Tyrosine Phosphorylation of CagA from Chinese *Helicobacter pylori* Isolates in AGS Gastric Epithelial Cells

Youli Zhang,† Richard H. Argent,2*† Darren P. Letley,2 Rachael J. Thomas,2 and John C. Atherton2

Division of Gastroenterology, The Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu, China,1 and Institute of Infection, Immunity, and Inflammation and Wolfson Digestive Diseases Centre, University of Nottingham, Queen’s Medical Centre, Nottingham, United Kingdom2

Received 20 July 2004/Returned for modification 13 September 2004/Accepted 18 October 2004

*Helicobacter pylori* strains possessing the *cag* pathogenicity island (PaI) are associated with the development of gastroduodenal diseases, including gastric cancer. *cag* PaI products induce the secretion of interleukin-8 (IL-8) from epithelial cells and facilitate the translocation of CagA into the cell cytosol. In East Asia, where the incidence of gastric cancer is high, most strains possess the *cag* PaI. To date, however, no *cag* PaI phenotypic data have been provided for strains isolated in mainland China. Here we used 31 Chinese strains to determine the genotypic and phenotypic status of the *cag* PaI. All strains possessed *cagA* and *cagE*, and we observed a variation in the length of *cagA* variable regions. Nucleotide sequencing of the *cagA* variable region revealed that CagA was of two types, a short “Western” form with two tyrosine phosphorylation sites and a longer “East Asian” form with three tyrosine phosphorylation sites. Coculture of strains with AGS epithelial cells showed that strains could induce IL-8 secretion from the cells and that CagA with three phosphorylation sites became more phosphorylated than that with two and could induce significantly (*P* < 0.001) more cells to elongate. We hypothesize that the preponderance of the more active East Asian form of *cagA* may underlie the high rate of gastric cancer in China.

*Helicobacter pylori* is a spiral, gram-negative, microaerophilic bacterium that is present in the stomachs of approximately half of the world’s population. *H. pylori* has been identified as a causative agent of chronic gastritis and of gastric and duodenal ulceration, and infection with this organism is an important risk factor for the development of gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma (12, 23). Several putative *H. pylori* virulence factors have been identified as additional risk factors involved in ulceration and gastric cancer, including the cytotoxin-associated gene (*cag*) pathogenicity island (PaI).

The *cag* PaI comprises a 40-kb DNA segment integrated into the *H. pylori* chromosome in some strains (9) and encodes a type IV secretory system, which is associated with enhanced virulence and increased mucosal inflammation. The *cagA* gene encodes the CagA protein and has been used as a marker for the presence of the *cag* PaI. The type IV secretory system facilitates the translocation of CagA into the cytosol of epithelial cells; CagA localizes to the inner surface of the plasma membrane (21) and subsequently undergoes tyrosine phosphorylation in the host cells (2, 7, 16, 19, 21) by Src family protein tyrosine kinases (2, 20, 22). The CagA tyrosine phosphorylation motifs (TPMs) have been mapped to the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs present in the C-terminal region of the protein (3, 13, 15, 22). CagA proteins generally possess two or more EPIYA motifs within the variable region, which accounts for the size variation observed between CagA proteins isolated from different *H. pylori* strains. CagA proteins with greater numbers of TPMs become more phosphorylated and lead to enhanced formation of the “hummingbird” phenotype (1, 13). Analysis of *cagA* in *H. pylori* strains isolated from patients in East Asia, where the incidence of stomach cancer is high, has shown differences between the *cagA* variable region of those isolates and that of “Western” isolates (24) and also an association between the number of CagA TPMs and the development of atrophic gastritis and gastric cancer in Japan (5, 24).

In this study we looked at the *cagA* status of *H. pylori* strains from Chinese patients. Previously, Zhou et al. (25) showed that all 18 *H. pylori* strains isolated from the Hangzhou region of China possessed *cagA* and that 17 of 18 were of the “East Asian” type, although no sequence data were provided to show differences between *cagA* genes from different strains. Zhou et al. (26) showed that 79 of 82 Chinese *H. pylori* strains possessed *cagA* genes which, as determined by PCR amplification of the 3′ variable regions, were of three types, although no data were provided to show the composition of each of these variable regions. Neither study presented phenotypic data regarding the expression and tyrosine phosphorylation of CagA from these strains. We therefore decided to look at the *cag* PaI status and *cagA* variable region in *H. pylori* strains isolated from eastern China in terms of the numbers of CagA TPMs, tyrosine phosphorylation, and hummingbird phenotype formation.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *H. pylori* strains were isolated from 31 Chinese patients, living in Jiangsu Province of mainland China, with chronic gastritis alone (12 cases), duodenal ulcers (11 cases), gastric ulcers (5 cases), both duodenal and gastric ulcers (2 cases), and gastric cancer (1 case), and were grown
on horse blood agar plates (Oxoid, Basingstoke, United Kingdom) at 37°C in a microaerobic environment.

PCR amplification of cagA and cagE genes. H. pylori genomic DNA was extracted from minimally passaged strains as previously described (4). PCR amplification of the cagA 3′ variable region with primers cag2 and cag3 as previously described for South African and European strains (18) was unsuccessful. The reverse primer was therefore redesigned as either cagA31 (5′-CGCTATGCTCAACCTGTTGGAACATCGAC-3′) or cagA26R (5′-GGCTT AGCTCTCGTATACC-3′), and the PCR was performed under otherwise identical conditions. For nucleotide sequencing of cagA variable regions, PCR-amplified products (with primers cag2 and cagA26R) were purified with a QIAquick purification kit (Qiagen, Crawley, United Kingdom) before sequence analysis by the Biopolymer Synthesis and Analysis Unit, Queen’s Medical Centre, University of Nottingham, United Kingdom. PCR amplification of cagE was carried out with primers PBRTF (5′-AAGGGTAAAAGATGGGACTGAAT) and PBTRR (5′-GGAAAGCTGATAAAAGACGAATGT). A reaction mixture containing a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.4 nM concentration of each primer, 0.5 U of Taq DNA polymerase (Roche Diagnostics, Penzberg, Germany) per μl, and 1 μl of genomic DNA, in buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM magnesium chloride, 50 mM potassium chloride, 1% [wt/vol] sodium dodecyl sulfate, 40% [vol/vol] glycerol, 0.4% [wt/vol] bromophenol blue). Samples were heated at 100°C for 5 min before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with the Biorad (Hemel Hempstead, United Kingdom) or anti-CagA polyclonal antibodies (Austral Biologicals, Santa Cruz, Calif.) or antiphosphotyrosine monoclonal antibodies (clone PY99; Santa Cruz Biotechnology, Inc.). Digestion of the samples loading buffer (0.2 M Tris-HCl [pH 6.8], 0.4 M dithiothreitol, 8% [wt/vol] sodium dodecyl sulfate, 40% [vol/vol] glycerol, 0.4% [wt/vol] bromophenol blue). Samples were heated at 100°C for 5 min before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antiphosphotyrosine monoclonal antibodies (clone PY99; Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-CagA polyclonal antibodies (Austral Biologicals, San Ramon, Calif.). Quantification of the CagA phosphorylation was performed by densitometry with a Bio-Rad (Hemel Hempstead, United Kingdom) GS-800 calibrated densitometer and Quantity One software and was expressed as a ratio of phospho-CagA to total CagA.

Expression and tyrosine phosphorylation of CagA. We next went on to look at the expression of CagA by Chinese H. pylori strains and whether they became phosphorylated within AGS cells. AGS human gastric epithelial cells were seeded into 25-cm² flasks (10⁵ cells/flask) in Ham F-12 nutrient mixture and incubated at 37°C in a 5% CO₂–air humidified atmosphere until almost confluent. H. pylori strains were resuspended in Ham F-12 nutrient mixture and diluted to an optical density at 550 nm of 0.1 (multiplicity of infection, ~100) before addition to AGS cells for 6 h at 37°C in a 5% CO₂–air humidified atmosphere. Infected AGS cells were washed twice with phosphate-buffered saline (PBS), and the cells were scraped from the flasks in 5 ml of PBS containing 1 mM sodium vanadate, harvested by centrifugation at 1,000 x g for 10 min, and then resuspended in 100 μl of PBS-vanadate and 5 μl of 4× sample loading buffer (0.2 M Tris-HCl [pH 6.8], 0.4 M dithiothreitol, 8% [wt/vol] sodium dodecyl sulfate, 40% [vol/vol] glycerol, 0.4% [wt/vol] bromophenol blue). Samples were heated at 100°C for 5 min before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antiphosphotyrosine monoclonal antibodies (clone PY99; Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-CagA polyclonal antibodies (Austral Biologicals, San Ramon, Calif.). Quantification of the degree of CagA phosphorylation was performed by densitometry with a Bio-Rad (Hemel Hempstead, United Kingdom) GS-800 calibrated densitometer and Quantity One software and was expressed as a ratio of phospho-CagA to total CagA.

RESULTS

PCR amplification of cagA and cagE genes. PCR amplification of cagA from Chinese H. pylori strains, using primers cag2 and cagA31, revealed that all (31 of 31) strains possessed this gene, although there was size variation in the lengths of products generated. Most strains possessed a cagA fragment of ~890 bp, whereas three of the strains possessed smaller fragments of ~840 bp (strain Z4) and ~770 bp (strains Z11 and Z33) (Fig. 1A). Amplification of cagE revealed that all (31 of 31) strains also possessed this gene.

Sequencing of the cagA variable region. As we found size variation within cagA between strains, we decided to perform nucleotide sequence analysis of the cagA variable regions from 10 strains (the three with shorter cagA genes and seven of those with longer cagA genes). The cagA variable region was amplified by PCR with primers cag2 and cagA26R prior to nucleotide sequencing. We found that CagA variable regions from these Chinese isolates fell into two types: a short one possessing two EPIYA motifs and a longer one possessing three EPIYA motifs (Fig. 2). Of the three shorter cagA genes, we found that one (strain Z4) possessed three variable-region TPMs but had a deletion between the second and third EPIYA motifs, whereas the other two (strains Z11 and Z33) possessed only two EPIYA motifs. We also found that the variable region possessing two TPMs had a Western-type sequence, whereas the variable region with three TPMs had an East Asian-type sequence, characterized by the EVSAINRKKIDRINKIAAS KGVGGFSGA pattern following the second EPIYA motif (Fig. 2).

Expression and tyrosine phosphorylation of CagA. We next went on to look at the expression of CagA by Chinese H. pylori strains and whether they became phosphorylated within AGS cells.
epithelial cells by coculture. We tested 18 of the strains and found that all of them expressed CagA, which became tyrosine phosphorylated within AGS cells (Fig. 3), and all of these strains induced interleukin-8 secretion from AGS cells (Fig. 4).

As expected, CagA proteins with two TPMs were of lower molecular weight than those with three TPMs, and densitometric analysis revealed that CagA proteins possessing three EPIYA motifs became more phosphorylated than those with only two EPIYA motifs (Fig. 3).

Induction of AGS cell hummingbird phenotype formation by CagA. CagA phosphorylation within epithelial cells leads to hummingbird phenotype formation, the extent of which appears to be increased in proportion to the number of EPIYA motifs (1). We found, following 24-h coculture of AGS cells with \textit{H. pylori} strains Z4, Z7, and Z23 (three variable-region EPIYA motifs) and with strains Z11 and Z33 (two variable-region EPIYA motifs) for 1 day before cells were visualized by microscopy. (A) Percentages of AGS cells displaying the hummingbird phenotype in randomly chosen microscopic fields. *, $P < 0.001$ (Z4, Z7, or Z23, compared to Z11, Z33, or cells alone). (B) Average lengths of protrusions characteristic of hummingbird phenotype of at least 100 randomly chosen hummingbird phenotype AGS cells cocultured with each strain. **, $P < 0.001$; #, $P > 0.05$. Errors bars represent standard errors of the mean.

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**FIG. 3.** CagA with two variable-region TPMs is less phosphorylated than CagA with three variable-region TPMs. (A) AGS cells were cocultured with \textit{H. pylori} strains possessing two (strain Z11) or three (strains Z4, Z7, and Z23) variable-region EPIYA motifs for 6 h at 37°C before the cells were lysed and the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antiphosphotyrosine monoclonal antibodies (lower panel). The blots were then stripped and reprobed with anti-CagA polyclonal antibodies (upper panel). (B) Densitometric analysis of the degree of CagA phosphorylation, expressed as a ratio of phospho-Tyr intensity to CagA protein intensity. The data shown are representative of those from three separate experiments.

**FIG. 4.** Interleukin-8 (IL-8) secretion induced by \textit{H. pylori} strains. Chinese \textit{H. pylori} strains were cocultured with AGS cells for 6 h before the amount of secreted interleukin-8 was determined by enzyme-linked immunosorbent assay. The results are expressed as percentages of the maximal value (for strain Z37). Errors bars represent standard errors of the means.

**FIG. 5.** AGS cell hummingbird phenotype formation is reduced by CagA with two variable-region TPMs. AGS cells were cocultured with \textit{H. pylori} strains Z4, Z7, and Z23 (three variable-region EPIYA motifs) and with strains Z11 and Z33 (two variable-region EPIYA motifs) for 1 day before cells were visualized by microscopy. (A) Percentages of AGS cells displaying the hummingbird phenotype in randomly chosen microscopic fields. *, $P < 0.001$ (Z4, Z7, or Z23, compared to Z11, Z33, or cells alone). (B) Average lengths of protrusions characteristic of hummingbird phenotype of at least 100 randomly chosen hummingbird phenotype AGS cells cocultured with each strain. **, $P < 0.001$; #, $P > 0.05$. Errors bars represent standard errors of the mean.
characteristic of the hummingbird phenotype induced were significantly \((P < 0.0001)\) shorter for the CagA proteins with two variable-region EPIYA motifs than for those with three EPIYA motifs (Fig. 5B).

**DISCUSSION**

*H. pylori* is recognized as a major causative agent of chronic gastritis and peptic ulcer disease and has been identified as a major risk factor for gastric cancer. In most studies, strains possessing cagA have been associated with an increased risk for the development of gastric cancer (8, 11), although some studies found no significant association (14, 17). This is especially true in East Asia, where more than 90\% of strains possess cagA. As CagA shows size differences due to variations in the number of TPMs within the variable region of the protein, the degree of CagA phosphorylation may be a more important determinant of virulence than possession of cagA (1, 5, 24). Indeed, studies using *H. pylori* strains isolated from Japanese subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24), and a study using *H. pylori* isolated from South Africans found that six of seven strains expressing CagA proteins from East Asian subjects have shown that strains expressing CagA with higher numbers of TPMs are more prevalent in patients with atrophic gastritis and gastric cancer (5, 24). CagA proteins vary in their length due to the presence of the pV-(S/T/A/V/I)-X-(V/I/L)-X-(W/F) phosphorylation motif (10) in the C-type motif, EPIYATIDDL (6, 13). CagA proteins lacking this motif (or the EPIYATIDDL motif present in CagA proteins from Western strains) do not appear to bind the phosphatase (6). Coculture of AGS cells with the Chinese *H. pylori* strains used in this study revealed that the CagA proteins with three EPIYA motifs induce significantly more cells to undergo transformation into the hummingbird phenotype and induce longer protrusions than CagA proteins with only two EPIYA motifs, due presumably to lack of the third (C-type) TPM.

In summary, we show that, in *H. pylori* strains from Jiangsu Province in China, there are two allelic forms of cagA: the more commonly occurring form of East Asian origin and the infrequently occurring form of Western origin. Strains with these different forms of cagA display marked differences in CagA phosphorylation and induction of the hummingbird phenotype. As all strains possess cagA, the differences in Cag phenotypes may represent important differences in the pathogenic potentials of strains infecting different patients. Large studies are now needed to examine the association between the number of tyrosine phosphorylation sites and disease, especially among populations where cagA is virtually ubiquitous, such as that in China.

**ACKNOWLEDGMENTS**

This work was funded by a grant from Cancer Research UK. Youli Zhang is funded by a scholarship from the Jiangsu Provincial Department of Education of China to conduct the research in the United Kingdom. John Atherton is funded by a Senior Clinical Fellowship from the Medical Research Council, United Kingdom.

We thank Joanne Rhead and Geraldine Flaujac-Lafontaine for technical assistance.

**REFERENCES**


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