Helicobacter pylori CagA upregulation of CIP2A is dependent on the Src and MEK/ERK pathways

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Helicobacter pylori is classified as a class I carcinogenic factor and its persistent colonization in the stomach induces gastric cancer. Cancerous Inhibitor of PP2A (CIP2A) is a newly identified oncoprotein overexpressed in gastric cancer. Serving as a key oncoprotein, CIP2A also participates in regulation of senescence and proliferation of gastric cells. The combination of aberrant CIP2A expression inducing unlimited cell proliferation, and *H. pylori* infection eliciting aberrant expression of some key proteins, results in the onset of gastric tumorigenesis. However, the relationship between *H. pylori* infection and CIP2A expression still remains undefined. The aim of our study was to verify the effect of *H. pylori* infection on CIP2A expression levels and identify *H. pylori* signalling molecules and corresponding pathways influencing CIP2A expression. Following plasmid-mediated expression of CagA in human gastric cell lines, the cells were infected with *H. pylori* and CIP2A expression levels were examined by immunoblotting. Signal inhibitors were used to verify which signal pathways were involved. We also performed CIP2A depletion and *H. pylori* infection after depletion in AGS cells. *H. pylori* infection-induced CIP2A expression was dependent on *cagA* gene expression and CagA phosphorylation. Bacterial oncoprotein CagA upregulated CIP2A expression and this upregulation effect was dependent on Src and Ras/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathways. *H. pylori* infection-induced Myc stabilization was partially attenuated by CIP2A depletion. The results of our study provide further information for understanding the mechanism of *H. pylori* carcinogenesis.

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INTRODUCTION

Helicobacter pylori has been defined as a class I carcinogenic factor by the World Health Organization and its persistent colonization in the stomach leads to an increased risk of peptic ulcers and gastric adenocarcinoma (Houghton & Wang, 2005; Peek & Blaser, 2002). Some *H. pylori* strains contain a 35–40 kb *cag* pathogenicity island (PAI) encoded by 27–33 genes (Akopyants *et al.*, 1998; Azuma *et al.*, 2004). One constituent of the *cag* PAI is *cagA*, which encodes a 120–140 kDa CagA protein. CagA protein, which has tyrosine phosphorylation (EPIYA) motifs, is injected into gastric epithelial cells by the type IV secretory system (TFSS) (Odenbreit *et al.*, 2000; Stein *et al.*, 2000). *cagA*-

positive *H. pylori* strains have a closer association with the progress of both peptic ulcers and gastric cancer than *cagA*-negative strains according to epidemiological research (Hatakeyama, 2003). Within gastric epithelial cells, tyrosine phosphorylation occurs at the C terminus of CagA by the Src family of tyrosine kinases (Kwok *et al.*, 2007; Stein *et al.*, 2002). CagA interacts with many signal molecules and elicits a series of cellular events. These events include activation of the Ras/mitogen-activated protein (MAP) kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, β -catenin, Src kinase, the NF- κ B pathway and the p38 MAP kinase pathway. Some changes related to cell morphology, cell scattering, cell proliferation and intercellular tight junctions have also been identified (Hatakeyama, 2003, 2004; Nguyen *et al.*, 2008; Backert & Selbach, 2008; Backert & Meyer, 2006; Handa *et al.*, 2007). According to these observations, bacterial oncoprotein CagA participates in gastric tissue injury caused by *cagA*-positive *H. pylori* infection.

Unlimited cellular proliferation is a hallmark of tumours. Tumorigenesis is related to the disordered expression of

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Abbreviations: CagA, cytotoxin-associated gene A; CIP2A, Cancerous Inhibitor of PP2A; MEK/ERK, Ras/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase; PP2A, protein phosphatase 2A; PR, phosphorylation-resistant; siRNA, small interfering RNA; TFSS, type IV secretory system; WT, wild-type.

some key factors which participate in the regulation of cell cycle progression, differentiation, apoptosis and senescence (Hanahan & Weinberg, 2000). Recent studies have reported that CIP2A (Cancerous Inhibitor of PP2A) serves as an important oncoprotein (Junttila *et al.*, 2007). Protein phosphatase 2A (PP2A) can facilitate the proteolytic degradation of oncoprotein Myc and prevent malignant cell growth (Yeh *et al.*, 2004). CIP2A stabilizes Myc protein through inhibiting PP2A activity and promotes tumour formation *in vivo* (Junttila *et al.*, 2007). Moreover, transformation of immortalized human cells is observed when CIP2A is overexpressed (Junttila *et al.*, 2007). More importantly, overexpression of CIP2A has been observed in gastric cancer (Li *et al.*, 2008). Using small interfering RNA (siRNA) to knock down CIP2A expression inhibits cell growth and clone formation, and promotes senescence in gastric cancer-derived AGS cell lines (Li *et al.*, 2008). Taken together, CIP2A is a newly identified oncoprotein in gastric tissues, and may serve as a diagnostic marker and therapeutic target for gastric tumours (Li *et al.*, 2008).

As mentioned above, *H. pylori* infection could affect the expression level of gastric carcinogenic factors, such as Myc (Zhu *et al.*, 2008), β -catenin (Franco *et al.*, 2005) and cyclin D1 (Chang *et al.*, 2006). However, up to now, the relationship between *H. pylori* infection and CIP2A expression has never been studied. However, both bacterial oncoprotein CagA and cellular oncoprotein CIP2A expression have been associated with gastric carcinogenesis. To figure out the effect of CagA on CIP2A may help us to understand the carcinogenic mechanism of *H. pylori* infection and the functional role of CIP2A in human gastric tumorigenesis. In order to elucidate the relationship between these two factors, we designed *H. pylori* infection and CagA transfection in gastric cells and tried to investigate whether main virulence factor CagA of *H. pylori* could increase the expression of oncoprotein CIP2A in cell lines. Furthermore, we used signal inhibitors to examine which pathway was involved in the CagA-mediated regulation of CIP2A expression.

METHODS

***H. pylori* and bacterial culture.** The *H. pylori* strains used in this study were *cagA*- and *vacA*-positive standard strains (NCTC 11637 and 26695). *H. pylori* NCTC 11637 has EPIYA-ABCCC type CagA, while *H. pylori* 22695 has EPIYA-ABC type CagA. Both strains were kindly provided by Dr Zhang Jianzhong (Chinese Disease Control and Prevention Center). Isogenic *cagA*⁻ mutants including *cagA*⁻ 26695 and *cagA*⁻ 11637 were constructed within strains 11637 and 26695 by insertional mutagenesis using the pILL570 vector. All strains were grown in Brucella broth with 5% fetal bovine serum (FBS) under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37 °C, harvested by centrifugation, and added to gastric cells at a bacteria-to-cell ratio of 100:1.

Plasmid. Wild-type (WT) *cagA*/pcDNA3.1(+) plasmid (WT-*cagA*) and a phosphorylation-resistant (PR) plasmid derivative (PR-*cagA*) were kindly provided by Zhu Yongliang (Zhejiang University, China). These two plasmids were characterized as described previously (Zhu *et al.*, 2004, 2005). The pcDNA3.1(+) mammalian expression vector was purchased from Invitrogen.

Cell lines and culture conditions. The human gastric epithelial AGS cells and immortalized GES-1 cells were maintained in our laboratory. AGS gastric cancer cells were cultured in F12 medium (Gibco Life Technologies) with 10% FBS (Gibco Life Technologies). GES-1 cells were cultured in RPMI 1640 medium (Gibco Life Technologies) with 10% newborn bovine serum (Gibco Life Technologies). All cells were cultured in 5% CO₂-air at 37 °C.

Transfection of AGS and GES-1 cells. FuGENE HD Transfection Reagent (Roche Applied Science) was used to transfect plasmid WT-*cagA*, PR-*cagA* or pcDNA3.1 blank vector into 5 × 10⁵ AGS or GES-1 cells in six-well plates. All transfection procedures were performed according to the manufacturers and all experiments were repeated three times. After the transfection, cells were harvested and lysed using cell lysis buffer (Beyotime) supplemented with protease inhibitors (Complete Mini; Roche Applied Science) before freezing at -80 °C. In order to investigate the signal pathways in the epithelial cells, we used the following reagents: BAY11-7082 (5 µM), PP1 (20 µM), PD98059 (50 µM) and SB203580 (10 µM) (all from Invitrogen). We used one of these reagents to pre-incubate cells for 30 min, and then transfected plasmids into cells. Protein collection methods were the same as without exposure to signal blocking reagents.

Western blot analysis. We used a BCA reagent kit (Merck) to measure protein concentrations and standardized samples in Western blot analysis according to the protein concentrations. Protein samples were separated by SDS-PAGE (8% SDS-acrylamide gels), and then proteins were transferred to a nitrocellulose membrane. After that, the membranes were incubated in the blocking buffer (TBS containing 0.1% Tween, 5% nonfat powdered milk) for 1.5 h, and immunoblotted for 1.5 h with antibodies against Myc, β -actin, C-CagA (Santa Cruz Biotechnologies) or CIP2A (Novus Biologicals). Membranes were then washed three times with TBS-Tween solution and incubated with the corresponding antibodies conjugated to horseradish peroxidase for 50 min. We then washed the membranes three times with TBS-Tween and used the Chemiluminescent ECL Detection System (Millipore) to detect the signals. Bio-Rad Quantity One 1-D Analysis software was used to analyse the Western blot results.

siRNA experiments. The siRNA targeting CIP2A and control siRNA were purchased from Invitrogen. The siRNA sequence for CIP2A was 5'-GACAACUGUCAAGUGUACCACUCUU-3'. Lipofectamine 2000 (Invitrogen) was used to transfect the gastric cells with siRNA.

Statistical analysis of data. The Statistical Package for the Social Sciences (SPSS) was used in the statistical analysis, and a Student's *t* test was used to determine statistical significance. *P*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of *H. pylori* infection on CIP2A expression level in AGS cells

H. pylori is a human gastric carcinogenic pathogen and CIP2A is a newly identified oncoprotein in gastric cancer (Li *et al.*, 2008; Khanna *et al.*, 2009). To elucidate whether *H. pylori* upregulated CIP2A expression, we designed *H. pylori* infection in human gastric epithelium AGS cells. AGS cells were incubated with *H. pylori* strain 26695 for 0.5, 1, 1.5 and 2 h, at a quantity of 100 bacteria per cell. Total cellular protein analysed by Western blotting indicated that the CIP2A expression level was elevated after infection for all time points compared to the expression in normal

AGS cells, but that the effect was not remarkably time-dependent (Fig. 1a). Furthermore, in order to elucidate which constituent of *H. pylori* is essential for promotion of CIP2A expression, we used *H. pylori* strains *cagA*⁻26695, *cagA*⁺26695, *cagA*⁻11637 and *cagA*⁺11637 to infect AGS cells for 6 h. The CIP2A expression level was elevated only after infection with strains *cagA*⁺26695 or *cagA*⁺11637 compared to the expression in normal AGS cells (Fig. 1b). However, there was no significant increase in CIP2A expression level in AGS cells infected with strain *cagA*⁻26695 and strain *cagA*⁻11637 compared to the expression in normal AGS cells (Fig. 1b). Taken together, *H. pylori* infection increased CIP2A expression in AGS cells, and this effect was not dependent on infection time but dependent on expression of the bacterial *cagA* gene.

CIP2A expression in AGS and GES-1 cells transfected with plasmid WT-*cagA* or PR-*cagA*

As CIP2A expression was demonstrated to be associated with CagA expression of *H. pylori*, we next evaluated the relationship between CagA and CIP2A in the absence of *H. pylori* infection. AGS and GES-1 cells were transfected with plasmid WT-*cagA* or PR-*cagA*. The PR-*cagA* plasmid encodes a CagA protein with a mutation in the EPIYA

motif, required for CagA tyrosine phosphorylation. Thus the PR-CagA mutant protein cannot be phosphorylated nor can it transmit the signal pathway (Zhu *et al.*, 2004, 2005; Yokoyama *et al.*, 2005). After 36 h transfection, the CIP2A expression level in AGS and GES-1 cells expressing WT-CagA was significantly greater than that in normal cells and cells transfected with blank vector (Fig. 2a). In contrast, there was no increase in CIP2A expression level in AGS and GES-1 cells transfected with vector expressing PR-CagA compared with cells alone or blank vector (Fig. 2b). Moreover, the Myc expression level was also elevated in cells expressing WT-CagA (Fig. 2a). Antibody against C-CagA ensured that WT-*cagA* and PR-*cagA* plasmid transfections and CagA protein expression were accomplished as expected (Fig. 2a, b). Taken together, bacterial oncoprotein CagA can upregulate the expression of CIP2A and this effect is closely dependent on the tyrosine phosphorylation of CagA.

Signal inhibitor effects on CIP2A expression in AGS cells transfected with WT-*cagA* plasmid

After translocating into epithelial cells through the TFSS, *H. pylori* CagA protein is known to activate the MEK/ERK pathway, Src kinase, the NF- κ B pathway and the p38 MAP kinase pathway regardless of its tyrosine phosphorylation

Fig. 1. Effect of *H. pylori* infection on CIP2A expression in AGS cells. (a) AGS gastric epithelial cells were incubated with medium alone, or with strain 26695 for 0.5 h, 1 h, 1.5 h or 2 h. Total cellular protein was extracted for Western blot analysis for the expression of CIP2A proteins. (b) AGS gastric epithelial cells were incubated with medium alone, or with strain 26695cagA⁻, 26695cagA⁺, 11637cagA⁻ or 11637cagA⁺ for 6 h. Total cellular protein was extracted for Western blot analysis for the expression of CIP2A. Representative results are shown. **P* > 0.05, ***P* < 0.01.

Fig. 2. Analysis of CagA transfection and its effects on CIP2A and Myc expression. (a) GES-1 and AGS gastric epithelial cells were transfected with blank vector pcDNA3.1 or WT-cagA plasmid for 36 h, respectively. Total cellular protein was extracted for Western blot analysis for the protein expression of CIP2A, Myc and C-terminal CagA. (b) GES-1 and AGS gastric epithelial cells were transfected with blank vector pcDNA3.1 or PR-cagA plasmid for 36 h, respectively. Total cellular protein was extracted for Western blot analysis for the expression of CIP2A and C-terminal CagA proteins. * $P > 0.05$, ** $P < 0.01$.

status. In order to elucidate which pathway participated in the upregulation of CIP2A by CagA, we used the MAP kinase inhibitor PD98059 to block MEK1 kinase activity, thereby inhibiting ERK1/2 phosphorylation (Alessi *et al.*, 1995), SB203580 to block p38 kinase activity (Young *et al.*, 1997), BAY11-7082 to inhibit the NF- κ B pathway (Pierce *et al.*, 1997) and PP1 to block Src-family tyrosine kinase activity (Hanke *et al.*, 1996). Each signal inhibitor was incubated with gastric cells for 30 min prior to plasmid transfection. After 36 h transfection, while CIP2A upregulation by CagA was attenuated by PP1 (Src kinase) and PD98059 (MEK1), CIP2A upregulation was maintained using SB203580 (P38) and BAY11-7082 (NF- κ B) (Fig. 3). In total, the results indicated that Src kinase and MEK/ERK pathways participated in CIP2A upregulation by CagA. In AGS cells, CagA protein was tyrosine phosphorylated by Src kinase, thereafter promoting the activated MEK/ERK pathway to upregulate CIP2A expression (Backert & Selbach, 2008).

Effect of CIP2A depletion and *H. pylori* infection on Myc expression in AGS cells

It has been reported that CIP2A depletion causes the downregulation of Myc expression in several cell lines (Li *et al.*, 2008). To investigate whether *H. pylori* infection could still upregulate Myc expression in AGS cells depleted

Fig. 3. Effects of signal inhibitors on CagA-induced CIP2A upregulation in AGS cells. AGS cells were transfected with blank vector or WT-cagA plasmid. The AGS cells were incubated with Src-family tyrosine kinase inhibitor PP1 (20 μ M), MEK1 inhibitor PD98059 (50 μ M), p38 MAP kinase inhibitor SB203580 (10 μ M) or NF- κ B pathway inhibitor BAY11-7082 (5 μ M). Signal inhibitors were used 30 min before transfection and total cellular protein was extracted for Western blot analysis for the expression of CIP2A after transfection for 36 h. * $P > 0.05$, ** $P < 0.01$.

of CIP2A, we performed siRNA-mediated CIP2A depletion and *H. pylori* strain 26695 infection after depletion in AGS cells. We found that the Myc expression level was higher in cells treated with CIP2A depletion and *H. pylori* infection than in cells only treated with CIP2A depletion, and this level was lower in cells treated with CIP2A depletion and *H. pylori* infection than in those only treated with *H. pylori* infection (Fig. 4). The results suggest that *H. pylori* infection-induced Myc stabilization was partially attenuated by CIP2A depletion.

CIP2A has recently been observed to be overexpressed in head and neck squamous cell carcinoma, colon cancer (Junttila *et al.*, 2007) and gastric cancer (Li *et al.*, 2008). CIP2A overexpression stabilized the Myc protein (Khanna *et al.*, 2009). The depletion of CIP2A inhibited growth and clonogenic capabilities, and induced senescence of AGS cells (Li *et al.*, 2008). CIP2A is also known to act as an endogenous PP2A inhibitor (Junttila *et al.*, 2007). *H. pylori*, a human gastric carcinogenic pathogen, has been classified as a class I carcinogenic factor by the World Health Organization and its long-term colonization in stomach initiates an increased risk of gastric cancer. Recent *in vitro* or *in vivo* data have demonstrated that *H. pylori* infection can upregulate cyclin D1 expression, stabilize Myc protein, increase β -catenin nuclear accumulation and induce release of cytokines such as interleukin-8 and CCL20 (Chang *et al.*, 2006; Yang *et al.*, 2003; Zhu *et al.*, 2008; Franco *et al.*, 2005; Kim *et al.*, 2006; Tomimori *et al.*, 2007). The expression level of murine double minute 2 was higher in gastric cancer than in chronic gastritis in *H. pylori*-infected gastric mucosa (Nakajima *et al.*, 2009). However, up to now, the relationship between gastric carcinogenic pathogen *H. pylori* infection and gastric cancer oncoprotein CIP2A expression has never been studied. Thus we have explored the relationship between *H. pylori* infection, CIP2A expression and signalling pathways leading to gastric cancer.

Some former studies showed that *H. pylori* infection could upregulate cyclooxygenase 2 expression *in vitro* (Seo *et al.*, 2007; Li *et al.*, 2009). In our study, we verified that CIP2A expression in AGS cells was stimulated by *H. pylori* infection, and that this upregulation effect was not

dependent on infection time but dependent on the *cagA* gene status of bacterial strains (Fig. 1). Kim *et al.* (2006) found that *H. pylori* CagA transfection of gastric epithelial cells induced IL-8 production, and Franco *et al.* (2005) found that CagA transfection increased β -catenin nuclear accumulation. In our study, we demonstrated that the CIP2A expression level was elevated by CagA transfection and that this elevation only occurred after tyrosine phosphorylation of CagA (Fig. 2). Moreover, Khanna *et al.* (2009) reported that Myc directly promoted CIP2A gene expression. Surprisingly, we found that CagA transfection also increased the Myc expression level (Fig. 2). These results offered more credible evidence for upregulation of CIP2A expression by CagA transfection. After translocation into epithelial cells, *H. pylori* CagA protein could activate four major pathways, including the MEK/ERK pathway, Src kinase, the NF- κ B pathway and the p38 MAP kinase pathway (Backert & Selbach, 2008). It was previously shown that signal inhibitors could be used to elucidate which pathway was involved in CagA-induced IL-8 production (Kim *et al.*, 2006) or *H. pylori* infection-induced cyclooxygenase 2 upregulation (Li *et al.*, 2009). By using four corresponding signal inhibitors, we identified that Src kinase and MEK/ERK pathways were involved in CIP2A upregulation by CagA (Fig. 3). Taken together, CagA protein was tyrosine-phosphorylated by Src kinase, and thereafter activated the MEK/ERK pathway to upregulate CIP2A expression in AGS cells. We also found that *H. pylori* infection-induced Myc stabilization was partially dependent on CIP2A expression, and CIP2A depletion caused increased Myc degradation (Fig. 4).

The combination of data from our research and others led us to propose the signalling mechanism outlined in Fig. 5. Kim *et al.* (2006) reported that CagA transfection activated Src kinase, and Zhu *et al.* (2007) proved that CagA transfection and phosphorylated CagA protein (P-CagA) activated the ERK1/2 pathway. Based on these two findings, we used two corresponding signal inhibitors to block Src kinase or MEK1 kinase, and figured out the following mechanisms. After translocating into gastric epithelial cells through the TFSS, bacterial oncoprotein CagA was tyrosine-phosphorylated by Src kinase. After

Fig. 4. Effects of CIP2A depletion and *H. pylori* infection on Myc expression in AGS cells. (a) AGS cells were transfected with control siRNA or CIP2A siRNA for 72 h. Western blotting was used to verify the efficiency of CIP2A depletion. (b) After transfection with control siRNA or CIP2A siRNA for 72 h, AGS cells were infected with *H. pylori* strain 26695 for 6 h. Western blotting was used for Myc expression analysis. ** $P < 0.01$.

Fig. 5. Schematic model of the upregulation effect of *H. pylori* CagA on CIP2A. Bacterial oncoprotein CagA was translocated into gastric epithelial cells through the TFSS, and then activated Src kinase and was phosphorylated by Src kinase. After that, P-CagA activated the MEK/ERK pathway and upregulated gastric oncoprotein CIP2A expression.

that, P-CagA activated the MEK/ERK pathway and upregulated gastric oncoprotein CIP2A expression. Upregulation of CIP2A expression by *H. pylori* provided a further explanation for the gastric carcinogenic effects of *H. pylori* infection.

Overall, this is the first time that the mechanism by which *H. pylori* infection and bacterial oncoprotein CagA upregulated CIP2A expression in gastric cell lines has been elucidated. CagA protein activated the Src and MEK/ERK signal pathways, resulting in the elevation of expression of CIP2A protein in AGS cells. This work has contributed to understanding the mechanism by which gastric tumours are caused by *H. pylori* infection in humans.

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