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# Acid activation of *Helicobacter pylori* vacuolating cytotoxin (VacA) results in toxin internalization by eukaryotic cells

**Mark S. McClain,[1] Wayne Schraw,[1] Vittorio Ricci,[2] Patrice Boquet[2] and Timothy L. Cover[1,3,4,*]**

*[1]Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232-2605, USA. [2]INSERM Unité 452, Faculté de Médecine, 06107 Nice Cedex 2, France. [3]Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232-2605, USA. [4]Department of Veterans Affairs Medical Center, Nashville, TN 37232-2637, USA.*

## Summary

***Helicobacter pylori*** **VacA is a secreted toxin that induces multiple structural and functional alterations in eukaryotic cells. Exposure of VacA to either acidic or alkaline pH ('activation') results in structural changes in the protein and a marked enhancement of its cell-vacuolating activity. However, the mechanism by which activation leads to increased cytotoxicity is not well understood. In this study, we analysed the binding and internalization of [$^{125}$I]-VacA by HeLa cells. We detected no difference in the binding of untreated and activated [$^{125}$I]-VacA to cells. Binding of acid-activated [$^{125}$I]-VacA to cells at 4°C was not saturable, and was only partially inhibited by excess unlabelled toxin. These results suggest that VacA binds either non-specifically or to an abundant, low-affinity receptor on HeLa cells. To study internalization of VacA, we used a protease protection assay. Analysis by SDS–PAGE and autoradiography indicated that the intact 87 kDa toxin was internalized in a time-dependent process at 37°C but not at 4°C. Furthermore, internalization of the intact toxin was detected only if VacA was acid or alkaline activated before being added to cells. The internalization of activated [$^{125}$I]-VacA was not substantially inhibited by the presence of excess unlabelled toxin, but was blocked if cells were depleted of cellular ATP by the addition of sodium azide and 2-deoxy-D-glucose. These results indicate that acid or alkaline pH-induced structural changes in VacA are required for VacA entry into cells, and that internalization of the intact 87 kDa toxin is required for VacA cytotoxicity.**

Accepted 11 May, 2000. *For correspondence. E-mail covertl@ctrvax.vanderbilt.edu; Tel. (+1) 615 322 2035; Fax (+1) 615 343 6160.



## Introduction

*Helicobacter pylori* are Gram-negative bacteria that colonize the gastric mucosa of humans. Infection with this organism is associated with an increased risk of peptic ulcer disease, gastric adenocarcinoma and gastric lymphoma (Warren and Marshall, 1983; Dunn *et al*., 1997). An important virulence factor produced by *H. pylori* is the vacuolating cytotoxin, VacA (Leunk *et al*., 1988; Atherton *et al*., 1995; Tee *et al*., 1995; Cover, 1996; Montecucco *et al*., 1999). This toxin is the product of a single gene, *vacA*, which encodes a precursor protein of approximately 140 kDa (Cover *et al*., 1994; Schmitt and Haas, 1994; Telford *et al*., 1994; Cover, 1996). Processing of this precursor at both the amino- and carboxyl-termini results in a mature, 87 kDa VacA protein. Based on similarities between the cleaved carboxyl-terminal portion of VacA and members of the IgA protease family of proteins, the carboxyl-terminal portion of VacA is believed to mediate secretion of the mature toxin across the bacterial outer membrane into the extracellular medium (Cover *et al*., 1994; Schmitt and Haas, 1994; Cover, 1996). VacA assembles into complex water-soluble oligomeric structures composed predominantly of 12 or 14 subunits (Lupetti *et al*., 1996; Cover *et al*., 1997).

When added to a variety of cultured cell lines *in vitro* in the presence of weak bases, the VacA cytotoxin causes extensive cytoplasmic vacuolation (Leunk *et al*., 1988; Cover *et al*., 1992; Papini *et al*., 1994). The vacuoles induced by the cytotoxin contain both late-endosomal and lysosomal markers, including the small GTP-binding protein, rab7, and Lgp110 (Papini *et al*., 1994; Molinari *et al*., 1997). A vacuolar-type ATPase and rab7 are required for VacA-induced vacuole formation (Cover *et al*., 1993; Papini *et al*., 1993; 1997). In addition to vacuolation, VacA-treated cells exhibit increased extracellular release of acidic hydrolases, alterations in antigen processing, defects in the degradative abilities of late endosomes and lysosomes and inhibition of antigen presentation (Satin *et al*., 1997; Molinari *et al*., 1998a). Many of these effects may result from VacA-induced alterations in endocytic trafficking. In addition, recent studies have demonstrated

that VacA can form anionic channels in lipid bilayers and in the plasma membrane of HeLa cells (Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999; Szabo *et al.*, 1999; Tombola *et al.*, 1999a, b).

The purified VacA cytotoxin exhibits minimal activity unless it is first exposed to acidic or alkaline pH before being added to eukaryotic cells (de Bernard *et al.*, 1995; Yahiro *et al.*, 1999). This pH activation of VacA is probably related to changes in protein structure (de Bernard *et al.*, 1995; Molinari *et al.*, 1998b; Pagliaccia *et al.*, 2000). Specifically, acidification to pH 3 or alkalinization to pH 10 result in the nearly complete disassembly of VacA oligomers into component monomers, which retain the capacity to reanneal into oligomers after neutralization (Cover *et al.*, 1997; Molinari *et al.*, 1998b; Yahiro *et al.*, 1999). Thus, it appears that VacA exists in an equilibrium between disassembled and fully assembled forms, such that the monomeric form predominates at acidic or alkaline pH and the oligomeric form predominates at neutral pH. This equilibrium is probably influenced by VacA concentration as well as by pH. Several studies have demonstrated that acid activation alters the interaction of VacA with membranes, promoting insertion of the toxin into lipid bilayers (Moll *et al.*, 1995; Molinari *et al.*, 1998b; Pagliaccia *et al.*, 2000). Moreover, the ability of purified VacA to form anion conductive channels is enhanced by acid activation (Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999; Tombola *et al.*, 1999a). However, the mechanism by which pH-induced activation of VacA leads to increased cytotoxicity is not completely understood.

At least three other pore-forming toxins (aerolysin, *Serratia* haemolysin and *Vibrio cholerae* haemolysin) are known to induce vacuolation in eukaryotic cells and have been compared with VacA (Abrami *et al.*, 1998; Hertle *et al.*, 1999; Coelho *et al.*, 2000). The vacuoles that form in response to these three pore-forming toxins seem to be different from those that form in response to VacA (Abrami *et al.*, 1998; Hertle *et al.*, 1999; Coelho *et al.*, 2000), and vacuolation induced by the former pore-forming toxins does not require the presence of weak bases such as ammonium chloride. Aerolysin, *Serratia* haemolysin, and *Vibrio* haemolysin are thought to induce vacuolation as a result of their interactions with the plasma membrane. In contrast, VacA has been detected within intoxicated cells (Garner and Cover, 1996; Fiocca *et al.*, 1999), and transient transfection of HeLa cells with plasmids encoding amino-terminal fragments of VacA results in cell vacuolation (de Bernard *et al.*, 1997; 1998; Vinion-Dubiel *et al.*, 1999; Ye *et al.*, 1999). These results suggest that the vacuolating activity of VacA might result from toxin activity in an intracellular site rather than from interaction with the plasma membrane. However, there is still uncertainty about the site of VacA action (Czajkowsky *et al.*, 1999; Szabo *et al.*, 1999; Pagliaccia *et al.*, 2000), and internalization of VacA by eukaryotic cells has not yet been studied in any detail.

In this study, we analysed the binding and internalization of radiolabelled VacA by HeLa cells (the cell line that has been used most widely to study VacA activity). The results indicate that acid or alkaline activation of VacA is required for toxin internalization by cells, and indicate that internalization of the intact 87 kDa toxin is required for cytotoxicity.

## Results

### *Binding of VacA to HeLa cells*

Purified VacA was radiolabelled with $^{125}$I, as described in *Experimental procedures*, with no detectable loss of biological activity (Vinion-Dubiel *et al.*, 1999). Using [$^{125}$I]-VacA, we examined whether the binding of acid-activated VacA to HeLa cells exhibited characteristics of a

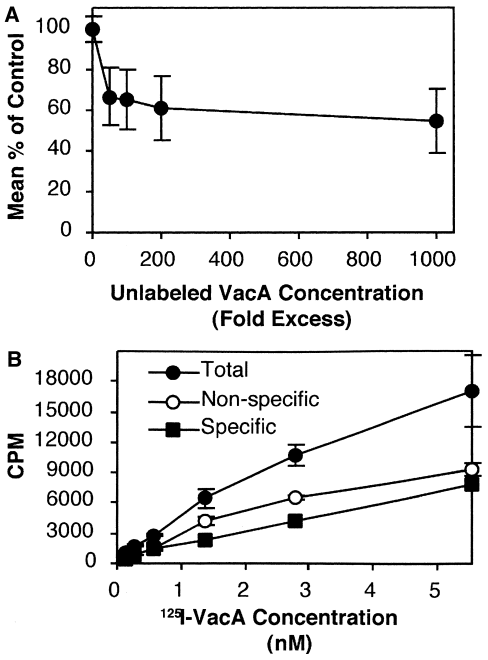


**Fig. 1.** Analysis of VacA binding to HeLa cells.
A. HeLa cells were incubated in binding buffer for 4 h at 4°C with 0.3 nM acid-activated [$^{125}$I]-VacA in the absence or presence of up to 1000-fold excess of unlabelled acid-activated toxin. Cells were then washed three times in binding buffer, and the cell-associated radioactivity was quantified. Results represent the mean and standard deviation of triplicate samples from three different experiments, each using a different [$^{125}$I]-VacA preparation. A 50-fold excess and a 1000-fold excess of unlabelled VacA inhibited [$^{125}$I]-VacA binding to similar extents ($P > 0.10$, Student's *t*-test).
B. HeLa cells were incubated in binding buffer for 4 h at 4°C with [$^{125}$I]-VacA (0.13–5.5 nM) in the absence (total binding, ●) or presence (non-specific binding, ○) of a 100-fold excess of unlabelled VacA. Cells were then washed three times in binding buffer, and the cell-associated radioactivity was quantified. Results represent the mean and standard deviation of triplicate samples. The specific binding (the difference between the total and non-specific binding, ■) is also shown.

high-affinity ligand–receptor interaction. Acid-activated [$^{125}$I]-VacA was incubated with cells in the absence or presence of up to a 1000-fold excess of unlabelled acid-activated toxin. Analysis of the radioactivity bound to cells indicated that a 50-fold excess of unlabelled VacA was able to inhibit binding of [$^{125}$I]-VacA by approximately 35%, with little additional inhibition at up to a 1000-fold excess of unlabelled toxin (Fig. 1A). This suggests that a large proportion of the detectable VacA binding is non-specific. To determine whether binding of VacA to HeLa cells was saturable, varying amounts (0.13–5.5 nM) of [$^{125}$I]-VacA were added to cell monolayers in the presence or absence of a 100-fold excess of unlabelled VacA at 4°C. Analysis of the radioactivity bound to cells indicated that VacA binding was not saturable within this range of toxin concentrations (Fig. 1B). In addition, less than 50% of the VacA bound to cells was released into the medium during prolonged incubations in large volumes, indicating that a substantial portion of VacA binding was not reversible (data not shown). These results suggest that VacA binds to multiple cell surface components or to an abundant, low-affinity receptor on HeLa cells. Similar results were observed using $^{14}$C-labelled VacA (data not shown).

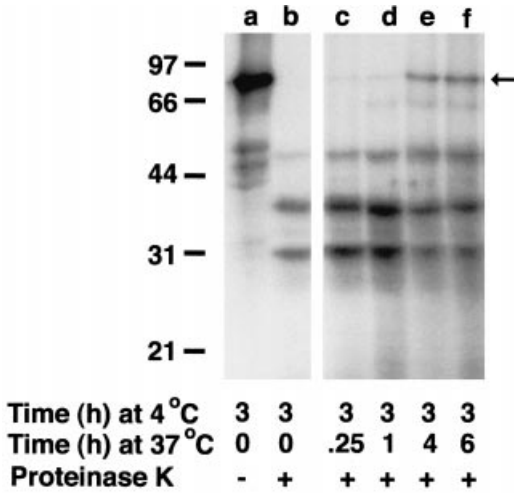


**Fig. 2.** Internalization of [$^{125}$I]-VacA by HeLa cells. HeLa cells were incubated in complete MEM with 6 nM acid-activated [$^{125}$I]-VacA for 3 h at 4°C (lanes a and b) or for 3 h at 4°C and then shifted to 37°C for 0.25–6 h (lanes c–f); ammonium chloride (10 mM) was added at the time of the temperature shift. After the incubations, cells were washed three times in TBS, treated with proteinase K (lanes b–f) or left untreated (lane a). Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. A protease-protected 87 kDa band (indicated by the arrow) could be visualized in lanes d–f. No protease-protected 87 kDa band was visualized in cells incubated with toxin for 7 h at 4°C before proteolysis (data not shown).

*Internalization of VacA by HeLa cells*

Several lines of evidence suggest that VacA may act on an intracellular target (Garner and Cover, 1996; de Bernard *et al*., 1997; 1998; Fiocca *et al*., 1999; Vinion-Dubiel *et al*., 1999; Ye *et al*., 1999). Therefore, we examined the internalization of [$^{125}$I]-VacA by HeLa cells using a protease protection assay. Acid-activated [$^{125}$I]-VacA was allowed to bind to cells at 4°C, and the cells then were shifted to 37°C. At various times after the temperature shift, extracellular [$^{125}$I]-VacA was proteolysed by the addition of proteinase K. Analysis by SDS–PAGE and autoradiography indicated that the intact, 87 kDa [$^{125}$I]-VacA was internalized in a time-dependent process at 37°C but not at 4°C (Fig. 2). A similar pattern of protease-resistant bands was observed when pronase was used in place of proteinase K (data not shown). The time course of [$^{125}$I]-VacA internalization was consistent with the time required for the formation of visible vacuoles and with the results of previous confocal or electron microscopy studies (Garner and Cover, 1996; Ricci *et al*., 1997; Sommi *et al*., 1998). In addition to the intact 87 kDa band, three lower molecular mass bands (approximately 52, 38 and 31 kDa) were consistently detected in these protease protection assays. These bands were also visualized in samples that were maintained at 4°C (Fig. 2, lane b). One possibility is that these bands represent toxin domains that were protected from proteolysis because they inserted into the plasma membrane. Alternatively, these bands may represent protease-resistant domains of VacA. Interestingly, proteolysis of VacA in solution with proteinase K produces a pattern of bands that is different from that shown in Fig. 2 (data not shown).

To determine whether the lower molecular mass bands were sufficient to induce cellular vacuolation, VacA was incubated with cells in the absence of ammonium chloride at 4°C or 37°C for 7 h. As expected, based on the requirement of ammonium chloride for VacA-induced vacuolation (Cover *et al*., 1992; Ricci *et al*., 1997), no vacuolation was detected in these cells upon microscopic examination. Cells were then washed, treated with proteinase K to remove extracellular toxin and incubated at 37°C in culture medium containing ammonium chloride. Analysis by SDS–PAGE confirmed the presence of a protease-protected 87 kDa VacA protein only in cells incubated at 37°C before protease treatment (Fig. 3, lane c). When examined microscopically, vacuoles formed rapidly upon addition of ammonium chloride to cells preincubated with VacA at 37°C, but did not form in cells preincubated with toxin at 4°C (Fig. 3). These results suggested that internalization of the intact 87 kDa VacA is required for vacuolation, and that the three lower molecular mass bands lack vacuolating activity.

As our binding studies indicated a large, non-specific component to VacA binding (Fig. 1), we determined whether [$^{125}$I]-VacA internalization could be inhibited by excess unlabelled toxin. Acid-activated [$^{125}$I]-VacA was

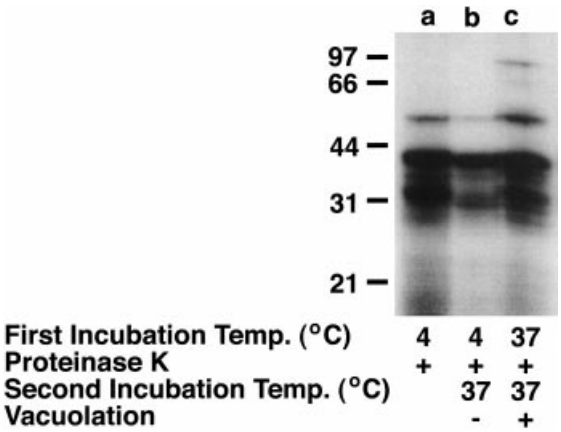


**Fig. 3.** Effect of proteolysis on VacA activity and internalization. HeLa cells were incubated (first incubation) with 24 nM acid-activated [$^{125}$I]-VacA for 7 h at 4°C (lanes a and b) or 37°C (lane c) in complete MEM. Cells were then washed three times in TBS and treated with proteinase K. The detached cells were stored at −70°C (lane a) or incubated (second incubation) overnight at 37°C in complete MEM containing 10 mM ammonium chloride (lanes b and c). Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. A protease-protected 87 kDa band was detected only in cells incubated at 37°C during the first incubation (lane c). Microscopic examination indicated that cells treated as in lane c vacuolated, whereas cells treated as in lane b failed to vacuolate.

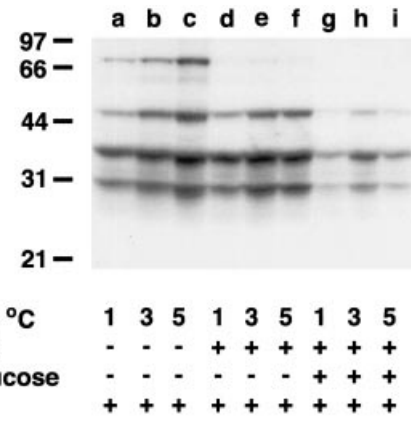


**Fig. 4.** Analysis of VacA internalization in ATP-depleted HeLa cells. HeLa cells were incubated for 1 h at 37°C in complete MEM (lanes a–c), in complete MEM containing 3 mg ml$^{-1}$ sodium azide (lanes d–f) or for 30 min in glucose-free DMEM with 10% FBS (dialysed) and 1 mg ml$^{-1}$ sodium azide followed by the addition of 13 mM 2-deoxy-D-glucose for an additional 30 min (lanes g–i). Ammonium chloride (10 mM) and 12 nM [$^{125}$I]-VacA were added to all samples, and the cells were incubated for 1 (lanes a, d and g), 3 (lanes b, e and h) or 5 h (lanes c, f and i) at 37°C. Cells were then washed three times in 0.9% sodium chloride and treated with proteinase K. Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. Treatment of cells with sodium azide and 2-deoxy-D-glucose resulted in reduced amounts of the protease-protected 87 kDa band.

allowed to bind to cells at 4°C in the presence or absence of a 100-fold excess of unlabelled toxin. After washing, cells were warmed to 37°C to permit cells to internalize the bound toxin, and the extracellular VacA was proteolysed by the addition of proteinase K. In agreement with previous results (Vinion-Dubiel *et al*., 1999), analysis by SDS–PAGE and autoradiography indicated that excess unlabelled VacA did not prevent internalization of the 87 kDa toxin (data not shown).

The absence of internalized 87 kDa VacA at 4°C suggested that active cellular processes are required for toxin entry. To investigate the mechanism of VacA internalization further, cellular ATP in HeLa cells was depleted by the addition of sodium azide and 2-deoxy-D-glucose before VacA addition (Kock *et al*., 1996). Acid-activated [$^{125}$I]-VacA was not internalized by cells treated with these metabolic inhibitors (Fig. 4). Similarly, VacA-induced vacuolation was inhibited by these agents. Moreover, VacA bound to the surface of rabbit erythrocytes, but was not internalized by these cells (Fig. 5). Thus, internalization of VacA by HeLa cells is mediated by an active cellular process and does not occur spontaneously after interaction with membranes.

### *Activation of VacA enhances toxin internalization*

The biological activity of purified VacA is greatly enhanced by treating the toxin at either acidic or alkaline pH before adding the toxin to cells, a process referred to as 'activation' (de Bernard *et al*., 1995; Yahiro *et al*., 1999) (Fig. 6A). Similar results were obtained in experiments using Vero or RK-13 cells (data not shown). To investigate the mechanism by which activation increases the activity of VacA, we examined the binding and internalization of untreated versus activated toxin. Neither acid nor alkaline

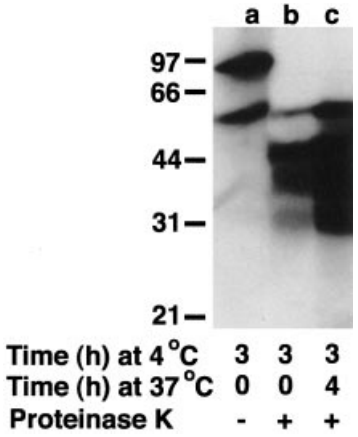


**Fig. 5.** Analysis of VacA interactions with rabbit erythrocytes. Rabbit erythrocytes were incubated in complete MEM with 6 nM acid-activated [$^{125}$I]-VacA for 3 h at 4°C (lanes a and b) or for 3 h at 4°C and then shifted to 37°C for 4 h (lane c). After the incubations, cells were washed in TBS and left untreated (lane a) or treated with proteinase K (lanes b and c). Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. No protease-protected 87 kDa band was detected.

activation led to a substantial increase in the 87 kDa VacA band bound to HeLa cells (Fig. 6B). Similar results were obtained by quantifying the total radioactivity bound to cells (data not shown). To investigate the effect of activation on VacA internalization by HeLa cells, either untreated or activated VacA was allowed to bind to HeLa cells in neutral pH medium at 4°C, and cells were then washed to remove unbound toxin. Cells were warmed to 37°C and treated with proteinase K (Fig. 6C). Both acid- and alkaline-activated [$^{125}$I]-VacA were internalized by HeLa cells (Fig. 6C, lanes g and k). In contrast, if VacA was not activated, very little if any untreated [$^{125}$I]-VacA (either the 87 kDa band or the lower molecular mass bands) could be detected after treatment of cells with proteinase K (Fig. 6C, lane c). The internalized 87 kDa band represented approximately 10% of the activated 87 kDa VacA bound to cells at 4°C. In contrast, less than 1% of the bound untreated 87 kDa band was internalized. These results indicate that activated VacA and untreated VacA interact differently with cells.

Cells treated with acid-activated VacA, but not untreated VacA, reportedly exhibit defects in the processing of procathepsin D and the degradation of epidermal growth factor (Satin *et al*., 1997), which suggests that acid-activated VacA may inhibit proteolytic activity in late endosomes and lysosomes. Consequently, we hypothesized that the failure to detect internalization of untreated VacA might reflect its rapid degradation in acidic endocytic compartments. We therefore examined the effect of the lysosomal inhibitors ammonium chloride and bafilomycin A1 on the internalization of VacA. Either untreated or acid-activated VacA was bound at 4°C to HeLa cells in the presence or absence of the lysosomal inhibitors. Cells were then incubated at 37°C for 4 h before being treated with proteinase K to proteolyse extracellular toxin. As reported previously (Cover *et al*., 1993; Papini *et al*., 1993), bafilomycin A1 inhibited the formation of vacuoles in response to acid-activated VacA, but the lysosomal inhibitors had no inhibitory effect on the internalization of acid-activated VacA (Fig. 7). Internalization of untreated VacA could not be detected, even in the presence of these inhibitors (Fig. 7). These results indicate that VacA must be activated to be internalized.

We next examined whether exposing cell-bound, untreated VacA to acidic extracellular pH would promote toxin internalization. Cells were incubated with untreated

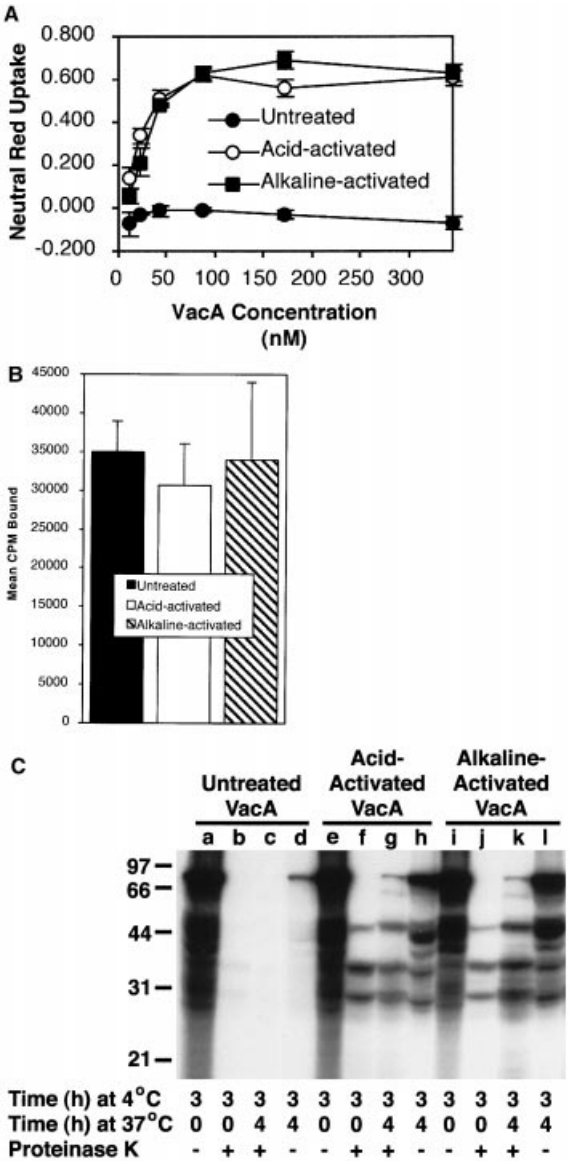


**Fig. 6.** Activation enhances VacA activity and is required for VacA internalization.
A. Varying concentrations of untreated (●), acid-activated (○), or alkaline-activated (■) VacA were added to the neutral pH culture medium (complete MEM containing 10 mM ammonium chloride) overlying HeLa cells. Cells were incubated for 16 h at 37°C, then visualized by light microscopy. Vacuolation was quantified using a neutral red uptake assay. Results represent the mean (± SD) net absorbance at 540 nm from triplicate samples. Activation of VacA results in increased cytotoxicity for HeLa cells.
B. HeLa cells were incubated in binding buffer with 6 nM untreated (filled bar), acid-activated (open bar) or alkaline-activated (hatched bar) [$^{125}$I]-VacA for 3 h at 4°C. Cells were then washed three times in TBS, lysed, and the proteins in the cell lysates were separated by SDS–PAGE and analysed by autoradiography. The 87 kDa bands were excised from the gel, and the radioactivity in each band was quantified. Results represent the mean and standard deviation from triplicate samples ($P > 0.3$, Student's *t*-test).
C. HeLa cells were incubated in binding buffer with 12 nM untreated (lanes a–d), acid-activated (lanes e–h) or alkaline-activated (lanes i–l) [$^{125}$I]-VacA for 3 h at 4°C and then washed three times in TBS. One group of samples was either left untreated (lanes a, e and i) or immediately treated with proteinase K (lanes b, f and j). The other group of samples was incubated at 37°C for 4 h in complete MEM containing 10 mM ammonium chloride and then treated with proteinase K (lanes c, g and k) or left untreated (lanes d, h and l). Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. A protease-protected 87 kDa band was detected only with activated toxin (lanes g and k).

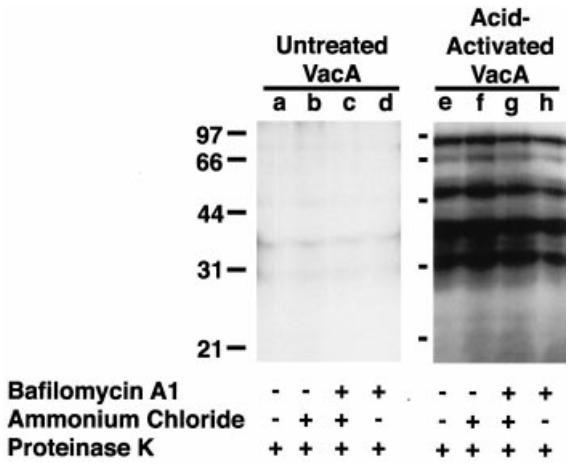


**Fig. 7.** Effect of lysosomal inhibitors on internalization of untreated VacA. HeLa cells were incubated in complete MEM for 15 min in the absence (lanes a, b, e and f) or presence (lanes c, d, g and h) of 10 nM bafilomycin A1. Either untreated (lanes a–d) or acid-activated (lanes e–h) [$^{125}$I]-VacA was then added at a concentration of 12 nM, and the cells were incubated for 3 h at 4°C. Ammonium chloride (10 mM, lanes b, c, f and g) or isotonic saline (lanes a, d, e and h) was then added, and the cells were incubated for 4 h at 37°C. Cells were then washed three times in TBS and treated with proteinase K. Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. The appearance of protease-protected forms of untreated VacA was not enhanced by treating cells with the lysosomal inhibitors.

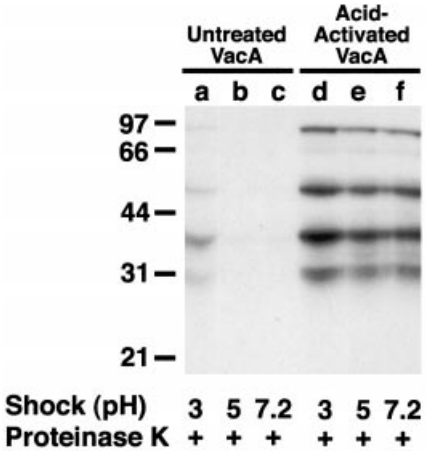


**Fig. 8.** Effect of acidic pH on the uptake of bound VacA. HeLa cells were incubated in binding buffer with 6 nM either untreated (lanes a–c) or acid-activated (lanes d–f) [$^{125}$I]-VacA for 3 h at 4°C. Cells were then washed three times in TBS and incubated for 2 min at 37°C in 100 mM citrate-buffered saline, pH 3 (containing 1 mM calcium chloride, 1 mM magnesium chloride and 100 $\mu$g ml$^{-1}$ BSA) (lanes a and d), in 100 mM citrate-buffered saline, pH 5 (containing 1 mM calcium chloride, 1 mM magnesium chloride and 100 $\mu$g ml$^{-1}$ BSA) (lanes b and e) or in binding buffer, pH 7.2 (lanes c and f). All samples were incubated for 4 h at 37°C in complete MEM (neutral pH) containing 10 mM ammonium chloride. Cells were then washed once in TBS and treated with proteinase K at pH 7.4. Proteins in cell lysates were separated by SDS–PAGE and analysed by autoradiography. Acidification of [$^{125}$I]-VacA that was bound to cells resulted in less efficient toxin internalization than did acid activation of [$^{125}$I]-VacA in solution.

[$^{125}$I]-VacA at 4°C, washed, incubated for 2 min at 37°C in either pH 3, pH 5 or pH 7.2 buffer and then incubated in neutral pH tissue culture medium at 37°C to allow toxin internalization to occur. Surface-exposed toxin was then degraded by proteinase K treatment, and the samples were analysed by SDS–PAGE. Untreated toxin bound to cells was internalized after an acid shock at pH 3 (Fig. 8, lane a), whereas little internalized toxin was observed after similar treatments at pH 5 or pH 7.2 (Fig. 8, lanes b and c). However, the activation of VacA in solution resulted in more efficient toxin internalization than did activation of VacA bound to cells (Fig. 8, lanes d–f).

## Discussion

The binding of many bacterial protein toxins to the surfaces of eukaryotic cells exhibits characteristics of high-affinity ligand–receptor interactions, including specificity, saturability and reversibility (Eidels *et al*., 1983; Vinayek and Gardner, 1990; Limbird, 1996). Several studies have investigated the binding of VacA to the surfaces of eukaryotic cells (Garner and Cover, 1996; Yahiro *et al*., 1997; 1999; Massari *et al*., 1998; Seto *et al*., 1998). Yahiro *et al*. (1997; 1999) reported that VacA binds to the 250 kDa receptor protein tyrosine phosphatase on AZ-521 cells (a human gastric adenocarcinoma cell line) and to an unidentified 140 kDa protein on the surface of a variety of epithelial cell lines, and Seto *et al*. (1998) suggested that VacA binds to the 170 kDa epidermal growth factor receptor on HeLa cells. Thus, there is currently considerable confusion about whether or not a specific VacA receptor exists and what its identity might be. As described in the present study, classical binding experiments using an iodinated VacA ligand suggest that VacA does not bind to a single high-affinity receptor. Classical experiments attempting to demonstrate a specific, saturable, reversible ligand–receptor interaction have also been problematic in studies of RTX toxins (Lally *et al*., 1997). The reported binding of VacA to multiple cell surface proteins (Yahiro *et al*., 1997; 1999; Seto *et al*., 1998) is perhaps consistent with the lack of saturable and specific binding observed in the current study. Furthermore, several previous studies have indicated that VacA binds to lipid membranes (Moll *et al*., 1995; Molinari *et al*., 1998b; Czajkowsky *et al*., 1999) and, thus, binding of VacA to multiple types of lipids on the cell surface may account for some of the binding we observed.

In previous studies, VacA has been detected within intoxicated cells (Garner and Cover, 1996; Fiocca *et al*., 1999), and intracellular expression of VacA results in cell vacuolation (de Bernard *et al*., 1997; Ye *et al*., 1999). Binding of VacA to a productive (i.e. specific) receptor is expected to result in efficient internalization of the toxin by cells, whereas binding to non-productive (i.e. non-specific)

receptors is expected to result in less efficient internalization. Therefore, in an effort to discriminate between VacA binding to a productive versus a non-productive receptor, we used a protease protection assay to investigate the internalization of VacA by cells.

One of the striking findings of this study was that VacA internalization was detected only if the toxin was acid or alkaline activated before contacting cells. We presume that pH-induced structural changes in VacA are required for toxin internalization (de Bernard *et al*., 1995; Cover *et al*., 1997; Molinari *et al*., 1998b; Yahiro *et al*., 1999; Pagliaccia *et al*., 2000). In particular, it seems likely that acid- or alkaline-induced disassembly of VacA oligomers is a critical requirement for VacA uptake by cells (Cover *et al*., 1997; Molinari *et al*., 1998b; Yahiro *et al*., 1999). One hypothesis is that there is a difference in receptor recognition by the untreated and activated forms of VacA, such that only the activated toxin binds to a productive receptor. In accordance with this model, Yahiro *et al*. (1999) reported that activated, but not untreated, VacA bound to a receptor on AZ-521 cells. A second hypothesis is that the fate of the ligand–receptor complex depends on the activation status of VacA. For example, the binding of some ligands to their cell surface receptors triggers oligomerization or cross-linking of the receptors, which, in turn, stimulates endocytosis (Pure and Tardelli, 1992; Parton *et al*., 1994; Bider and Spiess, 1998). Binding of VacA monomers (produced by activation) and subsequent oligomerization of VacA on the cell surface potentially stimulates cross-linking of receptors, triggering endocytosis of VacA–receptor complexes. A third hypothesis is that the capacity of activated VacA to insert into the plasma membrane is relevant for toxin internalization. The pH-induced structural changes in VacA perhaps result in a 'molten globule' form of the toxin, in which protein secondary structure is preserved, but tertiary structure is altered (Bychkova *et al*., 1988). The molten globule model has been used to explain how various bacterial toxins and other proteins, which are water soluble in their native conformation, are able to insert into membranes (Lazdunski and Benedetti, 1990). Presumably, increased exposure of VacA hydrophobic domains upon activation (Molinari *et al*., 1998b) could facilitate membrane insertion and subsequent internalization.

Potentially, a phenomenon similar to VacA activation *in vitro* could occur *in vivo*, in which secreted VacA may encounter acidic gastric pH before contact with gastric epithelial cells. However, we speculate that there may be important differences between our *in vitro* cell culture experiments and events in the human stomach. For example, concentrations of VacA are likely to be considerably lower *in vivo* than in the laboratory. Therefore, if oligomerization of VacA is a concentration-dependent phenomenon, secreted VacA may exist *in vivo* primarily as an active monomer that does not require activation. Alternatively, VacA may be translocated directly from adherent bacteria or bacterial membrane blebs (Fiocca *et al*., 1999; Keenan *et al*., 2000) into gastric epithelial cells *in vivo* without any intermediate steps of oligomerization and acid-induced disassembly.

Multiple lines of evidence from the current study indicate that, after binding of VacA to cells, internalization of the intact 87 kDa VacA protein is required for VacA-induced cytotoxicity. Evidence from the present study implicates an active cellular process in the internalization of activated VacA, as internalization did not occur at 4°C and was inhibited by depleting cells of ATP. Furthermore, VacA does not become protease resistant when incubated with rabbit erythrocytes, suggesting that VacA internalization does not occur spontaneously after binding to membranes. Once internalized, the 87 kDa protein persists inside the cell for hours with little noticeable degradation, extending the previous findings of Sommi *et al*. (1998). However, it is not known whether VacA is internalized as an 87 kDa monomer or as an oligomer, nor is it known where the intact 87 kDa VacA localizes inside the cell. Investigation of the mechanism by which VacA is internalized by cells is a topic of ongoing research in our laboratories.

There are still many unanswered questions regarding events after VacA internalization. There has been speculation that VacA may possess an enzymatic activity but, thus far, no enzymatic activity of VacA has been identified. A recent report (de Bernard *et al*., 2000) using a yeast two-hybrid selection identified an interaction between VacA and a previously unknown protein associated with intermediate filaments, but it is not known whether this interaction is required for VacA activity. One current hypothesis is that VacA forms anion channels in the membrane of endosomes, and that the influx through such channels results in endosomal swelling (i.e. vacuolation) (Czajkowsky *et al*., 1999; Tombola *et al*., 1999a). Further studies directed towards characterizing the intracellular actions of VacA may contribute to a better understanding of the role that this unique toxin plays in *H. pylori* infection.

## Experimental procedures

### *Purification of VacA*

VacA was purified from broth culture supernatant of *H. pylori* strain 60190, containing a type s1a/m1 *vacA* allele, by ammonium sulphate precipitation and gel filtration chromatography using a Superose 6 HR 16/50 column (Amersham Pharmacia Biotech), as described previously (Cover *et al*., 1997), except that the buffer was phosphate-buffered saline (PBS; pH 7.5) containing 1 mM EDTA and 0.02% sodium azide. VacA concentrations were determined using the BCA

protein assay (Pierce) and calculated based on a molecular mass of 87 kDa.

### *Radioiodination of VacA*

Purified VacA was iodinated using the iodogen method (Pierce). Iodogen (2 μg) in chloroform was plated onto the wall of a microfuge tube, and the chloroform was evaporated under a stream of $N_2$. To the iodogen-containing tube, 1 mCi [$^{125}$I]-iodide in 50 mM $NaPO_4$ buffer, pH 7.2, and 50 μg of purified VacA were added in a final volume of 100 μl and incubated for 10 min at 25°C. The liquid phase of the reaction was then removed and added to 10 mM non-radioactive iodide, and the free $^{125}$I was removed by gel filtration on a 10 ml G-25 Sephadex column equilibrated with 10 mM Tris-buffered saline (TBS), pH 7.4, containing 25 μg ml$^{-1}$ BSA. Fractions containing [$^{125}$I]-VacA were identified using a Packard Cobra II γ-counter. The radiolabelled VacA preparations retained biological activity as determined by cell culture and neutral red uptake assays (Cover *et al*., 1991).

### *Activation of VacA*

Purified VacA was acid activated by dropwise addition of 0.1 N HCl to ≤ pH 3, as described previously (de Bernard *et al*., 1995; Cover *et al*., 1997). Purified VacA was alkaline activated by dropwise addition of 0.1 N NaOH to ≥ pH 10 (Yahiro *et al*., 1999). Activated VacA was then added to the neutral pH medium overlying cells, unless otherwise indicated.

### *Quantification of vacuolating activity*

HeLa cells were cultured in minimum essential medium (Eagle, modified, containing Earle's salts; ICN Pharmaceuticals) containing 10% fetal bovine serum (FBS; complete MEM). Either unlabelled or [$^{125}$I]-VacA was added to replicate HeLa cell monolayers in complete MEM containing 10 mM ammonium chloride. The cell-vacuolating effects of VacA were quantified by neutral red uptake assay (Cover *et al*., 1991).

### *VacA binding assays*

Iodinated toxin was added to HeLa cell monolayers at 4°C in binding buffer (50 mM HEPES, 100 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium chloride and 100 μg ml$^{-1}$ BSA, pH 7.2) in the presence or absence of unlabelled VacA. After incubation for the specified period of time, cells were washed rapidly three times with binding buffer and removed from the culture plate by lysis in 1% SDS. Radioactivity was quantified by γ-counting.

### *Protease protection assays*

Unless otherwise indicated, samples were proteolysed with 250 μg ml$^{-1}$ proteinase K in TBS for 30 min at 4°C. Proteolysed cells were recovered by centrifugation and lysed in boiling SDS sample buffer to inactivate the proteinase K. The protease protection studies reported in this manuscript have been normalized internally such that, for any given experiment, equal amounts of VacA were added to equal numbers of cells, the cells were lysed in equal volumes and equal volumes of cell lysate were separated by SDS–PAGE.

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