RESEARCH LETTER

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

Chih-Ho Lai[1,2], Hung-Jung Wang[3], Yun-Chieh Chang[3], Wan-Chen Hsieh[3], Hwai-Jeng Lin[4], Chih-Hsin Tang[1], Jim Jinn-Chyuan Sheu[5], Chun-Jung Lin[1], Mei-Shiang Yang[1], Shu-Fen Tseng[2] & Wen-Ching Wang[3]

[1]Graduate Institute of Basic and Clinical Medical Science, School of Medicine, China Medical University, Taichung, Taiwan; [2]Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX, USA; [3]Institute of Molecular and Cellular Biology and Department of Life Sciences, National Tsing Hua University,Hsinchu, Taiwan; [4]Division of Gastroenterology and Hepatology, Department of Internal Medicine,Taipei Medical University Hospital, Taipei, Taiwan; and [5]Human Genetic Center, China Medical University Hospital, Taichung, Taiwan

Correspondence: Wen-Ching Wang, Institute of Molecular and Cellular Biology, National Tsing Hua University, No. 101 Sec. 2, Kung-Fu Road, 300 Hsinchu, Taiwan. Tel.: +886 3 5742766; fax: +886 3 5742766; e-mail: wcwang@mx.nthu.edu.tw

Received 14 May 2011; revised 5 July 2011; accepted 25 July 2011.
Final version published online 24 August 2011.

DOI: 10.1111/j.1574-6968.2011.02372.x

Editor: Arnoud van Vliet

Keywords
Helicobacter pylori; cytotoxin-associated gene A; cholesterol; interleukin-8.

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, induces 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, 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.

Introduction

Helicobacter pylori is a spiral-shaped Gram-negative bacterium that 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

FEMS MICROBIOLOGY LETTERS

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 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

Helicobacter pylori strains

Helicobacter 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). *Helicobacter 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 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 Fig. 3a. *cagA* fragments were amplified using 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 645–1186 using primers CagA-CTD59F and CagA-CTDR (Table 1). The primers used for PCR introduced a BamHI site at the 5′ end and an XbaI site at the 3′ end. The BamHI–XbaI 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 1–358 was amplified using primers CagA1-F and CagA-1R. The primers used for PCR introduced a BamHI site at the 5′ end and an EcoRI site at the 3′ end. The BamHI–EcoRI fragment was then inserted into pEF1 to derive pEF1-CagA1. A fragment encoding amino acids 357–707 was amplified using primers CagA2F and CagA2R. The primers used for PCR introduced an NdeI site at the 5′ end and an MfeI site at the 3′ end. After digestion, the NdeI–MfeI fragment was then inserted into the NdeI and MfeI 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 555–1186 was amplified using primers CagA-CTD69F and CagA-

Table 1. PCR primers used in this study

Primers	Nucleotide sequences (5′–3′)*	RE
CagA-CTD59F	GCGGGATCCAAATGGAAGCAAAAGCTCAAGCTAAC	BamHI
CagA-CTD42F	GCGGGATCCCAATGGGCGATTTCAGTAGGGTAGAG	BamHI
CagA-CTDR	GCGTCTAGAAGATTTTTGGAAACCACCTTTTG	XbaI
CagA1F	GCGGGATCCATGACTAACGAAACTATTGATC	BamHI
CagA1R	GCGGAATTCCTCGAGCATATGCACATTAATGAGTG	EcoRI
CagA2F	TGTGCATATGAAAAACGGCAGTG	NdeI
CagA2R	AGCCAATTGCTCCTTTGAGAAG	MfeI
CagACTD69F	GCGACTAGTTTCGTAAGGCGGAATTTAGAG	SpeI

*Underlined sequence indicates restriction enzyme (RE) sites.

CTDR. After digestion with BglII (at nucleotide 1851) and XbaI, the BglII–XbaI fragment was then inserted into the BglII and XbaI 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 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 (10–50 μM) and infected with 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

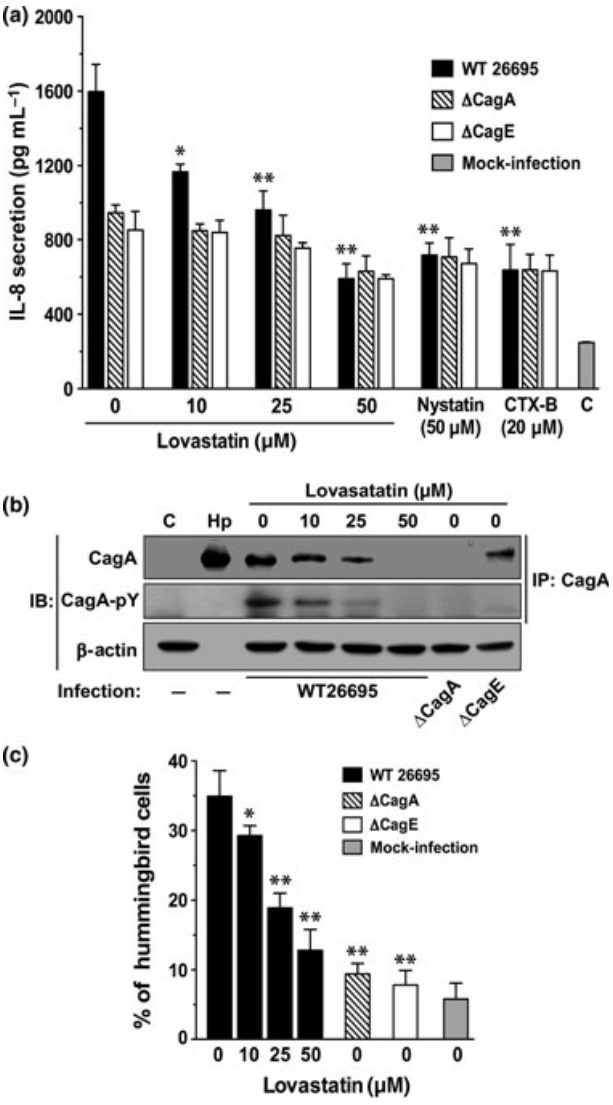

Fig. 1. *Helicobacter pylori* CagA-induced responses in AGS cells are dependent on the levels of cellular cholesterol. (a) AGS cells pretreated with lovastatin (0–50 μM), nystatin (50 μg mL^{-1}), or CTX-B (20 μg mL^{-1}), subsequently infected with wild-type, ΔCagA, or ΔCagE *Helicobacter pylori*. After incubation for 6 h, the concentration of IL-8 in the culture supernatant was analyzed using 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 *Helicobacter pylori*-infected cells was inhibited by treatment with lovastatin. C, untreated cells; mock-infection, uninfected cells; Hp, bacterial lysate; WT 26695, wild-type *Helicobacter 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.

whether cholesterol was necessary for CagA-mediated IL-8 secretion by use of two CagA functional deficiency mutants (ΔCagA and ΔCagE). When compared with 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 being 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.

Helicobacter 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) that 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 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.

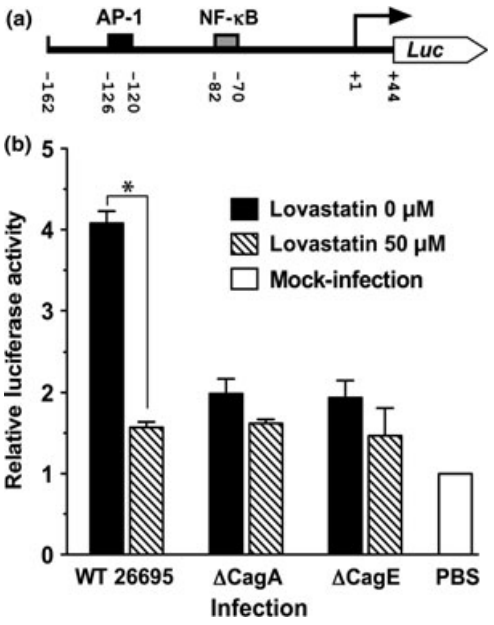

Fig. 2. Cholesterol is required for *Helicobacter pylori* CagA-induced *IL-8* promoter activity. (a) A schematic representation of IL8-Luc construct. AP-1, activator protein-1; NF-κB, nuclear factor-kappaB; Luc, luciferase reporter. In all numbering, the transcription initiation site is denoted by +1.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

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

We then sought to investigate whether the C-terminal domain (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 threefold increase in luciferase activity compared with 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 when 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 CagA-FL or CagA-ΔN induced

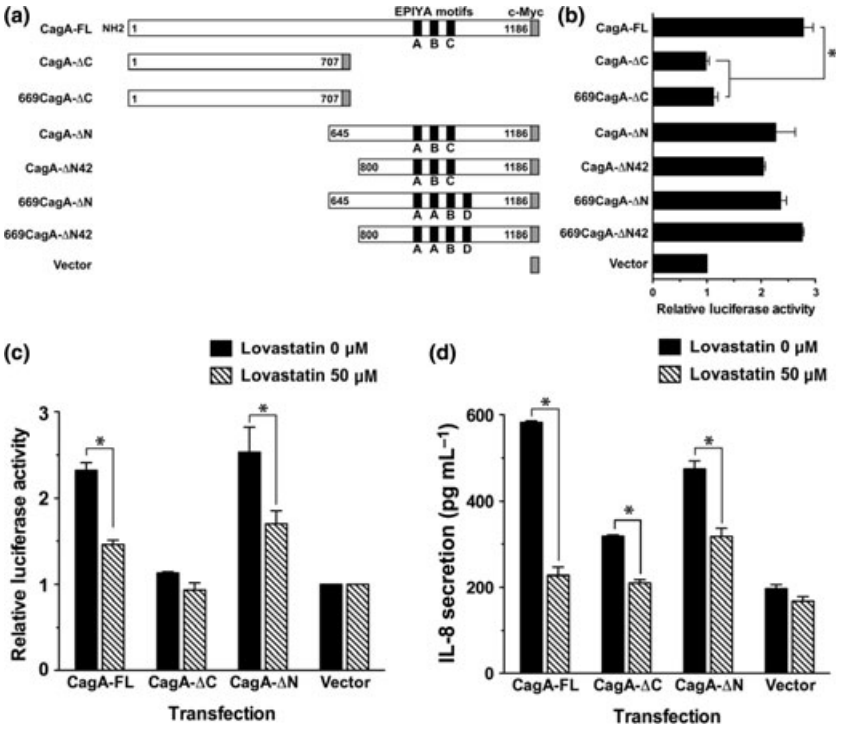

Fig. 3. The CagA C-terminal domain containing EPIYAs is crucial for the CagA-induced IL-8 promoter activation (b and c) and IL-8 secretion (d) in AGS cells. (a) Schematic representation of wild-type 26695 (ABC-type EPIYAs) and a series of truncated mutants based on wild-type 26695 and v669 isolate (AABD-type 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 (*$P < 0.05$).

significantly higher IL-8 production than vector alone. 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. Figure 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 using a cold-detergent extraction method to isolate 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 not known 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 Fig. 4b, CagA-ΔN was primarily localized in DRM (~82%) in the absence of the MβCD treatment, but shifted toward 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

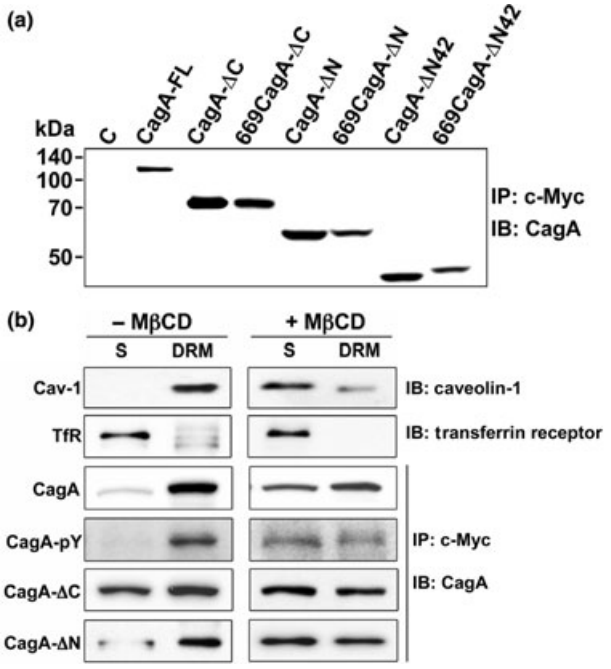

Fig. 4. CagA C-terminal domain interacts with cholesterol-rich microdomains. (a) Detection of different CagA expression in HEK-293T cells. Cell lysates were prepared and subjected to immunoprecipitation (IP) using anti-Myc antibody followed by immunoblotting (IB) analysis using anti-CagA antibody. Molecular 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 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 TfR antibodies were used to mark DRM and S fractions, respectively.

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 affect CagA membrane localization (Fig. S3). Cells were then transfected with CagA-FL, and CagA (red) and GM1 (green) were co-localized around in the plasma membrane (Fig. 5, first row). An analogous pattern was seen for CagA-ΔN-transfected cells (Fig. 5, second 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, third 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 madin-darby kidney cells (MDCK), 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 N-terminal domain (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

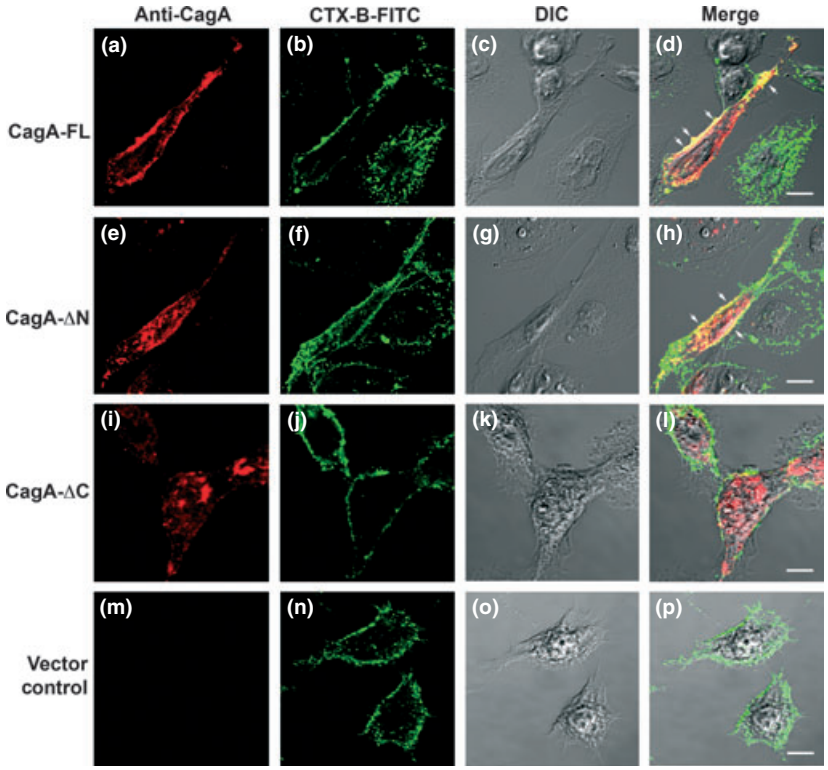

Fig. 5. The CagA C-terminal domain targets to membrane GM1-containing microdomains in confocal images. AGS cells were transfected with CagA-FL, CagA-ΔN, CagA-ΔC expression vectors, or empty vectors, respectively, as indicated. The transfected cells were fixed and stained with anti-CagA (red) and FITC-conjugated CTX-B to visualize GM1 (green) and analyzed using 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.

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.* (1994) reported an epidemiological association between *H. pylori* infection and coronary heart diseases. 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.

Acknowledgements

This work was supported by the National Science Council, Taiwan (NSC97-3112-B-007-005, 97-2313-B-039-003-MY3, 98-3112-B-007-004), China Medical University, Taiwan (CMU97-116, 97-346), and the Tomorrow Medical Foundation. We thank Shu-Chen Shen (Agricultural Biotechnology Research Center, Academia Sinica) for confocal microscopy analysis, and Yu-Ting Sing, Min-Chuan Kao, and Jo-Han Tseng for their expert technical assistance. None of the authors had any conflicts of interests.

Authors' contribution

H.-J.W. is co-first author.

References

Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ & Falkow S (2003) Disruption of the epithelial apical–junctional complex by *Helicobacter pylori* CagA. *Science* **300**: 1430–1434.

Anderson HA, Chen Y & Norkin LC (1996) Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. *Mol Biol Cell* **7**: 1825–1834.

Argent RH, Kidd M, Owen RJ, Thomas RJ, Limb MC & Atherton JC (2004) Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. *Gastroenterology* **127**: 514–523.

Armitage J (2007) The safety of statins in clinical practice. *Lancet* **370**: 1781–1790.

Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, Naumann M & Meyer TF (2000) Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* **2**: 155–164.

Bagnoli F, Buti L, Tompkins L, Covacci A & Amieva MR (2005) *Helicobacter pylori* CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *P Natl Acad Sci USA* **102**: 16339–16344.

Brown DA & London E (1998) Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* **14**: 111–136.

Chang YJ, Wu MS, Lin JT, Pestell RG, Blaser MJ & Chen CC (2006) Mechanisms for *Helicobacter pylori* CagA-induced cyclin D1 expression that affect cell cycle. *Cell Microbiol* **8**: 1740–1752.

Eck M, Schmausser B, Haas R, Greiner A, Czub S & Muller-Hermelink HK (1997) MALT-type lymphoma of the stomach is associated with *Helicobacter pylori* strains expressing the CagA protein. *Gastroenterology* **112**: 1482–1486.

Endo A (1981) 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors. *Methods Enzymol* **72**: 684–689.

Gunn M, Stephens JC, Thompson JR, Rathbone BJ & Samani NJ (2000) Significant association of *cagA* positive *Helicobacter pylori* strains with risk of premature myocardial infarction. *Heart* **84**: 267–271.

Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M & Hatakeyama M (2002) SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**: 683–686.

Higashi H, Yokoyama K, Fujii Y, Ren S, Yuasa H, Saadat I, Murata-Kamiya N, Azuma T & Hatakeyama M (2005) EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. *J Biol Chem* **280**: 23130–23137.

Hutton ML, Kaparakis-Liaskos M, Turner L, Cardona A, Kwok T & Ferrero RL (2010) *Helicobacter pylori* exploits cholesterol-rich microdomains for induction of NF-kappaB-dependent responses and peptidoglycan delivery in epithelial cells. *Infect Immun* **78**: 4523–4531.

Kwok T, Zabler D, Urman S *et al.* (2007) *Helicobacter* exploits integrin for type IV secretion and kinase activation. *Nature* **449**: 862–866.

Lai CH, Kuo CH, Chen YC, Chao FY, Poon SK, Chang CS & Wang WC (2002) High prevalence of *cagA*- and *babA2*-positive *Helicobacter pylori* clinical isolates in Taiwan. *J Clin Microbiol* **40**: 3860–3862.

Lai CH, Chang YC, Du SY, Wang HJ, Kuo CH, Fang SH, Fu HW, Lin HH, Chiang AS & Wang WC (2008) Cholesterol depletion reduces *Helicobacter pylori* CagA translocation and CagA-induced responses in AGS cells. *Infect Immun* **76**: 3293–3303.

Leitinger B & Hogg N (2002) The involvement of lipid rafts in the regulation of integrin function. *J Cell Sci* **115**: 963–972.

Marshall B (2002) *Helicobacter pylori*: 20 years on. *Clin Med* **2**: 147–152.

Mendall MA, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Camm AJ & Northfield TC (1994) Relation of *Helicobacter pylori* infection and coronary heart disease. *Br Heart J* **71**: 437–439.

Mimuro H, Suzuki T, Tanaka J, Asahi M, Haas R & Sasakawa C (2002) Grb2 is a key mediator of *Helicobacter pylori* CagA protein activities. *Mol Cell* **10**: 745–755.

Murata-Kamiya N, Kikuchi K, Hayashi T, Higashi H & Hatakeyama M (2010) *Helicobacter pylori* exploits host membrane phosphatidylserine for delivery, localization, and pathophysiological action of the CagA oncoprotein. *Cell Host Microbe* **7**: 399–411.

Naroeni A & Porte F (2002) Role of cholesterol and the ganglioside GM(1) in entry and short-term survival of *Brucella suis* in murine macrophages. *Infect Immun* **70**: 1640–1644.

Nusrat A, Parkos CA, Verkade P, Foley CS, Liang TW, Innis-Whitehouse W, Eastburn KK & Madara JL (2000) Tight junctions are membrane microdomains. *J Cell Sci* **113**: 1771–1781.

Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W & Haas R (2000) Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* **287**: 1497–1500.

Pear WS, Nolan GP, Scott ML & Baltimore D (1993) Production of high-titer helper-free retroviruses by transient transfection. *P Natl Acad Sci USA* **90**: 8392–8396.

Pike LJ, Han X, Chung KN & Gross RW (2002) Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry* **41**: 2075–2088.

Poppe M, Feller SM, Romer G & Wessler S (2007) Phosphorylation of *Helicobacter pylori* CagA by c-Abl leads to cell motility. *Oncogene* **26**: 3462–3472.

Segal ED, Cha J, Lo J, Falkow S & Tompkins LS (1999) Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *P Natl Acad Sci USA* **96**: 14559–14564.

Simons M, Kramer EM, Macchi P, Rathke-Hartlieb S, Trotter J, Nave KA & Schulz JB (2002) Overexpression of the

myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus–Merzbacher disease. *J Cell Biol* **157**: 327–336.

Singh RK, McMahon AD, Patel H, Packard CJ, Rathbone BJ & Samani NJ (2002) Prospective analysis of the association of infection with CagA bearing strains of *Helicobacter pylori* and coronary heart disease. *Heart* **88**: 43–46.

Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ & Covacci A (2002) c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol* **43**: 971–980.

Wunder C, Churin Y, Winau F, Warnecke D, Vieth M, Lindner B, Zahringer U, Mollenkopf HJ, Heinz E & Meyer TF (2006) Cholesterol glucosylation promotes immune evasion by *Helicobacter pylori*. *Nat Med* **12**: 1030–1038.

Zeaiter Z, Cohen D, Musch A, Bagnoli F, Covacci A & Stein M (2008) Analysis of detergent-resistant membranes of *Helicobacter pylori* infected gastric adenocarcinoma cells reveals a role for MARK2/Par1b in CagA-mediated disruption of cellular polarity. *Cell Microbiol* **10**: 781–794.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Materials and Methods.
Fig. S1. The level of cellular cholesterol in AGS cells was reduced by treatment with lovastatin.
Fig. S2. Cellular cholesterol is essential for CagA CTD interaction with detergent-resistant membrane.
Fig. S3. Myc-tags do not influence CagA membrane localization.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.