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Helicobacter pylori Exploits Host Membrane Phosphatidylserine for Delivery, Localization, and Pathophysiological Action of the CagA Oncoprotein

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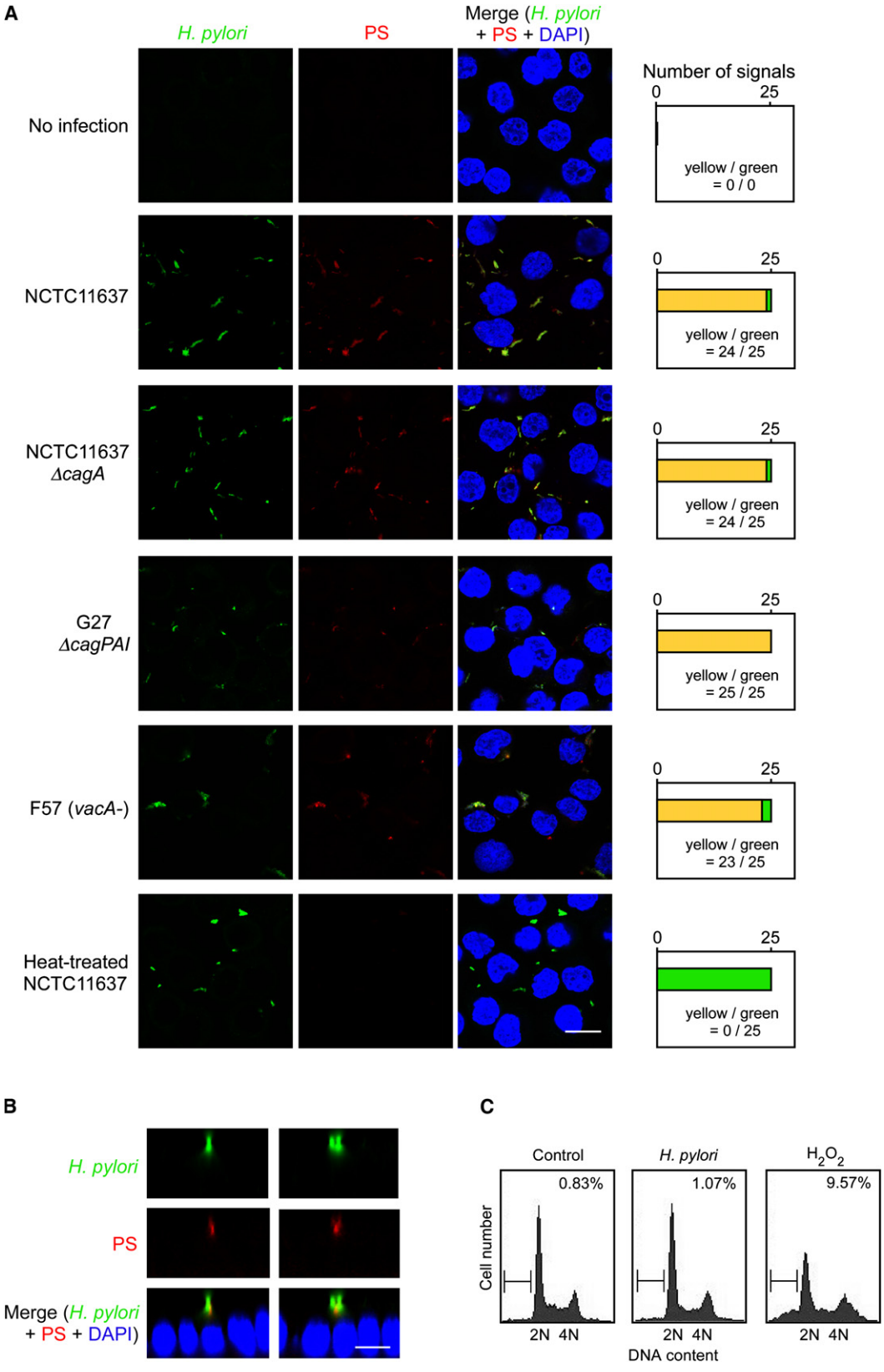
SUMMARY

When delivered into gastric epithelial cells via type IV secretion, *Helicobacter pylori* CagA perturbs host cell signaling and thereby promotes gastric carcinogenesis. However, the mechanisms of CagA delivery, localization, and action remain poorly understood. We show that direct contact of *H. pylori* with epithelial cells induces externalization of the inner leaflet enriched host phospholipid, phosphatidylserine, to the outer leaflet of the host plasma membrane. CagA, which is exposed on the bacterial surface via type IV secretion, interacts with the externalized phosphatidylserine to initiate its entry into cells. CagA delivery also requires energy-dependent host cell processes distinct from known endocytic pathways. Within polarized epithelial cells, CagA is tethered to the inner leaflet of the plasma membrane through interaction with phosphatidylserine and binds the polarity-regulating host kinase PAR1/MARK to induce junctional and polarity defects. Thus, host membrane phosphatidylserine plays a key role in the delivery, localization, and pathophysiological action of CagA.

INTRODUCTION

Infection with *Helicobacter pylori cagA*-positive strains is associated with the development of gastric carcinoma, the second-leading cause of cancer-related death worldwide (Peek and Blaser, 2002; Hatakeyama, 2008). The oncogenic bacterium delivers the *cagA* gene-encoded CagA protein into gastric epithelial cells via the *cag* pathogenicity island (PAI)-encoded type IV secretion system (TFSS) (Segal et al., 1999; Asahi et al., 2000; Backert et al., 2000; Odenbreit et al., 2000; Stein et al., 2000). Delivered CagA localizes to the inner surface of the plasma membrane, where it undergoes tyrosine phosphorylation at the C-terminal EPIYA motifs by host cell kinases (Hatakeyama, 2004; Backert et al., 2008). Tyrosine-phosphorylated CagA acquires the ability to specifically interact with and aberrantly activate SHP-2 tyrosine phosphatase, a bona fide oncoprotein whose mutations have been found in a variety of human malignancies (Higashi et al., 2002; Hatakeyama, 2004). CagA-deregulated SHP-2 elicits sustained Erk MAP kinase activation while inducing an extremely elongated cell shape known as the hummingbird phenotype (Higashi et al., 2004). CagA also binds and deregulates Csk kinase and Crk adaptor in a tyrosine phosphorylation-dependent manner and c-Met receptor kinase and Grb2 adaptor in a phosphorylation-independent manner (Tsutsumi et al., 2003; Suzuki et al., 2005; Churin et al., 2003; Mimuro et al., 2002). Furthermore, CagA interacts with partitioning-defective 1 (PAR1)/microtubule affinity-regulation kinase (MARK) in a tyrosine phosphorylation-independent fashion and inhibits the kinase activity, resulting in induction of junctional and polarity defects (Amieva et al., 2003; Bagnoli et al., 2005; Saadat et al., 2007). Hence, the bacterial protein functionally mimics mammalian scaffolding/adaptor proteins and thereby manipulates host signaling molecules to promote gastric carcinogenesis (Peek, 2005; Hatakeyama, 2008). Consistent with this notion, CagA activities reported to date are strictly dependent on the ability of CagA to localize at the inner surface of the plasma membrane in the host cells (Higashi et al., 2002, 2005; Bagnoli et al., 2005; Saadat et al., 2007). Oncogenic potential of CagA was recently confirmed by a study showing that transgenic mice systemically expressing CagA spontaneously develop gastrointestinal and hematological malignancies (Ohnishi et al., 2008).

The presence of TFSS in *H. pylori* was first revealed by the homology of *cag* PAI-encoded proteins to basic components of an archetypal TFSS, the *Agrobacterium tumefaciens* VirB/D4 system (Christie et al., 2005). TFSS is a supramolecular protein assembly that constitutes a substrate translocation channel spanning the inner and outer membranes of Gram-negative bacteria and a pilus-like structure protruding from the bacterial surface (Christie et al., 2005; Fronzes et al., 2009). Electron microscopic analyses have indicated that TFSS of *H. pylori* is present in the form of needle-like projections, which may serve as a conduit for CagA transport (Rohde et al., 2003). Alternatively, CagA might move along the outer surface of the TFSS needle to the tip of the pilus before delivery into the host cells (Rohde et al., 2003). A recent study has also


A
H. pylori
PS
Merge (H. pylori + PS + DAPI)
Number of signals
No infection
NCTC11637
NCTC11637 ΔcagA
G27 ΔcagPAI
F57 (vacA-)
Heat-treated NCTC11637
0
25
yellow / green = 0 / 0
yellow / green = 24 / 25
yellow / green = 24 / 25
yellow / green = 25 / 25
yellow / green = 23 / 25
yellow / green = 0 / 25
B
H. pylori
PS
Merge (H. pylori + PS + DAPI)
C
Control
H. pylori
H2O2
0.83%
1.07%
9.57%
Cell number
2N 4N
DNA content

shown that the interaction of CagL, a pilus component of *H. pylori* TFSS, with integrins $\alpha5/\beta1$ is crucial for the translocation of CagA into host cells (Kwok et al., 2007). However, molecular mechanisms through which CagA transverses across the plasma membrane remain poorly understood. Also, little is known about the mechanism by which delivered CagA is tethered to the inner surface of the plasma membrane, where it acts as a scaffolding/adaptor.

In eukaryotic cells, the outer leaflet of the plasma membrane is enriched with choline-containing phospholipids and glycosphingolipids, whereas the inner leaflet is enriched with amine-containing phospholipids such as phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidic acid (PA), and phosphatidylinositol (PI) (Daleke and Lyles, 2000). Recent studies have shown that asymmetric distribution of the membrane phospholipids plays an important role in signal transduction (Lemmon, 2008). When cells begin to undergo apoptosis, a collapse of lipid asymmetry occurs with concomitant exposure of PS at the outer leaflet of the plasma membrane, which serves as an "eat me" signal for phagocytes (Zwaal et al., 2005). A transient and reversible PS externalization, which is induced by a nonapoptotic signaling, has also been demonstrated in various cellular situations (Bevers et al., 1983; Goth and Stephens, 2001; Gautier et al., 2003; Mercer and Helenius, 2008; Soares et al., 2008).

In this work, we found that physical interaction of *H. pylori* CagA with host membrane PS, which is aberrantly externalized at the site of *H. pylori* attachment, plays a key role in the delivery, intracellular localization, and pathophysiological activity of CagA.

RESULTS

Exposure of PS by *H. pylori*

Several obligate and facultative intracellular bacteria can induce rapid and transient PS exposure upon infection (Goth and Stephens, 2001). Given this, we sought to investigate whether infection with *H. pylori* is also capable of inducing nonapoptotic PS externalization in host cells. To test this possibility, AGS human gastric epithelial cells or Madin-Darby canine kidney (MDCK) cells were infected by *H. pylori* and stained with annexin V (a probe of externalized PS) or propidium iodide (a measure of cell death). The results of the experiment revealed the appearance of PS on the outer leaflet of the host plasma membrane, specifically at sites of *H. pylori* attachment (Figures 1A and 1B). As previously reported, *H. pylori* preferentially adhered to the tight junctions during infection with polarized MDCK cells (Figure S1A) (Amieva et al., 2003), where externalized PS was specifically detected (Figure 1B). Surface exposure of PS by *H. pylori* was rapid and transient, with no overt signs of apoptosis as inferred by propidium iodide staining (Figure 1C). PS externalization required infection with viable *H. pylori*, but was independent of CagA, *cag* PAI, or VacA (Figure 1A).

Transiently externalized PS can serve as a portal of cell entry of macromolecules via endocytosis (Kenis et al., 2004). We thus postulated that *H. pylori*-triggered PS externalization is associated with the translocation of CagA into host cells. To test this hypothesis, we examined the effect of treatment with annexin V or anti-PS antibody on CagA delivery during *H. pylori* infection. To quantitate the CagA protein delivered into host cells, *H. pylori*-infected cells were extracted with phosphate-buffered saline (PBS)/0.1% saponin, which lyses mammalian cells but not bacteria (Figure S2A) (Odenbreit et al., 2000). The results of the experiments revealed that the level of CagA translocation was significantly decreased upon treatment of cells with annexin V or anti-PS antibody (clone 1H6) (Figures 2A, S2B, and S2C). Hence, delivery of CagA by *H. pylori* is impaired when surface-exposed PS is concealed by annexin V or anti-PS antibody. Incomplete inhibition of CagA delivery may be due to insufficient amount of annexin V or anti-PS antibody used. Indeed, a dose-response experiment showed that the inhibition of CagA delivery by annexin V was yet to be saturated within the concentrations that we examined (Figure S2D). However, partial inhibition could also be due to multivalent binding of the bacterium to the cell surface, which might override the ability of annexin V or antibody to compete. Also notably, whereas both annexin V and anti-PS antibody (1H6) efficiently bind to PS, their lipid-binding activities are not strictly specific to PS. The result, therefore, does not formally exclude the possibility that other minor phospholipids, which are also recognized by annexin V or anti-PS antibody (1H6), cooperate with PS during the entry process of CagA. Treatment of cells with an anti-CagA antibody also reduced the level of CagA delivered into host cells (Figures 2B and S2C). The results indicated that CagA is exposed on the bacterial surface or released from the bacterium and that the exposed or released CagA is delivered into host epithelial cells via a mechanism that utilizes externalized PS. Because CagA was not detectable in the supernatant of *H. pylori* culture (data not shown), it was unlikely that large amounts of CagA proteins were secreted from *H. pylori* into the culture. On the other hand, electron microscopic analysis using immunogold-labeled anti-CagA antibody showed the presence of CagA on the surface of *H. pylori* (Figure 2C). Although CagA has been reportedly localized to pilus-like structures on the surface of *H. pylori* (Rohde et al., 2003; Kwok et al., 2007), such structures were not observed in our electron microscopic study. We suspect that the pilus-like structures were fragile and were destroyed during fixation processes that we employed for sample preparation.

Figure 1. Externalization of PS by *H. pylori* Infection

(A) Confocal x-y plane views of *H. pylori* (green, anti-*H. pylori* antibody), PS (red, annexin V), and nuclear (blue, DAPI) staining. AGS cells were uninfected or infected with the indicated *H. pylori* strains for 3 hr at a multiplicity of infection (moi) of 100. Scale bar, 10 μm. For the quantitation study of colocalization, 25 green signals were arbitrarily chosen without any selection bias and the number of red signals colocalized with the green signals was counted. Yellow indicates colocalization of green (*H. pylori*) and red (PS) signals (bar graphs in right panels). The results were reproducible in three independent experiments.

(B) Confocal x-z plane views of *H. pylori* (green), PS (red), and DAPI (blue) staining. Polarized MDCK cells were infected with *H. pylori* NCTC11637 strain for 3 hr at a moi of 200. Scale bar, 10 μm.

(C) DNA histogram of AGS cells with or without *H. pylori* infection using propidium iodide staining. AGS cells were uninfected or infected with *H. pylori* NCTC11637 strain for 3 hr at a moi of 100. H_2O_2 was used to induce apoptosis as a control.

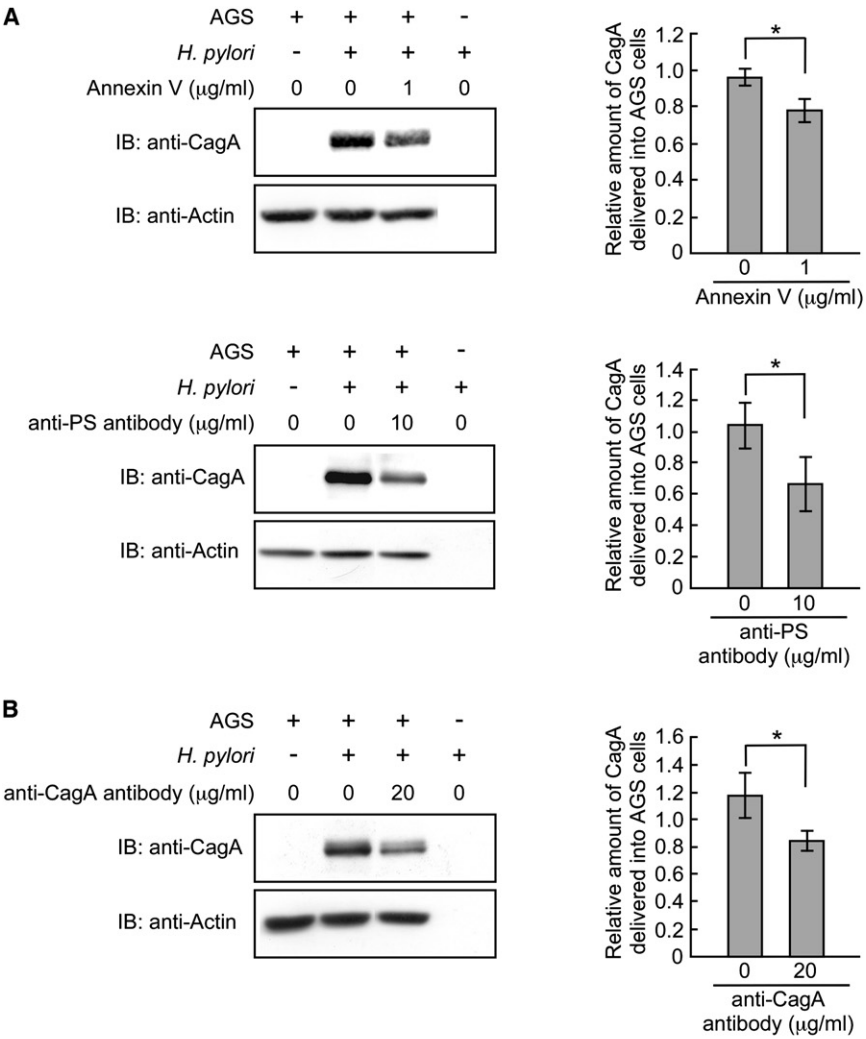


Figure 2. Role of PS in CagA Delivery

(A) AGS cells were uninfected or infected with *H. pylori* NCTC11637 strain for 2 hr at a moi of 100 in the presence or absence of annexin V (upper) or anti-PS antibody (lower). Cells were resuspended in PBS/0.1% saponin, and protein extracts were subjected to immunoblotting with the indicated antibodies. Error bars, ±SD (n = 3); *p < 0.05, Student's t test.

(B) AGS cells were uninfected or infected with *H. pylori* NCTC11637 strain for 2 hr at a moi of 100 in the presence or absence of anti-CagA antibody. Cells were resuspended in PBS/0.1% saponin, and protein extracts were subjected to immunoblotting with the indicated antibodies. Error bars, ±SD (n = 3); *p < 0.05, Student's t test.

(C) AGS cells were infected with *H. pylori* NCTC11637 strain (left) or NTCT11637-derived *ΔcagA* isogenic strain (right) for 5 hr at a moi of 100 and then subjected to immunoelectron microscopy. Arrows indicate CagA on the surface of *H. pylori*. Scale bar, 200 nm.

CagA-PS Interaction

The above-described observations raised the possibility that CagA, which is exposed on the bacterial surface via TFSS, directly interacts with PS that is aberrantly externalized at the plasma membrane of host cells upon *H. pylori* infection. To investigate this idea, we set out to determine whether CagA can directly bind phospholipids, especially PS. To do so, recombinant CagA protein was prepared and overlaid onto nitrocellulose membrane filters spotted with 100 pmol of each phospholipid. As shown in Figure 3A, full-length CagA (ABC-type CagA derived from *H. pylori* 26695 strain) strongly bound to PS. Full-length CagA also bound to PI, although the interaction was much weaker than the CagA-PS interaction (Figure 3A). Like full-length CagA, the N-terminal CagA fragment (residues 1–876), CagA-NT, strongly bound to PS, and the CagA-PS interaction was inhibited by excess PS (Figure 3A and data not shown). Hence, CagA specifically interacts with PS via the N-terminal CagA region (residues 1–876). Notably, CagA-NT acquired the ability to bind to PI3-phosphate (PI3P), PI4-phosphate (PI4P), and PI5-phosphate (PI5P), though much less efficiently compared to the interaction between CagA-NT and PS (Figure 3A). This observation indicated that the artificial deletion of the C-terminal region reduces CagA specificity to phospholipids, enabling interaction of CagA-NT with phospholipids other than PS. In contrast to CagA-NT, the C-terminal CagA fragment (residues 877–1186), CagA-CT, gave only faint signals for several phospholipids, which were much weaker than that generated by the interaction of full-length CagA or CagA-NT with PS (Figure 3A). Since phospholipids are hydrophobic while negatively charged, such weak signals may be due to nonspecific protein-phospholipid interactions through a hydrophobic bond or ionic bond.

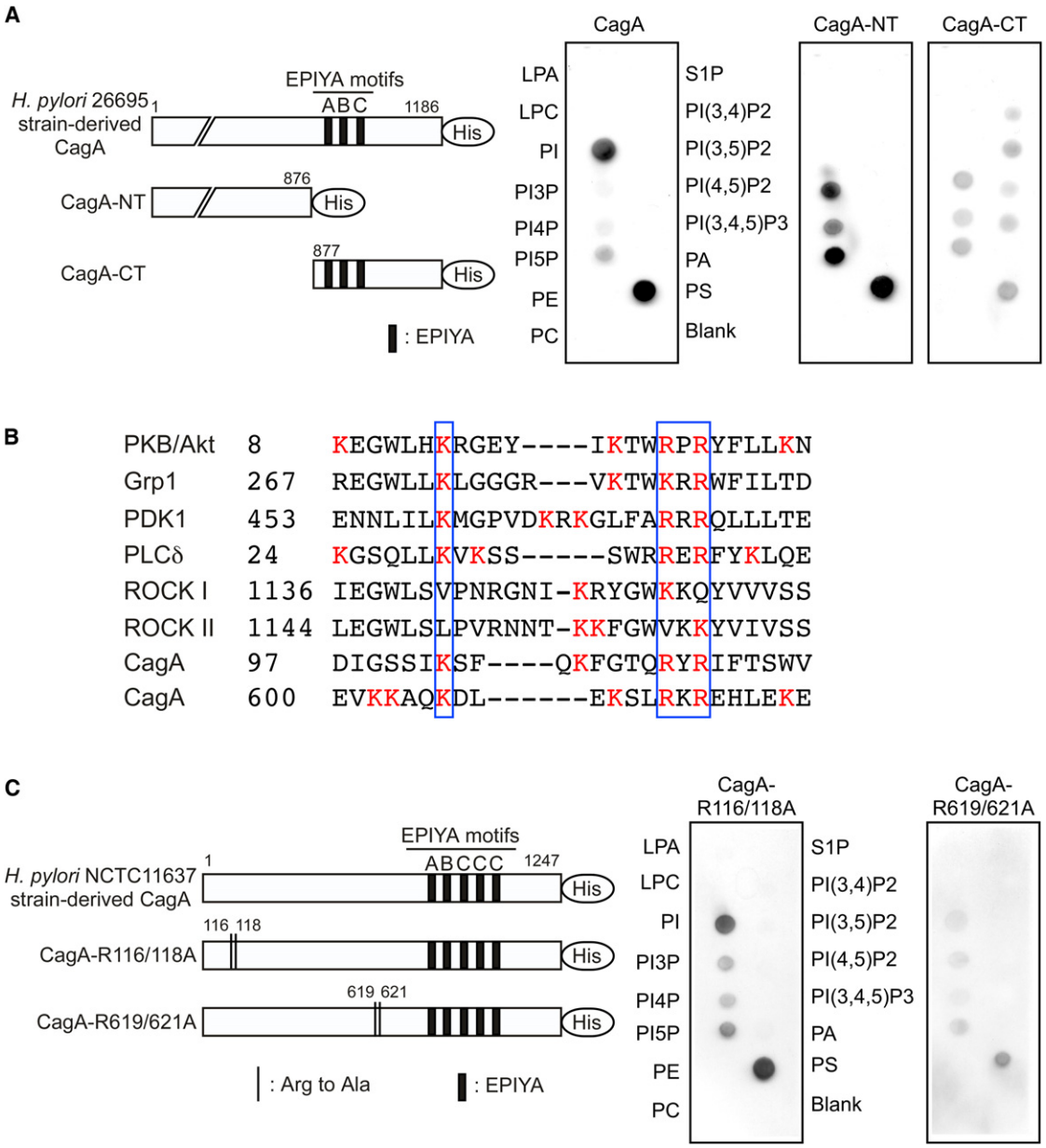


Figure 3. Direct Binding of CagA to PS

(A) Schematics of *H. pylori* 26695 strain-derived CagA (ABC-type CagA) and its derivatives, CagA-NT and CagA-CT. His indicates 6xHistidine epitope-tag (left). Lipid-binding assay on filters was performed using recombinant CagA proteins. The three filters were treated simultaneously and the filter exposure times were the same (right).

(B) Comparison of K-Xn-K/RXR-containing sequences in CagA with those of several PH domains. Sequences of the PH domains (PKB/Akt, Grp1, PDK1, and PLCδ), the split PH domains (ROCK), and regions surrounding "K-Xn-K/RXR" motifs in NCTC11637-derived CagA were aligned. Conserved K/R residues are shown in red.

(C) Schematics of *H. pylori* NCTC11637 strain-derived CagA (ABCCC-type CagA) and its derivatives, CagA-R116/118A and CagA-619/621A. His indicates 6xHistidine epitope-tag (left). Lipid-binding assay on filters was performed using mutant CagA proteins. The two filters were treated simultaneously, and the filter exposure times were the same (right).

There are several protein domains that can bind phospholipids. Among those, pleckstrin homology (PH) domain is best known for its ability to bind acidic phospholipids, usually PI and/or PS (Lemmon, 2008). Canonical PH domain possesses a basic consensus motif (K-Xn-K/R-X-R) within the β1-β2 loop region, in which the basic side chains from lysine and arginine mediate interaction with most phosphate groups (Wen et al., 2008). Although CagA does not possess canonical PH domains, closer inspection of the sequences revealed that the CagA protein derived from the *H. pylori* NCTC11637 strain (ABCCC-type CagA) possesses two K-Xn-R-X-R motifs, R116 and R118 and R619 and R621 (Figure 3B). To investigate whether the

identified K-Xn-R-X-R motifs are involved in the CagA-PS interaction, we replaced each of the two R-X-R sequences with the A-X-A sequence and generated recombinant CagA-R116/118A and CagA-R619/621A proteins in *E. coli* (Figure S3). Using the recombinant CagA mutants, we performed a lipid-binding assay for phospholipids and found that whereas CagA-R116/118A bound PS like wild-type CagA, CagA-R619/621A lost the ability to bind PS (Figure 3C). These observations indicated that membrane-externalized PS serves as a specific receptor for CagA and that the CagA-PS interaction requires the K-Xn-R-X-R motif present in the central region of CagA.

To substantiate the importance of the CagA-PS interaction in CagA delivery by *H. pylori*, we generated NCTC11637-derived isogenic *H. pylori* mutants that produce HA-tagged wild-type CagA (strain HA-WT-CagA-1) and HA-tagged CagA-R619/621A (strains HA-R619/621A-1 and HA-R619/621A-2) by homologous recombination (Figure 4A). The saponin-extraction experiment revealed that infection of AGS cells with strain HA-R619/621A-1 or HA-R619/621A-2 failed to deliver the CagA-R619/621A mutant into gastric epithelial cells (Figure 4B, upper). Antiphosphotyrosine immunoblotting analysis, which specifically detects the CagA proteins that have been tyrosine phosphorylated by host cell kinases, confirmed that CagA-R619/621A was not delivered into host cells from the isogenic *H. pylori* strains producing the mutant CagA (Figure 4B, lower left). To rule out the possibility that the impaired CagA delivery was due to a defect in TFSS, we tested the ability of the *H. pylori* strains to induce IL-8 secretion. Induction of IL-8 in host cells was almost comparable among the isogenic *H. pylori* strains producing wild-type and mutant CagA species (Figure 4B, lower right), indicating that they have functional TFSS (Fischer et al., 2001). These observations indicated that interaction of CagA with PS is critically involved in the delivery of CagA into the host cell.

Requirement of Active Host Cell Processes for CagA Delivery

PS externalization is reportedly accompanied by increased membrane permeability (Bouchier-Hayes et al., 2008). Accordingly, we postulated that the CagA-PS interaction on the plasma membrane activates host cell machineries that mediate CagA delivery into cells. Consistent with this idea, treatment of host cells with vanadate, which inhibits ATPases, or depletion of ATP from host cells prior to infection with *cagA*-positive *H. pylori* substantially reduced the level of CagA translocation (Figures 5A, 5B, S4A, and S4B). Also, depletion of membrane cholesterol reduced delivery of CagA into host cells by *H. pylori*, as previously described (Figures 5C and S4C) (Lai et al., 2008). Furthermore, latrunculin B, which binds monomeric actin and thereby inhibits actin polymerization that mediates endosomal trafficking, impaired CagA delivery into host cells (Figures 5D, S4D, and S4F). These observations collectively indicated that CagA is delivered into host cells by a mechanism that is dependent on host cell machineries.

Endocytosis is a basic cellular process used to internalize a variety of molecules in both physiological and pathological conditions (Tréhin and Merkle, 2004; Mayor and Pagano, 2007; Doherty and McMahon, 2009). Accordingly, we next investigated the possible involvement of endocytic processes in CagA delivery. Mechanistically, endocytosis can be divided into those that are clathrin dependent and those that are clathrin independent (Figure S4G) (Mayor and Pagano, 2007; Doherty and McMahon, 2009). Knockdown of the clathrin heavy chain (CHC) or AP-2 μ chain (AP50), which is critical for clathrin-dependent endocytosis, did not affect the translocation of CagA into host cells (Figure 5E). Hence, CagA delivery is independent of clathrin. Although clathrin-independent endocytic mechanisms are not well understood, they can be subdivided into four subtypes: RhoA-regulated, CDC42-regulated, Arf6-dependent, and caveolar-mediated mechanisms (Figure S4G) (Mayor and Pagano, 2007). Of these, the RhoA-regulated endocytosis and caveolar-mediated endocytosis also require dynamin. Inhibition of dynamin by a specific inhibitor dynasore or siRNA, however, did not affect CagA translocation (Figures 5F and S4E and data not shown). Likewise, knockdown of RhoA, CDC42, or Arf6 did not affect CagA internalization (Figures S4H–S4J). Inhibition of PAK1, a downstream effector of Rho family GTPases involved in certain types of endocytic processes (Doherty and McMahon, 2009), also failed to affect CagA internalization (Figure S4K). Furthermore, treatment of cells with amiloride, a specific inhibitor of macropinocytosis, an actin-dependent endocytic process used to internalize large amounts of fluid, had no effect on the translocation of CagA (Figures S4L–S4N). From these observations, we concluded that host cell processes involved in CagA delivery are distinct from known endocytic processes.

Mechanism of Intracellular Localization of CagA

After delivery into the host cells, CagA is localized to the inner leaflet of the plasma membrane. It was previously reported that the C-terminal EPIYA motif acts as a membrane-targeting signal of CagA in nonpolarized epithelial cells (Higashi et al., 2005). In contrast, in polarized epithelial cells, a CagA region that is distinct from the C-terminal EPIYA-containing region is thought to be important for the membrane association of CagA (Bagnoli et al., 2005; Saadat et al., 2007). To investigate the subcellular distribution of CagA-NT and CagA-CT, we expressed CagA-NT or CagA-CT in polarized MDCK cells and costained with PAR1b, which is membrane localized under polarized condition (Figure 6A). We also expressed CagA-NT or CagA-CT together with a fusion protein composed of green fluorescence protein and the C2 domain of lactadherin (GFP-Lact-C2), which specifically recognizes PS and therefore acts as a membrane probe in nonpolarized MDCK cells (Figure 6B) (Yeung et al., 2008). As a result, CagA-NT, which contains the N-terminal and central PS-binding regions (residues 1–876), was associated with the plasma membrane under a polarized condition, whereas it was localized to the cytoplasm under a nonpolarized condition (Figures 6A, 6B, and S5A). Conversely, CagA-CT, consisting of residues 877–1186, was present in the cytoplasm under a polarized condition, but was membrane associated under a nonpolarized condition (Figures 6A, 6B, and S5A). Hence, CagA employs at least two distinct mechanisms for membrane association, depending on the status of epithelial polarity. Since PS content represents up to 20%–30% of the total phospholipids in the inner leaflet of the plasma membrane (Lemmon, 2008), we postulated that PS is again utilized for the membrane tethering of CagA after delivery into polarized epithelial cells. To test this idea, we expressed

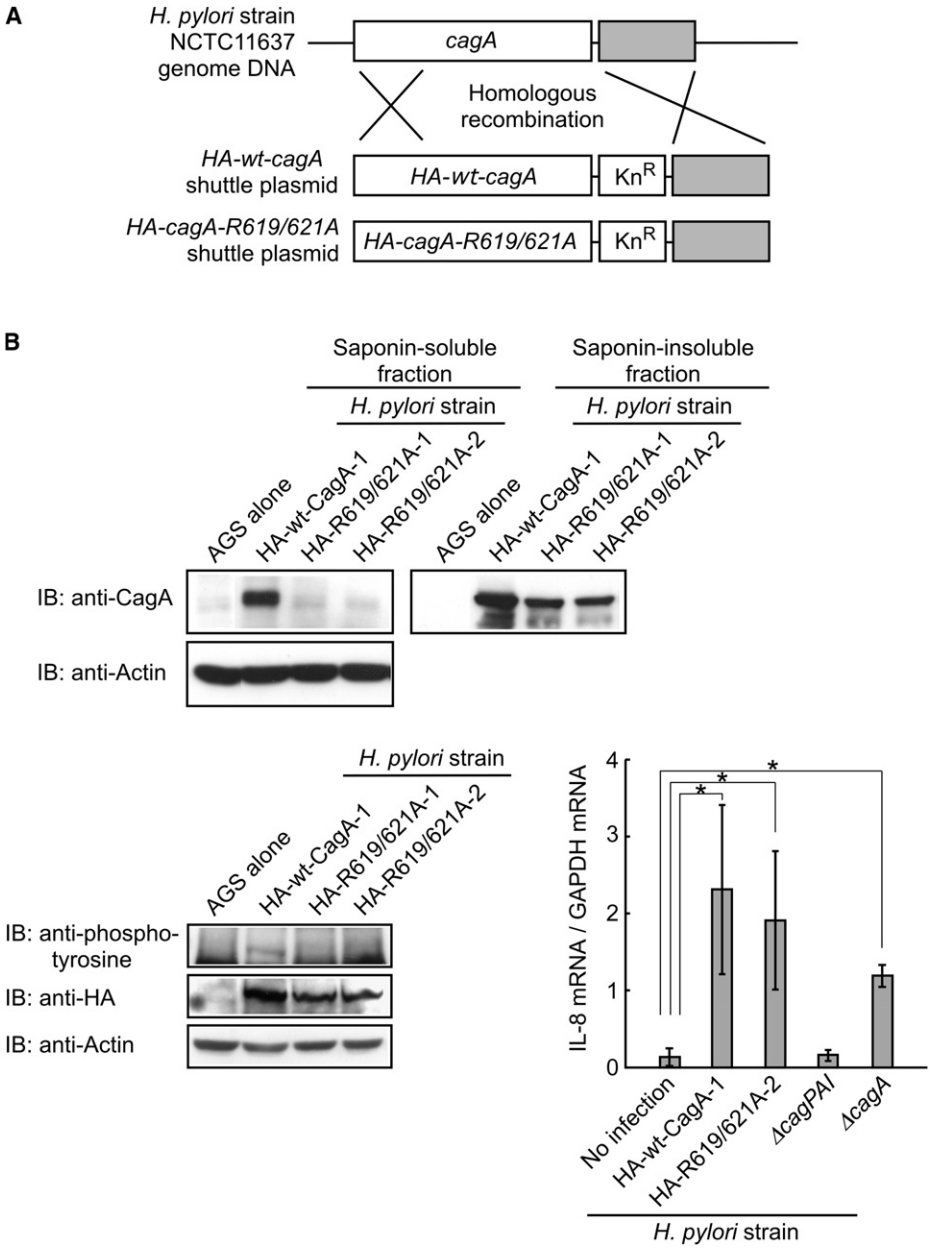


Figure 4. Role of CagA-PS Interaction in CagA Delivery

(A) Generation of *H. pylori* NCTC11637 isogenic strains carrying mutant *cagA* gene. Schematic view of targeting DNA constructs for hemagglutinin (HA) epitope-tagged wild-type CagA (HA-WT-CagA) and HA-tagged CagA-R619/621A (HA-CagA-R619/621A). Kanamycin-resistance gene (Kn^R) was used for the selection of recombinant strains.

(B) AGS cells were infected with an *H. pylori* NCTC11637 isogenic strain producing HA-WT-CagA (strain HA-WT-CagA-1) or HA-CagA-R619/621A (strains HA-R619/621A-1 and HA-R619/621A-2 generated independently) for 3 hr at a moi of 100. Host cell proteins were extracted with PBS/0.1% saponin and were subjected to immunoblotting with the indicated antibodies (upper). Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% Triton X-100) containing 2 mM sodium orthovanadate, and cell lysates were subjected to immunoblotting with the indicated antibodies (lower left). Expression of *IL-8* mRNA relative to *GAPDH* mRNA was determined by quantitative RT-PCR. Error bars, ±SD (n = 3); *$p < 0.05$, Student's t test (lower right).

CagA-R116/118A and CagA-R619/621A mutants, the latter of which cannot bind PS, in polarized MDCK cells. Upon staining, CagA-R116/118A was associated with the plasma membrane. In contrast, CagA-R619/621A was predominantly distributed to the cytoplasm (Figures 6C, S5B, and S5D). We also expressed the membrane marker GFP-Lact-C2 together with wild-type CagA or CagA-R619/621A in polarized MDCK cells and found that GFP-Lact-C2 colocalized with wild-type CagA, but not with CagA-R619/621A (Figure S5C). Although the possibility remains that a small fraction of CagA-R619/621A was also bound to the membrane, these results indicated that CagA-R619/621A is primarily distributed to the cytoplasm.

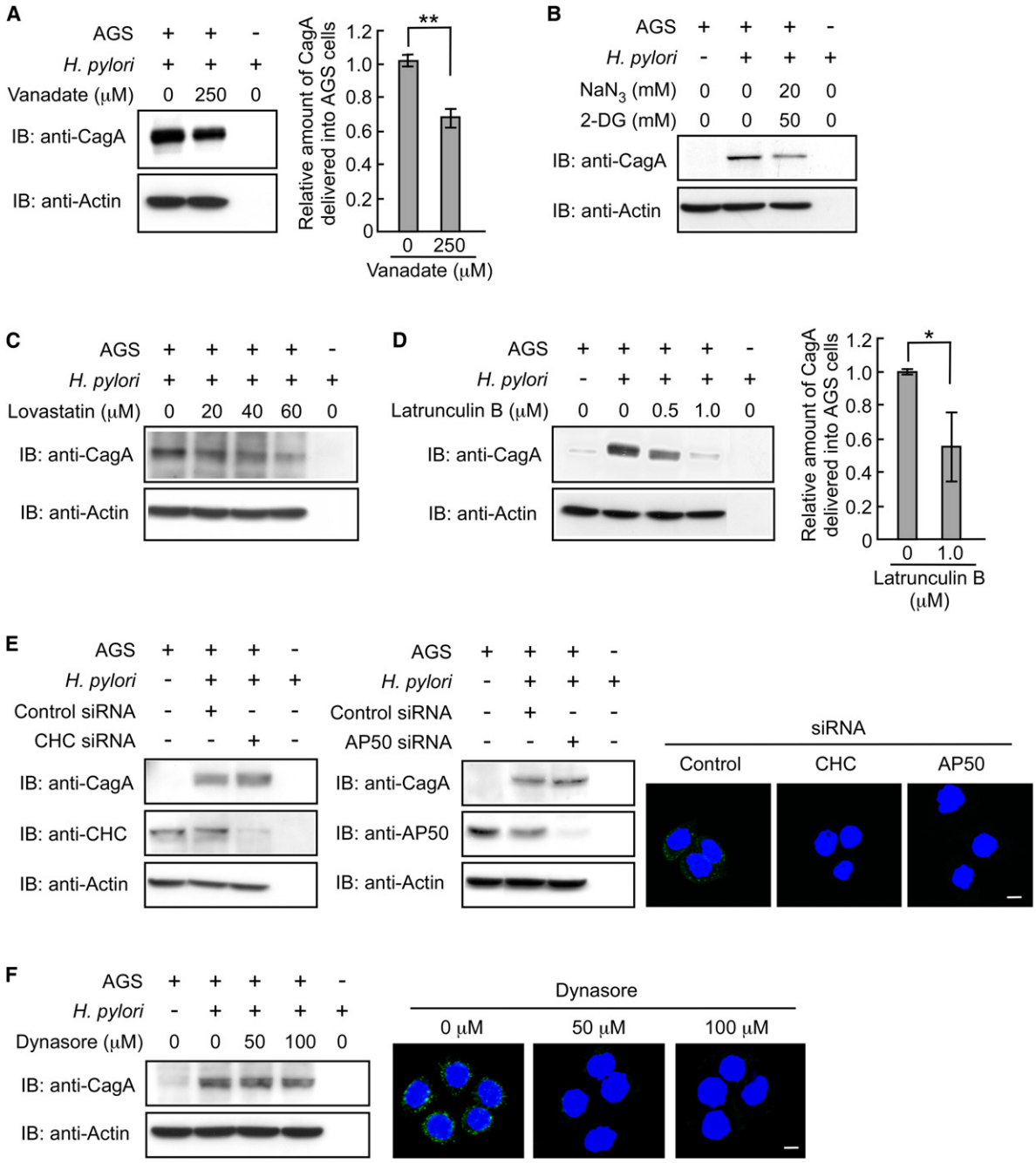


Figure 5. Requirement of Host Cell Machinery for CagA Delivery

(A) AGS cells were treated with vanadate for 15 min prior to infection with *H. pylori* NCTC11637 strain for 1 hr at a moi of 100. Error bars, ±SD (n = 4); **$p < 0.01$, Student's t test.

(B) AGS cells were treated with 20 mM sodium azide (NaN_3) and 50 mM 2-deoxy-D-glucose (2-DG) for 1 hr prior to infection with *H. pylori* NCTC11637 strain for 1 hr at a moi of 100.

(C) AGS cells were treated with lovastatin for 1 hr prior to infection with *H. pylori* NCTC11637 strain for 1 hr at a moi of 100.

(D) AGS cells were infected with *H. pylori* NCTC11637 strain for 1 hr at a moi of 100 in the presence or absence of latrunculin B. Error bars, ±SD (n = 3); *$p < 0.05$, Student's t test.

(E) AGS cells were transfected with clathrin heavy chain (CHC)-specific siRNA, AP50-specific siRNA, or luciferase-specific siRNA (control siRNA). At 48 hr after siRNA transfection, cells were uninfected or infected with *H. pylori* NCTC11637 strain at a moi of 100 and cultured for an additional 3 hr (left). At 48 hr after siRNA transfection, cells were treated with Alexa 488-conjugated transferrin (green) for 5 min at 37°C. Nuclei were visualized by DAPI staining (blue). Scale bar, 10 μm (right).

This, in turn, suggested that in polarized epithelial cells, delivered CagA is tethered to the inner leaflet of the plasma membrane via CagA-PS interaction.

Requirement of CagA-PS Interaction in Polarity-Dependent CagA Activity

Membrane localization has been shown to play a crucial role in the pathophysiological actions of CagA (Higashi et al., 2002, 2005; Bagnoli et al., 2005; Saadat et al., 2007). The finding that the CagA-R619/621A mutant cannot stably associate with the membrane in polarized epithelial cells therefore raised the possibility that the CagA mutant lacks polarity-dependent CagA functions. It has previously been reported that expression of CagA in polarized epithelial cells causes extrusion of CagA-expressing cells from the polarized monolayer (Amieva et al., 2003). This CagA activity is dependent on CagA-PAR1 complex formation and subsequent inhibition of the kinase activity of PAR1 in polarized epithelial cells (Saadat et al., 2007). Accordingly, we expressed CagA-R116/118A and CagA-R619/621A in polarized MDCK monolayer and found that whereas CagA-R116/118A caused extrusion of cells from the monolayer like wild-type CagA, CagA-R619/621A failed to do so (Figure 6D). The result indicated that CagA-R619/621A could not inhibit PAR1 kinase activity below the critical threshold in polarized epithelial cells. It has also been demonstrated that CagA-mediated PAR1 inhibition provokes junctional and polarity defects, which should contribute to the extrusion of CagA-expressing cells from the polarized epithelial monolayer (Saadat et al., 2007). Accordingly, we next investigated the effect of the CagA mutants on tight junctions and epithelial polarity. Again, both wild-type CagA and CagA-R116/118A were capable of mislocalizing the tight junction markers ZO-1 and cingulin to the apical membrane (Figures 6D and S5E). Moreover, CagA-116/118A induced aberrant distribution of the apical marker gp135 to the basolateral membrane and the lateral marker E-cadherin to the apical membrane, like wild-type CagA. On the other hand, expression of CagA-R619/621A did not have any impact on tight junction integrity and epithelial polarity (Figures 6D and S5E). These observations indicated that the CagA-R619/621A mutant failed to inhibit PAR1 in polarized epithelial cells and therefore could not induce junctional and polarity defects and subsequent extrusion of cells from the polarized monolayer. Hence, PS-mediated membrane tethering is crucial for CagA to provoke biological activities that are specific to polarized epithelial cells. Notably, however, CagA-R619/621A still retained the ability to bind SHP-2 and induced the hummingbird phenotype in nonpolarized AGS cells (Figure 7), where a phosphorylation-resistant CagA mutant (PR-CagA) neither bound SHP-2 nor induced the morphological change as previously reported (Higashi et al., 2002). Accordingly, CagA-PS interaction is primarily responsible for CagA delivery and subsequent junctional and polarity defects in polarized epithelial cells, pathophysiologically relevant cell targets for *H. pylori* in vivo.

DISCUSSION

H. pylori CagA is the only effector protein thus far shown to be translocated via *H. pylori* TFSS. Upon delivery, CagA manipulates host cell signaling by acting as a bacterium-derived scaffolding/adaptor protein and thereby promotes gastric carcinogenesis (Peek, 2005; Hatakeyama, 2008). The present work uncovered an interaction between *H. pylori* CagA and host plasma membrane PS and showed that the CagA-PS interaction plays a key role in mediating delivery, intracellular localization, and pathophysiological action of CagA (Figure S6).

We first found that direct contact of *H. pylori* with the host cell membrane induces rapid and transient externalization of PS, which is independent of apoptotic process, at the site of bacterial attachment. A previous study also demonstrated that *Chlamydia* infection elicits PS externalization on the surface of host cells (Goth and Stephens, 2001). Hence, the change in membranous PS distribution is not unique to *H. pylori* and may be triggered via physical interaction of the host plasma membrane with certain types of live bacteria. Interestingly, externalized PS remained constrained to the patch of the membrane that subtended the bacteria. One possible mechanism for this observation is that lateral diffusion of PS in the outer leaflet of the plasma membrane is specifically inhibited by bacterial attachment. Alternatively, flippase activity may be specifically inhibited upon bacterial contact, and thus externalized PS remains at the site of bacterial attachment, whereas laterally diffused PS is rapidly translocated to the inner leaflet of the membrane by flippases. We also found that CagA is exposed on the bacterial surface. Surface-exposed CagA then binds to the externalized PS, which serves as a portal site for CagA internalization. Although CagA does not possess protein domains that can bind phospholipids, it contains the K-Xn-R-X-R motif, a sequence motif conserved among various PH domains that is directly involved in the interaction with acidic phospholipids such as PI and/or PS (Lemmon, 2008). Indeed, the CagA-PS interaction requires the K-Xn-R-X-R motif present in the central region of CagA. As is the case of the PH domain, the basic side chains from lysine and arginine present in the K-Xn-R-X-R motif of CagA may mediate interaction with phosphate groups of PS (Wen et al., 2008). Expectedly, two arginine residues, R619 and R621, that constitute the K-Xn-R-X-R motif in the central region of *H. pylori* NCTC11637 strain-derived CagA are highly conserved throughout CagA species, including those from Western *H. pylori* strains (26695, G27, and J99) and East Asian *H. pylori* strain (F75). On the other hand, arginine residues (R116 and R118) that constitute another K-Xn-R-X-R motif in NCTC11637 strain-derived CagA are neither required for the CagA-PS interaction nor conserved among CagA species.

A. tumefaciens TFSS is the archetypal TFSS, which mediates delivery of T-DNA and associated proteins (Christie et al., 2005; Juhas et al., 2008). Even in the case of *A. tumefaciens* TFSS, however, detailed mechanisms by which effector molecules are delivered across the host membrane remain poorly

(F) AGS cells were treated with dynasore for 30 min prior to infection with *H. pylori* NCTC11637 strain and throughout the infection period (1 hr) at a moi of 100 (left). AGS cells were treated with dynasore for 30 min prior to addition of Alexa 488-conjugated transferrin (green) for 5 min at 37°C. Nuclei were visualized by DAPI staining (blue). Scale bar, 10 μm (right). In (A)–(F), host cell proteins were extracted with PBS/0.1% saponin, and cell extracts were subjected to immunoblotting with the indicated antibodies.

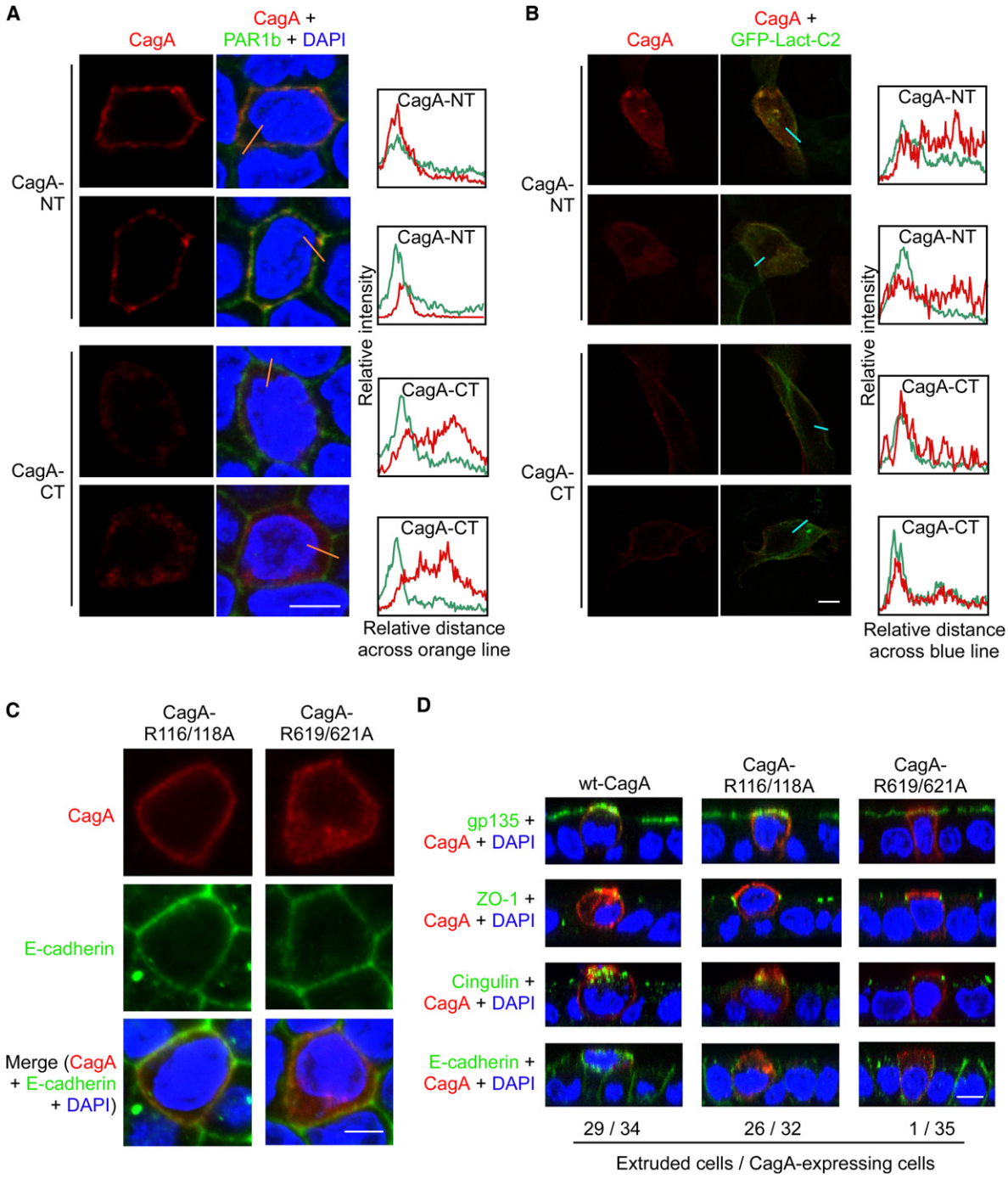


Figure 6. Mechanism of CagA Membrane Localization

(A) Confocal x-y plane views of CagA (red), PAR1b (green), and DAPI (blue) staining. Polarized MDCK cells were transfected with Flag-tagged CagA-NT or HA-tagged CagA-CT vector and stained with anti-PAR1b antibody together with anti-HA or anti-Flag antibody. Nuclei were visualized by DAPI staining. In polarized epithelial cells, PAR1b localizes to the membrane. Scale bar, 5 μm. The fluorescence intensity changes of CagA and PAR1b signals across the orange line were plotted as line intensity histograms in the right panels to evaluate membrane or cytoplasmic distribution of the CagA mutants.

(B) Confocal x-y plane views of CagA (red) and GFP-Lact-C2 (green) staining. Nonpolarized MDCK cells were transfected with Flag-tagged CagA-NT or HA-tagged CagA-CT vector together with an expression vector for the membrane marker GFP-Lact-C2. Transfected cells were stained with anti-HA or anti-Flag antibody. Scale bar, 10 μm. The fluorescence intensity changes of CagA and GFP-Lact-C2 signals across the blue line were plotted as line intensity histograms in the right panels to evaluate membrane or cytoplasmic distribution of the CagA mutants.

(C) Confocal x-y plane views of CagA (red), E-cadherin (green), and DAPI (blue) staining. Polarized MDCK cells were transfected with HA-tagged CagA-R116/118A or HA-tagged CagA-R619/621A vector and stained with anti-HA and anti-E-cadherin antibodies. In polarized epithelial cells, E-cadherin localizes to the membrane. Scale bar, 5 μm.

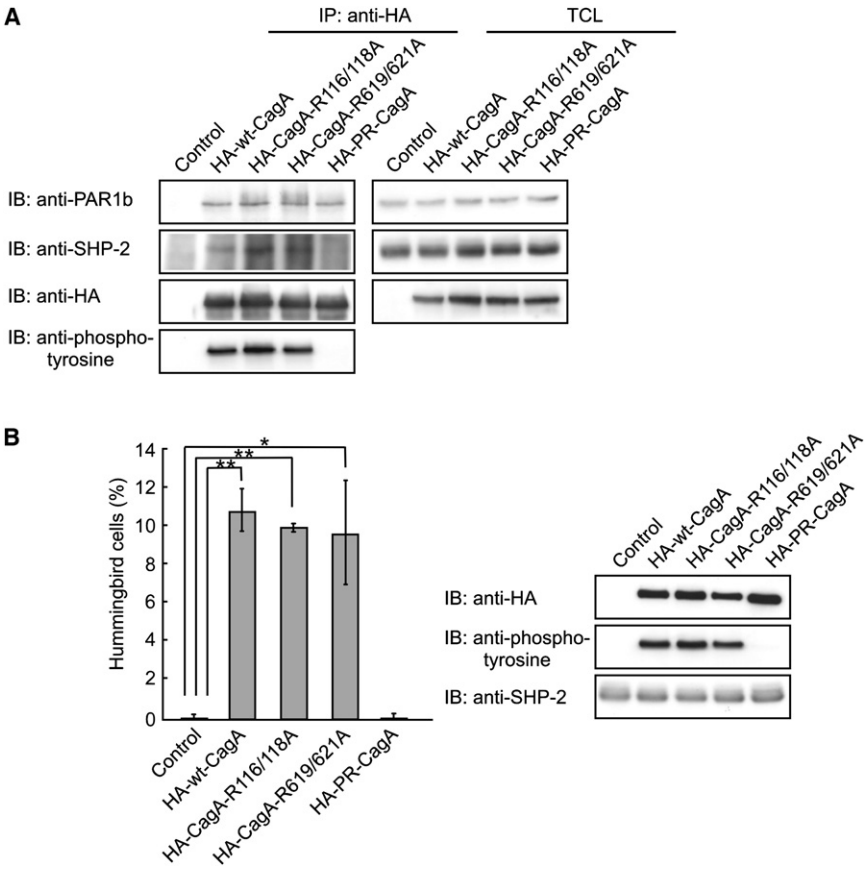


Figure 7. Biological Activities of CagA Mutants that Cannot Bind PS

(A) Nonpolarized MDCK cells were transfected with the indicated CagA vector. pSP65SRα empty vector was used as a control. HA-PR-CagA, HA-tagged phosphorylation-resistant CagA. Total cell lysates (TCL) were immunoprecipitated (IP) or immunoblotted (IB) with the indicated antibodies.

(B) AGS cells were transfected with the indicated CagA vector, and the number of hummingbird cells was counted. Control, pSP65SRα vector; HA-PR-CagA, HA-tagged phosphorylation-resistant CagA. Error bars, ±SD (n = 3); *p < 0.005, **p < 0.001, Student's t test (left). Expression levels of each CagA species are shown (right).

understood. We showed in the present work that CagA-PS interaction is necessary for CagA delivery into host cells. We also showed that *H. pylori* exploits a eukaryotic cellular mechanism for the translocation of CagA across the host plasma membrane. Although the molecular nature of the host machineries involved in CagA delivery is presently unclear, it does not require clathrin, dynamin, RhoA, CDC42, or ARF6. Hence, the process of CagA delivery does not utilize endocytic pathways that have so far been elucidated in mammalian cells. Furthermore, the process is not inhibited by amiloride, excluding the involvement of macropinocytosis in CagA delivery. Assuming that the interaction of CagA with externalized PS creates a membrane pore or channel that allows CagA translocation across the membrane, formation of such an abnormal structure may require nonphysiological membrane dynamics and actin cytoskeletal rearrangements in host cells, which are aberrantly triggered by the CagA-PS interaction, to assist the cell surface remodeling (Figure S6). In this regard, it is tempting to speculate that the reported CagL-β1-integrin interaction stabilizes the CagA-PS interaction (Kwok et al., 2007). Since integrin activation has been shown to stimulate entry of bacteria and viruses during infection (Isberg et al., 2000; Meier et al., 2002), CagL-β1-integrin interaction might also contribute to internalization of the PS-bound CagA into host cells through activation of integrin signaling. We note, however, that attachment of *H. pylori* to the apical membrane did not affect the basolateral localization of β1-integrin in polarized epithelial cells (Figure S1B).

In the host epithelial cells, delivered CagA is tethered to the inner surface of the plasma membrane. Our present work indicated that CagA may utilize distinct mechanisms for membrane association depending on the status of epithelial cell polarity. In polarized epithelial cells, interaction of delivered CagA with PS that is selectively distributed to the inner leaflet of the plasma membrane plays an important role in CagA membrane association. The observation is in good agreement with a previous report showing that the N-terminal region of CagA, which contains the K-Xn-R-X-R motif that mediates PS binding, is responsible for the membrane association of CagA in polarized epithelial cells (Bagnoli et al., 2005). Membrane-tethered CagA then initiates disruption of tight junctions and causes loss of epithelial apico-basal polarity by inhibiting kinase activity of PAR1 through physical complex formation (Amieva et al., 2003; Bagnoli et al., 2005; Saadat et al., 2007). In nonpolarized epithelial cells, however, CagA is localized to the plasma membrane via a mechanism that is dependent on the C-terminal EPIYA motif of CagA (Higashi et al., 2005). In this case, CagA might be membrane tethered through interaction with an EPIYA motif-binding protein, the expression and/or membrane localization of which is again regulated in a polarity-dependent manner. Also, in nonpolarized epithelial cells, interaction of the N-terminal CagA region with PS must be attenuated. It is tempting to speculate that concentration or distribution of PS on the inner leaflet of the plasma membrane may change depending on the degree of epithelial polarity status,

(D) Confocal x-z plane views of CagA (red), gp135, ZO-1, cingulin, and E-cadherin staining (green). Polarized MDCK cells were transfected with HA-tagged WT-CagA, HA-tagged CagA-R116/118A, or HA-tagged CagA-R619/621A vector and stained with anti-gp135, anti-ZO-1, anti-cingulin, or anti-E-cadherin antibody. Nuclei were visualized by DAPI staining (blue). Scale bar, 10 μm. Cells extruded from the polarized monolayer were defined as those in which a part of the cell nucleus (stained blue) was positioned on top of the nuclei of neighboring cells in confocal x-z images. Number of cells extruded from the layer and number of cells expressing CagA are shown at the bottom.

thereby enabling polarity-dependent regulation of the CagA-PS interaction.

The present work has revealed a mechanism by which *H. pylori* CagA oncoprotein sneaks into host cells and subverts host cell functions. Strikingly, CagA exploits PS at both the outer and inner leaflets of the host plasma membrane for entry and localization. This makes it possible for CagA to act as a bacterium-derived scaffolding/adaptor that contributes to neoplastic transformation of gastric epithelial cells. Kierbel et al. recently reported that *Pseudomonas aeruginosa* binds cell-cell junctions and activates PI3K to generate PI3,4,5-trisphosphate (PIP3) at the apical membrane (Kierbel et al., 2007). However, their work did not demonstrate specific interaction of a particular *Pseudomonas* protein with phospholipids. Thus, the present work establishes physical and functional interaction of a bacterial virulence factor with phospholipids. Our study underscores the importance of the host membrane phospholipids during infection with microbial pathogens, which may have great therapeutic implications.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

MDCK II cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). AGS cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA). MDCK II cells were cultured on Transwell (#3413, Corning; Lowell, MA) at 3×10^5 cells/cm^2 for 3 days to prepare polarized epithelial cells.

Expression Vectors

pSP65SRα-derived expression vectors for hemagglutinin (HA)-tagged wild-type CagA (*H. pylori* NCTC11637 strain-derived ABCCC-type CagA) and its phosphorylation-resistant mutant (HA-PR-CagA), in which all the EPIYA motifs were replaced by EPIAA sequence, have been described previously (Higashi et al., 2002). HA-tagged CagA-R116/118A and HA-tagged CagA-R619/621A were made from NCTC11637 strain-derived CagA (ABCCC-type CagA) by site-directed mutagenesis. *H. pylori* 26695 strain-derived *cagA*, which encodes an ABC-type CagA, was utilized to generate gene fragments encoding Flag-tagged CagA-NT and HA-tagged CagA-CT, which correspond, respectively, to residues 1–876 and 877–1186 of *H. pylori* 26695 strain-derived CagA (ABC-type CagA). Resulting *cagA* gene fragments were inserted into pSP65SRα. An expression vector for GFP-Lact-C2 was made by inserting *Lact-C2* cDNA fragment (provided by K. Tanaka) into pEGFP-C1 (Clontech; Mountain View, CA).

Bacteria and Production of Isogenic Mutant Strains

H. pylori NCTC11637 strain and its isogenic mutant *ΔcagA* have been reported previously (Higashi et al., 2004). *H. pylori* F57 strain carries an inactivation mutation in the *vacA* gene. *H. pylori* G27 *ΔcagPAI* strain was provided by S. Ding. *H. pylori* NCTC11637 strain-derived isogenic mutants carrying a gene encoding HA-tagged wild-type CagA or HA-tagged CagA R619/621A were generated by using natural transformation method (Haas et al., 1993).

Antibodies

Antibodies used in this work are described in the Supplemental Experimental Procedures.

Immunoprecipitation and Immunoblotting

The detergent saponin (Sigma) was used for selective lysis of mammalian cells. Saponin destroys AGS cells but leaves *H. pylori* intact (Odenbreit et al., 2000). Protein extracts were prepared by treating cells with phosphate-buffered saline (PBS) containing 0.1% saponin.

Cell Staining

For immunostaining, cells with or without *H. pylori* infection were fixed with Mildform 10N (Wako; Osaka, Japan) for 20 min and then permeabilized with 0.5% Triton X-100 for 20 min. The fixed samples were then treated with a primary antibody and were visualized with Alexa Fluor-conjugated secondary antibodies (Invitrogen). The nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI). Images were acquired using confocal microscope systems (FluoView, Olympus [Tokyo] and TCS-SPE, Leica [Mannheim, Germany]). *H. pylori* was detected using an anti-*H. pylori* antibody (AbD Serotec; Oxford, UK). PS exposure was detected using Annexin V-Cy3 (Sigma; St. Louis) according to the manufacturer's instructions.

Purification of Recombinant CagA Proteins

Recombinant CagA proteins were purified as described in the Supplemental Experimental Procedures.

Lipid-Binding Assay

PIP strips (P-6001, Echelon Biosciences; Salt Lake City, UT) were incubated with 32 nM His-tagged recombinant CagA in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 15 hr at 4°C. Blots were incubated with the anti-His antibody as a primary antibody. The signals were visualized using western blot chemiluminescence reagent.

Quantitative RT-PCR

Data were acquired using ABI PRISM 7700 Sequence detector (Perkin-Elmer). Detailed procedures are described in the Supplemental Experimental Procedures.

RNA Interference

Nucleotide sequences and transfection procedures are described in the Supplemental Experimental Procedures.

Statistical Analysis

All data were evaluated using Student's t test. $p < 0.05$ was considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.chom.2010.04.005.

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