Binding and internalization of *Helicobacter pylori* VacA via cellular lipid rafts in epithelial cells

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Abstract

In this study we investigated the roles of lipid rafts and glycosylphosphatidylinositol-anchored proteins (GPI-APs) in the process of VacA binding and internalization into epithelial cells. Vacuolating activity analysis in AGS, CHO cells, and a CHO-derived line that highly expresses GPI-linked fasI proteins indicated the significance of cholesterol and GPI-APs for VacA activity. Flow cytometric analysis along with VacA-cholesterol co-extraction experiments showed a cholesterol-dependent manner for VacA cell-binding activity, while GPI-APs were not related to it. Differential detergent extraction and fractionation in sucrose density gradient showed co-association of VacA and fasI with rafts on cell membranes. Subcellular distribution of fasI visualized by confocal microscope suggested that fasI trafficked via a newly defined endocytic pathway for GPI-APs in the derived line. Upon VacA intoxication, VacA was visualized to co-migrate along with fasI and finally induced vacuolation coupled with dramatic redistribution of fasI molecules. These results suggest that VacA exploits rafts for docking and entering the cell via the endocytic pathway of GPI-APs.

Keywords: VacA; GPI-AP; Lipid raft; Endocytosis; Fasciclin I; *Helicobacter pylori*

*Abbreviations: VacA, vacuolating toxin; GPI-AP, glycosylphosphatidylinositol-anchored protein; fasI, fasciclin I; MCD, methyl-β-cyclodextrin; PI-PLC, phosphatidylinositol-specific phospholipase C; DRM, detergent-resistant membrane; WGA, wheat germ agglutinin; CHX, cycloheximide; Tf, transferrin; RE, recycling endosome; GEEC, GPI-AP-enriched early endosomal compartment; PAGE, polyacrylamide gel electrophoresis; PK, proteinase K.

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activity by purified VacA and the process of VacA internalization into cells by confocal microscopic experiments.

Our biochemical experiments with purified VacA suggest that VacA associates with rafts in the cell-binding level and that GPI-APs might not be the primary receptors for VacA. Confocal microscopic analysis reveals that raft-associated fasI traffics along a newly defined recycling route for GPI-APs [12,13] and that VacA co-migrates with fasI in the process of binding and entering into perinuclear Golgi-localized regions. VacA-intoxicated cells then develop into large intracellular vacuoles surrounding with fasI and VacA. We interpret these findings in the context that VacA enters into cells by hijacking cellular functions of lipid rafts, particularly trafficking along the endocytic pathway for the delivery of GPI-APs [12,13].

Materials and methods

Reagents and antibodies. VacA was purified from the bacterial broth culture supernatants of H. pylori ATCC 49503 according to Wang et al. [14]. HRP-conjugated CTXB was purchased from Sigma. Oregon Green 488-conjugated wheat germ agglutinin (WGA), tetramethylrhodamine-conjugated WGA, and FITC-conjugated transferrin (Tf) were purchased from Molecular Probes. The polyclonal rabbit anti-VacA antisera were prepared with the recombinant VacA protein consisting of 33–856 amino acids of ATCC 49503 VacA. The anti-fasI monoclonal antibody 3B11 was a gift from K. Zinn. The Cy3-conjugated anti-rabbit antibody was purchased from Amersham Biosciences, and FITC-conjugated and Alexa Fluor 633-conjugated anti-mouse antibodies were from Molecular Probes.

Cell culture. AGS cells were grown in F-12 nutrient medium. CHO and CHO-fasI cells were grown in α-MEM with 10% fetal bovine serum (Hyclone) [11].

Vacuolating and VacA–cell binding assays. Cell vacuolating activity induced by VacA was determined by neutral red uptake assay [15]. VacA–cell binding activity was determined with 12 nM VacA at 4°C for 8 h, followed by flow cytometric assay [14].

DRM association assay. Cells (2 × 10^6) were lysed by 1% Triton X-100 in 10 μl TNE buffer (25 mM Tris–HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.5) at 4°C for 30 min. Cell lysates were adjusted to contain 40% sucrose in TNE buffer to a total volume of 200 μl, overlayed with 0.5 ml 16% sucrose, and centrifuged for 18 h at 38,000 rpm at 4°C in a RPS56T rotor (Hitachi). Fractions were collected from the top and assayed for GM1 [16], fasI, or VacA. The signals shown on films were further quantified by Computing Denstio meter (300E, Molecular Dynamics).

Cell labeling and other treatments. To visualize endosomal compartments, FITC-conjugated Tf was incubated with CHO-fasI cells at 50 μg/ml at 37°C for 1 h. FasI was stained with mouse anti-fasI mAb 3B11 (1:100) and with Alexa Fluor 633-conjugated or FITC-conjugated antimouse secondary antibody (1:200). Golgi apparatus was labeled with Oregon Green 488-conjugated or tetramethylrhodamine-conjugated WGA. VacA was labeled with a rabbit anti-VacA antibody and with Cy5-conjugated or Cy3-conjugated anti-rabbit secondary antibody. Cycloheximide (CHX) was used to inhibit protein synthesis in CHO-fasI cells (100 μg/ml CHX for indicated times at 37°C). To detect endocytosed fasI, CHO-fasI cells were labeled with mouse anti-fasI mAb 3B11 (1:100) on ice for 3 h, washed, and incubated at 37°C for indicated times. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 30 min. Cells were observed under confocal laser scanning microscope (Zeiss LSM 500) with the objective 100×1.3 oil.

Results and discussion

Association of VacA activity and fasI with lipid rafts

We first investigated the direct role of lipid rafts in intracellular vacuolating activity by purified VacA. Neutral red uptake assay revealed that AGS cells were highly sensitive to VacA, CHO cells were relatively unresponsive, and the derived CHO-fasI cells indeed were more sensitive to VacA as compared with parental cells [9] (Fig. 1A). Similar results were also found for another CHO derived line expressing Drosophila GPI-linked fasI (data not shown). Treatment of methyl-β-cyclodextrin (MCD) to sequester cholesterol from plasma membranes led to a specific decrease in VacA activity for all lines; PI-PLC digestion that removed GPI-APs from cell surface also specifically blocked VacA activity (Fig. 1A). We then analyzed VacA bind-
ing activities between CHO and derived CHO-fasI cells by flow cytometric analysis [17]. Unexpectedly, there were comparable VacA binding activities between the CHO and CHO-fasI cells (Fig. 1B). PI-PLC digestion did not reduce VacA binding activity. MCD treatment nevertheless resulted in a major drop for both lines (Fig. 1B), unlike that seen for aerolysin [18]. Moreover, cell-bound VacA could be extracted with MCD in a dose-dependent manner (Fig. 1C), indicating that VacA is associated with membranes via a cholesterol-dependent manner.

We then tested whether fasI was present in detergent-resistant membranes (DRMs) by a cold detergent extraction, followed by a sucrose density gradient centrifugation method [7]. GM1 and fasI were enriched in DRMs at the top lighter fractions (Fig. 2A), indicating that fasI is indeed a raft-associated molecule. When cells were incubated with VacA at 4°C for 3 h, VacA was also concentrated in DRMs. Similar results were also obtained when temperature was shifted to 37°C for 4 h (Fig. 2B), suggesting that cell-bound VacA was also concentrated in DRMs. The co-migration of fasI and VacA with DRMs suggests that both molecules associate with lipid rafts. However, no direct interaction between VacA and fasI could be found by using sandwich Western blot analysis (Fig. 2C), unlike that for aerolysin and α toxin, two channel-forming toxins [19].

Distribution of GPI-linked fasI between plasma membrane and perinuclear regions in CHO-fasI cells

Since fasI is a GPI-linked glycoprotein, we sought to test whether fasI recycled via a newly defined route for GPI-APs [12,13]. Confocal microscopic analysis of CHO-fasI cells revealed that fasI was distributed in two pools: one at the cell surface that had a punctate pattern at some locations, reminiscent of lipid rafts [20], and one in perinuclear compartments (Figs. 3a–c). A major fraction of fasI in the perinuclear region was colocalized with a Golgi probe, WGA, consistent with the high expression

![Fig. 2. Association of fasI and VacA with DRMs. (A) Isolation of GPI-linked fasI in DRMs. CHO-fasI cell lysates were subjected to fractionation in sucrose density gradient at 4°C. Each fraction was assayed for GM1 with HRP-conjugated CTXB (0.2 μg/ml) and fasI with anti-fasI antibodies, respectively, using dot blot analysis. (B) Coincidence of VacA and fasI in DRMs. CHO-fasI cells were incubated with 24 nM VacA at 37°C for 4 h prior to sucrose gradient fractionation. Each fraction was subjected to SDS–PAGE and Western blot analysis for VacA (open circles) and fasI (close circles), respectively. Inset: Western blot analysis of VacA distribution. (C) Sandwich Western blot analysis of VacA-fasI interaction. Samples were separated on a SDS–PAGE gel and blotted onto nitrocellulose paper. Blots were incubated with 24 nM VacA at 4°C for 8 h and stained with anti-VacA (upper panel) and anti-fasI (lower panel), respectively. Lane 1, CHO-fasI cell lysates; lane 2, CHO cell lysates; lane 3, purified fasI from CHO-fasI cells by PI-PLC treatment; lane 4, purified fasI in a secreted form [11]; and lane 5, purified VacA.](image1)

![Fig. 3. Distribution of GPI-linked fasI between the plasma membrane and perinuclear regions in CHO-fasI cells. CHO-fasI cells were treated in the absence (a–c) or presence (d–f) of 100 μg/ml CHX for 4 h at 37°C, then probed with specific markers for fasI (a, d) and Golgi (b, e) and viewed by confocal microscope. (g–i) CHO-fasI cells were treated with CHX for 8 h, incubated with FITC-Tf for 1 h (h), and then probed with specific markers for fasI (g) and Golgi (i) for confocal microscopic analysis. FasI is present in peripheral compartments (arrowheads) devoid of Tf but is localized with Tf in the perinuclear RE (open triangle). Golgi-localized fasI is hardly seen. Yellow in the merged images indicates colocalization (c, f, k). In (k) the framed region is magnified as shown in (l) with triple colors merged, indicating that fasI primarily colocalizes with RE in the perinuclear region. Bar, 10 μm.](image2)
of fasI. However, Golgi-localized fasI staining was greatly reduced with the CHX treatment for 4 h to inhibit protein synthesis (Figs. 3d–f). In the presence of CHX for 8 h, there was few Golgi-localized fasI fluorescence for a large proportion of cells (Figs. 3g, j, k). Instead, the perinuclear fasI staining was colocalized with internalized transferrin (Tf), a probe of recycling endosome (RE) (Figs. 3g–i). Moreover, a fraction of fasI remained segregated from Tf and WGA (Fig. 3i), suggesting that fasI was indeed endocytosed via GPI-AP-enriched early endosomal compartments (GEECs) as for several GPI-APs recently reported in Sabharanjak et al. [12].

Visualization of VacA internalization into CHO-fasI cells

We next explored the possibility whether VacA was endocytosed along with raft-associated fasI, since high sensitivity relied on GPI-APs and since VacA was endocytosed via a clathrin-independent pathway [9]. We first investigated the process of VacA internalization in CHO (Fig. 4a) and CHO-fasI cells (Figs. 4b–d) by confocal microscopic analysis. After incubation of VacA with CHO-fasI cells at 4°C for 3 h, VacA was clearly seen on the plasma membrane (Fig. 4b), displaying a pronounced raft-associated punctate pattern. Merged images showed that a portion of VacA was colocalized with fasI (denoted by arrows). When the temperature was shifted to 37°C, higher proportion of cell-bound VacA was colocalized with fasI within the first 2 h (Fig. 4c). Moreover, a certain fraction of VacA was internalized and migrated into the fasI-enriched perinuclear compartments (Figs. 4e–h). After 3–4 h at 37°C, a significant proportion of VacA was internalized into CHO-fasI cells (Fig. 4d). The bright fasI staining in perinuclear regions, however, essentially disappeared after 4 h at 37°C (Figs. 4i–l), analogous to that seen in cells with CHX treatment for 8 h (Figs. 3g–l). Instead, there were many intracellular vacuoles surrounding with fasI and VacA (Fig. 4d). For parental cells, much less VacA was internalized (Fig. 4a), even though there were comparable VacA binding activities at first (Fig. 1B). As a result, fewer vacuoles were seen after 4 h of intoxication at 37°C in parental cells, consistent with the results in Fig. 1A. By use of protease protection assay, a larger fraction of VacA was protected from proteinase K digestion in CHO-fasI cells than in parental cells (Fig. 4m), confirming that increasing amounts of VacA were internalized into CHO-fasI cells. These results collectively suggest that VacA co-migrates with fasI on rafts for efficient internalization and subsequently triggers cytotoxic activity that requires fusion of membranes from intracellular compartments like fasI-enriched perinuclear regions, hence altering the plasma membrane–Golgi distribution of fasI.

Fig. 4. Internalization of VacA by CHO and CHO-fasI cells. CHO (a) and CHO-fasI (b–d, e, i) were incubated with 24 nM VacA at 4°C for 3 h and were then shifted to 37°C for indicated times, and then probed with specific markers for fasI (a–d, e, i), VacA (a–d, e, i), and Golgi (e, i). Phase contrast was used in d to visualize vacuoles. In (e), the framed region is magnified as shown in (f–h). In (i), the framed region is magnified as shown in (j–l). Arrows point to colocalization of VacA and fasI. Bar, 10 μm. m, Analysis of VacA internalization by protease protection assay. CHO-fasI or CHO cells were incubated with 24 nM VacA at 4°C for 3 h and were then shifted to 37°C for 4 h (+) or kept at 4°C (–). Prior to immuno-detection of VacA, cells remained untreated or treated with 30 μg/ml proteinase K (PK) on ice for 20 min, followed by addition of 1 mM phenylmethysulfonyl fluoride.
Given these results, the likely mode of VacA action is that VacA binds to lipid rafts via a cholesterol-dependent manner without primary interaction with GPI-APs on the cell surface, migrates with GPI-APs-recruited rafts, and internalizes into cells via the endocytic pathway for delivering GPI-APs. The striking finding is that the steady-state distribution of GPI-linked fasI between the plasma membrane and the Golgi complex was essentially altered in VacA-intoxicated CHO-fasI cells that produced large intracellular vacuoles (Fig. 4j). An analogous disappearance of Golgi-co-localized fasI was seen in CHO-fasI cells treated with CHX for 8 h (Fig. 3g). Although it remains to be investigated, one possible mechanism is that VacA may affect an essential component of the relevant membrane trafficking machinery as it traffics along with fasI-associated rafts, hence inducing fusion of membranes coming from intracellular compartments including fast-enriched Golgi regions for the development of large vacuoles. In support of this view, rafts exhibit dynamic assemblies via selectively incorporating or excluding various molecules, which have been hypothesized to function in cellular signal transduction, membrane transport/trafficking, and membrane curvature [21,22]. The formation of an “immunological synapse” from the clustering of dispersed rafts in an activated T cells highlights the role of the dynamic nature in cellular functions [23]. In addition to clarifying VacA intoxication process, the sophisticated interactions among VacA, lipid rafts, and GPI-APs revealed in the present study might contribute to further comprehensions in VacA-induced alteration of raft-associated cellular functions.

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