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Association of *Helicobacter pylori* Vacuolating Toxin (VacA) with Lipid Rafts*

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A variety of extracellular ligands and pathogens interact with raft domains in the plasma membrane of eukaryotic cells. In this study, we examined the role of lipid rafts and raft-associated glycosylphosphatidylinositol (GPI)-anchored proteins in the process by which *Helicobacter pylori* vacuolating toxin (VacA) intoxicates cells. We first investigated whether GPI-anchored proteins are required for VacA toxicity by analyzing wild-type Chinese hamster ovary (CHO) cells and CHO-LA1 mutant cells that are defective in production of GPI-anchored proteins. Whereas wild-type and mutant cells differed markedly in susceptibility to aerolysin (a bacterial toxin that binds to GPI-anchored proteins), they were equally susceptible to VacA. We next determined whether VacA physically associates with lipid rafts. CHO or HeLa cells were incubated with VacA, and Triton-insoluble membranes then were separated by sucrose density gradient centrifugation. Immunoblot analysis revealed that a substantial proportion of cell-associated toxin was associated with detergent-resistant membranes (DRMs). DRM association required acid activation of the purified toxin prior to contact with cells, and acid activation also was required for VacA cytotoxicity. Treatment of cells with methyl-β-cyclodextrin (a cholesterol-depleting agent) did not inhibit VacA-induced depolarization of the plasma membrane, but interfered with the internalization or intracellular localization of VacA and inhibited the capacity of the toxin to induce cell vacuolation. Treatment of cells with nystatin also inhibited VacA-induced cell vacuolation. These data indicate that VacA associates with lipid raft microdomains in the absence of GPI-anchored proteins and suggest that association of the toxin with lipid rafts is important for VacA cytotoxicity.

Helicobacter pylori are Gram-negative bacteria that colonize the human gastric mucosa. Infection with these organisms consistently results in gastric inflammation and is a risk factor for the development of peptic ulcer disease, distal gastric adenocarcinoma, and gastric lymphoma (1, 2). Many *H. pylori* strains secrete a toxin (VacA) that exerts a variety of effects on epithelial cells *in vitro*, including the formation of large intracellular vacuoles, formation of anion-selective pores in the plasma membrane, apoptosis, and epithelial monolayer permeabilization (3–5).

Several studies in animal models have suggested that VacA is an important virulence factor produced by *H. pylori*. In a mouse model, a VacA-producing strain exhibited an enhanced capacity to colonize the stomach compared with an isogenic *vacA*-mutant strain, particularly in co-infection experiments (6). Studies in mouse and gerbil models also have suggested that VacA contributes to gastric mucosal injury (7, 8). Analyses of *H. pylori* isolates from humans have revealed that strains isolated from patients with peptic ulcer disease typically produce VacA proteins with detectable cytotoxic activity *in vitro* (encoded by *vacA* alleles belonging to the type s1 family), whereas strains isolated from patients with no history of peptic ulcer disease commonly produce VacA proteins that lack detectable cytotoxic activity *in vitro* (encoded by *vacA* alleles belonging to the type s2 family) (9–14).

The *vacA* gene encodes a 140-kDa protoxin, which undergoes cleavage of an amino-terminal signal sequence and cleavage of a carboxyl-terminal peptide to yield an 88 kDa secreted toxin (3–5,15). Secretion of VacA occurs via an autotransporter mechanism (16). When isolated in a purified form from broth culture supernatant, VacA is in an oligomeric state consisting predominantly of dodecameric or tetradecameric flower-shaped structures (17–19). These oligomeric forms of VacA are relatively inactive when added to eukaryotic cells *in vitro* (18, 20, 21). However, exposure of purified VacA to acidic or alkaline pH conditions results in disassembly of VacA oligomers into monomeric subunits and is associated with a marked increase in its cytotoxicity (18, 20–22). Thus, it is presumed that VacA toxicity requires binding of monomeric forms of the toxin to the plasma membrane.

Following binding of VacA to the surface of eukaryotic cells, the toxin can insert into the plasma membrane to form anion-selective channels (23–26) and can also be internalized (20, 27, 28). Intracellular expression of VacA by transient tranfection with VacA-encoding plasmids results in the formation of intracellular vacuoles that are indistinguishable from those that form when VacA is added to the outside of cells (29, 30), suggesting that this toxin acts intracellularly. HEp-2 cells expressing dominant negative mutants of two proteins required for clathrin-dependent endocytosis (Eps 15 and dynamin II) develop cellular vacuoles in response to VacA (31), which suggests that VacA internalization can occur via a clathrin-independent endocytic pathway. It has been proposed that VacA toxicity requires localization of the toxin in either endosomes or mitochondria (28, 32). One current model for understanding the cytotoxic effects of VacA proposes that its cell-vacuolating activity results from the formation of anion-selective channels in

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endosomal membranes (5, 23–26). Alternatively, it is possible that VacA might have novel intracellular activities distinct from membrane channel formation (5, 33, 34). In summary, there continues to be considerable uncertainty about the site of VacA action and the molecular mechanisms underlying its toxic activity.

In recent years, it has been recognized that a variety of pathogens and toxins interact with microdomains in the plasma membrane known as lipid rafts (35). Lipid rafts are membrane microdomains that are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)[1]-anchored proteins (36–39). Several bacterial, viral, and parasitic pathogens seem to use rafts as a site for gaining entry into mammalian cells (40, 41). In addition, certain bacterial toxins, including aerolysin, perfringolysin O, cholera toxin, and tetanus toxin, utilize rafts as either a site for high affinity binding and oligomerization on the surface of cells or as a site for internalization into host cells (42–46). A recent study reported that treatment of HEp-2 cells with phosphatidylinositol-specific phospholipase C (PI-PLC), an agent that removes GPI-anchored proteins from the cell surface, inhibited the capacity of VacA to induce cell vacuolation (31). It also was reported that incubation of the cells with nystatin (a cholesterol-binding agent) inhibited VacA-induced cell vacuolation (31). Based on these results, it was proposed that the presence of one or more GPI-anchored proteins and intact membrane lipid rafts are required for VacA cytotoxicity. Specifically, it was hypothesized that VacA monomers might bind to GPI-anchored proteins and that lipid rafts might act as concentrating platforms enabling VacA to concentrate locally and oligomerize efficiently (31). However, there has not yet been any direct evidence indicating that VacA physically interacts with either GPI-anchored proteins or lipid rafts. In the current study, we provide biochemical evidence indicating that VacA associates with lipid raft microdomains and report that the presence of GPI-anchored proteins is not required for either association of the toxin with rafts or VacA-induced cell vacuolation. In addition, we present data suggesting that VacA interaction with lipid rafts is an important feature of the process by which VacA intoxicates cells.

MATERIALS AND METHODS

Purification of H. pylori VacA and Aeromonas Proaerolysin—VacA was purified in an oligomeric form from culture supernatant of *H. pylori* strain 60190, as described previously (18, 20, 23). In most experiments, purified VacA was acid-activated by the slow addition of 200 mM HCl to the toxin preparation until a pH of 3 was reached (20, 21). Proaerolysin was purified from culture supernatant of *Aeromonas salmonicida* as described previously (47).

Cell Culture Methods—HeLa cells were grown in Eagle's medium containing 10% fetal bovine serum, and CHO cells were grown in Ham's F-12 medium containing 10% fetal bovine serum. Mutant CHO-LA1 cells (defective in production of GPI-anchored proteins) and mutant cells recomplemented with the *PIG-L* gene (*i.e.* with restored capacity for production of GPI-anchored proteins, hereafter designated as wild-type CHO cells) have been described previously (48). In assays to test VacA-induced vacuolation of cells, the tissue culture medium was supplemented with 5 mM ammonium chloride (49, 50), and VacA was acid-activated as described above. Acid-activated VacA then was diluted in neutral pH tissue culture medium and was added directly to the neutral pH medium overlying cells.

Preparation of a VacA Affinity Column—Purified VacA (~1.2 mg) from *H. pylori* 60190 (in 25 mM HEPES buffer, pH 7.2, containing 150 mM sodium chloride) was incubated with 1 ml of a 1:1 mixture of Affi-Gel 10:Affi-Gel 15 (Bio-Rad) at 4 °C for 4 h, followed by addition of 200 μl of 0.5 M ethanolamine. This preparation then was loaded onto a 2-ml Poly-prep column (Bio-Rad), and the column was sequentially washed with 10 mM potassium phosphate buffer (pH 7.2) containing 150 mM sodium chloride and 0.02% sodium azide, with 20 mM Tris (pH 8.0) containing 500 mM sodium chloride, with 100 mM glycine (pH 2.5), with 50 mM Tris (pH 8.8), with 100 mM ammonium hydroxide (pH 11.5), and finally with 10 mM potassium phosphate buffer (pH 7.2) containing 150 mM sodium chloride and 0.02% sodium azide.

Preparation of Affinity-purified anti-VacA Rabbit Serum—Anti-VacA rabbit serum 958 (about 1 ml), prepared by immunizing with purified oligomeric VacA from *H. pylori* strain 60190, was passed over CM Affi-Gel Blue (Bio-Rad) to remove albumin, according to the manufacturer's instructions. The immunoglobulin fraction was concentrated by precipitation with ammonium sulfate and resuspended in 10 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 150 mM sodium chloride and 0.02% sodium azide. The de-albuminated serum was then applied three times to the VacA affinity column described above. The column was washed two times with 5 column volumes of 10 mM potassium phosphate buffer (pH 7.2) containing 150 mM sodium chloride and 0.02% sodium azide and then washed two times with 5 column volumes of 20 mM Tris (pH 8.0) containing 500 mM sodium chloride. Bound immunoglobulin was eluted with ten 0.5-ml aliquots of 100 mM glycine (pH 2.5) and collected into 100 μl of 1 M Tris (pH 8.0) containing 2.5 mg/ml ovalbumin. Aliquots were tested for anti-VacA immunoreactivity by immunoblotting, and the reactive aliquots were pooled. EDTA (10 mM) and sodium azide (0.02%) were added to the reactive aliquots to facilitate storage. Residual nonspecific reactivity in the serum was removed by adsorbing the affinity-purified serum against an Affi-Gel column (prepared as described above) containing both *H. pylori* proteins (from a *vacA* null mutant strain) and HeLa cell proteins. The purified serum was concentrated to a volume of 2 ml in a Centricon-30 device, diluted 1:2 with glycerol, and stored at either 4 °C or −20 °C.

Detergent Extraction and Sucrose Density Gradient Centrifugation of Triton X-100-insoluble Cell Components—HeLa or CHO cells were grown to confluence in 75-mm plastic dishes, using Eagle's medium containing 10% fetal bovine serum or Ham's F-12 medium containing 10% fetal bovine serum, respectively. Cells were washed three times with 0.9% NaCl to remove serum, and serum-free Eagle's containing 1 mg/ml ovalbumin then was added to cells. Approximately $1–2 \times 10^7$ cells then were incubated for 1 h at 25 °C with 10 μg/ml *H. pylori* VacA. Triton-insoluble cell components were then isolated according to previously published protocols (51). In brief, cells were washed twice with 0.9% NaCl and once with Tris-buffered saline (pH 7.4) (TBS) to remove unbound toxin and then were lysed at 4 °C in 2 ml of TBS (pH 7.4) containing 1% Triton X-100, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and a eukaryotic cell protease inhibitor mixture (Sigma). The lysate was Dounce-homogenized (10 strokes) and incubated for 30 min on ice. An equal volume of 80% w/v sucrose in TBS was added to 2 ml of lysate. This was overlayered on a 0.35 ml of 80% sucrose cushion. The 40% sucrose-containing lysate was successively overlayered with 4 ml of 30% (w/v) sucrose in TBS and 4.2 ml of 5% (w/v) sucrose in TBS. Samples were centrifuged for 21 h at 208,000 × *g* at 4 °C in a Beckman SW 41 TI rotor. Afterward, 1-ml fractions were collected from the top of the gradient (fraction 1) to the bottom (fraction 12). Pelleted material recovered from the bottom of the tube was resuspended in fraction 12. Fractions were stored frozen at −20 °C prior to further analysis.

Analysis of Sucrose Density Gradient Fractions—Equal volumes of sucrose density gradient fractions (45 μl) were analyzed by SDS-PAGE and immunoblotting. Gradient fractions 1 and 2, 9 and 10, and 11 and 12 were routinely pooled (as *lanes a*, *h*, and *i*, respectively, in Figs. 3–5) to permit immunoblot analysis of all gradient fractions on a single polyacrylamide gel. The transferrin receptor was detected in gradient fractions by using a mouse anti-human transferrin receptor antibody (Zymed Laboratories Inc.). VacA was detected by using the affinity-purified rabbit polyclonal anti-VacA antibody preparation described above. CD55 was detected using rabbit anti-human CD55 affinity-purified antibody (Research Diagnostics Inc.). Immunoblot analyses were performed using enhanced chemiluminescence (Amersham Biosciences) with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. Alkaline phosphatase activity was detected by mixing equal volumes of gradient fractions with a solution of *p*-nitrophenyl phosphate (2 mg/ml) in 0.1 M Tris-Cl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$. After incubation at 37 °C for 10 min, the optical density of samples was analyzed at 410 nm. Triplicate reactions were performed for each gradient fraction.

Immunofluorescence Microscopy—HeLa or CHO cells were grown to near confluence on glass coverslips. Cells were incubated for 4 h at 37 °C with purified VacA (either acid-activated or untreated) in tissue

[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; CHO, Chinese hamster ovary; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DRM, detergent-resistant membrane.

culture medium containing 5 mM ammonium chloride. The cells then were washed with PBS containing 3% bovine serum albumin (PBS-BSA), fixed with 3.7% formaldehyde for 5 min, and permeabilized with 100% methanol for 2 min. The fixed, permeabilized cells were incubated for 30 min at room temperature with affinity-purified anti-VacA rabbit serum (diluted 1:5000 in PBS-BSA) and after washing were incubated for 30 min at room temperature with Cy3-conjugated anti-rabbit immunoglobulin G (Sigma) diluted 1:250 in PBS-BSA (27). Alternatively, in some experiments VacA was detected using anti-VacA monoclonal antibody 5E4 (52), followed by Cy3-conjugated anti-mouse immunoglobulin G (Sigma). Coverslips were mounted on slides in Aqua-Polymount (Polysciences), and the cells were visualized with a Zeiss 410 laser scanning confocal microscope (27).

Depletion of Cellular Cholesterol by Methyl-β-cyclodextrin—HeLa cells were incubated for 30 min at 37 °C in serum-free Eagle's medium containing 4 mM methyl-β-cyclodextrin (Sigma) prior to addition of VacA. Control experiments were performed using methyl-β-cyclodextrin-cholesterol complex (27 mg of cholesterol per gram methyl-β-cyclodextrin), prepared as described (53, 54). The cholesterol content of cells was measured using either thin layer chromatography (48) or an Infinity cholesterol reagent kit (Sigma).

Analysis of Membrane Potential—Analyses of membrane potential were performed as described by Szabo *et al.* (24), with several modifications. Briefly, HeLa cells were pretreated with 4 mM methyl-β-cyclodextrin at 37 °C for 30 min or left untreated, washed with 0.9% NaCl, and then detached with trypsin/EDTA. Cells were washed with 0.9% NaCl containing 13 mM D-glucose and resuspended in 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 13 mM D-glucose, 20 mM HEPES buffer (pH 7.4) at 1–3 × 10^6 cells/ml. Cells were then incubated with bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol (oxonol VI) (Molecular Probes) (final concentration 2.5 μM) for 15 min at 37 °C. A cell suspension (2 ml) was placed in a stirred quartz cuvette at 37 °C in a PerkinElmer Life Sciences LS50B fluorimeter. After stabilization of the fluorescence signal (excitation 585 nm, slit 10 nm; emission, 645 nm, slit 5 nm), either an acidified buffer control, acid-activated VacA (final concentration 5 μg/ml), or non-activated VacA (5 μg/ml) was added to the cells. Further depolarization was induced by addition of gramicidin A (20 μg/ml).

RESULTS

Analysis of the Role of GPI-anchored Proteins in VacA Cytotoxicity—A recent study reported that VacA cytotoxicity for HEp-2 and HeLa cells was inhibited by treatment of the cells with PI-PLC, an enzyme that cleaves GPI-anchored proteins from the cell surface (31). Based on this result, it was proposed that the high sensitivity of some cell types to VacA may be dependent on the presence of intact GPI-anchored proteins. To investigate further whether VacA cytotoxic activity requires the presence of GPI-anchored proteins, we analyzed the interactions of VacA with CHO-LA1 cells, which are defective in production of GPI-anchored proteins. A previous study has shown these cells are deficient in production of an enzyme encoded by the *PIG-L* gene that is responsible for the *N*-deacetylation of *N*-acetylglucosamine phosphatidylinositol, which is the second step in GPI biosynthesis (48). For comparison, we examined mutant CHO cells recomplemented with the *PIG-L* gene (*i.e.* with restored capacity for production of GPI-anchored proteins) (48), hereafter designated as wild-type CHO cells. VacA was purified from the culture supernatant of *H. pylori* 60190, and then both cell types were incubated for 18 h at 37 °C with varying concentrations of acid-activated VacA, ranging from 5 to 100 μg/ml. In agreement with previous studies, the minimum concentration of VacA required to induce vacuolation in >50% of CHO cells was about 10-fold higher than that required to induce similar vacuolation of HeLa cells (31, 55, 56). Importantly, wild-type CHO cells and CHO-LA1 mutant cells did not differ substantially in susceptibility to VacA cytotoxicity (Fig. 1). As a control, we compared the susceptibility of wild-type CHO cells and CHO-LA1 cells to the toxicity of aerolysin (a bacterial pore-forming toxin that binds to GPI-anchored proteins) (48, 57). As expected, wild-type CHO cells were highly susceptible to the toxicity of aerolysin,

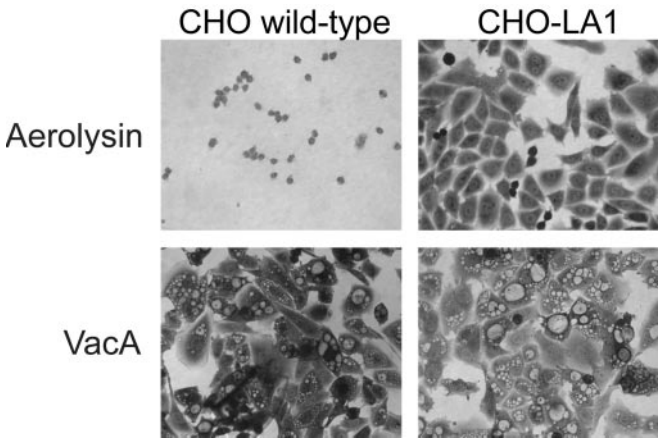

FIG. 1. **Cytotoxic effects of aerolysin and VacA for wild-type CHO cells and CHO-LA1 mutant cells.** Wild-type CHO cells and CHO-LA1 mutant cells were incubated with proaerolysin (150 ng/ml) or with purified acid-activated VacA (25 μg/ml) in the presence of 5 mM ammonium chloride for 18 h at 37 °C. The cells then were fixed with formaldehyde, stained with crystal violet, and visualized by light microscopy. As expected, wild-type cells were susceptible to aerolysin toxicity, whereas CHO-LA1 cells were resistant. Both cell types were susceptible to the vacuolating cytotoxic activity of VacA.

whereas the mutant CHO cells were resistant to aerolysin toxicity (Fig. 1). To examine possible internalization of VacA by wild-type CHO and CHO-LA1 cells, these cells types were incubated with acid-activated VacA for 4 h at 37 °C, and internalized toxin then was detected by indirect immunofluorescence and confocal microscopy. Acid-activated VacA was internalized to a similar extent by wild-type CHO and CHO-LA1 mutant cells, and no differences in the intracellular distribution of VacA were detectable in these two cell types (Fig. 2). Thus, in CHO cells, interaction of VacA with GPI-anchored proteins is not required for either internalization of the toxin or vacuolating cytotoxic effects.

Interaction of VacA with Detergent-resistant Membrane Microdomains—Despite the lack of a requirement of GPI-anchored proteins for VacA cytotoxicity or VacA internalization, we nevertheless investigated whether VacA was able to associate with lipid rafts and whether such an association might be important for VacA cytotoxicity. Lipid rafts are membrane microdomains that are enriched in cholesterol, sphingolipids, and various membrane proteins, including GPI-anchored proteins. Notable characteristics of these membrane microdomains are insolubility at 4 °C in the presence of certain nonionic detergents and a low buoyant density (36–39). To determine whether or not *H. pylori* VacA interacts with detergent-resistant membranes (DRMs), HeLa cells were incubated with purified acid-activated VacA for 1 h at room temperature (25 °C). The cells then were lysed, and Triton-insoluble cell components were separated by sucrose density gradient centrifugation, as described under "Materials and Methods." As expected, the transferrin receptor was detected almost exclusively in fractions at the bottom of the gradient (Fig. 3*A*). In contrast, most of the cell-associated VacA was found in low density fractions (Fig. 3*B*, *lanes c* and *d*). Similar results were obtained if VacA was incubated with HeLa cells at 4 °C instead of 25 °C, to prevent possible internalization of the toxin (data not shown). The low density fractions that contained VacA were enriched in alkaline phosphatase and CD55, two GPI-anchored proteins known to be constituents of lipid rafts (Fig. 3*D* and data not shown). When cell lysates were treated with the combination of 0.5% Triton X-100 plus 0.5% saponin (instead of Triton X-100 alone) and then analyzed by sucrose density gradient centrifugation, VacA was not detected in low

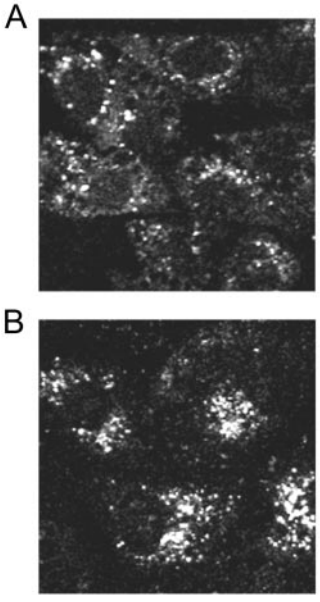

FIG. 2. **Internalization of VacA by wild-type CHO and CHO-LA1 cells.** Wild-type CHO cells and CHO-LA1 cells were incubated with acid-activated VacA (1 μg/ml) for 4 h at 37 °C. The cells were fixed and permeabilized, and the localization of VacA then was detected by indirect immunofluorescence microscopy using a monoclonal anti-VacA antibody. VacA was localized in a punctate pattern within the wild-type cells (*A*). The intracellular localization of VacA in the mutant cells (*B*) was indistinguishable from that in the wild-type CHO cells.

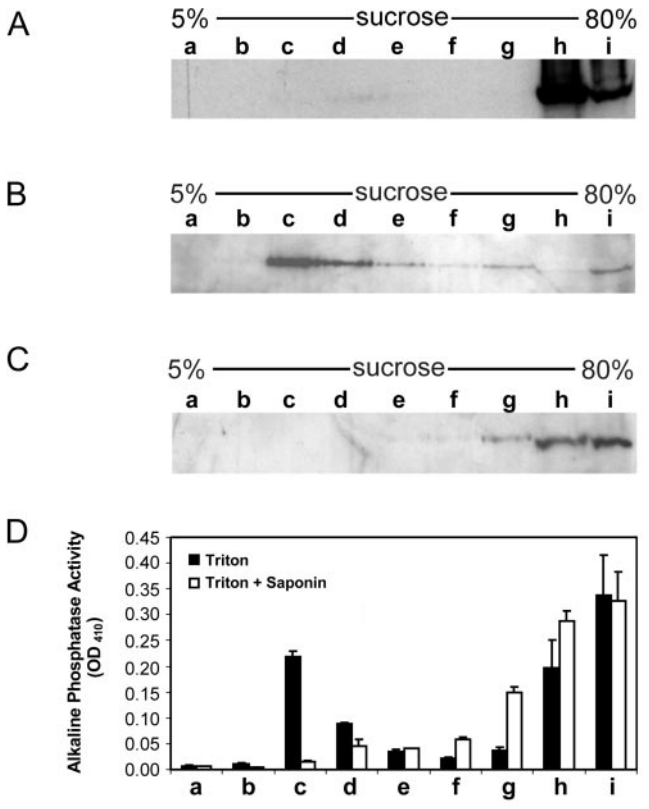

FIG. 3. **Interaction of VacA with lipid rafts.** Acid-activated VacA from *H. pylori* 60190 was incubated with HeLa cells for 1 h at room temperature (25 °C). Cells were lysed with 1% Triton X-100, and insoluble cell components then were separated by sucrose density gradient centrifugation. The gradient fractions were analyzed by immunoblotting with either anti-transferrin receptor antibodies (*A*) or anti-VacA serum (*B*). The transferrin receptor was present in fractions at the bottom of the gradient (*A*, *lanes h* and *i*), whereas in contrast, a substantial proportion of the 88-kDa VacA band was present in low density fractions (*B*, *lanes c* and *d*). When VacA-treated cells were lysed in a mixture of 0.5% Triton X-100 plus 0.5% saponin and insoluble cell components then were analyzed, VacA was detected predominantly in fractions at the bottom of the gradient (*C*). Alkaline phosphatase activity was detected in low density fractions from cells treated with Triton X-100, but not in low density fractions (*lanes c* and *d*) from cells treated with the combination of saponin plus Triton X-100 (*D*).

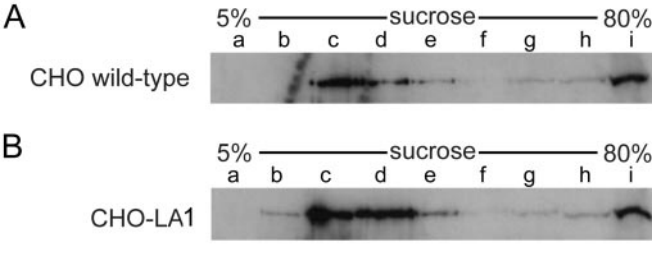

FIG. 4**. Interaction of VacA with lipid rafts in wild-type CHO cells and CHO-LA1 mutant cells.** Acid-activated VacA was incubated with wild-type CHO cells and with CHO-LA1 cells (defective in production of GPI-anchored proteins) for 1.5 h at 25 °C. After lysing the cells, Triton-insoluble fractions were analyzed by sucrose gradient centrifugation and immunoblotting of fractions with anti-VacA serum. VacA was detected in low density fractions prepared from both wild-type CHO cells (*A*) and CHO-LA1 mutant cells (*B*).

density fractions, but instead was detected predominantly in fractions at the bottom of the gradient (Fig. 3*C*). Similarly, under these conditions alkaline phosphatase activity was not detected in low density fractions, but was detected exclusively in fractions at the bottom of the gradient (Fig. 3*D*). These results are consistent with the known capacity of saponin to disrupt lipid rafts (51). As expected, when VacA was analyzed directly on gradients (*i.e.* without first being incubated with eukaryotic cells), all of the toxin was found at the bottom of the gradient (data not shown). In parallel studies, we analyzed interactions of VacA with DRMs of CHO cells and observed that acid-activated VacA associated with DRMs of both wild-type CHO cells and CHO-LA1 mutant cells (Fig. 4). In summary, these data show that VacA associates with DRMs of the plasma membrane and indicate that GPI-anchored proteins are not required for association of the toxin with DRMs.

Acid Activation of Purified VacA Increases Its Association with DRMs—Multiple previous studies (20–22) have shown that VacA purified from *H. pylori* broth culture supernatants has very little cytotoxic activity for cells unless the toxin is first exposed to low pH or high pH conditions prior to contacting the cells (a process known as "acid activation" or "alkaline activation"). Experiments using ^{125}I-labeled VacA have shown that both acid-activated and non-activated forms of the toxin can bind to HeLa cells at 4 °C, but acid-activated VacA is internalized by cells to a significantly greater extent than non-activated toxin (20). In agreement with these results, analysis of the binding of the non-labeled VacA to cells at 4 °C by immunoblotting confirmed that both acid-activated and non-activated forms of the toxin can bind to cells, although the acid-activated form bound to a somewhat greater extent than the non-activated form (Fig. 5*A*). Confocal microscopy studies indicated that when acid-activated VacA (1 μg/ml) was incubated with HeLa cells for 4 h at 37 °C, it was internalized and localized in a focal punctate distribution within the cells (Fig. 5*B*). In contrast, when non-activated VacA (1 μg/ml) was incubated with cells under the same conditions, it was not detected in such a distribution (Fig. 5*C*). When cells were incubated with a 10-fold higher concentration of non-activated VacA (10 μg/ml), a small proportion of cells demonstrated a detectable signal, but in most cells VacA was again not detected in a focal punctate distribution (data not shown).

Based on this evidence that acid-activated VacA and non-

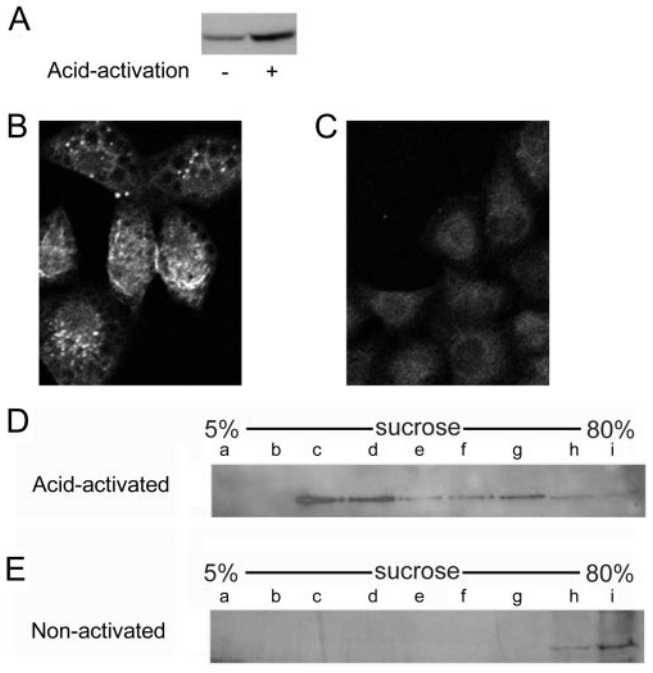

FIG. 5. **Acid activation of VacA enhances its localization in lipid rafts.** *A*, purified VacA was either acid-activated or left untreated and then incubated with HeLa cells at 4 °C for 1 h. Analysis of total cell lysates by immunoblotting with anti-VacA serum indicated that both forms of the toxin bound to cells and that the total binding of acid-activated VacA to cells was greater than that of non-activated VacA. *B* and *C*, HeLa cells were incubated with either acid-activated VacA (1 μg/ml) or non-activated VacA (1 μg/ml) for 4 h at 37 °C. The cells were fixed and permeabilized, and the localization of VacA then was detected by indirect immunofluorescence microscopy, using affinity-purified rabbit anti-VacA serum. Acid-activated VacA was localized in a focal punctate pattern within the cells (*B*), whereas this pattern of localization was not detected in cells treated with non-activated VacA (*C*). *D* and *E*, Triton-insoluble cell components were separated by sucrose density gradient centrifugation, and gradient fractions were analyzed by immunoblotting with anti-VacA serum. Acid-activated VacA was detected in low density gradient fractions (*D*), whereas non-activated VacA was detected predominantly in fractions at the bottom of the gradient (*E*).

activated VacA interact differently with cells, we hypothesized that these two forms of the toxin might interact differently with lipid rafts. To determine whether interaction of VacA with lipid rafts requires activation of the toxin, VacA was either acid-activated or left untreated and then added directly to the neutral pH medium overlying HeLa cells. Cells were incubated with VacA for 1 h at 25 °C. The association of VacA with DRMs was then analyzed, as described above. Acid-activated VacA was found in the low density gradient fractions corresponding to DRMs (Fig. 5*D*), whereas non-activated VacA was found almost exclusively at the bottom of the gradient (Fig. 5*E*). Similar results were obtained if acid-activated VacA and non-activated VacA were incubated with cells at 4 °C instead of room temperature (data not shown). Thus, acid activation of the purified toxin resulted in a marked increase in its association with DRMs. Because acid activation markedly enhances the cytotoxic activity of purified VacA, the foregoing observation suggested that VacA association with DRMs might be an important feature of the intoxication process.

Inhibitory Effects of Methyl-β-cyclodextrin on VacA Cytotoxicity—To investigate further the role of lipid rafts in the process by which VacA intoxicates cells, we examined the effects of methyl-β-cyclodextrin, an agent known to disrupt lipid rafts by extracting cholesterol from membranes (44). In preliminary experiments, we found that lipid rafts were more effectively disrupted by treatment of intact cells with high concentrations (≥10 mM) of methyl-β-cyclodextrin than by treatment with lower concentrations of the drug, based on the distribution of alkaline phosphatase activity in gradient fractions prepared from Triton-insoluble membranes. However, treatment of intact HeLa cells with ≥10 mM methyl-β-cyclodextrin resulted in marked alterations in cell morphology, whereas lower concentrations of methyl-β-cyclodextrin (≤4 mM) did not. Therefore, subsequent experiments were performed using a methyl-β-cyclodextrin concentration of 4 mM. The total cholesterol content of cells treated with 4 mM methyl-β-cyclodextrin at 37 °C for 30 min was 77 ± 2% of that in untreated cells.

HeLa cells were pretreated with 4 mM methyl-β-cyclodextrin in serum-free medium for 30 min and then were incubated with acid-activated VacA. VacA induced marked vacuolation in control (untreated) cells, but in contrast, no vacuoles were detected when VacA was added to cells that had been pretreated with methyl-β-cyclodextrin (Fig. 6, *A–C*). To test whether the observed inhibitory effects resulted specifically from cholesterol depletion rather than from direct inhibitory effects of methyl-β-cyclodextrin, cells were pretreated with 2 mM methyl-β-cyclodextrin plus 2 mM methyl-β-cyclodextrin-cholesterol complex, as described by Thiele *et al.* (53), prior to addition of acid-activated VacA. Thus, in this control experiment, the cells were exposed to 4 mM methyl-β-cyclodextrin, but there was no depletion of cellular cholesterol content (data not shown). Under these conditions, VacA induced prominent cell vacuolation (Fig. 6*D*). Additional control experiments were done in which cholesterol was depleted by incubating cells with 4 mM methyl-β-cyclodextrin for 30 min, and then methyl-β-cholesterol was removed and cells were incubated with 5 mM methyl-β-cyclodextrin-cholesterol complex. Analysis of cellular cholesterol content confirmed that this procedure effectively restored cholesterol to its original levels. When added to cells manipulated as described above, VacA induced prominent cell vacuolation (Fig. 6*E*). These experiments indicate that depletion of cellular cholesterol renders cells resistant to the vacuolating activity of VacA.

In parallel experiments, we analyzed the effects of methyl-β-cyclodextrin on the association of VacA with cells. In cells pretreated with methyl-β-cyclodextrin, the total amount of cell-associated VacA was detectably reduced compared with the amount of cell-associated VacA in untreated cells (Fig. 7*A*). Next, DRMs were prepared from VacA-treated cells that had been either pretreated or not pretreated with methyl-β-cyclodextrin. A reduction in alkaline phosphatase activity in low density fractions indicated that DRMs were partially disrupted by methyl-β-cyclodextrin treatment (Fig. 7*B*). Also, the amount of VacA detected in low density fractions was reduced, but not completely eliminated, by methyl-β-cyclodextrin treatment (Fig. 7*C*). These results are consistent with the existence of heterogeneity among DRMs (58), such that certain types of DRMs may be differentially affected by cholesterol depletion.

Additional experiments were done to investigate further the mechanism by which methyl-β-cyclodextrin inhibits VacA cytotoxicity. VacA is known to form anion-selective channels in the plasma membrane of HeLa cells, and the formation of these channels results in partial depolarization of the resting membrane potential (24). To determine whether methyl-β-cyclodextrin pretreatment of cells inhibited the capacity of VacA to induce depolarization of the plasma membrane, we used bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol as a probe to monitor the membrane potential of HeLa cells. Consistent with previously published results (24), we found that following the addition of acid-activated VacA, the resting membrane potential of HeLa cells was rapidly altered (Fig. 8*A*). Following depolarization of the membrane by VacA, further depolariza-

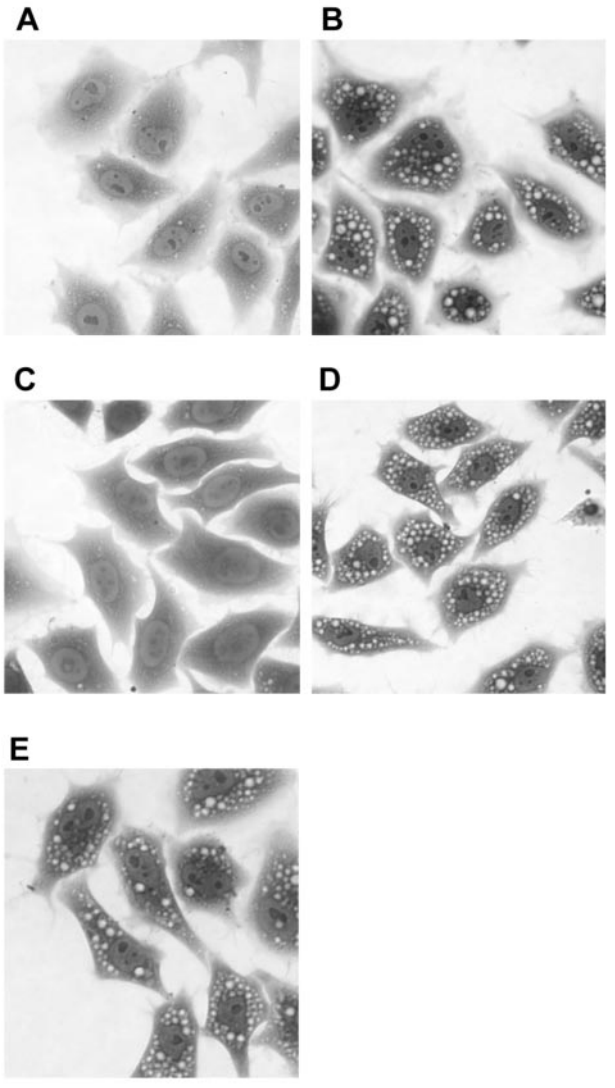

FIG. 6. **Inhibitory effects of methyl-β-cyclodextrin on VacA-induced cell vacuolation.** HeLa cells were pretreated as described below, incubated with acid-activated VacA (5 μg/ml) for 2 h at 37 °C, stained with crystal violet, and then visualized by microscopy (magnification ×40). *A*, no pretreatment and no addition of VacA. *B*, no pretreatment, followed by addition of VacA. *C*, pretreatment with 4 mM methyl-β-cyclodextrin for 30 min prior to addition of VacA. *D*, pretreatment with 2 mM methyl-β-cyclodextrin plus 2 mM methyl-β-cyclodextrin-cholesterol complex for 30 min prior to addition of VacA. *E*, pretreatment with 4 mM methyl-β-cyclodextrin for 30 min, followed by removal of methyl-β-cyclodextrin and incubation with 5 mM methyl-β-cyclodextrin-cholesterol complex prior to addition of VacA.

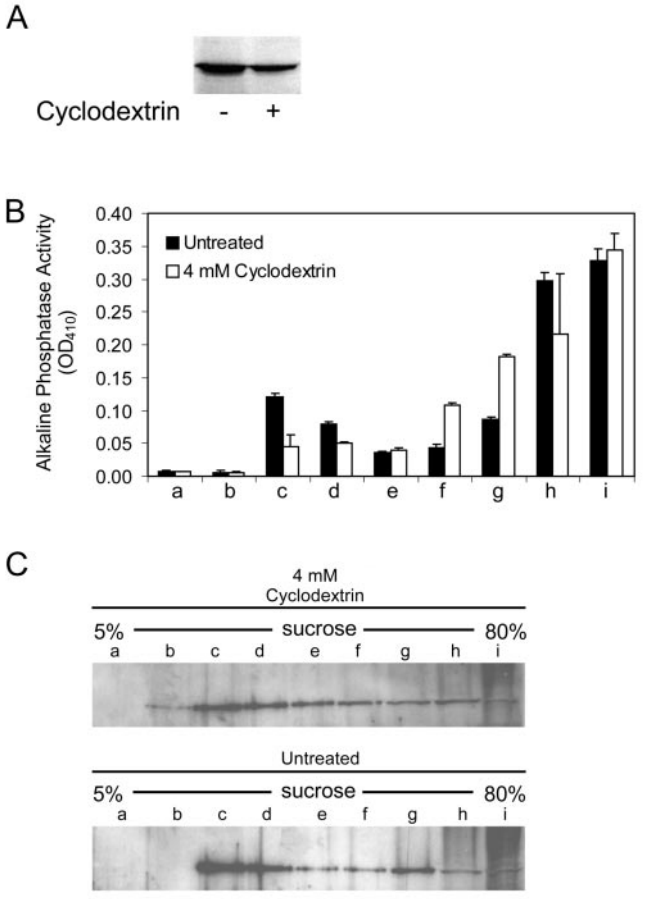

FIG. 7. **Effects of methyl-β-cyclodextrin on VacA interactions with cells.** *A*, HeLa cells were either pretreated with 4 mM methyl-β-cyclodextrin or left untreated as a control and then were incubated with acid-activated VacA for 4 h at 37 °C. Cells lysates then were analyzed by immunoblotting with anti-VacA serum. *B* and *C*, HeLa cells were either pretreated with 4 mM methyl-β-cyclodextrin or left untreated and then were incubated with acid-activated VacA for 1 h at 25 °C. Triton-insoluble cell components were separated by sucrose density centrifugation, and the alkaline phosphatase activity in fractions was quantified (*B*). Gradient fractions were analyzed for the presence of VacA by immunoblotting with anti-VacA serum (*C*).

tion could be induced by addition of gramicidin A (20 μg/ml) (data not shown). Addition of non-activated VacA or an acidified buffer control to the cells did not result in any detectable alterations of the membrane potential (Fig. 8*A* and data not shown). Interestingly, treatment with methyl-β-cyclodextrin had no effect on the membrane depolarization induced by acid-activated VacA (Fig. 8*A*). These results indicate that treatment of the cells with methyl-β-cyclodextrin does not interfere with the capacity of VacA to form channels in the plasma membrane.

Since methyl-β-cyclodextrin treatment inhibited VacA-induced vacuolation but not channel formation by VacA at the plasma membrane, we hypothesized that internalization or trafficking of the toxin might be altered in cholesterol-depleted cells. We therefore assessed the localization of VacA in methyl-β-cyclodextrin-treated cells. Whereas VacA was detected in an intracellular focal punctate distribution in untreated cells, only a diffuse distribution could be seen in cyclodextrin-treated cells (Fig. 8*B*), even when using a 10-fold higher concentration of acid-activated VacA (data not shown). To determine whether the altered distribution of VacA resulted from cholesterol depletion or from direct effects of the methyl-β-cyclodextrin, control experiments were performed in which cells were incubated with the same dose of cyclodextrin in the presence of excess cholesterol. Under these conditions, VacA localized in an intracellular focal punctate distribution (Fig. 8*B*). Similarly, if cellular cholesterol was depleted and subsequently replaced, VacA localized in the same intracellular focal punctate distribution (data not shown). These results indicate that cholesterol depletion induced by methyl-β-cyclodextrin disrupts the cellular processes normally utilized for internalization or intracellular trafficking of VacA.

In agreement with a previous report (31), we found that treatment of cells with nystatin also inhibited VacA-induced vacuolation (Fig. 9*A*). Nystatin binds to cholesterol but does not deplete cellular cholesterol levels, and therefore, as expected, the cholesterol content of nystatin-treated cells was not different compared with that of untreated cells. Treatment of cells with nystatin produced fairly modest effects on raft integrity and VacA association with rafts (data not shown), similar to the

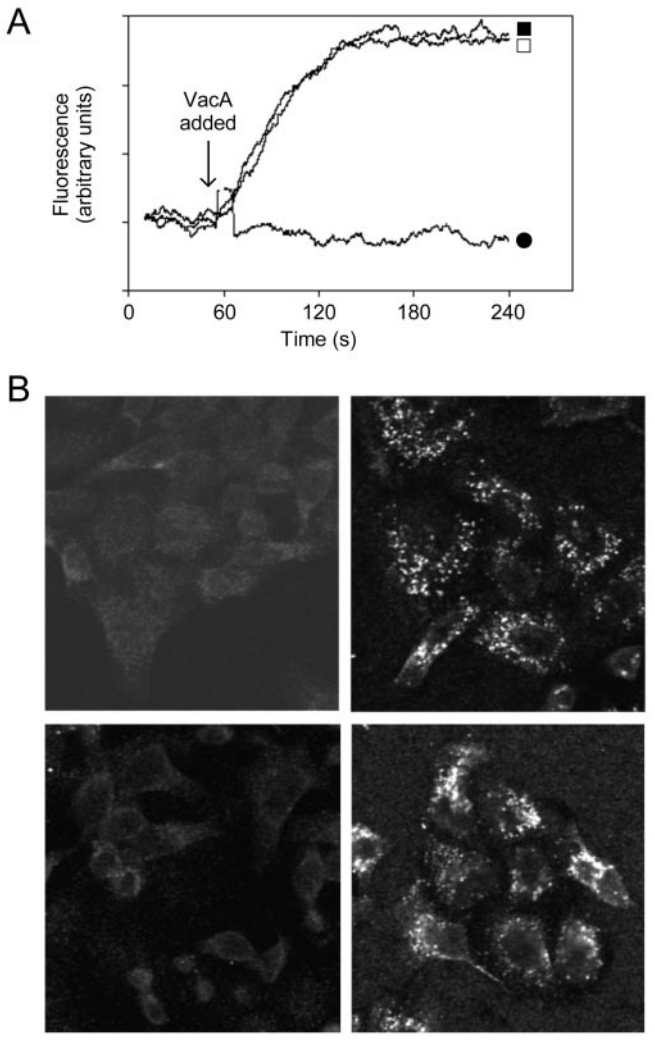

FIG. 8. **Effects of methyl-β-cyclodextrin pretreatment on VacA-induced alterations in membrane potential and VacA localization within cells.** *A*, HeLa cells were pretreated with 4 mM methyl-β-cyclodextrin or left untreated and then were loaded with oxonol VI (a probe used to monitor membrane potential). Following addition of acid-activated VacA (5 μg/ml), the resting membrane potential of both control (*open square*) and cyclodextrin-treated cells (*filled square*) was rapidly altered and reached a plateau within 2–3 min. Addition of non-activated VacA (5 μg/ml) (*filled circle*) or acidified buffer control (data not shown) did not induce any alteration in the membrane potential. *B*, HeLa cells were pretreated as described below and then incubated with acid-activated VacA for 4 h at 37 °C. The localization of VacA was assessed by indirect immunofluorescence methodology and confocal microscopy, using a monoclonal anti-VacA antibody. *Top left panel*, no pretreatment and no addition of VacA. *Top right panel*, no pretreatment, followed by addition of VacA. *Bottom left panel*, pretreatment with 4 mM methyl-β-cyclodextrin for 30 min prior to addition of VacA. *Bottom right panel*, pretreatment with 2 mM methyl-β-cyclodextrin plus 2 mM methyl-β-cyclodextrin-cholesterol complex for 30 min prior to addition of VacA.

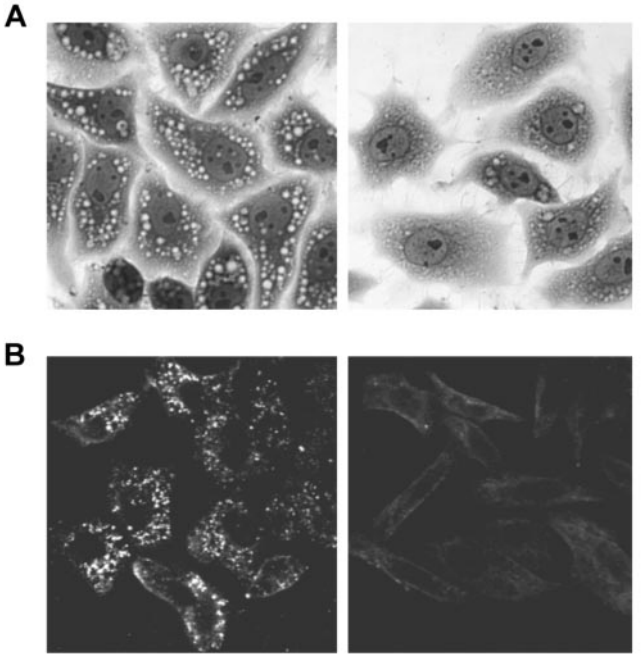

FIG. 9. **Inhibitory effects of nystatin on VacA-induced cell vacuolation.** HeLa cells were either pretreated with nystatin (40 μg/ml) for 1 h at 37 °C or not pretreated and then incubated with acid-activated VacA (5 μg/ml) at 37 °C. *A*, cells were fixed and stained with crystal violet. Addition of VacA induced vacuolation in untreated cells (*left panel*), but not in cells pretreated with nystatin (*right panel*). *B*, the localization of VacA was assessed by indirect immunofluorescence methodology and confocal microscopy, using a monoclonal anti-VacA antibody. VacA was localized in a focal punctate intracellular distribution in untreated cells (*left panel*), but not in cells that had been pretreated with nystatin (*right panel*).

results observed following treatment with 4 mM methyl-β-cyclodextrin (Fig. 7). However, treatment of cells with nystatin interfered with the internalization or intracellular localization of VacA (Fig. 9*B*). Thus, binding of nystatin to cellular cholesterol produced essentially the same effects on VacA activity and localization as those that were observed following depletion of cellular cholesterol.

DISCUSSION

Binding of bacterial protein toxins to eukaryotic cells often is mediated by an interaction of the toxin with a specific receptor on the cell surface. Several previous studies have provided evidence for saturability and specificity of VacA binding to cells (59–61). In contrast, studies with radiolabeled VacA have not demonstrated convincing evidence of a saturable binding process, and the binding of radiolabeled VacA was only partially inhibited by excess unlabeled VacA (20, 31). At least five different putative receptors for VacA have been reported, including receptor protein tyrosine phosphatase β, an unidentified 140-kDa protein, the epidermal growth factor receptor, heparan sulfate, and various lipids (22, 23, 62–65). It has also been reported that that treatment of cells with either nystatin or PI-PLC inhibits VacA cytotoxicity, and based on this observation, it was proposed that intact lipid rafts and GPI-anchored proteins are required for VacA susceptibility (31). Thus, at present, there is considerable confusion about which cell surface components are most relevant for VacA binding and cytotoxicity. It remains unclear whether VacA cytotoxicity is dependent on binding of VacA to one specific receptor or whether cytotoxicity results from nonspecific binding of VacA to multiple cell-surface components.

Several different bacterial protein toxins seem to utilize lipid rafts or raft-associated GPI-anchored proteins to intoxicate cells. Two pore-forming toxins, aerolysin and *Clostridium septicum* alpha toxin, each bind to GPI-anchored proteins, which are enriched in lipid raft microdomains of the plasma membrane (57, 66). Other pore-forming toxins, such as perfringolysin, bind to cholesterol components of lipid rafts (43). It has been proposed that lipid rafts serve as concentrating platforms to promote oligomerization of these toxins on the cell surface, a process that is required for membrane channel formation (57). Two toxins with intracellular enzymatic activity, cholera toxin and tetanus toxin, bind to GM1 and a specific GPI-anchored protein, respectively, which are enriched in lipid raft microdo-

mains (43–45, 58, 67). It has been proposed that lipid rafts play an important role in the internalization and intracellular trafficking of these toxins (44, 58, 67). The conclusion that these bacterial protein toxins require lipid rafts for intoxication of cells has been based mainly on experiments that utilize cyclodextrin or other cholesterol-interacting drugs to alter lipid rafts.

In the current study, we provide direct biochemical evidence indicating that VacA associates with lipid rafts. Moreover, we find that VacA is still able to associate with lipid rafts, undergo internalization, and induce cytotoxic effects in the absence of GPI-anchored proteins. Although our conclusions about VacA interactions with GPI-anchored proteins in CHO cells are unequivocal, these data do not completely exclude the possibility that GPI-anchored proteins might contribute to the high sensitivity of various other cell types to VacA cytotoxicity. Notably, a previous report found that doses of PI-PLC sufficient to block VacA activity in HEp-2 cells had no significant effect on ^{125}I-VacA binding to these cells (31). This result is consistent with the concept that there may be multiple binding sites and/or multiple internalization pathways by which VacA intoxicates cells (31). We speculate that the inhibitory effect seen with PI-PLC could be due to changes in raft properties that occur when GPI-anchored proteins are acutely removed by the enzymatic treatment. In contrast, GPI-deficient cells grow continuously in the absence of GPI-anchored proteins and therefore are perhaps able to compensate for this defect.

In agreement with a previous report (31), we found in the current study that treatment of cells with nystatin inhibits the activity of VacA. Our demonstration that acid-activated (active) forms of VacA associate with lipid rafts, whereas non-activated (inactive) forms do not, provides further evidence suggesting that interaction of VacA with lipid rafts is required for cytotoxicity. Finally, we found that treatment of cells with methyl-β-cyclodextrin, a cholesterol-depleting agent known to disrupt rafts, inhibits VacA cytotoxicity. Interestingly, the doses of cholesterol-interacting drugs used in this study resulted in nearly complete inhibition of VacA-induced cell vacuolation, but only partially disrupted DRMs and incompletely inhibited VacA association with DRMs. Moreover, methyl-β-cyclodextrin did not inhibit the capacity of VacA to depolarize the plasma membrane of cells. However, treatment of cells with cholesterol-interacting drugs strongly interfered with either the internalization or intracellular localization of VacA. Thus, it seems likely that early events (*e.g.* binding of the toxin to cells) in the VacA intoxication process are not substantially affected by these compounds but that their inhibitory effects are mainly due to interference with later events (*e.g.* internalization and intracellular trafficking of VacA). This interpretation is consistent with the concept that raft-dependent mechanisms are important for intracellular sorting events (68–71). We speculate that there is heterogeneity among DRMs (58) and that functional properties of certain types of DRMs relevant for intracellular trafficking processes may be especially susceptible to disruption by drugs such as methyl-β-cyclodextrin and nystatin. In future studies, it will be important to investigate further the role of lipid rafts and cellular cholesterol content on the intracellular trafficking of various bacterial protein toxins, viruses, and bacterial pathogens.

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