Analysis of Translocation of the CagA Protein and Induction of a Scattering Phenotype in AGS Cells Infected with *Helicobacter pylori*\(^\text{1}\)

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**Objective** To investigate whether the presence of structured CagA proteins in Western- and Eastern-type *Helicobacter pylori* (*H. pylori*) induces different incidences of gastric diseases. **Methods** CagA and phosphorylated CagA were expressed in AGS gastric epithelial cells infected with wild type and mutant strains. The ability of individual CagA was determined by immunoprecipitation and Western blot assay. Morphological changes of these cells were observed under microscope to evaluate the appearance of elongation hummingbird phenotype. **Results** The sizes of CagA proteins in different strains were different, and no phosphorylated CagA proteins were detected in wild-type strains. Meanwhile, the kinetics of CagA status in AGS infected with *H. pylori* was detected. The molecular weight of phosphorylated CagA with the same size of CagA proteins in *H. pylori* was different in infections with different wild-type strains. CagA and phosphorylated CagA increased in a time-dependent manner after the infection. The hummingbird phenotype with *H. pylori* for time-course was observed under microscope. Instead of HPK5 strain, the wild-type 26695 strain induced hummingbird phenotype in a time-dependent manner. **Conclusion** Translocation and phosphorylation of CagA are necessary, but not sufficient, for the induction of hummingbird phenotype in AGS cells.

**Key words**: CagA; Western CagA-specific sequence (WSS); Eastern CagA-specific sequence (ESS); Hummingbird phenotype

**INTRODUCTION**

Although *Helicobacter pylori* (*H. pylori*) was found by Marshall and Warren about 20 years ago\(^\text{[1]}\), and over 50% of the world’s population have been infected with this pathogen, it remains poorly understood in many aspects. *H. pylori* preferentially colonizes the human stomach, modulates the mucosal immune system to produce disease, and plays an important role in the pathogenesis of chronic gastritis, peptic ulcers, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma\(^\text{[2-3]}\). The World Health Organization has declared *H. pylori* as a class I carcinogen.

On the basis of the presence or absence of the multigene 40-kb cag pathogenicity island (cagPAI), *H. pylori* strains are grouped in types I and II, respectively\(^\text{[4-5]}\). *H. pylori* type I strains harbor cagPAI which is associated with increased bacterial virulence inducing severe inflammatory response in the host mucosa and plays an essential role in the induction of gastric disease. cagPAI is a continuous sequence, which can be divided by an insertion sequence, *IS605*, into two regions termed cagI and cagII\(^\text{[4]}\). The cagA gene encodes an immunodominant size-variant protein with unknown physiological function\(^\text{[4, 6]}\). During the bacterium-gastric epithelial cell interaction, *H. pylori* delivers virulent factors such as a CagA into the attached cells by a type IV secretion system (T4SS)\(^\text{[7-10]}\).

Translocated CagA protein is localized in the inner surface of plasma membrane and subsequently undergoes tyrosine phosphorylation in host cells by the Src family of protein tyrosine kinases. The phosphorylated protein is recruited to the membrane close to the location at which the bacteria attach\(^\text{[10-12]}\). The tyrosine phosphorylation of CagA is restricted to a identified repeated sequence and contains the

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five-amino-acid motif EPIYA (Glu-Pro-Ile-Tyr-Ala). Once tyrosine is phosphorylated, CagA perturbs cellular functions by deregulating SHP-2 and triggers a signal cascade which results in cell scattering and proliferation (hummingbird phenotype) characterized by dramatic cell elongation\textsuperscript{[13-15]}. CagA is also capable of interacting with C-terminal Src kinase (Csk), which in turn inactivates the Src family of protein-tyrosine kinases. CagA-Csk interaction prevents cell damage caused by the deregulated activation of SHP-2 and may enable cagA-positive \textit{H. pylori} to persistently infect the human stomach for decades while avoiding excess CagA toxicity to the host\textsuperscript{[16]}.

The five-amino-acid motif EPIYA of CagA is subclassified into EPIYA-A, -B, -C, and -D based on the sequences surrounding EPIYA motifs. The ‘A-B-C’ type (Western CagA-specific sequence (WSS)) is prevalent in North America, Europe, Africa, and Australia, whereas the ‘A-B-D’-type (Eastern CagA-specific sequence (ESS)) is common in East Asia. The ESS-type CagA exhibits stronger SHP-2 binding, greater morphogenetic activity and more severe inflammation than the WSS-type CagA because EPIYA-D matches the high-affinity binding sequence for the SH2 domains of SHP-2, while EPIYA-C differs in the binding sequence at the pY-5 position. However, bacterium-cell interaction is indispensable to the injection of CagA into target cells, suggesting that other bacterial factors can contribute to the alteration of molecular behavior in the infected cells. Furthermore, \textit{H. pylori} strains carrying more active CagA are more virulent than those carrying less active CagA and are more closely associated with gastric carcinoma\textsuperscript{[17]}. The EPIYA-repeat polymorphism of CagA greatly influences the magnitude and duration of phosphorylation-dependent Western- and Eastern-type CagA biological activity, which underlie the striking difference in gastric cancer incidence between these two geographic areas\textsuperscript{[18-19]}.

IL-8 is produced in the early and late phases after infection in a CagA-dependent and independent manner, irrespective of CagA-type\textsuperscript{[20]}. There are two bands of CagA in 26695 strains. The kinetics of CagA and its phosphorylation in AGS infected with \textit{H. pylori} were detectable, suggesting that other bacterial factors might contribute to injecting CagA into target cells.

**MATERIALS AND METHODS**

**Cell Lines and \textit{H. pylori} Strains**

AGS cells (a human gastric adenocarcinoma epithelial cell line, ATCC CRL1739c) were cultured following the standard procedures in the RPMI1640+7 medium containing L-glutamine, NaHCO\textsubscript{3}, Kanamycin (60 \(\mu g/mL\)), and streptomycin (20 \(\mu g/mL\). Nikken Biomedical, Japan) supplemented with 10% (vol/vol) fetal bovine serum (Biochrom, Ltd) at 37 °C in an atmosphere containing 5% CO\textsubscript{2}.

HPK5 (ESS-type), a clinical isolate derived from a patient with gastric ulcer. HPKT510, is a cdrA-disrupted mutant carrying xylE-Km cassette in cdrA\textsuperscript{[21-22]}. 26695 (WSS-type) and 11637(WSS-type) have been used in experiments\textsuperscript{[23]}. \textit{H. pylori} mutant strains with disrupted cagA (HPK5\textDelta cagA, 26695\textDelta cagA) were a kind gift from Dr. Takeuchi (Kochi University, Japan). The isogenic cagA knockout mutant was constructed by inserting a kanamycin resistance gene cassette in the cagA locus, as previously described\textsuperscript{[21]}. PCR was performed to detect these mutant strains with specific primers (cagA-F1, 5'-TGCATGCAGGAGAAACAATGACT AACG-3' and cagAR, 5'-TGCCTTAATAGATTT TTGG-AAACC-3') and Western blotting was carried out to determine the cagA gene knockout. All strains were grown on Brucella-serum agar supplemented with 10% horse serum and vancomycin (10 \(\mu g/mL\)) at 37 °C under microaerobic conditions containing a gas mix of 5% O\textsubscript{2}, 10% CO\textsubscript{2}, and 85% N\textsubscript{2}. For sub-culture experiments, bacteria were grown in 10 mL of Brucella-broth medium supplemented with 10% horse serum in 10 mL of 100-mL conical flasks which were shaken at 120 rpm as previously described\textsuperscript{[22]}. The bacterial growth was detected by determining the optical density at 600 nm (OD\textsubscript{600}) with a spectrophotometer (GeneQuant, Biochrom Ltd), and colony-forming units (CFU) were determined for bacterial viability.

**Infection of AGS Cells**

AGS cells (0.5\( \times 10^8\)) were cultured in 6-well culture plates (Sumitomo Bakelite) containing RPMI 1640+7 medium for 2 days, washed twice with phosphate-buffered saline (PBS), then cultured alone as control or co-cultured with \textit{H. pylori} strains at a multiplicity of infection (MOI) of 150 in RPMI 1640 medium (Invitrogen) for time-course at 37 °C under microaerobic conditions. The cells were collected at appropriate time points (such as 12, 24, and 48 h) after co-culture and utilized for Western blotting.

**Preparation of Whole-Cell Extracts**

At appropriate time points, AGS cells were washed vigorously with phosphate-buffered saline (PBS), and incubated in a PRIA buffer containing 50 mmol/L Tris-HCl (pH7.4), 1% Noidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L...
ethylene glycol bis (β-aminoethyl ether)-N, N,N’,N’-tetraacetic acid (EGTA), 1 mM/L NaF, 100 μmol/L sodium orthovanadate, 100 μmol/L phenylmethylsulfonyl fluoride, and a commercially available protease inhibitor mixture tablet (Roche Molecular biochemical) for 15 min and then harvested by scraping. Debris was removed by centrifugation at 10 000 rpm for 10 min and the lysates were collected. The lysates were added in 2 × lysis buffer containing 50 mmol/L Tris (pH6.8), 10 % (wt/vol) sodium dodecyl sulfate, 12% (vol/vol) 2-mercaptoethanol, 0.1% (vol/vol) bromophenol blue (BPB), and 20% (wt/vol) glycerol and then boiled for 5 min for Western blot analysis.

Western Blot Analysis

Equivalent amounts of total cell extract (200 μg) were fractioned into sodium dodecyl sulfate-polyacrylamid gel electrophoresis (5% SDS-PAGE) and electro-transferred onto nitrocellulose membranes (Millipore Corp.) with the apparatus (Marysol). The blots were blocked with 4% (wt/vol) dried skim milk (Nacalai tesque) in Tris-phosphate buffered saline with Tween 20 (TBST) at room temperature. Phosphorylated tyrosine proteins were detected using antibody PY99 (1:1000 Santa Cruz). The membrane was then incubated with ECL anti-mouse IgG peroxidase-linked species-specific antibody (1/1000, Amersham Biosciences) and developed using the ECL plus Western blotting detection reagents (Amersham Biosciences). The membrane was stripped and reprobed with anti-CagA antibody (1:1000 Austral Biologicals) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000, Jackson Immunoresearch). The membrane was developed again.

Immunoprecipitation

Immunoprecipitation was performed using 1 mg of lysate protein. The sample was incubated for 1 h at 4 °C with either polyclonal rabbit anti- H. pylori CagA antibody or monoclonal PY99 antibody, followed by an overnight incubation at 4 °C with a 20 μL aliquot of protein G plus-agarose beads (Santa Cruz). The beads were washed three times with a lysis buffer, and proteins were eluted by boiling for 10 min in 2 × electrophoresis sample buffer containing 50 mmol/L Tris, 10% sodium dodecyl sulfate, 12% 2-mercaptoethanol, 20% glycerine, and 1% Bromophenol blue. Immunoprecipitated proteins were subjected to Western blot analysis.

Synchronized Infection Assays

AGS cells were grown in 6-well plates containing RPMI 1640+7 medium complemented with 10% fetal bovine serum for 2 days to reach monolayers with about 30% cell confluence. The cells were washed with phosphate-buffered saline (PBS). H. pylori strains were suspended in RPMI1640 medium and added to AGS cells at a multiplicity of infection (MOI) of 150. Synchronized infections were done by centrifugation at 600 xg for 5 min and incubation for time-course.

Morphological Examination

AGS cells (1×10^5) were grown in 6-well plates containing RPMI 1640+7 medium supplemented with 10% fetal bovine serum for 2 days to reach monolayers at approximately 30% cell confluence. The cells were washed with PBS and four H. pylori strains (26695, 26695CA, HPK5, and HPK5CA) suspended in RPMI 1640 medium without serum and antibiotics were added to AGS cells at a MOI of 150. Morphological changes of these cells were observed under microscope for 2 successive days to evaluate the appearance of elongation phenotype (hummingbird) characterized by the production of thin needle-like cell protrusions. Smaller protrusions that were occasionally seen in the uninfected control cells were not counted.

RESULTS

Identification of CagA Protein in H. pylori by Western Blotting

The status of CagA proteins in H. pylori was examined by Western blotting. The sizes of CagA proteins in different strains were different, but neither CagA proteins nor phosphorylated CagA proteins were detected in mutant and wild type strains (Fig. 1). The strains HPK5, HPK510 showed one band of CagA proteins while the strain 26695 showed two significant bands of CagA protein as strain 11637.

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![Western Blotting Analysis of CagA Protein and Phosphorylation of CagA Protein in H. pylori.](image-url)

**Fig. 1.** Western Blotting Analysis of CagA Protein and Phosphorylation of CagA Protein in H. pylori. Proteins were isolated from H. pylori. The blot was probed with an anti-CagA antibody (top) and reprobed with an anti-phosphotyrosine antibody (PY99) (bottom). AGS cells were infected with 26695 strains as positive controls.
Identification of Tyrosine-phosphorylated CagA and CagA Proteins in AGS by Western Blotting and IP

To assess whether CagA proteins were translated into and phosphorylated in cells, we collected the AGS cell lysates infected with *H. pylori* strains to analyze the phosphotyrosine of CagA proteins (Fig. 2). CagA proteins and phosphorylated CagA proteins derived from wild-type strains increased gradually in a time- and strain-dependent manner during infection. The molecular weight of phosphorylated CagA with the same size of CagA proteins in *H. pylori* was different in infection with wild-type strains. CagA proteins increased gradually and kept their peak level until 48 h, while phosphorylated CagA proteins reached their peak level at 24 h and then degraded at 48 h. To determine whether bacterial motility affects the time-course status of CagA proteins during infection, we performed synchronized infection, a method that gives strains the same outer power to propel the strains to infect the cells at the same speed. Results revealed no difference in the status of CagA or phosphorylated CagA proteins (data not shown). The phosphorylation of CagA protein in AGS cells infected with HPK5 strain possessed one band. However, the phosphorylation of CagA protein in AGS cells infected with 26695 strain showed the upper band instead of the lower band (Fig. 2).

![Fig. 2. Western Blotting Analysis of AGS Cells Infected without or with *H. pylori* strains. The blot was probed with an anti-CagA antibody and reprobed with an anti-phosphotyrosine antibody (PY99). In each experiment, *H. pylori* infection was performed for time-course using a MOI of 150.](image)

To further confirm the identification of CagA and phosphorylated CagA, we performed immunoprecipitation. The phosphorylated protein or CagA protein was precipitated using anti-PY99 or anti-CagA antibody, and then detected by Western blotting (Fig. 3), showing that the upper band of phosphorylated protein of CagA was present and the lower band of phosphorylated protein of CagA was absent.

![Fig. 3. Immunoprecipitation analysis of AGS cells infected without or with *H. pylori* strains. In each experiment, *H. pylori* infection was performed for time-course using a MOI of 150.](image)

Morphological Characteristics

Interaction of host cells with *H. pylori* induced a growth factor-like response in AGS (hummingbird), characterized by spreading and elongated growth of the cells. AGS gastric epithelial cells were infected with the wild-type strains and CagA knock-out strains. The appearances of such phenotypes with four strains were observed under microscope for 2 successive days. AGS were used as controls. AGS infected with the cagA knock out strains, HPK5CA and 26695CA, did not express hummingbird, suggesting that CagA was necessary to induce the hummingbird phenotype. Rather than HPK5 or mutant strains, only 26695 induced the hummingbird phenotype in a time-dependent manner (Fig. 4). AGS cells infected with 26695 showed the hummingbird at 6 h, irrespective of synchronized infection. The hummingbird phenotype was observed. AGS cells infected with HPK5 carrying CagA did not show the hummingbird. HPK5 (ESS-type) should exhibit stronger morphogenetic activity than 26695 (WSS-type). However, the hummingbird of AGS cells infected with HPK5 was absent in our study, suggesting that CagA was not sufficient for inducing the hummingbird. Occasionally, elongated AGS cells were also seen in the controls and mutant strains, but its number was usually less than 1%. Vacuoles were seen from 12 h in all strains, irrespective of CagA mutant strains (Table 1).
TABLE 1
Morphology of AGS Cells Infected with *H. pylori* Strains. AGS Cells were Infected with *H. pylori* Strains at a MOI of 150 for Time-Course.

<table>
<thead>
<tr>
<th>Time-course</th>
<th>AGS</th>
<th>26695</th>
<th>26695CA</th>
<th>HPK5</th>
<th>HPK5CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>E-V-</td>
<td>E-V-</td>
<td>E-V-</td>
<td>E-V-</td>
<td>E-V-</td>
</tr>
<tr>
<td>6 h</td>
<td>E±V-</td>
<td>E-V-</td>
<td>E-V</td>
<td>E-V</td>
<td>E-V</td>
</tr>
<tr>
<td>12 h</td>
<td>E+V++</td>
<td>E-V++</td>
<td>E-V+</td>
<td>E-V</td>
<td>E-V</td>
</tr>
<tr>
<td>24 h</td>
<td>E+V++</td>
<td>E-V++</td>
<td>E-V+</td>
<td>E-V</td>
<td>E-V</td>
</tr>
<tr>
<td>48 h</td>
<td>E+V++</td>
<td>E-V++</td>
<td>E-V+</td>
<td>E-V</td>
<td>E-V</td>
</tr>
</tbody>
</table>

*Note.* E-, the number of hummingbird phenotypes is <1%. E±, the number of hummingbird phenotypes is 1%-5%. E+, the number of hummingbird phenotypes is >5%. V-, the number of hummingbird phenotypes is <5%. V+, the number of hummingbird phenotypes is 5%-40%. V++, the number of hummingbird phenotypes is >40%. E: elongation; V: vaculeae.

**Fig. 4.** Morphology of AGS Cells Infected with Wild-type Strains and Mutant Strains under Microscope. AGS cells were infected with wild-type 26695, HPK5 or 26695△cagA mutant, HPK5△cagA mutant strains. The morphology of AGS cells was investigated (magnification 360 ×). AGS cells were used as controls. Spreading and elongation of AGS cells to form needle-like structures were the typical changes in phenotypes of 26695 wild-type strains. Arrows indicate the hummingbird phenotype not observed in AGS cells infected with HPK5 and mutant strains.

**DISCUSSION**

The fact that CagA protein is different depending on *H. pylori* strains suggests that differences in CagA proteins may underlie the different prevalences of gastric carcinoma in different geographic areas[25]. CagA and phosphorylated CagA proteins increased in a time- and strain-dependent manner in AGS cells infected with wild-type strains. However, CagA proteins from 26695 showing the lower but not the upper band, was not phosphorylated in AGS cells, indicating that the lower band of CagA is not
efficiently translocated into AGS cells, and CagA protein can selectively enter a specialized type IV secretion system in the cag pathogenicity island; or CagA molecules are translocated, but not phosphorylated. The versatility of type IV secretion systems is reflected in recognition of the CagA translocation signal [26]. C-terminal of CagA is sufficient to determine the recruitment, and enters the translocation channel. Translocation of the CagA protein may depend on the presence of a secretion chaperone-like protein.

The hummingbird appeared in AGS cells infected with 26695 (WSS-type) but not with 26695ΔcagA, supporting that the morphological features are associated with phosphorylated CagA and the delivery of CagA into host cells, which is necessary for the virulence to induce the hummingbird phenotype [18,24]. Based on the binding affinities of different CagA-types to SHP-2, the ESS-type CagA should induce changes in hummingbird phenotype more effectively than the WSS-type CagA [18]. However, the phenotype induced by HPK5 (ESS-type) was absent in our experiments, suggesting that the phosphorylated CagA interaction might not be sufficient to induce morphological features and/or might depend on signal cascade of strains per se. The degree of colonization in mice infected with the cagA mutant is not significantly lower than in those infected with corresponding parental strains [27], demonstrating that cagA mutants are able to infect animal models with the same efficiency as the wild-type strains.

We sequenced HPK5 cagA and compared the amino acid sequence of 26695 cagA as previously described [20]. The aliment of amino residues demonstrated that three EPIYA motifs, critical for the tyrosine phosphorylation of CagA [28], existed in C-terminal of half HPK5 CagA, resulting in ESS type. 26695 CagA possessing two EPIYA and one EPIYT motifs is the WSS type. EPIYA is changed by EPIYT in 26695 strains at the position 918. The two strains possess a different number of EPIYA even though they are mostly homologous. Phosphorylation of CagA at Y-972 has a function in actin-based cytoskeletal rearrangements. However, other factors may contribute to the CagA-phosphorylation-dependent or CagA independent hummingbird phenotype in AGS cells induced with H. pylori strains per se.

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