Cellular Microbiology (2007) **9**(6), 1481–1490
doi:10.1111/j.1462-5822.2006.00886.x
First published online 23 January 2007

The concerted action of the *Helicobacter pylori* cytotoxin VacA and of the v-ATPase proton pump induces swelling of isolated endosomes

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Summary

The vacuolating cytotoxin (VacA) is a major virulence factor of *Helicobacter pylori*, the bacterium associated to gastroduodenal ulcers and stomach cancers. VacA induces formation of cellular vacuoles that originate from late endosomal compartments. VacA forms an anion-selective channel and its activity has been suggested to increase the osmotic pressure in the lumen of these acidic compartments, driving their swelling to vacuoles. Here, we have tested this proposal on isolated endosomes that allow one to manipulate at will the medium. We have found that VacA enhances the v-ATPase proton pump activity and the acidification of isolated endosomes in a Cl^- dependent manner. Other counter-anions such as pyruvate, Br^-, I^- and SCN^- can be transported by VacA with stimulation of the v-ATPase. The VacA action on isolated endosomes is associated with their increase in size. Single amino acid substituted VacA with no channel-forming and vacuolating activity is unable to induce swelling of endosomes. These data provide a direct evidence that the transmembrane VacA channel mediates an influx of anions into endosomes that stimulates the electrogenic v-ATPase proton pump, leading to their osmotic swelling and transformation into vacuoles.

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Introduction

The vacuolating cytotoxin A (VacA) is a major virulence factor of *Helicobacter pylori*, a bacterium that colonizes the gastric mucosa. This pathogen is associated with gastritis, peptic ulcer (Marshall and Warren, 1984) and with the development of gastric carcinoma and mucosa-associated lymphoid tissue lymphoma (Parsonnet *et al*., 1994). *In vivo*, VacA plays an important role during the initial bacterial colonization of the stomach (Salama *et al*., 2001). *In vitro*, it can have pleiotropic effects (Cover and Blanke, 2005). After internalization by cells, VacA has been reported to induce release of cytochrome *c* in the cytosol, leading to apoptosis (Galmiche *et al*., 2000; Cover *et al*., 2003; Willhite *et al*., 2003; Yamasaki *et al*., 2006). VacA also interferes with the immune system by inhibiting antigen presentation (Molinari *et al*., 1998) and T cell proliferation (Boncristiano *et al*., 2003; Gebert *et al*., 2003; Sundrud *et al*., 2004). Moreover, VacA has the capacity to increase the permeability of polarized epithelial cell monolayers to various ions and small uncharged molecules (Papini *et al*., 1998).

The best characterized activity of VacA is its ability to induce the formation of large cytoplasmic vacuoles (2–5 μm) in cultured cells (Cover and Blaser, 1992). Vacuoles originate from late endosomes (Papini *et al*., 1994) and their formation requires the active small GTPase Rab7 (Papini *et al*., 1997) and is blocked by bafilomycins, specific inhibitors of the electrogenic vacuolar-type ATPase proton pump (v-ATPase) (Cover *et al*., 1993; Papini *et al*., 1993a,b). The secreted VacA toxin oligomerizes into rosettes (Lupetti *et al*., 1996; Adrian *et al*., 2002) and inserts into biological membranes forming an anion-selective channel (Czajkowsky *et al*., 1999; Szabo *et al*., 1999; Tombola *et al*., 1999; Debellis *et al*., 2001). Cell vacuolation is inhibited by preventing the oligomerization of the toxin (Genisset *et al*., 2006) or by using chloride channel blockers (Szabo *et al*., 1999). This pathological phenotype can also be induced by expressing the toxin in the cell cytosol (de Bernard *et al*., 1997; Ye *et al*., 1999), as expected if vacuolization is associated with the activity of the VacA channel inserted in the external membrane of endosomes.

Studies of organellar pH in living cells and in isolated vesicles have provided evidence that Cl^- entry may be the rate-limiting passive conductance in allowing active H^+ entry into endosomes (Barasch *et al*., 1991; Sonawane *et al*., 2002). Activation of Cl^- channels might regulate endosomal acidification by providing a shunt to dissipate the positive internal potential produced by the electrogenic mode of operation of the H^+ pump. In fact, defects in the Cl^- channel activity of the cystic fibrosis transmembrane conductance regulator (CFTR) and of ClC-5 impair acidification of intracellular compartments and are associated with cystic fibrosis (Barasch *et al*., 1991) and Dent's disease (Gunther *et al*., 1998) respectively.

It was proposed that: (i) endocytosed VacA channels could stimulate the H^+ pumping activity of the v-ATPase by increasing the permeability of the endosomal membrane to anions, thus boosting the H^+ entry; and (ii) the consequent accumulation of osmotically active species, including membrane permeable weak bases that remain trapped within the lumen, would lead to water influx from the cytosol and swelling of these acidic compartments (Montecucco and Rappuoli, 2001). Moreover, vacuolization induced by VacA does not depend on soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that mediate endosomal homotypic fusion (de Bernard *et al*., 2002) suggesting that the limiting membranes of vacuoles derive from internal membranes and not from the fusion of neighbouring endosomes.

To test this model of VacA-induced vacuolization, we have investigated the direct effects of VacA on purified endosomes that allow one to modify at will the external medium, which topologically corresponds to the cytosol in cells. We found that the toxin stimulates the acidification of endosomes in a manner depending on the nature of the external anion but not of the cation. Acidification in the presence of VacA was associated with a concomitant increase of the endosomal size. The present set up and validation of this model system provides strong direct evidence in favour of the osmotic swelling mechanism of vacuolization, and, at the same time, defines a novel method to study the activity of other anion channel forming toxins.

Results

VacA colocalizes with the v-ATPase in the membrane of endosomes

When expressed in the cytosol of HeLa cells, VacA colocalizes with the v-ATPase in the membrane of vacuoles (de Bernard *et al*., 1997). To test if, after internalization by cells, the toxin localizes to cellular compartments endowed with the v-ATPase proton pump, confocal immunofluorescence localization studies with specific antibodies against VacA and the v-ATPase were performed. After 4 h of incubation with acid-activated concentrated supernatant from a *H. pylori* SPM 326 culture, cells showed large vacuoles in their cytoplasm. The membranes of these vacuoles were stained with both a polyclonal antibody against VacA and a monoclonal antibody specific for the human 116 kDa regulatory subunit of vacuolar ATPase (Fig. 1A), indicating the coexistence of these two molecules on the same vacuolar membranes. A complete colocalization between the two antigens is not expected because the v-ATPase is present on several intracellular organelles that are not targeted by VacA. On the other hand, colocalization clearly indicates their presence on the same vacuolar membrane, a prerequisite for the biochemical analysis object of the present work.

Given its importance, this point was further investigated by subcellular fractionation of HeLa cells that had been treated for 4 h with acid-activated supernatant from a *H. pylori* SPM 326 culture or from the VacA isogenic mutant strain, and immunoblotting with specific antibodies for the endosomal fraction, VacA and the cytosolic A subunit of the v-ATPase V1 domain. Figure 1B shows that, indeed, the endosomal fraction of treated cells included all three proteins, while in endosomes from mock-treated cells only Rab7 and the v-ATPase were present.

VacA increases the ATP-dependent acidification of endosomes

To test the model of the VacA-driven osmotic swelling of endosome via stimulation of the electrogenic v-ATPase proton pump activity, a simple *in vitro* system was used. Endosomes were isolated on a sucrose step gradient from BHK cells, a cell line that gives a high yield in endosomes and was previously used to study the biochemical composition of VacA-induced vacuoles (Molinari *et al*., 1997). The ATP-dependent acidification of these vesicles, in the presence of purified VacA, was measured using the extrinsic pH probe acridine orange (AO). This is a membrane-permeant weak base that accumulates in acidic compartments with ensuing concentration-dependent decrease of its fluorescence (Dell'Antone, 1979; Fuchs *et al*., 1994). Thus, a decrease in AO fluorescence signal accompanies acidification of the organelle lumen. As shown in Fig. 2, the uptake of AO is enhanced by VacA, but only in the presence of KCl. The dose–response profiles for KCl-dependent stimulation of endosome acidification by VacA revealed a maximum at around 60 mM KCl (Fig. 3A) and at 5 nM of toxin (Fig. 3B). The VacA-dependent increased acidification was strictly dependent on $[Cl^-]$ and/or $[K^+]$ because: (i) no effect was seen in a buffer containing only sucrose

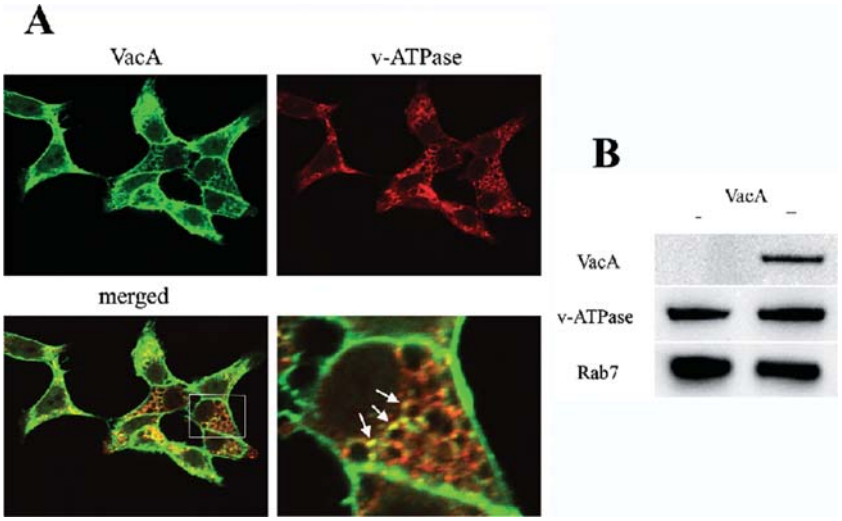

Fig. 1. VacA and the v-ATPase colocalize in the endosomes of intoxicated cells.
A. HeLa cells were intoxicated with acid-activated concentrated supernatant from *H. pylori* WT strain. After 4 h of incubation, the cells were fixed, permeabilized and the VacA toxin was immunostained with a rabbit anti-VacA antibody and a secondary FITC anti-rabbit antibody. The v-ATPase was detected with a murine monoclonal primary antibody and a secondary TRITC anti-mouse antibody. Lower right panel: zoom from the merged picture. Arrows indicate spots of colocalization (yellow) between VacA (green) and v-ATPase (red). A complete overlap between the two antigen is not expected because the v-ATPase is present on several intracellular organelles that are not targeted by VacA.
B. Western blotting performed on endosomes purified from HeLa cells incubated for 4 h with acid-activated concentrated supernatant from *H. pylori* WT strain (+VacA) or from the VacA null strain (–VacA) revealed the concomitant presence of VacA, the v-ATPase and the endosomal marker Rab7 in endosomes of intoxicated cells.

(Fig. 2A); and (ii) increasing KCl concentration enhanced the VacA-dependent acidification of isolated vesicles (Figs 2B and 3A). In some experiments, purified VacA was used with results closely similar to those obtained with partially purified VacA, as expected on the basis of the established findings that cell vacuolization is only due to VacA and not to other bacterial components (not shown). These experiments show the high sensitivity of this system, which allows one to test the effect of minute amounts of toxin, which are by far lower than those necessary for the cell vacuolization assay (Cover and Blaser, 1992).

To further investigate whether this phenomenon was due to Cl^- that had been previously accumulated in endosomes, isolated vesicles were equilibrated overnight at 4°C in an acidification buffer containing 0.25 M sucrose, in order to remove most of the internal Cl^- (Fuchs *et al.*, 1989). Under these conditions of ionic equilibrium, the addition of ATP led to a plateau of AO quenching within 10 min (Fig. 4). At this time point, addition of acid-

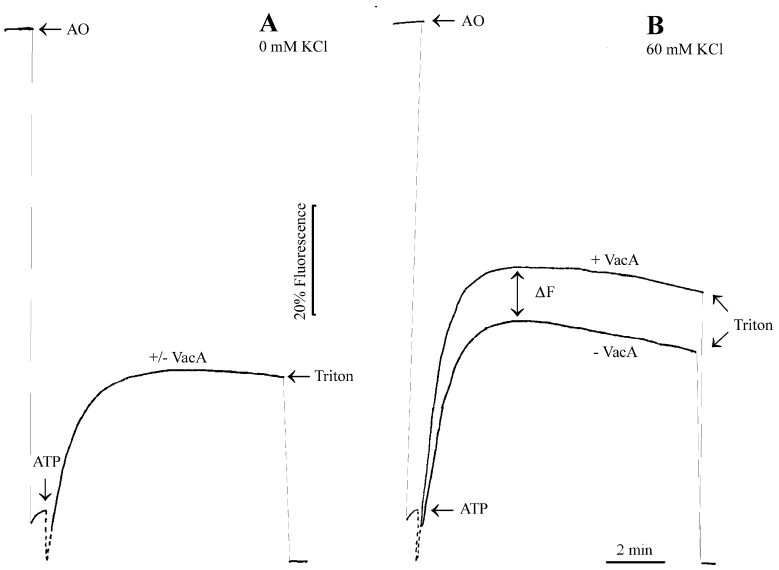

Fig. 2. ATP-dependent acidification of late endosomes requires VacA and KCl.
A. Endosomes (34 μg ml^{-1}) were equilibrated in a Hepes buffer containing either 0 mM KCl and 0.25 M sucrose, or 60 mM KCl and 0.13 M sucrose. Following 5 min of incubation with 50 μl of acid-activated supernatant dialysed against Hepes buffer devoid of chloride (+VacA), the acidification of endosome was monitored at 37°C after sequential addition of 6 μM acridine orange and of 3.3 mM ATP, in the absence (A) or in the presence (B) of 60 mM KCl. At the end of the run, the pH gradient was collapsed by addition of 0.025% of Triton X-100. Acidification was appraised as decrease in AO fluorescence. For the control, acidification of endosomes was recorded after addition of the same volume of acid-activated supernatant devoid of the toxin (–VacA). The data are representative of four separate sets of experiments.

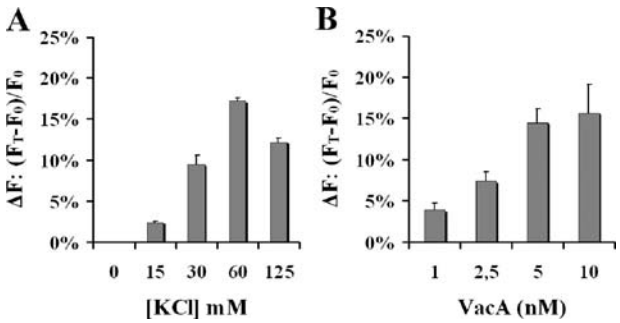

Fig. 3. Dose–response profiles of the KCl and the VacA concentrations effects on endosome acidification.
A. ATP- and VacA-dependent acidification of endosomes as a function of the KCl concentration. Acidification of isolated endosomes (37 μg ml^{-1}) was recorded as in Fig. 2, in a Hepes buffer containing varying concentrations of KCl and of sucrose in order to maintain the osmotic pressure constant. The difference in fluorescence (ΔF) is reported as a function of KCl concentration. F_T: maximal AO quenching in the presence of 100 μl of concentrated acid-activated supernatant containing VacA. F_0: maximal AO quenching in the presence of 100 μl of concentrated acid-activated supernatant devoid of VacA. The data are representative of two separated sets of experiments.
B. The ATP- and KCl-dependent acidification of endosomes as a function of VacA concentration. Acidification of isolated endosomes (0.42 μg ml^{-1}) was recorded as in Fig. 2, in a Hepes buffer containing 60 mM KCl and 0.13 M sucrose along with varying amounts of purified acid-activated VacA in PBS. The difference in fluorescence (ΔF) is reported as a function of VacA concentration. F_T: maximal AO quenching in the presence of acid-activated VacA. F_0: maximal AO quenching in the presence of acid-activated PBS. The data are representative of two separate sets of experiments.

activated, chloride-free VacA did not cause endosome acidification, demonstrating that the toxin alone does not affect the system. Yet, after another 5 min of incubation, addition of 30 mM KCl induced a net acidification (left panel of Fig. 4A). Acidification was also observed upon addition of 30 mM of KCl in the presence of the supernatant of a culture of *H. pylori* VacA isogenic mutant, but the initial velocity of AO quenching was much lower because in this case only endogenous Cl$^-$ channels were operating (Fig. 4A right panel and Table 1).

Table 1. Effect of cations and anions on VacA-dependent acidification.

	Activity (% quenching min^{-1} mg^{-1})	
	+VacA	–VacA
Na^{+a}	711	335
K^{+a}	686	400
Choline^{+a}	982	194
TEA^{+a}	616	205
F^{-b}	0	0
Cl^{-b}	711	335
Cl^{-c}	686	400
SCN^{-b}	672	422
Br^{-c}	833	272
Pyruvate^b	848	0
I^{-c}	511	156
Gluconate^c	0	0

a. Anion: Cl.
b. Cation: Na$^+$.
c. Cation: K$^+$.

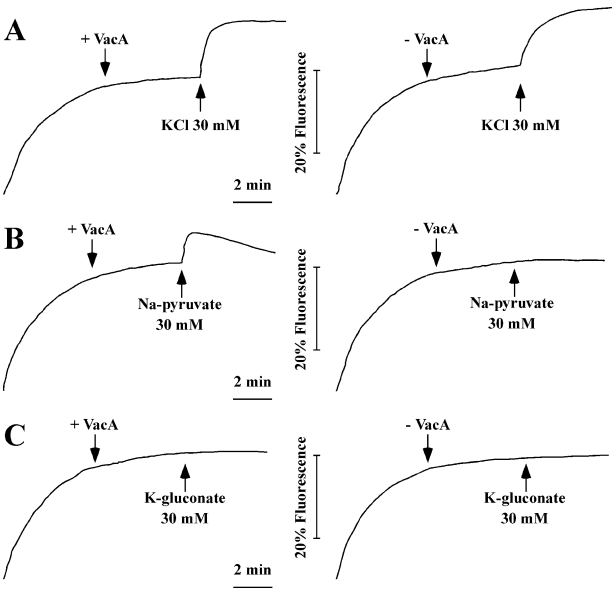

Fig. 4. Effect of anions on VacA-dependent acidification. In order to perform experiments under conditions of ionic equilibrium, isolated endosomes (50 μg ml^{-1}) were equilibrated overnight at 4°C in a Hepes buffer containing 0.25 M sucrose. Acidification was then recorded after addition of 6 μM AO and 3.3 mM ATP. When the AO fluorescence decrease had reached a plateau, acid-activated concentrated supernatant, containing or not containing VacA, was added to the assay. After further 5 min of incubation, the acidification was monitored upon addition of 30 mM KCl (A), 30 mM Na$^+$-pyruvate (B), or 30 mM K$^+$-gluconate (C). The initial rate of fluorescence quenching upon salt addition was extrapolated from fluorescence curves and reported as percentage of quenching per minute per milligram of endosomal protein in Table 1. The data are representative of three separate sets of experiments.

Role of anions on VacA-dependent acidification

Because the positive internal membrane potential due to H$^+$ transport can be dissipated either by influx of anions or by efflux of cations (Fuchs *et al*., 1989), both anion and cation permeability may be involved in the ATP-driven acidification of purified endosomes. To test whether the acidification observed in the presence of VacA was dependent on the nature of the anion and/or the cation, the initial quenching rate of AO fluorescence after addition of 30 mM of various ion species was determined (Fig. 4). Similar increases of activity were observed employing chloride salts with counter ions of different molecular sizes, both with high (Na$^+$, K$^+$) or low (choline, tetraethylammonium – TEA) membrane permeability characteristics (Table 1). These data demonstrate that the VacA-induced internal acidification is independent of the nature of the cation added and that it is exclusively due to facili-

tated Cl^- entry. On the contrary, the action of VacA on endosomes was dependent on the nature of the anion. Endosome acidification was stimulated by Cl^-, Br^-, I^- or SCN^-, which is much higher in the presence of VacA (Table 1 and Fig. 4A), indicating that endosomal channels are able to transport these anions and that the VacA toxin increases their rate of entry into endosomes. Further, pyruvate was able to stimulate the proton pump, but only when VacA had been added (Table 1 and Fig. 4B), showing that VacA is a pore that allows the passage of pyruvate, while endosomal membranes are impermeable to this anion. Instead the F^- and the organic anion gluconate had no effect on acidification, either in the presence or absence of VacA (Table 1 and Fig. 4C).

The channel activity of VacA is associated with an increase in endosome size

The model of VacA-induced vacuolization, here under test, proposes that vacuoles derive from the osmotically driven enlargement of endosomes supported by fusion of internal membranes with the outer organelle membrane (Montecucco and Rappuoli, 2001; de Bernard *et al*., 2002). Electron microscopy was used to assess whether the anion-, ATP- and VacA-dependent acidification of isolated endosomes was associated with their increase in size. Samples were equilibrated in acidification buffer containing gluconate, and the AO fluorescence signal was recorded after addition of ATP. When the fluorescence had reached a plateau, acid-activated VacA supernatant, or of the VacA deficient strain or of the VacA P9A mutant with defective channel activity (Ye and Blanke, 2000; McClain *et al*., 2003), in phosphate-buffered saline (PBS) was added to the reaction, in order to have the toxin and 20 mM Cl^- present at the same time. As shown in Fig. 5A, the addition of the toxin caused a rapid acidification followed by an increase of AO fluorescence (curve bending downwards), which might be due to a H^+ leakage caused by the volume increase of the endosomes. This occurs only in the presence of a functional toxin anion channel, as addition of a concentrated supernatant of a *vacA* null strain or of VacA P9A, a mutant form of the toxin with defective channel activity (Ye and Blanke, 2000; McClain *et al*., 2003), was without effect, i.e. only a slow initial rate of fluorescence change, due to the introduced chloride, was recorded and this was not followed by a fluorescence increase (Fig. 5B and C). The same phenomenon was also observed when the toxin was incubated with endosomes before the addition of ATP (data not shown). After 30 min of reaction, samples were negatively stained and examined by transmission electron microscopy (TEM). Although this staining method may alter the actual size and shape of the organelle, a comparative analysis of the differently treated endosomes provided a clear result.

Table 2. Effect of VacA on endosome size.

	Mean size (nm)	*n*
+VacA	1157 ± 393	37
–VacA	719 ± 382	30
+VacA P9A	794 ± 239	34
Crtl-	694 ± 295	31

Figure 5D–G shows that the purified late endosomes used in the assays were largely intact. A simple inspection of their diameter indicates that the presence of active VacA toxin causes an increase of endosome size (Fig. 5H and Table 2). Collectively, these data indicate that the AO fluorescence recovery observed after addition of VacA and acidification was associated with endosome swelling, owing to an increased entry of chloride ions. Moreover, a single-point VacA mutant that lacks channel function was not able to induce swelling.

The VacA-dependent vacuolization is enhanced by the presence of weak bases in the culture medium (Cover *et al*., 1992; Ricci *et al*., 1997). VacA-dependent acidification of isolated endosomes in the presence of 5 mM NH_4Cl was also associated with a volume increase (data not shown), suggesting that the accumulation of a weak base in the lumen of endosomes contributes to the osmotic swelling observed.

Discussion

Cell vacuolization induced by VacA is strictly dependent on the activity of the electrogenic v-ATPase proton pump. In fact, addition of the specific inhibitors bafilomycins to the culture medium (Cover *et al*., 1993; Papini *et al*., 1993a,b) or of a specific monoclonal antibody directed against the 116 kDa ATPase regulatory subunit (Papini *et al*., 1996), prevents vacuole formation. Bafilomycins also induce vacuolated cells to revert to a normal phenotype, suggesting that a continuous operation of the proton pump is necessary to maintain swelling (Papini *et al*., 1993a). Here we have shown that the proton pump and VacA, presented to cells from the outside, as it happens during *Helicobacter* infection, colocalize in the limiting membranes of the vacuoles. However, also cells transfected with a plasmid coding for mature VacA develop vacuoles whose limiting membrane includes both VacA and the v-ATPase (de Bernard *et al*., 1997; 1998; Ye *et al*., 1999), showing that VacA can assemble into active channel also from the cytoplasmic side of the endosomal membrane. On this basis, we have developed an *in vitro* model system using isolated endosomes, which allow one to modify the biochemical parameters of the toxin channel activity. The assay is simple to be carried out and the read-out is well documented and very fast, making it a

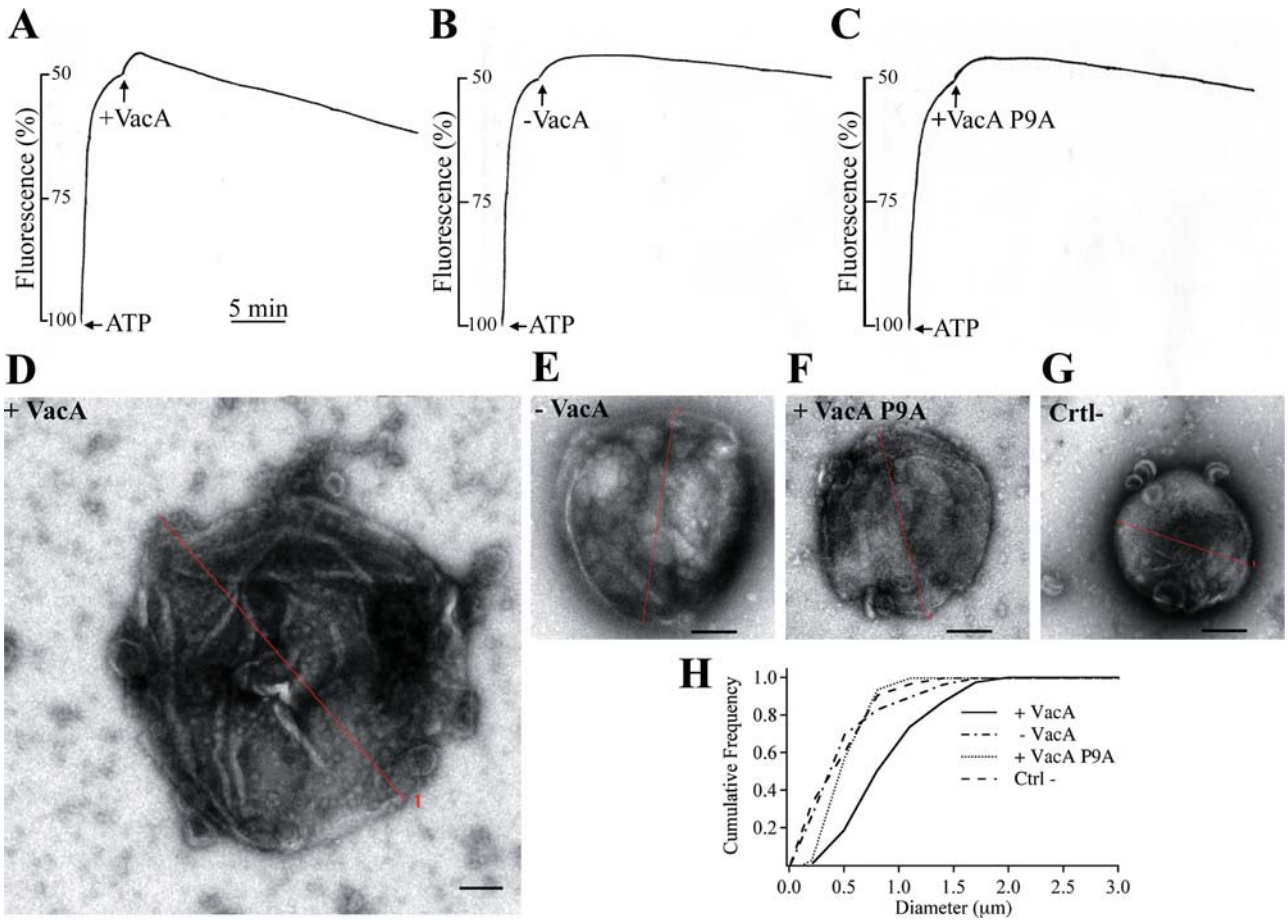

Fig. 5. Endosomal acidification in the presence of VacA is associated with an increase in size.
A–C. Late endosomes (8 μg ml^{-1}) were equilibrated in acidification buffer containing 125 mM K^+-gluconate, and the AO fluorescence signal was recorded at 37°C after addition of ATP. At plateau, an acid-activated concentrated supernatant in PBS containing VacA (A), no toxin (B), or VacA P9A (C), was added to the reaction and the AO fluorescence was recorded for 30 min.
D–G. Electron micrographs of the samples A–C, after 30 min of reaction. Crtl– (G) corresponds to late endosome incubated with acid-activated concentrated supernatant containing VacA in the absence of ATP. Each scale bar corresponds to 200 nm.
H. Cumulative frequency distributions of endosome diameter. Diameters recorded from endosomes treated as described in A were divided into groups and charted as histograms. The bin size was chosen equal to standard deviations (~300 nm,Table 2). The area of the graphs was integrated numerically with a commercial software (IGOR Pro, Wavemetrics) to obtain the cumulative frequency distribution. The tendency of the curves was independent of the bin size selected. Data shown are of one experiment representative of three sets performed.

useful tool for the study of other vacuolating toxins such as the *Vibrio cholerae* vacuolating cytotoxin (Moschioni *et al*., 2002).

Using this assay we have provided here direct evidence for the model of the VacA-induced cell vacuolization mentioned above and discussed in details elsewhere (Montecucco and Rappuoli, 2001). VacA effectively stimulated the v-ATPase activity with consequent quenching of acridine orange fluorescence. The effect of VacA was maximal at a concentration of 5 nM (Fig. 3B), while a full cellular vacuolation requires a higher concentration of toxin (Willhite *et al*., 2003; Blanke, 2005; Cover and Blanke, 2005). This *in vitro* VacA activity was strictly dependent on the Cl^- concentration and reached a maximum value at around 60 mM Cl^- (Fig. 3A), which is close to the physiological chloride concentration in the cytoplasm (Sonawane *et al*., 2002). Other anions such as Br^-, I^- and SCN^- but not F^-, can be transported by the VacA channel. On the contrary, the large-size gluconate anions failed to stimulate the v-ATPase either in the presence or in the absence of VacA, at variance with the gluconate permeation through the VacA channel obtained with artificial membranes (Tombola *et al*., 1999). This may be taken as an indication of a higher reliability of the present model system that uses the same cellular membranes as those targeted by the toxin *in vivo* (Tombola *et al*., 1999). Of potential interest is the finding that pyruvate had an effect only in presence of VacA, demonstrating that membranes of isolated endosomes are impermeable to pyruvate and that VacA can transport this

anion. VacA can be delivered to host cells via a contact-dependent transfer (Ilver *et al*., 2004), suggesting that *in vivo*, VacA is mainly released in the limited volume sandwiched between the mucus layer and the apical domain of epithelial cells of the stomach. Our finding indicates that VacA inserted in the plasma membrane of the host cell could transport pyruvate from the cytoplasm to *H. pylori*, where it could be used as energy source (Mendz *et al*., 1994). This would contribute to explain how the *vacA* gene confers a strong advantage to wild-type strains over VacA-defective mutants in the colonization of the stomach (Salama *et al*., 2001).

VacA-induced formation of vacuoles is promoted by the accumulation of membrane permeable weak bases that are trapped, after protonation, in the lumen of acidic compartments (Ricci *et al*., 1997). Protons themselves are not so effective because they have a higher tendency to leak out of the lumen. The electron microscopic observations performed in this study clearly show that also isolated endosomes swell in the presence of VacA, though we have not recorded the dimensions observed previously in intact cells (Ricci *et al*., 1997; de Bernard *et al*., 2002), possibly because of the lack of the structural complexity of the cell cytosol. Indeed, vacuole biogenesis appears to be a complex process that requires also several cellular factors like the small GTPase Rab7 (Papini *et al*., 1997) and the member of the Rho family, Rac1 (Hotchin *et al*., 2000). Indeed, the present model system appears very suitable to test a variety of cytosolic factors for their ability to support vacuole formation. This model system is very sensitive, in accordance with the fact that there is a very limited membrane-free space available within late endosomal compartments (Gruenberg, 2001). Therefore, a limited uptake of osmotic species causes a significant increase in osmotic pressure. This model system should be useful in testing several other anion channel-forming toxins acting inside cells.

Experimental procedures

Bacteria and culture conditions

Helicobacter pylori strains SPM 326 (Marchetti *et al*., 1995) and isogenic *vacA* mutant strain SPM 326KO2 (Genisset *et al*., 2006) were maintained in 5% CO_2 at 37°C on Columbia agar plates supplemented with 5% horse blood. Colonies were inoculated into brain heart infusion broth containing 5% fetal calf serum and were cultured for 2 days with rotary shaking at 180 r.p.m. at 37°C under microaerophilic conditions.

Proteins of supernatant from broth culture, diluted to an optical density at 600 nm (OD_{600}) of 0.8, were precipitated with 50% ammonium sulfate and resuspended in the appropriate volume of 20 mM Hepes pH 7.4 to be concentrated 15-fold as previously described (Skibinski *et al*., 2006). Concentrated supernatants were dialysed against the same buffer. When indicated, they were prepared in PBS. VacA was further purified by chromatography as described (Manetti *et al*., 1995). The VacA content was estimated by Coomassie blue staining after SDS-PAGE and by Western blotting with rabbit anti-VacA serum (Telford *et al*., 1994). Its vacuolating activity was evaluated in HeLa cells by measuring the uptake of neutral red after 7 h of incubation at 37°C as previously described (Cover and Blaser, 1992). For activation, the toxin was incubated with 7.5 mM H_2SO_4 for 5 min at room temperature and neutralized with 15 mM NaOH, as previously described (de Bernard *et al*., 1995).

Construction of an H. pylori *strain expressing mutant VacA P9A protein*

The *H. pylori* SPM 326KO2 strain was transformed with pBlue-Script KS p33 P9A, and a sucrose-resistant, kanamycin-sensitive transformant that was obtained by homologous recombination, was selected, as previously described (Genisset *et al*., 2006). The strain was analysed by nucleotide sequencing to verify that the desired mutation was present and the expression of VacA P9A was confirmed by Western blotting using a rabbit anti-VacA serum (Telford *et al*., 1994). The plasmid pBluescriptKS p33 P9A was obtained from pBluescriptKS p33, a plasmid containing a substantial portion of the *vacA* SPM 326 gene, by introduction of a single amino-acid mutation using the QuickChange site-directed mutagenesis kit (Stratagene). Briefly, oligonucleotide primers P9A_f (ACAACCGTGATCATTgcAGCCATTGTTGGGGG) and P9A_r (CCCCCAACAATGGCTgcAATGATCACGGTTGT) were extended by polymerase chain reaction (PCR) using Pfu DNA polymerase. Following thermal cycling, template DNA was eliminated by DpnI and the PCR products were used to transform *Escherichia coli* XL1-blue.

Immunofluorescence and confocal microscopy

HeLa cells seeded on coverslips (3×10^4 cells per well on 24-wells plates) were incubated with acid-activated SPM 326 concentrated supernatant (dilution 1:4) in DMEM containing 2% FCS for 4 h before fixation with 3.7% paraformaldehyde in PBS for 30 min. Cells were permeabilized with 0.01% Nonidet P40 for 20 min at room temperature and blocked with a solution of PBS containing 0.5% bovine serum albumin (BSA). VacA was stained with an anti-VacA antibody (de Bernard *et al*., 1997) and with a fluorescein isothiocyanate (FITC) anti-rabbit secondary antibody. The v-ATPase was labelled with a murine monoclonal antibody (OSW2) specific for the regulatory subunit (Sato and Toyama, 1994; Papini *et al*., 1996) followed by a tetramethylrhodamine isothiocyanate (TRITC) goat anti-mouse antibody. Confocal images were obtained with an Ultraview microscope (PerkinElmer).

Cell culture and isolation of endosomes by sucrose step gradient

Baby hamster kidney (BHK) cells were seeded at the density of 10^5 per millilitre in 10 cm tissue culture dishes and were grown in Dulbecco's modified medium containing 10% FCS at 37°C in 5% CO_2 for 48 h. Endosomes were isolated at 4°C as described previously (Gorvel *et al*., 1991; Molinari *et al*., 1997; Kobayashi *et al*., 1998) and were used soon after for the fluorescence

assays. Cells were washed with ice-cold PBS, detached with a cell scraper and pelleted by centrifugation (500 *g*, 5 min). They were then resuspended in 3 ml of homogenization buffer [HB-EDTA, 0.25 M sucrose (8%) 3 mM imidazole, 0.5 mM EDTA pH 7.4], pelleted (1300 *g*, 10 min), resuspended in 0.6 ml of HB-EDTA and homogenized by 10 passages through a 22-gauge 1[1/4] needle. After centrifugation (1300 *g*, 10 min), the post nuclear supernatant (PNS) was collected and its sucrose concentration increased to 40.6% by slow addition of 62% sucrose in HB-EDTA. PNS was then carefully overlaid with 1.5 ml of 35% and 1 ml of 25% sucrose in EDTA. The tubes were filled with HB, and centrifuged at 35 000 r.p.m. for 1 h at 4°C in a SW60 Ti rotor (Beckman Instruments). The late endosome-enriched fraction was collected between the 25% sucrose and HB (upper interface), the early endosome-enriched fraction between the 35% and 25% sucrose (middle interface). In some cases, early and late endosome fractions were pooled.

Immunoblot analysis

HeLa cells were seeded at the density of 10^5 per millilitre in 10 cm tissue culture dishes and grown in DMEM containing 10% FCS at 37°C in 5% CO_2 for 48 h. Cells were incubated for 4 h with acid-activated SPM 326 or SPM 326KO2 supernatant (dilution 1:2) in the same medium containing only 2% FCS and endosomes were isolated, as detailed above. The coexistence of VacA and v-ATPase in endosomes was assayed by Western blot analysis using a rabbit anti-VacA serum, polyclonal IgY (chicken) against subunit A of the V1 domain of the v-ATPase (Genway) and a polyclonal rabbit antibody against the endosomal marker Rab7.

Cell-free acidification assay

Purified endosomes (30–50 μg ml^{-1} of protein) were equilibrated with 6 μM AO in 0.5 ml of acidification buffer (20 mM Hepes pH 7.4, 5 mM $MgSO_4$) containing different concentrations of KCl and sucrose, in order to vary [Cl^-] without changing the osmotic pressure. After incubation with 50–100 μl of acid-activated VacA, the operation of the v-ATPase was initiated by addition of 3.3 mM ATP from an ATP-regenerating stock solution (100 mM ATP, 250 mM creatine phosphate and 250 U ml^{-1} creatine phosphokinase) adjusted to pH 7.4, and the change of the fluorescent signal of AO was recorded at 492 nm excitation and 530 nm emission wavelength using a Perkin-Elmer MPF 2A spectrofluorimeter, as previously described (Dell'Antone and Piergallini, 1997). Under these conditions, a decrease in AO fluorescence, due to accumulation-dependent concentration quenching caused by the uptake of the dye into the lumen, indicates acidification of the organelles. To recover the quenched fluorescence, the transmembrane pH gradient was collapsed at the end of each run by addition of Triton X-100 at the final concentration of 0.025%. For VacA-mediated ion permeability studies, endosomes were equilibrated overnight at 4°C in acidification buffer containing 0.25 M sucrose.

Transmission electron microscopy

Thin carbon films on 400 mesh copper grids were used as supports for TEM. Specimens from fluorescence assays were spread onto the carbon film pretreated with glow discharge, incubated for 10 min at 37°C inside a humid chamber and thoroughly rinsed with warm acidification buffer containing 125 mM K^+-gluconate. Excess liquid was carefully removed and samples were immediately stained with two drops of 1% uranyl acetate and dried with filter paper. Images were recorded at 100 kV on a Tecnai G^2 electron microscope (Fei Company, Eindhoven, the Netherlands) equipped with a FastScan F114B-CS-HS-4 CCD Camera with an F114-Tecnai shutter control (TVIPS, Gauting, Germany) using a LaB_6-filament. Endosome size was measured using the software provided by the producer.

Acknowledgements

We thank Giuseppe Tognon (University of Padua, Italy) for providing TEM grids and technical assistance, and Stéphane Méresse (University of Marseille, France) for the anti-Rab7 antibody. This work was supported by the MIUR Cofin 2005060371 project and from AIRC regional grant 2005 – Veneto.

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