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Helicobacter pylori CagA-mediated IL-8 induction in gastric epithelial cells is cholesterol-dependent and requires the C-terminal tyrosine phosphorylation-containing domain

Running title: Association of CagA and cholesterol

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Abstract

Upon infection of the gastric epithelial cells, the *Helicobacter pylori* cytotoxin-associated gene A (CagA) virulence protein is injected into the epithelial cells via the type IV secretion system (TFSS), which is dependent on cholesterol. Translocated CagA is targeted by the membrane-recruited c-Src family kinases in which a tyrosine residue in the Glu-Pro-Ile-Tyr-Ala (EPIYA)-repeat region, which can be phosphorylated, inducing cellular responses, including interleukin-8 (IL-8) secretion and hummingbird phenotype formation. In this study, we explored the role of EPIYA-containing C-terminal domain (CTD) in CagA tethering to the membrane lipid rafts and in IL-8 activity. We found that disruption of the lipid rafts reduced the level of CagA translocation/phosphorylation as well as CagA-mediated IL-8 secretion. By CagA truncated mutagenesis, we identified that the CTD, rather than the N-terminal domain (NTD), was responsible for CagA tethering to the plasma membrane and association with detergent-resistant membranes, leading to CagA-induced *IL-8* promoter activity. Our results suggest that CagA CTD-containing EPIYAs directly interact with cholesterol-rich microdomains that induce efficient IL-8 secretion in the epithelial cells.

Keywords: *Helicobacter pylori*, cytotoxin-associated gene A, cholesterol, interleukin-8

Introduction

Helicobacter pylori is a spiral-shaped Gram-negative bacterium which inhabits approximately half of the world's human population (Marshall, 2002). Persistent *H. pylori* infection in human gastric mucosa induces gastritis and leads to the progression of several types of gastrointestinal diseases, including duodenal and gastric ulcers and gastric cancer or lymphoma (Eck, *et al.*, 1997).

Virulent *H. pylori* strains carry the *cag* pathogenicity island (*cag* PAI), which encodes members of the type IV secretion system (TFSS) and an immunodominant antigen called cytotoxin-associated gene A (CagA) (Backert, *et al.*, 2000). The TFSS mediates translocation of CagA into host cells (Segal, *et al.*, 1999), where tyrosine phosphorylation of CagA is mediated by c-Src family tyrosine kinases (SFKs) (Odenbreit, *et al.*, 2000). In addition, c-Abl, along with c-Src, has been shown to phosphorylate CagA, which leads to cell migration (Poppe, *et al.*, 2007). Phosphorylated CagA binds to and activates the Src homology 2 (SH2) domain of the protein tyrosine phosphatase SHP-2 and deregulates SHP-2 phosphatase activity (Higashi, *et al.*, 2002), which subsequently stimulates the RAS/ERK pathway and induces host cell scattering and proliferation (Mimuro, *et al.*, 2002).

One mechanism by which *H. pylori* escapes immune surveillance is by assimilating and modifying cellular cholesterol (Wunder, *et al.*, 2006), an important component of lipid rafts, which are dynamic microdomains in the exoplasmic leaflet of lipid bilayer membranes (Brown & London, 1998). For *in vitro* studies, the integrity of lipid rafts is usually preserved using the cold-detergent extraction method by in the presence of non-ionic detergents such as Triton X-100, whereas

disruption of lipid rafts is performed using the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) (Simons, *et al.*, 2002). Analysis of proteins in detergent-resistant membranes (DRMs) indicates that CagA alters the expression and cellular distribution of host proteins in lipid rafts, and that CagA co-fractionates with DRMs (Zeaiter, *et al.*, 2008). Translocation of CagA and by which induced IL-8 production in infected AGS cells is also blocked by cholesterol depletion (Lai, *et al.*, 2008, Murata-Kamiya, *et al.*, 2010).

The presence of a single Glu-Pro-Ile-Tyr-Ala (EPIYA) motif in the C-terminal region of CagA was shown to be crucial for membrane localization (Higashi, *et al.*, 2005). Delivery of CagA with more phosphorylation motifs was found to induce a higher level of phosphorylation in epithelial cells, which may therefore influence the severity of the clinical outcomes (Argent, *et al.*, 2004). However, the detailed role of lipid rafts in membrane tethering of CagA remains to be elucidated. In this study, we investigated the effects of various CagA truncation mutants on the association between CagA and lipid rafts and on *IL-8* induction. Our results provide evidence that the CagA C-terminal EPIYA-containing region is targeted to membrane rafts, which allows CagA-mediated induction of *IL-8*.

Materials and Methods

H. pylori strains

H. pylori 26695 (ATCC 700392) was used as a reference strain and contains a *cagA* gene with three C-terminal EPIYA motifs (ABC-type) (Higashi, *et al.*, 2005). Clinical strain v669 was isolated from a patient with gastric cancer and contains a *cagA* gene with four C-terminal EPIYA motifs

(AABD-type) (Lai, *et al.*, 2002). *H. pylori* strains were recovered from frozen stocks on Brucella blood agar plates (Becton Dickinson). Construction of the *cagA* (ΔCagA) and *cagE* (ΔCagE) knockout strains were performed using the kanamycin resistance cassette (*Km*^{r}) from pACYC177 and the erythromycin resistance cassette (*Ery*^{r}) from pE194, respectively, by the natural transformation method as we described previously (Lai, *et al.*, 2008). PCR and western blot analysis were employed to confirm that the correct insertion of antibiotic resistance cassettes into the target genes.

Construction of CagA expression plasmids

Various expression constructs encoding CagA truncation mutants were generated based on the *H. pylori* 26695 *cagA* sequence and v669 as illustrated in Figure 3A. *cagA* fragments were amplified by PCR from *H. pylori* 26695 and v669 genomic DNA as described previously (Lai, *et al.*, 2002). The CagA-ΔN mutant was generated from strain 26695 by amplification of sequence encoding amino acids 645 to 1186 using primers CagA-CTD59F and CagA-CTDR (Table 1). The primers used for PCR introduced a *BamH*I site at the 5' end and an *XbaI* site at the 3' end. The *BamH*I–*Xba*I fragment was then ligated into pEF1 expression vector (Invitrogen). Similar procedures were used to obtain the 669CagA-ΔN mutant from strain v669 using primers CagA-CTD59F and CagA-CTDR.

To generate the CagA-ΔC mutant, a fragment encoding amino acids 1 to 358 was amplified using primers CagA1-F and CagA-1R. The primers used for PCR introduced a *BamH*I site at the 5' end and an *EcoR*I site at the 3' end. The *BamH*I–*EcoR*I fragment was then inserted into pEF1 to

derive pEF1-CagA1. A fragment encoding amino acids 357 to 707 was amplified using primers CagA2F and CagA2R. The primers used for PCR introduced an *Nde*I site at the 5' end and an *Mfe*I site at the 3' end. After digestion, the *Nde*I-*Mfe*I fragment was then inserted into the *Nde*I and *Mfe*I sites of pEF1-CagA1 to obtain the CagA-ΔC mutant. Similar procedures were used to construct the 669CagA-ΔC mutant from strain v669 as described above. To create the full-length CagA construct, CagA CTD69, a fragment encoding amino acids 555 to 1186 was amplified using primers CagA-CTD69F and CagA-CTDR. After digestion with *Bgl*II (at nucleotide 1851) and *Xba*I, the *Bgl*II–*Xba*I fragment was then inserted into the *Bgl*II and *Xba*I sites of pEF1-CagAΔC to obtain the full-length CagA construct.

Transient transfection of IL-8 promoter-luc constructs and luciferase activity assay

AGS cells were grown to 90% confluence in 12-well plates and transfected using Lipofectamine 2000 (Invitrogen). After a 24-h incubation for transfection, cells were infected with wild-type or ΔCagA *H. pylori* in the absence or presence of various concentrations of lovastatin (Sigma-Aldrich) for 6 h. To prepare total cell lysates, 100 µl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. An equal volume of luciferase substrate was added to all samples, and luminescence was measured using a microplate luminometer (Biotek). Luciferase activity was normalized to transfection efficiency, which was determined by the β-galactosidase activity generated from a co-transfected β-galactosidase expression vector (Promega).

Statistical analysis

The Student's *t*-test was used to calculate the statistical significance of experimental results

between two groups. $P < 0.05$ was considered significant.

RESULTS

Cholesterol is essential for *H. pylori* CagA-induced responses in AGS cells

We first examined that whether sufficient cellular cholesterol plays a crucial role for *H. pylori* CagA-induced IL-8 secretion in AGS cells. Several lipid raft disruptors and usurpers were used to treat cells including: lovastatin (which is a HMG-CoA reductase inhibitor) (Endo, 1981), nystatin (which chelates to cholesterol and removes cholesterol from membrane) (Anderson, *et al.*, 1996), and cholera toxin subunit B (CTX-B, which binds to GM1 in rafts) (Naroeni & Porte, 2002). When cells were pretreated with lovastatin (10 to 50 μM) and infection of wild-type *H. pylori* (strain 26695), the levels of IL-8 secretions were significantly decreased compared with untreated cells (Fig. 1A). Lovastatin-treated cells contained lower levels of cellular cholesterol as the concentration of lovastatin increased (Fig. S1A). However, the viability of *H. pylori* and cells were barely affected under treated with lovastatin (Fig. S1B). In parallel, pretreatment of cells with nystatin and CTX-B also resulted in significant reduction of *H. pylori*-induced IL-8 production. We next evaluated whether cholesterol was necessary for CagA-mediated IL-8 secretion by use of two CagA functional deficiency mutants (ΔCagA and ΔCagE). As compared to cells infected with the wild-type strain, there was a lower level of IL-8 secretion in either ΔCagA- or ΔCagE-infected cells (Fig. 1A). However, there were no significant differences in the IL-8 secretion upon cells pretreated with lovastatin, nystatin, and CTX-B following by infection of ΔCagA and ΔCagE,

respectively.

We next assessed whether lower level of cellular cholesterol had any influence on translocation/phosphorylation of CagA in *H. pylori*-infected AGS cells. The level of translocated/tyrosine-phosphorylated CagA (Fig. 1B), and the proportions of elongated cells (Fig. 1C) were reduced significantly in a concentration-dependent manner after pretreatment of cells with different concentrations of lovastatin. Together, these results suggest that an adequate amount of cellular cholesterol is required for CagA-induced responses in *H. pylori*-infected cells.

H. pylori CagA-induced IL-8 promoter activity requires cholesterol

We further evaluated whether the level of endogenous cholesterol influenced the IL-8 transcriptional activation using a human *IL-8* promoter construct (IL8-Luc) which contains AP-1 and NF-κB sites, fused with a luciferase reporter gene (Fig. 2A) (Chang, *et al.*, 2006). Following transfection with the IL8-Luc, AGS cells were treated with lovastatin to reduce the level of endogenous cholesterol and then infected with wild-type, ΔCagA, or ΔCagE *H. pylori*. Our data show that a significant attenuation in the stimulation of *IL-8* promoter activity in cells infected with the wild-type strain, but not with ΔCagA or ΔCagE *H. pylori* (Fig. 2B). These results suggest that CagA-mediated *IL-8* promoter activity was dependent on host endogenous cholesterol in epithelial cells.

The CagA C-terminal region containing EPIYAs is required for CagA-mediated IL-8 induction

We then sought to investigate whether the CTD of CagA that contains EPIYAs was involved in

CagA-mediated IL-8 activation. Various expression constructs were constructed based on the strain 26695 that contains three EPIYA motifs (ABC type): a CagA full-length expression construct (CagA-FL) and CagA truncation mutants including two mutants with N-terminal deletions (CagA-ΔN and CagA-ΔN42) and a mutant with the C-terminal deletion (CagA-ΔC) (Fig. 3A). In parallel, a clinical isolate v669, which contains *cagA* sequence with AABD-type EPIYA repeats, was utilized to generate the analogous N-terminal deletion mutants (669CagA-ΔN and 669CagA-ΔN42) as well as a C-terminal deletion mutant (669CagA-ΔC) (Fig. 3A). When cells were co-transfected with IL8-Luc and CagA-FL, there was an approximately 3-fold increase in luciferase activity compared to cells transfected with IL8-Luc alone (Fig. 3B). Cells co-transfected with IL8-Luc and either CagA-ΔC or 669CagA-ΔC constructs exhibited basal level luciferase activity. In contrast, cells co-transfected with any of the N-terminal deletion mutants (CagA-ΔN, CagA-ΔN42, 669CagA-ΔN, and 669CagA-ΔN42) exhibited no significantly different luciferase activity as compared with cells co-transfected with CagA-FL (Fig. 3B).

We next evaluated whether *IL-8* promoter activity was influenced by lovastatin treatment. Cells co-transfected with IL8-Luc and either CagA-FL or CagA-ΔN expression constructs, lovastatin-treated cells exhibited a significant decrease in luciferase activity (Fig. 3C). In contrast, cells co-transfected with IL8-Luc and the construct lacking the CTD (CagA-ΔC), lovastatin had no significant effect on *IL-8* promoter activity (Fig. 3C). These results suggest that both the C-terminal EPIYA-containing domain of CagA and cholesterol are crucial for induction of *IL-8* promoter activity. We further assessed that whether the presence of cholesterol affects *IL-8* activity

also influences IL-8 production. Transfection with either CagA-FL or CagA-ΔN induced significantly more than the vector alone (Fig. 3D). However, in lovastatin-treated cells, the CagA-FL or CagA-ΔN induced production of IL-8 was reduced. These results together provide further evidence that *IL-8* promoter activity and IL-8 secretion induced by CagA is cholesterol-dependent.

CTD CagA target to cholesterol-enriched microdomains

We further assessed the association of CagA with lipid rafts using HEK-293T cells because of its high transfection efficiency (Pear, *et al.*, 1993). Cells were transfected with the Myc-tagged CagA expression vectors, followed by immunoblot analysis with anti-CagA antibody. Fig. 4A shows the expression of full-length CagA and various CagA truncation proteins in transfected HEK-293T cells. To assess whether the expressed CagA proteins were associated with lipid rafts, transfected cells were fractionated by a cold-detergent extraction method to isolate detergent-resistant membrane (DRM) and -soluble membrane (S) fractions, followed by immunoprecipitation and immunoblot analysis (Fig. 4B). We probed caveolin-1 (Cav-1), a 22-kDa transmembrane scaffolding protein of lipid rafts and caveolae, and transferrin receptor (TfR), which is known not to be associated with lipid rafts as internal controls. In cells transfected with CagA-FL, CagA was also enriched in DRM (92%) rather than S (8%) as expected (Fig. 4B). The distribution of CagA shifted from DRM to S when cells were pretreated with 5.0 mM MβCD. A parallel DRM-to-S shift of tyrosine-phosphorylated CagA was also observed with MβCD treatment. We then performed the same experiment using each of the CagA deletion mutants (CagA-ΔC and CagA-ΔN), respectively.

As shown in Figure 4B, CagA-ΔN was primarily localized in DRM (~82%) in the absence of the MβCD treatment, but shifted towards the S fraction upon MβCD treatment (Fig. 4B). On the other hand, a substantial proportion of CagA-ΔC was found in the S fraction independent of MβCD treatment. In addition, the distributions of 669CagA-ΔC and 669CagA-ΔN were similar to CagA-ΔC and CagA-ΔN, respectively (Fig. S2), suggesting that the number of EPIYA sites did not affect the ability of CagA to associate with membrane rafts. These results demonstrate that sufficient cholesterol as well as the CTD-containing EPIYAs are required for CagA tethering to cholesterol-rich microdomains.

Confocal microscopy was used to ascertain whether CagA proteins colocalized with the raft-enriched ganglioside GM1, marked by CTX-B-FITC. We first examined that Myc-tagged did not affected CagA membrane localization (Fig. S3). Cells were then transfected with CagA-FL, CagA (red) and GM1 (green) were co-localized around in the plasma membrane (Fig. 5, 1st row). An analogous pattern was seen for CagA-ΔN-transfected cells (Fig. 5, 2nd row). In cells transfected with CagA-ΔC, an evident cytoplasmic distribution of CagA (red) was seen. On the other hand, hardly any GM1 co-localized signal was detected in the plasma membrane (Fig. 5, 3rd row). These observations support that CagA CTD containing the EPIYA repeats is important for CagA tethering to the membrane raft microdomains.

Discussion

Several lines of evidence suggest that tethering of CagA to membrane-associated components is

crucial for its subsequent functions: (i) Following *H. pylori* infection, translocated CagA binds to raft-associated SFKs and undergoes tyrosine phosphorylation in the EPIYA motifs (Stein, *et al.*, 2002); (ii) CagA associates with the epithelial tight-junction scaffolding protein ZO-1 (Amieva, *et al.*, 2003); (iii) CagA interacts with membrane-externalized phosphatidylserine (PS) to initiate its entry into cells in epithelial cells (Murata-Kamiya, *et al.*, 2010); and (iv) Depletion of cellular cholesterol blocks internalization of CagA into host cells (Lai, *et al.*, 2008). Of note, those identified CagA partners including c-Src (Lai, *et al.*, 2008), ZO-1 (Nusrat, *et al.*, 2000), and PS (Pike, *et al.*, 2002) have been shown to associate with DRMs. In addition to CagA, the *H. pylori* TFSS component CagL was found to bind and activate $\alpha_5\beta_1$ integrin (Kwok, *et al.*, 2007), which is abundantly localized in cholesterol-rich microdomains (Leitinger & Hogg, 2002). This interaction was further demonstrated to trigger the delivery of peptidoglycans across the cell membrane, resulting in the induction of NF-κB and IL-8 responses in the epithelial cells (Hutton, *et al.*, 2010). Collectively, these results suggest that TFSS, as well as internalized CagA, can reside primarily in cholesterol-enriched microdomains, where they interact with various signaling molecules, inducing multiple cellular responses, including IL-8 secretion, cell motility, proliferation, and polarity.

Our study shows that the CTD of CagA containing EPIYA repeats, either ABC-type (Western type) or AABD-type (East Asian type), is important for raft tethering and for IL-8 induction in AGS cells. Mutants that lacked the CTD lost their normal ability to associate with membrane rafts, in accord with the finding from Higashi *et al* (Higashi, *et al.*, 2005). In polarized MDCK cells, however, the N-terminal rather than the C-terminal region of CagA tethered to the cell-cell

junctions (Bagnoli, *et al.*, 2005). Of note, a recent report using polarized and non-polarized cells to demonstrate that CagA utilized at least two distinct mechanisms for membrane association, relying on the status of epithelial polarity (Murata-Kamiya, *et al.*, 2010). Moreover, CagA contacts with the inner leaflet of cell membrane through interaction with PS largely via its NTD and binds to PAR1/MARK in polarized cells or interacts with membrane-externalized PS in non-polarized epithelial cells (Murata-Kamiya, *et al.*, 2010). Given these results, CagA might act as a resilient protein and employ its NTD or CTD to associate with a range of molecules for its functions.

The present investigation demonstrated that CagA-induced *IL-8* promoter activity was inhibited by lovastatin, an inhibitor of HMG-CoA reductase, which catalyzes the rate-limiting step in cholesterol biosynthesis (Endo, 1981). This cholesterol-lowering agent has provided valuable treatment for cardiovascular diseases for over two decades (Armitage, 2007). Examination of clinical associations between *H. pylori* infection and cholesterol-related diseases is therefore of interest. Mendall *et al.* reported an epidemiological association between *H. pylori* infection and coronary heart diseases (Mendall, *et al.*, 1994). Infection with CagA-positive strains of *H. pylori* has also been linked to coronary heart disease and premature myocardial infarction (Gunn, *et al.*, 2000, Singh, *et al.*, 2002), supporting the likelihood that cholesterol levels influence *H. pylori* pathogenesis.

In conclusion, we have demonstrated that the levels of cellular cholesterol play a central role in CagA-induced *IL-8* activity and IL-8 secretion in epithelial cells. We also showed that the CagA CTD that consists of EPIYA repeats is crucial for recruiting CagA to lipid rafts of AGS cells.

Modulation of cellular cholesterol levels may alter the partitioning of CagA into membrane lipid microdomains, thereby reducing CagA-induced inflammation and perhaps slowing the progression of *H. pylori*–associated diseases.

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Figure legends

Fig. 1. *H. pylori* CagA-induced responses in AGS cells are dependent on the levels of cellular cholesterol. (A) AGS cells pretreated with lovastatin (0 to 50 μM), nystatin (50 μg/ml), or CTX-B (20 μg/ml), subsequently infected with wild-type, ΔCagA, or ΔCagE *H. pylori*. After incubation for 6 h, the concentration of IL-8 in the culture supernatant was analyzed by a standard ELISA method. (B) Total cell lysates from infected cells were immunoprecipitated (IP) for CagA following by subjected to immunoblot (IB) for analysis of the translocated or tyrosine-phosphorylated CagA. (C) Hummingbird phenotype induced by CagA in *H. pylori*-infected cells was inhibited by treatment with lovastatin. C, untreated cells; Mock-infection, uninfected cells; Hp, bacterial lysate; WT 26695, wild-type *H. pylori* strain 26695; CTX-B, cholera toxin subunit B. Statistical analysis was evaluated using Student's *t*-test. *, $P < 0.05$ and **, $P < 0.01$ compared with WT 26695 infected cells without pretreatment of agents.

Fig. 2. Cholesterol is required for *H. pylori* CagA-induced *IL-8* promoter activity. (A) A schematic representation of IL8-Luc construct. AP-1, Activator protein-1; NF-κB, nuclear factor-kappaB; Luc, luciferase reporter. In all numbering, the transcription initiation site is denoted by +1. (B) AGS cells were transfected with IL8-Luc vector. After 24 h transfection, the cells were then treated with lovastatin prior *H. pylori* infection. Cells were infected with wild-type (WT 26695), ΔCagA, or ΔCagE mutant *H. pylori* for 6 h and then subjected to luciferase activity assays. The significant of the difference was assessed by Student's *t*-test. *, $P < 0.05$.

Fig. 3. The CagA C-terminal domain containing EPIYAs is crucial for the CagA-induced IL-8 promoter activation (B and C) and IL-8 secretion (D) in AGS cells. (A) Schematic representation of wild-type 26695 (ABC-type EPIYAs) and a series of truncated mutants based on wild-type 26695 and v669 isolate (AABD-type EPIYAs). The EPIYA motifs and c-Myc tag are marked. The numbering indicates the number of amino acid residues. (B) AGS cells were co-transfected with the CagA expression constructs and IL8-Luc for 24 h, and then subjected to luciferase activity assays. For quantification, each luciferase activity is shown relative to the luciferase activity of the empty vector (mock-infection)-transfected AGS cells. Data represented as means and standard deviations of three independent experiments. (C) and (D), AGS cells were co-transfected with the indicated CagA constructs and IL-8-Luc followed by incubation in the absence or presence of lovastatin. On the following day, cell lysates and cultured supernatants were subjected to luciferase activity (C) and IL-8 ELISA assay (D), respectively. Statistical significance was evaluated using Student's *t*-test (*, $P < 0.05$).

Fig. 4. CagA C-terminal domain interacts with cholesterol-rich microdomains. (A) Detection of different CagA expression in HEK-293T cells. Cell lysates were prepared and subjected to immunoprecipitation (IP) using anti-Myc antibody followed by immunoblotting (IB) analysis using anti-CagA antibody. Molecular mass markers (kDa) are indicated. (B) HEK-293T cells were transfected with various CagA expression constructs for 24 h as indicated and then treated with or

without 5 mM MβCD. The cell lysates were fractionated by the cold-detergent extraction using 1% Triton X-100, followed by centrifugation to separate detergent-resistant membrane (DRM) and detergent-soluble (S) fractions. Each fraction was subjected to IP using anti-Myc antibody and IB analysis using antibodies against CagA, phosphorylated CagA (CagA-pY). IB by Caveolin-1 (Cav-1) and transferrin receptor (TfR) antibodies were used to mark DRM and S fractions, respectively.

Fig. 5. The CagA C-terminal domain targets to membrane GM1-containing microdomains in confocal images. AGS cells were transfected with CagA-FL, CagA-ΔN, CagA-ΔC expression vectors or empty vectors, respectively, as indicated. The transfected cells were fixed and stained with anti-CagA (red) and FITC-conjugated CTX-B to visualize GM1 (green) and analyzed by confocal microscopy. Regions of CagA co-localization appear in yellow in the overlay as denoted by arrows. DIC: differential interference contrast. Representative images are shown. Scale bars, 10 μm.

Table 1. PCR primers used in this study

Primers	Nucleotide sequences (5′ to 3′)[a]	RE
CagA-CTD59F	GCGGGATCCAAATGGAAGCAAAAGCTCAAGCTAAC	*BamH*I
CagA-CTD42F	GCGGGATCCCAATGGGCGATTTCAGTAGGGTAGAG	*BamH*I
CagA-CTDR	GCGTCTAGAAGATTTTTGGAAACCACCTTTTG	*Xba*I
CagA1F	GCGGGATCCATGACTAACGAAACTATTGATC	*BamH*I
CagA1R	GCGGAATTCCTCGAGCATATGCACATTAATGAGTG	*EcoR*I
CagA2F	TGTGCATATGAAAAACGGCAGTG	*Nde*I
CagA2R	AGCCAATTGCTCCTTTGAGAAG	*Mfe*I
CagACTD69F	GCGACTAGTTTCGTAAGGCGGAATTTAGAG	*Spe*I

[a] Underlined sequence indicates restriction enzyme (RE) sites.

Fig. 1

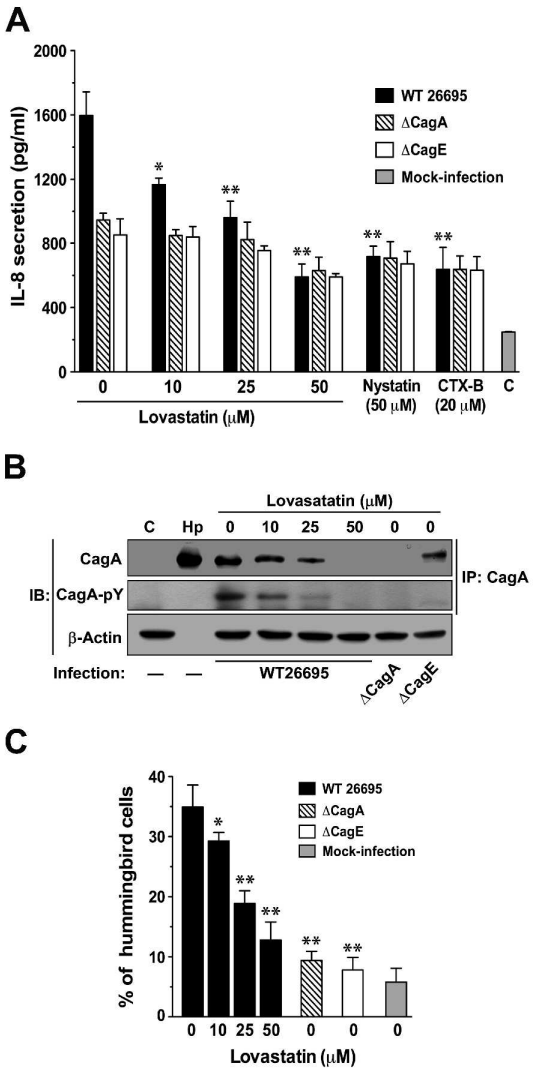

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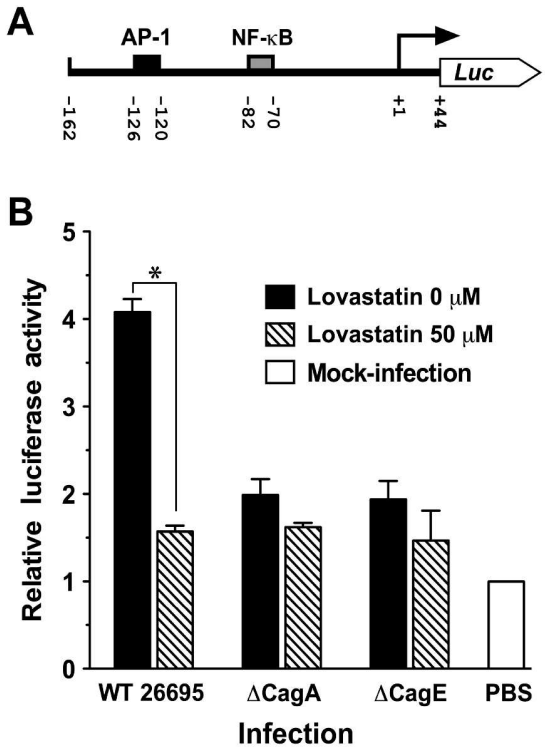
Fig. 2



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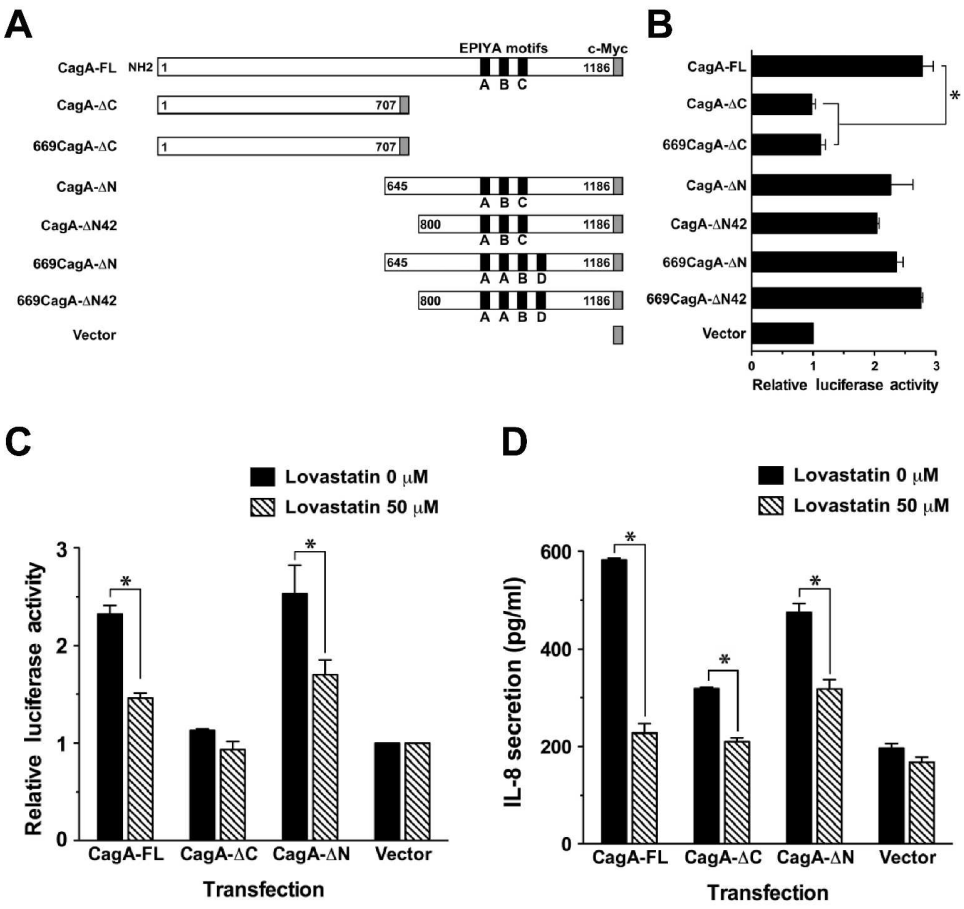

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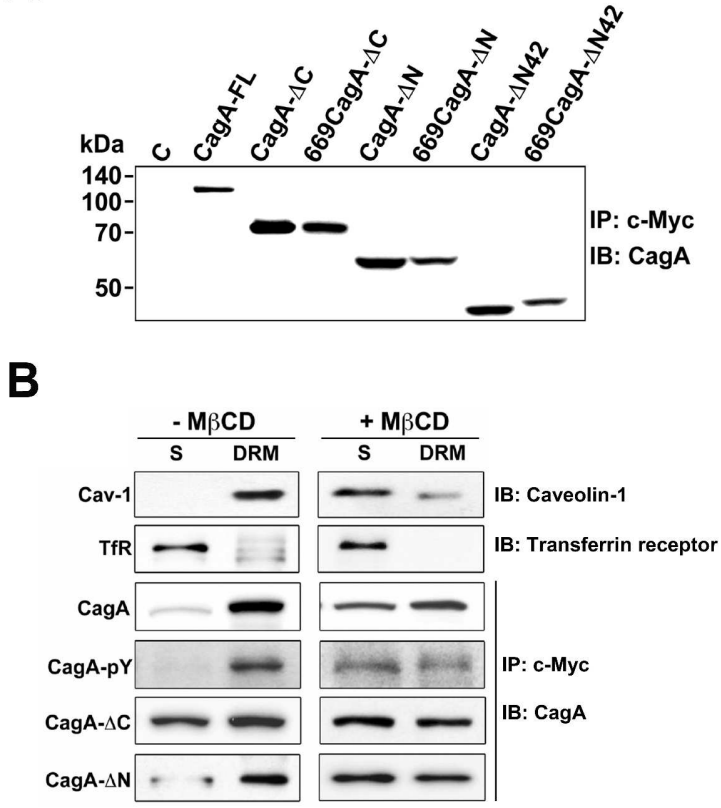
Fig. 4



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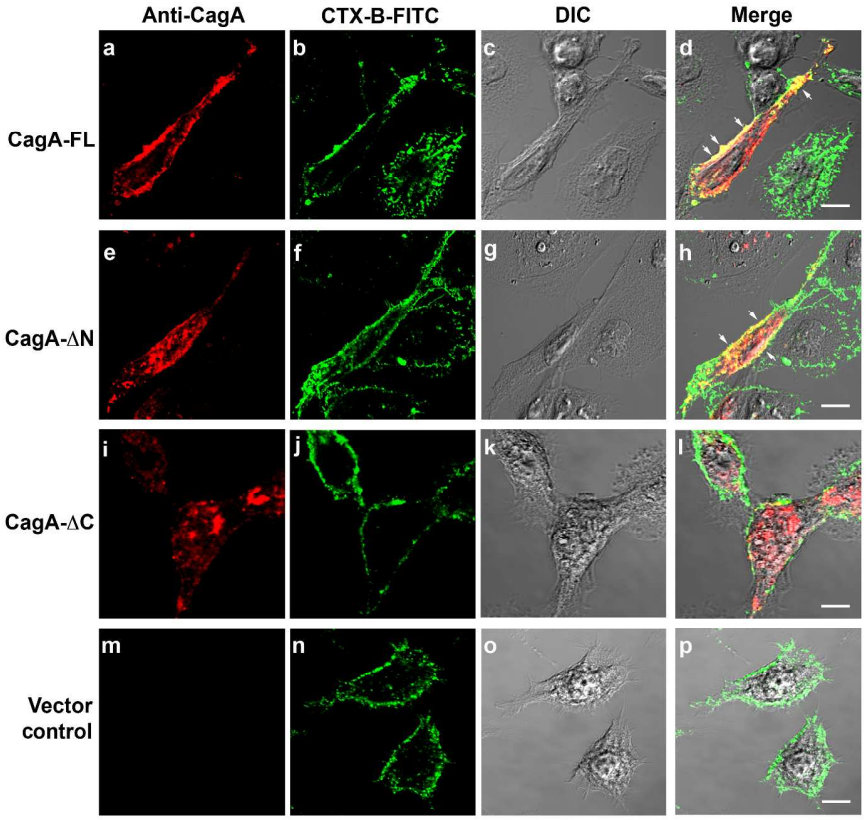

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Supporting Information

Materials and Methods

Reagents and antibodies

CagA polyclonal antibody and phosphotyrosine (4G10) antibody were purchased from Santa Cruz Biotechnology. Myc (9E10) and actin mouse monoclonal antibodies were from Upstate Biotechnology. FITC-conjugated CTX-B was purchased from Sigma-Aldrich, and DAPI was from Molecular Probes (Invitrogen). Lipofectamine 2000 was from Invitrogen, and the luciferase assay kit was from Promega. The IL-8 promoter construct containing NF-κB and AP-1 (IL8-luc; nucleotides −162 to +44) was described previously (Fong, *et al.*, 2008).

Mammalian cell culture

AGS cells (human gastric adenocarcinoma epithelial cells; ATCC CRL 1739) were cultured in F12 medium (HyClone) and HEK-293T cells (human embryonic kidney epithelial cells; ATCC CRL 11268) were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone). All culture medium was supplemented with 10% complement-inactivated FBS (HyClone) and maintained at 37°C. For transient transfection, AGS cells or HEK-293T cells were cultured in six-well plates and incubated in 2 ml OPTI-MEM (Invitrogen), 4 µg total DNA, and 4 µl Lipofectamine 2000 for 6 h at 37°C. Transfected cells were cultured in complete medium for 48 h before further analysis.

Determination of hummingbird phenotype of *H. pylori*-infected cells

The quantitative analysis of *H. pylori*-induced hummingbird activity has been reported elsewhere (Backert, *et al.*, 2004). Briefly, AGS cells (1 x 10^6 cells) were cultured in 12-well plates at 37°C for

20 h. After one wash with PBS, cells were pretreated with various concentrations of lovastatin (0 to 50 μM) and then infected with wild-type, ΔCagA, or ΔCagE *H. pylori* at a MOI of 50 for 6 h. Elongated cells were defined as cells that had thin needlelike protrusions that were >20 μm long and a typical elongated shape. All samples were determined in triplicate in at least three independent experiments. The proportion of elongated cells was calculated the numbers of cells having the hummingbird phenotype.

Analysis of cellular cholesterol and cell viability

AGS cells were pretreated or un-pretreated with various concentration of MβCD or lovastatin for 1 h. After that the treated cells were washed three times with PBS, and disrupted by ultrasonication (three 10-sec bursts at room temperature). The cholesterol contents were then measured by using an Amplex Red cholesterol assay kit (Molecular Probes) as described in the manufacturer's instructions. The trypan blue staining was used to measure the effects of MβCD or lovastatin on cell viability as described previously (Lai, *et al.*, 2008).

Measurement of IL-8

To detect the secretion of IL-8 from AGS cells during *H. pylori* infection, the concentrations of IL-8 were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Lai, *et al.*, 2008). Briefly, AGS cells were pretreated with various concentrations of lovastatin (0 to 50 μM), nystatin (50 μg/ml), or CTX-B (20 μg/ml) in cell culture medium before *H. pylori* infection. The treated cells were then infected with *H. pylori* at a MOI of 100 for 6 h. The supernatants were collected and stored at −80°C before analysis. To measure IL-8 secretion from

AGS cells with transfection of various CagA mutants, all transfections were cultured for 24 h. As such, culture supernatants were harvested and stored at −80°C until further analysis. The level of IL-8 in supernatants from AGS cell cultures was determined by using a sandwich ELISA kit (R&D systems) according to the manufacturer's instruction.

Immunoprecipitation and immunoblotting

Untransfected and transfected HEK-293T cells were lysed in ice-cold immunoprecipitation buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF; Roche), 2 µg/ml aprotinin, and 2 µg/ml leupeptin. Cell lysates were centrifuged at 16,000 × *g* for 30 min. An aliquot of the resulting supernatant containing 1 mg total protein was subjected to immunoprecipitation at 4°C overnight, using 10 µg monoclonal mouse anti-Myc (9E10) according to manufacturer's instructions (Upstate Biotechnology). Precipitates were heated in the presence of SDS-PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.05% Brilliant blue R) at 100°C for 10 min, resolved by 10% SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% skim milk in TBS buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM $CaCl_2$) containing 0.01% Tween-20 at room temperature for 1 h and then incubated overnight with goat anti-CagA (1:2000) or mouse anti-phosphotyrosine (4G10; 1:2000) antibodies at 4°C. The blots were washed and then incubated with horseradish peroxidase (HRP)−conjugated secondary antibody (Millipore) at a dilution of 1:3000. Proteins of interest were visualized using the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia). To analyze

translocated or tyrosin-phosphorylated CagA in *H. pylori*-infected cells, AGS cells were pretreated with various concentrations of lovastatin and infection of cells with wild-type, ΔCagA, or ΔCagE *H. pylori*. Total cell lysates were subjected to immunoprecipitation using 10 µg polyclonal anti-CagA (Santa Cruz Biotechnology) following by immunoblot analysis of CagA and tyrosin-phosphorylated CagA as described above.

Isolation of detergent-soluble and detergent-resistant fractions

To isolate detergent-soluble and -resistant fractions, 1×10^6 HEK-293T cells were transfected with full-length CagA or the indicated deletion construct using Lipofectamine 2000 as described above. Transfected cells were lysed with ice-cold TNE buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 and incubated on ice for 30 minutes. Cell lysates were centrifuged at 18,000 × *g* at 4°C for 30 min to separate detergent-soluble and -resistant fractions as described previously (Simons & Toomre, 2000). Each fraction was assayed by immunoblotting.

Construction of CagA-EGFP expression plasmid

EGFP (enhanced green fluorescence protein) from pEGFP-C1 (Clontech) was cloned into pEF1 expression vector (Invitrogen) or CagA-FL-pEF1. Briefly, the EGFP was obtained from pEGFP-C1 digested with *EcoR*I and *Pml*I. The digested fragment was ligated into pEF1 or CagA-FL-pEF1, and the sequences were confirmed by DNA sequencing.

Immunofluorescence labeling of cells

To visualize localization of wild-type CagA and CagA deletion fragments in epithelial cells, AGS cells (0.5×10^6) were seeded on coverslips in 6-well plates and incubated for 20 h. Cells were

transfected with full-length CagA or the indicated CagA deletion construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After a 24-h incubation, cells were labeled with FITC-conjugated CTX-B (10 μg/ml) for 30 min, washed three times with PBS, and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) for at least one hour at 4°C. To label the full-length CagA or CagA deletion fragments, cells were incubated for at least 4 h with anti-CagA antibodies (Santa Cruz Biotechnology) followed by Cy3-conjugated anti-goat IgG (Santa Cruz Biotechnology). Samples were analyzed under a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss) with a 63× oil immersion objective.

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Lai CH, Chang YC, Du SY, *et al.* (2008) Cholesterol depletion reduces *Helicobacter pylori* CagA translocation and CagA-induced responses in AGS cells. *Infect Immun* **76**: 3293-3303.

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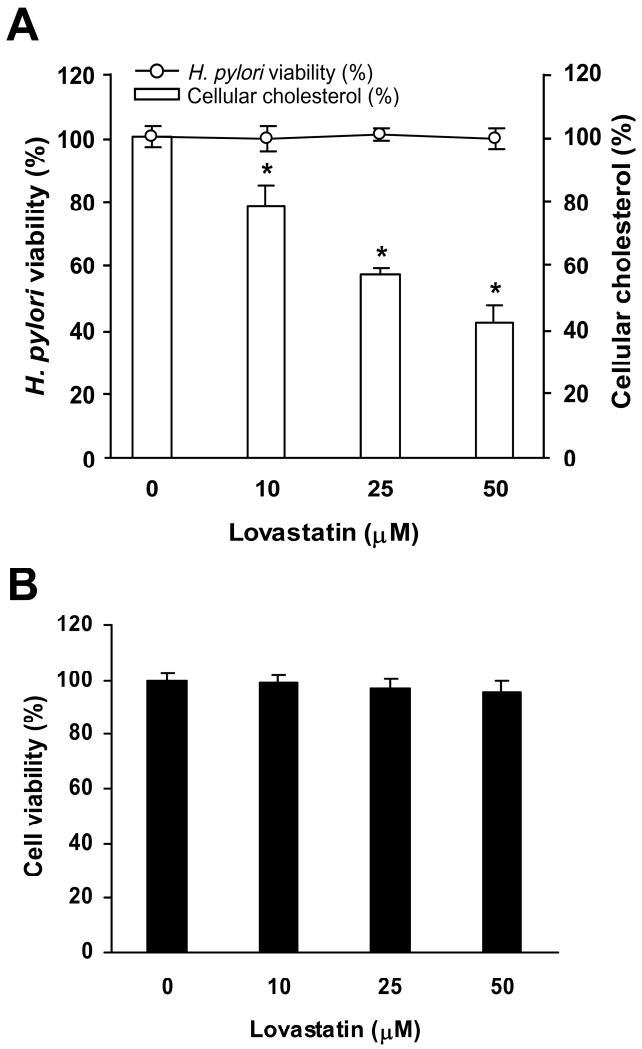

Fig. S1. The level of cellular cholesterol in AGS cells was reduced by treatment with lovastatin. (A) AGS cells were treated with various concentrations of lovastatin (0–50 μM) followed by infection with *H. pylori* at a MOI of 50 for 6 h. Bacterial suspension were plated onto Brucella blood agar plates and incubated for 3–4 days, after which the CFUs were counted for assessment of bacterial viability (open circle). Whole cell lysates were then prepared for cholesterol level analysis (open bar). (B) Cell viability was barely influenced after treatment with lovastatin, as determined by the trypan blue exclusion assay. Statistical significance was evaluated using Student's *t*-test (*, $P < 0.05$).

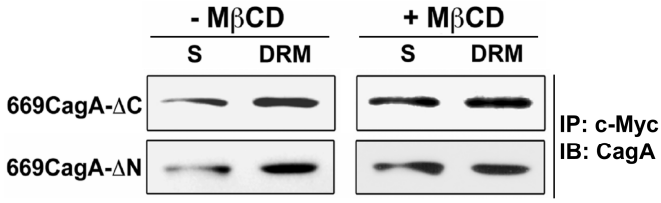

Fig. S2. Cellular cholesterol is essential for CagA CTD interacts with detergent-resistant membrane. Cells were transfected with 669CagA-ΔC or 669CagA-ΔN for 24 h and treated with or without 5 mM MβCD. The cell lysates were fractionated by the cold-detergent extraction using 1% Triton X-100, followed by centrifugation to separate detergent-resistant membrane (DRM) and detergent-soluble (S) fractions. Each fraction was subjected to IP and IB using anti-Myc and anti-CagA, respectively.

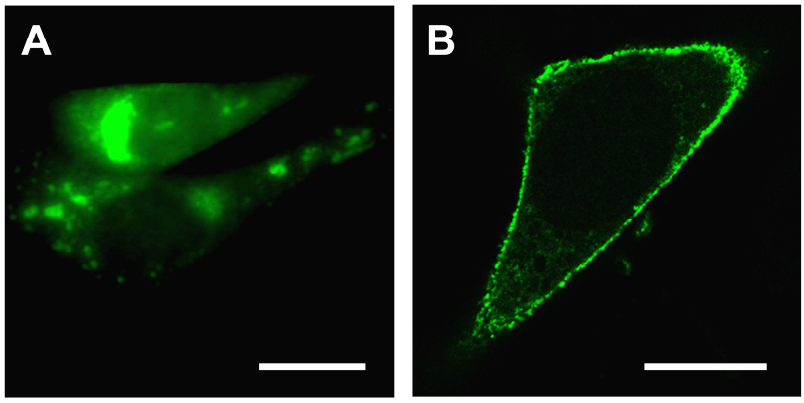

Fig. S3. Myc-tags do not influence CagA membrane localization. AGS cells were transfected with (A) EGFP-pEF1 or (B) CagA-EGFP-pEF1. After incubation for 24 h, cells were fixed and analyzed by confocal microscopy. (A) Transfection of cells with EGFP-pEF1, the EGFP was dispersedly distributed in the cytoplasm. (B) In the cells transfection with CagA-EGFP-pEF1, CagA was largely localized on the cell membrane. Scale bars, 10 μm.