. S4C). Together, these data indicate that Hp- and VacA-dependent mitochondrial fragmentation requires Drp1 GTPase activity, thereby implicating the involvement of the cellular mitochondrial fission machinery.

**Drp1-Mitochondrial Localization Is Important for VacA-Dependent Mitochondrial Fragmentation.** Further validation of Drp1 involvement came from studies using AZ-521 cells overexpressing either Drp1 fused to enhanced green fluorescence protein, EGFP-Drp1 or a dominant-negative form of Drp1, EGFP-DN-Drp1 (K38A), which inhibits Drp1 association with the mitochondrial outer membrane (36). VacA-intoxicated cells overexpressing EGFP-DN-Drp1 (K38A) demonstrated significantly reduced mitochondrial fragmentation than those overexpressing EGFP-Drp1 (Fig. 4B and Fig. S4D). These results further support the functional importance of the cellular fission machinery for VacA-mediated mitochondrial fragmentation.

**Drp1-Dependent Mitochondrial Fission Precedes and Is Important for VacA-Induced Bax Activation.** To evaluate whether VacA-mediated mitochondrial fragmentation may be linked to cell death, we first investigated whether Drp1-dependent mitochondrial fission is required for activation of the proapoptotic Bcl-2 effector, Bax, as reported within VacA-intoxicated cells (19). Notably, several studies have reported significant cross-talk between Drp1- and Bax-mediated cellular processes (37, 38). Within this study, we observed a significant decrease in Bax activation in AZ-521 cells incubated with VacA in the presence of the Drp1 inhibitor mdivi-1 (Fig. 5). The overall cellular levels of Bax were unaltered in cells that had been incubated with mdivi-1 and/or VacA (Fig. S5A). Consistent with the results obtained using mdivi-1, VacA-dependent Bax activation was also inhibited in AZ-521 cells overexpressing EGFP-DN-Drp1 (K38A) (Fig. S5B). Although mitochondrial fragmentation was clearly visible by 60 min in AZ-521 cells incubated with VacA (Fig. S2E), a significant increase in the fraction of cells with activated Bax was not detected until 2 h, and the fraction of cells with activated Bax continued to increase through 8 h (Fig. S5C). Together, these results suggest that within VacA-intoxicated cells, Drp1-mediated mitochondrial fission precedes and is important for Bax activation.

**Bax Is Not Essential for Drp1-Dependent Mitochondrial Fission in VacA-Intoxicated Cells.** Studies to evaluate the essentiality of cellular Bax for mitochondrial network fragmentation revealed clearly visible fragmentation in both bax+/− and bax−/− mouse embryonic fibroblasts (MEFs) that had been incubated with VacA (Fig. S5D). These results suggest that Bax is not required for Drp1-dependent mitochondrial fission within VacA-intoxicated cells.
Drp1-Dependent Fission Is Important for VacA-Induced MOMP. Within VacA-intoxicated cells, Bax activation is associated with MOMP, as manifested by release of mitochondrial cytochrome c (Cyt c) into the cytosol (19). Studies to evaluate the relationship between MOMP and Drp1-dependent mitochondrial fission revealed that the fraction of AZ-521 cells with Cyt c released from mitochondria into the cytosol had not increased after 1-h incubation with VacA (Fig. S5E), although mitochondrial fragmentation was evident at this same time point (Fig. S2D). However, there was a significant increase in the fraction of cells with Cyt c released from mitochondria into the cytosol by 2 h, and the fraction continued to increase through 8 h (Fig. S5E), similar to the kinetics of Bax activation (Fig. S5C), indicating that VacA-mediated mitochondrial fragmentation precedes MOMP. Cyt c release was significantly inhibited in the presence of the Drp1 inhibitor mdivi-1 (Fig. 6 A and B). In addition, Cyt c release in response to VacA was visibly reduced in cells overexpressing EGFP-DN-Drp1 (K38A) (Fig. S5F). These results suggest that enhanced mitochondrial fission promotes MOMP within VacA-intoxicated cells.

VacA-Mediated Drp1 Activation Is Important for Toxic-Dependent Cell Death. VacA-dependent MOMP precedes and is necessary for cell death (16, 19). Studies to evaluate the importance of Drp1 revealed that cell death was significantly reduced in AZ-521 (Fig. 7) or AGS cells (Fig. S6) that were incubated with VacA in the presence of mdivi-1. These results indicate that Drp1-dependent fission is important for VacA-induced cell death and suggest that the disruption of cellular mitochondrial dynamics plays an important role in activation of the cell death mechanism following Hp infection.

VacA Anion Channel Activity Is Important for Mitochondrial Fragmentation. Anion-selective, membrane channels formed by VacA were earlier reported to be important for toxic-dependent MOMP and cell death (16). Studies to evaluate whether VacA channel activity is also required for toxic-dependent disruption of mitochondrial dynamics revealed the absence of detectable mitochondrial fragmentation in cells exposed to two mutant forms of toxin (Fig. 8 A and B). VacA (P9A) or VacA (G14A) (39), each of which had been previously shown to be attenuated in membrane channel-forming activity (40). Additionally, ectopic expression of the amino-terminal p34 domain of VacA, which contains the residues required for VacA channel activity (40), directly within the cytosol of transiently transfected AZ-521 cells, resulted in morphological changes, including apparent fragmentation in the cellular mitochondrial network (Fig. S7A). Finally, we confirmed that there was significantly less Bax activation (Fig. S7B) and cell death (Fig. S7C) in AZ-521 monolayers exposed to VacA (P9A) or VacA (G14A) than cells exposed to wild-type toxin. These data indicate that VacA membrane channel activity is important for the activation of Drp1-dependent mitochondrial fission within intoxicated cells.

Discussion

Mitochondrial health is closely linked to cell viability, as these organelles produce the energy required for cellular function while at the same time functioning as regulators of programmed cell death (41). Accordingly, mitochondrial dysfunction has been increasingly linked to several human pathologies, including those associated with cancer (42), inflammatory disorders (43), and degenerative diseases (44).

Here, we demonstrated that Hp infection of gastric epithelial cells disrupts the morphological dynamics of mitochondria by a mechanism dependent on the mitochondrial acting exotoxin, VacA. Whereas mitochondrial fragmentation has been previously observed in cells infected with Hp (23) or intoxicated with VacA (29), our studies revealed that Drp1-mediated mitochondrial fission precedes and is important for induction of VacA-dependent cell death. For Hp, an increase in cell death within the gastric mucosa may alter the host niche in several ways, including the loss of specialized cells, such as gastric parietal cells, but also increased cellular proliferation and gastric atrophy that precedes metaplasia, dysplasia, and ultimately cancer (45, 46).

A causal link between Drp1 and the cellular apoptotic machinery has been reported within diverse organisms such as yeast (47) and Drosophila (48). In contrast, down-regulation of Drp1 expression within HeLa or COS-7 cells did not prevent Bax-de-
pendent apoptosis induced by UV radiation or actinomycin D (49). In addition, a recent report indicated that cell death does not result from the perturbation of mitochondrial dynamics in cells infected with *Listeria monocytogenes* (50), indicating that cell death is not an obligate outcome of disrupting mitochondrial dynamics. Thus, potential roles for Drp1 in mitochondrial-dependent cell death may ultimately be dictated by factors such as cell/tissue type or the nature of the prodeath stimulus.

Our results indicate that Drp1-mediated mitochondrial localization and GTPase activity is required for activation of Bax in VacA-intoxicated cells (Fig. 5). Recent studies have demonstrated that functional crosstalk between Drp1 and Bax exists in some cases (51). One study identified Drp1 as the factor within rat brain extracts that stimulates Bax activity and mitochondrial Cyt c release, but in a manner that is independent of its GTPase activity (52), suggesting fundamental differences with Drp1-dependent Bax activation in VacA-intoxicated cells, which requires Drp1 GTPase activity (Fig. 5). On the other hand, Bax was reported to colocalize with Drp1 at mitochondrial scission sites (37) and promote sumoylation of Drp1, which is required for stable association of Drp1 with mitochondria (38). Furthermore, Bax was reported to influence redistribution of Drp1 to mitochondria through release of the mitochondrial effector DDP/TIMM8a (53). Our data indicate that Bax is not required for Drp1-mediated fission, suggesting that within VacA-intoxicated cells, crosstalk between Drp1 and Bax may be unidirectional (e.g., Drp1-dependent fission as a trigger for Bax-mediated MOMP). Currently, we are investigating the mechanism by which Drp1-dependent fission results in Bax activation and MOMP within VacA-intoxicated cells.

The mechanism by which VacA induces Drp1-dependent mitochondrial fission is not clear. Our data indicate that VacA channel activity is required for toxin-dependent mitochondrial fragmentation (Fig. 8), but the cellular site at which toxin channel activity is required for fragmentation has not been identified. Because ectopic expression of p34-EGFP directly within the cytosol of AZ-521 cells alters mitochondrial morphology (Fig. S7A), it is unlikely that VacA membrane channels formed on the surface of mammalian cells (54) are required for toxin-mediated activation of Drp1-dependent mitochondrial fission. Alternatively, VacA might act directly at mitochondria, as several previous studies reported that a portion of VacA taken up from the cell surface localizes to this organelle (16, 29, 55). Preliminary studies to address a possible relationship between the location of intracellular VacA and the perturbation of mitochondrial dynamics revealed that VacA localization to mitochondria (Fig. S8) is evident within AZ-521 cells at 30 min, before the earliest time (60 min) that visible mitochondrial fragmentation was detected after exposure to toxin (Fig. S2E).

Whereas these data are consistent with the idea that VacA may act directly at mitochondria to induce Drp1-mediated fission, we cannot currently rule out the possibility that VacA-mediated mitochondrial fragmentation is triggered independently of toxin localization to mitochondria. In support of this latter possibility, a recent study reported that localization of VacA to mitochondria is delayed within MEFs lacking both Bax and the related effector Bel-2 homologous antagonist/killer (Bak) (55), although our data clearly indicate that Bax is not required for VacA-induced Drp1-mediated mitochondrial fission (Fig. SSD).

Preliminary studies to address the relationship between mitochondrial dysfunction and fission within VacA-intoxicated cells revealed that Drp1 activity is not required for VacA-mediated dissipation of mitochondrial transmembrane potential (ΔΨm) (Fig. S9), suggesting that fission in VacA-intoxicated cells is not likely the trigger for mitochondrial dysfunction. However, it remains possible that VacA-induced ΔΨm dissipation induces Drp1-dependent fission. Support for this idea comes from unrelated studies that demonstrated that mitochondrial depolarization in HeLa cells induced a sustained cytotoxic calcium rise, followed by calcineurin-mediated dephosphorylation of Drp1 at Ser637 as a mechanism to drive Drp1 translocation to mitochondria (56). VacA is sufficient to induce ΔΨm dissipation (19) and increases in cytotoxic calcium (57), but a potential link between elevated cellular calcium or calcineurin action and Drp1-dependent mitochondrial fission within VacA-intoxicated cells remains to be evaluated.

In summary, these results demonstrate that apoptosis of gastric epithelial cells during *Hp* infection is triggered by VacA-induced disruption of mitochondrial morphological dynamics through Drp1-mediated fission, which both precedes and is required for Bax-dependent remodeling of the mitochondrial outer membrane. Future work will be required to reveal whether other pathogens also promote host cell death by targeting the morphological dynamics of mitochondria.

**Materials and Methods**

The sources of reagents, cell lines, bacterial strains, and plasmids used in this study are provided in *SI Materials and Methods*. In addition, a detailed description of experimental methods is provided in *SI Materials and Methods*. Finally, statistical analyses of the data are described in the *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Bacterial Strains. *Hp* 60190 (cag PAI+, *vacA* s1/m1; 49503; ATCC) was cultured in bisulphite- and sulfite-free *Brucella* broth (BSFB) containing 5 μg vancomycin/mL (Sigma Aldrich), on a rotary platform shaker for 48 h at 37 °C, under 5% CO₂ and 10% O₂. *Hp* VM022 (Δ*vacA*) (1) and *Hp* VM084 (Δ*vacA*:Δ*vacA*) were kind gifts from Timothy Cover (Vanderbilt University Medical Center, Nashville, TN) (2). *Hp* 60190-derived strains producing *VacA* (P9A) and *VacA* (G14A) were constructed and cultivated as described previously (2, 3). *Hp* 26695 (cag PAI+, *vacA* s1/m1) was obtained from ATCC (700392). *Hp* G27 (cag PAI+, *vacA* s1/m1) (4) was obtained as a kind gift from Karen Guillemin (University of Oregon, Eugene, OR).

Mammalian Cells. AZ-521 human gastric cancer-derived cell line was obtained from Japan Health Science Foundation (3940) and maintained in MEM (Sigma Aldrich), which when supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (1 mg/mL), and 10% fetal bovine calf serum, was referred to as “supplemented MEM.” HeLa (CCL-2; ATCC), HEP-2 (CCL-23; ATCC), and Madin-Darby canine kidney (MDCK) II cells (CCL-34; ATCC) were maintained in supplemented MEM. AGS cells (CRL-1739; ATCC) were maintained in Ham’s F-12 Kaighn’s modification medium (Cellgro), which when supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (1 mg/mL) (Sigma Aldrich), and 10% fetal bovine calf serum (JRH Biosciences), was referred to as “supplemented Ham’s F-12 medium.” *vacA*+/+ and *vacA*−/− mouse embryonic fibroblasts (MEFs), obtained as a kind gift from Wei-Xing Zong (Stony Brook University, Stony Brook, NY), were maintained in DMEM (Cellgro), which when supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (1 mg/mL), and 10% fetal bovine calf serum, was referred to as supplemented DMEM. All cell lines were maintained at 37 °C within a humidified atmosphere and under 5% CO₂.

Plasmids. The plasmids pDsRed2-Mito and pAcGFP1-N1 were obtained from Clontech Laboratories. Plasmids pEGFP-Drp1 and pEGFP-DN-Drp1 (K38A) were obtained as a kind gift from Marina Jendrach (Goethe University; Frankfurt/Main, Germany). The plasmid encoding p34(1-319)-EGFP was obtained as a kind gift from Marina Jendrach (Goethe University; Frankfurt/Main, Germany).

Transfection. Cells were transfected with the indicated plasmids using Lipofectamine-2000 transfection reagent (Invitrogen), according to the manufacturer’s instructions.

*Hp* Infection of Mammalian Cells. Monolayers of mammalian cells, at 37 °C within a humidified environment under 5% CO₂, were incubated with the indicated *Hp* strains and at the indicated multiplicity of infection (MOI). At the end of incubation, monolayers were washed with PBS pH 7.2 and processed for the indicated analyses.

Analysis of Mitochondrial Fragmentation within AZ-521, AGS, and Mouse Embryonic Fibroblast Cells. Mammalian cells plated in eight-well culture slides (BD Biosciences) that had been transiently transfected with pDsRed2-Mito, a mammalian expression vector that encodes a fusion between red fluorescent protein and the mitochondrial targeting sequence from subunit VIII of the human cytochrome c oxidase, were infected with *Hp* (at the indicated MOI), or incubated with *VacA* (at the indicated concentration) or *Hp* culture filtrate (HPCF) (at the indicated concentration), or mock treated with PBS pH 7.2. At the indicated times, the cells were washed three times with PBS pH 7.2 and then fixed by incubation with paraformaldehyde (4%) at 37 °C for 20 min. Mitochondrial morphology was analyzed by DIC-epifluorescence microscopy. Images were processed using DeltaVision SoftWoRx 3.5.1 software suite (Applied Precision). Mitochondrial lengths were measured using Imaris 5.7 software (Bitplane). Data were rendered as the average mitochondrial lengths obtained by combining data from at least two or three independent experiments. In each independent experiment, 15 cells were analyzed from at least four randomly chosen fields for each treatment. Within each cell, at least three mitochondria were analyzed and mitochondrial lengths measured. Relative mitochondrial lengths were calculated as the fold change in average mitochondrial length measured in cells treated as indicated, relative to those measured in mock-treated cells.

Generation of Polarized Madin-Darby Canine Kidney (MDCK) Monolayers. Polarized MDCK monolayers were generated and maintained, as described previously (5). Briefly, MDCK cells (2.5 × 10° cells/0.5 mL in supplemented MEM) were seeded onto 12 mm, 3.0 μm-pore polyester tissue culture transwell inserts (Corning) and incubated at 37 °C and under 5% CO₂ with 1.5 mL supplemented MEM added in the basal chamber. Cell culture medium in the apical chambers was replaced once, 24 h after seeding, with fresh supplemented MEM, whereas the medium in the basolateral chamber was replaced with fresh supplemented MEM daily. The plates were maintained at 37 °C in a humidified atmosphere and under 5% CO₂ for at least 4 d before the experiments.

To assess the integrity of polarized monolayers, transwell inserts with MDCK cells or mock-seeded transwell inserts (incubated with PBS pH 7.2 alone) were incubated at 37 °C and under 5% CO₂ with 50 μg/mL biotin-albumin (5 mM; Sigma Aldrich) in supplemented MEM in the transwell basolateral chamber and supplemented MEM alone (0.5 mL) in the apical chamber. After 1 h, medium from the apical and basolateral chambers was collected and resolved by SDS gel electrophoresis, followed by Western blot analysis to probe for biotin-albumin using streptavidin-HRP (GE Healthcare). The presence of biotin-albumin was detected by chemiluminescence using the Supersignal West Femto chemiluminescence detection kit (Thermo Scientific).

Analysis of Mitochondrial Fragmentation within HeLa, HEP-2, and Polarized MDCK Cells. HeLa and HEP-2 cells plated in eight-well culture slides, and transmembrane polyester inserts containing polarized MDCK monolayers were washed twice with PBS pH 7.2 and fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization in PBS pH 7.2 containing Triton-X 100 (0.1%), 1.5 μL/mL Sigma Aldrich) in supplemented MEM in the transwell basolateral chamber and supplemented MEM alone (0.5 mL) in the apical chamber. After 1 h, medium from the apical and basal chambers was collected and resolved by SDS gel electrophoresis, followed by Western blot analysis to probe for biotin-albumin using streptavidin-HRP (GE Healthcare). The presence of biotin-albumin was detected by chemiluminescence using the Supersignal West Femto chemiluminescence detection kit (Thermo Scientific).
Preparation of HPCF. The indicated Hp strains were grown in 200 mL bisulfite- and sulfite-free Brucella broth (BSFB) containing 5 μg vancomycin/mL, in 1-L culture flasks, on a rotary platform shaker at 37 °C, under 5% CO₂ and 10% O₂. After 48 h, Hp cultures were harvested by centrifugation at 8,000 g for 30 min at 4 °C. The supernatants were collected, and pellets were decated by autolysis. The supernatants were cooled to 4 °C, and the total protein was precipitated by slowly dissolving ammonium sulfate (Sigma Aldrich) to 90% saturation with stirring, followed by stirring overnight at 4 °C. The precipitates were collected by centrifugation at 8,000 g for 30 min at 4 °C, and the pellets were resuspended in 10 mM sodium phosphate buffer pH 7.0. The samples were dialyzed at 4 °C into 10 mM sodium phosphate buffer pH 7.0 using the Spectra/Por membrane (molecular weight cutoff (MWCO) 50,000 Da; Spectrum Laboratories), concentrated approximately fivefold using an Amicon Ultra centrifugal filter unit (MWCO 50,000 Da; Sigma Aldrich), and filter sterilized using a 0.2-μm vacuum filtration unit (Corning) to obtain the final HPCF.

The presence of full-length VacA within the HPCFs was confirmed by Western blot analysis, using VacA rabbit antisera (Rockland Immunochemicals), followed by incubation with HRP-conjugated antirabbit IgG secondary antibody (Cell Signaling Technology). The presence of VacA was detected by chemiluminescence using the Supersignal West Femto chemiluminescence analysis software; Silk Scientific) to compare the total pixels of each band against those obtained using known concentrations of purified VacA. The HPCFs were used within several days of preparation, during which time there was no detectable loss of VacA-induced vacuolation of AZ-521 cells, as determined by quantification of cellular vacuolation (6, 7).

Heat Inactivation of HPCF. HPCFs (5 mg/mL) were incubated in a 37 °C or 95 °C water bath for 30 min, followed by incubation for 10 min at 37 °C. The HPCFs were immediately activated by adding 0.1 vol/vol 300 mM HCl to HPCF preparation and incubation for 30 min at 37 °C, followed by neutralization with the same volume of 300 mM NaOH. Activated HPCFs were incubated with AZ-521 cells at a final concentration of 0.05 mg/mL. At the indicated times, the AZ-521 cells were analyzed for changes in mitochondrial morphology relative to cells that were mock intoxicated with PBS pH 7.2.

Intoxication of Mammalian Cells with VacA or HPCF. Mammalian cells were incubated at 37 °C and within a humidified environment under 5% CO₂ with purified VacA or HPCF (both at the indicated concentrations). At the indicated times, the cells were processed for specific analyses.

Heat Inactivation of Hp. Hp 60190 (12.5 × 10⁶ cfu/mL in 1 mL PBS pH 7.2) were incubated in a 37 °C or 65 °C water bath for 30 min and then further incubated for 10 min at 37 °C. Immediately, 37 °C- or 65 °C-preincubated Hp was incubated with AZ-521 cells (MOI 100) or, alternatively, enumerated by serially diluting in PBS pH 7.2, followed by spread plating onto fresh F-12 agar plates (supplemented with 5% FBS and 5 μg vancomycin/mL), and incubating the plates at 37 °C, and under 5% CO₂ and 10% O₂. After 72 h, cfu/mL were determined by direct counting of colonies on the F-12 plates and back calculating the appropriate dilution factor.

Analysis of Hp Association with AZ-521 Cells. Monolayers (85–95% confluence) of AZ-521 cells, plated at 0.75 × 10⁶ cells per well, were incubated with Hp 60190, Hp VM022 (ΔvacA), or Hp VM084 (ΔvacA::vacA) (all at MOI 100) at 37 °C and under 5% CO₂ and 10% O₂. After 8 h, the cell monolayers were washed twice with PBS pH 7.2. Each monolayer was gently lysed by incubating with 0.1% Triton X-100 in PBS pH 7.2 (50 μL) for 3 min on ice and collected in 950 μL PBS pH 7.2. The cell-associated Hp was plated on F-12 media plates supplemented with 10% FBS and 5 μg vancomycin/mL and incubated at 37 °C under 5% CO₂ and 10% O₂. After 72 h, the cfu/mL was determined by the direct counting of colonies on F-12 plates and back calculating using the appropriate dilution factor.

Purification of VacA. VacA (1 mg/mL) was purified from Hp 60190 and activated, as previously described (8). Hp culture filtrate (from Hp broth culture) was prepared as previously described (9).

Heat Inactivation of VacA. Purified VacA (4 μL) was incubated in a 37 °C or 95 °C water bath, followed by further incubation of both samples at 37 °C for 10 min. Immediately, VacA was activated as described previously (3, 8, 10) and incubated with AZ-521 cells (at a final concentration of 250 nM). At the indicated times, the AZ-521 cells were analyzed for changes in mitochondrial morphology relative to cells that were mock intoxicated with PBS pH 7.2.

Inactivation of VacA with Anti-VacA Antibody. Purified VacA (0.1 mg/mL) was preincubated on ice with VacA rabbit antisera (2 mg/mL), a nonspecific rabbit antisera against Haemophilus ducreyi cytotoxyl distending toxin A (CdtA; 2 mg/mL) or PBS pH 7.2. After 30 min, the VacA-containing samples were activated as described previously (3, 8, 10) and immediately incubated at 37 °C with AZ-521 cells (250 nM VacA in each sample). At the indicated times, the AZ-521 cells were analyzed for changes in mitochondrial morphology relative to cells that were mock intoxicated with PBS pH 7.2.

Expression and Purification of Recombinant VacA Proteins. The recombinant VacA fragments, p33 (residues 1–321) and p55 (residues 312–821), were expressed in Escherichia coli (DH5α) and purified as described previously (11).

Analysis of Drp1 Localization to Mitochondria. Mitochondrial localization of Drp1 was analyzed as described previously (12). Mammalian cells that had been transiently transfected with pDsRed2-Mito and plated in eight-well culture slides, were incubated with Hp (at the indicated MOI), or purified VacA (at the indicated concentrations), or Hp culture filtrates (at the indicated concentrations). At the indicated times, the monolayers were washed three times with PBS pH 7.2, fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, permeabilized by incubation with PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C, immunostained for Drp1 using anti-Drp1 mAb (BD Biosciences), and, finally, incubated at 25 °C for 1 h with mouse anti-IgG conjugated to Alexa Fluor 647 (Invitrogen). The cells were imaged using DCl-epifluorescence microscopy. Drp1 localization to mitochondria was quantified using the colocalization module of the DeltaVision SoftWoRx 3.5.1 software suite. Results were expressed as the colocalization index, derived from calculating Pearson’s coefficient of correlation, which in this study was a measure of colocalization between Drp1 and mitochondria in each z plane of the cell. For each cell, images from an average of 10–20 z planes at a thickness of 0.2 μm were collected. A colocalization index of 1.0 indicates 100% colocalization of Drp1 to mitochondria, whereas a colocalization index of 0.0 indicates the absence of detectable colocalization between Drp1 and mitochondria. Data were rendered as the average colocalization index obtained from analyzing 30 cells from over the course of three independent experiments. In each independent experiment, 10 cells were analyzed from at least four randomly chosen fields for each treatment.
Flow Cytometry Assay for Determination of Total Cellular and Activated Bax. AZ-521 cells were detached from tissue culture wells by mild trypsinization for 3 min at 37 °C with trypsin EDTA (Cellgro), fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization on ice with saponin (0.1%) in PBS pH 7.2 containing BSA (0.5%) and anti-Bax 2D2 mAb (1 μg/mL; BD Biosciences) to stain for total cellular Bax. Alternatively, the cells were fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization on ice with saponin (0.1%) in PBS pH 7.2 containing BSA (0.5%) and anti-Bax Clone 3 mAb to stain for activated Bax. After 45 min, the cells were washed three times with PBS pH 7.2, followed by incubation with mouse anti-IgG conjugated to Alexa Fluor 488 (1 μg/mL) in PBS pH 7.2 containing saponin (0.1%) and BSA (0.5%) for 30 min on ice and in the dark. As a negative control, cells were incubated in the presence of mouse anti-IgG conjugated to Alexa Fluor 488 (1 μg/mL) alone. Cells were washed in PBS pH 7.2 containing saponin (0.1%) and BSA (0.5%) and resuspended in PBS pH 7.2. Alexa Fluor 488 fluorescence was quantified by flow cytometry in the FL1 channel (525/40 nm band pass filter). A total of 10,000 cells were analyzed for each sample.

Microscopic Analysis of Intracellular Activated Bax. Cells were fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization on ice with saponin (0.1%; Sigma Aldrich) in PBS pH 7.2 containing BSA (0.5%) and anti-Bax (Clone 3) mAb (1 μg/mL; BD Biosciences) to stain for activated Bax. After 45 min, the cells were washed three times with PBS pH 7.2, followed by incubation with mouse anti-IgG conjugated to Alexa Fluor 647 or Alexa Fluor 488 (1 μg/mL) in PBS pH 7.2 containing saponin (0.1%) and BSA (0.5%) for 1 h on ice in the dark. The cells were visualized by DIC-epifluorescence microscopy, with visible fluorescence indicative of activated Bax. To enumerate the number of cells displaying active Bax, imaging was carried out at a constant time of exposure. All images were deconvolved using SoftWoRx constrained iterative deconvolution tool (ratio mode) to remove out-of-focus signal. Following deconvolution, cells displaying visible fluorescence were considered to contain active Bax, as opposed to the absence of Bax activation in cells that did not display fluorescence. Data were rendered as the percentage of cells displaying active Bax within the entire population and were obtained by analyzing over 700 cells from randomly chosen fields over the course of two independent experiments.

Analysis of Cyt c Release. Cyt c release was analyzed as described previously (13). Briefly, mammalian cells were fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, permeabilized by incubation with PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C, immunostained for Cyt c by incubating with anti-Cyt c mAb (BD Biosciences), and then further incubated with one of two secondary antibodies. To visualize Cyt c within cells intoxicated with VacA in the presence or absence of midi-1, cells were further incubated with mouse anti-IgG conjugated to Alexa Fluor 488. Alternatively, to visualize Cyt c within VacA-intoxicated cells overexpressing either EGFP-Drpl or EGFP-DN-Drpl (K38A), cells were further incubated with mouse anti-IgG conjugated to Alexa Fluor 647. The cells were imaged using DIC-epifluorescence microscopy. Cells with diffuse, nonlocalized fluorescence were scored as having released (cytosolic) Cyt c, whereas cells with punctate fluorescence localized in the perinuclear regions were scored as having mitochondrial-localized Cyt c.

Cell Death. Cell death was measured by flow cytometry, using the Live-Dead viability/cytotoxicity assay kit (Invitrogen) according to the manufacturer’s instructions.

Analysis of VacA Localization to Mitochondria. AZ-521 cells plated in eight-well culture slides were incubated with purified VacA (100 nM). After 30 min, the monolayers were washed three times with PBS pH 7.2, fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, permeabilized by incubation with PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C, immunostained for VacA using anti-VacA rabbit polyclonal Ab and mitochondria using anti-Tom 20 mAb, and incubated at 25 °C for 2 h. The cells were washed three times with PBS pH 7.2 followed by incubation with rabbit anti-IgG conjugated to Alexa Fluor 568 and mouse anti-IgG conjugated to Alexa Fluor 488 for 1 h at 25 °C. The cells were washed three times with PBS pH 7.2 and imaged using DIC-epifluorescence microscopy.

Analysis of Mitochondrial Transmembrane Potential (ΔΨm). AZ-521 cells were incubated with Tetramethylrhodamine ethyl ester perchlorate (TMRE) (50 nM; Sigma Aldrich) for 30 min before the end of each experiment. The cells were detached by mild trypsinization for 3 min at 37 °C with trypsin-EDTA and washed two times with PBS pH 7.2. TMRE fluorescence was quantified by flow cytometry in the FL2 channel (575/30-nm band pass filter). A total of 10,000 cells were analyzed for each sample.

Flow Cytometry. Analytical flow cytometry was carried out using a BD FACSCanto II flow analyzer (BD Biosciences) located at the R. J. Carver Biotechnology Center Flow Cytometry Facility (University of Illinois at Urbana–Champaign). The flow cytometer was equipped with a 70-μm nozzle, 488-nm line of an air-cooled argon-ion laser, and 400 mW output. The band pass filters used for analysis were 525/40 nm, 575/30 nm, and 675/30 nm. Cell analysis was standardized for scatter and fluorescence by using a suspension of fluorescent beads (Beckman Coulter). Events were recorded on a log fluorescence scale and the geometric mean and percent events were determined using FCS Express analysis software (De Novo Software). Forward and side scatter properties were considered to exclude noncellular (debris) events from viable and (or) dead cell populations.

DIC-Epifluorescence Microscopy. Fluorescence and DIC images were collected using a Delta Vision RT microscope (Applied Precision), EX 490/20 and EM 528/38, EX 555/28 and EM 617/73, EX 640/20 filters used for analysis were 525/40 nm, 575/30 nm, and 675/30 nm. Cell analysis was standardized for scatter and fluorescence by using a suspension of fluorescent beads (Beckman Coulter). Events were recorded on a log fluorescence scale and the geometric mean and percent events were determined using FCS Express analysis software (De Novo Software). Forward and side scatter properties were considered to exclude noncellular (debris) events from viable and (or) dead cell populations.

Statistical Analysis. Unless otherwise indicated, each experiment was performed at least three independent times. For those data requiring statistical analysis, data were combined from two or three independent experiments, as indicated, with each independent experiment carried out in triplicate. Statistical analyses were performed using Microsoft Excel (version 11.0). Unless otherwise noted, error bars represent SD. All P values were calculated with the Student’s t test using paired, two-tailed distribution. P < 0.05 indicates statistical significance.

Additional cells were mock infected and incubated at either 65 °C or 37 °C for 30 min. Additionally, cells were mock infected with PBS pH 7.2 (ΔvacA) (all at MOI 100) or mock-infected H. pylori strains with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was evaluated.

### Analysis of H. pylori-dependent mitochondrial fragmentation

AZ-521 cells (A and E–H) or AGS cells (B) that had been previously transfected with pDsRed2-Mito or polarized MDCK cells (C) were incubated at 37 °C and under CO₂ with H. pylori 60190 at MOI 100 (A–C and F) or for the indicated MOI (E), or incubated with H. pylori 26695 (A) or H. pylori G27 (A) (all at MOI 100). In G, monolayers were incubated with culture filtrate that had been preincubated at either 37 °C or 95 °C for 30 min. In H, monolayers were incubated with H. pylori 60190 (MOI 100) that had been preincubated at either 65 °C or 37 °C for 30 min. Additionally, cells were mock infected with PBS pH 7.2 (A–C and E–H). After 8 h (A–C, E, G, and H) or at the indicated times (F), the cells were fixed (A, B, and E–H) or fixed, permeabilized, and immunostained for Tom-20 as a mitochondrial marker (C). The cells were evaluated for mitochondrial fragmentation using DIC-epifluorescence microscopy. The images reveal the morphology of fluorescently stained mitochondria (Scale bar, 5 μm) and are representative of those collected from two (A and C) or three (B and E–H) independent experiments. Mitochondrial lengths were measured using Imaris 5.7 (Bitplane) software. (2) The integrity of the polarized MDCK monolayer was evaluated by monitoring the passage of biotin-BSA (50 μg/mL) from the transwell basal chamber through either the MDCK monolayer or mock-seeded transwell inserts (in the absence of cells) into the apical chamber. The presence of biotin-BSA within the apical or basal chamber was assessed by Western blot analysis, using the streptavidin-HRP conjugate and chemiluminescent signal development. The Western blot data are representative of those collected from two independent experiments, each with two independent MDCK monolayers. (1) Association of vacA− and vacA+ H. pylori strains with AZ-521 cells was evaluated. AZ-521 cells were incubated at 37 °C and under CO₂ with H. pylori 60190, H. pylori VM022 (ΔvacA), or H. pylori VM084 (ΔvacA::vacA) (all at MOI 100) or mock infected with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and H. pylori associated with AZ-521 cells in each treatment was quantified by dilution plating and direct CFU counting. Data are rendered as the average CFU/mL obtained from combining data collected from two independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for differences in CFU/mL between H. pylori 60190 (WT) and H. pylori VM022 (ΔvacA) or H. pylori VM084 (ΔvacA::vacA).
**Fig. S2.** Characterization of VacA induced mitochondrial fragmentation. AZ-521 cells (A and D) or AGS cells (B) that had been previously transfected with pDsRed2-Mito or HeLa (B), HEP-2 (B) and polarized MDCK cells (C), were incubated at 37 °C and under CO₂ with VacA (250 nM), AZ-521 (A and D), and AGS (B) or 500 nM HeLa (B), HEP-2 (B), and MDCK (C). In A, cells were also incubated with VacA (250 nM) that had been pretreated for 30 min at 37 °C or 95 °C or for 30 min on ice with VacA antiserum (2 mg/mL) or a nonspecific H. ducreyi CdtA antiserum (2 mg/mL). Additionally, the cells were mock intoxicated with PBS pH 7.2 (A–D). After 4 h AZ-521 (A), AGS (B), and MDCK (C) or 8 h HeLa (B) and HEP-2 (B), the cells were fixed AZ-521 (A), AGS (B), or fixed, permeabilized, and immunostained for Tom-20 as a mitochondrial marker HeLa (B), HEP-2 (B), and MDCK (C), and cellular mitochondria were visualized by DIC-epifluorescence microscopy. (D) Mitochondria within each representative cell of AZ-521 monolayer that was previously intoxicated with VacA or mock treated with PBS pH 7.2 were monitored for the indicated time periods using live cell imaging. The images reveal the morphology of fluorescently stained mitochondria and are representative of those collected from two (C and D) or three (A and B) independent experiments. (Scale bar, 5 μm.)

**Fig. S3.** Evaluation of VacA-dependent Drp1 localization to mitochondria. AZ-521 cells that had been previously transfected with pDsRed2-Mito were incubated at 37 °C and under CO₂ with VacA (at the indicated concentrations) or mock intoxicated with PBS pH 7.2. After 4 h, the cells were fixed, permeabilized, and immunostained for cellular Drp1. Localization of Drp1 to mitochondria was determined using DIC-epifluorescence microscopy followed by colocalization analysis. The data are rendered as the average Drp1-mitochondrial colocalization obtained by combining data from two independent experiments. In each independent experiment, 15 randomly chosen, pDsRed2-Mito transfected cells were analyzed. Error bars indicate SD. Statistical significance was calculated for differences in colocalization indices between cells incubated with VacA at the indicated concentrations and those cells mock intoxicated with PBS pH 7.2.
Fig. S4. Effects of inhibiting Drp1 GTPase activity on mitochondrial fragmentation. AZ-521 cells (A, C, and D) or AGS cells (B), previously transfected with pDsRed2-Mito (A–C) and cotransfected with either pEGFP-Drp1 or pEGFP-DN-Drp1 (K38A) (D), were incubated at 37 °C and under CO2 with VacA (250 nM) (A, B, and D) or infected with Hp 60190 (MOI 100) (C) or mock treated with PBS pH 7.2 (A–D), both in the absence or presence of mdivi-1 (50 μM) (A–C). After 4 h (A, B, and D) or 8 h (C), the cells were fixed and cellular mitochondria were visualized by DIC epifluorescence microscopy. The images reveal the morphology of fluorescently stained mitochondria and are representative of those collected from three independent experiments. (Scale bar, 5 μm.)
Fig. S5. Relationships between Drp1 GTPase activity, Bax activation, and mitochondrial Cyt c release. AZ-521 cells (A, C, and E), AZ-521 cells that had been transfected with pEGFP-Drp1 or pEGFP-Drp1 (K38A) (B and F) or bax+/+ or bax–/– MEFs that had been previously transfected with pDsRed2-Mito (D) were incubated at 37 °C and under CO₂ with VacA (250 nM) (A–C, E, and F) or PBS pH 7.2 (B and F). For A, the data were rendered as the fold change in total cellular Bax between cells incubated with VacA and those mock intoxicated with PBS pH 7.2, both in the presence or absence of mdivi-1 and were obtained by combining data from two independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for fold differences in Bax levels between cells incubated with PBS pH 7.2 alone versus cells pretreated with mdivi-1 or cells treated with VacA in the presence or absence of mdivi-1. For A, the cells were fixed, permeabilized, and immunostained for activated Bax. Intracellular-activated Bax within each transfected cell was visualized by DIC-epifluorescence microscopy. (Scale bar, 5 μm.) (B) Images are representative of those collected from three independent experiments. (C) Data were rendered as the fraction of the entire population of cells incubated with VacA or PBS pH 7.2 that display active Bax, at the indicated time periods. Data were obtained by analyzing over 700 cells from randomly chosen fields over the course of two independent experiments. Error bars indicate SD. Statistical significance was calculated for differences in fraction of total cells that display Bax activation between cells treated with VacA and those treated with PBS pH 7.2 or between cells intoxicated with VacA for indicated time periods versus those intoxicated for 1 h. For D, the cells were fixed, and cellular mitochondria were visualized using DIC-epifluorescence microscopy. The images reveal the morphology of fluorescently stained mitochondria and are representative of those collected from three independent experiments. (Scale bar, 5 μm.) For E and F, the cells were fixed, permeabilized, and immunostained for Cyt c. Cellular localization of Cyt c within each transfected cell was visualized by DIC-epifluorescence microscopy. (Scale bar, 5 μm.) For E, data were rendered as the fraction of the entire population of cells incubated with VacA or PBS pH 7.2 that display Cyt c release, at the indicated time periods. Data were obtained by analyzing over 1,000 cells from randomly chosen fields over the course of two independent experiments. Error bars indicate SD. Statistical significance was calculated for differences in fraction of total cells that display Cyt c release between cells intoxicated with VacA and those treated with PBS pH 7.2 or between cells intoxicated with VacA for indicated time periods versus those intoxicated for 1 h. (F) Images are representative of those collected from three independent experiments.
**Fig. S6.** Drp1 GTPase activity and VacA induced cell death in AGS cells. AGS cells were incubated at 37 °C and under CO₂ with VacA (250 nM) or mock treated with PBS pH 7.2, both in the absence or presence of mdivi-1 (50 μM). After 24 h, cell viability was determined with the Live-Dead viability/cytotoxicity assay kit, using flow cytometry. The data were rendered as the fold increase in dead cells following VacA intoxication relative to cells mock treated with PBS pH 7.2, obtained by combining data collected from two independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for fold differences in cell death between cells intoxicated with VacA versus cells treated with PBS pH 7.2 in the presence or absence of mdivi-1.

**Fig. S7.** Role of VacA anion channel function in mitochondrial morphology changes, Bax activation, and cell death within AZ-521 cells. AZ-521 cells were transfected with plasmids encoding p34-EGFP (residues 1–319) or EGFP (A) or incubated at 37 °C and under CO₂ with HPCFs (0.05 mg/mL) prepared from Hp 60190 or Hp strains expressing VacA (P9A) or VacA (G14A) (B and C), which are mutant forms of VacA deficient in membrane channel activity (1, 2). (A) After 24 h or 48 h the cells were fixed, permeabilized, and immunostained for Tom-20 as a mitochondrial marker, followed by visualization of cellular mitochondria and p34-EGFP and EGFP using DIC-epifluorescence microscopy. Images include, as indicated, mitochondria (red), p34-EGFP, or EGFP (green). Arrows (white) indicate cells positive for p34-EGFP or EGFP expression. Images are representative of those collected over the course of two independent experiments (n = 60). (Scale bar, 5 μm.) (B) After 18 h, the cells were fixed, permeabilized, and immunostained for activated Bax. The data were rendered as the fold change in activated Bax levels between cells incubated with the indicated HPCF compared with monolayers mock intoxicated with PBS pH 7.2, obtained by combining data collected from three independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for differences in fold-activated Bax between cells mock intoxicated with PBS pH 7.2 and those cells incubated with HPCF, HPCF-VacA (P9A), or HPCF-VacA (G14A).

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Fig. S8. VacA localizes to mitochondria. AZ-521 cells were incubated with VacA (100 nM) at 37 °C and under CO₂. After 30 min, the cells were fixed, permeabilized, and immunostained for VacA, and Tom-20 as a mitochondrial marker, and analyzed for localization of VacA to mitochondria. Image includes, as indicated, mitochondria (red), VacA (green), and VacA colocalized to mitochondria (yellow). Image is representative of those collected over the course of three independent experiments. (Scale bar, 5 μm.)

Fig. S9. The effects of inhibiting Drp1 GTPase activity on VacA-dependent dissipation of mitochondrial inner membrane potential (ΔΨₐ). AZ-521 cells were incubated at 37 °C and under CO₂ with VacA (250 nM) or mock intoxicated with PBS pH 7.2, both in the absence or presence of mdivi-1 (50 μM). After 4 h, the relative ΔΨₐ was determined using flow cytometry analysis of TMRE (50 nM)-stained cells. The data are rendered as the fold change in TMRE uptake in cells intoxicated with VacA relative to cells mock intoxicated with PBS pH 7.2, obtained by combining data collected from three independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for differences in ΔΨₐ between cells mock intoxicated with PBS pH 7.2 versus cells incubated with mdivi-1 or cells incubated with VacA in the presence or absence of mdivi-1.