Influence of EPIYA-repeat polymorphism on the phosphorylation-dependent biological activity of Helicobacter pylori CagA

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Dear Dr. Liddle and Dr. Brenner,

Thank you very much for conducting the review of our manuscript GASTRO-D-05-00617. I am delighted to hear from you that the manuscript is acceptable for publication in Gastroenterology. I greatly appreciate your efforts and the time and very helpful comments of the reviewers. I am returning herewith the revised manuscript, in which modified parts are indicated in Red. Followings are our point-by-point responses to the reviewers’ comments:

Reviewer #1
In accordance with the reviewer’s comment, we discussed the potential role of SHP-2 polymorphism on the CagA-SHP-2 interaction and the H. pylori-mediated gastric carcinogenesis, by referring a recently published work by Goto et al (Int J Cancer 118, 203-208, 2006). Although there are reported polymorphisms in the csk gene (Klootwijk et al. J Med Genet 40, e43, 2003), its relationship with gastric carcinoma is currently unknown.

Reviewer #2
1. We agree with the reviewer that we cannot exclude the possibility raised by the reviewer. Accordingly, we modified the discussion by incorporating the reviewer’s idea in the revised manuscript.
2. In accordance with the reviewer’s comment, we discussed the pathophysiological relevance of the EPIYA-repeat polymorphism from...
the bacterial side in the revised manuscript.

I hope that our responses to the reviewers’ comments/concerns are appropriate and satisfactory. I again thank you very much for your time and continued interest with our work.

Sincerely yours,

Masanori Hatakeyama, M.D., Ph.D.
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Influence of EPIYA-repeat Polymorphism on the Phosphorylation-dependent Biological Activity of Helicobacter pylori CagA

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Short title: EPIYA POLYMORPHISM ON H. PYLORI CagA ACTIVITY

The abbreviations used are: Csk, C-terminal Src kinase; HA, hemagglutinin; IB, immunoblotting; IP, immunoprecipitate; SH2, Src homology 2; SFK, Src family kinase; TCL, total cell lysate.
Abstract

Background & Aims: Helicobacter pylori CagA-positive strain is associated with gastric adenocarcinoma. CagA is delivered into gastric epithelial cells, where it undergoes tyrosine phosphorylation at the EPIYA sites by Src family kinases. Due to homologous recombination within the 3'-region of the cagA gene, four distinct EPIYA sites, each of which is defined by surrounding sequences, are variably assembled in both number and order among CagA proteins from different clinical Helicobacter pylori isolates. Tyrosine-phosphorylated CagA specifically binds and deregulates SHP-2 via the Western CagA-specific EPIYA-C or East Asian CagA-specific EPIYA-D site, and Csk via the EPIYA-A or EPIYA-B site. Here we investigated the influence of EPIYA-repeat polymorphism on the CagA activity.

Methods: A series of EPIYA-repeat variants of CagA were expressed in AGS gastric epithelial cells and the ability of individual CagA to bind SHP-2 or Csk was determined by the sequential immunoprecipitation and immunoblotting method. Results: CagA proteins carrying multiple EPIYA-C or EPIYA-D sites bound and deregulated SHP-2 more strongly than those having a single EPIYA-C or EPIYA-D. Furthermore, the ability of CagA to bind Csk was correlated with the number of
EPIYA-A and EPIYA-B sites. Because Csk inhibits Src family kinase, CagA with greater Csk-binding activity more strongly inhibited Src-dependent CagA phosphorylation and more effectively attenuated induction of cell elongation caused by CagA-SHP-2 interaction.

**Conclusions:** EPIYA-repeat polymorphism of CagA greatly influences the magnitude and duration of phosphorylation-dependent CagA activity, which may determine the potential of individual CagA as a bacterial virulence factor that directs gastric carcinogenesis.
Infection with *Helicobacter pylori* (*H. pylori*) is associated with chronic gastritis and peptic ulcer and is the strongest risk factor for gastric adenocarcinoma.\(^1,2\) Recent studies have shown that the development of gastric carcinoma is dependent on genetic factors in both the host and the pathogen. For instance, host genetic polymorphisms in proinflammatory cytokine genes affect the risk of gastric carcinoma.\(^3,4\) Genetic diversity in *H. pylori* strains also plays a crucial role in gastric cancer development. Especially, infection with *H. pylori* strains harboring the *cag* pathogenicity island (*cag* PAI) results in the development of severe gastric mucosal inflammation and is most closely associated with the development of gastric adenocarcinoma.\(^5,6\)

*cag* PAI is a ~40-kilobase (kb) DNA fragment that contains a group of genes that encode the bacterial type IV secretion system (TFSS).\(^7,8\) The DNA fragment also contains a gene called *cagA*, whose product is a 125–140-kilodalton (kDa) CagA protein.\(^9,10\) CagA is directly translocated from *H. pylori* into the bacteria-attached gastric epithelial cells via TFSS, and, upon localizing to the plasma membrane, undergoes tyrosine phosphorylation by Src family kinases (SFKs).\(^11-17\) Tyrosine-phosphorylated CagA then binds specifically to SHP-2 tyrosine phosphatase and deregulates phosphatase activity.\(^18-20\) CagA-activated
SHP-2 dephosphorylates focal adhesion kinase (FAK) and inhibits kinase activity, which elicits elevated cell motility by reducing active focal adhesion spots.\textsuperscript{21} CagA-activated SHP-2 also causes sustained Erk MAP kinase activation, which stimulates cell-cycle progression.\textsuperscript{22} Because abnormal proliferation as well as abnormal cell motility are characteristic of transformed cells, deregulation of SHP-2 by CagA may play an important role in gastric cancer development.\textsuperscript{20,23} Indeed, recent studies have shown that gain-of-function mutations in \textit{PTPN11}, the gene encoding SHP-2, are associated with various human malignancies,\textsuperscript{24,25} indicating that SHP-2 is a \textit{bona fide} oncoprotein that is substantially involved in human malignancies.

Whereas the majority of CagA proteins expressed in gastric epithelial cells interact with SHP-2, a fraction of them also interact with the C-terminal Src kinase (Csk), again, in a tyrosine phosphorylation-dependent manner.\textsuperscript{18,26} Through the complex formation, CagA activates Csk, which in turn inhibits SFK kinase activity by phosphorylating the C-terminal inhibitory tyrosine residues. Since SFKs are responsible for CagA phosphorylation,\textsuperscript{16,17} CagA-Csk interaction is considered to attenuate tyrosine phosphorylation-dependent pathophysiological activity of CagA.\textsuperscript{26} Such a feedback regulation may
contribute to the long-term equilibrium between *cagA*-positive *H. pylori* and the host without causing excess CagA toxicity.

CagA is tyrosine-phosphorylated at multiple Glu-Pro-Ile-Tyr-Ala (EPIYA) sites present in the C-terminal region.\(^{18-20}\) Due to frequent homologous recombination within the 3’-region of the *cagA* gene, the EPIYA-repeat region of CagA is highly divergent among various CagA species and is composed of various combinations of four discrete segments termed EPIYA-A, -B, -C and -D.\(^{19,20,27}\) Thus, the EPIYA-repeat regions of prevalent CagA proteins from *H. pylori* isolated in Western countries consist of EPIYA-A, EPIYA-B and variable numbers of EPIYA-C segments (ABC-, ABCC- or ABCCC-type CagA). In contrast, the EPIYA-repeat regions of prevalent East Asian CagA proteins consist of EPIYA-A, EPIYA-B and EPIYA-D segments (ABD-type CagA). Each of the EPIYA segments contains a single EPIYA site (EPIYA-A, -B, -C or -D site), which undergoes tyrosine phosphorylation.\(^{27}\) SHP-2 binds to the EPIYA-C and EPIYA-D sites of Western and East Asian CagA species, respectively, in a phosphorylation-dependent manner. The EPIYA-D site exhibits greater SHP-2-binding activity and therefore stronger ability to induce cell elongation known as the hummingbird phenotype than does the EPIYA-C site.\(^{19}\) Consequently, *H. pylori* strains carrying East Asian CagA elicit
stronger mucosal inflammation and are more closely associated with gastric carcinoma than are those carrying Western CagA. Furthermore, Western *H. pylori* strains carrying CagA with multiple EPIYA-C sites, which bind SHP-2 more effectively than those having a single EPIYA-C, are more frequently isolated from patients with gastric adenocarcinoma. Hence, the degree of CagA to deregulate SHP-2 appears to play an important role in determining oncogenic potential of individual *H. pylori* cagA-positive strain.

In this work, we investigated the influence of EPIYA-repeat polymorphism on CagA activities to bind SHP-2 and Csk as well as to induce the hummingbird phenotype and found that the degree of phosphorylation-dependent CagA activities is variably altered by the diversity of EPIYA repeats. Our results provide a functional link between the EPIYA-repeat polymorphism of CagA and the virulence of individual cagA-positive *H. pylori*. 


Materials and Methods

Construction of Expression Vectors

A gene encoding the ABDD-type CagA of F75 East Asian strain\(^1\) was synthesized and was cloned into pSP65SR\(\alpha\). EPIYA-repeat variants of East Asian CagA were generated from the ABDD-type CagA by using a Chameleon site-directed mutagenesis kit (Stratagene). Similarly, EPIYA-repeat variants of Western CagA were generated from the gene encoding ABCCC-type CagA of NCTC11637 origin.\(^1\) A membrane-targeting mutant of Csk was made by adding the myristoylation signal sequence from avian c-Src to the N-terminus and was cloned into pSP65SR\(\alpha\).\(^3\)

Cell Culture and Transfection

AGS human gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and at 37°C in 5% CO\(_2\). Cells (2.0 \(\times\) 10\(^6\) cells/10-cm\(^2\) dish) were transfected with 30 \(\mu\)g of plasmids by using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

Antibodies

Anti-HA monoclonal antibody 3F10 (Roche Applied Science) was used as primary antibody for immunoprecipitation. Anti-HA polyclonal
antibody Y-11 (Santa Cruz Biotechnology), anti-SHP-2 polyclonal antibody C-18 (Santa Cruz Biotechnology), anti-Csk polyclonal antibody C-20 (Santa Cruz Biotechnology), anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology), anti-phospho-Src (Tyr-416) polyclonal antibody (Cell Signaling), anti-c-Src polyclonal antibody N-16 (Santa Cruz Biotechnology) and anti-Myc-epitope monoclonal antibody 9E10 were used as primary antibodies for immunoblotting.

**Immunoprecipitation and Immunoblotting**

For immunoprecipitation, cells were harvested at 36 h after transfection and total cell lysates were prepared as described previously. Total cell lysates and immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Protein transferred to polyvinylidene difluoride membrane filters (Millipore) were incubated in primary antibodies and then visualized by using Western blot chemiluminescence reagent (PerkinElmer Life Sciences). Intensities of chemiluminescence on the immunoblotted membrane were quantitated by using a luminescence image analyzer (LAS1000, FUJIFILM).

**Cell Morphological Analysis**

AGS cells were seeded into 35-mm² dishes (1.2 x 10⁵ cells/dish) and 8 µg of plasmids was transfected into cells. Cell morphology was examined
by light microscopy.
Results

Diversity in the Order and Number of EPIYA Sites among CagA Proteins

Based on the structure of the EPIYA-repeat region, most of the Western CagA proteins are categorized as the ABCn-type (where n indicates the number of EPIYA-C sites),\textsuperscript{27-29} whereas prevalent East Asian CagA species belong to the ABD-type (Figure 1). Notably, however, a small number of East Asian CagA proteins show complicated variations in the EPIYA-repeat region. The presence of duplicated EPIYA-A and/or EPIYA-B segments in some of these CagA variants suggested that the EPIYA-repeat polymorphism of CagA not only affects CagA-SHP-2 interaction but also CagA-Csk interaction. To address the effect of EPIYA diversity on CagA function, we generated a series of EPIYA-repeat variants of CagA that represent reported variations in the order and number of EPIYA segments (Figure 1). All of the CagA constructs generated were C-terminal hemagglutinin (HA)-tagged.

Influence of EPIYA-repeat Polymorphism on the Level of CagA Tyrosine Phosphorylation

To investigate the effect of EPIYA-repeat polymorphism on the level of CagA tyrosine phosphorylation, AGS human gastric epithelial cells were
transfected with each of the CagA variants and cell lysates prepared were immunoblotted with an anti-HA antibody or an anti-phosphotyrosine (pY) antibody. The protein bands detected by chemiluminescence were quantitated with the luminescence image analyzer. The intensity of the image obtained by the luminescence image analyzer is directly proportional to the light intensity, indicating that it is broader in dynamic range and has better linearity and is therefore more accurate than densitometric analysis for quantitation.

In Western CagA proteins, the level of CagA tyrosine phosphorylation was proportional to the number of EPIYA-C sites as reported previously (Figure 2A). In East Asian CagA species, CagA tyrosine phosphorylation was correlated to the number of EPIYA-D sites (Figure 2B). Since EPIYA-A and EPIYA-B sites were also tyrosine-phosphorylated, though less effectively, CagA with more EPIYA-A and/or EPIYA-B sites underwent higher levels of tyrosine phosphorylation than did CagA with less EPIYA-A and/or EPIYA-B sites (Figure 2B).

Influence of EPIYA-repeat Polymorphism on CagA-SHP-2 Interaction

Next, to investigate the effect of EPIYA-repeat polymorphism on the complex formation between CagA and SHP-2, lysates of cells expressing
each of the EPIYA-repeat variants of CagA were immunoprecipitated with an anti-HA antibody. The anti-HA immunoprecipitates were then immunoblotted with an anti-SHP-2 antibody. The amount of SHP-2 co-precipitated with CagA was proportional to the number of EPIYA-C sites in Western CagA as previously reported (Figure 3A, left). Quantitation analysis revealed that ABCC-type CagA and ABCCC-type CagA respectively co-precipitated 4-fold and 8-fold greater amounts of SHP-2 than the ABC-type CagA did (Figure 3A, right). In East Asian CagA species, those having two EPIYA-D sites co-precipitated 8-fold greater amounts of SHP-2 than did CagA with a single EPIYA-D site (Figure 3B). These results indicated that the presence of multiple EPIYA-C or -D sites synergistically potentiates the ability of CagA to bind SHP-2.

Influence of EPIYA-repeat Polymorphism on CagA-Csk Interaction

The effect of EPIYA-repeat polymorphism on the complex formation between CagA and Csk was next investigated. Consistent with the observation that Csk binds to the EPIYA-A or EPIYA-B site of CagA in gastric epithelial cells, the amount of Csk co-precipitated with CagA was proportional to the number of EPIYA-A and EPIYA-B sites in both Western CagA (Figure 4A) and East Asian CagA (Figure 4B), except for the cases
with ABDABD-type CagA and ABDBD-type CagA. These two East Asian CagA variants co-precipitated significantly larger amounts of Csk compared with other CagA species (Figure 4B). As they exhibited the highest level of tyrosine phosphorylation among the CagA variants (Figure 2B), we examined the possibility that the increased level of CagA tyrosine phosphorylation affected the Csk-binding activity. To this end, a tyrosine residue within the first EPIYA-D site of ABDABD-type CagA was replaced by phenylalanine residue. The resulting ABdABD-type CagA showed levels of tyrosine phosphorylation and SHP-2 binding similar to those observed with ABABD-type CagA in AGS cells (Figure 5). However, the CagA mutant still retained the ability to co-precipitate Csk to a level comparable to that of Csk co-precipitated by ABDABD-type CagA. These results excluded the role of tyrosine-phosphorylated EPIYA-D in the CagA-Csk interaction and suggested that the presence of two EPIYA-B segments, both of which are directly followed by EPIYA-D segments, confers strong Csk-binding activity to the tyrosine-phosphorylated EPIYA-B sites.

Direct Comparison of SHP-2- and Csk-binding Activities between Western CagA and East Asian CagA

Comparison of the SHP-2-binding activity confirmed that ABD-type
East Asian CagA interacts with SHP-2 more strongly than does ABC-type Western CagA (Figure 6A). On the other hand, ABC-type CagA was found to bind Csk more efficiently than ABD-type CagA (Figure 6A). Given that CagA binds to SHP-2 and Csk in a mutually exclusive manner, the observation indicated that CagA-SHP-2 interaction affects CagA-Csk complex formation. To address this possibility, we replaced the tyrosine residue in the EPIYA-C site of ABC-type CagA or in the EPIYA-D site of ABD-type CagA with alanine residue. The resulting ABc-type and ABd-type CagA mutants bound Csk at comparable levels (Figure 6B). Thus, degree of CagA-Csk interaction is affected by the degree of strength of the CagA-SHP-2 interaction.

**Inhibition of CagA-SHP-2 Interaction by Csk**

To determine whether elevated Csk activity is capable of inhibiting tyrosine phosphorylation of CagA through SFK inhibition, we generated a Csk mutant that possesses a membrane-targeting signal derived from avian c-Src at the N-terminus (Myr-Csk). The Csk mutant associates with the plasma membrane and acts as a constitutively active form of Csk. Co-expression studies of abD-type CagA, which has tyrosine-to-phenylalanine substitutions in both EPIYA-A and EPIYA-B sites and thus does not bind Csk, together with Myr-Csk revealed that the
level of tyrosine phosphorylation at the EPIYA-D site as well as the amount of SHP-2 associated with CagA were significantly reduced in AGS cells expressing Myr-Csk (Figure 7). The result indicated that elevated Csk activity inhibits SFK-mediated CagA phosphorylation and subsequent CagA-SHP-2 complex formation.

Influence of EPIYA-repeat Polymorphism on Induction of the Hummingbird Phenotype by CagA

CagA-activated SHP-2 induces an elongated cell shape termed the hummingbird phenotype.\textsuperscript{18,22} Thus, CagA with greater ability to bind SHP-2 exhibits stronger activity to induce the hummingbird phenotype than does CagA with less SHP-2-binding activity.\textsuperscript{19} On the other hand, elevated Csk activity inhibits SFK and subsequent CagA-SHP-2 complex formation (Figure 7). These results collectively indicated that stimulation of Csk by CagA downregulates CagA-SHP-2 signaling. To investigate whether such a feedback regulation of the CagA activity indeed operates in cells expressing CagA, we directly compared the ability of ABD-type CagA, ABDD-type CagA, and ABDABD-type CagA to induce the hummingbird phenotype. When expressed in AGS cells, ABDD-type CagA and ABDABD-type CagA bound more SHP-2 than did ABD-type CagA (Figure 3B). As shown in Figure 8A, kinetic studies of the hummingbird-phenotype
induction by these CagA species revealed that, at 15 h after transfection, 15% of AGS cells transfected with ABD-type CagA exhibited the hummingbird phenotype, whereas 20% of cells transfected with ABDD-type CagA or ABDABD-type CagA developed the hummingbird phenotype. At 17 h after transfection, 20% of the transfected cells showed the hummingbird phenotype in all CagA cases. At this time point, the level of ABD-type CagA expression was greater than that of ABDD- or ABDABD-type CagA (Figure 8B). Thus, CagA with greater SHP-2-binding activity induced the hummingbird phenotype significantly earlier than did CagA with less SHP-2-binding activity. Next duration of the hummingbird phenotype with these CagA species was examined. At 48 h after transfection, the number of cells with the hummingbird phenotype induced by ABDABD-type CagA, which bounds more Csk than did ABD-type CagA or ABDD-type CagA (Figure 4B), was significantly less than that induced by ABD- or ABDD-type CagA (Figure 8A). When the ratio of tyrosine-phosphorylated CagA to total CagA was compared at the time points of 15 h and 48 h after transfection, CagA tyrosine phosphorylation was found to decrease more rapidly in cells expressing ABDABD-type CagA than in cells expressing ABD-type CagA or ABDD-type (Figure 8B). These results indicated that CagA that binds more Csk downregulates CagA
tyrosine phosphorylation and thereby attenuates CagA-SHP-2 interaction more strongly than does CagA that binds less Csk.

To determine whether stronger attenuation of CagA-SHP-2 signaling by ABDABD-type CagA was due to greater inhibition of SFK kinase activity, we examined the level of active SFKs in cells expressing each of these CagA species with the use of anti-p-Src (Tyr-416) antibody, which specifically recognizes active forms of SFKs. As shown in Figure 8C, ABDABD-type CagA inhibited SFK kinase activity more strongly than did ABD- or ABDD-type CagA. From these observations, we concluded that CagA-Csk interaction causes inhibition of SFKs, which in turn results in reduced levels of CagA phosphorylation and subsequent downregulation of CagA-SHP-2 interaction.
Discussion

In the present work, we demonstrated that the level of tyrosine phosphorylation, the degree of SHP-2 binding activity, and the magnitude of the hummingbird phenotype-inducing activity are proportional to the number of EPIYA-C and EPIYA-D sites in Western CagA and East Asian CagA, respectively. Thus, the EPIYA-D site of East Asian CagA is the functionally equivalent of the EPIYA-C site of Western CagA in terms of CagA-SHP-2 interaction.\(^{19,20}\) We also showed that the CagA-SHP-2 interaction is synergistic rather than additive with regard to the number of EPIYA-C or EPIYA-D sites. The observation indicates that simultaneous interaction between the two SH2 domains of a single SHP-2 protein and the two tyrosine-phosphorylated EPIYA-C or EPIYA-D sites in a CagA molecule dramatically stabilizes CagA-SHP-2 complex formation.

We also found in this work that the ability of CagA to interact with Csk is proportional to the number of EPIYA-A and EPIYA-B sites. Notably, certain East Asian CagA proteins such as ABDABD-type CagA and ABDBD-type CagA exhibit extremely strong activities to bind Csk compared to other CagA species. These CagA variants are characterized by the presence of two EPIYA-B segments, each of which is directly connected by the EPIYA-D segment. The structure made by the two
EPIYA-B/EPIYA-D stretches may confer strong Csk-binding affinity to the EPIYA-B sites of CagA. If Csk can form a homodimer as are the cases with other protein kinases, such a Csk homodimer might form a stable complex with CagA carrying the two EPIYA-B/EPIYA-D stretches. Whereas the EPIYA-A or EPIYA-B site of Western CagA shows ability to bind Csk comparable to the ability of that of East Asian CagA, ABC-type Western CagA binds Csk more efficiently than does ABD-type East Asian CagA. This is because stronger SHP-2-binding of ABD-type CagA to ABC-type CagA more competitively inhibits CagA-Csk interaction.

CagA that binds more SHP-2 exhibits stronger activity to induce the hummingbird phenotype.\textsuperscript{19} At the same time, CagA that binds more Csk attenuates the hummingbird phenotype more efficiently. These observations suggest functional interplay between the CagA-SHP-2 complex and the CagA-Csk complex. Indeed, our result showing that stronger CagA-Csk interaction shortens the duration of the hummingbird phenotype provides evidence for the presence of a feedback regulatory mechanism of CagA-SHP-2 signaling by the CagA-Csk interaction. Since CagA-Csk interaction is also dependent on CagA tyrosine phosphorylation,\textsuperscript{21,26} such a feedback regulatory loop may generate oscillation of deregulated SHP-2 activity in cells expressing CagA. Given
that ABD-type East Asian CagA binds SHP-2 more strongly but Csk less effectively than does ABC-type Western CagA, East Asian CagA may cause a greater magnitude of oscillation of SHP-2 activity, which may contribute to its more virulent nature.

*H. pylori* strains carrying East Asian CagA are more closely associated with gastric carcinoma than those carrying Western CagA. Among Western *cagA*-positive strains, those with CagA having multiple EPIYA-C sites are more frequently isolated from patients with gastric carcinoma. Although a possibility remains that such Western *H. pylori* strains are positively selected under conditions that predispose gastric carcinoma, the selected variants are biologically more active and thus more potent in inducing gastric mucosal damages. These clinical observations indicate that *H. pylori* strains possessing CagA with stronger SHP-2 activity are more critically involved in the development of gastric carcinoma. However, such *H. pylori* strains can also be isolated from patients with atrophic gastritis or peptic ulcers (Figure 1).

Accordingly, we consider that most if not all *cagA*-positive *H. pylori* strains are capable of inducing atrophic gastritis and peptic ulcers, depending on host genetic factors such as cytokine gene polymorphisms, and only a portion of *H. pylori* strains that possess CagA proteins with stronger SHP-2-binding activities can
direct progression of multi-step gastric carcinogenesis. Extending this idea, host genetic polymorphisms that potentiate the interaction between CagA and SHP-2 or Csk may also affect the oncogenic role of cagA-positive H. pylori. Indeed, it has recently been reported that the G/A single nucleotide polymorphism (SNP) in the PTPN11 gene that encodes SHP-2 is a risk factor for gastric atrophy, a precancerous mucosal change, in individuals infected with H. pylori cagA-positive strains.31

From the bacterial point of view, a critical role of CagA may be to provoke gastric mucosal damages that result in increase in gastric pH, providing better environmental conditions for H. pylori settlement. The observed CagA EPIYA-repeat polymorphism might have been developed to fine-tune the individual CagA activity within certain ranges in both magnitude and duration so as to ensure long-term colonization of H. pylori, without causing acute and fatal damages to the host.

SHP-2 and Csk bind tyrosine-phosphorylated CagA via distinct EPIYA sites. Since Csk acts as an inhibitor of CagA-SHP-2 signaling, the degree of individual CagA to deregulate SHP-2 cannot be simply determined by the total number of EPIYA sites or by the net level of CagA tyrosine phosphorylation. Obviously, CagA with more EPIYA-C and EPIYA-D sites and, at the same time, with less EPIYA-A and EPIYA-B sites is biologically
more active in deregulating SHP-2 in both magnitude and duration. Accordingly, EPIYA-repeat polymorphism of CagA substantially influences the degree of virulence as well as the oncogenic potential of individual cagA-positive H. pylori strain.
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Footnotes

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Figure legends

Figure 1. EPIYA-repeat polymorphism of CagA. Schematic views of EPIYA-repeat variations in Western CagA (A) and East Asian CagA (B) species isolated from patients with various gastrointestinal diseases, such as chronic gastritis (CG), gastric ulcer (GU), duodenal ulcer (DU) and gastric cancer (GC). Color boxes and black bars indicate the EPIYA segments and EPIYA sites, respectively. Names of H. pylori strains and the diseases from which they were isolated are shown in parentheses. Strains 26695, GC78 and F32 are described by Higashi et al.27, Argent et al.29 and Asahi et al.13, respectively. The other strains are described by Higashi et al.19

Figure 2. Tyrosine-phosphorylation of EPIYA-repeat variants of CagA. (A) Total cell lysates (TCL) prepared from AGS cells transfected with EPIYA-repeat variants of Western CagA were subjected to immunoblotting (IB) with anti-phosphotyrosine (pY) antibody or anti-HA antibody (left). Relative amounts of phosphorylated CagA were calculated from the immunoblotting data, defining the values in ABC-type CagA as 1 (right). Experiments were performed in triplicates, and error bars indicate 2x SD. (B) AGS cells were transfected with expression vectors for EPIYA-repeat
variants of East Asian CagA. Cell lysates prepared from the transfected AGS cells were subjected to immunoblotting with anti-phosphotyrosine (pY) or anti-HA antibody (upper). Relative amounts of phosphorylated CagA were calculated from the immunoblotting data, defining the values in ABD-type CagA as 1 (lower). Experiments were performed in triplicates, and error bars indicate 2x SD.

Figure 3. Interaction of EPIYA-repeat variants of CagA with SHP-2. (A) Lysates prepared from AGS cells transfected with EPIYA-repeat variants of Western CagA were immunoprecipitated (IP) with an anti-HA antibody. Anti-HA-immunoprecipitates and total cell lysates were subjected to immunoblotting with anti-SHP-2 antibody or anti-HA antibody (left). Relative amounts of CagA-bound SHP-2 were calculated from the immunoblotting data, defining the values in ABC-type CagA as 1 (right). Experiments were performed in triplicates, and error bars indicate 2x SD.

(B) AGS cells were transfected with expression vectors for EPIYA-repeat variants of East Asian CagA. Cell lysates prepared from the transfected AGS cells were immunoprecipitated with an anti-HA monoclonal antibody, which recognizes HA-tagged CagA. Anti-HA-immunoprecipitates and total cell lysates were subjected to immunoblotting with anti-SHP-2 antibody or
anti-HA antibody (upper). Relative amounts of CagA-bound SHP-2 were calculated from the immunoblotting data, defining the values in ABD-type CagA as 1 (lower). Experiments were performed in triplicates, and error bars indicate 2x SD.

Figure 4. Interaction of EPIYA-repeat variants of CagA with Csk. (A) Lysates of AGS cells transfected with EPIYA-repeat variants of Western CagA were immunoprecipitated with an anti-HA antibody. Anti-HA-immunoprecipitates and total cell lysates were subjected to immunoblotting with anti-Csk antibody or anti-HA antibody (left). Relative amounts of CagA-bound Csk were calculated from the immunoblotting data, defining the values in ABC-type CagA as 1 (right). Experiments were performed in triplicates, and error bars indicate 2x SD. (B) AGS cells were transfected with expression vectors for EPIYA-repeat variants of East Asian CagA. Cell lysates prepared from the transfected AGS cells were immunoprecipitated with an anti-HA monoclonal antibody, which recognizes HA-tagged CagA. Anti-HA-immunoprecipitates and total cell lysates were subjected to immunoblotting with anti-Csk antibody or anti-HA antibody (upper). Relative amounts of CagA-bound Csk were calculated from the immunoblotting data, defining the values in ABD-type
CagA as 1 (lower). Experiments were performed in triplicates, and error bars indicate 2x SD.

**Figure 5.** Effect of EPIYA-D phosphorylation on CagA-Csk interaction. Lysates prepared from AGS cells transfected with the indicated CagA variant or empty vector were immunoprecipitated with anti-HA antibody. ABdABD-type CagA was made from ABDABD-type CagA by replacing the tyrosine residue in the first EPIYA-D site with phenylalanine. The immunoprecipitates and total cell lysates were immunoblotted with the indicated antibodies (upper). Relative amounts of tyrosine-phosphorylated (pY) CagA (lower, left), bound SHP-2 (lower, middle), and bound Csk (lower, right) were calculated from the immunoblotting data, defining the values in ABD-type CagA as 1. Experiments were performed in triplicates, and error bars indicate 2x SD.

**Figure 6.** SHP-2- and Csk-binding activities of ABD-type CagA and ABC-type CagA. (A) Lysates prepared from AGS cells transfected with ABC-type CagA, ABD-type CagA, or empty vector were immunoprecipitated with anti-HA antibody. The immunoprecipitates and total cell lysates were immunoblotted with indicated antibodies (upper).
Relative amounts of tyrosine-phosphorylated (pY) CagA (lower, left), bound SHP-2 (lower, middle), and bound Csk (lower, right) were calculated from the immunoblotting data, defining the values in ABD-type CagA as 1. Experiments were performed in triplicates, and error bars indicate 2x SD.

(B) Lysates prepared from AGS cells transfected with ABc-type CagA, ABd-type CagA or empty vector were immunoprecipitated with anti-HA antibody. The anti-HA immunoprecipitates and total cell lysates were immunoblotted with the indicated antibodies (left). Relative amounts of tyrosine-phosphorylated (pY) CagA (middle) and bound Csk (right) were calculated from the immunoblotting data, defining the values in ABd-type CagA as 1. Experiments were performed in triplicates, and error bars indicate 2x SD.

**Figure 7.** Inhibition of CagA-SHP-2 interaction by Csk. AGS cells were co-transfected with the indicated CagA mutants and Myr-Csk-Myc, or empty vector. Total cell lysates and the anti-HA immunoprecipitates of the lysates were immunoblotted with indicated antibodies (upper). Relative amounts of tyrosine-phosphorylated (pY) CagA (lower, left) and bound SHP-2 (lower, right) were calculated from the immunoblotting data, defining the values in ABD-type CagA as 1. Experiments were performed in
triplicates, and error bars indicate 2x SD.

**Figure 8.** Kinetics of hummingbird-phenotype induction by EPIYA-repeat variants of CagA. (A) AGS cells were transfected with the EPIYA-repeat variant of CagA and, at indicated time-points after transfection, the cells showing hummingbird phenotype were counted in 10 different 0.25-mm² fields in each of three different dishes (*left*). Induction of hummingbird phenotype was compared at 15 h and 48 h post-transfection among the EPIYA-repeat variants of CagA (*right*). Experiments were performed in triplicates, and error bars indicate 2x SD. *, $P < 0.05$. (B) Transfected AGS cells were harvested at 15 h or 48 h after transfection and the cell lysates were immunoblotted with indicated antibodies (*left*). Relative amounts of tyrosine-phosphorylated CagA were calculated from the immunoblotting data, defining the values in each of tyrosine-phosphorylated CagA at 15 h post-transfection as 1 (*right*). Experiments were performed in triplicates, and error bars indicate 2x SD. (C) AGS cells were transfected with 7.5 µg of ABD-type CagA, 9.0 µg of ABDD-type CagA or 30 µg of ABDABD-type CagA expression vector (total amounts of plasmids transfected were adjusted to 30 µg by adding empty control vector) in order to achieve comparable levels of CagA expression. Transfected cells were incubated
with or without 5 µM PP2 for 2 h before harvest. The cell lysates were immunoblotted with indicated antibodies (left). Position of c-Src, p-c-Src and SFKs are indicated by arrows. Relative amounts of phosphorylated c-Src at tyrosine 416 (Y416) were calculated from immunoblotting data by luminescence image analyzer and indicated in graphs defining the values in the absence of CagA as 1. Experiments were performed in triplicates, and error bars indicate 2x SD. *, P < 0.05 (right).
Figure 7.

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IP: anti-HA

TCL
Figure 8.

A

Hummingbird cells (%)

- Control
- ABD
- ABDABD
- ABDD
- ABDABD

(time (hr))

13 15 17 24 36 48

B

CagA-HA

IB: anti-HA

IB: anti-pY

IB: anti-SHP-2

C

DMSO

PP2

CagA-HA ABD

CagA-HA ABDD

CagA-HA ABDABD

IB: anti-HA

IB: anti-p-Src Y416

IB: anti-c-Src

IB: anti-SHP-2

TCL
This piece of the submission is being sent via mail.