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Clustering of *Helicobacter pylori* VacA in Lipid Rafts, Mediated by Its Receptor, Receptor-Like Protein Tyrosine Phosphatase β, Is Required for Intoxication in AZ-521 Cells▽

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Helicobacter pylori **vacuolating cytotoxin, VacA, induces multiple effects on epithelial cells through different cellular events: one involves pore formation, leading to vacuolation, mitochondrial damage, and apoptosis, and the second involves cell signaling, resulting in stimulation of proinflammatory responses and cell detachment. Our recent data demonstrated that VacA uses receptor-like protein tyrosine phosphatase β (RPTPβ) as a receptor, of which five residues (QTTQP) at positions 747 to 751 are involved in binding. In AZ-521 cells, which mainly express RPTPβ, VacA, after binding to RPTPβ in non-lipid raft microdomains on the cell surface, is localized with RPTPβ in lipid rafts in a temperature- and VacA concentration-dependent process. Methyl-β-cyclodextrin (MCD) did not block binding to RPTPβ but inhibited translocation of VacA with RPTPβ to lipid rafts and all subsequent events. On the other hand, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), which disrupts anion channels, did not inhibit translocation of VacA to lipid rafts or VacA-induced activation of p38 mitogen-activated protein (MAP) kinase, but inhibited VacA internalization followed by vacuolation. Thus, p38 MAP kinase activation did not appear to be required for internalization. In contrast, phosphatidylinositol-specific phospholipase C (PI-PLC) inhibited translocation, as well as p38 MAP kinase/ATF-2 activation, internalization, and VacA-induced vacuolation. Neither NPPB nor PI-PLC affected VacA binding to cells and to its receptor, RPTPβ. Thus, receptor-dependent translocation of VacA to lipid rafts is critical for signaling pathways leading to p38 MAP kinase/ATF-2 activation and vacuolation.**

Infection with *Helicobacter pylori* plays a major role in the development of chronic gastritis and peptic ulcer and is a risk factor for gastric cancer (13, 31, 34). Pathogenic strains of *H. pylori* produce a potent cytotoxin, VacA, which causes progressive vacuolation of epithelial cells and gastric injury (4, 7, 33, 34). Purified VacA, under denaturing conditions, has a molecular mass of about 90 kDa, whereas the native toxin is an oligomer of about 1,000 kDa (24). The VacA protein consists of two functional domains: the 58-kDa C-terminal domain (p58) is responsible for binding to the VacA receptor (25), whereas the 37-kDa N-terminal domain (p37) plus 150 amino acids of p58 is cytotoxic when transiently expressed in cultured cells (11, 46).

It is well known that VacA induces multiple effects on epithelial cells, including mitochondrial damage (15, 20, 41) and apoptosis (8, 15, 20, 22, 41). These actions of VacA appear to result from activation of cellular pathways, independent of those leading to vacuolation (45). Similarly, phosphorylation of Git1 (G protein-coupled receptor kinase-interactor 1), which may be responsible for epithelial cell detachment caused by VacA, results from a mechanism different from that leading to vacuolation (14).

Analysis of VacA receptors provided new insights into the molecular basis of VacA function. We reported that two VacA proteins, termed m1VacA and m2VacA, which were defined by sequence differences in the middle of the molecules, interacted with target cells by binding to two types of receptor-like protein tyrosine phosphatases (RPTPs; i.e., RPTPα and RPTPβ), resulting in toxin internalization and vacuolation of the human gastric adenocarcinoma cell lines AZ-521 and G401 (12, 30, 43, 44).

Following binding and internalization, VacA forms channels in the limiting membranes of intracellular organelles such as late endosomes (10, 46). The amino-terminal hydrophobic region of the p37 fragment, which is essential for pore formation by VacA, has three tandem GxxxG motifs (27). Alanine replacement of glycine residues at positions 14 and 18 in VacA diminishes VacA oligomerization, vacuolating activity, and anion-selective membrane channel-forming activity in lipid bilayers (27). Channel formation by VacA was observed in artificial lipid bilayers (9, 19, 38, 39) as well as in VacA-treated HeLa cells (37, 39). In living cells, however, both the number of toxin oligomers and the molecular ultrastructure of the VacA channel differed from those formed in artificial lipid bilayers (2).

Although there is not yet any direct evidence that VacA physically interacts with glycosylphosphatidylinositol (GPI)-

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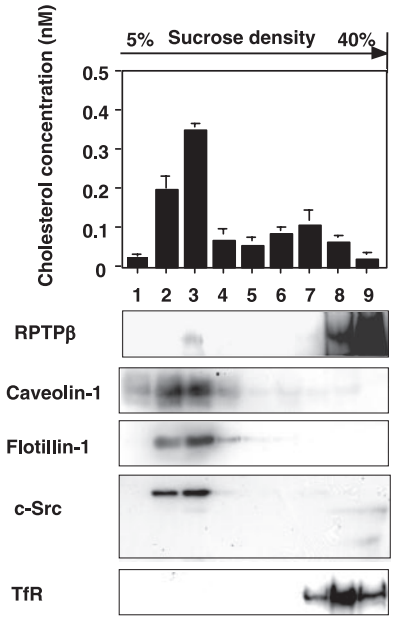

FIG. 1. RPTPβ in lipid rafts of AZ-521 cells. AZ-521 cells were lysed in 1% Triton X-100 and subjected to sucrose density gradient centrifugation to isolate lipid rafts as described in Materials and Methods. Proteins in equal volumes of the indicated fractions were separated by SDS-PAGE in 5% gels and subjected to Western blotting using specific antibody against RPTPβ. Lipid rafts were identified by cholesterol content and by Western blotting with antibodies against caveolin-1, flotillin-1, c-Src, and TfR, respectively. No protein bands were seen in the absence of a primary antibody. Data are representative of three separate experiments.

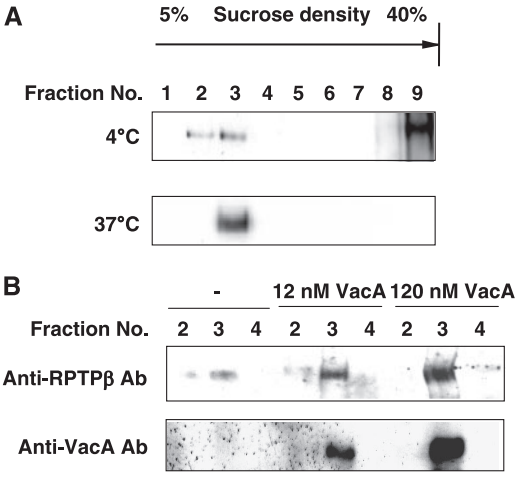

FIG. 2. Temperature- and concentration-dependent accumulation of VacA in lipid rafts of AZ-521 cells. (A) AZ-521 cells incubated with 12 nM VacA at 4°C or 37°C for 30 min were lysed in 1% Triton X-100 and subjected to sucrose density gradient centrifugation to isolate lipid rafts. Proteins (0.5 μg) in equal volumes of the indicated fractions were separated by SDS-PAGE in 6% gels and subjected to Western blotting with specific antibody (Ab) against VacA. (B) AZ-521 cells incubated with the indicated amounts of VacA at 37°C for 30 min were subjected to sucrose density gradient centrifugation after lysis in 1% Triton X-100. Translocation of RPTPβ to lipid rafts was analyzed by Western blotting with anti-RPTPβ antibody subsequent to SDS-PAGE in 6% gels. Lipid rafts were identified by cholesterol content as shown in Fig. 1. Data are representative of three separate experiments.

anchored proteins, it was suggested that VacA endocytosis might occur via a GPI-protein-dependent pathway, independent of clathrin. Incubation of Hep-2 cells with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that removes GPI-anchored proteins from the cell surface, inhibited VacA-induced cell vacuolation (23, 29, 35). Schraw et al. (36) showed, however, that VacA associates with lipid raft microdomains in the absence of GPI-anchored proteins using CHO-LA1 mutant cells that are defective in production of GPI-anchored proteins. In addition, treatment of cells with a cholesterol-sequestering drug, nystatin, or methyl-β-cyclodextrin (MCD), which is known to disrupt lipid rafts, resulted in a reduction of VacA activity, suggesting that lipid rafts participate in VacA-induced vacuolation (32, 35, 36). However, it remains unclear whether VacA-induced vacuolation and signal transduction events are dependent on binding of VacA to RPTPα or RPTPβ, translocation to lipid rafts, or raft-associated GPI-anchored proteins in toxin-sensitive cells.

Here we report that in AZ-521 cells, which mainly express RPTPβ, VacA is concentrated in lipid rafts after binding to RPTPβ in non-lipid raft microdomains on the AZ-521 cell surface. Furthermore, translocation of VacA with RPTPβ to lipid rafts and VacA-induced vacuolation were inhibited by treatment with MCD, which did not block binding of VacA to its receptor, suggesting that VacA localization with RPTPβ to lipid rafts is critical for activation of signaling pathways leading to vacuolation. 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), which disrupts anion channels, reduced VacA internalization and VacA-induced vacuolation, but not translocation of VacA to lipid rafts or activation of p38 mitogen-activated protein (MAP) kinase in AZ-521 cells, whereas PI-PLC inhibited VacA translocation, internalization, and VacA-induced vacuolation, as well as activation of p38 mitogen-activated protein (MAP) kinase/ATF-2. Neither NPPB nor PI-PLC significantly affected VacA binding to AZ-521 cells.

MATERIALS AND METHODS

Cell culture. AZ-521 cells, human gastric cancer cell lines obtained from the Japan Health Sciences Foundation, were cultured in Earle's minimal essential medium (Sigma) containing 10% fetal calf serum.

Purification of VacA. The toxin-producing *H. pylori* strain ATCC 49503 was the source of VacA for purification by a modification of our published procedure (21). In brief, after growth of *H. pylori* in brucella broth containing 0.1% β-cyclodextrin at 37°C for 3 to 4 days with vigorous shaking in a controlled microaerobic atmosphere of 10% O_2 and 10% CO_2, VacA was precipitated from culture supernatant with 50% saturated ammonium sulfate. Precipitated proteins were dialyzed against RX buffer (10 mM KCl, 0.3 mM NaCl, 0.35 mM $MgCl_2$, 0.125 mM EGTA, 1 mM HEPES, pH 7.3) and applied to an anti-VacA-specific immunoglobulin G (IgG) antibody column equilibrated with RX buffer. After washing the column with RX buffer, VacA was eluted with 50 mM glycine-HCl buffer (pH 1.0), which was promptly neutralized with 1 M Tris-HCl (pH 10). After gel filtration on Superose 6HR 10/30 equilibrated with Tris-buffered saline (TBS) buffer (60 mM Tris-HCl buffer, pH 7.7, containing 0.1 M NaCl), purified VacA was stored at −20°C.

Assay for vacuolating activity. Vacuolating activity was assessed using AZ-521 cells as previously described (44). Cells (1×10^5 cells/well, 250 μl) were grown in 48-well culture plates as monolayers for 24 h in a 5% CO_2 atmosphere at 37°C. VacA was added, and cells were incubated at 37°C for the indicated times. To quantify vacuolating activity, the uptake of neutral red into vacuoles was determined as described previously (44).

Preparation of biotin-labeled VacA. VacA binding to cells and internalization were quantified using VacA labeled with sulfo-NHS-SS-biotin (Pierce), according to the instructions provided by the manufacturer. In brief, 25 μl of 1 M

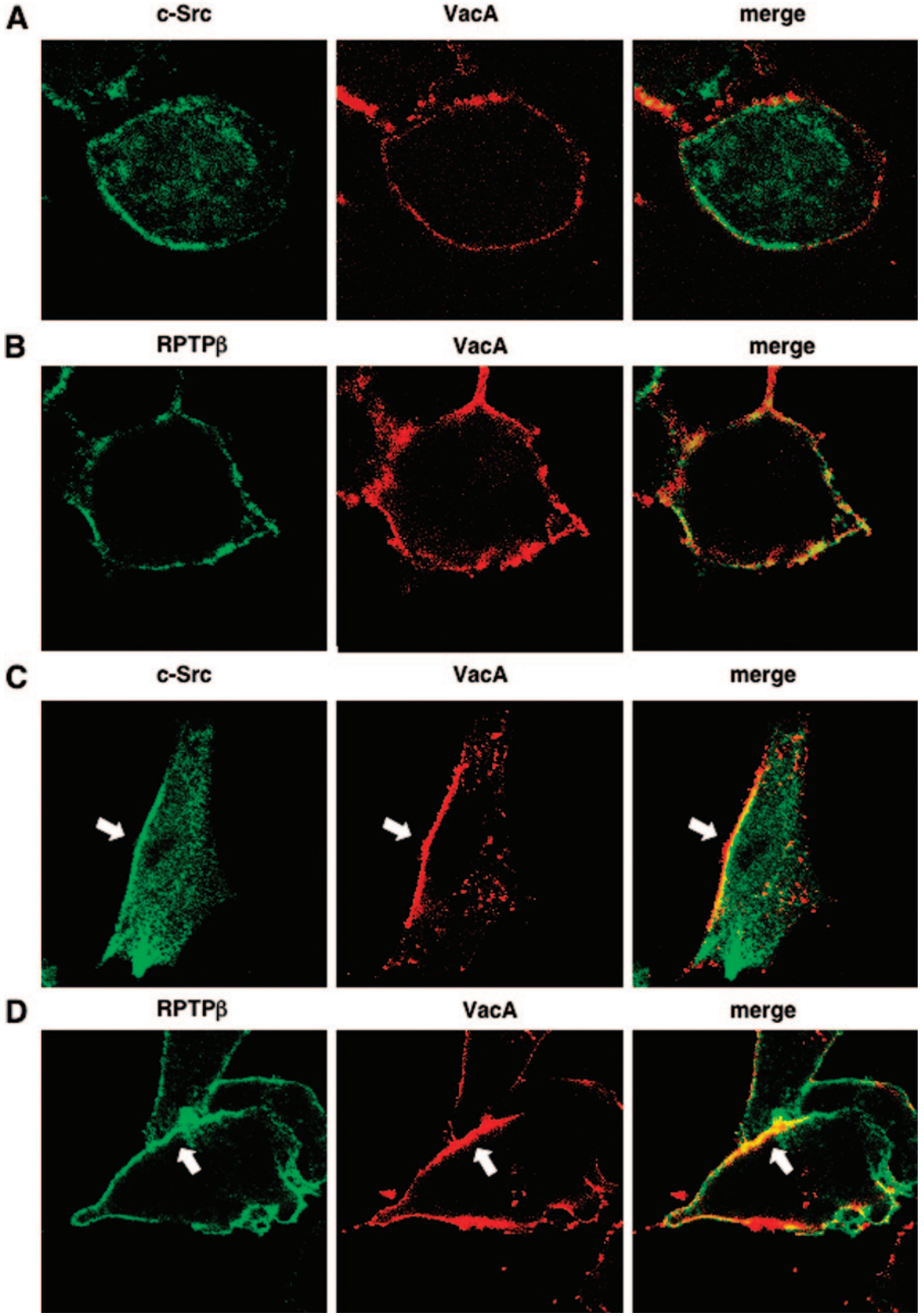

FIG. 3. Confocal microscopy analysis of VacA and RPTPβ colocalization on plasma membrane. AZ-521 cells were incubated with 120 nM Cy3-labeled VacA for 30 min at 4°C (A and B) or 37°C (C and D). The first image (green) in each set shows c-Src (A and C) or RPTPβ (B and D), the second shows Cy3-labeled VacA (red), and the third represents the merged picture. c-Src was stained as lipid raft-associated marker protein. Yellow areas shown by arrow in the merge panel show a region of overlap, consistent with the colocalization of VacA with RPTPβ in lipid rafts after incubation at 37°C.

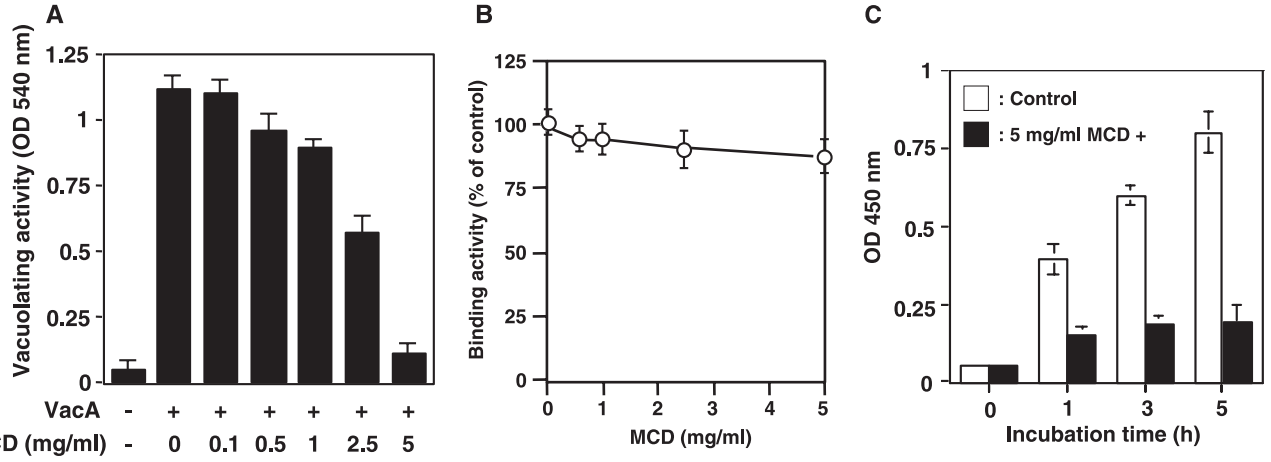

FIG. 4. Effect of MCD of VacA on binding, internalization, and vacuolation in AZ-521 cells. (A) Z-521 cells were incubated with or without MCD at 37°C for 1 h, followed by incubation with 120 nM VacA at 37°C for 5 h. Vacuolation of cells was quantified by neutral red uptake assay. (B) AZ-521 cells were treated with the indicated concentration of MCD at 37°C for 1 h followed by incubation with VacA-SS-biotin (120 nM) at 4°C for 30 min. After cells were fixed with 0.25% glutaraldehyde, VacA-SS-biotin bound to cells was detected with avidin-HRP. (C) AZ-521 cells were pretreated with (■) or without (□) MCD (5 mg/ml) at 37°C for 1 h, before incubation with VacA-SS-biotin (120 nM) for the indicated times. After incubation with 0.25% glutaraldehyde followed by treatment with 2-mercaptoethanesulfonic acid, internalized VacA was detected with avidin-HRP. Data are mean ± standard error of values from triplicate experiments. OD, optical density.

sodium bicarbonate buffer (pH 8.5) was added to 500 μl (100 μg in phosphate-buffered saline [PBS]) of VacA, followed by addition of 10 μl (10 μg) of sulfo-NHS-SS-biotin and incubation on ice for 2 h. After addition of 465 μl of 1 M Tris-HCl (pH 7.5) and dialysis against PBS, VacA-SS-biotin (100 μg/ml) was stored at 4°C (43).

Binding assay. VacA binding to cells was determined using VacA-SS-biotin. AZ-521 cells (0.8×10^5 cells) were incubated at 37°C for 1 h with or without MCD at the indicated concentration before incubation at 4°C with VacA-SS-biotin (120 nM) for 30 min. The cells were washed twice with PBS and fixed with 0.25% glutaraldehyde for 20 min. The cells were washed twice with PBS and blocked with PBS containing 3% bovine serum albumin (BSA), then incubated with horseradish peroxidase (HRP)-conjugated streptavidin (1:500; Amersham Pharmacia) in PBS containing 3% BSA for 1 h at room temperature. The cells were washed four times with PBS containing 3% BSA and incubated with 50 μl of soluble 3,3′,5,5′-tetramethylbenzidine (BM blue POD substrate; Roche Diagnostics) for 20 min, following which the reaction was stopped by addition of 50 μl of 1 M H_2SO_4. The HRP reaction produced by bound VacA was detected at 450 nm (43).

Internalization assay. Internalization of VacA into cells was quantified using VacA-SS-biotin. AZ-521 cells (0.8×10^5 cells) were incubated at 37°C for 1 h with or without 5 mg/ml MCD before incubation at 37°C with VacA-SS-biotin (120 nM) for the indicated times. The cells were washed twice with PBS and fixed with 0.25% glutaraldehyde for 20 min. The cells were washed twice with PBS, and biotin was cleaved from VacA-SS-biotin on the surface with 0.5 M 2-mercaptoethanesulfonic acid (Sigma). Internalized VacA labeled with biotin was protected from 2-mercaptoethanesulfonic acid cleavage. After being washed twice with PBS, cells were incubated with 50 μl of PBS containing 1% Triton X-100 and then washed twice with PBS; after being blocked with PBS containing 3% BSA, cells were incubated for 1 h at room temperature with HRP-conjugated streptavidin (1:500; Amersham Pharmacia) in PBS containing 3% BSA. The cells were washed four times with PBS containing 3% BSA and incubated with 50 μl of soluble 3,3′,5,5′-tetramethylbenzidine for 20 min; the reaction was stopped by addition of 50 μl of 1 M H_2SO_4. The HRP reaction produced by internalized VacA was detected at 450 nm (43).

Isolation of detergent-resistant membrane lipid rafts. Lipid rafts were isolated by ultracentrifugation in a discontinuous sucrose density gradient (6). Cells (1×10^7 cells) were washed with PBS and lysed in 450 μl of 25 mM MES buffer (25 mM 2-morpholinoethanesulfonic acid, pH 7.4, 150 mM NaCl with protease inhibitors) containing 1% Triton X-100. The lysate was incubated on ice for 30 min and adjusted to 40% (wt/vol) sucrose with an equal volume of 80% (wt/vol) sucrose in 25 mM MES buffer. The mixture was overlaid with 2.4 ml of 30% sucrose and then 1.2 ml of 5% sucrose. After centrifugation at 170,000 × *g* in an SW55 Ti rotor (Beckman Instruments) at 4°C for 16 h, 0.5-ml fractions were collected from the top of the gradient. Fractions were analyzed to identify lipid rafts based on the quantification of cholesterol with Cholesterol E-test Wako using cholesterol standard solution.

Detection of phosphorylation of p38 MAP kinase and ATF-2 induced by VacA in AZ-521 cells treated with MCD, NPPB, or PI-PLC. AZ-521 cells were treated without or with 5 mg/ml MCD for 1 h or with 0, 0.5, or 1 U/ml PI-PLC for 1 h or with 0, 25, or 50 μM NPPB for 30 min at 37°C, followed by incubation with 120 nM VacA for 0, 10, or 30 min. Cells were solubilized by incubation for 10 min on ice in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin (10 μg/ml). After centrifugation (15 min, 15,000 × *g*), samples (20 μg protein) of supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels and Western blotting using anti-p38 MAP kinase, anti-phospho-p38 MAP kinase, anti-ATF-2, or anti-phospho-ATF-2 antibodies (28).

Immunofluorescence confocal microscopy. For immunofluorescent staining of VacA colocalized with RPTPβ in lipid rafts, Cy3-labeled VacA was prepared using a Cy3 Mono-Reactive dye pack (Amersham Biosciences). After AZ-521 cells (0.8×10^5 cells) were incubated with 120 nM Cy3-labeled VacA for the indicated time, cells were fixed with 2% paraformaldehyde at room temperature for 10 min, washed with PBS twice, and then incubated with blocking buffer (Block Ace solution; Snow Brand Milk Products, Tokyo, Japan) at room temperature for 30 min. To stain for c-Src, cells were treated with 0.1% Triton X-100 in PBS for 10 min for permeabilization before incubation with antibody. Cells were further incubated with primary antibodies for 1 h at room temperature and washed twice with PBS containing 10% Block Ace solution, followed by incubation at room temperature with Alexa Fluor 488-conjugated anti-rabbit IgG for 1 h. Rabbit antibodies were used to detect c-Src and RPTPβ; rabbit antibody to RPTPβ was raised against its extracellular domain, corresponding to the N-terminal amino acids of the human protein. Stained cells were inspected by confocal microscopy (Leica). Cy3-VacA was detected at 543 nm, and c-Src and RPTPβ were quantified at 488 nm. To examine the effect of PI-PLC treatment on VacA internalization, AZ-521 cells (0.8×10^5 cells), which were treated with 1 U/ml PI-PLC at 37°C for 1 h, were incubated for 30 min at 4°C or 37°C with 120 nM VacA. After incubation, cells were fixed with 2% paraformaldehyde–PBS, washed with PBS twice, and then incubated with blocking buffer at room temperature for 30 min. The cells were incubated with anti-VacA antibodies for 1 h at room temperature and washed with PBS twice, followed by incubation with Alexa Fluor 546-conjugated anti-rabbit IgG for 1 h. VacA was detected at 543 nm by confocal microscopy. The images were analyzed using Leica confocal software version 2.6.1, arranged with Adobe Photoshop Elements version 2.0.

Other methods and chemicals. Protein was measured by the method of Bradford using bovine serum albumin as a standard (5). Mouse anti-RPTPβ, anti-flotillin-1, anti-transferrin receptor, and anti-p38alpha/SAPK2a mono-

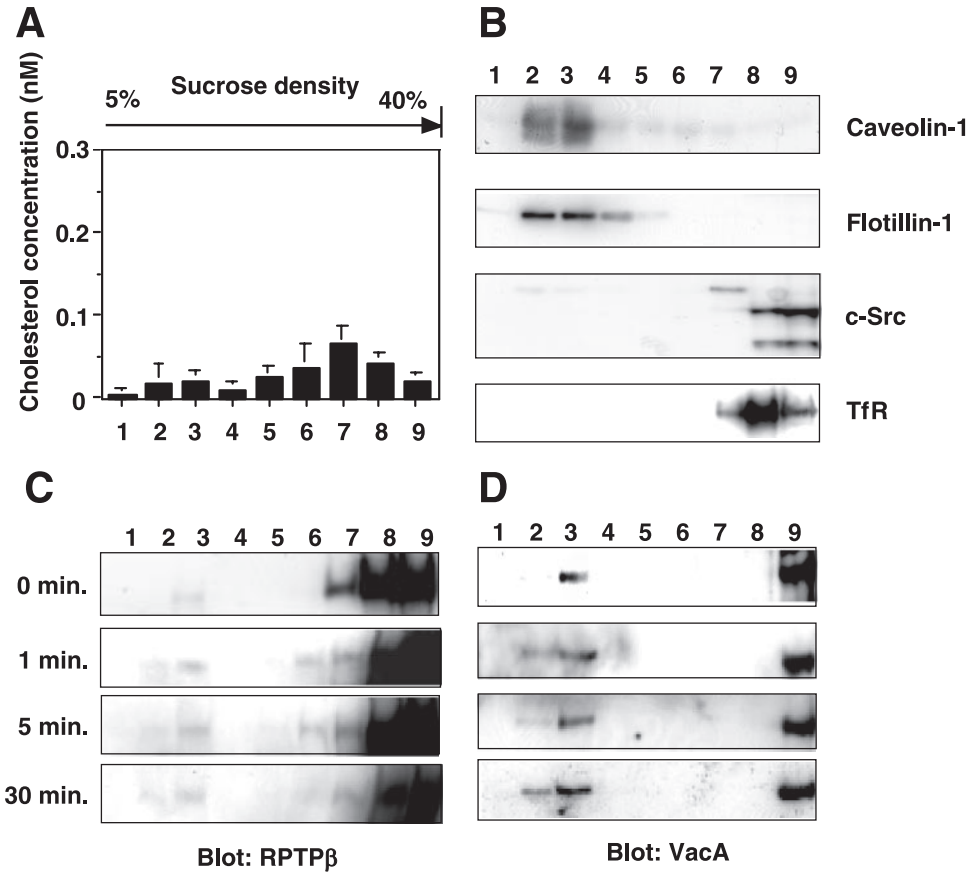

FIG. 5. VacA and RPTPβ in lipid rafts of AZ-521 cells treated with MCD. AZ-521 cells were incubated with MCD (5 mg/ml) for 1 h at 37°C and then with 12 nM VacA at 37°C for the indicated times and then lysed in 1% Triton X-100, and lysates were subjected to sucrose density gradient centrifugation to isolate lipid rafts. Proteins from equal volumes of the indicate fractions were separated by SDS-PAGE in 5% gels. Lipid rafts were identified by cholesterol (A) and by Western blotting using antibodies against caveolin-1, flotillin-1, c-Src, and TfR (B). Data are representative of three experiments. RPTPβ (C) or VacA (D) was identified by Western blotting.

clonal antibodies and rabbit anti-caveolin-1 polyclonal antibody were obtained from BD Transduction Laboratories; rabbit anti-c-Src polyclonal antibodies were from Santa Cruz Biotechnology; and anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibodies were from Cell Signaling. Anti-rabbit IgG Alexa Fluor 488 was purchased from Molecular Probes; the Cholesterol E-test Wako was from Wako Pure Chemical Industries, Ltd.; and NPPB and PI-PLC were from Sigma. Other reagents were of analytical grade.

RESULTS

Translocation of VacA and RPTPβ to lipid rafts of AZ-521 cells. VacA causes vacuolation of AZ-521 cells through its binding to RPTPβ, followed by internalization (12, 30, 44). To determine whether RPTPβ was constitutively present in membrane lipid rafts, plasma membrane microdomains were isolated from AZ-521 cells on the basis of their insolubility in Triton X-100 and low buoyant density in sucrose gradients (6). AZ-521 cells were treated with 25 mM MES buffer containing 1% Triton X-100 for 30 min on ice and then subjected to sucrose density centrifugation as described in Materials and Methods. Equivalent proportions of each fraction were analyzed to identify lipid rafts based on quantification of cholesterol. Samples of fractions were also subjected to SDS-PAGE and immunoblotting using antibodies against RPTPβ, caveolin-1, flotillin-1, c-Src, and transferrin receptor (TfR). In agreement with the fact that lipid rafts in the Triton X-100-insoluble fraction are enriched in cholesterol, raft-associated proteins (e.g., caveolin-1, flotillin-1, and c-Src) were found near the top of the gradient (fractions 2 to 4) and were separated from the TfR, which is present in non-lipid raft microdomains; this procedure effectively separated lipid rafts from the rest of the membranes (Fig. 1). RPTPβ was recovered in fractions 8 and 9 with a minor percentage in fraction 3; thus, RPTPβ was mainly present in non-lipid raft membranes.

To identify the location of cell-bound VacA, we incubated AZ-521 cells with VacA at 37°C prior to sucrose density gradient fractionation. Following incubation of cells with toxin for 0 to 5 min, VacA was located in the raft fraction and the nonlipid raft fraction; after incubation for 30 min, most of the VacA appeared in lipid rafts (data not shown). In parallel with VacA translocation to lipid rafts after 30 min of incubation, RPTPβ also accumulated in lipid rafts from non-lipid raft microdomains. This VacA translocation to rafts was temperature dependent; it occurred at 37°C, but not 4°C (Fig. 2A). Translocation of RPTPβ to lipid rafts after a 30-min incubation at 37°C occurred concurrent with a concentration-dependent increase of VacA in lipid rafts (Fig. 2B).

By confocal microscopy, colocalization of Cy3-labeled VacA

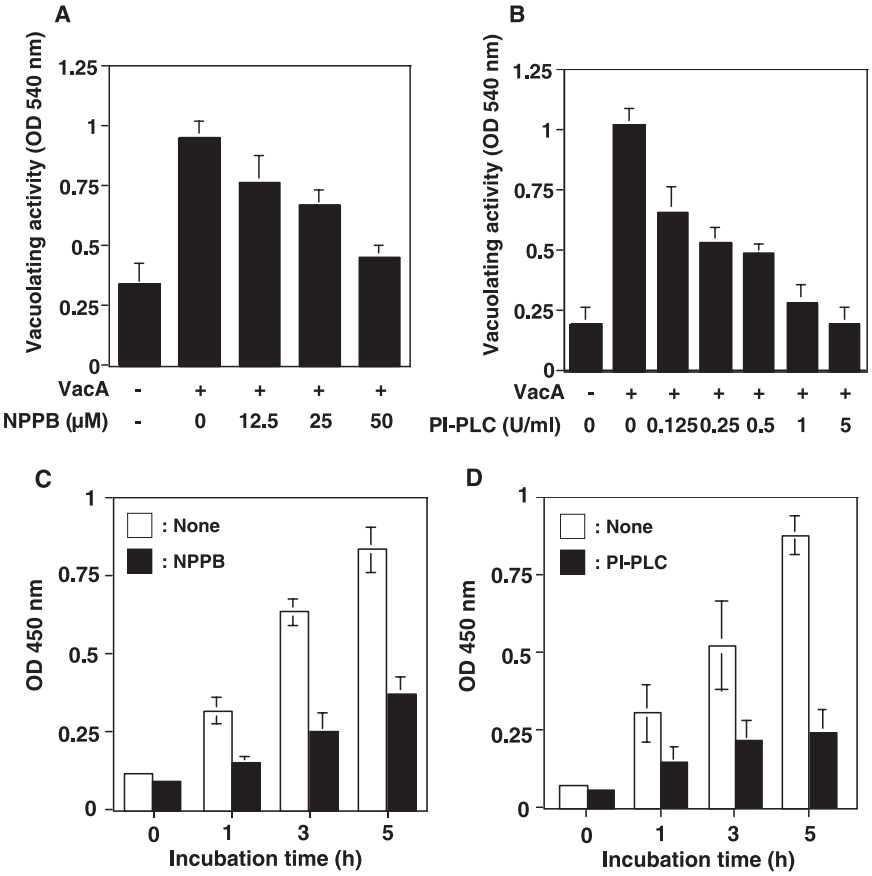

FIG. 6. Inhibition of VacA-induced vacuolation and its internalization by NPPB and PI-PLC. AZ-521 cells were incubated with or without the indicated concentrations of NPPB for 30 min (A) or of PI-PLC for 1 h (B) at 37°C, before incubation with 120 nM VacA for 5 h at 37°C for the indicated time and measurement of vacuolation. AZ-521 cells were incubated with (■) or without (□) 50 μM NPPB for 30 min (C) or with PI-PLC (1 U/ml) for 1 h (D) at 37°C, followed by incubation with VacA-SS-biotin (120 nM) at 37°C for 1, 3, or 5 h. After incubation with 0.25% glutaraldehyde followed by treatment with 2-mercaptoethanesulfonic acid, internalized VacA labeled with biotin was detected with avidin-HRP. Data are mean ± standard error of values from triplicate experiments. OD, optical density.

with RPTPβ in non-lipid raft microdomains of cell membrane was observed. Incubation at 37°C induced accumulation of VacA colocalized with RPTPβ in lipid rafts. Indeed, in AZ-521 cells treated with 120 nM Cy3-labeled VacA at 4°C for 30 min, colocalization of VacA with c-Src was undetectable on the cell membrane, whereas VacA and RPTPβ were colocalized on membranes including non-lipid rafts as well as lipid rafts (Fig. 3A and B). However, after incubation at 37°C for 30 min, colocalization of VacA with RPTPβ and c-Src was primarily detected on the plasma membrane, in particular, in lipid rafts (Fig. 3C and D).

Implication of lipid rafts in VacA-induced vacuolation and VacA internalization, but not binding activity of VacA in AZ-521 cells. Treatment of Hep-2 (human larynx carcinoma) cells with nystatin (35) or HeLa (human cervix carcinoma) cells with MCD (32, 36) strongly interfered with the internalization or intracellular localization of VacA, resulting in a reduction of VacA-induced vacuolation, suggesting that VacA associated with sphingolipid- and cholesterol-enriched microdomains, followed by cellular vacuolation. There is, however, no direct evidence of the physical interaction of VacA receptor RPTPβ with VacA in lipid rafts and where VacA associates with its receptor. Therefore, we determined whether lipid rafts were involved with VacA binding, internalization, and vacuole formation in AZ-521 cells. AZ-521 cells were treated with MCD at the indicated concentration before incubation with 120 nM VacA. Vacuolation of AZ-521 cells was significantly decreased by prior treatment of cells with MCD in a concentration-dependent manner (Fig. 4A). Cells incubated with 5 mg/ml MCD did not show cellular vacuolation, and about 75% of treated cells were alive (data not shown). Furthermore, this decrease in vacuolating activity was not associated with a decrease in VacA binding to AZ-521 cells treated with MCD (Fig. 4B). In contrast, the internalization of VacA in MCD-treated AZ-521 cells was significantly inhibited compared to that in untreated cells (Fig. 4C). These data indicate that VacA associates with lipid raft microdomains and suggest that association of the toxin with lipid rafts is important for VacA-induced vacuolation in AZ-521 cells. Under these conditions, disruption of lipid rafts by MCD (Fig. 5A and B) also results in inhibition of VacA and RPTPβ translocation from non-lipid raft microdomains to lipid rafts (Fig. 5C and D). Thus, the lipid raft was essential for translocation of VacA and RPTPβ, but not for VacA cell-surface binding. Our conclusion is consistent with the inhibitory effect of cholesterol-depleting agents (MCD and hydroxypropyl-β-cyclodextrin), but not sterol-binding

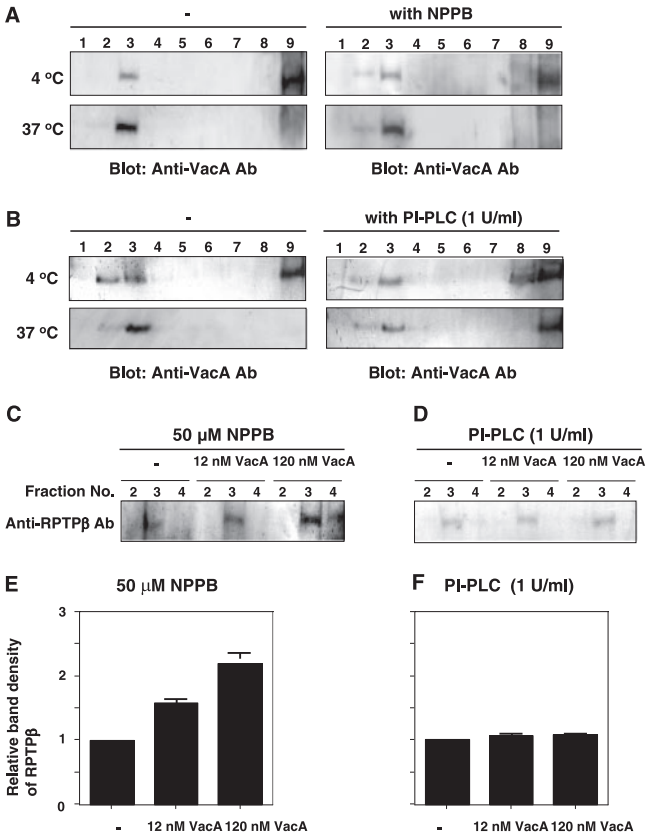

FIG. 7. Effect of NPPB and PI-PLC on recruitment of VacA and RPTPβ in lipid rafts. To determine effects on VacA localization, AZ-521 cells were incubated with or without 50 μM NPPB for 30 min (A) or 1 U/ml PI-PLC for 60 min (B) at 37°C before incubation with 120 nM VacA for 30 min at 4°C or 37°C. To determine effects on RPTPβ localization, AZ-521 cells were incubated with or without 50 μM NPPB (C) or 1 U/ml PI-PLC (D) before incubation with 12 nM or 120 nM VacA for 30 min at 37°C. After incubation with VacA, the cells were washed with PBS and lysed in 450 μl of 25 mM MES buffer containing 1% Triton X-100. Lysates were fractionated and analyzed by discontinuous sucrose density gradient ultracentrifugation. After centrifugation, 0.5-ml fractions were collected from the top of the gradients. Fractions containing lipid rafts were subjected to SDS-PAGE and Western blotting using anti-VacA (A and B) or anti-RPTPβ antibodies (Ab) (C and D). Relative densities of RPTPβ and VacA as determined by densitometry scan analysis (E and F) were compared to densities obtained without VacA. Data are mean ± standard error of values from triplicate experiments.

agents (filipin, saponin, digitonin, and nystatin), on VacA internalization and vacuolation in HeLa cells (32).

Effects of NPPB and PI-PLC treatments on VacA and RPTPβ translocation to lipid rafts. Both NPPB (39) and PI-PLC (23, 29, 35) abolished VacA internalization and subsequent vacuolation by HeLa cells. In AZ-521 cells, NPPB and PI-PLC reduced VacA-induced vacuolation in a concentration-dependent manner (Fig. 6A and B), consistent with the inhibition of VacA internalization by NPPB or PI-PLC (Fig. 6C and D). There was a clear difference between NPPB- and PI-PLC-treated cells in translocation of VacA and RPTPβ to lipid rafts (Fig. 7). NPPB did not alter translocation of VacA and RPTPβ to lipid rafts, whereas PI-PLC inhibited it, suggesting that GPI-anchored proteins in AZ-521 cells were critical for the accumulation of VacA in lipid rafts from non-lipid raft microdomains. In addition, following PI-PLC treatment of AZ-521 cells, internalization of VacA was inhibited and vacuolation reduced (Fig. 8). It appears that the accumulation of VacA in lipid rafts is significant for internalization and vacuolating activity of VacA.

Effects of MCD, NPPB, and PI-PLC treatments on VacA-induced p38 MAP kinase/ATF-2 activation. VacA-induced activation of p38 MAP kinase/ATF-2-mediated signal pathway is independent of cellular vacuolation, decrease in mitochondrial membrane potential, or cytochrome *c* release from mitochondria caused by VacA (28). Therefore, we examined whether translocation of VacA to lipid rafts was involved in p38 MAP kinase signaling pathway. AZ-521 cells were treated with NPPB or PI-PLC prior to incubation with VacA, and then activation of p38 MAP kinase and ATF-2 was determined by Western blotting using specific antibodies against phospho-p38 and ATF-2. MCD and PI-PLC inhibited VacA-induced p38 MAP kinase as well as ATF-2 phosphorylation in a concentration-dependent manner, whereas NPPB did not (Fig. 9). Taken together with the findings shown in Fig. 6, the inhibitory effect of PI-PLC treatment on VacA-induced p38 MAP kinase activation was due to disruption of VacA/RPTPβ translocation to lipid rafts of AZ-521 cells. Thus, release of GPI-anchored proteins from the cell surface inhibited VacA translocation, p38 MAP kinase/ATF-2 activation, and vacuolation. NPPB inhibited internalization and vacuolation, but not translocation to lipid rafts and the p38 MAP kinase/ATF-2-mediated signal pathway (Fig. 10).

DISCUSSION

Data from multiple lines of evidence are consistent with the conclusion that VacA interaction with RPTPβ is responsible for cell vacuolation in AZ-521 cells (42) and phorbol myristate acetate-treated HL-60 cells (30) as well as effects on signal transduction pathways such as activation of p38 MAP kinase/ATF-2 cascade in AZ-521 cells via a mechanism different from that leading to vacuolation (28). Like other bacterial protein toxins, VacA seem to utilize lipid rafts or raft-associated GPI-anchored proteins to intoxicate cells. In this study, we investigated the importance of lipid rafts and GPI-anchored proteins in the multiple steps (e.g., binding to RPTPβ) and signaling pathways (e.g., phosphorylation of p38 MAP kinase and ATF-2) involved in VacA action.

The plasma membrane of cells comprises patches and microdomains or lipid rafts, which are enriched in glycosphingolipids and cholesterol and have been implicated in cellular processes such as membrane sorting and signal transduction (26). Lipid molecules in the plasma membrane are not homogeneously distributed, but are arranged in patches as a result of association of sphingolipid and cholesterol in lipid rafts, to which specific membrane proteins become incorporated. Our results suggest that VacA binds RPTPβ in nonraft regions of the plasma membrane and the VacA-RPTPβ complex becomes concentrated in lipid rafts (Fig. 2 and 3). Consistent with earlier reports (32, 35, 36), lipid raft integrity is essential for VacA-induced vacuolation in AZ-521 cells, since cholesterol depletion with MCD and disruption of lipid rafts inhibited VacA-induced vacuolation (Fig. 4).

Similar to MCD treatment, incubation of AZ-521 cells with NPPB or PI-PLC resulted in significantly reduced VacA internal-

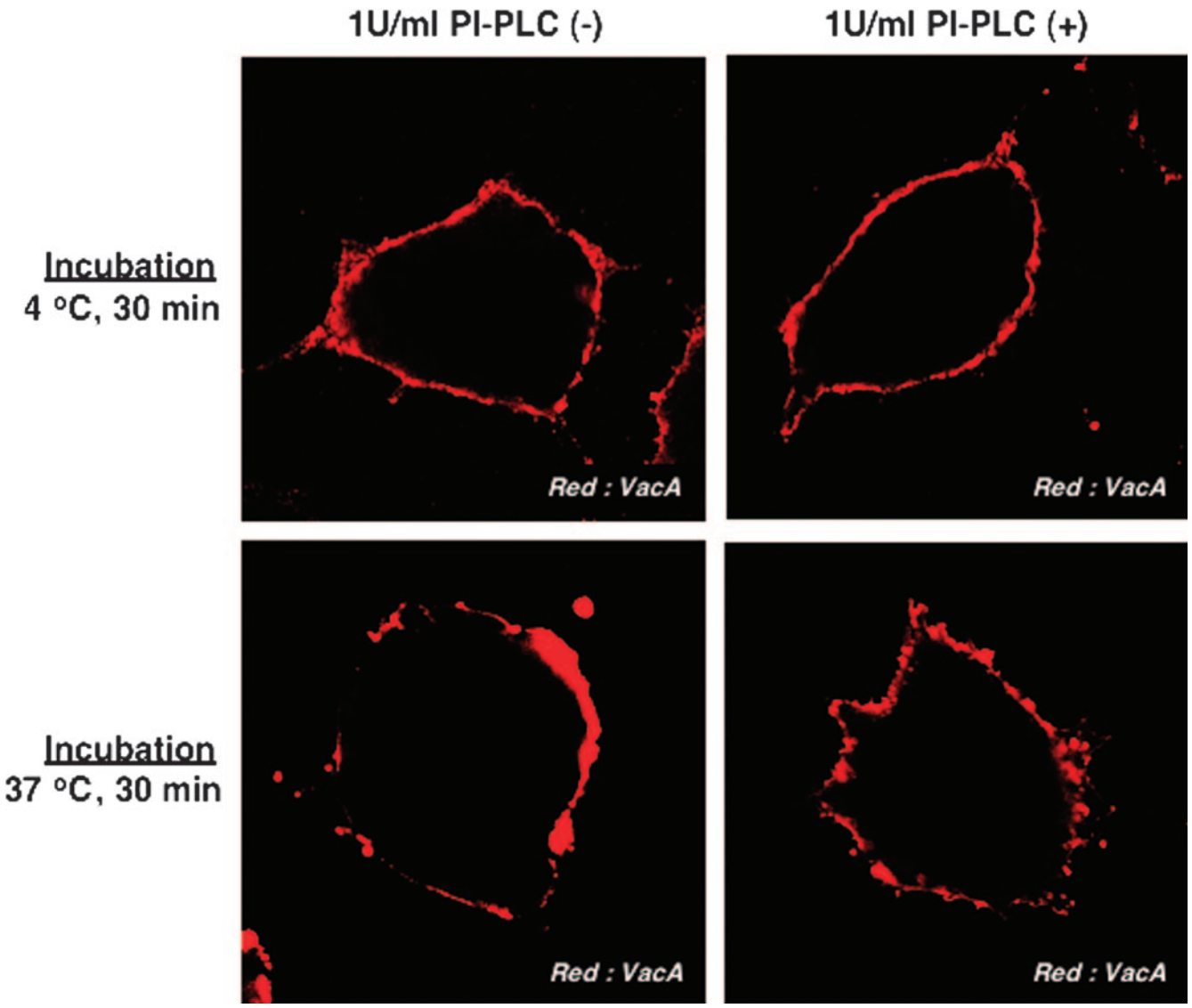

FIG. 8. Inhibitory effect of PI-PLC on VacA internalization in AZ-521 cells. AZ-521 cells which were treated with PI-PLC for 1 h before incubation with 120 nM VacA at 4°C or 37°C for 30 min as described in the text. After incubation, cells were incubated with anti-VacA antibodies, followed by incubation with Alexa Fluor 546-conjugated anti-rabbit IgG for 1 h. VacA (red) was detected by confocal microscopy. Data are representative of at least two experiments.

ization and vacuole formation (Fig. 6). However, PI-PLC decreased the VacA concentration in lipid rafts, whereas NPPB did not. To avoid unexpected effects of contaminating proteases in PI-PLC, we repeated this experiment in the presence of protease inhibitors and obtained the same result (data not shown). In AZ-521 cells, GPI-anchored proteins are required for VacA translocation to lipid rafts from non-lipid rafts, in addition to VacA-induced vacuolation and p38 MAP kinase activation (Fig. 7, 8, and 9). This finding supports an earlier observation that GPI-anchored proteins are required for formation of functional VacA channels at the cell surface and that endocytosis of these channels provokes vacuolation (17). In addition, it is likely that VacA may colocalize with GPI-anchored proteins in the cell peripheral endocytic compartments (16). GPI-anchored proteins were not required for VacA binding to RPTPβ; they apparently are necessary for translocation to lipid rafts and pore formation. PI-PLC treatment, which cleaves GPI-anchored proteins, releasing the extracellular domain from the cell surface, reduced VacA translocation to lipid rafts without inhibiting VacA binding to AZ-521 cells. Pore-forming toxins (aerolysin and hemolysin) from *Aeromonas hydrophila* (1) and *Aeromonas sobriae* (40) bind to and utilize GPI-anchored proteins (e.g., placental and intestinal alkaline phosphatases) associated with lipid rafts to facilitate channel formation (18). VacA uses a non-lipid raft protein, RPTPβ, in binding, but translocation of the complex to a lipid raft is necessary for further activity.

Previously, we demonstrated that VacA caused the phosphorylation of MAP kinases, p38 MAP kinase and Erk1/2, as well as ATF-2. Moreover, p38 MAP kinase and MKK3/6 were phosphorylated and activated in T cells after stimulation by VacA (3). From our data, it appears that movement of the VacA-RPTPβ complex to lipid rafts is sufficient to induce p38

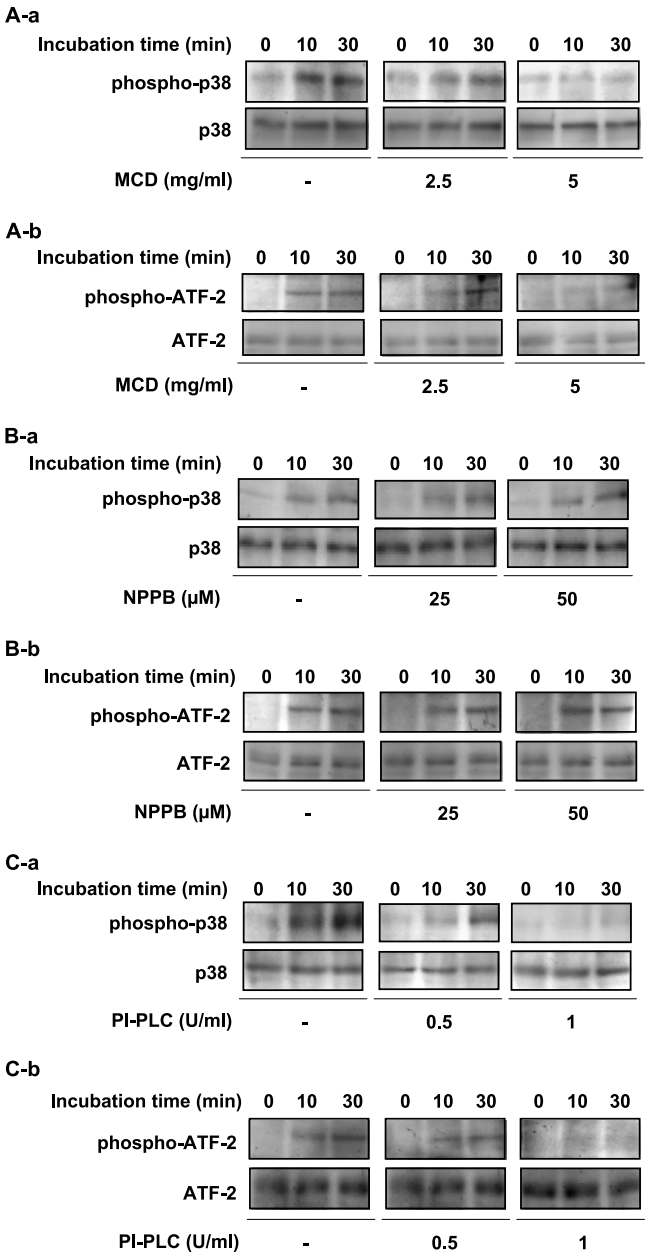

FIG. 9. Effect of NPPB and PI-PLC on VacA-induced p38 MAP kinase (p38) phosphorylation in AZ-521 cells. AZ-521 cells were incubated with 0, 2.5, and 5 mg/ml MCD for 1 h at 37°C (A), or 0, 25, and 50 μM NPPB for 30 min at 37°C (B) or with 0.5 and 1 U/ml PI-PLC at 37°C for 1 h (C) before addition of 120 nM VacA. After incubation for 10 and 30 min, the cells were solubilized, followed by SDS-PAGE in 10% gels and Western blotting using anti-p38, anti-phospho-p38, anti-ATF-2, or anti-phospho-ATF-2 antibody. Results are representative of three independent experiments.

activation in the absence of internalization or vacuolation. Thus, inhibition of VacA action by NPPB is selective and targets internalization and vacuolation.

Our results suggest that GPI-anchored proteins may serve to stabilize rafts and enable them to concentrate VacA. In agreement, a CHO-fasI line that constitutively expresses fasciclin I is more sensitive to VacA than wild-type CHO cells, consistent with

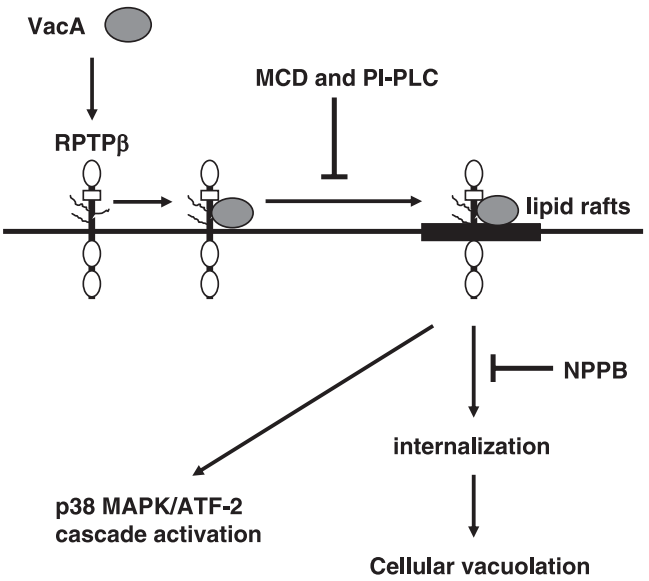

FIG. 10. Multiple actions of VacA on cell surface of AZ-521 cells. After clustering of VacA and RPTPβ in lipid rafts of AZ-521 cells, cytotoxic effects of VacA include alterations in the endocytic compartment resulting in vacuolation and stimulation of proinflammatory signaling, such as p38 MAP kinase/ATF-2 cascade activation. VacA-induced p38 MAP kinase/ATF-2 cascade activation, which is not inhibited by vacuolation inhibitor (NPPB), is dependent on the presence of GPI-anchored proteins in lipid rafts.

a role for GPI-anchored proteins in VacA activity (23). However, Schraw et al. (36) showed that VacA associates with lipid raft microdomains in the absence of GPI-anchored proteins, using CHO-LA1 mutant cells that are defective in production of GPI-anchored proteins. The discrepancy between CHO and AZ-521 cells in the function of GPI-anchored proteins in lipid rafts remains to be resolved. It appears that the lipid rafts may play an important role in VacA-induced vacuolation by concentrating the VacA-RPTPβ complex, serving as an efficient carrier to transport VacA to the cytosol of AZ-521 cells. Based on our data, however, translocation of VacA to lipid rafts, independent of internalization, is sufficient for activation of some signaling events.

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