Structural Basis and Functional Consequence of Helicobacter pylori CagA Multimerization in Cells*

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Helicobacter pylori cagA-positive strains are associated with gastric adenocarcinoma. The cagA gene product CagA is delivered into gastric epithelial cells where it localizes to the plasma membrane and undergoes tyrosine phosphorylation at the EPIYA-repeat region, which contains the EPIYA-A segment, EPIYA-B segment, and Western CagA-specific EPIYA-C or East Asian CagA-specific EPIYA-D segment. In host cells, CagA specifically binds to and deregulates SHP-2 phosphatase via the tyrosine-phosphorylated EPIYA-C or EPIYA-D segment, thereby inducing an elongated cell shape known as the hummingbird phenotype. In this study, we found that CagA multimerizes in cells in a manner independent of its tyrosine phosphorylation. Using a series of CagA mutants, we identified a conserved amino acid sequence motif (FPLXXXVXDL-SKVG), which mediates CagA multimerization, within the EPIYA-C segment as well as in a sequence that located immediately downstream of the EPIYA-C or EPIYA-D segment. We also found that a phosphorylation-resistant CagA, which multimerizes but cannot bind SHP-2, inhibits the wild-type CagA-SHP-2 complex formation and abolishes induction of the hummingbird phenotype. Thus, SHP-2 binds to a preformed and tyrosine-phosphorylated CagA multimer via its two Src homology 2 domains. These results, in turn, indicate that CagA multimerization is a prerequisite for CagA-SHP-2 interaction and subsequent deregulation of SHP-2. The present work raises the possibility that inhibition of CagA multimerization abolishes pathophysiological activities of CagA that promote gastric carcinogenesis.

Helicobacter pylori is a micro-aerophilic spiral-shaped bacterium. It colonizes the human stomach and is estimated to inhabit at least half of the world’s human population. Chronic infection in the stomach with H. pylori causes gastric diseases such as chronic gastritis and peptic ulceration later in life. Epidemiological and pathological studies have further indicated that infection with cagA-positive H. pylori strains is associated with a high risk of gastric cancer (1–3).

The cagA gene encodes an ~120–145-kDa CagA protein that is delivered into gastric epithelial cells via the type IV secretion system and localizes to the plasma membrane (4–10). Translocated CagA then undergoes tyrosine phosphorylation by members of the Src family of protein tyrosine kinases such as c-Src, Fyn, Lyn, and Yes (11–13). Tyrosine phosphorylation of CagA occurs at the EPIYA motif, a five-amino-acid sequence (Glu-Pro-Ile-Tyr-Ala) that is present in variable numbers in the C-terminal EPIYA-repeat region of the protein (13). Because of homologous recombination that occurs within the cagA gene, four distinct EPIYA segments (EPIYA-A to -D), each of which possesses a single EPIYA motif, have been identified (14–16). The EPIYA-repeat region of the CagA protein of H. pylori isolated in Western countries contains the EPIYA-A, EPIYA-B, and Western CagA-specific EPIYA-C segments. The EPIYA-C segment variably multiples among distinct Western CagA proteins, mostly ranging from 1 to 3. On the other hand, the EPIYA-repeat region of the CagA protein of H. pylori isolated in East Asian countries contains the EPIYA-A, EPIYA-B, and East Asian CagA-specific EPIYA-D segments. EPIYA motifs present in the EPIYA-C and EPIYA-D segments are major sites of CagA tyrosine phosphorylation, to which SHP-2 protein tyrosine phosphatase specifically binds in a phosphorylation-dependent manner (14, 16). Upon complex formation, CagA deregulates SHP-2 phosphatase activity, which, in turn, dephosphorylates and inactivates focal adhesion kinase, thereby inducing an elongated cell shape with elevated cell motility (17). CagA-deregulated SHP-2 also provokes sustained Erk mitogen-activated protein kinase activation, which plays a crucial role in cell cycle progression (18).

The CagA-SHP-2 interaction requires both the N-SH2 and C-SH2 domains of SHP-2 (14, 16). In contrast, a single EPIYA-C or EPIYA-D segment is sufficient for CagA to form a physical complex with SHP-2. These observations indicate that a SHP-2 protein simultaneously interacts with two tyrosine-phosphorylated CagA proteins via the SH2 domains. Consistent with this idea, we previously reported that CagA multimerizes in mammalian cells (14). In this work, we examined the structural basis of CagA multimerization and identified the multimerization motif that is highly conserved between Western and East Asian CagA proteins. We also investigated the biological role of the CagA multimerization and found that SHP-2 specifically binds to a preformed CagA multimer in a tyrosine phosphorylation-dependent manner, and we discuss the pathophysiological relevance of CagA multimerization in cells.
EXPERIMENTAL PROCEDURES

Expression Vectors—Mammalian expression vectors for hemagglutinin (HA)²-tagged, wild-type CagA (CagA-ABCCC derived from H. pylori NCTC11637 Western strain) and its mutants, CagA-ΔABCC, CagA-ΔABCCC-s, CagA-ΔABCCC, CagA-ΔABCCC+EPIYA, CagA-ΔABCCC+3EPIYA, CagA-ΔABCCC-EPIYA, and CagA-ABD have been described previously (14, 15). Genes encoding a series of mutant CagA-ABD molecules were generated from cagA-ABD, which was isolated from H. pylori F32 East Asian strain, by restriction enzyme digestions and/or site-directed mutagenesis as previously described (14). Structures of the mutant CagA molecules made are schematically represented in the figures in which they are used. The amino acid residue numbers shown in the schematics are from NCTC11637 CagA in the cases of CagA-ABCCC derivatives and are from F32 CagA in the cases of CagA-ABD derivatives. The mutant cagA genes were cloned into a pSP65Srα mammalian expression vector. Human SHP-2 cDNA and its derivatives were tagged with Myc epitope as previously described (14, 15).

Cell Culture and Transfection—Monkey COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. AGS human gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. COS-7 cells (1.5 × 10⁶ cells) were transfected with 30 μg of plasmids by using the calcium phosphate method as previously described (19). AGS cells were seeded into 35-mm² dishes (1.2 × 10⁵ cells/dish), and 8 μg of plasmid was transfected into cells as previously described (13). Cell morphology was examined by light microscopy at 18 h after transfection.

Antibodies—Anti-HA monoclonal antibody 3F10 (Roche Applied Science) and anti-FLAG monoclonal antibody M2 (Sigma) were used for immunoblotting and immunoprecipitation of HA-tagged CagA and FLAG-tagged CagA. Anti-Myc monoclonal antibody 9E10 was used for immunoblotting and immunoprecipitation of Myc-tagged CagA. Anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology) and anti-SHP-2 polyclonal antibody C-18 (Santa Cruz Biotechnology) were used for immunoblotting.

Immunoprecipitation and Immunoblotting—COS-7 cells were harvested at 36 h after transfection and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Brij-35) containing 2 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 10 μg/ml aprotinin (14). Total cell lysates and immunoprecipitates were subjected to SDS-PAGE. Proteins 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 filter were quantitated using a luminescence image analyzer (LAS-1000, FUJIFILM).

RESULTS

Phosphorylation-independent Multimerization of CagA—In a previous study, we demonstrated that ABCCC-type Western CagA (CagA-ABCCC) can multimerize and thus exist as a dimer, oligomer, or a higher order of protein complex in cells (14). However, we also noticed that the recombinant CagA-ABCCC protein produced in Escherichia coli does not spontaneously multimerize in vitro, indicating that CagA multimerization occurs only in
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To understand the biological role of CagA multimerization, we first investigated whether East Asian CagA, another major type of CagA, can also multimerize in cells. Because Western CagA and East Asian CagA are primarily characterized by the difference in the structure of the EPIYA-repeat region, we employed a CagA-ABD mutant that was made from CagA-ABCCC by replacing the entire EPIYA-repeat region with that derived from F32 East Asian CagA (Fig. 1A) (14). CagA-ABD was tagged with a FLAG or HA epitope, and the resulting FLAG- and HA-tagged CagA-ABD molecules were co-expressed in COS-7 cells. Sequential immunoprecipitation and immunoblotting of the cell lysates with anti-HA and -FLAG antibodies revealed that CagA-ABD, which possesses the EPIYA-repeat region from East Asian CagA, multimerizes in cells (Fig. 1B). Quantitation of each of the CagA bands indicated that only a small fraction (<5%) of FLAG-tagged CagA-ABD proteins expressed in COS-7 cells were associated with HA-tagged CagA-ABD proteins. Thus, the CagA multimers may be labile and easily dissociate into monomers during protein extraction and immunoprecipitation in our experimental conditions. Alternatively, predominant CagA proteins may exist as monomers, with a small amount of CagA existing as multimers, in cells. In this case, the formation of CagA multimer may require an additional cellular component whose expression level determines the amount of CagA multimers.

Next, to address the role of tyrosine phosphorylation in CagA multimerization, we made a phosphorylation-resistant form of CagA-abd, by replacing tyrosine residues that are present in the EPIYA-A, -B, and -D segments with alanine residues. The CagA-abd mutant was HA-tagged and was co-expressed together with FLAG-tagged CagA-ABD in COS-7 cells. As was the case for CagA-ABD, CagA-abd, which did not undergo tyrosine phosphorylation, was capable of interacting with CagA-ABD (Fig. 1C). Based on this result, we concluded that CagA multimerizes independently of tyrosine phosphorylation. This result also excluded the possibility that CagA multimerization requires cellular proteins (such as SHP-2 and Csk) that interact with CagA in a tyrosine phosphorylation-dependent manner (11, 19).

**Delineation of the CagA Region Involved in Multimerization**—To investigate the mechanism of CagA multimerization in more detail, we narrowed down the CagA region that is

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**FIGURE 2. Role of the EPIYA-repeat region in CagA multimerization.** A, FLAG-tagged CagA-ABD was co-expressed with HA-tagged CagA-ΔABCCC, which lacks the entire EPIYA-repeat region, or its derivative in COS-7 cells. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody. Anti-HA immunoprecipitates and total cell lysates (TCL) were immunoblotted (IB) with the indicated antibodies. In a control experiment, SHP-2 formed a physical complex with CagA-ABD but not other CagA-derivatives. The data shown are representative of those from three separate experiments. Schematics of the CagA derivatives used are shown at the top. B, FLAG-tagged CagA-ABD was co-expressed with HA-tagged CagA-ABD-D or HA-tagged CagA-ABD-ΔAB in COS-7 cells. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. Anti-HA immunoprecipitates and total cell lysates were immunoblotted with the indicated antibodies. The data shown are representative of those from three separate experiments. Schematics of the CagA derivatives used are shown at the top. *pY*, phosphotyrosine.

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3 H. Higashi, T. Hayashi, M. Horio, and M. Hatakeyama, unpublished observation.
involved in multimerization by using a series of CagA deletion mutants. When CagA-ABD was co-expressed with CagA-ΔAβCCCC, which lacks the entire EPIYA-repeat region in COS-7 cells, the two CagA derivatives failed to interact with each other (Fig. 2A). This result revealed a critical role of the EPIYA-repeat region in CagA multimerization. Restoration of a single EPIYA motif or triple EPIYA motifs in the CagA-ΔAβCCCC mutant did not confer CagA multimerization, arguing against an active role of the EPIYA motif in the multimerization process (Fig. 2A).

We then generated two CagA-ABD derivatives, CagA-ABD-ΔD and CagA-ABD-ΔAB. CagA-ABD-ΔD contains EPIYA-A and EPIYA-B segments but lacks the EPIYA-D segment. Conversely, CagA-ABD-ΔAB lacks the EPIYA-A and EPIYA-B segments (Fig. 2B). Studies on co-expression in COS-7 cells show that CagA-ABD-ΔAB is capable of interacting with CagA-ABD, whereas CagA-ABD-ΔD failed to do so (Fig. 2B). Accordingly, the 91-amino-acid sequence (residues 922–1012) in the EPIYA-repeat region of CagA-ABD is responsible for CagA multimerization.

To further delineate the CagA sequence that is required for CagA multimerization, the above-identified 91-amino-acid residues were subdivided into D1 and D2 sequences (Fig. 3A), and a CagA-ABD-ΔAB mutant that also lacks the D1 or D2 sequence was generated. When co-expressed in COS-7 cells, FLAG-tagged CagA-ABD-ΔAB was capable of interacting with HA-tagged CagA-ABD-ΔABD1 but not HA-tagged CagA-ABD-ΔABD2 (Fig. 3B). Again, deletion of the EPIYA sequence from CagA-ABD-ΔAB did not influence CagA multimerization. A reciprocal immunoprecipitation experiment confirmed the interaction between CagA-ABD-ΔABD1 and CagA-ABD-ΔAB (Fig. 3C). These observations indicated that the D2 sequence is responsible for CagA multimerization in ABD-type East Asian CagA species.

Identification of the CagA Multimerization Motif That Is Conserved between East Asian and Western CagA Species—We have previously shown that Western CagA multimerizes in mammalian cells (14). Identification of the D2 sequence in East Asian CagA therefore prompted us to investigate the sequence that is utilized for Western CagA multimerization. The observation that the EPIYA-A and EPIYA-B segments, which are highly conserved between Western and East Asian CagA species, are not involved in CagA multimerization suggested that the EPIYA-C segment is involved in the multimerization of Western CagA. Accordingly, we expressed a Western CagA mutant (CagA-ΔAβCCC-s) that lacks the amino acid sequence between 869 and 1008 together with CagA-ABCCC and found that they interact with each other (Fig. 4A). Taken together with the previous finding that CagA-ΔAβCCC (which was made from CagA-ABCCC by deleting the entire EPIYA-repeat region) lost the ability to multimerize, we concluded that the sequence between residues 1009 and 1086, which contains a single repeat of the EPIYA-C segment, is responsible for Western CagA multimerization.

Assuming that CagA multimerization is mediated by the homophilic interaction of an integrated CagA structure, we wondered whether such a structure is conserved between Western CagA and East Asian CagA proteins. Accordingly, we expressed CagA-ABD-ΔAB together with CagA-ΔAβCCC, which was made from CagA-ΔAβCCC-s by deleting a 44-amino-acid stretch located immediately after the last repeat of the EPIYA-C segment in the EPIYA-repeat region of CagA-ABCCC, in COS-7 cells (Fig. 4B). The co-expression study
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**A**

NCTC11637-CagA (CagA-ABCCC)

CagA-ΔABCC-s

IP: anti-Flag
IB: anti-Flag
IB: anti-HA
IB: anti-SHP-2

**B**

NCTC11637-CagA (CagA-ABCCC)

CagA-ΔABCC

CagA-ΔABCC-ΔEPIYA

CagA-ΔABΔ-ΔAB-Flag

IP: anti-HA
IB: anti-Flag
IB: anti-HA

**C**

EPIYA-C segment

1009

**D**

EPIYA-C segment

1009

CagA-ABD-

IB: anti-Flag
IB: anti-HA

IP: anti-HA
TCL

EPIYA-D segment

922
revealed that the two CagA derivatives mutually interact with each other in cells, indicating that the mechanism of CagA multimerization is conserved between Western and East Asian CagA proteins. Furthermore, the result indicated that the sequence responsible for multimerization of Western CagA is present within the EPIYA-C segment. However, as we reported previously, the EPIYA-C and EPIYA-D segments exhibit only a limited homology around the EPIYA motif (Fig. 4C, upper panel) (14). As noted, the EPIYA motif itself does not seem to play a role in CagA multimerization (see Figs. 2A and 3B). Consistent with this notion, deletion of the EPIYA motif from CagA-ΔABC-CC did not abolish CagA multimerization (Fig. 4B). Furthermore, deletion or substitution of the TID sequence, which is also conserved between the EPIYA-C and EPIYA-D segments, did not affect CagA multimerization (Fig. 4C). Meanwhile, we noticed that the first 16-amino-acid residues of the EPIYA-C segment are closely related to the residues that located immediately downstream of the EPIYA-D segment in the D2 sequence of East Asian CagA (Fig. 4D, upper panel, gray boxes). This observation raised the idea that the conserved 16-amino-acid sequence mediates CagA multimerization. To investigate this possibility, we deleted the 16-amino-acid stretch from CagA-ABD-ΔABD1 to make CagA-ABD-ΔABD1–16AA. When co-expressed in COS-7 cells, CagA-ABD-ΔABD1–16AA lost the ability to interact with CagA-ABD-ΔAB (Fig. 4D, lower panel). Thus, the conserved 16-amino-acid sequence is essential for East Asian CagA multimerization. The identified sequences between the EPIYA-C segment of Western CagA and the D2 sequence of East Asian CagA are characterized by the presence of a conserved FPLLRRXXXVXDSLKVG motif, which we designate as the CagA multimerization (CM) motif. It should also be noted that the sequences that immediately follow the EPIYA-C and EPIYA-D segments are well conserved between Western and East Asian CagA species. Accordingly, Western CagA has an additional CM motif that follows the last repeat of the EPIYA-C segment (Fig. 4D, upper panel) (see also “Discussion”).

Role of CagA Multimerization in CagA-SHP-2 Complex Formation—We previously reported that both the N-SH2 and C-SH2 domains of SHP-2 are indispensable for the complex formation of SHP-2 with Western CagA (14). We wished to know whether this is also the case with East Asian CagA. To this end, we co-expressed SHP-2 and a series of EPIYA mutants for CagA-ABD in COS-7 cells and confirmed that the CagA-SHP-2 interaction is strictly dependent on the phosphorylation of the EPIYA-D site in the case of East Asian CagA (Fig. 5A). We also co-expressed East Asian CagA and a series of SHP-2 mutants and confirmed that the CagA-SHP-2 interaction requires both of the SH2 domains of SHP-2 (Fig. 5B). Notably, each of the SH2 domains of SHP-2 independently binds to tyrosine-phosphorylated peptides with similar specificity (20). In contrast, major CagA species, such as ABD-type CagA and ABC-type CagA, possess only a single SHP-2-binding site, the EPIYA-C or EPIYA-D segment. These findings collectively indicated that a single SHP-2 molecule binds to a preformed CagA dimer or multimer. If this is the case, then the phosphorylation-resistant interaction is strictly dependent on the phosphorylation of the EPIYA-D site in the case of East Asian CagA (Fig. 5A). We also co-expressed East Asian CagA and a series of SHP-2 mutants and confirmed that the CagA-SHP-2 interaction requires both of the SH2 domains of SHP-2 (Fig. 5B).

FIGURE 4. Identification of the CagA-multimerization motif. A, lysates from COS-7 cells co-transfected with FLAG-tagged CagA-ABCCC-α and HA-tagged CagA-ΔABC-CCs or control empty vector were immunoprecipitated (IP) with anti-HA antibody. Anti-HA immunoprecipitates and total cell lysates (TCL) were subjected to immunoblotting with the indicated antibodies. The data shown are representative of those from three separate experiments. Schematics of the CagA derivatives used are shown at the top. In a control experiment, physical complex formation between SHP-2 and CagA is shown. B, FLAG-tagged CagA-ABD-ΔAB was co-expressed with HA-tagged CagA-ΔABC or CagA-ΔABC-ΔEPIYA in COS-7 cells. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. Anti-HA immunoprecipitates and total cell lysates were immunoblotted with the indicated antibodies. The data shown are representative of those from three separate experiments. Schematics of the CagA derivatives used are shown at the top. C, upper panel, comparison of the EPIYA-C segment of Western CagA with the EPIYA-D segment of East Asian CagA. Asterisks show the conserved amino acids between the two sequences. Lower panel, FLAG-tagged CagA-ABD was co-expressed with HA-tagged CagA-ABD-ΔAB, HA-tagged CagA-ABD-mTID that lacks the conserved TID sequence, or HA-tagged CagA-ABD-mTID that has QVN at the conserved TID sequence in COS-7 cells. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. Anti-HA immunoprecipitates and total cell lysates were immunoblotted with the indicated antibodies. Experiments were done twice, and the results were reproducible. D, upper panel, alignment of the sequences between the EPIYA-C segment of Western CagA and the D2 sequence of East Asian CagA. Gray boxes are the 16-amino-acid sequences conserved between the EPIYA-C segment and the D2 sequence of CagA-ABD. Lower panel, FLAG-tagged CagA-ABD-ΔAB was co-expressed with HA-tagged CagA-ABD-ΔAB or its derivative, CagA-ABD-ΔABD1–16AA that lacks the 16-amino-acid residues conserved between the EPIYA-C segment of Western CagA and the D2 sequence of East Asian CagA. Cell lysates were immunoprecipitated with an anti-HA antibody. Anti-HA immunoprecipitates and total cell lysates were subjected to immunoblotting with the indicated antibodies. The data shown are representative of those from three separate experiments.
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CagA-abd, which multimerizes but cannot bind SHP-2, should act as a dominant-negative CagA that inhibits CagA-SHP-2 complex formation as a component of the CagA dimer (or multimer). To this end, we co-transfected CagA-ABD and CagA-abd in \( COS-7 \) cells and compared the levels of endogenous SHP-2 that formed complexes with CagA-ABD. The results of the experiment showed that CagA-SHP-2 complex formation was abolished by co-expression of CagA-abd. Thus, the phosphorylation-resistant CagA-abd acted as a dominant-negative mutant in the CagA-SHP-2 complex formation (Fig. 6A). The result, in turn, indicated that CagA multimerization is a prerequisite for the interaction of SHP-2 with ABC- or ABD-type CagA. Furthermore, efficient inhibition of CagA-SHP-2 complex formation by phosphorylation-resistant CagA suggested that the molecular nature of the CagA multimer is an oligomer such as a dimer. Finally, to consolidate the role of CagA multimerization in the pathophysiological activity of CagA, we examined the effect of the dominant-negative CagA-abd mutant on the induction of cell elongation known as the hummingbird phenotype by CagA-ABD (6). The morphological change has been shown to be induced by the complex formation between CagA and SHP-2 in AGS cells (13, 18). As shown in Fig. 6B, expression of CagA-ABD together with CagA-abd significantly inhibited induction of the hummingbird phenotype by CagA-ABD in AGS cells. The result indicated that CagA multimerization plays an important role in the morphogenetic activity of CagA in gastric epithelial cells.

**DISCUSSION**

In this work, we demonstrated that CagA, either Western or East Asian species, multimerizes in cells independently of its tyrosine phosphorylation status. We also identified a CM motif that is conserved between Western and East Asian CagA species. Finally, we showed that CagA multimerization is critically involved in the formation of the CagA-SHP-2 signaling complex, which plays an important role in cagA-positive \( H. \) pylori-mediated gastric pathogenesis.

Upon delivery into gastric epithelial cells, CagA functionally mimics mammalian scaffolding adaptor proteins, such as Gab family proteins, through its interaction with multiple cellular proteins by both tyrosine phosphorylation-dependent and -independent mechanisms (21). As a result, CagA perturbs intracellular signaling that regulates cell growth, cell motility, and cell polarity and thereby predisposes CagA-injected gastric epithelial cells to transformation. The best-characterized cellular target of CagA is the SH2 domain containing tyrosine phosphatase SHP-2 (13, 21). CagA specifically interacts with SHP-2 in a tyrosine phosphorylation-dependent manner and deregulates the phosphatase activity. The results of our previous and present works provide compelling evidence that both the N- and C-SH2 domains of SHP-2 are required for the CagA-SHP-2 interaction. This finding, in turn, indicates that two phosphorytrosine-containing CagA sequences must interact with the two SH2 domains of a single SHP-2 molecule. In this regard, prevalent Western or East Asian CagA proteins possess only a single SHP-2-binding site, EPIYA-C in the case of the ABC-type Western CagA and EPIYA-D in the case of the ABD-type East Asian CagA. Given this notion, there are two possible mechanisms that explain the interaction of SHP-2 with CagA carrying a single SHP-2-binding site. One possible mechanism is that CagA multimerizes, or dimerizes, at the host cell plasma membrane, and the preformed CagA multimer (dimer) interacts with a single SHP-2 molecule. The other possible mechanism is that translocated CagA exists as a monomer and a single SHP-2 molecule bridges two tyrosine-phosphorylated CagA monomers to make a stable tripartite complex. The latter possibility is
excluded by the results of our present work showing that CagA multimerizes regardless of its tyrosine phosphorylation status and that a phosphorylation-resistant CagA, which multimerizes but cannot bind SHP-2, inhibits the interaction between wild-type CagA and SHP-2. Hence, CagA-SHP-2 complex formation is impaired when the phosphorylation-resistant CagA is incorporated into the CagA multimer, such as the dimer. It should be also noted that there are a few CagA species that possess multiple EPIYA-C or EPIYA-D segments (4, 14, 16). Our present work does not exclude the possibility that such CagA proteins bind to SHP-2 via the multiple SHP-2-binding sites regardless of CagA multimerization.

Our study, employing a series of CagA mutants, uncovered a critical role of the 16-amino-acid sequence motif in CagA multimerization, which we termed as the CM motif. It is possible that two CagA proteins dimerize via the homophilic interaction of the structure that is created by the CM motif. Given that CagA multimerization occurs only in mammalian cells, however, it is also possible that the multimerization process requires additional cellular proteins. Such proteins, if they exist, may exist as a multimer (dimer) to which CagA binds via the CM motif.

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REFERENCES

1. Blaser, M. J., Perez-Perez, G. I., Kleanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., Stemmermann, G. N., and Nomura, A. (1995) Cancer Res. 55, 2111–2115
2. Parsonnet, J., Friedman, G. D., Orentreich, N., and Vogelman, H. (1997) Gut 40, 297–301
3. Rugge, M., Busatto, G., Cassaro, M., Shiao, Y. H., Russo, V., Leandro, G., Avellini, C., Fabiano, A., Sidoni, A., and Covacci, A. (1999) Cancer 85, 2506–2511
4. Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Chiancone, E., and Covacci, A. (2000) Cancer Res. 60, 5470–5478
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Massone, A., Papini, E., Xiang, Z., Figura, N., and Rappuoli, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5791–5795
5. Tummuru, M. K., Cover, T. L., and Blaser, M. J. (1993) Infect. Immun. 61, 1799–1809
6. Segal, E. D., Cha, J., Lo, J., Falkow, S., and Tompkins, L. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14559–14564
7. Asahi, M., Azuma, T., Ito, S., Ito, Y., Suto, H., Nagai, Y., Tsubokawa, M., Tohyama, Y., Maeda, S., Omata, M., Suzuki, T., and Sasakawa, C. (2000) J. Exp. Med. 191, 593–602
8. Backert, S., Ziska, E., Brinkmann, V., Zimny-Arndt, U., Fauconnier, A., Jungblut, P. R., Naumann, M., and Meyer, T. F. (2000) Cell. Microbiol. 2, 155–164
9. Odenbreit, S., Puls, J., Sedlmaier, B., Gerland, E., Fischer, W., and Haas, R. (2000) Science 287, 1497–1500
10. Stein, M., Rappuoli, R., and Covacci, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1263–1268
11. Selbach, M., Moese, S., Hauck, C. R., Meyer, T. F., and Backert, S. (2002) J. Biol. Chem. 277, 6775–6778
12. Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W. J., and Covacci, A. (2002) Mol. Microbiol. 43, 971–980
13. Higashi, H., Tsutsuki, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M., and Hatakeyama, M. (2002) Science 295, 683–686
14. Higashi, H., Tsutsuki, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T., and Hatakeyama, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14428–14433
15. Higashi, H., Yokoyama, K., Fujii, Y., Ren, S., Yuasa, H., Saadat, I., Murata-Kamiya, N., Azuma, T., and Hatakeyama, M. (2005) J. Biol. Chem. 280, 23130–23137
16. Naito, M., Yamazaki, T., Tsutsuki, R., Higashi, H., Onoe, K., Yamazaki, S., Azuma, T., and Hatakeyama, M. (2006) Gastroenterology 130, 1181–1190
17. Tsutsuki, R., Takahashi, A., Azuma, T., Higashi, H., and Hatakeyama, M. (2006) Mol. Cell. Biol. 26, 261–276
18. Higashi, H., Nakaya, A., Tsutsuki, R., Yokoyama, K., Fujii, Y., Higuchi, M., Takahashi, A., Kurashima, Y., Ishikawa, S., Tanaka, S., Azuma, T., and Hatakeyama, M. (2004) J. Biol. Chem. 279, 17205–17216
19. Tsutsuki, R., Higashi, H., Higuchi, M., Okada, M., and Hatakeyama, M. (2003) J. Biol. Chem. 278, 3664–3670
20. De Souza, D., Fabri, L. J., Nash, A., Hilton, D. J., Nicola, N. A., and Baca, M. (2002) Biochemistry 41, 9229–9236
21. Hatakeyama, M. (2004) Nat. Rev. Cancer 4, 688–694
22. Neel, B. G., Gu, H., and Pao, L. (2003) Trends Biochem. Sci. 28, 284–293
23. Bentires-Alj, M., Paez, J. G., David, F. S., Keilback, H., Halmos, B., Naoki, K., Maris, J. M., Richardson, A., Bardelli, A., Sugarbaker, D. J., Richards, W. G., Du, J., Girard, L., Minna, J. D., Loh, M. L., Fisher, D. E., Velculescu, V. E., Vogelstein, B., Meyerson, M., Sellers, W. R., and Neel, B. G. (2004) Cancer Res. 64, 8816–8820
24. Azuma, T., Yamazaki, S., Yamakawa, A., Ohtani, M., Muramatsu, A., Suto, H., Ito, Y., Dojo, M., Yamazaki, Y., Kuriyama, M., Keida, Y., Higashi, H., and Hatakeyama, M. (2004) J. Infect. Dis. 189, 820–827
25. Argent, R. H., Kidd, M., Owen, R. J., Thomas, R. J., Limb, M. C., and Atherton, J. C. (2004) Gastroenterology 127, 514–523