Structure and interaction of VacA of *Helicobacter pylori* with a lipid membrane

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In its mature form, the VacA toxin of *Helicobacter pylori* is a 95-kDa protein which is released from the bacteria as a low-activity complex. This complex can be activated by low-pH treatment that parallels the activity of the toxin on target cells. VacA has been previously shown to insert itself into lipid membranes and to induce anion-selective channels in planar lipid bilayers. Binding of VacA to lipid vesicles and its ability to induce calcein release from these vesicles were systematically compared as a function of pH. These two phenomena show a different pH-dependence, suggesting that the association with the lipid membrane may be a two-step mechanism. The secondary and tertiary structure of VacA as a function of pH and the presence of lipid vesicles were investigated by Fourier-transform infrared spectroscopy. The secondary structure of VacA is identical whatever the pH and the presence of a lipid membrane, but the tertiary structure in the presence of a lipid membrane is dependent on pH, as evidenced by H/D exchange.

**Keywords:** calcein release; Fourier-transform infrared spectroscopy; membrane interaction; VacA toxin.

The toxin VacA produced by pathogenic *Helicobacter pylori* strains is a major virulence factor in human gastroduodenal diseases [1–5]. The protein is synthesized as a 140-kDa precursor from which a 40-kDa C-terminal domain necessary for extracellular secretion is removed on the bacterial surface [6]. The mature protein (95 kDa) is found both at the bacterial cell surface and in the extracellular medium. This 95-kDa protein purified from the supernatants of *H. pylori* often contains two fragments of 37 kDa (p37) and 58 kDa (p58) [6]. The full-length protein released is a low-activity complex formed by up to 12 monomers [6,7]. This complex can be activated by low-pH treatment, which most probably results in oligomer disassembly [7,8]. Treatment of cells with activated VacA leads to formation in the cell cytoplasm of large V-ATPase-positive, rab 7-positive and Lgp-positive vacuoles originating from late endosomal and lysosomal compartments [9,10] and to a size-selective increase in the permeability of polarized epithelial monolayers [11]. Very recently, it has been demonstrated that low-pH-activated VacA was able to induce anion-selective channels in planar lipid bilayers [12]. The molecules transported by the channel include Cl⁻, HCO₃⁻ and small organic molecules such as pyruvate [12]. On the basis of these observations, a model of activity of VacA was proposed in which VacA may be part of an strategy to survive on the surface of the gastric epithelium by extracting nutrients from the host cells. In this model, vacuolation may be a late consequence of the increase in anion permeation. Whether VacA needs to reach the cytosol to exert a more direct effect on a cytoplasmic target is still under debate.

The pH-dependence of VacA-induced vacuolation reveals that maximum activity is reached slightly below pH 5.0 and remains stable down to pH 2 [13]. Using sucrose-density-gradient centrifugation, it has been demonstrated that, at pH 5.0, the protein undergoes a transition between an oligomeric and a monomeric structure [13]. This transition was characterized by exposure at the surface of the protein of hydrophobic sites, as evidenced by binding of 8-anilino-naphthalene-1-sulfonate, a fluorescent hydrophobic probe [13]. Furthermore, activation at mildly acidic pH leads to deep insertion of VacA into the lipid membrane, as demonstrated by labeling by hydrophobic photoactivatable lipid probes [14]. Although it seems clear that low-pH activation is required for the interaction of VacA with the lipid membrane, very little is known about the molecular mechanism of this interaction and the associated structural modifications. In this paper, we have systematically compared the binding of VacA and its ability to destabilize membranes as a function of pH using large unilamellar vesicles (LUV). We have also characterized the structural changes mediated by low-pH activation and lipid binding by Fourier-transform infrared (FTIR) spectroscopy.

**MATERIALS AND METHODS**

**Materials**

L-α-Phosphatidylcholine from egg yolk (egg PtdCho; type V) and asolectin (mixed soybean phospholipids) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Asolectin
was purified by the method of Kawaga & Racker [15]. D_2O was from Merck (Darmstadt, Germany).

Protein purification
VacA from H. pylori strain CCUG 17847 was purified from the broth culture supernatants as described [16]. For all experiments, the protein was purified by gel filtration on a Superose 6 column in 2 mm Hepes/2 mm NaCl, pH 7.2, and stored at 4 °C.

Liposome preparation
Asolectin and PtdCho were kept as a stock solution in Liposome preparation column in 2 ments, the protein was purified by gel filtration on a Superose 6 broth culture supernatants as described [16]. For all experi-
tIobserved after addition of Triton X-100, and by lysing the LUV with Triton X-100 (0.25% final concen-
tration) to the sample, at a flow rate of 75 mL·min^{-1} controlled with a Brooks flowmeter. The spectrophotometer was driven by a computer program. The spectra at each time point were the computer program. The spectra at each time point were the control spectra of the 2 m

Infrared spectroscopy
Attenuated total reflection infrared spectra (resolution of 4 cm\(^{-1}\)) were obtained on a Perkin-Elmer 1720X FTIR spectrophotometer as previously described [19]. Measurements were carried out at room temperature. Thin films were obtained by slowly evaporating a sample under a stream of nitrogen on one side of the attenuated total reflection plate (germanium plate) [20,21]. The plate was then sealed in a universal sample holder (Perkin-Elmer 186-0354) and deuterated by flushing the sample compartment with D_2O-saturated N_2 at room temperature for 90 min. The hydrogen/deuterium exchange allows differentiation of \(\alpha\)-helix from random structure, the absorption bands of which shift from \(\approx 1655\) cm\(^{-1}\) to \(\approx 1642\) cm\(^{-1}\). Secondary structure was determined by analysis of the deuterated amide I region as described previously [18,22–24]. The frequency limits for the different structures were as follows: \(1662–1645\) cm\(^{-1}\), \(\alpha\)-helices; \(1689–1682\) cm\(^{-1}\) and \(1637–1613\) cm\(^{-1}\), \(\beta\)-sheets; \(1644–1637\) cm\(^{-1}\), random; \(1682–1662\) cm\(^{-1}\), \(\beta\)-turn. The control spectra of the 2 mm Hepes/2 mm NaCl, pH 7.4, buffer and asolectin vesicles at pH 3.0, 5.0, 7.4 showed no absorbance between 1700 and 1600 cm\(^{-1}\) (data not shown).

Kinetics of deuteration
The experimental procedure was carried out as previously described [22,25]. The pH of each sample was checked to ensure that it was 3.0, 5.0, or 7.4. If it was not, the pH was adjusted with diluted HCl or NaOH. The sample was spread on a germanium plate as described previously [20,21]. Before the deuteration was started, 10 spectra were recorded to verify the reproducibility of the measurements and the stability of the system. At time zero, a D_2O-saturated N_2 flux was applied to the sample, at a flow rate of 75 mL·min^{-1} controlled with a Brooks flowmeter. The spectrophotometer was driven by a computer program. The spectra at each time point were the accumulation of 12 scans with a resolution of 4 cm\(^{-1}\). The background due to the atmospheric water contribution was computed as described previously [22] and was subtracted from each spectrum. The amide I and II band areas were measured between 1702 and 1596 and 1585–1502 cm\(^{-1}\), respectively. The amide II area was divided by the amide I area for each spectrum to take into account any change in the total intensity of the spectra during the deuteration process. This ratio, which was expressed between 0 and 100%, was plotted against deuteration time. The 100% value is defined by the amide II/amide I ratio obtained before deuteration, whereas the 0% value corresponds to a zero absorbance in the amide II region. It has been shown previously [26,27] on a series of proteins that can be fully denatured (and therefore fully deuterated in the denatured state) and then refolded to the original structure, that
complete H/D exchange resulted in $0 \pm 5\%$ absorbance in amide II region. We are therefore confident that a zero absorbance in the amide II region reflects the full deuteration of the protein.

**RESULTS**

**pH-dependent lipid association of VacA protein**

To characterize the association with lipid membranes, VacA was incubated with asolectin LUV at different pH values (7.4, 6.0, 5.0, 4.5, 4.0, 3.0), and the samples were subjected to sucrose-density-gradient centrifugation to separate the lipid-bound proteins from the free proteins. The amount of protein and lipid was evaluated in each fraction by Trp fluorescence and choline dosage, respectively. The association of VacA with the lipid membrane was strongly pH-dependent, most of the protein being bound to the lipid membrane between pH 3 and 5 (Fig. 1A,B). At pH 7.4 and 6 there is very little association of VacA (Fig. 1C,D) with the lipid, and protein distributions were characteristic of the protein or the lipid vesicles alone, whatever the pH (not shown). To test the reversibility of association of VacA with the lipid membrane, the protein was mixed with asolectin vesicles at pH 5.0 or 3.0. After 10 min of incubation, the samples were divided into two: half was kept at pH 5.0 or 3.0 and the other half was brought back to pH 7.4. The samples were then run in a sucrose-density gradient as described. Whatever the initial pH of the incubation mixture, we observed only partial release of the protein from the lipid membrane when the pH was brought back to neutral (shown in Fig. 2 for pH 3.0), as evidenced by the presence of free protein at the bottom of the gradient (arrow Fig. 2B).

**pH-dependent calcein release mediated by VacA**

Figure 3 shows the effect of pH on the leakage of calcein from LUV composed of either asolectin or egg PtdCho induced by VacA. From pH 5.5 to 7.2, addition of the protein to the vesicles did not cause any release of calcein, whatever the lipid composition (Fig. 3, inset). In contrast, a sharp transition was observed between pH 5 and 4 when VacA was added to negatively charged vesicles of asolectin. Below pH 4, there was a slight increase in calcein release until pH 3.0. The transition was shifted downwards by about 0.75 pH unit when VacA was added to neutral vesicles of PtdCho. Such a shift has been previously observed in the interaction of diphtheria toxin with asolectin and PtdCho membranes [28]. It was suggested that the shift may originate from the negatively charged surface of the asolectin vesicles giving rise to high cation concentrations in the diffuse double layer existing at the membrane–solution interface. In fact, according to the Gouy–Chapman equation and considering a mean surface charge density of $3 \times 10^{-3}$ charged group per $A^2$ for asolectin [29,30] (corresponding to around 20% of negative lipids), the calculated surface pH is at least 0.5 pH unit lower than that of the bulk solution.

A characteristic of some bacterial toxins such as diphtheria toxin, is that they aggregate on incubation in conditions that promote their interaction with the lipid membrane. This aggregation leads to a decreased ability of the protein to destabilize lipid vesicles [28]. When preincubated for 10 min at pH 4 and 3 before addition to PtdCho vesicles, the VacA protein loses part of its ability to induce calcein release (Fig. 3), suggesting that, on acidification and in the absence of a lipid membrane, the protein undergoes a conformational change that impairs its ability to interact with the lipid membrane.

**Effects of pH and the presence of lipids in structure of VacA**

FTIR spectroscopy is based on analysis of the vibration bands of protein and in particular the amide I band ($\nu_{C=O}$) of the
peptidic bond), the frequency of absorption of which is strongly dependent on the secondary structure. This method has been successfully used to investigate the structure of soluble and membrane proteins [19,22±27]. The spectra of pure VacA were similar whatever the pH (Fig. 4; spectra A±C), which suggests that no significant secondary-structure change took place when the pH was lowered. The main absorption band (1634±1630 cm$^{-1}$) within the amide I band was located in a region associated with the $\beta$-sheet structure (Fig. 4, insert), suggesting that this structure is predominant in VacA. A curve-fitting procedure performed as described by Cabiaux et al. [24] gives values of 24±5% and 35±5%, respectively, for the $\alpha$-helical and $\beta$-sheet structures of VacA.

To study the effect of the presence of a lipid membrane on the secondary structure of VacA, the protein was incubated with asolectin LUV at pH 5.0 and 3.0, the values at which most of the protein is lipid-bound. Membrane-bound protein was separated from free protein by centrifugation on a sucrose-density gradient. Evaluation of secondary structure in the presence of lipids at both pH 3.0 and 5.0 gave a content of $\alpha$-helical and $\beta$-sheet structure similar to those found in the absence of lipids (Fig. 4, spectrum D and inset at pH 5.0; pH 3.0 not shown). To characterize further the conformational changes that accompany the binding of VacA to the lipid membrane, we followed the kinetics of deuteration of VacA in the presence or absence of lipid membrane at pH 3.0 and 5.0. With constant experimental conditions (pH, secondary structure, temperature), the rate of H/D exchange is related to the solvent accessibility of the NH amide groups of the protein and consequently to the tertiary structure of the protein and to the stability of the secondary structure. Peptide hydrogen...
exchange was followed by monitoring the amide II absorption peak [v(N–H maximum in the 1596–1502 cm⁻¹] decrease due to its shift to the 1460 cm⁻¹ region [amide II’], v(N–D)] on deuteration. The percentages of non-exchanged residues calculated from the ratio of amide II/amide I, as described in Materials and methods, are given in Fig. 5. The presence of the lipid vesicles did not significantly modify the exchange of H/D undergone by VacA at pH 3.0 (Fig. 5B) whereas it increased the extent of deuteration when the samples were incubated at pH 5.0 (Fig. 5A).

DISCUSSION

Structural and functional studies have led to the suggestion that VacA may be a member of the A–B family of di-chain toxins [8,10,14]. In this family, a B domain (putatively p58) binds to specific cell surface receptors, is internalized by endocytosis, reaches internal compartments where, because of a modification of the environment (such as pH in the case of diphtheria toxin or the toxins of Bacillus anthracis), it inserts itself into the lipid membrane and mediates the translocation of an enzymatically active A domain (which would correspond to p37) responsible for the biological activity of the toxin (reviewed in [31]). In support of this hypothesis, the purified p58 gave saturable binding to target cells with a dose-response similar to that of the full-length protein (J. M. Reyrat, personal communication). However, unlike the B domain of most A–B type toxins, the p58 domain alone cannot be made responsible for the channel formation in the lipid bilayer observed with the full-length protein [12]. Furthermore, according to the proposed model of Tombola et al. [12], VacA may also exert its activity without being endocytosed. These last two properties suggest that VacA may not be a true A–B-type toxin and that it may share some properties with the family of the pore-forming toxins which act by permeabilizing the target cell membrane, such as the α-toxin of Staphylococcus aureus or aerolysin (for a review, see [32]). Our data will be discussed in view of this hypothesis.

All of the VacA was already bound to asolectin vesicles at pH 5.0, whereas calcein release took place between pH 5.0 and 4.0. This suggests that the interaction with the lipid membrane could involve at least two steps: binding to the lipid membrane followed by a structural change leading to membrane leakage. Such a two-step process has been described for the α-toxin of S. aureus (pore-forming toxin) in which the soluble monomer diffuses towards the target membrane where it oligomerizes to form a heptamer (for a review, see [32]). At this stage, the heptamer is non-lytic and undergoes a conformational change (folding of a surface loop into an antiparallel β-hairpin) which allows its insertion into the lipid membrane [32]. Although the conformational change in α-toxin involved very few residues, it was characterized by a shift of the β-sheet frequency of absorption from 1638 cm⁻¹ for the free protein to 1634 cm⁻¹ for the lipid-bound protein, as evidenced by FTIR spectroscopy (X.-M. Wang, unpublished results). Such a shift has never been observed with VacA, and our FTIR spectroscopic data suggest that the secondary structure of VacA (35% β-sheet, 24% α-helix) is independent of the presence of lipid vesicles whatever the pH. This suggests that if a ‘pre’ pore structure is formed by VacA, its ‘maturation’ to the pore structure does not require any secondary-structure modification. This does not, however, exclude a tertiary or quaternary conformational change (see below). An alternative mechanism for a two-step process is a pH-dependent association of the two subunits, p37 and p58, with the lipid membrane. It has been previously demonstrated that the pH-dependence of the interaction of the two separated subunits with a lipid membrane was slightly different. For example, p58 induced the fusion of small unilamellar vesicles at a higher pH than p37 [14]. As channel formation requires both p58 and p37 [12], it is conceivable that p58 could bind to the membrane around pH 5.0 but that the channel structure (and the ability to induce calcein release) requires the presence of the p37 domain which insert below pH 5.0. In any case, a two-step mechanism is supported by the H/D exchange. At pH 5.0, the presence of the lipid vesicles increases the H/D exchange which indicates that the protein has a more open configuration when lipid-bound than in solution. In contrast, at pH 3.0 there is no difference in H/D exchange in the absence or presence of vesicles. As exchange itself pH-dependent, no conclusion can be drawn from a comparison between two pH values, but it is nonetheless clear that the presence of lipid vesicles does not lead to the same behavior at pH 5 and 3. This suggests that either the tertiary or quaternary structure of VacA is different at pH 5.0 and 3.0. The structure modification could be responsible for the ability of the protein to destabilize the membrane and to induce channel formation. This is in agreement with the finding that preincubation of VacA at pH 3.0 decreases its ability to induce calcein release from PtdCho vesicles.

A model of insertion of pore-forming toxins with a β-sheet structure can be derived from the crystal structure of the α-toxin of S. aureus in the presence of a detergent [33]. The toxin inserts itself into the lipid membrane as a heptamer with a mushroom shape, the stem constituting the pore-forming domain. This pore-forming domain has a β-barrel topology, each monomer contributing to two β-strands in the structure. The presence of a high percentage of β-sheets in the VacA structure suggests that this toxin could interact with the lipid membrane similarly to the α-toxin. Furthermore, the presence of an oligomeric form (hexamer) of VacA in the lipid membrane has been suggested by recently published atomic force microscopy images [34]. The following model could therefore be proposed for the pore formation induced by VacA toxin. VacA needs low-pH activation to induce monomerization of the toxin in solution. This monomerization may uncover hydrophobic domains that would allow the protein to bind to the lipid membrane in a low-lytic configuration (low calcein release and low channel formation; E. Papini, personal communication). In this configuration, the protein would have greater accessibility to H/D exchange than when in solution at the same pH. At a slightly lower pH, the protein interacts with the lipid membrane through amphipathic β-sheets, leading to the structure responsible for channel formation and calcein release. This would be related to an oligomerization step. Whether oligomerization takes place at the surface of or in the membrane remains to be determined.

ACKNOWLEDGEMENTS

This work was supported by the EC grant Biomed2 BMH4 CT97-2410. C. P. was supported by a short-term EMBO fellowship. V. C. is a research associate of the Fonds National pour la Recherche Scientifique (Belgium).

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