博士論文

Regulation of the *Helicobacter pylori* CagA oncoprotein by the SH2 domain containing protein tyrosine phosphatase SHP1

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ABSTRACT:

Infection with *Helicobacter pylori* (*H. pylori*) *cagA*-positive strain is associated with human malignancies such as gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (MALT) of B-lymphocyte origin. The *cagA* gene product, CagA is injected by the bacterium into gastric epithelial cells, where it subsequently undergoes tyrosine phosphorylation. Upon tyrosine phosphorylation, CagA acquires the ability to specifically binds to Src homology2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2), activates the phosphatase activity and thereby promotes neoplastic transformation of cells. Here I show that CagA is also capable of interacting with the SHP2 homologue, SHP1 in gastric epithelial cells. However unlike SHP2, CagA-SHP1 interaction is completely independent of CagA tyrosine phosphorylation. I also demonstrated that CagA directly binds to SHP1 and the interaction requires the N-terminal SH2 domain-containing region of SHP1 but not the functional SH2 domains. And such CagA-SHP1 interaction results in the activation of the phosphatase. I also found that SHP1 is a major and specific phosphatase that catalyzes the dephosphorylation of tyrosine-phosphorylated CagA. In striking contrast, expression of SHP2 did not result in the dephosphorylation of CagA both in gastric epithelial cells and *in vitro*. Furthermore, SHP1 but not SHP2 inhibits induction of the unique cell morphology termed 'Hummingbird phenotype', which is caused by tyrosine-phosphorylated CagA. Taken together these results indicate that SHP1 tyrosine phosphatase is a specific inhibitor for the phosphorylation-dependent oncogenic action of *H. pylori* CagA that directs gastric carcinogenesis.

Introduction:

Infection with *Helicobacter pylori* (*H. pylori*) is associated with upper gastrointestinal diseases, including chronic gastritis, peptic ulcer diseases, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach. Among various pathogenic bacterial effectors, which are delivered into host mammalian cells, special attention has been paid to those that undergo tyrosine phosphorylation by host cell kinases (6). *Helicobacter pylori* CagA is a prototype of such bacterial effectors. The *cagA* gene, which located in the *cag* pathogenicity island (PAI) in the *H. pylori* genome, encodes a 120-to 145-kDa immunodominant protein CagA (13, 72). Compared with *cag*-negative *H. pylori* strains, *cag*-positive strains significantly increase the risk of developing severe diseases including gastric carcinoma (33,58). The CagA protein is injected directly from the bacterium into gastric epithelial cells via type IV secretion system and localized to the inner side of the plasma membrane, where it undergoes tyrosine phosphorylation by Src family kinases (SFKs) such as c-Src, Lyn, Fyn, and Yes or c-Abl kinase at the Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence-motif that is present in variable numbers in its C-terminal region (24,69,73). Depending on sequences surrounding each of the EPIYA-motif, four distinct EPIYA segments EPIYA-A, -B, -C, and –D have been described (23). Whereas the EPIYA-A and EPIYA-B segments are virtually present in all the CagA proteins, the EPIYA-C segment is specific to CagA of *H. pylori* isolated in Western countries (Europe and North America), Africa, Australia, and several Asian countries (Middle East, India and Malaysia), while the EPIYA-D segment is unique to CagA of *H. pylori* isolated in the far East (China, Japan and Korea). These EPIYA segments can appear in different numbers and configurations in different CagA species, making C-terminal CagA variations (7). Tyrosine-

phosphorylated CagA specifically binds to the Src-homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2; also termed as PTPN11, Syp, SH-PTP3, PTP-1) (2,19,76), a bona fide oncoprotein, gain-of-function mutations of which are associated with various human malignancies (106). CagA-deregulated SHP2 perturbs the Erk-MAP kinase (27) and also dephosphorylates focal adhesion kinase (FAK) to induce an elongated cell-shape known as the hummingbird phenotype (89,90). CagA transgenic mice studies showed induction of abnormal proliferation of gastric epithelial cells as well as hematopoietic cells, followed by the development of gastrointestinal carcinomas and leukemias /lymphomas in a tyrosine phosphorylation-dependent manner (92).

Tyrosine phosphorylation plays an important role in the signal transduction from the cell surface to the nucleus. Major member of proteins that down-regulate tyrosine-based signaling pathways by coordinately antagonizing the actions of protein-tyrosine kinases are protein tyrosine phosphatases (PTPs) (40). Because of their opposite catalytic functions, PTPs control a wide range of phosphotyrosine-mediated signaling pathways in metazoan. In mammalian genome, there are approximately 100 genes that potentially encode PTPs (reviewed in 87). Among these, two structurally related intracellular PTPs, SHP1 (also termed as PTPN6, SH-PTP1, PTP1C, HCP, or Hcph) (65,55,78,44) and SHP2 are unique in that they contain SH2 domains (3,22,52). They are capable of interacting with tyrosine-phosphorylated proteins via SH2 domains and indeed they have been shown to bind to multiple receptor molecules including cytokine receptors (18,34,80), receptor-type protein tyrosine kinases (19,65,75,76,79), antigen receptors (16,41,59), and adhesion molecules (31). In the absence of adequate stimuli, SHPs are considered to exist in their inactive configurations (104). SHP1 and SHP2 are

approximately 50% identical at their amino-acid levels (12,87,104). Although they belong to the same family and have similar catalytic and regulatory mechanisms, SHP1 and SHP2 have different biological functions *in vivo* (104). SHP1 is expressed predominantly in hematopoietic cells of all lineages and all stages of maturation, however it is also expressed in epithelial cells (44, 49, 55, 65, 78). On the other hand SHP2 is a ubiquitously expressed PTP and positively regulates signal transduction events from a variety of activated receptor tyrosine kinases (2,19,21). Both SHP1 and SHP2 are non-transmembrane protein tyrosine phosphatases with a central catalytic domain, two SH2 domains at the N-terminus and a C-terminus with potential tyrosine phosphorylation sites (8, 32, 39, 51, 57, 71, 83) and involved in the regulation of cellular proliferation and survival (50). SHP1 is considered to act as a negative regulator of signal transduction in hematopoietic cells, terminating signals from a diverse range of signaling molecules including interleukin-3 receptor, c-Kit, receptor-associated JAK kinases, colony-stimulating factor-1 receptor and B and T cell antigen receptors (reviewed in 84). The essential role of SHP1 as a negative regulator of signal transduction is consistent with the multiple defects in hematopoietic cells observed in moth eaten mice, which lack functional SHP1 (93). Motheaten mice die soon after birth due to over-proliferation and accumulation of macrophages in the lungs (105). However functional role of SHP1 expressed in non-hematological cells including epithelial cells remains largely unknown (reviewed in 17).

In this study I explored the role of SHP1 in *H. pylori* CagA-mediated gastric carcinogenesis. I found a novel interaction between SHP1, the SHP2 homologue and *Helicobacter pylori* CagA in gastric epithelial cells. Unlike CagA-SHP2 interaction however, the association between CagA-SHP1 is totally independent of the EPIYA-

tyrosine phosphorylation of CagA. Consistent with this the interaction is independent of functional SH2 domains of SHP1, although it requires the SH2 domain-containing N-terminal region of SHP1. Using an *in vitro* binding assay, I also found that CagA directly interacts with SHP1 and activates its phosphatase activity in *in vitro* phosphatase assay system. Additionally, I show that SHP1 directly tyrosine-dephosphorylates CagA both *in vitro* and in gastric epithelial cells. In striking contrast, SHP2 is not capable of dephosphorylating CagA under the same experimental conditions, highlighting that CagA is a specific substrate of SHP1. Finally, I show that ectopic expression of SHP1 not SHP2 in gastric epithelial cells inhibit induction of the 'Hummingbird phenotype', an extremely elongated cell morphological change caused by CagA-SHP2 interaction, which is concomitantly associated with the reduced tyrosine phosphorylation levels of CagA in cells. These findings indicate a critical role of SHP1 in counteracting pathogenic/oncogenic action of *H. pylori* CagA through dephosphorylation. The work also reveals opposing roles of SHP1 and SHP2 in CagA-mediated neoplastic transformation of cells.

Purpose

SHP1 tyrosine phosphatase is proposed as a tumor suppressor gene candidate in various hematopoietic cancers. The purpose of this study was to explore the role of SHP2-related SHP1 tyrosine phosphatase in *H. pylori* CagA-mediated gastric carcinogenesis.

Material and Methods:

Expression vectors: Human *SHP1* cDNA (gift from Dr. T. Matozaki, Kobe University of Medicine, Japan) was Flag-tagged and cloned into pcDNA3-based mammalian expression vectors. All Flag-tagged SHP1 point mutants used in this work were generated from the human *SHP1* cDNA by site-directed mutagenesis (Fig 7A). To disable SH2 domains of SHP1, arginine-30 of N-SH2 and arginine-136 of C-SH2 domains were substituted with lysine (SHP1 R30K, SHP1 R136K, SHP1 R30, 136K) (30,71,83). To disable the PTP domain of SHP1, aspartic acid-419, cysteine-453 and arginine-459 were substituted with alanine, serine and methionine respectively (SHP1 D419A, SHP1 C453S, SHP1 R459M) (45, 67,74). Hemagglutinin (HA)-tagged, wild type CagA (CagA- ABCCC derived from *H. pylori* NCTC11637 western strain) were cloned into pSP65SRα mammalian expression vectors (HA-CagA-ABCCC or HA-CagA) (26,70). Generation of HA-tagged phosphorylation-resistant CagA (HA-PR CagA) in which all the tyrosine in the EPIYA motifs were substituted with phenylalanine (here denoted as HA-PR CagA) and CagA mutant lacking the EPIYA motif-containing region: deletion of amino acid residues from 868-1087 (HA-CagA ΔABCCC) has been described previously (26,70). HA-tagged PR CagA and CagA ΔABCCC were cloned into pSP65Srα mammalian expression vectors (26,70). Human *SHP2* cDNA (derived from AGS cells) was Flag-tagged and cloned into pcDNA3-based mammalian expression vectors. For knockdown experiment, duplex small interference RNA (SiRNA) for human SHP1 (111) was synthesized by operon (Japan) and SiRNA for luciferase was synthesized by Greiner bio-one (Japan). Nucleotide sequence used were SHP1, sense 5'-AGCCUGGAGACUUCGUGCUUU-3' and antisense 5'-AGCACGAAGUCUCCAGGCUUU-3' and luciferase-specific SiRNA was used as

silencer negative control, sense 5'-CGUACGCGGAAUACUUCGATT-3' and antisense 5'-UCGAAGUAUUCCGCGUACGTT-3'.

Antibodies and Reagents: Anti-SHP1 polyclonal antibody C-19 (Santa Cruz Biotechnology), anti-Flag monoclonal antibody (M2) (Sigma-Aldrich), anti-HA polyclonal antibody (3F10) (Roche) were used as primary antibodies for immunoblotting and immunoprecipitation. Anti-HA polyclonal antibody (3F10) (Roche), Anti-HA monoclonal antibody (C29F4) (Cell Signaling), Anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology), anti-CagA polyclonal antibody (Austral Biological), anti-His polyclonal antibody (6C4) anti-GST polyclonal antibody (Santa Cruz Biotechnology) and anti-Actin polyclonal antibody C-11 (Santa Cruz Biotechnology) were used as primary antibodies for immunoblotting.

Cell culture and transfection: AGS human gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), at 37°C, under 5% CO_2 humidified atmosphere. 20 μg expression vectors were transiently transfected into 1.2×10^6 AGS cells in a 100-mm dish by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instructions. For immunoprecipitation, cell lysates were prepared 24 h after the transfection. For SHP1 knockdown experiment, 100 pmol of SiRNA were transiently transfected into $4x10^5$ AGS cells in a 60-mm dish by using lipofectamine 2000. After 24 h the cells were reseed, 12 h after reseeding, HA-CagA expression vectors were transfected into AGS cells by using lipofectamine 2000 (Invitrogen). BJAB cells (EBV-negative human burkitt's lymphoma cells, Kindly provided by Dr. Ping-Ning Hsu, National Taiwan University, Taiwan) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C, under 5% CO_2 humidified atmosphere. BJAB cells were infected with *H. pylori* for 24 h at a

multiplicity of infection (MOI) of 100 before harvest. For immunoprecipitation, cell lysates were prepared 24 h after the infection.

Confocal microscopy: AGS cells were cultured in RPMI 1640 medium supplemented with 10% FBS. For transfection, 240 ng of plasmids was transfected into AGS cells (2×10^4 cells/0.8 cm^2) by using 0.4 μl of Lipofectamine 2000 reagent (Invitrogen). After 24 h incubation, cells were fixed with Mildform 10N (Wako) for 20 min and permeabilized with 0.5% Triton X-100 for 20 min. The cells were then treated overnight with primary antibody and were visualized with Alexa Flour 488-conjugated and 546-conjugated anti-rabbit and anti-mouse antibody (Invitrogen) respectively. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Images were acquired using confocal microscope system (FV 1200, Olympus).

Immunoprecipitation and immunoblotting: AGS cells or BJAB cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 0.25 % Sodium-deoxycholate, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin). Cell lysates were treated with specific antibodies, and immune complexes were trapped on protein G-Sepharose beads. Total cell lysates and immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins transferred to polyvinylidene difluoridemembrane filter (Millipore) were soaked in solutions of primary antibodies (1:1000, 90 min at room temperature) and then with secondary antibody (1:10,000, 45 min at room temperature). Proteins were visualized by using western blot chemiluminescence reagent (Perkin-Elmer Life Sciences). Intensity of protein band was quantitated by a luminescence image analyzer (LAS-4000, FUJIFILM).

Bacteria: *H. pylori* NCTC11637 strain and its *cagA*-defective isogenic strains (Δ*cagA*) have been reported previously (26). BJAB cells (Burkitt's Lymphoma Cells) were infected with *H. pylori* NCTC11637 or the isogenic Δ*cagA* strain for 24 h at a multiplicity of infection (MOI) of 100 before harvest.

Construction and expression of recombinant proteins: Bacterial expression vectors for the glutathione *S*-transferase (GST), fused SHP1 proteins used in this study were made by sub cloning the following cDNA- or PCR-amplified fragments into pGEX6P-1 using *EcoR1* (5'-end) and *Not1* (3'-end) enzyme site: the full-length human *SHP1* cDNA (1-595), a full length human *SHP1* cDNA containing a Cys453-Ser mutation (SHP1 C453S), the human *SHP1* cDNA fragment encoding the COOH-terminal region (247-595) (SHP1 ΔN). Bacterial expression vectors for GST fused SHP2 proteins were made by sub cloning the full-length human *SHP2* cDNA into pGEX6P-2 using *Bam1* (5'-end) and *Sal1* (3'-end) enzyme site. GST fused SHP1 or SHP2 expression plasmids were transformed into *Escherichia coli* BL21 (DE3) and GST fusion proteins were purified as described previously (29). Briefly a single colony was inoculated into 100 ml of LB medium containing 100 μg/μl ampicillin and cultured overnight. Eighty ml of this overnight culture were added to 1.5 L of LB media containing, ampicillin and grown at 37°C to OD_{600} = 0.8. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 100 mM, and the bacteria were kept 16 h at 25°C under shaking. Then the bacteria were pelleted at 6,000 × *g* and 4°C, for 10 min. The pellet was resuspended in 150 ml of equilibration buffer containing 25 mM Tris-HCl, (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM β-mercaptoethanol, with 0.3 mg/ml benzamidine. To the suspension lysozyme was added (0.5 mg/ml final concentration), and the mixture was incubated at 25°C for 20 min. Subsequently EDTA and Triton X-

100 were then added at a final concentration of 20 mM and 0.5 %, respectively, and the mixture was incubated for another 20 min. The bacterial lysate was centrifuged at 30,000 × *g* and 4°C for 40 min and the supernatant was transferred to a fresh 50-ml polypropylene tube containing 3 ml bed of Glutathione-Sepharose 4B (GE Healthcare). This suspension was mixed by end-over-end rotation for 1 h at 4°C and then charged this bead slurry into empty column (Bio-Rad) and beads were washed 3 times at 4°C with 30 ml of W1 buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 10 mM β-mercaptoethanol) and 3 times with 30 ml of W2 buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM β-mercaptoethanol). The bound proteins were incubated with 15 μl of preScission protease (GE Healthcare) in 3 ml of W2 buffer at 4°C for 16 h. Released protein fraction (3 ml) and additional washout fraction with 7 ml of W2 buffer were collected, and the protein concentration was determined using Bradford method. Details on the purification of recombinant CagA have been described previously (28). In brief, the full-length *cagA* gene was isolated by PCR from *H. pylori* 26695 and was inserted into pGEX-6P-2 (GE Healthcare) with a C-terminal hexahistidine-tag sequence. Recombinant CagA proteins expressed by *E. coli* BL21 (DE3) were cultured with Terrific-broth at 37°C. Protein expression was induced at OD_{600} of 0.7-1.0 by 0.4 mM IPTG for 2 h at 30°C. Bacterial pellets were re-suspended in 20 mM Tris-HCl (pH 8.0), 100 mM MgCl2, 10 mM imidazole, and 0.3 mg/ml benzamidine and lysed by ultrasonication. The soluble fraction of the sample was applied to nickel-nitrilotriacetic-acid (Ni-NTA) agarose beads (QIAGEN), washed with 20 mM imidazole containing buffer, and eluted with 250 mM imidazole containing buffer. The elutes were applied to Glutathione Sepharose 4B beads (GE Healthcare) and washed with 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 5 mM EDTA, and 2 mM DTT followed by 50 mM Tris-HCl

(pH 7.0), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT. The GST-tags were excised by the in-gel digestion method with PreScission protease (GE Healthcare) and the unbound fractions were collected. After buffer change in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl on a Hiprep 26/10 Desalting column (GE Healthcare), the proteins were applied to a HisTrap HP Ni-affinity column (GE Healthcare), washed with a linear gradient of 0-40 mM imidazole, and eluted with 250 mM Imidazole. Finally, gel-filtration chromatography was performed using Superose 6 (GE Healthcare) in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl. The purified proteins were concentrated to 50-60 mg/ml using Amicon Ultra-4 (50,000 MWCO, Millipore). For the expression of tyrosine-phosphorylated CagA (pY CagA-His) in *E. coli*, bacterial expression vectors for recombinant v-Src was introduced together with the recombinant CagA expression vector into *E. coli* strain BL21 (DE3). Briefly, the *v-src* gene was cloned into a bacterial expression vector pACYCDuet1 using *Not1* enzyme site and the resulting pACYCDuet1-v-Src were co expressed with pGEX-6P-2 CagA in BL21 (DE3) for generating tyrosine-phosphorylated CagA. Resulting *E. coli*, BL21 (DE3) were cultured with Terrific-broth at 37°C and protein expression was induced at OD600 of 1.0 by 0.1 mM IPTG, for shaking culture overnight at 18°C. For the purification of the recombinant tyrosine-phosphorylated CagA protein (pY CagA-His) from the bacterial pellets, I followed the same protocol as mentioned above for recombinant CagA protein (Lisa Nagase et al., manuscript under preparation).

***In vitro* phosphatase assay.** *In vitro* phosphatase assay were carried out as described previously (46,83). In brief, *para*-nitrophenylphosphate (*p*NPP) (Wako) was used as a substrate to determine the phosphatase activity of the recombinant SHP1. The phosphatase assay were carried out at 37°C for 30 min in 50 µl of reaction buffer (100

mM Na-HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 10 mM dithiothreitol, 10 mM *p*NPP) with 1 μM SHP1 in the absence or presence of 10 μM CagA-His. The reactions were quenched with 950 μl of 1N NaOH. The phosphatase activity was determined and quantitated by measuring the spectrophotometric absorbance of the reaction product *p*-nitrophenolate at A_{405}. For *in vitro* CagA phosphatase assay, phosphorylated CagA (pY CagA-His) was mixed with wild-type SHP1, wild-type SHP2, or phosphatase-dead SHP1 C453S. Phosphatase reactions were carried out at 37°C for 60 min in 30 μl of phosphatase buffer (25 mM HEPES (pH 7.4), 2.5 mM EDTA, 5 mM dithiothreitol, 50 mM NaCl, 65 ng/μl BSA) and reaction was stopped by adding SDS sample buffer and boiling at 100°C. Samples were analyzed by SDS-PAGE and western blotting with an anti-phosphotyrosine (anti-pY) antibody.

***In vitro* binding assay.** For *in vitro* binding assay, GST, GST-pY CagA-His, or GST-CagA-His was incubated with wild-type SHP1 or phosphatase-dead SHP1 (SHP1 C453S) or C-terminal region of SHP1 (amino-acid residues 247-595) (SHP1ΔN) at 4°C for 1 h and washed 5 times in pull down buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.01%Triton X-100). Protein complexes were collected with GST-fused beads and were analyzed on SDS-PAGE, followed by Western blotting with an anti-phosphotyrosine (anti-pY), anti-SHP1, anti-His or anti-GST antibody.

Cell-morphological analysis & Hummingbird assay. The morphology of the AGS cells was observed at 17 h after transfection as described previously (48,69). AGS cells were transiently transfected with expression vectors and after 17 h incubation cell morphology was examined by light microscopy. Cells showing the hummingbird phenotype were counted in 10 different fields in each of four dishes (the area of one field = 0.25 mm^2). To investigate cell-morphology in more detail, the images of the

AGS cells were captured at 24 h after transfection by using confocal microscope system (FV 1200, Olympus). Hummingbird phenotype was defined as an elongated cell-shape, in which the ratio of the longest protrusion of cell to the shortest cell diameter is more than 2-fold.

Results:

1. CagA associates with SHP1 tyrosine phosphatase in AGS cells and B cells.

It was demonstrated previously that upon delivery into the host cell, *H. pylori* CagA forms a physical complex with SHP2 (24) in gastric epithelial cells. To examine whether CagA can also form a physical complex with the SHP2 homologue SHP1, I transiently co-transfected the HA-tagged wild-type CagA (HA-CagA) vector and the Flag-tagged SHP1 (Flag-SHP1) vector in AGS cells and through sequential immunoprecipitation-western blotting experiment, it was found that CagA forms a physical complex with SHP1 (Fig 1A). Recent reports demonstrated that *H. pylori* can deliver CagA directly into B cells, via the type IV secretion system (35,42) and the translocated CagA formed a complex with SHP2 (35,42). Since SHP1 is abundantly expressed in hematopoietic cells including B cells (reviewed in 84), I wondered if infection with *cagA*-positive *H. pylori* could also give rise to the generation of the CagA-SHP1 complex in B cells. I therefore performed *H. pylori* infection experiment (moi: 100) using human lymphoma derived B cell line (BJAB). BJAB cells were infected with *H. pylori cagA*-positive strain or isogenic *cagA*-negative strain as a control and it was found that bacterially delivered CagA efficiently interacted with endogenous SHP1 in B cells (Fig 1B). Taken together, these results indicated that *H. pylori* oncoprotein CagA interacts with SHP1 in B cells as well as in gastric epithelial cells.

Figure 1

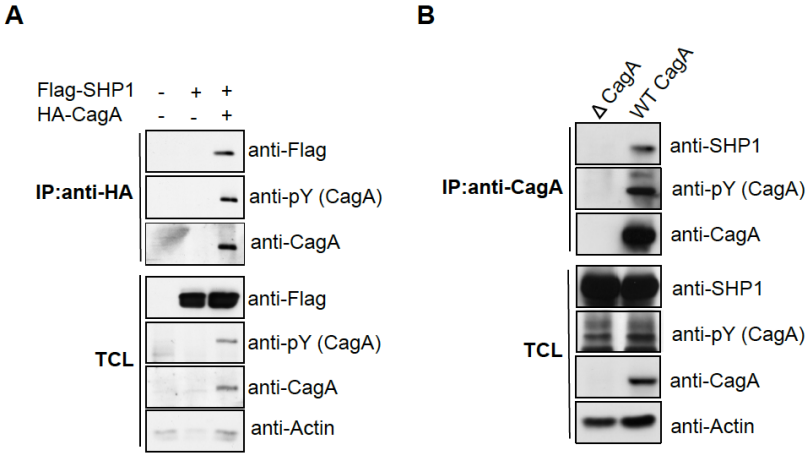


Figure 1. Interaction between CagA and SHP1 in AGS and B cells. (A) AGS cells were co-transfected with Flag-SHP1 and HA-CagA vectors. Total cell lysates (TCLs) were immunoprecipitated with anti-HA antibody and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. **(B)** BJAB cells were infected with the *H. pylori cagA*-positive or the *cagA*-negative isogenic strain (Δ *cagA*) for 24 h. Total cell lysates (TCLs) were immunoprecipitated with an anti-CagA antibody and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. Each experiment was repeated at least three times and the representative data were shown.

2. CagA-SHP1 interaction is independent of tyrosine phosphorylation of CagA at EPIYA motif.

The *cagA* gene product CagA undergoes tyrosine phosphorylation at the C-terminal EPIYA motifs when expressed in mammalian cells (24,69). Since SHP2 binds to CagA in a manner that is dependent on CagA EPIYA tyrosine phosphorylation and next I investigated whether the observed CagA-SHP1 interaction was also dependent on CagA tyrosine phosphorylation. To this end, I transiently transfected a Flag-SHP1 vector together with an expression vector for HA-tagged wild type CagA (HA-CagA) or HA-tagged phosphorylation resistant CagA (HA-PR CagA, in which all the tyrosine's in the EPIYA motifs were substituted by phenylalanine residues) in AGS cells and found that

PR CagA was also capable of binding with SHP1 (Fig 2A). To consolidate phosphorylation-independent interaction of CagA with SHP1, HA-PR CagA or HA-CagA vector was transfected into AGS cells and the cell lysates were immunoprecipitated with an anti-SHP1 antibody. The resulting anti-SHP1 immunoprecipitates contained WT and PR CagA (Fig 2B). These observations indicated that CagA forms a complex with endogenous SHP1 in a CagA phosphorylation-independent manner. In addition to CagA phosphorylation, the EPIYA-containing region of CagA exerts multifaceted functions such as PAR1 interaction (91). Accordingly I next investigated whether the EPIYA-containing region is still responsible for SHP1 interaction independent of EPIYA tyrosine-phosphorylation. To do so, I transiently transfected an expression vector for HA-CagA or a HA-CagA mutant in which the EPIYA-containing region was deleted (HA-CagA ΔABCCC) (25) together with the Flag-SHP1 vector. The result of the experiment revealed that HA-CagA ΔABCCC was still capable of binding SHP1 (Fig2C). I thus concluded that the EPIYA-containing region does not play a major role in the CagA-SHP1 interaction. From these observations, I concluded that unlike CagA-SHP2 interaction, *H. pylori* oncoprotein CagA interaction with SHP1 tyrosine phosphatase is totally independent of CagA tyrosine phosphorylation and EPIYA-containing region of CagA.

Figure 2

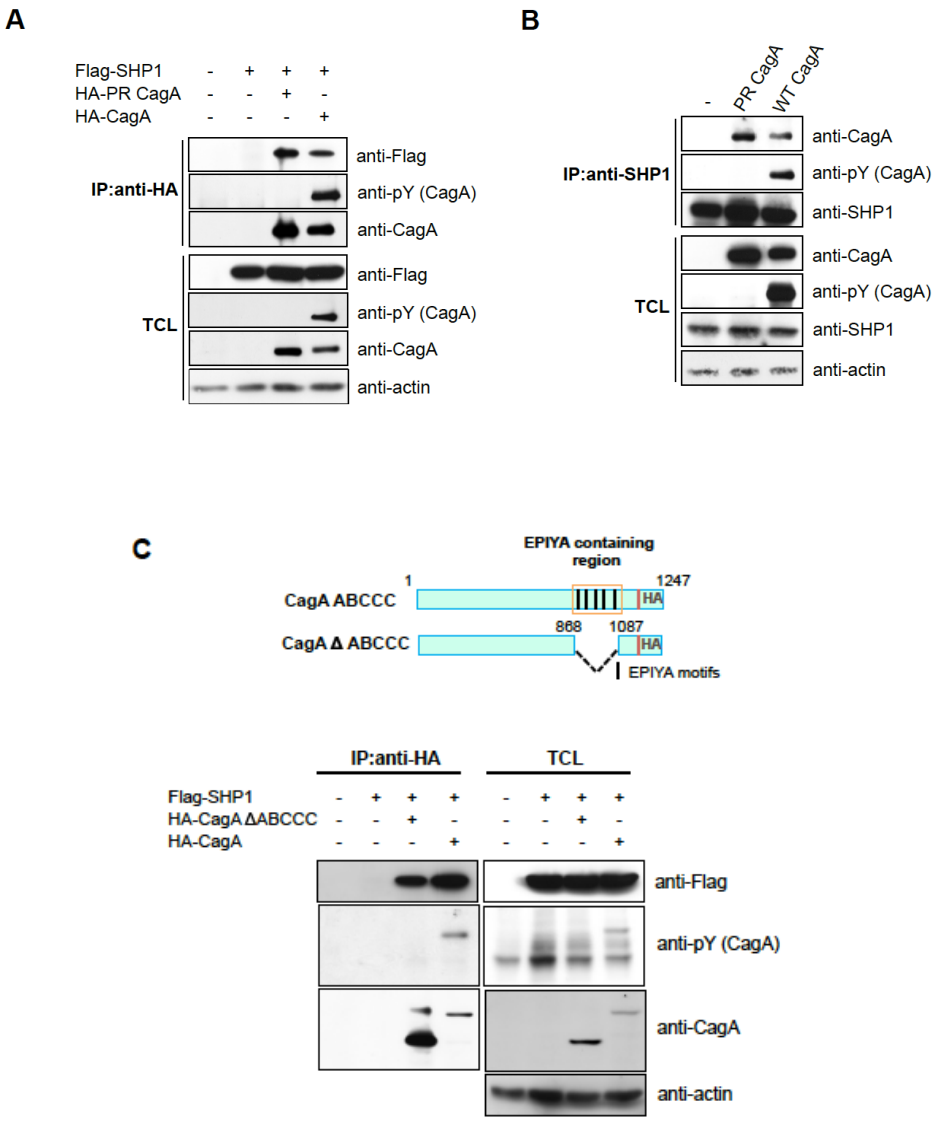


Figure 2. CagA-SHP1 interaction is independent of tyrosine phosphorylation of CagA at EPIYA motifs. (A) AGS cells were transfected with Flag-SHP1 and HA-CagA or HA-PR CagA (in which all the tyrosine in the EPIYA-motifs were substituted by phenylalanine). Total cell lysate (TCLs) were immunoprecipitated with anti-HA antibody, and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. **(B)** AGS cells were transfected with HA-CagA or HA-PR CagA. Total cell lysates (TCLs) were immunoprecipitated with anti-SHP1 antibody and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibody. **(C)** AGS cells were transfected with Flag-SHP1 and HA-CagA or HA-CagA ΔABCCC (in which the EPIYA-containing region were deleted as shown in the schematics). Total cell lysates (TCLs) were immunoprecipitated with anti-HA antibody and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibody. Each experiment was repeated at least three times and the representative data were shown.

3. *H. pylori* CagA interact directly with SHP1 tyrosine phosphatase.

To determine whether the CagA-SHP1 interaction I observed in the cells were direct or not, I carried out an *in vitro* binding assays using recombinant SHP1 and GST, GST-fused phosphorylated CagA (GST-pY CagA-His) or GST-fused unphosphorylated CagA (GST-CagA-His) (described in Materials and Methods) immobilized on glutathione beads. Consistent with my cells data, both phosphorylated (GST-pY CagA-His) and unphosphorylated (GST-CagA-His) CagA bound to SHP1 upon *in vitro* mixing of the two proteins (Fig 3A). These results indicate that SHP1 directly interacts with CagA and the interaction is entirely independent of the tyrosine phosphorylation of CagA.

Figure 3

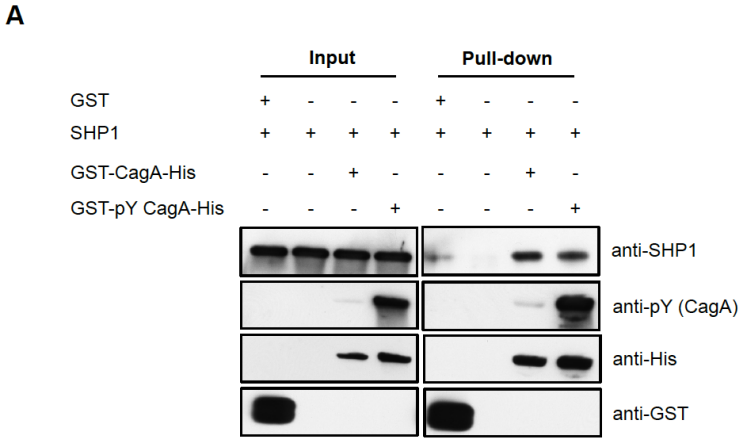


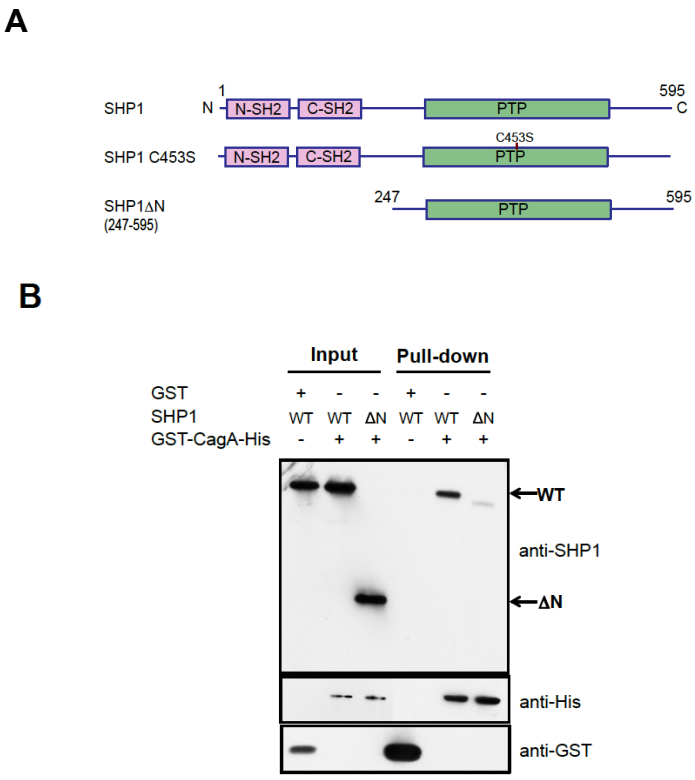
Figure 3. *H. pylori* CagA interact directly with SHP1 tyrosine phosphatase. (A) *In vitro* pull down assay were performed using recombinant SHP1 and GST, GST-pY CagA-His (phosphorylated CagA) or GST-CagA-His and pull down samples were subjected to immunoblotting with the indicated antibodies. Experiment was repeated at least three times and the representative data was shown.

4. SHP1 interacts with CagA through its N-terminus, independent of SH2 domain-phosphotyrosine interaction.

Since SHP1 is occasionally recruited to its substrates by utilizing one or both of its SH2

domains (60), I next asked if the SH2 domains are responsible for mediating direct interaction of SHP1 with CagA. To this end, I generated a truncated form of recombinant SHP1 consisting exclusively of the carboxyl region [aa 247 to 595 (SHP1ΔN)]. The result of the *in vitro* binding assay using full-length SHP1 or SHP1ΔN revealed that full-length SHP1 but not SHP1ΔN bound to GST-CagA-His, indicating that N-terminal region of SHP1, which contains the two SH2 domains is required for CagA binding (Fig 4B). Given this, I next performed an *in vitro* binding assay using GST or GST-pY CagA-His and SHP1 or catalytically inactive-mutant of SHP1 (SHP1 C453S), which has been described as a substrate-trapping mutant (45). It was found that SHP1 C453S exhibited strongly elevated binding with GST-pY CagA-His (Fig 4C). These data together suggest that SHP1 binds to CagA via N-terminus and mutation in the PTPase domain may further facilitate or stabilize SHP1-CagA interaction.

Figure 4



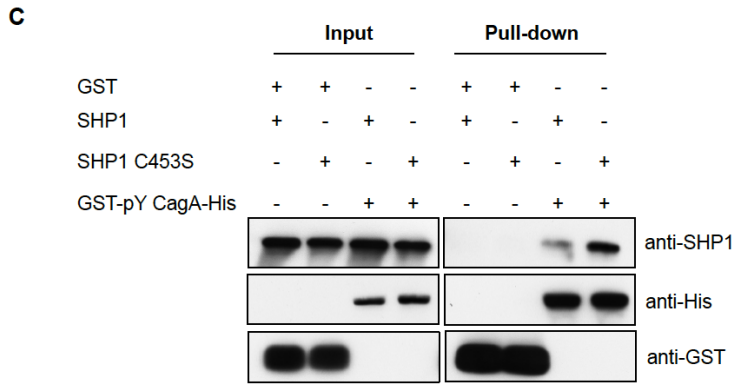


Figure 4. N-terminus of SHP1 directly binds to CagA independent of SH2 domain-phosphotyrosine interaction. (A) Different SHP1 constructs used in this experiments is shown in the schematics. **(B)** *In vitro* pull down assay was performed using recombinant full length SHP1 or truncated SHP1 with N-terminus deleted (denoted as SHP1ΔN) and GST or GST-CagA-His and samples were subjected to immunoblotting with the indicated antibodies. **(C)** *In vitro* pull down assay was performed using recombinant SHP1 or catalytically inactive-SHP1 C453S and GST, GST-pY CagA-His (phosphorylated CagA) and samples were subjected to immunoblotting with the indicated antibodies. Each experiment was repeated at least three times and the representative data were shown.

5. Co-localization of CagA-SHP1 complex on the cell membrane.

It is widely recognized that SHP1 is a cytoplasmic phosphatase (3,20,61,84). However, a novel nuclear localization signal for SHP1 has also been demonstrated in non-hematopoietic cells (12). Therefore, intracellular localization of SHP1 may differs between hematopoietic and non-hematopoietic cells, in fact SHP1 protein is virtually exclusively cytoplasmic in hematopoietic cell lines and in the nucleus in some epithelial cells (12). Given this, I wished to know the subcellular localization of SHP1 in AGS human gastric epithelial cells that I used in this study. I overexpressed Flag-SHP1 in AGS cells and performed immunostaining experiment and staining images were acquired using confocal microscope system (FV 1200, Olympus). It was found that SHP1 broadly distributed in the nucleus and in the cytoplasm in AGS cells (Fig 5A). Next, to examine the co-localization of CagA-SHP1 complex in the cells, I co-express

HA-CagA and Flag-SHP1 in AGS cells. It was demonstrated previously that once delivered into the host cells *H. pylori* CagA localized to the cell membrane and undergoes tyrosine phosphorylation by Src family kinases (24,69). Here I show that in the presence of CagA, SHP1 was co-localized with CagA on the membrane periphery (Fig 5B). The results indicate cytoplasmic/nuclear localization of SHP1 in gastric epithelial cells, and at the same time provide further evidence that CagA-SHP1 forms a complex in the membrane periphery.

Figure 5

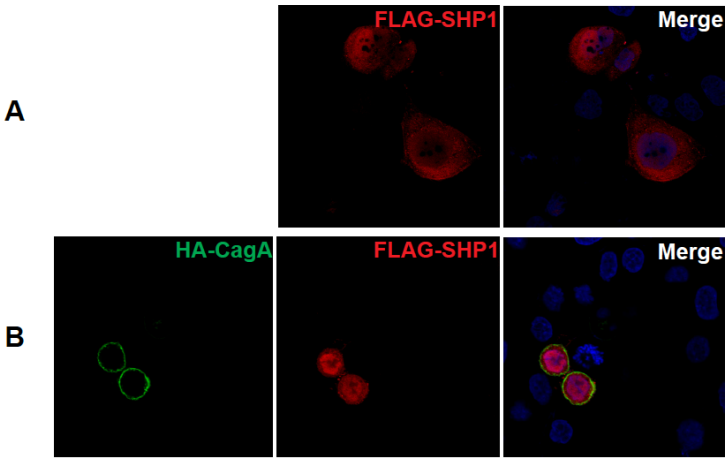


Figure 5. Localization of CagA-SHP1 complex. (A) AGS cells were transfected with a Flag-SHP1vector and were stained with an anti-Flag (SHP1: red) antibody. **(B)** AGS cells co-transfected with Flag-SHP1 and HA-CagA vectors were double stained with anti-Flag (SHP1: red) and anti-HA (CagA: green) antibodies. Nuclei (blue) were visualized by DAPI staining. Each experiment was repeated at least three times and the representative images were shown.

6. CagA activates SHP1 phosphatase activity.

The N-terminal region of SHP1 is involved in CagA binding. Previous studies have already shown that the phosphatase activity of SHP1 is up-regulated by peptide-binding

to the SH2 domains at the N-terminal region (57,108,109,110). Interaction of CagA with the N-terminal region of SHP1 suggested that the complex formation affects SHP1 phosphatase activity. I therefore examined the phosphatase activity of the recombinant SHP1 (1 μM) protein in the absence or presence of recombinant CagA protein (10 μM). As shown in Figure 6, SHP1 phosphatase activity was significantly increased in the presence of CagA. These data indicates that CagA activates phosphatase activity of SHP1 through its binding to the N-terminal region of SHP1.

Figure 6

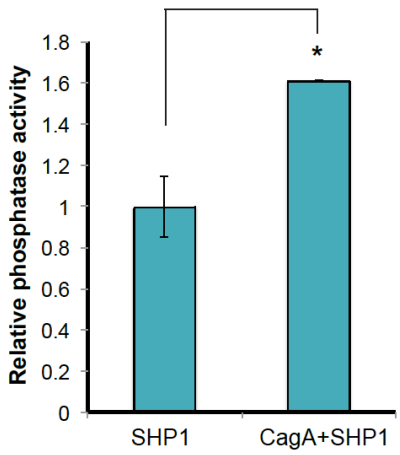


Figure 6. CagA activates SHP1 phosphatase activity. The phosphatase activity of recombinant SHP1 protein was measured using *p*NPP as a substrate in the absence or presence of recombinant CagA as indicated. Error bars, mean ± S.D. (n=3), $^{*}p < 0.01$ (student's *t* test).

7. SHP1 expression reduces the tyrosine phosphorylation level of *H. pylori* CagA in gastric epithelial cells.

Since SHP1 is a protein tyrosine phosphatase, an intriguing idea is that CagA is tyrosine-dephosphorylated by SHP1. I therefore investigated the effect of SHP1 on the tyrosine phosphorylation status of CagA in epithelial cells by ectopically expressing wild-type SHP1 or its mutants (shown in Fig 7A). Overexpression of HA-CagA with

Flag-SHP1 in AGS cells caused a substantial reduction in the level of CagA tyrosine phosphorylation (Fig 7B). Next to see whether the reduced CagA phosphorylation was dependent on the catalytic activity of SHP1, HA-CagA was co-expressed with Flag-SHP1 or a catalytically inactive-SHP1 mutants in which aspartic acid 419, cysteine 453 and arginine 459 in the catalytic center was replaced by alanine, serine and methionine respectively (D419A, C453S and R459M) (45, 67,74). Upon expression in AGS cells, wild-type SHP1 reduced the level of CagA tyrosine phosphorylation, whereas the catalytically inactive-mutants (D419A, C453S, R459M) all failed to do so (Fig 7B, C). These data provided compelling evidence that SHP1 phosphatase activity is essentially required for the reduced levels of CagA tyrosine phosphorylation in cells. SH2 domains of SHP1 are important in recruiting phosphotyrosine-containing proteins, controlling substrate specificity as well as cellular localization of SHP1. Mutations in the SH2 domains have been shown to influence the functions and activities of SHP1 (30,57, 71,83). To investigate the potential involvement of the SH2 domains of SHP1 in the tyrosine dephosphorylation of CagA, I next investigated a series of SH2 domain-mutants of SHP1. R30K mutation abolishes the function of N-SH2 domain of SHP1 whereas R136K substitution abolishes the function of C-SH2 domain of SHP1 (Fig 7A) (30,71,83). I generated an expression vector for a Flag-tagged SHP1 mutant carrying R30K, R136K or both and transfected it into AGS cells together with HA-CagA and found that these SH2 domain-mutants were still capable of inducing CagA dephosphorylation in AGS cells (Fig 7D). The results argue against the idea that the functional SH2 domains of SHP1 is involved in the tyrosine dephosphorylation of CagA upon ectopic expression of SHP1.

Figure 7

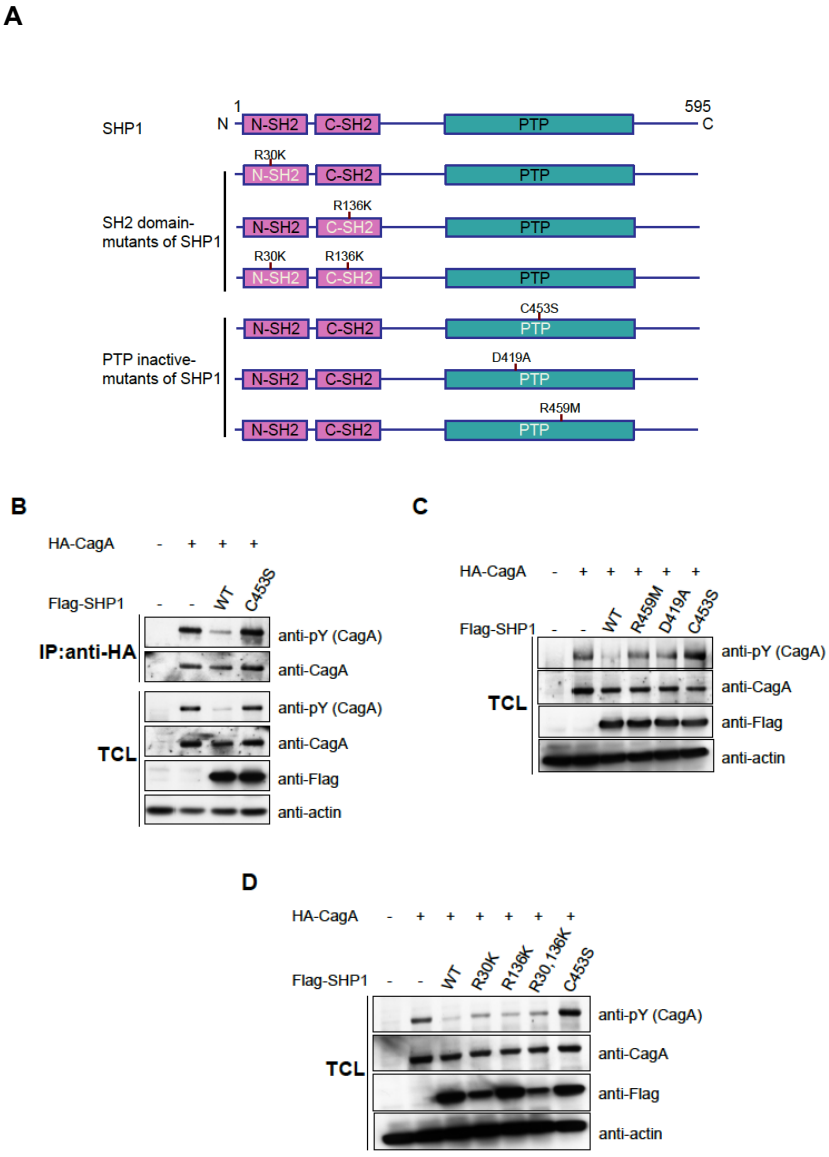


Figure 7. SHP1 dephosphorylates CagA in AGS cells. (A) Schematics of human SHP1 and its various point mutants used in this experiments. (**B)** AGS cells were transfected with HA-CagA alone or together with Flag-SHP1 or catalytically inactive-Flag-SHP1 C453S vectors. Total cell lysates (TCLs) were immunoprecipitated with anti-HA antibody and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. **(C)** AGS cells were transfected with HA-CagA vector alone or together with Flag-SHP1, or Flag-SHP1 R459M, Flag-SHP1 D419A and Flag-SHP1 C453S vectors. Cell lysates were subjected to immunoblotting with the indicated antibodies. **(D)** AGS cells were transfected with HA-CagA vector alone or together with Flag-SHP1, or Flag-SHP1 R30K, Flag-SHP1 R136K, Flag-SHP1 R30, 136K, Flag-SHP1 C453S vectors. Cells lysates were subjected to immunoblotting with the indicated antibodies. Each experiment was repeated at least three times and the representative data were shown.

8. Dephosphorylation of *H. pylori* CagA oncoprotein by SHP1 *in vitro*.

These above mentioned observations indicated that SHP1 is a phosphatase that tyrosine-dephosphorylates *H. pylori* oncoprotein CagA. To investigate this possibility, I carried out an *in vitro* phosphatase assays using recombinant SHP1 and tyrosine-phosphorylated CagA (pY CagA-His) at 37°C for 60 min in 30 µl of phosphatase buffer. The CagA-His protein was phosphorylated using the v-Src kinase co-expressed in *E. coli* and after purification was used as a substrate of *in vitro* phosphatase assays. Consistent with the cells data, *in vitro* incubation of pY CagA-His protein with SHP1 efficiently dephosphorylated pY CagA (Fig 8A), indicating that CagA is a dephosphorylation substrate of SHP1 *in vitro*. *In vitro* phosphatase assay was also performed using phosphorylated CagA (pY CagA-His) and a phosphatase-dead mutant of SHP1 in which cysteine 453 in the catalytic center was replaced with serine (SHP1 C453S, hereby referred to as CS). The CS mutant lacks the PTP activity while retaining the ability to bind substrates (45). In contrast to wild-type SHP1, the SHP1 CS mutant failed to dephosphorylate CagA (Fig 8B) indicating that the intact catalytic activity is required for dephosphorylation of CagA by SHP1. However, the possibility still existed that any phosphatases could non-specifically dephosphorylate CagA in the *in vitro* phosphatase assay. Accordingly I also investigated the ability of the SHP1-related SHP2 in dephosphorylating CagA in the *in vitro* phosphatase assay. Notably, there was no change in the phosphorylation status of pY CagA-His in the presence of wild-type SHP2 (Fig 8C). On the other hand, SHP1 efficiently dephosphorylated pY CagA-His at 1 µM (*lane 3*) and almost totally dephosphorylated pY CagA-His at 5 µM (*lane 5*). The results of the experiment clearly indicated that SHP2 did not dephosphorylate CagA and argue for a strict substrate specificity of SHP1 towards CagA.

Figure 8

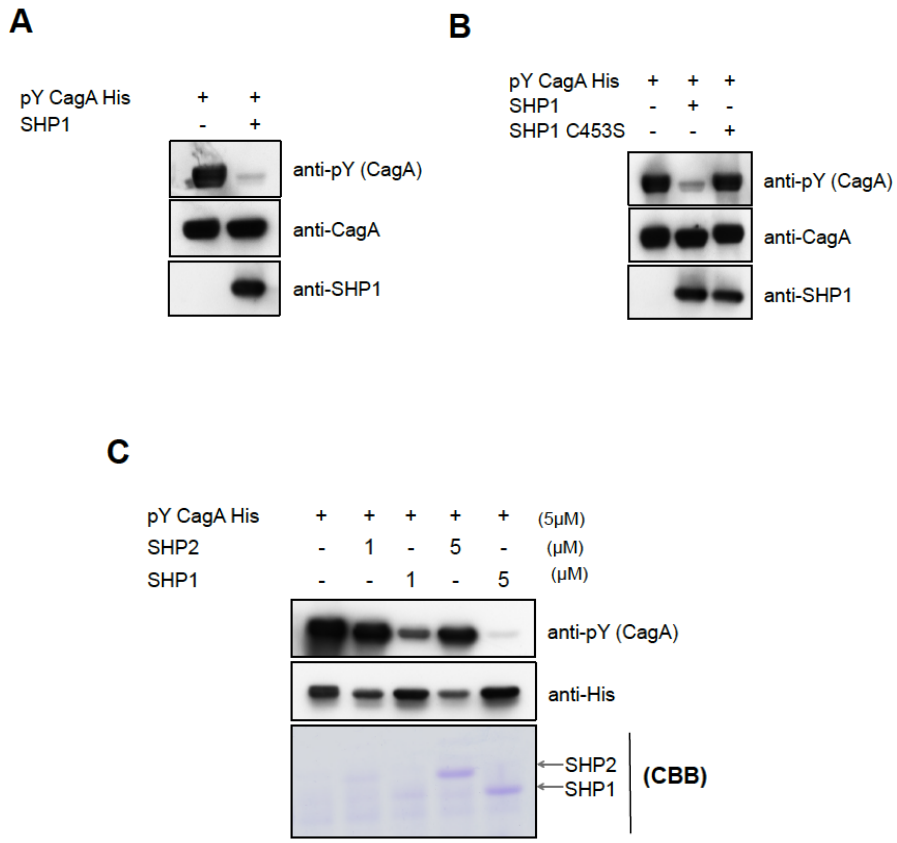


Figure 8. SHP1 specifically dephosphorylates CagA *in vitro*. (A) Tyrosine phosphorylated CagA-His (pY CagA-His) (1 μM) was used as the substrates in phosphatase assay with recombinant wild type SHP1 (1 μM) and samples were subsequently analyzed for residual tyrosine phosphorylation (pY) by immunoblotting with the indicated antibodies. **(B)** Equal aliquots (1 μM) of tyrosine-phosphorylated CagA-His (pY CagA-His) were used as the substrates in phosphatase assay with the bacterially expressed, purified WT (1 μM) or C453S mutant of SHP1 (1 μM) and samples were analyzed for residual tyrosine phosphorylation by immunoblotting with the indicated antibodies. **(C)** Equal aliquots of tyrosine phosphorylated CagA-His (pY CagA-His) (5 μM) were used as a substrates in phosphatase assays with the indicated amounts of recombinant SHP2 (1 μM, 5 μM) and SHP1 (1 μM, 5 μM). Samples were subsequently analyzed for residual tyrosine phosphorylation by immunoblotting with indicated antibodies. Each experiment was repeated at least three times and the representative data were shown.

9. Elevated tyrosine phosphorylation of CagA in SHP1-knockdown epithelial cells.

From the result of the above-described experiments, over expression of SHP1 was found to cause tyrosine dephosphorylation of CagA. Next to see whether the observed phenomenon is physiologically relevant, I performed SHP1-knockdown experiment in

AGS cells using SHP1-specific SiRNA. As shown in figure 9, knockdown of SHP1 in AGS cells resulted in the increase of tyrosine phosphorylation of CagA indicating that SHP1 is indeed acting as the phosphatase that dephosphorylates *H. pylori* oncoprotein CagA.

Figure 9

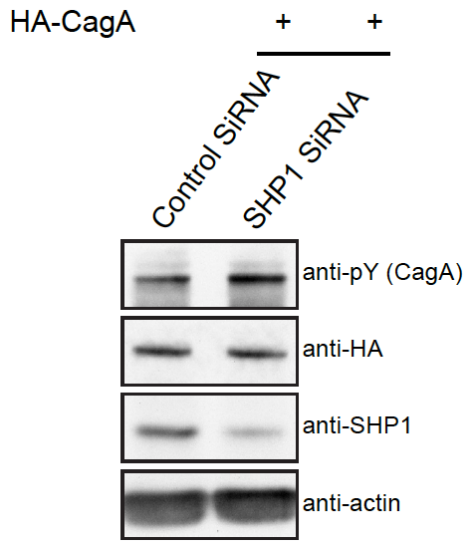


Figure 9. Effects of SHP1 knockdown on the level of CagA tyrosine phosphorylation in AGS cells. AGS cells were transfected with the indicated specific SiRNA. Cell lysates were then prepared and subjected to immunoblotting with the indicated antibodies. Experiment was repeated at least three times and the representative data was shown.

10. Expression of SHP1 inhibits a unique cell-morphological change caused by tyrosine-phosphorylated *H. pylori* CagA.

Expression of CagA in AGS cells, either by infection with *cagA*-positive *H. pylori* or transfection of a CagA expression vector induces an elongated cell morphology termed the 'hummingbird phenotype'. The morphological change, which is characterized by development of one or two long protrusions, requires aberrant activation of SHP2 by CagA that has been tyrosine-phosphorylated at the EPIYA-C or EPIYA-D segment. This cell-morphological change is associated with cell scattering and increased cell

motility (24,48,63). From the results of the above-described experiments, SHP1 was found to act as a specific phosphatase of CagA. This in turn indicated that SHP1 counteracts the ability of CagA to induce the hummingbird phenotype as this phenomenon is entirely depend on the tyrosine phosphorylation of CagA. To investigate this, I transfected a HA-CagA vector alone or together with a Flag-SHP1 vector in AGS cells. After 17 h, cell morphology was examined by light microscopy (Fig 10A). It was found that the numbers of hummingbird cells were significantly reduced in cells expressing SHP1 (Fig 10A). At this time point cells were harvested and immunoblotted. Again as shown in (Fig 10A) the level of CagA tyrosine phosphorylation was reduced upon SHP1 expression. To investigate cell-morphology in more detail, the images of the AGS cells were captured at 24 h after transfection by using confocal microscope system. AGS cells were transfected with EGFP or HA-CagA alone or together with Flag-SHP1 or with Flag-SHP2 vectors. The hummingbird phenotype induced by CagA was inhibited in cells expressing SHP1 but not SHP2 (Fig 10B). These results indicated that SHP1 dampens the pathogenic/oncogenic potential of *H. pylori* CagA upon tyrosine dephosphorylation.

Figure 10

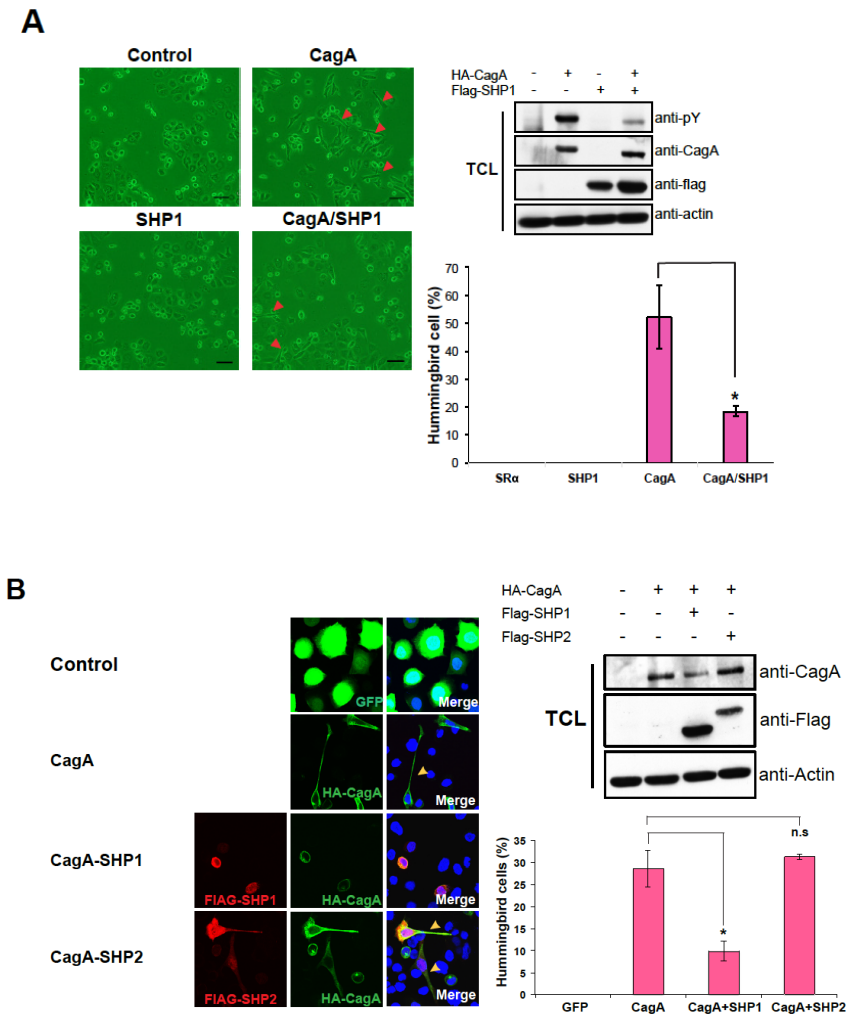


Figure 10. SHP1 inhibits cell-morphological change caused by tyrosine-phosphorylated CagA. **(A)** AGS cells were transfected with a HA-tagged CagA alone or together with Flag-tagged SHP1 vector and cell morphology was observed by light microscopy at 17 h after transfection. Red arrows indicate hummingbird cells induced by CagA. The number of hummingbird cells induced by CagA alone or in the presence of SHP1 were counted and percentage of hummingbird cells was quantified. Error bars, mean ±S.D. (n=3), $^{*}p < 0.01$ (student's *t* test). At this time points, total cell lysates (TCL) were also prepared and were subjected to immunoblotting (IB) with the indicated antibodies. **(B)** AGS cells were transfected with HA-CagA alone or together with Flag-SHP1 or Flag-SHP2 vectors and cells were stained with anti-HA (CagA: green), and anti-Flag (SHP1 and SHP2: red) antibody. Nuclei were stained with DAPI (blue). Confocal images are shown. Yellow arrows indicate hummingbird cells induced by CagA. The number of hummingbird cells induced by HA-CagA alone or together with Flag-SHP1 and Flag-SHP2 were counted and percentage of hummingbird cells was quantified. Error bars, mean ±S.D. (n=3), $^{*}p < 0.01$ (student's *t* test). Total cell lysates (TCL) were also prepared and were subjected to immunoblotting (IB) with the indicated antibodies.

DISCUSSION

CagA is a major virulence factor of *Helicobacter pylori,* a causal agent for various gastro duodenal diseases ranging from gastritis to gastric cancer. This bacterial protein is delivered into the host gastric epithelial cells via the bacterial Type IV secretion system (1,4,53,62,63). Upon delivery, CagA disturbs the intrinsic signal transduction by targeting the signal molecules of the host cells (4). The best-characterized cellular target of CagA is SHP2 tyrosine phosphatase, an oncoprotein, gain-of-function mutation of SHP2 has been found in a variety of human cancers (24,88). The CagA-SHP2 interaction is totally dependent on the tyrosine phosphorylation of CagA at the EPIYA-segment (24). CagA-deregulated SHP2 elicits augmented activation of the promitogenic/prooncogenic Erk-MAP kinase signaling pathway while inducing the morphological changes in gastric epithelial cells, which is characterized by the elongation of cell shape with thin protrusion, is known as hummingbird phenotype. This cell-morphological change is associated with cell scattering and increased cell motility (24, 27, 48,63).

Two SH2 domain-containing phosphotyrosine phosphatases, SHP1 and SHP2 are widely implicated in the regulation of signaling pathways involved in cell proliferation, differentiation and survival (96). These PTPs are involved in the pathogenesis of lymphoma, leukemia and other types of cancers (97,98). Despite their high degree of homology, SHP1 and SHP2 have distinct expression profiles and functions. In contrast to SHP2, which is ubiquitously expressed, SHP1 is mainly found in hematopoietic cells (44). Multiple binding partners and substrates of SHP1 have been identified. For example, SHP1 acts as a negative regulator of several signal transduction proteins

including cytokine receptors, by dephosphorylating the receptor itself and/or receptor-associated kinases such as Src and JAK family kinases (30,82). This PTP can also down regulate the activation of STATs (99). SHP1 has been implicated in negative signaling through inhibitory receptors (ITIM). Inhibitory receptors play a vital role in regulating the cells of immune system (106). The role of the ITIM was first elucidated in B cells. The importance of the inhibitory signals delivered by SHP1 is highlighted by the motheaten (*me)* mouse, which has a natural mutation in the SHP1 locus (106). Mice with this deficiency are characterized by a widespread autoimmune phenomenon, caused by an inability to negatively regulate immune responses (59, 79, 80, 93, 94, 95). Furthermore, recent reports have clearly shown that the absence of SHP1 or decrease in the level of SHP1 protein expression is frequent in malignant T cells and it results from the methylation of SHP1 gene promoter (100,101,102). Because the enforced expression of SHP1 reverses the malignant phenotype, the phosphatase has been proposed as a tumor suppressor gene candidate in various hematopoietic cancers (103).

In contrast to hematopoietic cells, little is known about the role of SHP1 in epithelial cells (55). In this study I explored the role of SHP1 in *H. pylori* CagA mediated carcinogenesis in gastric epithelial cells. The key findings in this study are the identification of SHP1 as a major and specific phosphatase that tyrosine-dephosphorylates *Helicobacter pylori* CagA oncoprotein and thereby attenuates CagA-mediated carcinogenesis in gastric epithelial cells. Here I found a novel interaction between SHP1 and *H. pylori* CagA, in gastric epithelial cells and B cells (Fig 1A, B). I also found that CagA acts as a binding partner and substrate for SHP1 in gastric epithelial cells. Unlike SHP2, the CagA-SHP1 interaction is independent of EPIYA-tyrosine phosphorylation of CagA (Fig 2A, B, 3A). I also demonstrated that SHP1

directly binds to CagA and further established that, this interaction is mediated via N-terminal region, which contains SH2 domains of SHP1 (Fig 3A, 4B). SH2 domains commonly recognize and bind phosphorylated-tyrosine, thereby trigger the formation of protein complexes in a ligand dependent manner (81). In the case of SHP1 SH2 domains, a preference for phosphorylated-CagA *in vitro* was not observed. However, this is not unusual because the SH2 domains of SHP1 are known to often bind interacting partners/substrates in a phosphotyrosine-independent manner (9,30,32,46,47,82). My preliminary experiments indicated that the CagA-SHP1 interaction, which is mediated by the N-terminal region of SHP1 does not seem to require functional-SH2 domains as the introduction of mutation into both of the SH2 domains did not abolish SHP1 binding with CagA (data not shown). This indicates that CagA-SHP1 interaction is mediated by a novel mechanism that is totally different from the SH2 domains-phosphotyrosine interaction.

The present study provided evidence that SHP1 efficiently dephosphorylates *H. pylori* oncoprotein CagA in gastric epithelial cells and *in vitro*. Expression of wild type SHP1 but not the phosphatase-dead form of SHP1 resulted in the dephosphorylation of CagA (Fig 7B-C, 8A-B) consolidating that the dephosphorylation depends on the catalytic activity of SHP1. Furthermore, knockdown of SHP1 in AGS cells resulted in the up-regulation of tyrosine phosphorylation of CagA (Fig 9). I also demonstrated that SHP1 but not its structurally related family member SHP2 is the specific phosphatase for CagA (Fig 8C) further highlighting the specificity of SHP1 on CagA as a substrate.

What are the biological significance for the functional interaction between SHP1 and *H. pylori* CagA oncoprotein? Expression of SHP1 resulted in the dephosphorylation and

down-regulation of tyrosine-phosphorylated CagA. Besides, ensuring a rapid down-regulation of phosphotyrosine-dependent signaling by CagA, another possible consequence of the constitutive proximity of SHP1 to its substrates could be that such setup allows the substrate to feedback regulate SHP1 catalytic activity, as seen in other instances (32,30,82,37). For example, CagA interaction with the N-terminal region of SHP1, which contains SH2 domains, could release the conformation-dependent auto-inhibition of SHP1 (57). It was found that such CagA-SHP1 interaction resulted in the significant activation of SHP1 phosphatase activity (Fig 6).

Inhibition of CagA tyrosine phosphorylation or disruption of the CagA-SHP2 complex formation might result in the attenuation of *H. pylori* CagA function. CagA-SHP2 complex formation plays an important role in the progression of the multistep gastric carcinogenesis that is associated with *cagA*-positive *H. pylori* infection (24). Tyrosine phosphorylation of CagA is an essential prerequisite for CagA-SHP2 complex formation and subsequent induction of unique cell morphology termed hummingbird phenotype, which is associated with cell scattering and increased cell motility (5,24,63). I found that expression of SHP1 inhibited the 'hummingbird phenotype' caused by CagA-SHP2 interaction (Fig 10A, B), highlighting the role of SHP1 in counteracting the oncogenic potential of CagA.

There are many reports with regard to expression level of SHP1 during cancer progression (10,14,15,36,38,68,77,85,86), in which expression of SHP1 is often suppressed and suggesting the important role played by SHP1 as a negative regulator of cell signaling. It was also reported recently that SHP1 expression is suppressed during the process of gastric carcinogenesis via promoter hyper-methylation, suggesting the

role of SHP1 as a tumor suppressor in the gastric epithelium (66). In this study, I found that SHP1 tyrosine phosphatase effectively tyrosine-dephosphorylates *H. pylori* oncoprotein CagA in gastric epithelial cells, thereby attenuating the pathophysiological action of CagA. Attenuation of *H. pylori* CagA activity by C-terminal Src kinase (CSK) has been reported previously (69). In that case CSK inhibits the kinase activity of Src, which tyrosine-phosphorylates CagA (69). The results of my study provides potentially important role of SHP1 in down-regulating the oncogenic activity of *H. pylori* CagA. In turn, the relatively low levels of SHP1 expression in gastric epithelial cells might contribute to the development of CagA-mediated carcinogenesis. At the same time, overexpression of SHP1 should almost completely abolish tyrosine phosphorylation of CagA and thus impairs the oncogenic potential of CagA, raising the idea that elevated levels of SHP1 in gastric epithelial cells could prevent gastric cancer development in patients infected by *cagA*-positive *H. pylori*. CagA-mediated cellular pathogenesis depends, at least partly, on relative levels of SHP2 expression in the host cells. In the present study, I found that high levels of SHP1 expression in gastric epithelial cells dampen the oncogenic potential of CagA. In normal gastric epithelial cells SHP2 expression is relatively high compared to SHP1 (http://157.82.78.238/refexa/main_search.jsp, Laboratory for Systems Biology and Medicine at RCAST, The University of Tokyo). Based on this, low expression of SHP1 in gastric epithelial cells might contribute to the development of CagA-mediated pathogenesis where SHP2 is predominantly expressed. Development of a small compound that stimulates the phosphatase activity of SHP1 may be applicable to the prophylaxis of gastric cancer in individuals infected by *H. pylori cagA*-positive strains. Since SHP1 is abundantly expressed in hematopoietic cells including B-lymphocytes to

which CagA can also be delivered by *H. pylori* (44). And it is possible that high expression levels of SHP1 in lymphocytes might be one reason that explains the low incidence of MALT lymphoma compared to gastric cancer in patients infected with *cagA*-positive *H. pylori*. Given that SHP1 is occasionally hyper-methylated in gastric cancer cases (66), it should be important to elucidate the role for the relative levels of SHP1 and SHP2 expression in gastric epithelial cells in the development of stomach cancer by *cagA*-positive *H. pylori.* At present, there is no *in vivo* evidence to show how efficiently SHP1 can regulate *H. pylori* CagA-associated gastric carcinogenesis. However in my future work, I am interested to focus more on those aspects and other influences of SHP1 on the gastric oncogenic development by *H. pylori* CagA.

Figure 11

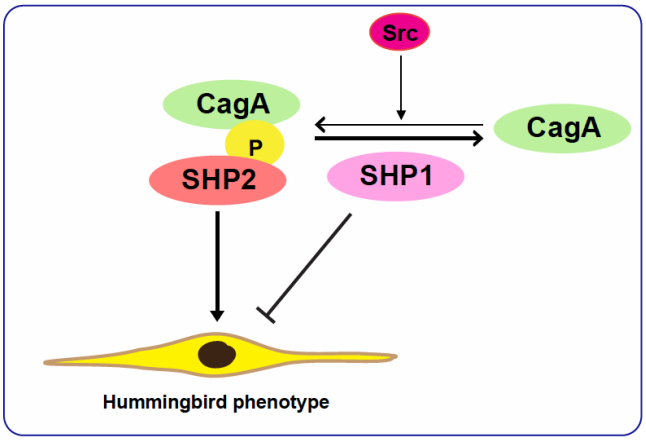


Figure 11. A proposed model for the negative regulation of *Helicobacter pylori* CagA by SHP1 tyrosine phosphatase. Once translocated into the host cell via type IV secretion system *H. pylori* CagA undergoes tyrosine phosphorylation at the EPIYA-motifs by Src family kinases and specifically forms complex with SHP2 tyrosine phosphatase in gastric epithelial cells and results in subsequent induction of unique cell-morphology termed hummingbird phenotype. On the other hand in my studies it was found that CagA forms direct complex with SHP1 tyrosine phosphatase and activates its enzymatic activity. Furthermore, expression of SHP1 results in efficient dephosphorylation of CagA and thereby inhibits the hummingbird phenotype caused by CagA-SHP2 complex formation.

Conclusion

In this study I demonstrated that the *H. pylori* oncoprotein CagA interacts directly with SHP1 tyrosine phosphatase. I also showed that expression of SHP1 in gastric epithelial cells results in the efficient tyrosine dephosphorylation of CagA and inhibits the unique cell morphology caused by tyrosine-phosphorylated CagA, thereby attenuates the pathogenic/oncogenic potential of *H. pylori* oncoprotein CagA in gastric epithelial cells. Given these it will be very helpful for our understanding of the mechanism of regulation of *H. pylori*-related gastric diseases, especially the gastric cancer, the second leading cause of cancer-related death worldwide and also MALT Lymphoma.

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