Natural variant of the Helicobacter pylori CagA oncoprotein that lost the ability to interact with PAR1

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Gastric cancer is the second most common cause of cancer-related death in the world.¹ Helicobacter pylori, a microaerophilic spiral-shaped bacterium, colonizes the human stomach and causes gastric diseases such as chronic atrophic gastritis and peptic ulcers.¹ Epidemiological studies have also shown that H. pylori is involved in the development of gastric cancer.²⁻⁴ However, the molecular mechanisms by which H. pylori trigger the process leading to gastric cancer remain largely uncertain.

Some H. pylori strains have a cag pathogenicity island (cag-PAI), an approximately 40-kb DNA segment integrated in their chromosome, which is associated with enhanced virulence as measured by mucosal inflammation.⁵⁻⁷ The cag-PAI DNA segment contains genes encoding a type IV secretion apparatus, as well as the cagA gene that codes for the 120–145-kDa CagA protein. Because transgenic mice expressing CagA throughout the body develop gastrointestinal and hematological malignancies, CagA is recognized as the first bacterial oncoprotein.⁸ During colonization, H. pylori carrying cag-PAI (cagA-positive H. pylori) injects the CagA protein into human gastric epithelial cells through a type IV secretion apparatus.⁴ On delivery into gastric epithelial cells, CagA undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs by Src family kinases and Abl kinase.⁹ Tyrosine-phosphorylated CagA acquires the ability to specifically bind to and aberrantly activate SHP2 (also known as Ptpn11) tyrosine phosphatase, thereby deregulating cell proliferation and motility.¹⁰⁻¹¹ CagA also binds to partitioning-defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK) via the CagA multimerization (CM) sequence in a tyrosine phosphorylation-independent manner.¹² In mammalian cells, there are four PAR1 isoforms, PAR1a/MARK3, PAR1b/MARK2, PAR1c/MARK1 and PAR1d/MARK4. Among them, PAR1b plays a primary role in the establishment and maintenance of epithelial cell polarity. CagA binding inhibits kinase activity of PAR1b, which leads to the disruption of epithelial cell polarity.¹² Because EPIYA motifs are present in multiple numbers in a single CagA protein, the EPIYA-containing C-terminal region is called the EPIYA-repeat region. H. pylori is genetically diverged among distinct strains and CagA is highly polymorphic at the EPIYA-repeat region.¹¹ The EPIYA-repeat region of CagA comprises various combinations of four distinct Helicobacter pylori strains carrying the cagA gene are associated with severe disease outcomes, most notably gastric cancer. CagA protein is delivered into gastric epithelial cells by a type IV secretion system. The translocated CagA undergoes tyrosine phosphorylation at the C-terminal EPIYA motifs by host cell kinases. Tyrosine-phosphorylated CagA acquires the ability to interact with and activate SHP2, thereby activating mitogenic signaling and inducing cell morphological transformation (hummingbird phenotype). CagA also interacts with PAR1b via the CM sequence, resulting in induction of junctional and polarity defects. Furthermore, CagA-PAR1b interaction stabilizes the CagA-SHP2 complex. Because transgenic mice systemically expressing CagA develop gastrointestinal and hematological malignancies, CagA is recognized as a bacterium-derived oncoprotein. Interestingly, the C-terminal region of CagA displays a large diversity among H. pylori strains, which influences the ability of CagA to bind to SHP2 and PAR1b. In the present study, we investigated the biological activity of v225d CagA, an American CagA of H. pylori isolated from a Venezuelan Piaroa Amerindian subject, because the variant CagA does not possess a canonical CM sequence. We found that v225d CagA interacts with SHP2 but not PAR1b. Furthermore, SHP2-binding activity of v225d CagA was much lower than that of CagA of H. pylori isolated from Western countries (Western CagA). v225d CagA also displayed a reduced ability to induce the hummingbird phenotype than that of Western CagA. Given that perturbation of PAR1b and SHP2 by CagA underlies the oncopgenic potential of CagA, the v225d strain is considered to be less oncogenic than other well-studied cagA-positive H. pylori strains.
EPIYA segments, EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D, each of which is defined by the amino-acid sequence that surrounds the individual EPIYA motif. CagA proteins derived from *H. pylori* isolated in Western countries (Western CagA) have EPIYA-A and EPIYA-B followed by EPIYA-C, whereas CagA proteins derived from *H. pylori* isolated in East Asian countries (East Asian CagA) have EPIYA-D instead of EPIYA-C. The CM sequence is present within the EPIYA-C segment (proximal CM sequence) as well as immediately after the repeats of EPIYA segments (distal CM sequence). Also notably, CM sequences are well conserved but not identical between Western CagA and East Asian CagA. (13)

Two SH2 domains of SHP2 bind to two EPIYA-C or EPIYA-D segments and CagA with only one EPIYA-C or EPIYA-D segment can stably interact with SHP2 through its dimerization by binding to PAR1b. (14) Previous studies have also shown that the diversity in the EPIYA-repeat region of CagA influences its binding ability to SHP2 and PAR1b. (12,15,16) Hence, genetic analysis of the EPIYA-repeat region is important for predicting the magnitude of CagA binding to SHP2 and PAR1b. The variation in the EPIYA-repeat region of CagA is thought to be associated with differences in pathogenicity, such as differences in the incidences of duodenal ulcer, gastric ulcer and gastric cancer, in *H. pylori*-infected patients. (17,18) However, a clinicoepidemiological study cannot link the correlation with actual mechanisms, which is pointing the importance of *in vitro* study. Currently, CagA is classified into several groups, including Western CagA, East Asian CagA, African CagA and Amerindian CagA, on the basis of its structural diversity in the EPIYA-repeat region.

*H. pylori* v225d strain was isolated from a gastric antral biopsy specimen obtained from a patient with Piaroa Amerindian acute superficial gastritis. (19) *H. pylori* v225d-derived CagA (v225d CagA) is classified as an Amerindian CagA on the basis of its geometric distribution and genetic configuration of cagA gene. (19) However, none of the reported CagA has an EPIYA-repeat region that is closely related to v225d CagA. (20) Accordingly, to address the oncogenic potential of the *H. pylori* v225d strain, we investigated the pathobiological activity of v225d CagA by focusing on its functions related to the unique EPIYA-repeat region.

**Materials and Methods**

**Antibodies.** Anti-hemagglutinin (HA) antibody (3F10) (Roche, Basel, Switzerland) was used as the primary antibody for immunoprecipitation, immunoblotting and immunostaining. Anti-FLAG (M2) (SIGMA, St. Louis, MO, USA) and anti-Omni (Santa Cruz, Dallas, TX, USA) antibodies were used for immunoprecipitation and immunoblotting and Anti-SHP2 (Santa Cruz), anti-Myc (9E10) (Santa Cruz), anti-phosphotyrosine (4G10) (Millipore, Billerica, MA, USA) and anti-Actin (Santa Cruz) antibodies were used for immunoblotting. Anti-ZO-1 antibody (Invitrogen, Carlsbad, CA, USA) was used for immunostaining.

**DNA construction.** The cagA gene isolated from the *H. pylori* v225d strain was C-terminal tagged with the HA epitope or FLAG epitope and cloned into pSP65SRz (v225d CagA). Phosphoreistsant v225d CagA, in which the tyrosine residue of the EPIYA-D/C^{ACM} segment or that of the partial EPIYA-D/C^{ACM} (pD/C^{ACM}) segment was substituted with phenylalanine (v225d YF1 and v225d YF2), was generated using site-directed mutagenesis. The cagA gene isolated from the *H. pylori* NCTC11637 strain was C-terminal tagged with the HA epitope and cloned into pSP65SRz (ABCCC CagA-HA). ABCCC CagA was generated from ABCCC CagA using site-directed mutagenesis.

**Cell culture and transfection.** AGS human gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Madin–Darby canine kidney (MDCK) II cells were cultured in DMEM supplemented with 10% FBS. Expression vectors were transiently transfected into appropriate cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 24 h after transfection. Monkey COS-7 cells were cultured in DMEM supplemented with 10% FBS. COS-7 cells were transfected with expression vectors using the calcium phosphate method. Cells were harvested 36 h after transfection.

**Immunoprecipitation and immunoblotting.** Immunoprecipitation and immunoblotting were performed as described previously. (10,11)

**Immunostaining.** Immunostaining was performed as described previously. (12)

**Cell morphological analysis.** AGS cells were transiently transfected with expression vectors. The morphology of AGS cells was observed at 24 h after transfection. Fluorescent microscopic observation and statistics were performed as described previously. (14)

**Results**

**PAR1b-binding activity of v225d CagA.** *H. pylori* v225d CagA has an EPIYA-A segment, an EPIYA-B segment and an unusual EPIYA segment comprising an EPIYA-D-like sequence lacking the proximal CM sequence (Fig. 1a, orange) on the right side of the EPIYA motif and an EPIYA-C-like sequence (Fig. 1a, yellow) on the right side of the EPIYA motif (EPIYA-D/C^{ACM}). Furthermore, the distal CM sequence of v225d CagA is interrupted by the insertion of the partial EPIYA-D/C^{ACM} (pD/C^{ACM}) segment (Fig. 1a, red dotted square), giving rise to the generation of a left-side CM fragment (L-CM) and a right-side CM fragment (R-CM) (Fig. 1a). (19) Because such an interrupted CM sequence is quite unique and its function is unknown, we first investigated the PAR1b-binding activity of v225d CagA. To do so, COS-7 cells were co-transfected with a HA-tagged CagA expression vector and an Omni-tagged PAR1b expression vector. *H. pylori* NCTC11637 strain-derived CagA (ABCCC CagA), which has three repeats of the EPIYA-C segment and four CM sequences, was used as a positive control. Total cell lysates (TCL) prepared were immunoprecipitated with an anti-Omni antibody followed by immunoblotting with an anti-HA antibody. Whereas ABCCC CagA was efficiently co-immunoprecipitated with PAR1b, v225d CagA was not (Fig. 2a). This result indicates that v225d CagA does not bind to PAR1b in cells, even when both proteins are overexpressed.

Expression of CagA in polarized epithelial cells causes mislocalization of the tight junction protein ZO-1 through CagA-PAR1b interaction. (12,13) Given the observations described above, we postulated that v225d CagA cannot elicit ZO-1 mislocalization. To test this, we expressed v225d CagA or ABCCC CagA in polarized MDCK cells and co-stained the cells with ZO-1. In contrast to ABCCC CagA, ZO-1 was not mislocalized in cells expressing v225d CagA (Fig. 2b). These results indicate that v225d CagA could not provoke junctional and polarity defects because of a lack of PAR1b-binding ability. Furthermore, v225d CagA was localized to the cytoplasm as well as to the membrane (Fig. 2b). We confirmed that
CagA did not colocalize with the autophagosomes (data not shown).

SHP2-binding activity of v225d CagA. CagA-PAR1b interaction has been shown to strengthen CagA-SHP2 interaction.\(^{13,14}\) Because v225d CagA did not bind to PAR1b, it was suggested that v225d CagA does not interact with SHP2 or, if it does, the binding ability would be very weak. To test this idea, we investigated the SHP2-binding activity of v225d CagA. COS-7 cells were co-transfected with a HA-tagged CagA expression vector and a FLAG-tagged SHP2 expression vector. Total cell lysates prepared were immunoprecipitated with an anti-HA antibody followed by immunoblotting with an anti-FLAG antibody. The results indicate that v225d CagA undergoes tyrosine phosphorylation and binds to SHP2 in cells overexpressing both proteins (Fig. 3a).

Next we investigated whether v225d CagA binds to endogenous SHP2. AGS human gastric epithelial cells were transfected with a HA-tagged CagA expression vector and then total cell lysates prepared were immunoprecipitated with an anti-HA antibody. Consistently, endogenous SHP2 was also co-immunoprecipitated with v225d CagA. However, the amount of SHP2 interacting with v225d CagA was much smaller than that with ABCCC CagA (Fig. 3b).

Mechanism of v225d CagA interaction with SHP2. We wanted to gain insights into the mechanism of v225d CagA-SHP2 interaction. The finding that v225d CagA bound to SHP2 without PAR1b interaction raised the possibility that most of the v225d CagA-SHP2 interaction was mediated by cis-interaction of a single CagA rather than by trans-interaction of dimerized CagA. To investigate this idea, we generated two phosphoresistant v225d CagA mutants, v225d YF1 and v225d YF2, in which the tyrosine residue was replaced with phenylalanine in the EPIYA-D\(^{13,14}\) segment, respectively (Fig. 4a). AGS cells were transfected with a phosphoresistant v225d CagA expression vector and then total cell lysates prepared were immunoprecipitated with an anti-HA antibody. Consistently, endogenous SHP2 was also co-immunoprecipitated with v225d CagA.
FLAG-tagged CagA expression vector and then total cell lysates prepared were immunoprecipitated with an anti-FLAG antibody. Because Western CagA with a greater number of EPIYA-C segments exhibits a better ability to bind to SHP2 than that with a lower number of EPIYA-C, ABCCC CagA with two EPIYA-C segments was used as a control for the experiment as v225d CagA has EPIYA-D/\(^{CM}\) and EPIYA-pD/\(^{CM}\) segments (Fig. 1b). Although SHP2 was co-immunoprecipitated with v225d CagA wild type (WT) and mutants (YF1 and YF2), the amount of SHP2 co-immunoprecipitated with v225d CagA was much smaller than that co-immunoprecipitated with ABCCC CagA (Fig. 4b). This result indicates that v225d CagA was capable of binding to SHP2 through both the EPIYA-D/\(^{CM}\) and EPIYA-pD/\(^{CM}\) segments. Also notably, the amount of SHP2 co-immunoprecipitated with v225d YF1 CagA was less than that co-immunoprecipitated with v225d YF2 CagA (Fig. 4b).

AGS cells expressing infected or transfected CagA display an extremely elongated cell shape known as the "hummingbird phenotype", which is due to deregulated SHP2 by CagA.\(^{(10,11)}\) A previous study also showed that infection of AGS cells with the \(H. pylori\) v225d strain induced the hummingbird phenotype.\(^{(19)}\) To consolidate this, we investigated the hummingbird-inducing activity of v225d CagA. Expression of wild-type v225d CagA or its phosphoresistant mutant, v225d CagA-YF, resulted in induction of the hummingbird phenotype. However, the efficiency of the hummingbird-cell induction was substantially lower than that induced by ABCCC CagA (Fig. 4c). In addition, protrusions of cells expressing v225d CagA, either WT or YF mutants, were significantly shorter than those of cells expressing ABCCC CagA (Fig. 4d). These results indicate that v225d CagA-SHP2 complex formation is not mediated by cis interaction. Instead, the result suggests that both the EPIYA-D/\(^{CM}\) and EPIYA-pD/\(^{CM}\) segments in v225d CagA can independently interact with one of the two SH2 domains of SHP2.

**Discussion**

The present study revealed that a CagA protein derived from the \(H. pylori\) v225d strain does not have a functional CM sequence and thus fails to interact with PAR1b. Because v225d CagA also lacks some critical amino acid residues that
are required for interaction with PAR1 family kinases, it is highly possible that the CagA variant does not bind to all PAR1 members, PAR1a–d. This is the first report demonstrating the presence of a natural CagA species that does not bind PAR1 and thus cannot disrupt epithelial cell polarity. Additionally, the present study showed that the SHP2-binding activity of v225d CagA is extremely weak. A previous study reported that the CagA-SHP2 interaction is substantially potentiated by the CagA-PAR1b interaction. Therefore, v225d CagA has an attenuated SHP2-binding activity due to its inability to interact with PAR1b.

Suzuki et al. recently reported that the CM sequence (CRPIA motif) of Amerindian CagA is distinct from that of Western and East Asian CagA, which is responsible for an attenuated binding activity of Amerindian CagA to host proteins. In contrast, the v225d strain does not fit into either group because v225d CagA carries a unique interrupted CM sequence that is not present in either AM-I or AM-II CagA. Because v225d CagA is the first reported CagA with a disrupted CM sequence, the H. pylori v225d strain might also be less oncogenic than other Amerindian strains that carry CagA with uninterrupted CM sequences. Because v225d CagA contains two SHP2-binding EPIYA motifs while lacking the functional CM sequence, it is possible that the v225d CagA interacts with two SH2 domains of SHP2 in cis by utilizing the EPIYA-D/CACM and EPIYA-pD/CACM segments of v225d CagA. However, both of the YF1 and YF2 mutants of v225d CagA retained the ability to interact with SHP2, ruling out the possibility of a cis-interaction while showing that each of the EPIYA-D/CACM and EPIYA-pD/CACM segments is functional in SHP2 binding. In this regard, a previous study demonstrated that an artificial mutant of

Fig. 4. Mechanism of v225d CagA-SHP2 interaction. (a) Schematic view of v225d wild-type CagA and mutant CagA proteins. Phosphoresistant v225d CagA was constructed by mutating tyrosine residue of the EPIYA-D/CACM or EPIYA-pD/CACM segment to phenylalanine (v225d YF1 and v225d YF2, respectively). (b) AGS cells were transiently transfected with the indicated CagA-FLAG expression vectors. Cell lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with the indicated antibodies. (c) Left panel: AGS cells were transfected with the indicated CagA expression vectors. Cell morphology was examined under a microscope at 24 h after transfection. Arrows indicate hummingbird cells induced by CagA. Bar, 100 μm. Right upper panel: the number of hummingbird cells at 24 h after transfection was counted. Error bars, ±SD (n = 3). *P < 0.01, Student’s t-test. Right lower panel: total cell lysates (TCL) were also prepared and subjected to immunoblotting with the indicated antibodies. (d) Upper panel: AGS cells were transfected with an EGFP expression vector together with a CagA expression vector. At 24 h after transfection, the cell morphological change was examined under a fluorescence microscope (left). The length of cells was measured using images analyzed with the ImageJ software (right). Bar, 100 μm. Lower panel: the length of cells was calculated from images presented in the upper right panels. Red dots show cells that are longer than the maximum value of control cells. n = 200. *P < 0.01, Mann-Whitney U-test.

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CagA-ABD lacking the CM sequence was still capable of binding to SHP2 despite the lack of PAR1b-mediated CagA dimerization, although the binding activity was markedly reduced compared with that of wild-type ABD CagA.\(^{(13,14)}\) Accordingly, v225d CagA has a property similar to that of the artificial CagA-ABD mutant lacking the CM sequence.

The v225d YF1 CagA protein was consistently less phosphorylated than v225d YF2 CagA in cells and had less ability to interact with SHP2 than v225d YF2 CagA did (Fig. 4b), indicating differential phosphorylation levels between the two EPIYA-C-related sequences. Such a difference in the level of phosphorylation was not observed in the case of three EPIYA-C segments in ABCC CagA.\(^{(15)}\) The results indicate that the EPIYA-D/C\(^{\text{CM}}\) segment was quantitatively more active than the EPIYA-pd/C\(^{\text{CM}}\) segment in SHP2 binding due to greater tyrosine phosphorylation. In this regard, the relationship between amino acid sequences spanning EPIYA motifs and SHP2 binding ability has also elucidated from the study comparing the EPIYA-C and EPIYA-D segments.\(^{(15)}\) Differential SHP2 binding activity between EPIYA-C and EPIYA-D segments was due to a difference in amino acid residue following the E-P-I-Y-A-T-I-D sequence (Y + 5 position). Aspartic acid (D) is located at the Y + 5 position in the EPIYA-C segment, whereas phenylalanine (F) is located at the Y + 5 position in the EPIYA-D segment. Hence, the EPIYA-D segment binds to SHP2 more strongly than the EPIYA-C segment does.\(^{(15)}\) We also found that the amino acid residue at the Y + 5 position was different between the EPIYA-D/C\(^{\text{CM}}\) and EPIYA-pd/C\(^{\text{CM}}\) segments of v225d CagA; aspartic acid (D) was located at the Y + 5 position in the EPIYA-D/C\(^{\text{CM}}\) segment, whereas leucine (L) was located at the Y + 5 position in the EPIYA-pd/C\(^{\text{CM}}\) segment (Fig. 1b). Thus, the difference in the Y + 5 residue amino acid might also contribute to the differential SHP2-binding ability between the EPIYA-D/C\(^{\text{CM}}\) and EPIYA-pd/C\(^{\text{CM}}\) segments of v225d CagA.

SHP2 positively regulates pro-mitogenic signaling pathways such as the Erk-MAP kinase pathway\(^{(21–24)}\). Because CagA interacts with SHP2 and deregulates the phosphatase activity, CagA-SHP2 interaction has been thought to play an important role in the development of gastric cancer. Interaction of CagA with PAR1b might also be involved in the oncogenic activity of CagA because CagA-mediated PAR1 inhibition disrupts epithelial cell polarity, which might potentiate mitogenic stimuli triggered by the CagA-SHP2 interaction.\(^{(13,14)}\) Transgenic mice systemically expressing H. pylori CagA develop both gastrointestinal neoplasia and hematological malignancy, whereas mice expressing a gain-of-function SHP2 mutant (Ptpn1/\(^{\text{E76K}}\)) develop hematological malignancy but not epithelial neoplasia.\(^{(8,25,26)}\) The observations point to the notion that both CagA-SHP2 interaction and CagA-PAR1b interaction are important for the development of epithelial neoplasia such as gastric cancer. Results of the present study indicate that the v225d CagA, a natural CagA variant isolated in the Amazon rainforest, is biologically less potent in terms of CagA activity that promotes pro-oncogenic action. The reason why such an ameliorated CagA oncoprotein was generated and has survived in the stomach of people from an isolated Amazon tribe warrants future investigation.

Previous studies have shown that H. pylori evolution is due to human migration.\(^{(27,28)}\) It is thought that H. pylori was migrated from Africa to Europe and Asia and then to America and East Asia by an infected human. During the migration, CagA has evolved and acquired the diversity in the EPIYA-repeat region, which influences the oncogenic activity of CagA. Therefore, we were interested in the evolutionary pathway of v225d CagA. We found that the EPIYA-repeat region of v225d CagA has significant similarity (approximately 97% in amino acid sequence identity) with that of CagA derived from the NA1764 strain isolated in Colombia.\(^{(29)}\) There is a possibility that v225d CagA evolved from NA1764 CagA through recombination at the 7-bp TGATCTC sequences (Supporting Information Fig. S1). Likewise, NA1764 CagA likely evolved from other Amerindian CagA, such as AM-I and AM-II CagA including Shi470 CagA, which has EPIYA-A, EPIYA-B and EPIYA-D/C\(^{\text{CM}}\) segments. A 4-bp sequence TTTC is the probable site for the recombination leading to the duplication of the D/C\(^{\text{CM}}\) segment (Fig. S1).

Multiple infections of H. pylori in a single stomach are well-known phenomena and the diversity of H. pylori is thought to arise through genetic exchanges during multiple infection.\(^{(30–33)}\) We speculate that less pro-oncogenic v225d strain has evolved and was conserved in the confined environment because it did not have opportunities to undergo further genetic exchanges. Amerindians in Venezuelan Piaroa have been separated from civilization for 15 000–20 000 years. Given the fact that Western and East Asian strains, but not Amerindian strains, are widely distributed, it seems it is hard for the Amerindian strains to survive during multiple infections. Extending this idea, it is possible that H. pylori strains like the v225d strain will be diminished quickly through extensive genetic exchanges with major H. pylori species in the human stomach in the near future. The variations in the EPIYA-repeat region are associated with the magnitude of oncogenic potential of individual CagA proteins.\(^{(13,15–18)}\) Accordingly, the anatomical structure of the EPIYA-repeat region of rare CagA variants obtained from geographically isolated regions, like v225d CagA, warrants intensive investigation because it should provide clues to our understanding of the evolution of the CagA oncoprotein in terms of the host-gastric pathogen interaction.

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Disclosure Statement

The authors have no conflict of interest.

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Possible recombination process of how v225d CagA has evolved.