Targeting of *Helicobacter pylori* vacuolating toxin to lipid raft membrane domains analysed by atomic force microscopy

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The *Helicobacter pylori* vacuolating toxin VacA causes several effects on mammalian cells *in vitro*, including intracellular vacuolation, formation of pores in the plasma membrane and apoptosis. When added to cells, VacA becomes associated with detergent-resistant membranes, indicating that it binds preferentially to lipid rafts. In the present study, we have used atomic force microscopy to examine directly the association of VacA with lipid domains in supported lipid bilayers. VacA did not bind to lipid bilayers at pH 7.6. In contrast, at pH 4.0, VacA associated with the bilayers in the form of 26-nm oligomeric complexes. VacA bound to bilayers produced from either brain lipids or SM (sphingomyelin) plus cholesterol, each of which lacked detectable lipid domains. Bilayers composed of DOPC (dioleoylphosphatidylcholine), SM and cholesterol contained clearly visible raft-like domains, and VacA preferentially associated with these rafts. VacA bound

poorly to raft-like domains in DOPC/SM bilayers, indicating that cholesterol is required for efficient association of VacA with lipid domains. When PS (phosphatidylserine), an anionic phospholipid that does not partition significantly into rafts, was added to the mixture of DOPC, SM and cholesterol, VacA was excluded from the rafts, indicating that it binds more avidly to PS than to the raft components. A typical plasma membrane exhibits pronounced lipid asymmetry, with SM enriched in the outer leaflet and PS in the inner leaflet. Therefore it is probable that the association of VacA with rafts in DOPC/SM/cholesterol bilayers represents a useful model for understanding the interactions of VacA with membranes *in vivo*.

Key words: atomic force microscopy, *Helicobacter pylori*, lipid bilayer, lipid raft, VacA, vacuolating toxin.

INTRODUCTION

Colonization of the human gastric mucosa with *Helicobacter pylori* results in gastric inflammation, and is a risk factor for the development of peptic ulcer and gastric carcinoma [1,2]. Many strains of *H. pylori* secrete a vacuolating toxin (VacA), which causes a number of effects on mammalian cells *in vitro*, including the formation of intracellular vacuoles, formation of anion-selective pores in the plasma membrane, apoptosis and permeabilization of epithelial monolayers [3–5]. In addition, VacA interferes with antigen processing [6] and inhibits T-lymphocyte activation [7].

VacA is synthesized as a 140 kDa protoxin that undergoes cleavage of an N-terminal signal sequence and a C-terminal peptide to yield an 88 kDa secreted toxin [3–5,8]. VacA is isolated from broth culture supernatants predominantly in the form of dodecameric or tetradecameric flower-shaped particles [9–11]. These oligomeric forms have relatively little cytotoxic activity [10,12,13]; however, acidification results in the disassembly of VacA oligomers into constituent subunits, along with a significant increase in cytotoxicity [10,12–15]. Analyses of acid-activated VacA in association with lipid bilayers, using either deep-etch EM (electron microscopy) or AFM (atomic force microscopy), have revealed the presence of hexameric structures that closely resemble the soluble dodecamers [9,16], suggesting that VacA monomers are capable of reassembling into membrane-spanning oligomers after association with the target bilayer.

It is well established that mixed lipid bilayers generate lipid domains or ‘rafts’ that are enriched in SM (sphingomyelin) and

cholesterol [17,18]. SM/cholesterol-enriched rafts are supposed to exist in a liquid-ordered state, in which the lipid molecules are tightly packed but are still capable of diffusing laterally [18]. The liquid-ordered phase domains are surrounded by a liquid-disordered phase (composed primarily of phospholipids with unsaturated, and therefore kinked, fatty acyl chains), which allows for a greater degree of lateral diffusion. There is growing evidence that lipid rafts exist in biological membranes, and that the preferential association of proteins with rafts is involved in crucial cellular functions such as cell signalling and membrane traffic and fusion [19–22]. In addition, several bacterial and viral pathogens appear to use raft binding as a means of entry into their target cells [23,24], and a variety of toxins, such as perfringolysin O, lysenin, *Vibrio cholerae* cytolysin, aerolysin and cholera toxin, also seem to require raft association for cytotoxic activity [25–30].

Recently, it has been shown that incubation of acid-activated VacA with Chinese-hamster ovary or HeLa cells results in the association of the toxin with DRMs (detergent-resistant membranes [31,32]), which are believed to be the biochemical equivalent of lipid ‘rafts’. Furthermore, the cell vacuolation normally caused by the toxin was inhibited by treatment of the cells with either the cholesterol-depleting agent methyl- β -cyclodextrin or the cholesterol-binding agent nystatin [31–34]. These results indicate that the association of VacA with rafts in the plasma membrane is important for its cytotoxic effects.

The kinked structure of the fatty acyl chains of phospholipids results in a shorter molecular length relative to the straight SM molecules. For instance, the thickness of a bilayer composed of C_{18:1} PC (phosphatidylcholine) is 3.5 nm, whereas a bilayer

Abbreviations used: AFM, atomic force microscopy; PC, phosphatidylcholine; DOPC, dioleoyl-PC; DRM, detergent-resistant membrane; EM, electron microscopy; GPI, glycosylphosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

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composed of C_{18:0} SM is 4.6 nm thick [20]. For this reason, rafts in lipid bilayers protrude slightly from the background liquid-disordered phase. We [35–37] and others [38–41] have shown that this protrusion can be detected by AFM imaging of supported lipid bilayers. Furthermore, the distribution of proteins between the raft and non-raft phases of the bilayers can be visualized. For instance, the GPI (glycosylphosphatidylinositol)-anchored protein placental alkaline phosphatase, which is enriched in DRMs prepared from cells [17], is efficiently targeted to rafts *in vitro* [35,39], whereas the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor syntaxin 1 is excluded from rafts provided that the bilayer contains cholesterol [37]. In the present study, we examined the binding of VacA to supported lipid bilayers of varied lipid composition, focusing particularly on its distribution between raft and non-raft domains. We show that efficient binding of VacA to lipid bilayers requires the presence of cholesterol. VacA preferentially associates with rafts in bilayers composed of DOPC (dioleoyl-PC), SM and cholesterol, but is found predominantly in the non-raft phase when PS (phosphatidylserine) is also added. The probable physiological significance of these findings is discussed.

EXPERIMENTAL

Purification of VacA

VacA was purified from a broth culture supernatant of *H. pylori* strain 60 190 as described previously [10,12].

Formation of supported lipid bilayers

Lipids [total brain lipids, DOPC, L- α -PS, SM and cholesterol] were purchased as chloroform solutions (Avanti Polar Lipids, Alabaster, AL, U.S.A.). The composition of the total brain lipids (specified by the suppliers) was 16.7 % (w/w) phosphatidylethanolamine; 10.6 % PS; 9.6 % PC; 2.8 % phosphatidic acid; 1.6 % phosphatidylinositol, and 58.7 % other lipids. The cholesterol content, previously assayed by us [42], was 28 % (w/w) of the total. A total of 2 mg of lipid was placed in a 1 ml volumetric flask (Sigma). To minimize contamination, all flasks were precleaned in MilliQ water, ethanol and chloroform, and all lipids were pipetted using a gas-tight, glue-free Hamilton syringe (Sigma). The chloroform was evaporated with a gentle stream of dry nitrogen for 1–2 h. Lipids were rehydrated in 500 μ l of MilliQ water for 16 h at room temperature (21 °C). The hydrated lipid suspension was vortex-mixed to form large multilamellar vesicles. This solution was then sonicated with a bath sonicator for 30 min at 45 °C to produce unilamellar vesicles, and an equal volume of 2-fold concentrated buffer was added to the liposome suspension to produce the desired final buffer composition (usually 1 mM citrate, pH 4.0).

For VacA incorporation into liposomes, 90 μ l of a 2 mg/ml suspension of liposomes in citrate buffer (pH 4.0), was incubated with 10 μ l of VacA (400 μ g/ml in PBS, pH 7.6). Because of the ratio of volumes used, the pH of the final incubation mixture remained at 4.0. The protein/lipid mixture was incubated at 4 °C with agitation for 36–60 h. (These conditions consistently provided the highest quality images of VacA in association with lipid bilayers.) Proteoliposomes (100 μ l) were deposited on to freshly cleaved muscovite mica (Goodfellow Cambridge, Huntingdon, Cambs., U.K.). The vesicles were left to adsorb at room temperature for 20 min. Mica discs were then rinsed gently in 8 ml of the appropriate buffer. Great care was taken to prevent the sample from drying out. Once rinsed, the sample was placed in the atomic force microscope and covered with 50–80 μ l of the buffer for imaging.

Figure 1 Biochemical and structural characterization of VacA

(A) The VacA preparation used in the experiments described in the present study was analysed by SDS/PAGE with Coomassie Blue staining. The VacA band occurred at a molecular mass of 88 kDa, as expected. Positions of the molecular-mass standards (kDa) are indicated on the right. (B–D) AFM images of unacidified VacA bound to mica and scanned under fluid. Scale bars: 100 nm (B), 50 nm (C) and 25 nm (D).

AFM imaging

AFM imaging was performed using a MultiMode atomic force microscope [Veeco (Digital Instruments), Santa Barbara, CA, U.S.A.] controlled by a Nanoscope IIIa controller with an in-line electronics extender module. The microscope was placed on a silicone-gel vibration isolation pad, which was subsequently mounted on an air table. An acoustic hood was placed over the microscope to reduce interference from ambient room sounds. All data were collected using tapping mode in fluid. The short (100 μ m), wide (21 μ m) oxide-sharpened silicon nitride probes (DNP-S; Digital Instruments) were tuned to 10–30 % below the peak of the resonance frequency (typically found between 7.5 and 9 kHz). The microscope was engaged with a 0-nm scan area to allow for tuning and a set point adjustment of 75–100 nm above the surface before scanning of the sample was performed. The set point was adjusted to the highest setting that allowed imaging with little noise, to minimize the force applied to the sample. All images were captured at 512 $\times$ 512 pixel resolution. Data were subjected to a zero- or first-order flattening with the software provided by the atomic force microscope manufacturer. No further data processing or modification was performed.

RESULTS

VacA was isolated from a broth culture supernatant of *H. pylori*, as described previously [10,12]. A Coomassie Blue-stained SDS/polyacrylamide gel of the protein sample used in the present study (Figure 1A) shows that it is highly purified, with the major band occurring at a molecular mass of 88 kDa, as expected from the known structure of the VacA monomer [3–5,8]. In an initial experiment, unacidified VacA was imaged on

Figure 2 Association of VacA with a supported bilayer composed of brain lipids

(A) A supported bilayer composed of total brain lipids was prepared by the collapse of a liposome suspension on to a mica support. The darker (lower) area at the lower left is the mica and the lighter (higher) area is the lipid bilayer. (B) Liposomes were incubated with VacA in citrate buffer (pH 4.0) before they were deposited on to the mica. Note the even spread of VacA particles. Scale bar, 100 nm. A shade-height scale is shown on the right.

a mica support. The images shown in Figures 1(B)–1(D) reveal the structure of the protein. At the lowest magnification (Figure 1B), the protein appeared as a homogeneous spread of particles, which had an outer diameter of approx. 30 nm. The higher-magnification images indicate the presence of a central pore (Figures 1C and 1D). Scans were taken at the highest magnification to determine the particle dimensions. Particle diameters were measured at half the maximal height to compensate for the tendency of AFM to overestimate this parameter because of the geometry of the tip. This procedure has been shown previously to produce accurate values of particle volume over a wide range of protein molecular masses [43]. It was found that the VacA particle had an outer diameter of 30.3 ± 0.7 nm (mean $\pm$ S.E.M., $n = 21$), a maximum pore diameter of 9.9 ± 0.4 nm ($n = 16$), a pore diameter at half depth of 4.7 ± 0.2 nm ($n = 16$) and a height above the mica substrate of 6.0 ± 0.1 nm ($n = 27$). These dimensions are similar to those obtained previously using either deep-etch EM [10] or AFM [16]. Note that these images were obtained by AFM scanning under fluid, and therefore probably provide a genuine representation of the structure of VacA under physiological conditions.

Figure 2 demonstrates the association of VacA (at pH 4.0) with a bilayer composed of brain lipids. In the absence of VacA, the bilayer was smooth and featureless (Figure 2A). Despite the pre-

sence of SM and cholesterol in the lipid mixture, no rafts were seen. The surface of the bilayer was approx. 5 nm higher than the mica substrate (the dark area in the bottom left). This height is slightly greater than the value predicted for a bilayer (approx. 4 nm) based on the known dimensions of the lipid molecules, and indicates the presence of a hydration layer between the bilayer and the mica. Similar observations were made in previous studies of supported lipid bilayers [35–37]. When VacA was added to the bilayer at pH 4.0, the resultant AFM image showed a spread of particles that appeared to be distributed randomly over the surface of the bilayer, with no signs of clustering (Figure 2B). It is clear that all of the particles in the image are not exactly of the same size. This variation is probably caused partly by differences in the interactions of the tip with the individual VacA particles and partly by factors such as protein aggregation (e.g. the small number of large particles seen might consist of more than one VacA multimer). The dimensions of all of the particles shown in Figure 2(B) were measured. The particles had an outer diameter, at half-height, of 26.1 ± 0.4 nm (mean $\pm$ S.E.M., $n = 78$) and a height above the bilayer surface of 2.1 ± 0.1 nm ($n = 78$). This height is 3.9 nm less than the value calculated for VacA particles imaged on mica, a difference that is approximately equal to the thickness of a lipid bilayer (approx. 4 nm). These results, therefore, are consistent with previous reports suggesting that acidified VacA is capable of forming membrane-spanning pores in lipid bilayers [16]. Alternatively, they may indicate that acidified VacA in association with the bilayer consists of hexamers, whereas non-acidified VacA bound to mica is dodecameric [9,16]. The observation that the measured diameter of the bilayer-associated VacA is less than that of the toxin bound to mica probably reflects the embedding of a significant part of the protein in the bilayer.

The imaging resolution of bilayer-associated VacA was typically lower than that achieved with VacA bound to mica. This is most probably because the complexes were not fixed in position, and so were being displaced slightly by the scanning tip. Nevertheless, within a single experiment, such as the one illustrated in Figure 2(B), the S.E.M. of the diameter of VacA particles (1.5 % of the mean) was well within the range reported previously for a variety of proteins bound to mica [43]. There was also some variation between experiments in the distribution of particle sizes, as judged by inspection of the images, which was probably caused by day-to-day differences in tip geometry and scanning parameters. However, in a typical experiment, the mean particle diameter (26.1 nm) was very close to values obtained previously for bilayer-associated VacA imaged either by deep-etch EM [9] or by AFM [16].

To learn more about the characteristics of the binding of VacA to lipid bilayers, images were obtained of VacA in association with bilayers of various lipid compositions. Figure 3 shows images of bilayers composed of SM (90 %) and cholesterol (10 %). These protein-free bilayers were again featureless (Figure 3A), whereas bilayers to which VacA had been added showed the characteristic spread of 26 nm particles (Figure 3B). Hence, VacA could bind to SM/cholesterol bilayers in the absence of negatively charged phospholipids. Again, there was no visible clustering of the VacA particles.

It is known that PC/SM/cholesterol bilayers generate rafts that are visible by AFM [35–37]. As shown in Figure 4(A), rafts in a DOPC (45 %)/SM (45 %)/cholesterol (10 %) bilayer appeared as raised (lighter) flat areas, surrounded by lower (darker) regions. The mica substrate is visible as a very dark area at the top right of the image. When such a bilayer was incubated with VacA at pH 4.0, the protein bound efficiently to it (Figure 4B). Strikingly, almost all of the VacA particles were associated with the raft areas,

Figure 3 VacA binds to a supported bilayer composed of SM/cholesterol

(A) A supported bilayer composed of SM (90 %)/cholesterol (10 %) was prepared by the collapse of a liposome suspension on to a mica support. The darker (lower) area is the mica and the lighter (higher) area is the lipid bilayer. (B) Liposomes were incubated with VacA in citrate buffer (pH 4.0) before they were deposited on to the mica. Note the association of VacA particles with the supported bilayer. Scale bar, 100 nm. A shade-height scale is shown on the right.

and the surrounding fluid phase was almost completely devoid of protein. Note that the rafts in bilayers prepared in the presence of VacA were more fragmented than those in protein-free bilayers and, in addition, more fenestrations were observed in the non-raft areas. The mechanisms underlying these apparent effects of VacA on the structure of the bilayer are at present obscure. The vertical dimensions of the features in Figure 4(B) are shown in the cross-section below (Figure 4C). The bilayer is approx. 5 nm higher than the mica substrate, as indicated above, and the rafts protrude by 0.80 ± 0.04 nm (mean $\pm$ S.E.M., $n = 5$) from the fluid-phase background. The VacA particle in the section has a diameter of approx. 26 nm. As stated in the Introduction section, acidification of purified VacA enhances its binding to membranes and results in increased cytotoxic effects on intact cells [10,12–16]. Figure 4(D) shows that when this acidification step was omitted and the toxin was added to the bilayer at pH 7.6 (i.e. in PBS), VacA binding to the bilayer was almost undetectable in the AFM images. It is known that the structure of isolated VacA changes significantly after acidification; for instance, the VacA oligomer dissociates into monomers [10]. In contrast, we and others [16], we observed no significant changes in the structure of the supported lipid bilayer. It is quite probable, therefore, that the dependence of VacA binding to the bilayer on acidification is a consequence of a change in the properties of the protein itself, rather than in the bilayer lipids. Some of the smaller particles bound directly to

Figure 4 VacA is targeted to SM-enriched domains in a supported lipid bilayer composed of DOPC/SM/cholesterol

(A) A supported bilayer composed of DOPC (45 %)/SM (45 %)/cholesterol (10 %) was prepared by the collapse of a liposome suspension on to a mica support. The dark (low) area at the top right is the mica; the mid-grey (higher) area is the fluid phase of the lipid bilayer; and the lightest (highest) area is the SM/cholesterol-enriched liquid-ordered raft phase. Horizontal scale bar, 100 nm. (B) Liposomes were incubated with VacA in citrate buffer (pH 4.0) before they were deposited on to the mica. Note the specific association of VacA particles with the raft areas and their exclusion from the non-raft areas. Horizontal scale bar, 100 nm. A shade-height scale is shown on the right. (C) Cross-section of the supported bilayer at the position indicated by the line in (B). Vertical scale bar, 2 nm. (D) Liposomes were incubated with VacA in PBS (pH 7.6) before they were deposited on to the mica. Note the absence of VacA particles from the surface of the bilayer and the presence of particles (most probably VacA and attached liposomes) bound directly to the mica. Horizontal scale bar, 100 nm. A shade-height scale is shown on the right.

the mica are probably VacA (as in Figure 1), although the larger particles probably represent liposomes that have not flattened out to form bilayers.

Figure 5 VacA binds weakly to a supported bilayer composed of DOPC/SM, in the absence of cholesterol

(A) A supported bilayer composed of DOPC (50 %)/SM (50 %) was prepared by the collapse of a liposome suspension on to a mica support. The dark (low) area is the mica; the mid-grey (higher) area is the fluid phase of the lipid bilayer; and the lightest (highest) area is the SM-enriched liquid-ordered raft phase. (B) Liposomes were incubated with VacA in citrate buffer (pH 4.0) before they were deposited on to the mica. Note the sparse association of VacA particles with the raft areas. Scale bar, 100 nm. A shade-height scale is shown on the right.

It has been shown before that the production of rafts in supported lipid bilayers does not require the presence of cholesterol; e.g. rafts appear in bilayers composed of an equimolar mixture of DOPC and SM [35–37]. Figure 5(A) shows an AFM image of a protein-free DOPC/SM bilayer, in which rafts are clearly visible. Note that the protrusion of the rafts from the fluid phase (1.10 ± 0.04 nm; mean $\pm$ S.E.M., $n = 6$) for this case was greater than that observed in the presence of cholesterol, as reported previously [35]. When a bilayer of this composition was incubated with VacA, very little protein association with the bilayer was observed, although the little VacA that did bind was again predominantly raft-associated (Figure 5B). This result indicates that efficient binding of VacA to lipid bilayers requires the presence of cholesterol.

VacA binds to most negatively charged phospholipids [16]. Therefore we tested the effect of including PS in the mixture of raft-forming lipids on the targeting of VacA within the supported bilayer. There is no evidence that PS is completely excluded from the SM-enriched domains. However, according to data provided by the suppliers, approx. half of the fatty acid chains present in the PS used in these experiments were unsaturated. Hence, most of the PS molecules would have contained at least one unsaturated chain, and would therefore have been excluded from

Figure 6 VacA is excluded from SM-enriched domains in a bilayer composed of PC/PS/SM/cholesterol

(A) Liposomes composed of DOPC (35 %)/L- α -PS (20 %)/SM (35 %)/cholesterol (10 %) were incubated with VacA in citrate buffer (pH 4.0) before they were deposited on to the mica to produce a supported lipid bilayer. The dark (low) area is the mica; the mid-grey (higher) area is the fluid phase of the lipid bilayer; and the lightest (highest) area is the SM/cholesterol-enriched liquid-ordered raft phase. Note the specific association of VacA particles with the non-raft area and their exclusion from the raft area. (B) Higher-magnification image of the region shown in (A). Scale bar, 100 nm. A shade-height scale is shown on the right.

the liquid-ordered, SM-enriched domains. As shown in Figure 6, VacA bound exclusively to non-raft regions in bilayers composed of DOPC (35 %)/L- α -PS (20 %)/SM (35 %)/cholesterol (10 %). Hence, VacA binds more avidly to PS in non-raft domains than to SM/cholesterol in lipid raft domains. The relevance of this observation for understanding interactions of VacA with membranes of intact cells is considered further in the Discussion section.

DISCUSSION

It is known that the biological activity of a number of bacterial toxins depends critically on their ability to interact with specific plasma-membrane domains. In some cases, the toxins form pores directly in the plasma membrane, and often this activity involves an interaction with the raft constituents, cholesterol and/or SM. For instance, perfringolysin O, produced by *Clostridium perfringens*, binds to cholesterol in rafts and then forms oligomeric pores [25]. Depletion of membrane cholesterol using cyclodextrin almost completely abolishes perfringolysin O binding [25]. On the

other hand, lysenin, from the coelomic fluid of the earthworm *Eisenia foetida*, binds to the plasma membrane of target cells via SM [26]. Interestingly, the oligomerization, but not the initial binding, of lysenin is affected by changes in the fatty acid composition of the SM, suggesting that the fluidity of the membrane is crucial to the ability of the toxin to form pores [26]. For *Vibrio cholerae* cytotoxin, binding to both cholesterol and sphingolipids is apparently required for pore formation [27]. The toxin aerolysin, secreted by *Aeromonas hydrophila*, interacts with rafts indirectly, through binding to GPI-anchored receptors [28], which are themselves concentrated in SM/cholesterol-enriched domains [17]. Only after this receptor-mediated binding step does aerolysin oligomerize to form pores. In this case, therefore, the toxin uses the rafts as concentration platforms [28]. Finally, some other toxins also need to be endocytosed to exert their cytotoxic activity. For example, cholera toxin binds to the ganglioside GM₁, which is also concentrated in SM/cholesterol-enriched rafts, before being endocytosed via caveolae-like domains [29,30].

For VacA, it was proposed that binding to GPI-anchored proteins might mediate its concentration in rafts, by a mechanism similar to that used by aerolysin [34]. However, it was shown recently that Chinese-hamster ovary cells deficient in GPI-anchored proteins were just as susceptible as wild-type cells to the toxic effects of VacA, but were much less sensitive to aerolysin [31]. It was further demonstrated that VacA was concentrated in DRMs prepared from the target cells, indicating a direct association of the toxin with rafts [31,32]. In support of this idea, methyl- β -cyclodextrin treatment inhibited the ability of VacA to cause vacuolation [31,33].

In the present study, we have used AFM to examine directly the binding of VacA to supported lipid bilayers of different compositions. All the experiments were performed under physiological conditions (i.e. in the unfixed state and under fluid). We show that the lipid composition of the bilayer is a key determinant not only of the absolute amount of VacA binding, but also of the distribution of the toxin among the various lipid domains within the bilayer. Specifically, we show that VacA is targeted almost exclusively to raised features that are apparent in bilayers composed of DOPC, SM and cholesterol. These features correspond to SM-enriched domains and are a consequence of the longer length of the saturated lipid chains of the SM molecules, relative to the length of the kinked chains of the DOPC molecules [20]. These artificial rafts are also enriched in cholesterol, as demonstrated by their decoration by the cholesterol-binding reagent filipin [36]. Results of the present study support the conclusion, drawn from previous experiments on biological membranes [31,32], that VacA interacts directly with raft lipids. In addition, the observation that VacA binding is almost abolished when cholesterol is omitted from the lipid mixture emphasizes the importance of this membrane constituent for VacA binding. There is no evidence that VacA interacts directly with cholesterol [33]. Hence, it is probable that it is a particular membrane state, e.g. the liquid-ordered state known to be prevalent in raft domains, that represents the favoured environment for binding and penetration of the toxin.

Why is raft association so important with respect to the toxicity of VacA? One possibility is that lipid rafts play a crucial role in the internalization and intracellular trafficking of VacA. Support for this suggestion has come from studies of the effects of methyl- β -cyclodextrin, a cholesterol-depleting agent known to disrupt rafts [36], on the activity of VacA. It was shown that concentrations of methyl- β -cyclodextrin that almost completely abolish VacA-induced cell vacuolation only partially disrupt DRMs and incompletely inhibit VacA association with the DRMs [31]. Furthermore, VacA-mediated cell depolarization still occurs at these concentrations of methyl- β -cyclodextrin. In contrast,

VacA internalization is strongly inhibited. Hence, the functional effect of methyl- β -cyclodextrin appears to depend on its ability to block VacA internalization and intracellular trafficking, an interpretation that is consistent with the view that rafts play a critical role in intracellular sorting events [19–22].

The preference of VacA for raft association was reversed when PS, an anionic phospholipid that does not partition into rafts, was added to the DOPC/SM/cholesterol mixture. This efficient binding of VacA to anionic lipids under acidic conditions has been demonstrated in a previous AFM study [16], and is clearly effective enough to be able to override the preference of VacA for binding to the liquid-ordered raft phase of the bilayer.

The method of preparation used in the present study results in bilayers that have a symmetrical lipid distribution between the two leaflets. This is in stark contrast with the situation in biological membranes, which are known to show pronounced lipid asymmetry [19,20]. In particular, in almost all plasma membranes that have been analysed, more than 80% of the total PS is present in the inner (cytoplasmic) leaflet, whereas more than 80% of the SM is in the outer (extracytoplasmic) leaflet [44]. *In vivo*, therefore, VacA will first encounter a plasma membrane exposing a leaflet containing PC, SM and cholesterol. On the basis of our results, we would expect that the VacA would become targeted almost exclusively to SM/cholesterol-enriched rafts, a step that probably mediates its efficient endocytosis [31]. Whether internalized VacA is ever capable of interacting with the cytoplasmic leaflet of the plasma membrane is not clear. Evidence has been presented recently that internalized VacA associates with mitochondria [45], which indicates that it is capable of gaining access to the cytoplasm. According to these results, therefore, the toxin might indeed be capable of interacting with PS-enriched domains in the cytoplasmic leaflet of the plasma membrane. However, the functional significance of such an association is at present completely obscure.

In conclusion, we suggest that the use of AFM to image the association of proteins with specific domains in artificial membranes having defined lipid composition can potentially provide a great deal of information about the mechanisms underlying the interaction of these proteins with biological membranes.

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