Reactive Oxygen Species-Induced Autophagic Degradation of *Helicobacter pylori* CagA Is Specifically Suppressed in Cancer Stem-like Cells

Hitoshi Tsugawa,1 Hidekazu Suzuki,1,* Hideyuki Saya,2 Masanori Hatakeyama,3 Toshiya Hirayama,4 Kenro Hirata,1 Osamu Nagano,2 Juntaro Matsuzaki,1 and Toshifumi Hibi1

1Division of Gastroenterology and Hepatology, Department of Internal Medicine
2Division of Gene Regulation, Institute for Advanced Medical Research
Keio University School of Medicine, Tokyo 160-8582, Japan
3Division of Microbiology, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan
4Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan
*Correspondence: hsuzuki@a6.keio.jp
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SUMMARY

Sustained expression of CagA, the type IV secretion effector of *Helicobacter pylori*, is closely associated with the development of gastric cancer. However, we observed that after translocation, CagA is degraded by autophagy and therefore short lived. Autophagy and CagA degradation are induced by the *H. pylori* vacuolating cytotoxin, VacA, which acted via decreasing intracellular glutathione (GSH) levels, causing reactive oxygen species (ROS) accumulation and Akt activation. Investigating this further, we found that CagA specifically accumulated in gastric cells expressing CD44, a cell-surface marker associated with cancer stem cells. The autophagic pathway in CD44-positive gastric cancer stem-like cells is suppressed because of their resistance to ROS, which is supported by increased intracellular GSH levels. These findings provide a molecular link between *H. pylori* and gastric carcinogenesis through the specific accumulation of CagA in gastric cancer stem-like cells.

INTRODUCTION

A possible link has been demonstrated between *Helicobacter pylori* infection and development of gastric cancer by epidemiological (Uemura et al., 2001) and animal studies (Suzuki et al., 2009). Although long-term *H. pylori* infections of the gastric mucosa might cause gastric cancer from severe inflammation, no direct molecular link was demonstrated until Hatakeyama reported that the transfer of *H. pylori*-derived CagA to epithelial cells through a bacterial type IV secretion system promotes an early event of gastric carcinogenesis (Hatakeyama, 2004). Ohnishi et al. (2008) also demonstrated that systemic expression of CagA in cagA-transgenic mice induced gastrointestinal malignancies, indicating the oncogenic potential of bacterial CagA in mammals. However, it was also reported that CagA, after translocation to gastric epithelial cells, does not persist for a long period (Ishikawa et al., 2009).

Recently, it has been reported that autophagy—a system for bulk protein degradation and the elimination of invaded pathogens (Deretic and Levine, 2009)—is induced in *H. pylori*-infected cells (Raju et al., 2012; Terebiznik et al., 2009). In the present study, we observed that intracellular CagA was degraded by autophagy induced by the accumulation of reactive oxygen species (ROS), suggesting that CagA may not promote carcinogenesis. Even if CagA escapes the autophagy system, intracellular CagA could never be transferred to daughter cells and would be lost after cell division. If translocated CagA does indeed trigger gastric carcinogenesis, it should be transferred to slow-cycling master-regulator cells and escape from autophagic degradation.

CD44 is a cell-surface marker associated with cancer stem cells in various tumors (Dalerba et al., 2007). Gastric cancer stem-like cells expressing the variant isoform of CD44 (CD44v9) suppress ROS accumulation by control of intracellular glutathione (GSH) levels by stabilizing xCT, a cystine transporter (Ishimoto et al., 2011). In the present report, we used CD44v9-expressing gastric cancer stem-like cells to study the ability of intracellular CagA to escape from autophagy and show a direct molecular link between *H. pylori*-derived CagA and gastric cancer stem-like cells.

RESULTS

CagA Is Degraded by Autophagy

To investigate the stability of intracellular CagA, we constructed an in vitro *H. pylori* infection model using a gastric cancer cell line (AGS). After 5 hr of *H. pylori* ATCC700392 infection, the AGS cells were incubated with kanamycin to kill extracellular bacteria. In AGS cells after *H. pylori* eradication, the levels of intracellular CagA and tyrosine-phosphorylated CagA (p-CagA) decreased in a time-dependent manner (Figure 1A). Therefore, intracellular CagA did not persist for a prolonged period in gastric epithelial cells and was soon degraded by host cell defenses.

To examine the mechanism of CagA degradation in host epithelial cells, we used proteasome inhibitors (MG132 and lactacystin [Lact]) and autophagy inhibitors (3-methyladenine [3MA] and wortmannin [Wort]). At 24 hr after *H. pylori* ATCC700392 eradication, while intracellular CagA and p-CagA levels were...
not affected by proteasome inhibitors (10 µM MG132 or 20 µM lactacystin [Lact]), they were significantly increased by exposure of the cells to autophagy inhibitors (5 mM 3MA or 50 nM wortmannin [Wort]), as compared with cells not exposed to these inhibitors (None) (Figure 1B). These results indicated that autophagy contributed to CagA degradation in host epithelial cells.

We then examined whether autophagy was activated within AGS cells after _H. pylori_ ATCC700392 eradication. A hallmark of autophagy is the carboxyl terminus modification of microtubule-associated protein light chain 3 (LC3), which becomes linked to phosphatidylethanolamine and associates with the autophagosomal membrane. LC3-I to LC3-II conversion was

Figure 1. Autophagy Induction Associated with Intracellular CagA Stability

(A) AGS cells infected with _H. pylori_ ATCC700392 for 5 hr were incubated at indicated times in a medium containing antibiotic to kill extracellular bacteria. Intracellular CagA and phosphorylated CagA (p-CagA) levels were quantified. Data represent the mean of three independent assays.

(B) AGS cells infected with _H. pylori_ for 5 hr were incubated in a medium containing antibiotic with or without a proteasome inhibitor (10 µM MG132 or 20 µM lactacystin [Lact]) or autophagy inhibitor (5 mM 3-methyladenine [3MA] or 50 nM wortmannin [Wort]) for 24 hr. Intracellular CagA and p-CagA levels were quantified. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01.

(C) AGS cells infected with _H. pylori_ ATCC700392 for 5 hr were incubated in a medium containing antibiotic for the indicated times, and intracellular CagA and LC3-I to LC3-II conversion were examined.

(D) After transfection of AGS cells with the EGFP-LC3B plasmid, cells infected with _H. pylori_ for 5 hr were incubated with a medium containing antibiotic for 24 hr with or without an autophagy inhibitor (10 µM MG132 or 20 µM Lact), and intracellular CagA was stained. EGFP-LC3B plasmid alone indicates the absence of _H. pylori_ infection. Scale bar = 25 µm.

(E) AGS cells infected with _H. pylori_ ATCC700392 for 5 hr were incubated with a medium containing antibiotic for 24 hr, and LysoTracker Red DND-99 staining was performed. Scale bar = 50 µm.
intracellular CagA levels were significantly decreased in the culture supernatant in a dose-dependent manner with LC3-I to LC3-II conversion (Figure 3A). In addition, in AGS cells at 24 hr after *H. pylori* eradication, intracellular CagA levels were significantly decreased, as compared to 15 hr after eradication; conversion of LC3-I to LC3-II was clearly evident (Figure 3B). Conversely, in WT-A10 cells exposed to *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) culture supernatant, there was no decrease in intracellular CagA levels, and no LC3-I to LC3-II conversion was detected (Figure S1A).

Moreover, in AGS cells at 24 hr after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) eradication, there was no decrease in intracellular CagA, and no LC3-I to LC3-II conversion was detected (Figure S1A). To investigate the function of CagA from each strain, we examined the tyrosine phosphorylation level of each CagA protein. All the CagA proteins were phosphorylated (Figure S1C), suggesting that those CagA species behaved similarly in delivered host cells.

In CagA-expressing WT-A10 cells incubated with m1VacA for 24 hr, a significant m1VacA-dependent decrease in intracellular CagA levels was observed along with LC3-I to LC3-II conversion (Figure 3C). Autophagy inhibitors (5 mM 3MA or 50 nM Wort) repressed the LC3-I to LC3-II conversion induced by m1VacA and significantly increased intracellular CagA levels (Figure 3D). In CagA-expressing WT-A10 cells incubated with m2VacA, intracellular CagA was not degraded and LC3-I to LC3-II conversion was not observed (Figure S1D). In addition, at 24 hr after *H. pylori* F57 (VacA-negative) eradication, there was a significant increase in CagA, as compared with cells infected with *H. pylori* ATCC700392 (s1m1VacA) (Figure S1E). The increase of intracellular CagA produced by *H. pylori* F57 (VacA-negative) was reduced by the addition of 60 nM m1VacA, in contrast to the addition of 60 nM m2VacA (Figure S1E). To evaluate the biological activity of VacA, we examined the vacuolation activity of m1VacA and m2VacA. Both proteins induced vacuolation in CagA-expressing WT-A10 cells in a dose-dependent manner, although m1VacA induced stronger vacuolation activity than m2VacA (Figure S1F). Our observations demonstrate that the autophagy responsible for CagA degradation is induced by m1VacA in gastric epithelial cells, independent of vacuolating cytotoxicity.

We recently found that low-density lipoprotein receptor-related protein-1 (LRP1) was one of the VacA receptors that...
mediate induction of autophagy (Yahiro et al., 2012). Then, to examine the relevance of LRP1 for the induction of autophagy-mediated CagA degradation, we constructed specific LRP1-knockdown AGS cells using small interfering RNAs (siRNAs) (Figure S1G). The LRP1 knockdown repressed the LC3-I to LC3-II conversion, resulting in the inhibition of CagA degradation (Figure S1H). This result indicates that LRP1 is required for the induction of autophagy-mediated CagA degradation in response to m1VacA. Next, to compare the binding ability of m1VacA and m2VacA, we incubated with an autophagy inhibitor (5 mM 3MA or 50 nM Wort) for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01.

(C) CagA-expressing WT-A10 cells were incubated with m1VacA for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *p < 0.05.

(D) CagA-expressing WT-A10 cells, stimulated by m1VacA, were incubated with an autophagy inhibitor (5 mM 3MA or 50 nM Wort) for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01. See also Figure S1.

**Figure 3. Autophagy, Causing CagA Degradation, Is Induced by m1VacA**

(A) CagA-expressing WT-A10 cells were stimulated with H. pylori ATCC700392 (s1m1VacA) culture supernatant, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *p < 0.05.

(B) AGS cells infected with H. pylori (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for 15 and 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; **p < 0.01.

(C) CagA-expressing WT-A10 cells were incubated with m1VacA for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01.

(D) CagA-expressing WT-A10 cells, stimulated by m1VacA, were incubated with an autophagy inhibitor (5 mM 3MA or 50 nM Wort) for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01. See also Figure S1.

We then examined the relationship between p53 downregulation and intracellular CagA stability. The specific
p53-knockdown using small interfering RNAs (siRNAs) accelerated LC3-I to LC3-II conversion, thereby enhancing CagA degradation in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection (Figure 4C). Moreover, in KATOIII cells, which are genetically deficient of p53 (p53/C0/C0/KATOIII cells), LC3-I to LC3-II conversion was clearly detected at 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), and intracellular CagA levels were significantly decreased, as compared with p53/C0/C0/KATOIII cells transfected with the WT p53 expression plasmid (Figure 4D). In addition, we examined the effect of nutlin-3—an inhibitor of MDM2-phosphorylation—on CagA stability. Treatment with 10 μM nutlin-3 repressed p53 downregulation and LC3-I to LC3-II conversion (Figure 4E), resulting in the inhibition of CagA degradation (Figure 4E). These results show that p53 downregulation, through the acceleration of MDM2-phosphorylation by m1VacA, induces autophagy, causing CagA degradation.

**ROS Accumulation Is Necessary for the Induction of Autophagy, Causing CagA Degradation**

An accumulation of intracellular ROS induces autophagy, and the generation of intracellular ROS is enhanced in gastric epithelial cells during *H. pylori* infection (Ding et al., 2007). We hypothesized that the enhanced generation of intracellular ROS participates in induction of autophagy, causing CagA...
degradation. AGS cells at 15 and 24 hr after the eradication of infected *H. pylori* were analyzed using fluorescence microscopy and flow cytometry after staining with CM-H$_2$DCFDA, an ROS-sensitive fluorescent probe. Hydrolyzed CM-H$_2$DCFDA is oxidized to dichlorofluorescin (DCF) by intracellular ROS (Suzuki et al., 1994). DCF fluorescence was apparent in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), as compared with AGS cells without *H. pylori* exposure (Figure 5A). The intensity of DCF fluorescence in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) was significantly increased, as compared to AGS cells without *H. pylori* exposure (Figure 5B). Conversely, in AGS cells after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or ss1 (s2m2VacA) infection, no increase in DCF fluorescence was observed (Figure S3A). These results show that the accumulation of intracellular ROS was enhanced during the induction of autophagy.

NADPH oxidase (NOX)-generated ROS is a key regulator of autophagy (Huang et al., 2009), while mitochondrial-superoxide (O$_2^-$) production is involved in the induction of autophagy (Scherz-Shouval and Elazar, 2007). To identify the source of enhanced ROS generation associated with the induction of autophagy through p53 downregulation, we examined the effects of an NOX inhibitor (acetovanillone), an MnSOD mimic compound (MnTMPyP), and an N-acetylcysteine (NAC). p53 downregulation was not inhibited by 250 µM acetovanillone or 20 µM MnTMPyP; therefore, LC3-I to LC3-II conversion was not repressed (Figure 5C). Conversely, p53 downregulation was inhibited by treatment with 10 mM NAC, and LC3-I to LC3-II conversion was repressed (Figure 5C). Moreover, intracellular CagA levels were significantly increased by treatment of AGS cells with 10 mM NAC at 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) (Figure S5D). These results show that the accumulation of intracellular ROS is necessary for induction of autophagy, causing CagA degradation, independent of NOX- and mitochondria-associated ROS generation.

Administration of NAC, a cysteine prodrug, replenishes intracellular GSH levels; therefore, NAC has been used to treat GSH deficiency (Atkuri et al., 2007). We hypothesized that the accumulation of intracellular ROS during the induction of autophagy was caused by decreased GSH levels. To prove this, we examined the change of GSH levels in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection. Intracellular GSH levels in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) were significantly decreased, as compared to AGS cells without *H. pylori* exposure (Figure 5E). Moreover, intracellular GSH levels in AGS and CagA-expressing WT-A10 cells were significantly decreased by m1VacA in a dose-dependent manner (Figure 5F). In AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), intracellular GSH was decreased, as compared to cells at 15 and 24 hr after eradication of *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or ss1 (s2m2VacA) (Figure S3B). Moreover, intracellular GSH levels in AGS and CagA-expressing WT-A10 cells were not decreased by treatment with m2VacA (Figure S3C). These results show that the accumulation of intracellular ROS associated with the induction of autophagy was induced by decreased GSH levels caused by m1VacA. Next, to provide the relevance of LRP1 in the reduction of intracellular GSH levels, we measured intracellular GSH levels in specific LRP1-knockdown AGS cells; they were significantly increased at 15 or 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), as compared with those in AGS cells transfected with control siRNA (Figure S3D). These results demonstrate that the binding of m1VacA to LRP1 is required for the reduction of intracellular GSH levels.

**Activation of the Akt Pathway Depends on the Accumulation of ROS for Autophagy Induction**

Phosphorylated Akt enhances the ubiquitination-promoting function of MDM2 by phosphorylation, resulting in p53 downregulation (Ogawara et al., 2002). In addition, exogenous and endogenous ROS enhance Akt phosphorylation (Dong-Yun et al., 2003). We hypothesized that the accumulation of intracellular ROS by decreased GSH levels enhances Akt phosphorylation, leading to the induction of autophagy through p53 downregulation by the activation of MDM2. To investigate this hypothesis, we examined Akt phosphorylation in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection. Although Akt expression was unaltered, the levels of phosphorylated Akt at Thr308 and Ser473 were significantly increased in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection (Figure 6A). To examine whether Akt phosphorylation depends on the accumulation of intracellular ROS, we examined the effect of NAC on Akt phosphorylation. Treatment with 10 mM NAC inhibited Akt phosphorylation at Ser473, but not at Thr308 (Figure 6A); therefore, Akt phosphorylation at Ser473 was dependent on accumulation of intracellular ROS after *H. pylori* ATCC700392 (s1m1VacA) infection. In addition, although Akt phosphorylation at Thr308 was increased in AGS cells after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or ss1 (s2m2VacA) infection, Akt phosphorylation at Ser473 was not increased (Figure S4A).

Moreover, Akt phosphorylation at Thr308 and Ser473 was not increased in CagA-expressing WT-A10 cells, suggesting that Akt phosphorylation was independent of intracellular CagA (Figure S4B).

**Accumulation of Translocated CagA in CD44v9-Expressing Gastric Cancer Stem-like Cells**

Intracellular CagA produced by m1VacA *H. pylori*, but not m2VacA *H. pylori*, was degraded by autophagy. Although some studies indicated that m1VacA *H. pylori* infection was at a greater risk of gastric cancer compared with m2VacA *H. pylori* infection (Basso et al., 2008; Miehlke et al., 2000), others have indicated that there is no correlation between virulence and the vacA
Figure 5. Reduced Intracellular GSH Levels Trigger Autophagy, Causing CagA Degradation

(A) AGS cells at 24 hr after \textit{H. pylori} (s1m1VacA) eradication were stained with CM-H$_2$DCFDA and MitoTracker Red FM and examined by fluorescence microscopy. Scale bar = 50 \textmu m.

(B) Flow cytometry of AGS cells at 15 and 24 hr after \textit{H. pylori} (s1m1VacA) eradication. H$_2$DCF fluorescence intensity was determined by using analysis software. Data represent the mean ± SD of three independent assays; **p < 0.01.

(C) H$_2$DCF fluorescence intensity was determined by using analysis software. Data represent the mean ± SD of three independent assays; **p < 0.01.

(D) CagA Accumulation in Gastric Cancer Stem Cells

(E) 15 hr after eradication

(F) 24 hr after eradication

Note: All data are expressed as mean ± SD. **p < 0.01.
CagA Accumulation in Gastric Cancer Stem Cells

genotype (Marshall et al., 1999; Yamaoka et al., 1998). In fact, m1 and m2 VacA strains are both observed in gastric cancer patients (Wang et al., 1998). From these reports, we hypothesized that there was a characteristic alteration in host cell associated with the inhibition of autophagy, which led to the accumulation of intracellular CagA. CD44v9-expressing gastric cancer cells are resistant to ROS, supported by increased intracellular GSH synthesis (Ishimoto et al., 2011). We hypothesized that accumulation of intracellular CagA resulted from inhibiting autophagy induction in CD44v9-expressing cells. To prove this hypothesis, we prepared MKN28 mutant cells by transfection of CD44 standard form (CD44s)- or CD44v9-expression vectors into CD44-negative MKN28 cells (Ishimoto et al., 2011). CD44s or CD44v9 expression in MKN28 cells was confirmed using flow cytometry (Figure S3A). Intracellular GSH levels in MKN28 cells expressing CD44s were significantly increased in comparison to MKN28 cells, whereas GSH levels in MKN28 cells expressing CD44v9 were increased in comparison to MKN28 cells expressing CD44s (Figure S5B). These results were consistent with previous observations that CD44v9 expression increases cellular GSH contents through the promotion of xCT-mediated cystine uptake, and CD44s expression increases cellular GSH levels through the maintenance of pentose phosphate pathway (PPP) flux and consequent NADPH production (Tamura et al., 2012). Intracellular GSH levels in MKN28 cells expressing CD44v9 were not decreased at 15 or 24 hr after the eradication of H. pylori ATCC700392 (s1m1VacA), in contrast to the reduction of GSH levels in MKN28 cells expressing CD44s (Figure 7A). Intracellular CagA levels were significantly increased in MKN28 cells expressing CD44v9, as compared with those in MKN28 cells expressing CD44s (Figure 7B). In addition, the increase of Akt and MDM2 phosphorylation and p53 degradation were not observed in MKN28 cells expressing CD44v9 (Figure 7B). As a result, LC3-I to LC3-II conversion was repressed (Figure 7B) and LysoTracker signals were markedly decreased in MKN28 cells expressing CD44v9 (Figure 7C). These results suggest that intracellular CagA accumulated in cells expressing CD44v9 through the inhibition of autophagy. We then examined the effect of sulfasalazine, a potent xCT inhibitor, on the stability of intracellular CagA in MKN28 cells expressing CD44v9. Intracellular CagA levels were decreased by the application of sulfasalazine in a dose-dependent fashion (Figure 7D). Moreover, Akt and MDM2 phosphorylation was significantly increased, and p53 downregulation was induced by treatment with sulfasalazine (Figure 7D), resulting in a significant increase in the conversion of LC3-I to LC3-II (Figure 7D).

To assess the effect of CD44v9-expression on the accumulation of intracellular CagA in human gastric adenocarcinoma, endoscopically resected early gastric cancer tissue from four patients (case 1: 62-year-old female, well-differentiated adenocarcinoma, H. pylori-positive; case 2: 68-year-old male, well-differentiated adenocarcinoma, H. pylori-positive; case 3: 72-year-old male, well-differentiated adenocarcinoma, H. pylori-positive; case 4: 78-year-old male, well-differentiated adenocarcinoma, H. pylori-positive), with written informed consent, was used. Remarkable intracellular CagA staining was detected with an anti-CagA antibody in the CD44v9-positive cells in each gastric adenocarcinoma (Figure 7E). It was confirmed using an anti-H. pylori antibody that these CagA-stained patterns were different from H. pylori-specific staining (not CagA) (data not shown), suggesting that only transported CagA, but not the H. pylori itself, was detected in CD44v9-expressing gastric cancer tissue. Endoscopically resected early gastric cancer tissue from an H. pylori-negative patient (80-year-old female, well-differentiated adenocarcinoma), with written informed consent, was used as a CagA-negative control. In this specimen, intracellular CagA staining was not detected in either CD44v9-positive or CD44v9-negative cells (Figure S5C). In addition, we detected the intracellular CagA-negative region in both CD44v9-positive and CD44v9-negative cells in endoscopically resected early gastric cancer tissue from a patient at 40 months after H. pylori eradication (72-year-old male, well-differentiated adenocarcinoma), with written informed consent (Figure S5D).

**DISCUSSION**

The present study reveals that the accumulation of intracellular CagA in CD44v9-expressing cancer stem-like cells is caused by the repression of autophagy. The autophagic pathway associated with CagA degradation is induced as follows: m1VacA-induced GSH deficiency via binding to LRP1 and then enhances Akt phosphorylation at Ser473. Activation of Akt induces MDM2-mediated p53 degradation through the ubiquitin-proteasome system and then activates autophagy.

Figures S1I and S3D indicated that binding of m1VacA to LRP1 was required for the reduction of intracellular GSH levels and the induction of autophagy, causing CagA degradation. In contrast,
the binding to LRP1 of m2VacA was not detectable by immunoprecipitation assay (Figure S1I). It has been reported that the mid-region of VacA has an important role in the binding of VacA to host cells (Cover and Blanke, 2005). Therefore, these findings suggest that the reason m2VacA could not induce autophagy was the lack of binding ability to LRP1, unlike m1VacA.

Our observations indicate that m1VacA reduces intracellular CagA levels via the induction of autophagy (Figure 3). Intracellular CagA deregulates SHP-2 and PAR1, which promote cell...
proliferation, thus causing loss of cell polarity (Saito et al., 2010). Therefore, an excess of intracellular CagA leads to cell damage that disturbs the attachment of bacteria to gastric epithelial cells. Recently, it was suggested that VacA can downregulate CagA-induced signal-transduction in gastric epithelial cells to some extent, thus minimizing the degree of cellular damage (Yokoyama et al., 2005). Therefore, this CagA degradation response to VacA is considered an important strategy for the long-term colonization of the gastric mucosa by H. pylori.

H. pylori ATCC700392–derived CagA contains the EPIYA-ABC motif, and CagA expressed in WT-A10 cells contains the EPIYA-ABCC motif derived from H. pylori NCTC11637. Our data showed that both of these types of CagA were degraded by autophagy induced by m1VacA (Figures 1, 2, and 3). These results suggest that CagA degradation by autophagy is not affected by differences in the EPIYA motif.

A number of studies demonstrated a link between CagA and gastric cancer development (Blaser et al., 1995; Huang et al., 2003). However, intracellular CagA was only detected in the gastric mucosa of H. pylori–infected patients with atrophic gastritis, and not in the gastric mucosa of patients with intestinal metaplasia or cancer (Yamazaki et al., 2003). Therefore, CagA was thought to play a causative role at a relatively early phase of gastric carcinogenesis. Our findings indicate that intracellular CagA is degraded by autophagy induced by the accumulation of intracellular ROS. Thus, even if CagA is translocated into a host cell, it does not persist for a long period. The accumulation of intracellular CagA is restricted to cells in which autophagy is suppressed. We demonstrated that intracellular CagA specifically accumulates in CD44v9-expressing human gastric cancer cells in which CagA degradation by autophagy has been suppressed by their resistance to ROS (Figure 7E). Thus, we show a direct molecular link between CagA and gastric cancer stem–like cells and suggest that the role of CagA in gastric carcinogenesis is not restricted to the early phase.

Chronic inflammation triggers the expression of CD44s (Ishimoto et al., 2010), suggesting that chronic severe inflammation after long-term H. pylori colonization induces CD44 expression in normal gastric epithelial cells. CD44-expressing cells have increased intracellular GSH levels, as compared to CD44-negative cells, by maintaining PPP flux and the consequent production of NADPH (Tamada et al., 2012) (Figure S5B), suggesting that CD44-positive cells are slightly resistant to oxidative stress. Conversely, CD44v9-expressing cells are more resistant to oxidative stress, compared with CD44s-expressing cells, by enhancing intracellular GSH levels through the promotion of xCT-mediated cystine uptake (Ishimoto et al., 2011) (Figure S5B). Thus, CagA specifically accumulates in CD44v9-expressing cells by escaping from the autophagy induced by ROS (Figure 7). Additionally, the mRNA expression of Igr5, one of the markers of stem cells besides CD44, was not detectable in the CD44- or CD44v9-expressing MKN28 cells (data not shown). Takaiishi et al. (2009) reported that the expression of other potential cell-surface markers did not show any correlation with CD44-expressing gastric cancer stem cells. From these findings, we conclude that the accumulation of intracellular CagA by inhibition of autophagy is a specific character of CD44v9-expressing gastric cancer stem-like cells because of their resistance of ROS, and it does not correlate with LGR5. A variety of CD44 isoforms are generated by alternative splicing of the pre-mRNA. CD44v9 is one of the CD44 isoforms and is expressed in gastric cancer stem cells (Mayer et al., 1993). In addition, H. pylori infection induced CD44v9 expression, suggesting that the development of cells that accumulate CagA can be caused by H. pylori infection (Fan et al., 1996). CD44v9 expression, which is regulated by epithelial spying regulatory protein 1, plays a functional role in carcinogenesis, differentiation, and metastasis (Yae et al., 2012). In addition, CagA oncogenic signals were maintained in CD44v9-expressing cancer stem-like cells in the present study. xCT, stabilized by CD44v9, plays an important role in maintaining intracellular redox balance (Patel et al., 2004). Sulfasalazine, a potent xCT inhibitor that has been used routinely for the treatment of inflammatory bowel disease and rheumatoid arthritis, suppresses metastasis of CD44v9-expressing lung cancer and inhibits hepatocellular carcinoma cell growth (Yae et al., 2012). In the present study, sulfasalazine also inhibited the accumulation of intracellular CagA in CD44v9-expressing cells by suppressing autophagy (Figure 7D), suggesting a prophylactic effect for sulfasalazine against CagA-dependent gastric cancer development, especially by targeting cancer stemness.

**EXPERIMENTAL PROCEDURES**

**In Vitro H. pylori Infection Model** Cells were incubated with s1m1VacA H. pylori, VacA-negative H. pylori, s1m2VacA H. pylori, and s2m2VacA H. pylori for 5 hr (multiplicity of infection of 50), and the cells were incubated with RPMI1640 culture medium containing 400 μg/ml kanamycin to kill extracellular bacteria with or without each inhibitor (MG132, Lact, 3MA, Wort, LY294002,nutlin-3, or sulfasalazine) or each antioxidant (acetoivanillone, MnTMPyP, or NAC) for the indicated incubation period (0, 3, 15, and 24 hr). The cells were then washed three times with PBS and harvested.

**Preparation of H. pylori Culture Supernatants** s1m1VacA, VacA-negative, s1m2VacA, and s2m2VacA H. pylori, normalized to an OD600 of 0.3, were transferred to cell culture medium (RPMI1640 medium supplemented with 10% FBS) and cultured for a further 15 hr. The supernatants were collected by centrifugation, passed through 0.22 μm filter units to remove any bacteria, and diluted with fresh medium.

**Immunohistochemistry** Tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm. The sections were deparaffined and then rehydrated in a graded series of ethanol solutions. For immunohistochemistry, the sections were washed in Tris-buffered saline with Tween-20 (TBS-T) and subjected to antigen retrieval by heating for 10 min at 105°C in Target Retrieval Solution (pH 9.0) (Dako, Tokyo). Nonspecific binding was blocked by Protein Block (Dako). The sections were incubated overnight at 4°C with primary antibody (see Supplemental Experimental Procedures). Immunoreactivity was detected using Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA), Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrogen), and Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen). The samples were examined using an FV10i fluorescence microscope (Olympus, Tokyo).

**Electron Immunocytochemistry** CagA-expressing WT-A10 cells stimulated with 100 nM rapamycin for 24 hr were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 80 min. The specimens were then dehydrated in a graded ethanol series and processed with the postembedding immunocytochemical technique using reduced osmium and acrylic resin. Immunogold labeling was performed by incubation with an anti-CagA goat polyclonal antibody (bk-300, 1:1000, Santa Cruz Biotechnology), followed by the addition of secondary antibodies conjugated...
Figure 7. Accumulation of Intracellular CagA Is Detected in CD44v9-Expressing Gastric Cancer Stem-like Cells

(A) MKN28 cells were transfected with the pRC/CMV-CD44s or pRC/CMV-CD44v expression plasmid; cells infected with *H. pylori* (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for 15 and 24 hr, and intracellular GSH levels were examined. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01; NS, not significant.
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Western Blotting
Total protein (10 μg/lane) was separated on a 4%–12% NuPAGE gradient gel (Invitrogen) and transferred to a PVDF membrane (Invitrogen), which was probed with each primary antibody, followed by reprobing with an anti-actin antibody (Sigma) as the loading control. Signal detection of the immunoreactive bands was facilitated by enhanced chemiluminescence using ECL plus (GE Healthcare, Piscataway, NJ). Signal quantification was performed using the ImageJ program (National Institutes of Health).

Statistical Analysis
All values are expressed as means ± SD. The statistical significance of differences between two groups was evaluated using Student’s t test. Analysis was performed using JSTAT statistical software (version 8.2). Statistical significance was accepted at p < 0.05, unless otherwise indicated.

Tissue Specimens
Human gastric adenocarcinoma tissue specimens were obtained from a 62-year-old female (case 1), a 68-year-old male (case 2), a 72-year-old male (case 3), a 78-year-old male (case 4), an 80-year-old female (H. pylori-negative patient), and a 72-year-old male (patient at 40 months after H. pylori eradication) who underwent endoscopic submucosal dissection at Keio University Hospital after receiving written informed consent before the procedure. Pathological diagnosis was well-differentiated adenocarcinoma according to the Japanese Gastric Cancer Association classification of gastric carcinoma (14th edition). The study protocol was approved by the ethics committees of Keio University School of Medicine and registered with the UMIN Clinical Trials Registry (UMIN000001057; http://www.umin.ac.jp/ctr/). The study was performed in accordance with the principles of the Declaration of Helsinki.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.10.014.

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(B) MKN28 cells were transfected with the pRC/CMV-CD44s or pRC/CMV-CD44v expression plasmid; cells infected with H. pylori (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for the indicated times, and intracellular CagA levels were quantified. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01. Akt and MDM2 phosphorylation, p53 expression, and LC3-II formation were quantified. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01, compared to each cell at 0 hr after eradication.

(C) Representative staining for LysoTracker Red DND-99 is shown. MKN28 cells were transfected with the pRC/CMV-CD44s or pRC/CMV-CD44v expression plasmid; cells infected with H. pylori (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for the indicated times, and the cells were stained using LysoTracker Red DND-99. Scale bar = 50 μm.

(D) MKN28 cells were transfected with the pRC/CMV-CD44v expression plasmid; cells infected with H. pylori ATCC700392 (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic with sulfasalazine for 24 hr; and intracellular CagA, pAkt (Ser473), pMDM2, p53, and LC3-II formation were examined. Data represent the mean ± SD of three independent assays; *p < 0.05, **p < 0.01.

(E) Immunostaining of CagA and CD44v9 in human gastric adenocarcinoma. Case 1, Case 2, Case 3, and Case 4 indicate each gastric adenocarcinoma tissue specimen from the four different patients. Red staining indicates intracellular CagA and green indicates CD44v9. Nuclei (blue) were stained with DAPI. Scale bar = 20 μm. See also Figure S5.
REFERENCES


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