中 國 醫 藥 大 學
基礎醫學研究所
碩士學位論文

胃幽門螺旋桿菌 CagA 上 EPIYA 重複區域與脂質筏的交互作用誘導 IL-8 分泌

Helicobacter pylori CagA EPIYA repeat regions interact with lipid raft for CagA-induced IL-8 activity

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中 華 民 國 100 年 4 月

誌謝

時光飛逝，研究所的兩年時光即將劃上句點，在基礎醫學研究所的大家庭中，不論遇到多少喜怒哀樂，身邊總有許多師長、同學及朋友的分享、幫助、鼓勵及陪伴，我才得以有今日的成果。

研究的時光匆匆，首先要感謝我的指導老師 賴志河副教授，感謝老師在學業上的教導與支持，更不厭其煩的與我討論實驗的進行方向，每一次的討論不僅僅使得研究流程更加順暢，老師更鼓勵我多提出自己新穎的想法，使得論文的內容更加充實與完整。同時，也要感謝基醫所 黃志揚所長及熱心的老師們在課業上及研究上給予的幫助與鼓勵，因為您們的支持與打氣，讓我在研究所的旅途中，備感溫馨。

在此，也要感謝這兩年一起打拼過來的同儕，雖然研究所只有這短短兩年，但在你們的陪伴下，才能有這些愉快且美好的回憶；謝謝醫學系藥理學科 湯智昕老師、分子系統生物醫學研究所 馬明琪老師、 張皓騰老師，在研究期間，受到他們及實驗室成員的幫忙及協助;謝謝永國學長在動物實驗時給予的指導與幫忙；也要謝謝實驗室裡的夥伴: 郁婷、梅湘、婉琦、思佑…，因為你們的提攜照顧，我才能有今天的甜美成果；另外，也要感謝所辦秘書麗如姐，在這基醫所的大家庭中，溫柔的看照著我們，讓我們無後顧之憂的往自己的路前進。

最後，我要感謝我的家人，感謝我的父母默默的在背後支持著我，適時的給予我鼓勵，並讓我知道你們以我為榮，謝謝弟弟妹妹的激勵，讓我知道我能夠做的更好，謝謝外婆及奶奶的加油打氣，因為有他們在背後為我撐腰，我才能夠這麼有自信的一路走來。在這研究的路上，要向太多太多的恩人及貴人致謝，未來的路還很漫長，我會永遠記得你們的提攜與鼓勵，繼續堅持下去!

謹以此文，獻給我感謝的大家。

林君榕 謹誌

中華民國一〇〇年七月

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中文摘要 (Abstract in Chinese)

胃幽門螺旋桿菌 (*Helicobacter pylori*)在近年來的研究中，被認為與人類胃部疾病相關，長期的感染會造成人類胃部發炎、胃潰瘍、十二指腸潰瘍，甚至腸胃道癌症及淋巴相關淋巴癌。胃幽門螺旋桿菌的毒力因子主要分為兩大類，第一類與細菌的黏附能力相關，如: 鞭毛；第二類則造成胃部組織的受損，如: Vacuolating toxin A (VacA)， Cytotoxin-associated gene A (CagA)等。研究指出，長期感染胃幽門螺旋桿菌，會使胃部細胞 nuclear factor (NF)−κB 活化，進一步促進細胞分泌 IL-8，產生發炎反應，而長期的胃部發炎就是導致胃部疾病的主要原因。

CagA 毒素是被幽門螺旋桿菌送入細胞體中的毒素之一，幽門螺旋桿菌中的 *cag PAI* gene 能夠製造第四型分泌系統 (Type IV Secretion System)，與 CagA 蛋白。當 CagA 蛋白透過第四型分泌系統進入細胞後，會與 Src family kinase 作用，造成 CagA 上 C 端的 EPIYA motif (Glu-Pro-Ile-Tyr-Ala) 被磷酸化，隨著訊息傳遞路徑，導致細胞型態改變，形成 hummingbird phenotypes。在我們的研究中發現，CagA 促使細胞分泌 IL-8，需要 activator protein-1 (AP-1) promoter 及 NF-κB promoter 兩個共同作用才能誘發，並且 CagA 所造成的訊息傳遞路徑，需要 cholesterol 的參與。另外，我們使用了 CagA 的缺失序列，進一步探討位於 CagA 上 EPIYA motif 所扮演的角色，研究結果也指出，CagA 上的 EPIYA motif 的存在對於細胞內 CagA 趨向細胞膜上的 lipid rafts 區域是重要的，並且 EPIYA motif 對於 CagA 誘發細胞分泌 IL-8 是必須存在的。因此，在我們的研究成果中，說明了 CagA 上的 EPIYA motifs 能夠促使細胞中的 CagA 與細胞膜上的 lipid rafts 作用，進一步促使訊息傳遞，造成細胞的變形及發炎反應。

關鍵字: 胃幽門螺旋桿菌，細胞毒性相關基因 A (cytotoxin-associated gene A), 細胞核轉錄因子-κB (nuclear factor -κB), 細胞白借素第八因子(interleukin-8), 膽固醇, 脂質筏

Abstract

The infection of *Helicobacter pylori* was thought to be related with human gastric diseases including peptic ulcer, lymphoma, gastric atrophy, and adenocaecinoma. Upon infection of gastric epithelial cells, *Helicobacter pylori* cytotoxin-associated gene A (CagA) is injected into epithelial cells via the type IV secretion system, which is dependent on cholesterol. Translocated CagA then co-localizes with the lipid raft marker GM1 and interacts with c-Src in which a tyrosine residue in the CagA Glu-Pro-Ile-Tyr-Ala (EPIYA) repeat region can be phosphorylated. In the present study, we found that CagA induction of IL-8 promoter activity in gastric epithelial cells was dependent on both of the presence of activator protein-1 (AP-1) and nuclear factor (NF)-κB binding sites, and cholesterol plays a crucial role in the pathway. Additionally, the EPIYA repeat region in the C-terminal domain was indispensable for CagA-induced interleukin *(IL)-8* promoter activity, and this activity was dependent on cholesterol. Using multiple CagA truncation constructs, we showed that the C-terminal domain containing the EPIYA repeats was responsible for CagA-induced *IL-8* promoter activity and for raft association. Our results suggest the importance of the EPIYA repeat region for interaction of CagA with lipid rafts and for CagA-induced pathogenesis.

Key words: *Helicobacter pylori*, cytotoxin-associated gene A (CagA), nuclear factor (NF)-κB, interleukin (IL)-8, cholesterol, lipid rafts

Introduction

Helicobacter pylori is a spiral shaped, gram-negative bacterium which inhabited in human stomach lining and infected nearly 3 billions people around the world (Marshall 2002; Zhou, Yamazaki et al. 2004). The bacterium was thought to transmit by close contact with carriers in childhood (Rothenbacher, Bode et al. 1999; Miyaji, Azuma et al. 2000). Persistent infection of *H. pylori* causes interleukin(IL)-8 expression through NF-kappa(κ)B activation and leads to pro-inflammatory effects which may induce chronic gastritis, duodenal and peptic ulcer, lymphoid tissue lymphoma, and even gastric adenocarcinoma (Eck, Schmausser et al. 1997; Covacci, Telford et al. 1999; Kuipers 1999; Brandt, Kwok et al. 2005).

The virulence factors of *H. pylori* have been studied in decades. Virulent strains carry the *cag* pathogenicity island (*cag* PAI), which encodes members of the type four secretion system (TFSS), and an immunodominant antigen called cytotoxin-associated gene A (CagA) (Backert, Ziska et al. 2000; Odenbreit, Puls et al. 2000; Stein, Rappuoli et al. 2000). Upon infection, *H. pylori* injects the CagA protein into host cell via the TFSS and this process was thought to mediate through the interaction between CagL and intergrins (Segal, Cha et al. 1999; Odenbreit, Puls et al. 2000; Kwok, Zabler et al. 2007). Subsequently, CagA undergoes a tyrosine phosphorylation at the EPIYA motif (Higashi, Yokoyama et al. 2005), which was mediated by Src homology 2 (SH2) and Abl kinase (Odenbreit, Puls et al. 2000; Selbach, Moese et al. 2002). Phosphorylated CagA then interacts with tyrosine phosphatase SHP-2 and regulates SHP-2 phosphatase activity (Higashi, Tsutsumi et al. 2002), which stimulates the RAS/ERK pathway and induces “hummingbird” phenotype and cell proliferation (Mimuro, Suzuki et al. 2002). Recently, several studies have been demonstrated that CagA can interact with partitioning-defective 1b (PAR1b)/microtubule

affinity-regulating kinase 2 (MAPK2), and inhibit their activity, resulting in disruption of cell membrane tight junctions as well as apical-basolateral polarity in polarized epithelial cell, such as MDCK cells (canine kidney tubular epithelial cell line) (Saadat, Higashi et al. 2007; Zeaiter, Cohen et al. 2008). On the other hand, in non-polarized epithelial cells, like human gastric adenocarcinoma AGS cells, CagA promotes disassembly of focal adhesions, which arises morphogenetic activity (Li, Chen et al. 2009). CagA-mediated inhibition of PAR1b influences microtubules stability and causes microtubule-based spindle dysfunction and subsequent disorientation (Umeda, Murata-Kamiya et al. 2009). Meanwhile, the increase frequency of gastrointestinal and hematological malignancies observed in transgenic mice carrying *cagA* suggested that CagA acts as a bacterial oncogene in mammals (Ohnishi, Yuasa et al. 2008; Hatakeyama 2009).

Helicobacter pylori escapes from human immune surveillance through multiple ways, one is by assimilating and modifying cellular cholesterol, and lipid rafts, which plays a dynamic role of microdomains in the exoplasmidc leaflet of cellular bilayer membrane (Brown and London 1998; Hooper 1999). Of interest, upon *H. pylori* infection, translocation of CagA, as well as delivery of vacuolating cytotoxin A (VacA), into host cell also requires lipid rafts (Ricci, Galmiche et al. 2000; Schraw, Li et al. 2002; Kuo and Wang 2003; Gauthier, Monzo et al. 2005; Nakayama, Hisatsune et al. 2006; Lai, Chang et al. 2008). In previously known *in vitro* studies, the integrity of lipid rafts is usually preserved using the cold-detergent extraction method by using non-ionic detergents such as Triton X-100 (Brown and Waneck 1992), whereas disruption of lipid rafts is preformed using the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) (Simons, Kramer et al. 2002)). Analysis of the protein in detergent-resistant membranes (DRMs) indicates that CagA alters both the expression and the cellular distribution of host protein in lipid rafts (Zeaiter, Huynh et

al. 2008), and that CagA co-fractionates with DRMs (Lai, Chang et al. 2008). CagA-induced IL-8 production in infected AGS cells is also blocked by cholesterol depletion (Lai, Chang et al. 2008). Base on previous study by Higashi *et al.*, the authors indicated that a single Glu-Pro-Ile-Tyr-Ala (EPIYA) motif in the C-terminal of CagA was crucial for CagA membrane localization (Higashi, Yokoyama et al. 2005). Interestingly, the EPIYA-containing region, or the EPIYA repeat region, is quite polymorphic. Analysis of various clinical *H. pylori* isolates revealed that this region typically contains three to five EPIYA repeats (Higashi, Tsutsumi et al. 2002; Azuma, Yamakawa et al. 2004). There are four types of EPIYA motifs that have been classified: A, B, C, and D; and the EPIYA-C motif were presented mostly in the western population while the EPIYA-D motif was presented in the East Asian. Additionally, clinical isolates carrying various EPIYA repeats also showed differing degrees of tyrosine phosphorylation as well as the risk of gastric carcinogenesis (Higashi, Tsutsumi et al. 2002; Higashi, Tsutsumi et al. 2002; Naito, Yamazaki et al. 2006; Basso, Zambon et al. 2008; Jones, Joo et al. 2009). Although the delivery of CagA with more phosphorylation motif was found to induce a higher level of phosphorylation state within epithelial cells, however, the actual mechanism of lipid rafts in membrane tethering of CagA remains unclear. In this study, we demonstrated the effects of various CagA truncation mutants on the association between CagA and lipid rafts by using membrane fractionation method and immunocytochemistry, and using an *IL-8* promoter activity assay to determine the IL-8 induction. Here, we provide evidence that the CagA C-terminal EPIYA-containing region is targeted to membrane raft microdomain, which allows CagA-mediated induction of IL-8.

Materials & Methods

Antibodies and materials

CagA polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphotyrosine (4G10) antibody was purchased from Millipore (Bedford, MA). Myc (9E10) and actin mouse monoclonal antibodies were from Upstate Biotechnology (Billerica, MA). Alexa Fluor 488-conjugated CTX-B, Alexa Fluor 555-conjugated CTX-B, and DAPI were from Molecular Probes (Invitrogen, Carlsbad, CA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA), and the luciferase assay kit was from Promega (Madison, WI). The *IL-8* promoter constructs, including the wild type construct (IL-8/wt; nucleotides −162 to +44), the AP-1 site mutation construct (IL-8/ΔAP-1), and the κB site mutation construct (IL-8/ΔκB), were obtained from Dr. Chih-Hsin Tang (Department of Pharmacology, China Medical University) (Fong, Maa et al. 2008).

Bacterial strain and cell culture

AGS cells (ATCC CRL 1739) were cultured in F12 medium (HyClone, Logan, UT) and HEK-293T cells (ATCC CRL 11268) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone). All culture medium was supplemented with 10% complement-inactivated fetal bovine serum (FBS; HyClone) and maintained at 37°C. For transient transfection, cells were cultured in 12-well plates and incubated in the 2 ml OPTI-MEM (Invitrogen) with the mixture of 4 μg total DNA and 4 μl of Lipofectamine 2000 for 6 h at 37°C. Transfected cells then cultured with complete medium overnight for further experiments.

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, Yokoyama et

al. 2005). Clinical strain v669 was isolated from a patient with gastric cancer (Lai, Kuo et al. 2002), which contains a CagA gene with four C-terminal EPIYA motifs (AABD-type). Construction of the *cagA* knock-out strain derived from *H. pylori* 26695 (ΔCagA) was performed using the kanamycin resistance cassette (Km^r) from pACYC177 (Rose 1988) by the natural transformation method described as Wang *et al*. (Wang, Zinn et al. 1993). *H. pylori* strains were recovered from frozen stocks on Brucella agar plates (Becton Dickinson, Franklin Lakes, NJ) containing 10% sheep blood. Storage and cultivation of *H. pylori* strains was performed as described (Yang, Wang et al. 1997; Kuo, Poon et al. 1999).

Construction of CagA expression plasmids

Expression constructs encoding CagA truncation mutants were generated based on the *H. pylori* 26695 *cagA* sequence (ABC type) and v669 (AABD type) as illustrated (Lai, Kuo et al. 2002). The *cagA* fragments were amplified by PCR from *H. pylori* strain 26695 and v669 genomic DNA as previous described (Lai, Kuo et al. 2002). The CagA ΔN mutant was generated from strain 26695 by amplification of sequence encoding amino acids 645 to 1186 using primers pEF1_CagA_CTD59_F and pEF1_CagA_CTD_R (Table 1). The primers used for PCR introduced a *BamH*I site at the 5' end and an *Xba*I site at the 3'end. Subsequently, the *BamH*I–*Xba*I fragment was ligated into pEF1 expression vector (Invitrogen, Carlsbad, CA) to produce the cagA ΔN mutant truncation. Similar procedures were used to obtain the vCagA ΔN mutant from strain v669, and by using pEF1_CagA_CTD42_F and pEF1_CagA_CTD_R primers.

To generate the CagA ΔC mutant, a fragment encoding amino acids 1 to 358 was amplified using primers pEF1_CagA1_F and R. 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 vector. A fragment encoding amino acids 357 to 707 was amplified using primers pEF1_CagA2_F and R. The primers used for PCR introduced an *Nde*I site at the 5' end and an *EcoR*I site at the 3'end. After digestion, the *Nde*I-*EcoR*I fragment was then inserted into the *Nde*I and *EcoR*I sites of pEF1-CagA1 to obtain the CagA ΔC mutant. To generate the full-length CagA construct, CagA CTD69, a fragment encoding amino acids 555 to 1186 was amplified using primers pEF1_CagA_CTD69_F and CagA_CTD_R. 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.

Detection of cellular cholesterol and cell viability assay

Cells were treated 1 h with/without various concentration of MβCD or lovastatin, and then cells were washed 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, Eugene, Oregon) as described as manufacturer's instructions. The crystal violet staining was used to measure the cell viability after cells treated with MβCD or lovastatin as described previously (Lai, Chang et al. 2008).

Immunoprecipitation and western blot

HEK-293T cells with and without transfection 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, Indianapolis, IN), 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, Billerica, MA). 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, Billerica, MA). The membranes were blocking 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, Little Chalfont, UK).

Immunofluorescence labeling of CagA-transfected cells

To visualize localization of wild-type CagA and CagA deletion fragments in epithelial cells, AGS cells (5×10^6) were seeded on coverslips in six-well plates and incubated overnight. Cells were then transfected with full-length CagA or the indicated CagA deletion construct using Lipofectamine 2000 according to the manufacturer's protocol. After 24 hours incubation, cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for at least one hour at 4°C. The cells were then permeabilized with 0.1% Triton X-100 for 30 min and stained with Alexa Fluor 488-conjugated CTX-B (5 μg/ml; Sigma-Aldrich). To label the full-length CagA or CagA deletion fragments, samples were incubated for 30min with anti-CagA (1:200), followed by fluorescein isothiocyanate

(FITC)-conjugated anti-goat IgG (1:200; Millipore). Samples were analyzed by confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss, Göttingen, Germany) with a 100× objective (oil immersion, aperture 1.3).

Isolation of detergent-soluble and detergent-resistant membrane fractions

To isolate detergent-soluble and -resistant fractions, 1×10^6 HEK-293T cells were transfected with full-length CagA or deletion construction plasmids by 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 and Toomre 2000). Each fraction was assayed by western blotting.

Transfection of IL-8 promoter constructs and Luciferase assay

AGS cells were grown to 90% confluence in 12-well plate. IL-8-Luc plasmid was transfect by using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen), after 6 hours of 37°C incubation, change the medium of fresh F12 medium with 10% fetal bovine serum and incubate overnight to further experiments.

To prepare cell lysates, 100 μl of reporter lysis buffer (Promega, Madison, WI) was added to each well, and samples were collected from dish by scraper. Supernatants were generated by 13000 *g* centrifugation for 10 min. The luciferase assay was performed by mixing 20 μl of supernatants and 80 μl luciferase substrate (Promega), and the luminescence was measured by using a microplate luminometer (Biotek, Winooski, VT). 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).

Determination of IL-8 secretion

After the treatment and incubated for assigned time, culture medium were centrifuged at 13000 *g* for 10 min, and the supernatant were collected and stored at -80℃ to further analysis.

Collected supernatant were diluted 1:10 with assay buffer right before the assay, and the IL-8 secretion in culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (Invitrogen, Carlsbad, CA), in brief, the anti-human IL-8 antibody was coated at 96 well plate at 4 °C overnight, and each well were blocked by assay buffer at room temperature for 1 hr to further process. Coated wells then washed with washing buffer and the standard reagent and diluted samples were added in indicated well, following that, wells then added with detection antibody, and plate was incubated at room temperature for 2 h with 700 *rpm* shaking. After incubation, aspirate wells and wash 5 times and each well then added streptavidin-HRP solution, incubate for 2 h at room temperature with continual shaking (700 *rpm*), subsequently, washed 5 times then added the TMB substrate, incubated for 30 min with shaking, finally added the stop solution and measure absorbance at 450 nm with reference absorbance at 650 nm within 30 min of adding stop solution.

Statistical analysis

Student's *t*-teat was used to estimate the statistical significance of the experimental results for two groups, and a P value of <0.05 was considered significant difference.

Results

1. Phosphorylation of CagA was correlated with cholesterol concentration in a dose-dependent manner

Upon infection of *H. pylori*, CagA was translocated into host cells, where it undergoes a tyrosine phosphorylation and subsequently induces IL-8 production (Brandt, Kwok et al. 2005). Additionally, cell lipid rafts were thought to involve in the process. Lipid rafts were a cholesterol-rich membrane microdomains , thus, in order to investigate whether lipid rafts participate in the translocation of CagA, cells were treated with lovastatin, a 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor, which may reduce the production of cholesterol in cells. Cells were treated with 50 μg/ml of Nystatin, which is a cholesterol depleting agent, and 20 μg/ml of Cholera enterotoxin subunit B (CTX-B), which is known to compete with the raft binding sites on the cell membrane. After 30 mins of treatment, cells were then infected with different types of *H. pylori*, wild type, *cagA* knockout mutant (ΔCagA), and *cagE* knockout mutant (ΔCagE) strains. As CagE protein worked as an ATPase in the TFSS, providing the energy for the translocation of substrates like CagA protein (Bourzac and Guillemin 2005). Following the experiment, cultured supernatant was utilized to analyze the production of IL-8 **(Figure 1A)**, and total protein was analyzed by western blotting to visualize the amount of CagA protein within cells **(Figure 1B)**. When AGS cells were pretreated with different concentration of lovastatin (0 to 50 μM), the levels of total CagA and phosphorylated CagA both decreased in a dose-dependent pattern, and reach a significant decrease at 50 μM concentration of lovastatin. On the other hand, no phosphorylated CagA signal was detected in cells that infected with ΔCagA and ΔCagE strains. Taken together, the amount of CagA translocation and tyrosine-phosphorylation were correlated with cholesterol

concentration in cells. After the translocation and tyrosine-phosphorylation of CagA, phosphorylated CagA then induces a signaling pathway which subsequently leads to hummingbird phenotype (Segal, Cha et al. 1999; Bagnoli, Buti et al. 2005). Thus, when cells were pretreated with 50 μM of lovastatin, the hummingbird phenotype significantly decreased, comparing with cells treated with wile-type *H. pylori* **(Figure 1C, 1D)**. Moreover, cells treated with either ΔCagA or ΔCagE *H. pylori* shown a minor amount of hummingbird phenotype, which indicated that, the translocation and the phosphorylation of CagA require a functional TFSS, and cholesterol is also participated in this process.

2. Induction of IL-8 is dependent both on AP-1 and NF-κB binding sites in the IL-8 promoter activity

In a previous report (Chang, Wu et al. 2006), CagA can induce AP-1 activation, resulting in the enhancement of cyclin D1 expression. Additionally, NF-κB is found to be involved in CagA-induced IL-8 release (Brandt, Kwok et al. 2005; Kim, Lee et al. 2006; Hutton, Kaparakis-Liaskos et al. 2010). Therefore, we sought to evaluate whether NF-κB or AP-1 signaling pathways were participated in CagA-induced IL-8 expression by using luciferase (luc) reporter assay (Chang, Wu et al. 2006). AGS cells were transfected with multiple types of human IL-8 promoter-luc constructs, including IL-8/wt (containing both AP-1 and NF-κB promoter binding sites), IL-8/ΔAP-1 (containing only NF-κB promoter binding site), and IL-8/ΔkB (containing only AP-1 promoter binding site), as shown in **Figure 2A**. Following transfection, cells were infected with wild-type *H. pylori* (strain 26695) or *cagA* knockout mutant strain (ΔCagA) or *cagE* knockout mutant strain (ΔCagE), and then samples were subjected to luciferase activity assay. The data showed that higher IL-8 promoter activity was observed in cells infected with wild-type *H. pylori* strain, comparing with cells infected with ΔCagA and ΔCagE mutant strain of *H. pylori* **(Figure 2A, bottom)**. On the other hand, cells transfected with different types of IL-8 promoter constructs, and followed by infection with wild-type or ΔCagA mutant type of *H. pylori*, IL-8 promoter activity significantly decreased in groups that transfected with either ΔAP-1 or ΔκB luciferase plasmid ($P < 0.01$) **(Figure 2A, bottom)**. These results indicated that both AP-1 and NF-κB signaling pathway were required in CagA-induced activation of IL-8 expression. Moreover, in order to reveal whether lipid rafts were participated in CagA-induced IL-8 expression, cells were transfected with IL-8/wt promoter constructs, following by the pretreatment with different concentration of lovastatin to deplete the level of endogenous cholesterol (Endo 1981),

and subsequently infected with wild-type *H. pylori*. As shown in **Figure 3A**, our data demonstrated a significant attenuation of IL-8 promoter activity in a lipid-concentration-dependent manner; on the other hand, lovastatin slightly attenuating the IL-8 promoter activity in cells infected with ΔCagA and ΔCagE strain of *H. pylori*. The amount of cholesterol in cells dropped progressively while the lovastatin concentration increased; however, the cell viability had no difference between lovastatin-untreated cells and cells treated with 50 μM of lovastatin (data not shown). Collectively, these results suggested that CagA was the main reactant resulting in the induction of IL-8 upon *H. pylori* infection, and the signal was transducted via both AP-1 and NF-κB signaling pathway, suggesting that this signaling pathway was strongly dependent on the concentration of cholesterol.

3. Both of CagA EPIYA motifs and cholesterol are required for CagA-induced IL-8 promoter activity

It has been reported previously that (Higashi, Yokoyama et al. 2005) the presence of a single EPIYA motif in CagA was crucial for membrane localization. In order to determine whether the EPIYA motif located in the C-terminal of CagA is targeting CagA to the membrane rafts, we performed *in vitro* experiments using various expression constructs. CagA truncation mutants were generated based on the *cagA* sequence of *H. pylori* 26695 strain, which contains three EPIYA motifs (ABC type). As shown in **Figure 4A, left panel**, two of the mutants had N-terminal deletions (CagA ΔN and CagA ΔN42), while the other two had a C-terminal deletion (CagA ΔC and CagA ΔN42). Similarly, clinical isolation strain v669, which contains *cagA* sequence with AABD-type EPIYA motifs, was utilized to generate the analogous N-terminal deletion mutants: vCagA ΔN and vCagA ΔN42. Moreover, all CagA mutant truncates were carried a Myc epitope at the C-terminal for further experiment.

When AGS cells were co-transfected with IL-8/wt luciferase reporter plasmid and the full-length CagA expression construct. There was an approximately 3-fold increase in luciferase activity compared to cells that transfected with only IL-8/wt plasmid **(Figure 2A)**. Cells co-transfected with IL-8/wt and constructs with C-terminal deletion (CagA ΔC, and vCagA ΔC) exhibited a basal level of luciferase activity, while cells co-transfected with any types of the N-terminal deletion truncates (CagA ΔN, CagA ΔN42, vCagA ΔN, and vCagA ΔN42) exhibited a comparable luciferase activity compared with cells co-transfected with full-length construct **(Figure 4, left panel)**. We next evaluated whether IL-8 promoter activity was influenced by the presence of lovastatin. Cells co-transfected with IL-8/wt and with either a full-length CagA or CagA ΔN expression constructs, presented a significant decrease in luciferase activity assay. In addition, cells co-transfected with IL-8/wt and

the plasmid which is lacking of C-terminal domain (CagA ΔC), lovastatin had no impact on IL-8 promoter activity **(Figure 5A)**. Even more, cells transfected with different types of CagA constructs, including CagA full-length, CagA ΔC, and CagA ΔN plasmid, and treated with or without lovastatin, then the culture medium were utilized to measure the production of IL-8, the outcome of IL-8 expression is consistent with IL-8 promoter activity **(Figure 5B)**. Therefore, we concluded that both C-terminal EPIYA-containing region of CagA and cholesterol are crucial for the induction of CagA-induced IL-8 expression.

4. Full-length CagA and CagA mutants that contain the EPIYA motifs are targeted to detergent-resistant membrane fractions

To assess the association of CagA with lipid rafts in HEK-293T cells, we used cold-detergent extraction method according to pervious report (Simons and Toomre 2000). HEK-293T cells were transfected with myc-tagged CagA expression constructs and both detergent-resistant and -soluble membrane fractions were isolated, then, confirming the expression efficiency, expressed CagA was immunoprecipitated by anti-Myc and visualized by western blotting using anti-CagA **(Figure 6)**. Following that, after transfection of the same myc-tagged CagA expression plasmid, cold-detergent extraction was applied upon those cells and the proteins were visualized using multiple antibodies. As shown in **Figure 7A**, caveolin-1, a palmitoylated lipid raft protein, was presented in detergent-resistant membrane (DRM) fractions while the transferring receptor (Tfr) was localized to clathrin-coated pit, which was enriched in detergent-soluble (S) fractions (Lai, Chang et al. 2008). As expected, in cells which were transfected with full-length CagA expression construct, the CagA protein appeared in the DRM fraction rather than the S fraction, as well as phosphorylated CagA. On the other hand, the distribution of CagA shifted from the DRM fraction to the S fraction when cells were pretreated with 5.0 mM MβCD, and this shifting phenomenon was reproduced in the phosphorylated CagA protein as well **(Figure 7A**, bottom row**)**. These data suggested that full-length CagA, containing the EPIYA motifs, was associated with detergent-resistant membrane in transfected cells or infected cells.

The influence of cells transfected with CagA deletion mutant was also examined. **Figure 7B** demonstrated that both CagA ΔN and vCagA ΔN protein were primarily localized in the DRM fractions in the absence of MβCD, but shifted to S fraction after treated with MβCD, analogous to full-length CagA transfected cells. On the other

hand, a substantial proportion of CagA ΔC was found in the S fraction independent of MβCD treatment. These results indicated that C-terminal domain containing the EPIYA repeat region is required for CagA association with detergent-resistant membranes.

5. EPIYA repeat regions are important for CagA targeting to membrane lipid rafts

Cholera toxin B subunit binds to ganglioside GM1, which has been extensively used as a membrane lipid rafts marker (Pang, Le et al. 2004). We next visualized the co-localization of ectopically expressed CagA and the raft marker GM1 in transfected AGS cells using confocal microscopy. As shown in **Figure 8A**, there was no CagA signal in uninfected cells, whereas GM1 was visualized around the plasma membrane and near the perinuclear region as expected (Lai, Kuo et al. 2002). Cells under the condition of transfection with full-length CagA expression construct, CagA (colored red) and GM1 (colored green) were co-localized in the plasma membrane and perinuclear region (**Figure 8A**, bottom row).

When cells were transfected with CagA ΔC expression plasmid, there was an evident cytoplasmic distribution of CagA (red) that was identified without any GM1 co-localization pattern (**Figure 8B**, upper row); yet, cells transfected with either CagA ΔN (Figure5B, middle row) or vCagA ΔN (**Figure 8B**, bottom row), the signal of expression were analogous to Figure 8A. The results from these observations suggested that the C-terminal region containing the EPIYA repeat region is important for CagA targeting to membrane lipid rafts.

Discussion

H. pylori have been pointed to relate with human gastric disease, since the discovery by Dr. Marshall and Dr. Warren (Marshall and Warren 1984). Although, *H. pylori* have infected in half of the world population, most of the carrier did not have any gastrointestinal symptoms (Peek and Blaser 2002). Nevertheless, long term of infection still raised the risk of related-disease development, for 10% develop to peptic ulcer, 1~3% can develop into gastric carcinoma (Peek and Crabtree 2006; Wroblewski, Peek et al. 2010). The virulence factors of *H. pylori* have been studied in over a century, however, the detail mechanism of how this bacterium leads to all these human gastrointestinal disease (Peek, Fiske et al. 2010). In *H. pylori*, most of the virulence factors were encoded from *cagPAI* gene, especially the type IV secretion system and the CagA protein, which is known to relate with the delivery and the translocation of CagA. CagA in cells then can further divide into two distinct pathways: unphosphorylated CagA pathway and the phosphorylated CagA pathway (Wroblewski, Peek et al. 2010). The unphosphorylated CagA in cells, can alter the activation of β-catenin, disrupting the apical junction complex, and contribute to the loss of cell polarity (Amieva, Vogelmann et al. 2003; Bagnoli, Buti et al. 2005; Franco, Israel et al. 2005; Suzuki, Mimuro et al. 2005; Murata-Kamiya, Kurashima et al. 2007; Saadat, Higashi et al. 2007); on the other hand, phosphorylated CagA undergoes a tyrosine phosphorylation at the EPIYA repeat region by interacts with Src family kinase, subsequently deregulating the SHP-2 oncoprotein (Hatakeyama 2004; Suzuki, Mimuro et al. 2005). Upon signaling transduction, phosphorylated CagA then leads to cell cytoskeletal rearrangements and activation of NF-κB, eventually inducing the expression of IL-8 via a Ras-Raf-Mek-Erk-NF-κB signaling pathway (Brandt, Kwok et al. 2005). Expression of IL-8 is known to be a key mediator of human inflammation, and a member of CXC chemokine family, which effect in human stomach led to

gastritis, peptic ulcer, and even gastric cancer, attributed to the infection of *H. pylori* (Sugimoto, Yamaoka et al. 2010). Notably, four EPIYA sites have been described (Higashi, Tsutsumi et al. 2002), specially, the EPIYA-A and EPIYA-B binding site were found in most of the *H. pylori* strain, while the EPYIA-C was public in Europe, North America, and Australia; and EPIYA-D was found in Asia commonly (Hatakeyama 2011). *H. pylori* containing CagA strains may carried varying numbers of EPIYA motifs, and the different combination of the EPIYA repeat regions also contributes to different degree of outcome in the clinical patients, for examples, in gastritis and duodenal ulcer patients the EPIYA-C were mostly found in the CagA EPIYA motifs (Argent, Kidd et al. 2004; Azuma, Yamakawa et al. 2004; Higashi, Yokoyama et al. 2005; Naito, Yamazaki et al. 2006; Quiroga, Huertas et al. 2010; Hatakeyama 2011).

In present study, we used multiple agents to reduce the amount of cholesterol or depleting raft binding sites in cell membrane, and both IL-8 expression (Figure 1A) and the immunoblotting data (Figure 1B) suggested that while cholesterol concentration decrease, the translocation and phosphorylation of CagA decrease in a dose-dependent manner. These results suggested that the role of cholesterol may play an important role in CagA-related pathways, which was consistent with the view from recent article (Murata-Kamiya, Kikuchi et al. 2010). Moreover, phosphorylated CagA in cell was found to associate with the hummingbird phenotype. Thus, we investigated the cell morphology, to demonstrate that after the depletion of cholesterol with lovastatin. As expected, upon cells treated with lovastatin, the CagA-induced hummingbird phenotype shown significantly decreased (Figure 1C, 1D).

We have further demonstrated that both AP-1 and NF-kB signaling pathways contributed to CagA-induced IL-8 promoter activity (Figure 2). Importantly, this activity was significantly depending on cholesterol (Figure 3), which is known to be a

main member of lipid rafts. Several evidences suggested that tethering of the translocated CagA to the plasma membrane is pivotal for its subsequent functions, including (i) Following *H. pylroi* infection, CagA interacts with SHP-2 to induce the deregulation of Ras-Erk MAP kinase cascade (Hatakeyama 2009); (ii) CagA binds to PAR1b, a member of the seven transmembrane G-protein-coupled receptor superfamily, and associates with epithelial tight-junction scaffolding protein ZO-1 (Amieva, Vogelmann et al. 2003), and disrupting epithelial polarity (Saadat, Higashi et al. 2007; Zeaiter, Cohen et al. 2008); (iii) CagA binds to C-terminal Src kinase (CSK) attenuating CagA-SHP-2 signaling (Tsutsumi, Higashi et al. 2003). Notably, CagA and Src family kinase (Lai, Chang et al. 2008), and ZO-1 (Nusrat, Chen et al. 2000) have been shown to associated with DRMs, in which fraction that lipid rafts located. By using the cold-detergent extraction method, revealed that not only CagA, but also the polarity-associated serine/threonine kinase MARK2 (EMK-1/Par-1b), which interacts with CagA, were enriched in the DRM fractions (Lai, Chang et al. 2008; Zeaiter, Cohen et al. 2008). Furthermore, the interaction between CagA and EMK-1/Par-1b lead to disruption of apical junctions and inhibition of tubulogenesis and cell differentiation (Zeaiter, Cohen et al. 2008). To sum up, these results suggested that the injected CagA resides primarily in membrane lipid rafts (Asahi, Fu et al. 2007; Lai, Chang et al. 2008), and lipid raft, is where CagA triggers various signaling molecules, altering cell motility, proliferation, and differentiation.

Furthermore, HEK-293T cell line was a universal platform to express ideal protein (Thomas and Smart 2005). In this study, we transfected various mutant constructs in to HEK-293T cells to enlarge the production of expression constructs, for AGS cells expressing efficiency was limited. As a result, mutants that lacked both types of EPIYA repeat region, i.e., CagA ΔN (ABC type) and vCagA ΔN (AABD type), can not reproduce the same IL-8 promoter activity (Figure 4, 5A), nor the

expression of IL-8 (Figure 5B), compare with the mutants constructs containing either types of EPIYA repeat regions. Finally, our data reveals that both ABC- and AABD-type EPIYA repeat regions of CagA mediate raft association (Figure 7A), that is to say, CagA lacked EPIYA repeat region, will lost their normal ability to associate with membrane rafts (Figure 7B).

Base on the results in the present study, it is reasonable to speculate that the CagA EPIYA tyrosine residues are phosphorylated by Src family kinase take place around the rafts (Selbach, Moese et al. 2002; Dykstra, Cherukuri et al. 2003). Moreover, the members of lipid rafts, including phosphoatidylserine and sphingomyelin, were pointed to act as a membrane receptor for the delivery and translocation of CagA in recent articles (Gupta, Patel et al. 2008; Gupta, Wilson et al. 2010; Murata-Kamiya, Kikuchi et al. 2010; Wandler, Parthasarathy et al. 2010). In addition, a 16-residue sequence located downstream of the EPIYA-C or EPIYA-D segment might facilitate the recruitment of CagA into rafts, because this sequence has been shown to mediate CagA multimerization for stable interaction between CagA and SHP-2, inducing CagA partitioning in rafts (Ren, Higashi et al. 2006). Further investigations will need to clarify whether other C-terminal regions of CagA are involved in raft association. Additionally, CagA was pointed out membrane localized in non-polarized cell, like MDCK cells (Saadat, Higashi et al. 2007; Zeaiter, Cohen et al. 2008), and the mechanism lead to this difference still remain unclear.

The present investigation demonstrated that CagA-induced IL-8 promoter activity was inhibited by lovastatin, and inhibitor of 3-hydroxy-3-methylutary CoA (HMG CoA) reductase, which catalyses the rate-limiting step in cholesterol biosynthesis (Endo 1981). This cholesterol-lowering agent has provided valuable treatment for cardiovascular disease for over two decades (Singh, McMahon et al. 2002; 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, Goggin et al. 1994). Infection with CagA-positive strains of *H. pylori* has also been linked to premature myocardial infarction (Gunn, Stephens et al. 2000), supporting the likelihood that cholesterol levels influence *H. pylori* pathogenesis.

In conclusion, we have demonstrated that membrane rafts play a central role in CagA-induced IL-8 activity, and both NF-κB and AP-1 signaling mediated the CagA induction of IL-8. We also showed that CagA C-terminal EPIYA repeat region, is crucial for tethering of CagA to lipid rafts, not the N-terminal region. Modulation of cellular cholesterol level may alter the partitioning of CagA into rafts, thereby reducing CagA-induced inflammation and perhaps slowing the progression of *H. pylori*-associated disease.

Tables

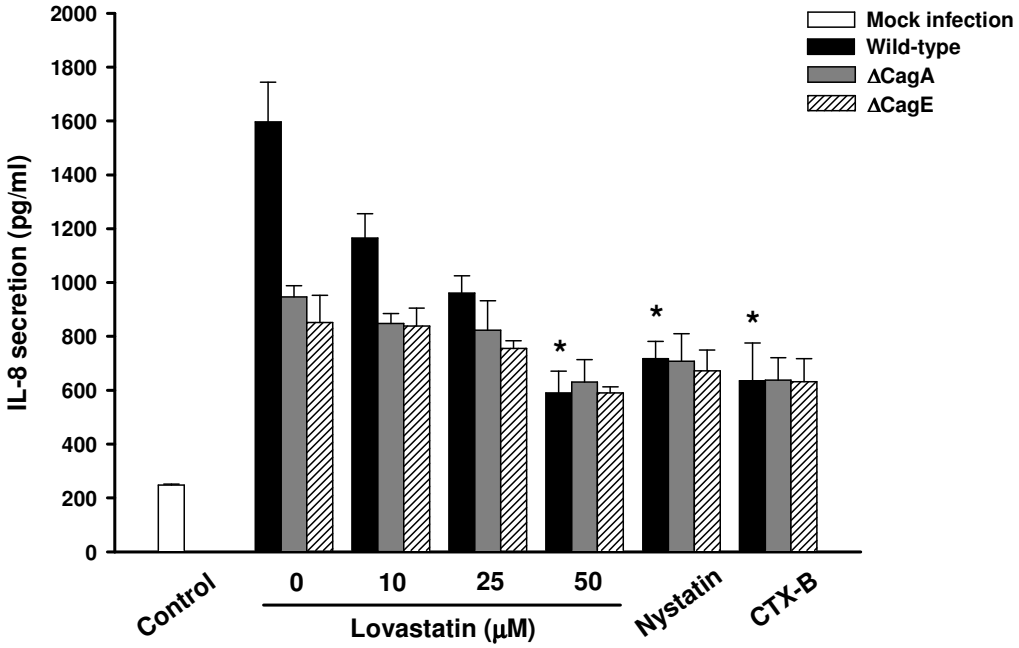
Table 1. PCR primers used in this study

Primers	Nucleotides sequences (5' to 3')[a]	Restriction enzyme
pEF1_CagA_CTD59_F	GCGGGATCCAAATGGAAGCAAAAGCTCAAGCTAAC	*BamH*I
pEF1_CagA_CTD42_F	GCGGGATCCCAATGGGCGATTTCAGTAGGGTAGAG	*BamH*I
pEF1_CagA_CTD_R	GCGTCTAGAAGATTTTTGGAAACCACCTTTTG	*Xba*I
pEF1_CagA1_F	GCGGGATCCATGACTAACGAAACTATTGATC	*BamH*I
pEF1_CagA1_R	GCGGAATTCCTCGAGCATATGCACATTAATGAGTG	*EcoR*I
pEF1_CagA2_F	TGTGCATATGAAAAACGGCAGTG	*Mfe*I
pEF1_CagA2_R	AGCCAATTGCTCCTTTGAGAAG	*EcoR*I
pEF1_CagA_CTD69_F	GCGACTAGTTTCGTAAGGCGGAATTTAGAG	*Spe*I

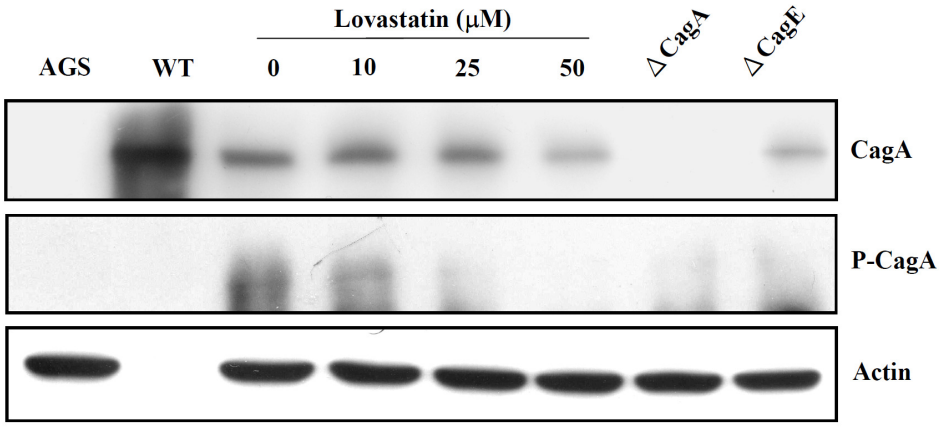
[a]Underlined sequence indicates restriction enzyme sites.

Figures

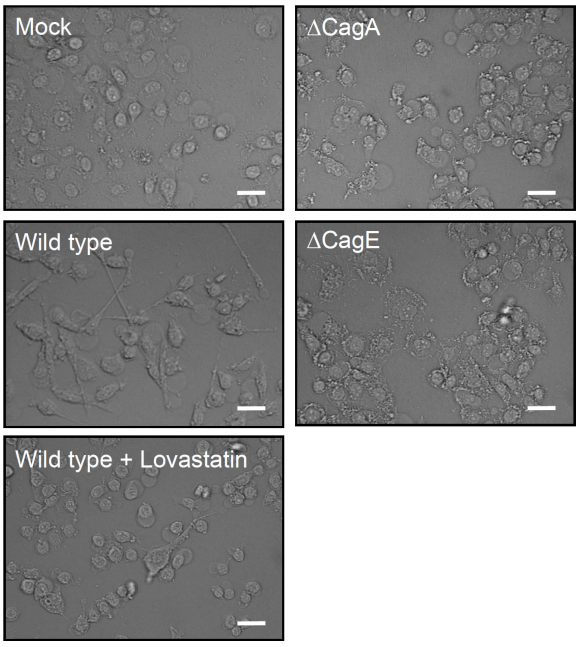
A


Mock infection
Wild-type
ΔCagA
ΔCagE
IL-8 secretion (pg/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
Control
0
10
25
50
Lovastatin (μM)
Nystatin
CTX-B

B


Lovastatin (μM)
AGS
WT
0
10
25
50
ΔCagA
ΔCagE
CagA
P-CagA
Actin

C



D

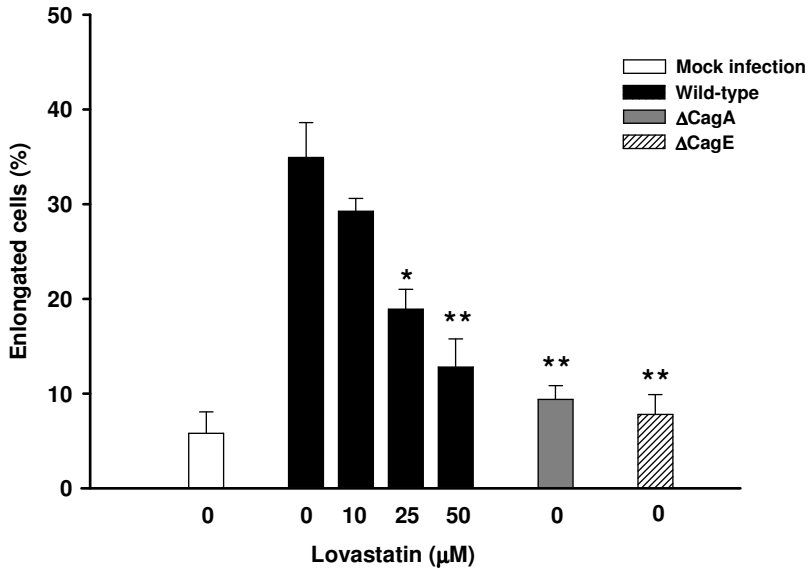


Figure 1. Cholesterol is participating in the process of CagA signaling.

(A) AGS cells were pretreated with lovastatin (0, 10, 25, 50 μM), Nystatin (gray bar), or CTX-B for 1 h, and infected cells with wild-type, ΔCagA, and ΔCagE strain of *H. pylori* (MOI 100) for 6 h. The level of IL-8 secretion was determined by ELISA. (B) Cells were pretreated with Lovastatin for 1 h, and infected cell with different types of

H. pylori (MOI 100), after 6 h of infection, protein expression were analyzed using western blot. (C, D) AGS cells were pretreated with 50 μM Lovastatin, and infected with various mutants of *H. pylori*. After 6 h of infection, cells were fixed by 1% paraformaldehyde for 30 min, and the hummingbird phenotype were observed by confocal microscopy, Scale bars, 10 μm, and the percentage of enlongated cells were caculated. The data represent means ± SD relative to basal signal (non-infected cells) from at least three independent experiments. Statistical significance was evaluated using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

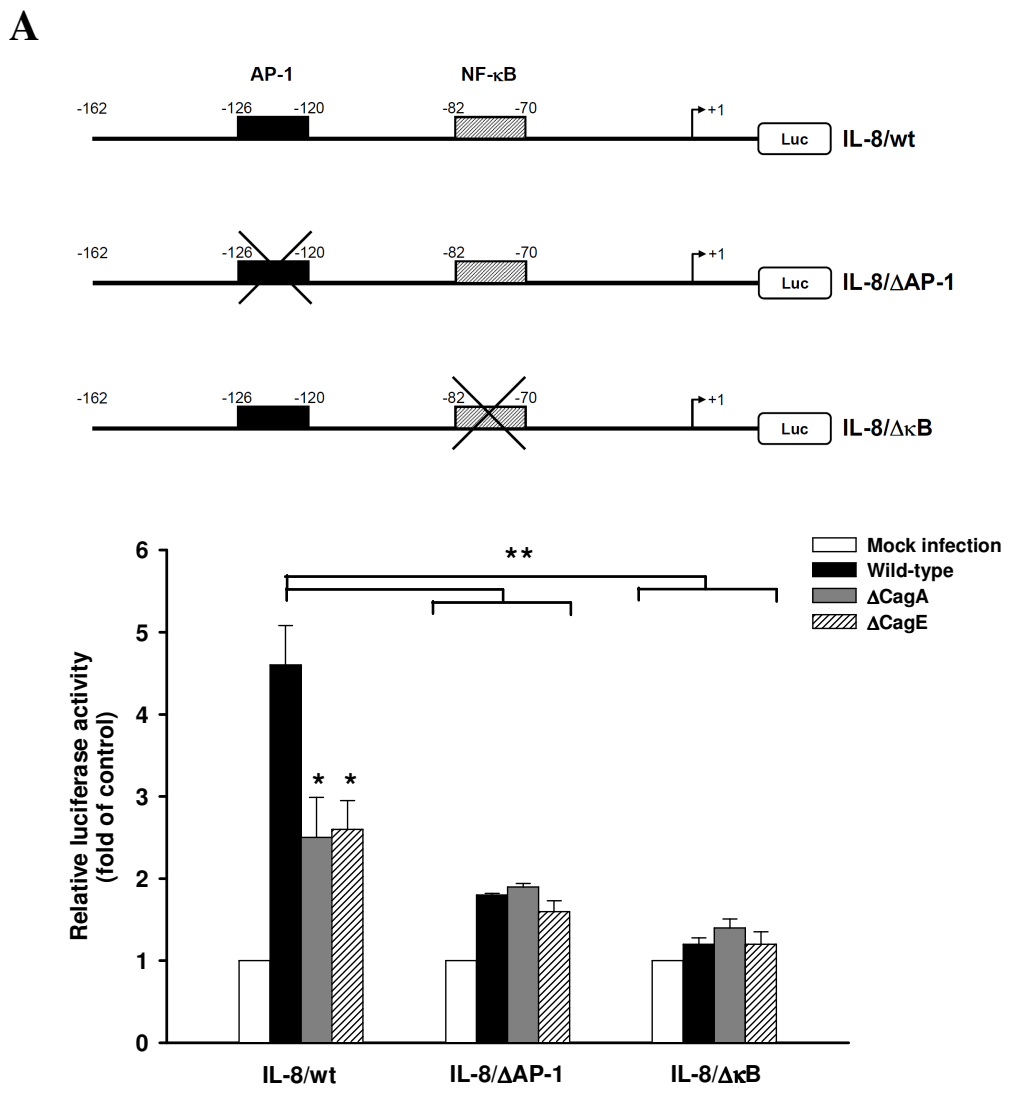


Figure 2. Both AP-1 and NF-κB signal are required for *H. pylori* CagA-induced *IL-8* promoter activity.

AGS cells were transfected with IL-8/wt, IL-8/ΔAP-1, or IL-8/ΔκB luciferase expression vectors. After 24 h, the cells were infected with wild-type *H. pylori* strain 26695 (filled bars), ΔCagA mutant (gray bars), or the ΔCagE mutant (slant bar) for 6 h and then subjected to luciferase activity assays. The data represent means ± SD relative to basal signal (transfected but uninfected cells) from at least three times of independent experiments. Statistical significance was evaluated using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

A

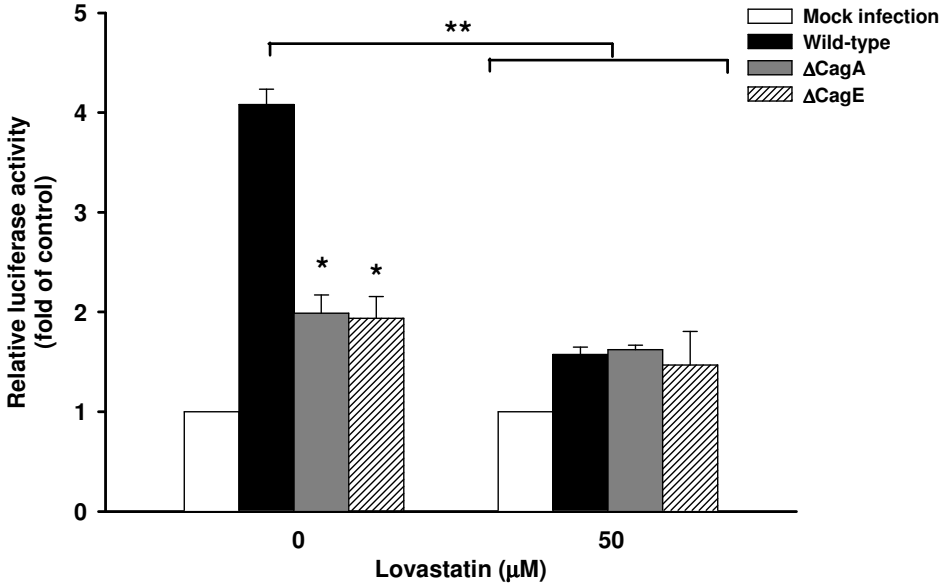


Figure 3. Induction of IL-8 promoter activity involves CagA and lipid rafts.

AGS cells were transfected with IL-8/wt luciferase expression vector, treated with lovastatin (0, 50 μM), and then infected with wild-type (filled bars), ΔCagA (gray bars), or ΔCagE (slant bar) *H. pylori*. After 6 h of infection, luciferase activity assays were performed. The data represent means ± SD relative to basal signal (transfected but uninfected cells) from at least three times of independent experiments. Statistical significance was evaluated using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

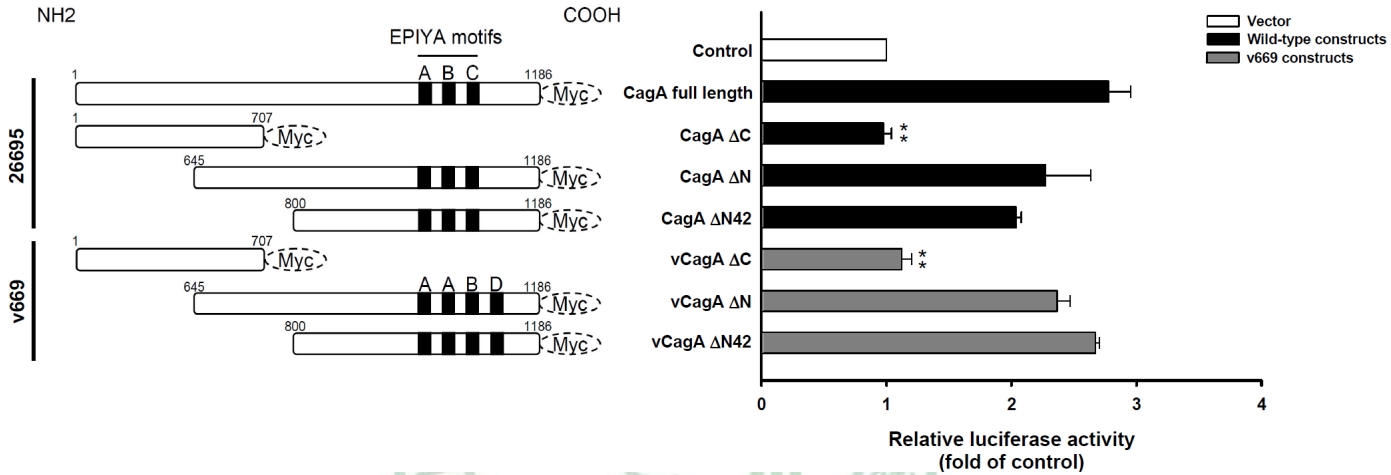


Figure 4. The EPIYA repeat region of CagA is crucial for CagA-induced IL-8 activity in AGS cells.

Schematic diagram of CagA expression constructs used (left panel), including full-length CagA, CagA ΔC, CagA ΔN, and CagA ΔN42. All constructs were prepared using pEF1 expression vector as described in material and methods. AGS cells were co-transfected with the indicated CagA construct and IL-8/wt, and cell lysates were subjected to luciferase activity assays. The cell viability was hardly influenced under these conditions, as determined by trypan blue staining. The data represent means ± SD relative to basal signal (cells transfected with IL-8/wt alone) from at least three independent experiments. Statistical significance was evaluated using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

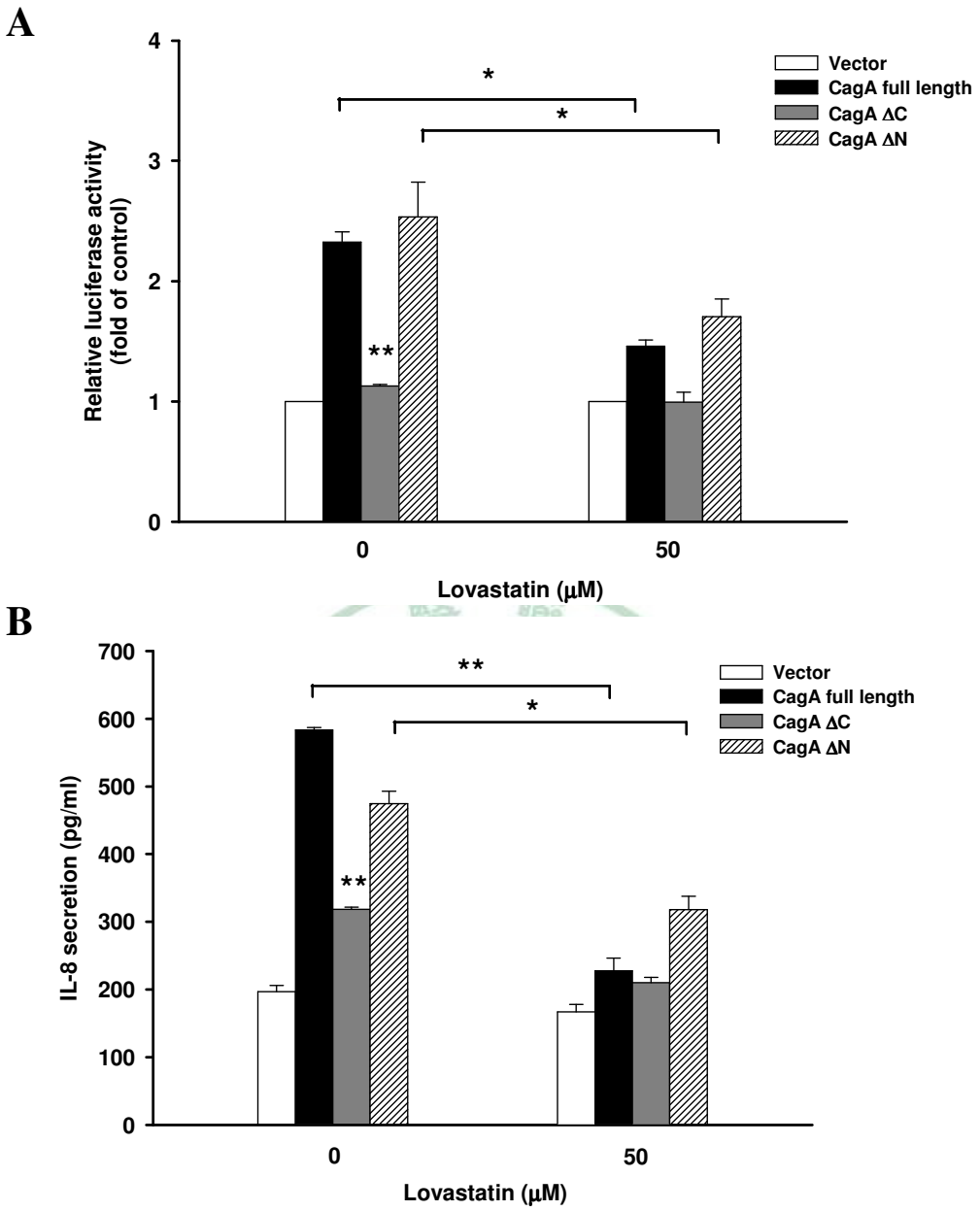


Figure 5. Cholesterol is crucial for the induction of CagA-induced IL-8 expression.

(A) AGS cells were cotransfected with indicated CagA construct and IL-8/wt luciferase plasmid, and treated with lovastatin (0, 50 μM). The cell lysates were subjected to luciferase activity assays. (B) Cells were transfected with indicated CagA constructs, and the cultered medium were collected, subjected to ELISA assay for IL-8 secretion.

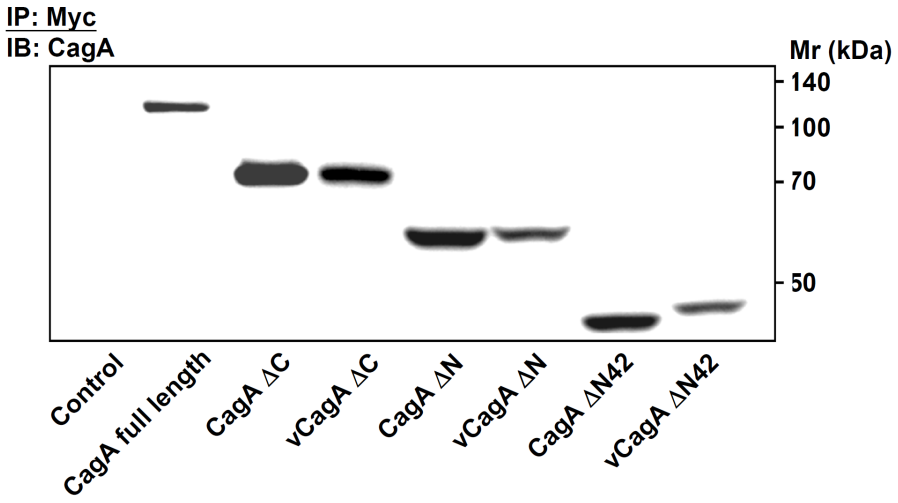


Figure 6. The CagA construct expression patterns in HEK-293T cells.

The indicated CagA expression constructs were transfected into HEK-293T cells. Cell lysates were prepared and immunoprecipitated using anti-Myc antibody, followed by immunoblot analysis using anti-CagA antibody. Molecular mass markers (Mr) are indicated at right.

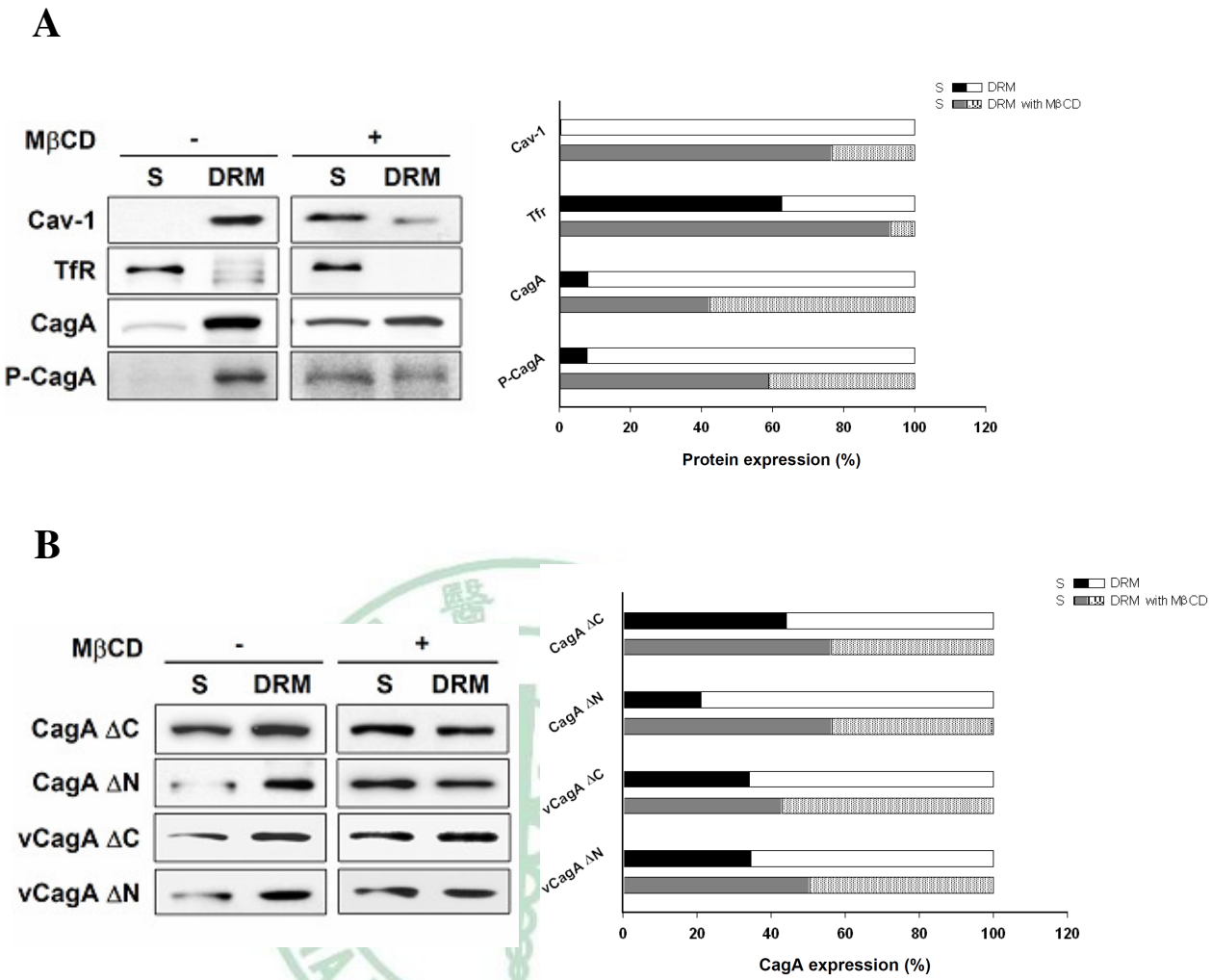


Figure 7. Wild-type CagA and the CagA ΔC mutant, both containing the EPIYA repeat region, are enriched in detergent-resistant-membrane fractions.

(A) HEK-293T cells were transfected with full-length CagA expression vector, treated with or without 5 μM MβCD, and then subjected to cold-detergent extraction using 1% Triton X-100 followed by centrifugation to separate detergent-resistant membrane fractions (DRM) and detergent-soluble fractions (S). Each fraction was subjected to immunoblot analysis using antibodies against caveolin-1 (Cav-1), transferring receptor (Tfr), CagA, or phosphorylated CagA (P-CagA). (B) HEK-293T cells were transfected with CagA ΔC, CagA ΔN, vCagA ΔC or vCagA ΔN, treated with or

without 5 μM MβCD, and fractionated by the cold-detergent extraction method to give detergent-resistant membrane fractions (DRM) and detergent-soluble fractions (S). Each fraction was subjected to immunoblot analysis using anti-CagA.

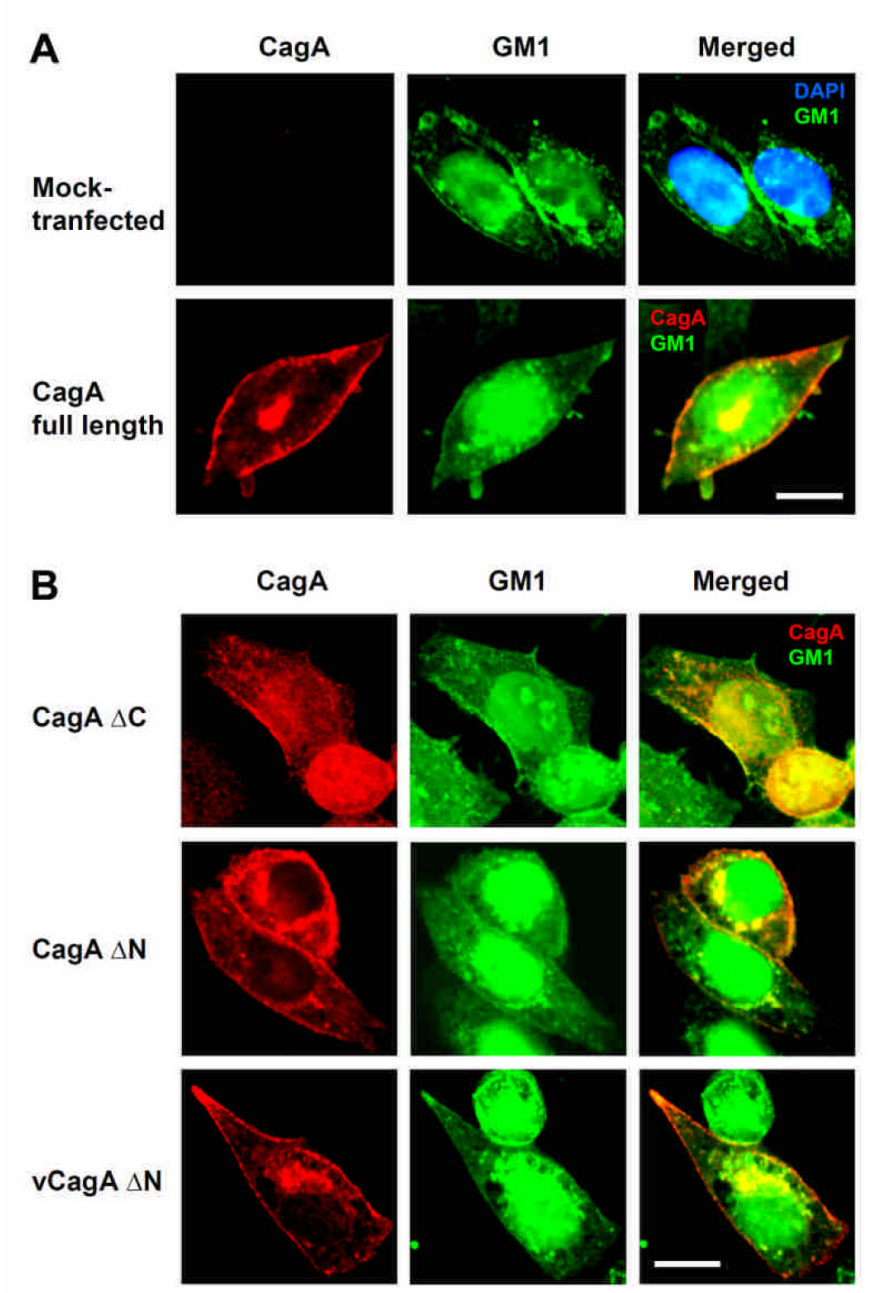


Figure 8. EPIYA repeat regions are required for CagA trafficking to membrane rafts in AGS cells.

AGS cells were transfected with or without the CagA full length expression vector (A) or with the indicated CagA truncation mutant expression vector (B). 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 and lipid raft co-localization appear in yellow in the overlay. Representative images are shown. Scale bars, 10 μm.

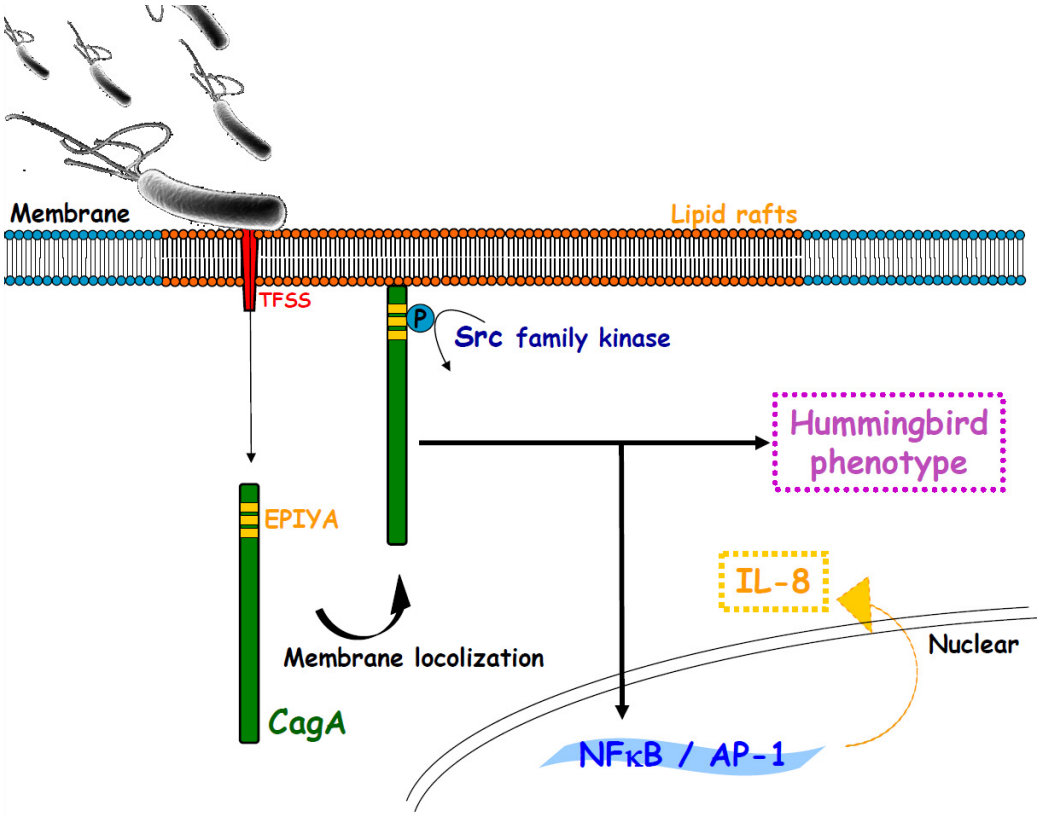


Figure 9. *Helicobacter pylori* CagA EPIYA repeat region interacts with lipid rafts, and induces IL-8 expression through NF-κB and AP-1 signaling pathway.

To our finding, *H. pylori* CagA could interact with lipid rafts by EPIYA motifs, and subsequently leading both NF-κB and AP-1 signaling activation, which may contribute to the expression of IL-8 and hummingbird phenotypes.

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