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ORIGINAL ARTICLE

Rebamipide abolishes *Helicobacter pylori* CagA-induced phospholipase D1 expression via inhibition of NFκB and suppresses invasion of gastric cancer cells

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Infection with *cagA*-positive *Helicobacter pylori* is a risk factor for the development of severe gastritis and gastric cancer (GC). CagA protein is injected into gastric epithelial cells and deregulates a variety of cellular signaling molecules. Phospholipase D (PLD) is elevated in many different types of human cancers and has been implicated as a critical factor in inflammation and carcinogenesis. In this study, we show that infection with *cagA*-positive *H. pylori* in GC cells significantly induces PLD1 expression via CagA-dependent activation of nuclear factor κB (NFκB). Interestingly, the level of PLD1 protein and IκBα phosphorylation is aberrantly upregulated in *H. pylori*-infected human GC tissues. Infection with *cagA*-positive *H. pylori* and expression of CagA enhanced the binding of NFκB to the PLD1 promoter, and two functional NFκB-binding sites were identified within the PLD1 promoter. Rebamipide, a mucosal-protective antiulcer agent, abolished *H. pylori cagA*-induced PLD1 expression via inhibition of binding of NFκB to the PLD1 promoter, and also inhibited PLD activity. Moreover, rebamipide suppressed *H. pylori*-induced matrix metalloproteinase-9, interleukin-8 and activation-induced cytidine deaminase expression as well as invasion of GC cells through downregulation of PLD1. Our data suggest that *H. pylori cagA* targets PLD1 for invasion of GC cells, and rebamipide might contribute to the antitumorigenic effect of GC cells via inhibition of the *H. pylori cagA*-NFκB-PLD1 signaling pathway.

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INTRODUCTION

Helicobacter pylori causes chronic gastritis and peptic ulcer disease and has been defined as a carcinogen for gastric cancer (GC).[1] The *H. pylori cag*PAI (*cag* pathogenicity island) is a strain-specific locus that encodes a type IV secretion system that mediates the translocation of bacterial virulence factor *cagA (cytotoxin-associated gene A)* into host epithelial cells.[2] Based on the presence or absence of a gene termed *cagA*, *H. pylori* can be divided into *cagA*-positive and *cagA*-negative strains.[3] Infection by *cagA*-positive *H. pylori* strains is associated with an increased risk for GC compared with infection by *cagA*-negative strains,[4–6] implying an important role for CagA in *H. pylori*-associated gastric diseases.

*H. pylori cag*PAI is required for activation of nuclear factor κB (NFκB),[7] but NFκB activation by CagA still remains controversial. Several lines of evidence suggest that CagA is able to induce NFκB activation.[8,9] In contrast, it has been reported that CagA has only a partial or minor role in NFκB activation.[10–12] Thus, the exact function of *H. pylori cagA* in the activation of NFκB and the NFκB-dependent response is yet to be addressed. Upon injection into epithelial cells, intracellular CagA targets host proteins that regulate various cellular responses, including inflammation, actin-cytoskeletal rearrangements and cell scattering, all of which are believed to be involved in *H. pylori*-mediated gastric carcinogenesis.[2,13] Furthermore, transgenic expression of CagA in mice induces hyperplasia in the gastric mucosa (GM) and polyps in the glandular stomach, highlighting the oncogenic potential of CagA in GC.[14]

Phospholipase D (PLD), an enzyme that converts phospholipid to phosphatidic acid (PA), is a critical component in the regulation of cell growth.[15] Two mammalian isoforms of phosphatidylcholine-specific PLD, PLD1 and PLD2 have been identified and characterized.[16] PLD is upregulated at protein and/or activity levels in various cancers.[17] Although increased expression of PLD has an important role in cell proliferation and oncogenesis, the molecular mechanisms mediating PLD expression in cancer cells are only beginning to emerge. We have recently reported that NFκB mediates mitogen-induced PLD1 expression via its binding on the PLD1 promoter.[18,19] PA activates NFκB, a bridge between inflammation and cancer, which induces PLD1 expression through its binding on the PLD1 promoter,[20] suggesting cooperation of the link between PLD1 and NFκB in induction of inflammatory signals, which drives transformation. Inhibition of PLD suppresses growth factor-induced matrix metalloproteinase (MMP) expression and invasion.[20] These studies provide compelling evidence that the elevated activity and expression of PLD observed in cancer are functionally linked with oncogenic signals and tumorigenesis. However, molecular mechanisms of the *PLD* gene expression are not well-understood.

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Rebamipide (2-[4-chlorobenzoylamino]-3-[2(1H) quinolinon-4-yl] propionic acid; OPC-12759) is a gastro-protective agent used in the treatment of gastritis and gastric ulcer.[21] Rebamipide functions as an anti-inflammatory agent in both acute and chronic inflammation.[21] It exerts protective effects against GM inflammation induced by *H. pylori* by inhibiting neutrophil function.[22,23] Moreover, rebamipide has been reported to inhibit the growth of GC cells[19,24] and prevent dextran sulfate sodium-induced colitis in rats.[25] Although these studies shed some light on the actions of rebamipide, the exact targets and molecular mechanism of rebamipide in cancer cells are still unknown.

Here, we demonstrate that CagA from a subset of *H. pylori* strains are able to induce PLD1 expression through NFκB activation in GC cells. Rebamipide abolishes *H. pylori cagA*-induced PLD1 expression via suppression of NFκB transactivation and then inhibits MMP-9, interleukin (IL)-8 and activation-induced cytidine deaminase (AICDA) expression and invasion of GC cells induced by the *H. pylori cagA*-NFκB-PLD1 signaling pathway. The identification of PLD1 as a novel target of CagA might provide a better understanding of the role of CagA as an oncoprotein in gastritis and GC.

RESULTS

CagA is required for *H. pylori*-induced activation of NFκB

To investigate the role of CagA in *H. pylori*-induced NFκB activation, we compared *cagA*-positive and -negative *H. pylori* for their ability to induce transactivation of NFκB in gastric epithelial cancer cells. Infection of AGS and MKN-1 GC cells with four different *cagA*-positive *H. pylori* strains (NCTC11637, G27, 60190-*cagA*$^+$, 26695-*cagA*$^+$) significantly increased NFκB activation as analyzed by the NFκB luciferase reporter assay, whereas *cagA*-negative *H. pylori* isogenic mutants (60190-*cagA*$^-$ and 26695-*cagA*$^-$) induced slight changes in NFκB activity, which might be due to other factors that originated from *H. pylori* (Figure 1a). CagA may influence on quantity of intracellular bacteria consequently leading to different levels of NFκB activation. Thus, we tried to quantify an intracellular bacteria using a gentamicin protection assay to examine whether *H. pylori cagA*-mediated NFκB activation in GC cells was dependent on the quantity of intracellular bacteria. After gentamycin treatment, NCTC11637- and 26695-*cagA*$^+$ *H. pylori* strains displayed somewhat an enhanced intracellular survival in AGS and MKN-1 GC cells (Figure 1b), compared with that of G27-, 60190-*cagA*$^+$ *H. pylori* strains, which still significantly transactivated NFκB (Figure 1a). Taken as a whole, *cagA-H. pylori* isogenic mutant bacteria (60190, 26695) displayed an intracellular survival similar with *cagA*$^+$ *H. pylori* at 12 h of infection (Figure 1b), suggesting that alterate levels of NFκB activation is not due to different numbers of intracellular bacteria in CagA$^-$and CagA$^+$-infected cells. Therefore, it can be excluded that CagA influence on quantity of intracellular bacteria consequently leading to different levels of NFκB activation. Moreover, gentamicin treatment did not reduce NFκB transactivation in GC cells infected with *H. pylori* (Supplementary Figure 1). Ectopic expression of CagA also increased transactivation of NFκB in a dose-dependent manner in both AGS and MKN-1 cells (Figure 1c). Moreover, infection with NCTC11637 or G27 in AGS cells increased phosphorylation (Ser-32 and Ser-36) and degradation of IκBα (Figure 1d). In addition, infection with the *cagA*-positive *H. pylori* for 30 min increased translocation of NFκB from the cytoplasm to the nucleus, as analyzed by western blot. Recently, it has been reported that the CRPIA (conserved repeat responsible for phosphorylation independent activity) motif, especially 952 and 986 amino-acid residues of CRPIA in CagA was responsible for the activation of NFκB.[26] We further confirmed that unlike wild-type CagA, ΔCRPIA (R952/986A) and ΔPY (deletion of CRPIA motif) mutant constructs from 26695-*cagA*$^+$ *H. pylori* strain showed a marginal effect on the transactivation of NFκB (Supplementary Figure 2). These data suggest that CagA is involved in the activation of NFκB. We next examined the status of phosphorylated IκBα using immunohistochemistry in 54 human gastric tissue specimens, including *cagA*-positive *H. pylori*-infected GC ($n=14$), *H. pylori*-uninfected GC ($n=20$) and normal GM lacking *H. pylori* infection ($n=20$). CagA and p-IκBα from *H. pylori*-infected GC showed very strong immunoreactivity (Figure 1e). *H. pylori*-infected GC tissues showed the immunoreactivity of strong ($n=9$) and moderate ($n=5$) level of p-IκBα, respectively, whereas *H. pylori*-uninfected GC tissues showed moderate ($n=3$), weak ($n=8$) and negative ($n=9$) immunoreactivity of p-IκBα (Figure 1e). In contrast, there was no immunoreactivity of p-IκBα in any of normal GM tissues (Figure 1e). In addition, *H. pylori*-infected GC tissues showed the immunoreactivity of strong ($n=4$), moderate ($n=6$) and weak ($n=4$) level of nuclear NFκB, whereas *H. pylori*-uninfected GC tissues showed moderate ($n=2$), weak ($n=7$) and negative ($n=11$) immunoreactivity of nuclear NFκB. In contrast, there was no immunoreactivity of nuclear NFκB in any of normal GM tissues. Thus, it is suggested that the nuclear localization of NFκB is correlated with the phosphorylation level of IκBα in GC patient tissues (Figure 1e). Taken together, these results suggest that CagA mediates NFκB activation in both *cagA*-positive *H. pylori*-infected GC cell lines and patient tissues.

H. pylori cagA selectively induces PLD1 expression via NFκB

Recently, we reported that mitogen and growth factors selectively induce PLD1 but not PLD2 expression via activation of NFκB.[20,27] As IκBα is aberrantly phosphorylated in *cagA*-positive *H. pylori*-infected GC, we examined expression of PLD1 in *H. pylori*-infected or –uninfected patient GC or normal GM, using immunohistochemistry. Immunoreactivity for PLD1 in *H. pylori*-infected GC tissues showed immunoreactivity of strong ($n=12$) and moderate ($n=2$) level of PLD1, respectively, in 14 specimens (Figure 2a). Normal GM tissues showed immunoreactivity of weak ($n=2$) and negative ($n=18$) status of PLD1. *H. pylori*-uninfected GC showed immunoreactivity of moderate ($n=4$), weak ($n=12$) and negative ($n=4$) level of PLD1, respectively (Figure 2a). These data indicate that PLD1expression is aberrantly upregulated in *H. pylori*-infected GC and also increased in *H. pylori*-uninfected GC, compared with that of normal GM. To further examine whether or not *H. pylori* regulates expression of PLD isozymes, AGS and MKN-1 cells were infected with *H. pylori* strains. Infection with four kinds of *cagA*-positive *H. pylori* strains significantly induced the expression of PLD1, compared with PLD2 as analyzed by quantitative (q)-PCR (Figure 2b), whereas two kinds of *cagA*-negative *H. pylori* isogenic mutants induced very slight changes in PLD1 or PLD2 levels (Figure 2b). These phenomena were also observed in the promoter assay of PLD1 or PLD2 (Figure 2b). Other factors from *cagA*-negative *H. pylori* isogenic strains may contribute to slight increases in PLD expression. Tx30A, *cag*PAI-negative *H. pylori* strain, did not affect in PLD expression (data not shown). A little increase in PLD2 expression induced by infection with NCTC11637, *cagA*-positive *H. pylori* strain, might not be due to CagA-induced NFκB activation, as ectopic expression of NFκB did not affect PLD2 expression and *H. pylori*-induced PLD2 expression was not suppressed by NFκB inhibitor (PDTC) or expression of a dominant-negative IκBα (S32A/S36A; Figure 2c). However, NFκB increased PLD1 expression and NCTC11637-induced PLD1 expression was substantially abolished by PDTC and expression of dominant-negative IκBα, as analyzed by q-PCR and promoter assays (Figure 2c). We further investigated the question of whether or not NFκB has a role in *H. pylori*-stimulated PLD1 induction. Transfection of small interfering RNAs (siRNAs) for two kinds of NFκB (p65) inhibited NCTC11637-induced PLD1 expression, as analyzed by western blot and promoter assays (Figure 2d). Taken together, these results indicate that

CagA-mediated NFκB activationis involved in *H. pylori*-induced PLD1 expression.

CagA increases the binding of NFκB to the PLD1 promoter

We have reported that mitogen and growth factors increase the binding of NFκB to two NFκB-binding sites in the PLD1 promoter.[20,27] We examined whether the NFκB-binding sites are responsible for transcriptional activation of PLD1 induced by infection with *H. pylori* strains (Figure 3a) or ectopic expression of CagA constructs (Figure 3b). Mutation of the NFκB-binding sites (NFκB-1 and NFκB-2) significantly inhibited NCTC11637 and 26695-*cagA*$^{+}$, or CagA-induced PLD1 expression in AGS cells, but not 26695-*cagA*$^{-}$ (Figures 3a and b). Furthermore, infection

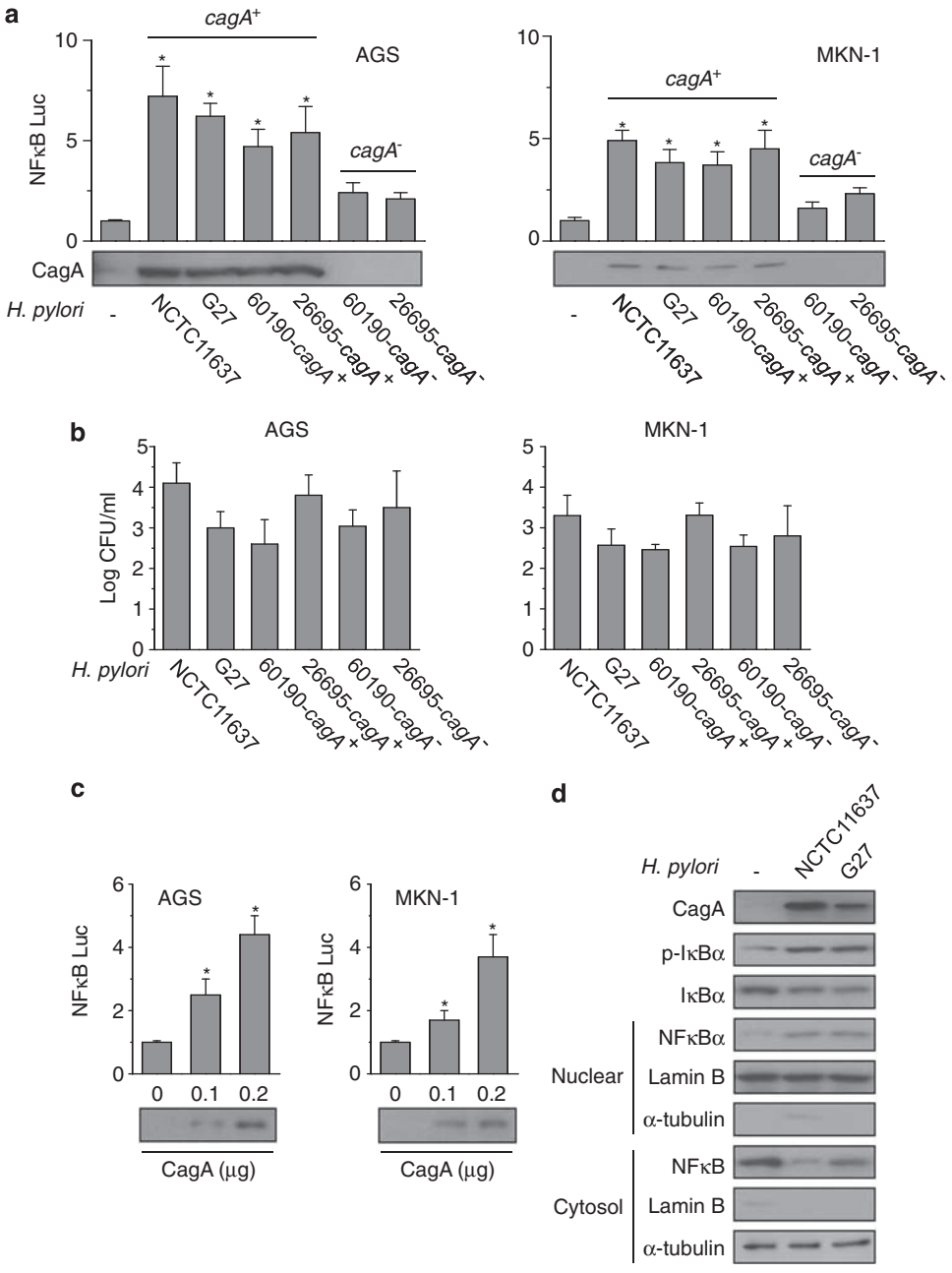


Figure 1. CagA is required for *H. pylori*-mediated activation of NFκB. (**a**) After AGS (left) and MKN-1 (right) GC cells were transfected with NFκB-dependent reporter plasmid pGL2-3X NFκB for 24 h, the cells were infected with various *H. pylori* strains for 12 h, and luciferase activity was measured. *$P<0.05$ versus no infection. Western blot was performed using antibody against CagA to validate infection efficiency. (**b**) After the GC cells were infected with various *H. pylori* strains for 12 h, intracellular survival (c.f.u.) of the bacteria was determined using the gentamicin protection assay. The bar represents the log value of the number of *H. pylori* survived in AGS and MKN-1 cells. (**c**) The cells were co-transfected with pGL2-3X NFκB and CagA for 36 h, and luciferase activity was measured. *$P<0.05$ versus mock. Data are presented as the mean ± s.d. of three independent experiments. (**d**) AGS cells were infected with *CagA*-positive *H. pylori* strains (NCTC11637 or G27) for 30 min, and cell lysates were analyzed by immunoblotting using the indicated antibodies. To detect nuclear localization of NFκB, the cells were fractionated into cytosol and nuclear fractions. As a control, lamin B or α-tubulin was used for nuclear and cytosolic marker, respectively. Data are representative of three independent experiments. (**e**) The images represent immunohistochemistry using the indicated antibodies and hematoxylin and eosin (H&E) staining in *H. pylori*-infected and –uninfected GC and normal GM (top). Intensity of immunoreactivity for p-IκBα or nuclear NFκB; +++, strong; ++, moderate; +, weak and −, no detection (bottom).

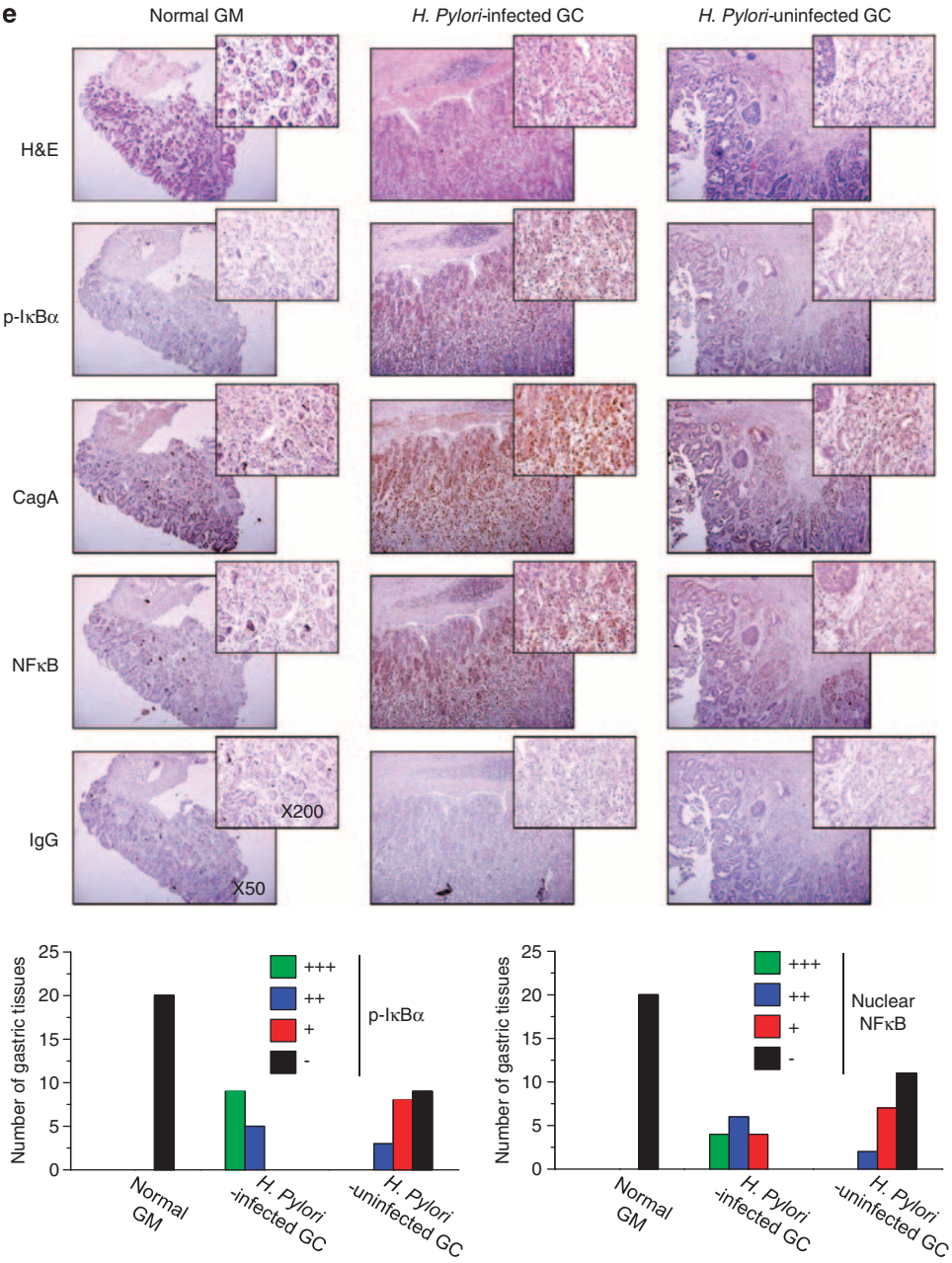


Figure 1. Continued.

with the 26695-*cagA*$^{+}$ in AGS cells significantly increased the binding activity of NFκB to the PLD1 promoter, whereas infection with the 26695-*cagA*$^{-}$-induced very small changes in the binding of NFκB to the PLD1 promoter, as analyzed by chromatin immunoprecipitation assays (Figure 3c). Moreover, ectopic expression of CagA (wild type) significantly promoted the binding of NFκB to the PLD1 promoter, compared with that of expression of the empty vector, *Δ*CRIPA or *Δ*PY (Figure 3d). These data implicate PLD1 as a direct transcriptional target of the *H. pylori cagA*-mediated NFκB signaling pathway.

Rebamipide suppresses *H. pylori* cagA-induced PLD1 expression via inhibition of the NFκB signaling pathway

Rebamipide, a mucosal-protective agent used for the treatment of gastritis and gastric ulcer, is known to inhibit GC cell growth via several signaling pathways.[19,24] To examine whether or not rebamipide regulates *H. pylori*-induced PLD1 expression, AGS cells were pretreated with rebamipide and infected with NCTC11637. Treatment with rebamipide for 20 h decreased the mRNA and protein levels of PLD1 induced by CagA (Figure 4a). Hummingbird phenotype induced by CagA in *H. pylori*-infected cells was not affected by treatment with rebamipide (Figure 4b). Rebamipide also suppressed both NCTC11637 and CagA-stimulated PLD1 promoter activity (Figure 4c). Moreover, rebamipide significantly inhibited both NCTC11637 and CagA-induced transactivation of NFκB (Figure 4c). In addition, rebamipide abolished the phosphorylation and degradation of IκBα induced by NCTC11637 infection and expression of CagA (Figure 4d). We further examined whether rebamipide regulates *H. pylori*-induced binding of NFκB to the PLD1 promoter. As shown in (Figure 4e), rebamipide remarkably inhibited the binding of NFκB to the PLD1 promoter, which was increased by infection with NCTC11637 as analyzed by chromatin immunoprecipitation assay. Furthermore, rebamipide significantly decreased the binding activity

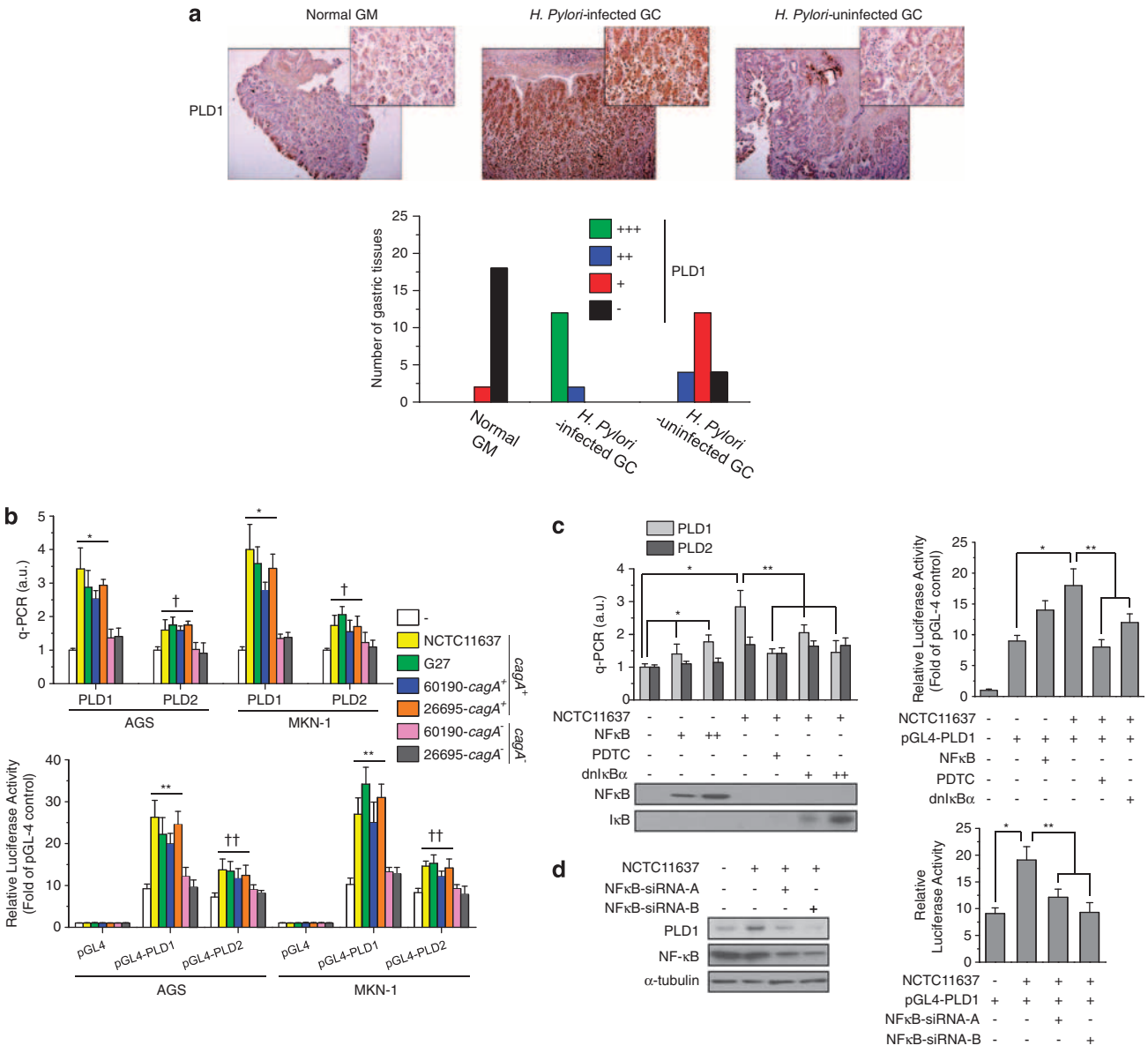


Figure 2. *H. pylori cagA* selectively induces PLD1 expression via NFκB. (**a**) The images represent immunohistochemistry of PLD1 in *H. pylori*-infected and -uninfected GC and normal GM (top). Intensity of immunoreactivity for PLD1; +++, strong; ++, moderate; +, weak and −, no detection (bottom). (**b**) AGS and MKN-1 cells were infected with the indicated *H. pylori* for 12 h. The expression of *PLD* was analyzed by q-PCR (top). After the cells were transfected with pGL4-PLD1 or pGL4-PLD2 for 30 h, the cells were infected with various *H. pylori*, and the luciferase activity was then measured (bottom). $^{*}P<0.05$; $^{**}P<0.05$; $^{\dagger}P<0.01$ and $^{\dagger\dagger}P<0.01$ versus no infection. (**c**) AGS cells were transfected with NFκB (1, 2 μg) or dominant negative (dn)IκBα (1, 2 μg) for 24 h, or preincubated with PDTC (50 μM) for 30 min and then infected with NCTC11637 for 12 h. The expression of *PLD* was examined using q-PCR (left). Under the same condition, the cells were transfected with pGL4-PLD1 and infected with NCTC11637. The relative luciferase activities were measured (right). $^{*}P<0.05$, $^{**}P<0.05$. (**d**) AGS cells were transfected with siRNAs for control or NFκB and then infected with NCTC11637. The lysates were immunoblotted with the indicated antibodies (left). Data are representative of three independent experiments. The cells were transfected with pGL4-PLD1 and siRNAs for NFκB and then infected with NCTC11637 for 12 h. The relative luciferase activities were measured (right). $^{*}P<0.05$, $^{**}P<0.05$. Data are presented as the mean ± s.d. of three independent experiments.

of NFκB to the PLD1 promoter in AGS cells expressing CagA (Figure 4e). These data suggest that rebamipide suppresses *cagA*-positive *H. pylori*- and CagA-induced PLD1 expression via the inhibition of NFκB transactivation.

Rebamipide inhibits *H. pylori* cagA-stimulated PLD activation

We examined whether *H. pylori*-induced PLD1 expression results in an increase of its enzymatic activity. Infection with two kinds of *cagA*-positive *H. pylori* in AGS cells significantly increased PLD activity compared with that of infection with *cagA*-negative *H. pylori* strains (Figure 5). Treatment with rebamipide significantly inhibited *cagA*-positive *H. pylori*-induced PLD activity in a dose-dependent manner. These data show that rebamipide inhibits *H. pylori cagA*-induced PLD activity.

Rebamipide suppresses *H. pylori*-induced MMP-9, IL-8 and AICDA expression and inhibits invasion of GC cells via downregulation of PLD1

MMPs, a family of tissue degrading enzymes, have an important role in cancer progression, and IL-8 has an important role in

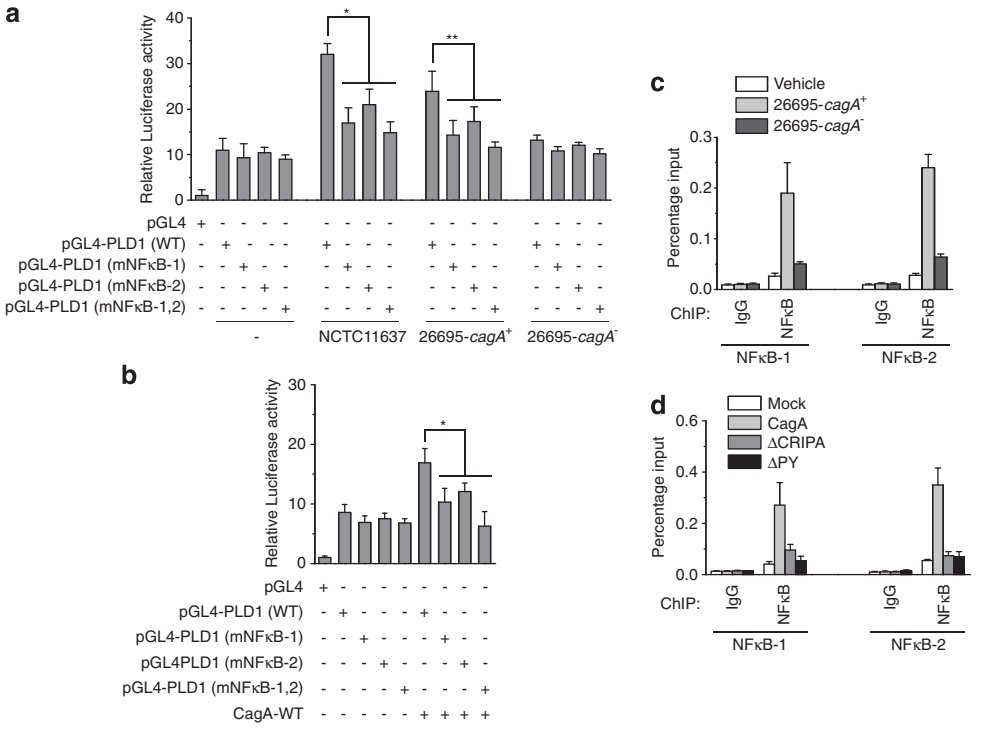


Figure 3. CagA promotes the binding activity of NFκB to the PLD1 promoter. (**a**) After AGS cells were transfected with wild-type (WT) pGL4-PLD1 or NFκB-binding site mutant pGL4-PLD1, the cells were infected with indicated *H. pylori* for12 h and then the luciferase activity was measured. $^{*}P<0.05$, $^{**}P<0.05$. (**b**) The cells were transfected with the indicated expression vectors and then the luciferase activity was measured. $^{*}P<0.05$, $^{**}P<0.05$. Data are presented as the mean ± s.d. of three independent experiments. (**c**, **d**) After AGS cells were infected with the indicated *H. pylori* (**c**) or transfected with indicated CagA constructs (**d**) chromatin immunoprecipitation (ChIP) assay was performed for binding of NFκB to the *PLD1* promoter. Data are representative of three independent experiments.

tumor growth, angiogenesis and metastasis. MMP-9 and IL-8 are upregulated in GC[28,29] and associated with an invasive phenotype.[29,30] *H. pylori* upregulates the expression of MMP-9 and IL-8 through activation of NFκB in GC cells.[9,31] Moreover, it was suggested that the aberrant AICDA expression caused by *cagA*-positive *H. pylori*-induced NFκB activation might be the mechanism of *p53* gene mutation in the GM during *H. pylor*-associated gastric carcinogenesis.[32] Thus, we examined whether rebamipide regulates *H. pylori*-induced MMP-9, IL-8 and AICDA expression. Infection with NCTC11637 significantly induced the expression of MMP-9, IL-8 and AICDA (Figure 6a). Rebamipide significantly abolished expression of the genes induced by infection of NCTC11637. Furthermore, ectopic expression of PLD1 rescued the expression of MMP-9, IL-8 and AICDA suppressed by rebamipide in AGS cells infected with NCTC11637 (Figure 6a). We further investigated the biological significance of rebamipide-induced PLD1 downregulation. Invasion of GC cells was remarkably increased by infection with NCTC11637, and *H. pylori*-induced invasion of AGS cells was significantly inhibited by treatment with rebamipide (Figure 6b). Moreover, NCTC11637-induced invasion inhibited by rebamipide was rescued by ectopic expression of PLD1 (Figure 6b), suggesting that rebamipide inhibits *cagA*-positive *H. pylori*-induced invasion via downregulation of PLD1. To further examine the correlation of PLD1 expression with invasion, immunocytochemistry was performed to analyze the level of PLD1 in the invaded GC cells. PLD1 expression in the invaded AGS cells was significantly increased by infection with NCTC11637, whereas treatment with rebamipide decreased *H. pylori*-induced PLD1-positive invaded AGS cell populations (Figure 6c). Furthermore, infection with NCTC11637 in AGS and MKN-1 cells, significantly increased high expression of PLD1 and MMP-9-double-positive cell population as analyzed by flow cytometry, whereas rebamipide inhibits *H. pylori*-induced high expression of PLD1 and MMP-9-double-positive cell population (Figure 6d). As a control, anti-PLD1 antibody recognized PLD1 (Supplementary Figure 3). These results suggest that *H. pylori*-induced PLD1 upregulation is intimately involved in the invasion of GC cells. In addition, apoptosis of AGS cells was significantly increased by infection with NCTC11637, and treatment with rebamipide further increased *H. pylori*-induced apoptotic effects (Figure 6e). Rebamipide-stimulated apoptosis was suppressed by ectopic expression of PLD1 in *H. pylori*-infected AGS cells, as analyzed by caspase 3 activity assay and immunoblot using antibody to cleaved caspase 3 (Figure 6e). It was reported that *H. pylori* can induce or prevent apoptosis of gastric epithelial cell.[33] Interestingly, knockdown of PLD1 remarkably promoted the apoptosis of AGS cells induced by infection with NCTC11637 (Figure 6f), suggesting that *H. pylori*-induced PLD1 expression might have a role in the regulation of apoptotic activity induced by *H. pylori*. Moreover, decrease in the cell viability by *H. pylori* was enhanced by rebamipide, whereas ectopically expressed PLD1 remarkably rescued the decreased cell viability (Figure 6g). Taken together, these results indicate that rebamipide suppresses invasion via downregulation of *H. pylori cagA*-induced PLD1 expression.

DISCUSSION

H. pylori induces active chronic gastric inflammation, which progresses to gastric adenocarcinoma in ~3% of affected individuals.[34] NFκB in human intestinal epithelial cells has a central role in regulating genes that govern the onset of mucosal inflammatory responses following microbial infection such as *H. pylori*.[2] The mammalian signaling pathways triggered by *H. pylori*

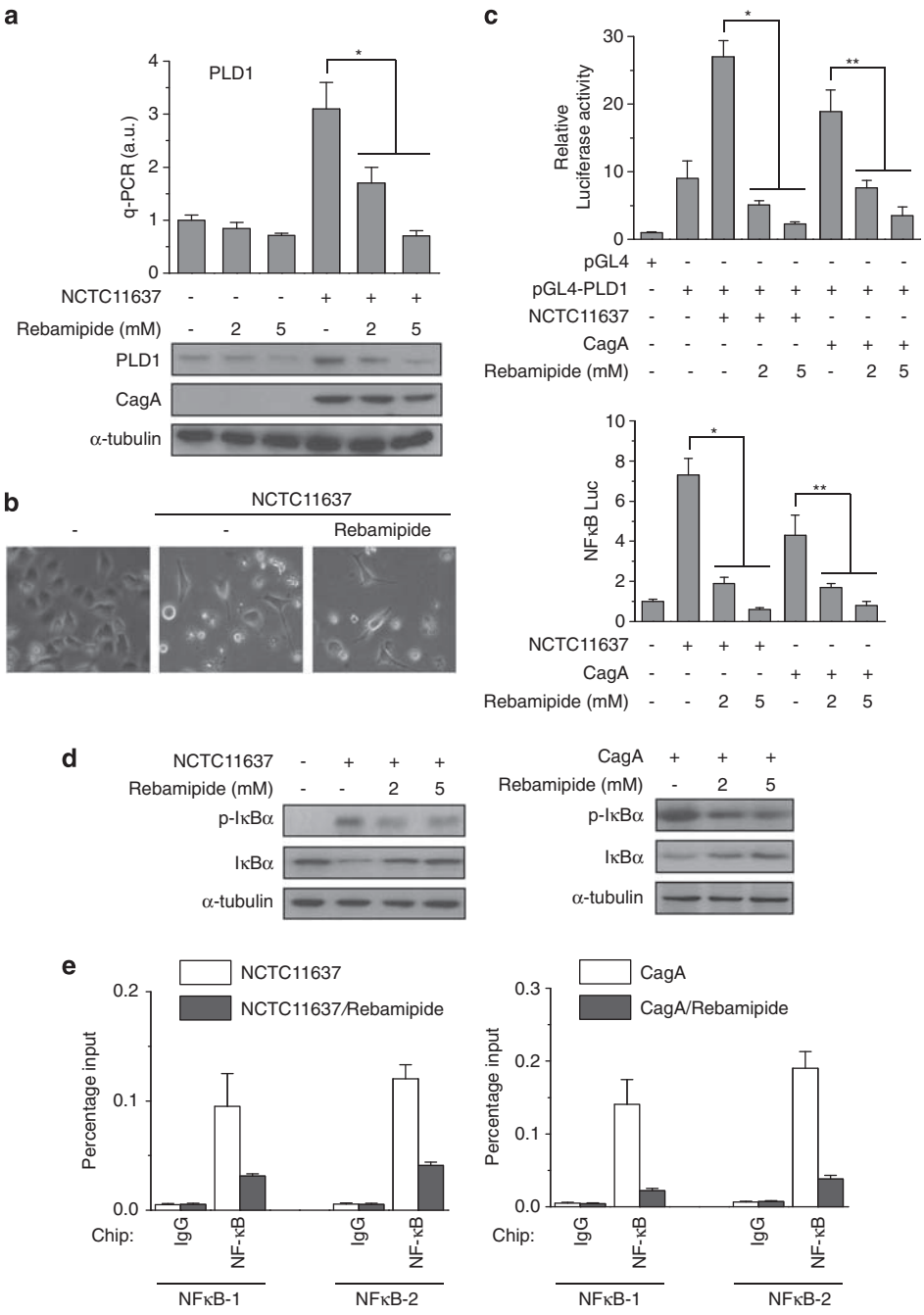


Figure 4. Rebamipide suppresses *H. pylori cagA*-induced PLD1 expression via inhibition of NFκB. (**a**) AGS cells were pretreated with rebamipide for 30 min and infected with NCTC11637 for 20 h. The expression of PLD1 was analyzed by q-PCR. The cell lysates were immunoblotted with the indicated antibodies. Data are representative of three independent experiments. (**b**) AGS cells were pretreated with rebamipide (5 mM) for 30 min and infected with NCTC11637 for 20 h. Hummingbird phenotype was analyzed by light microscopy. (**c**) After AGS cells were transfected with pGL4-PLD1 (top), pGL2-3X NFκB (bottom) and/or CagA, the cells were pretreated with rebamipide for 30 min and infected with NCTC11637 for 12 h. Luciferase activity was then measured. $^{*}P < 0.01$, $^{**}P < 0.05$. Data are presented as the mean ± s.d. of three independent experiments. (**d**) AGS cells were pretreated with rebamipide and infected with NCTC11637 for 30 min (left). CagA-expressing AGS cells were treated with rebamipide (right). The cell lysates were analyzed by immunoblotting using the indicated antibodies. (**e**) AGS cells were pretreated with 5 mM rebamipide and infected with NCTC11637 (left). AGS cells were transfected with CagA and treated with 5 mM rebamipide for 1 h (right). Chromatin immunoprecipitation (ChIP) assay was performed for binding of NFκB to the *PLD1* promoter and analyzed by q-PCR. Data are representative of four independent experiments.

remain largely unknown. In this study, we identified PLD1 as a new target of *H. pylori cagA*-dependent NFκB activation in human GC cells and tissues.

Multiple protein targets both upstream and downstream of PLD have been linked to propagation of survival signals and metastasis in cancer progression. Elevated expression and activity of PLD has been detected in various human tumors such as breast, colorectal, gastric, renal and papillary thyroid cancers, compared with adjacent non-neoplastic tissues.[16] Overexpression of the PLD isozyme has been reported to induce anchorage-independent

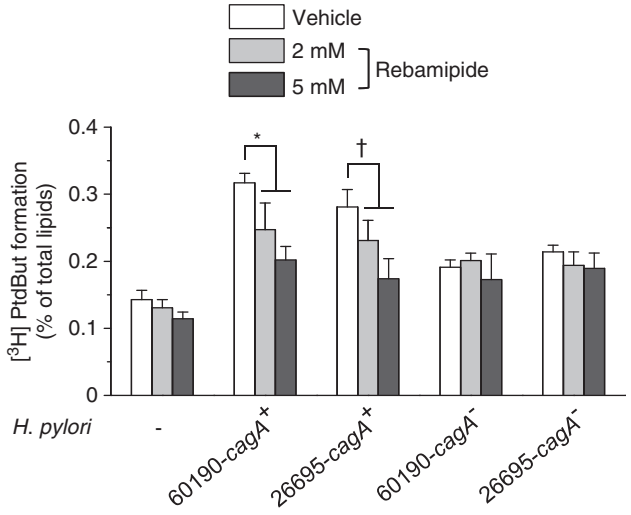


Figure 5. Rebamipide inhibits *H. pylori cagA*-stimulated PLD activity. AGS cells were labeled with [^{3}H] myristate and pretreated with the indicated concentrations of rebamipide and infected with the indicated *H. pylori*. PLD activity was measured as described in Materials and methods. $^*P<0.01$, $^\dagger P<0.01$. Data are presented as the mean ± s.d. of three independent experiments.

growth, tumor cell invasion and formation of metastases in syngeneic mice.[35,36] PLD1 tends to be overexpressed in tumors expressing high levels of cytokeratins 5/17, markers of basal-like tumors, which are frequently associated with poor prognosis.[37] A sustained and increased level of PLD can be deleterious and can contribute to the development of cancer. As of its important role in progression of cancer, PLD could be a target for cancer therapy. However, regulation of expression of PLD remains poorly understood. PLD-generated PA participates in downstream signal transduction from growth factor receptors via mitogen-activated protein kinase and the NFκB pathway.[27]

We recently observed that PA activates NFκB and increases the expression of PLD1, but not PLD2.[20] Thus, a positive feedback loop dependent on enzymatic activity of PLD isozymes selectively enhances expression of PLD1. At an early time, *H. pylori*-induced PLD activation generates PA, which acts as a second messenger to trigger downstream signaling cascades including NFκB activation, and induces transcriptional activation of PLD1 over the long term, and ultimately enhances invasion by upregulation of MMP-9, IL-8 and AICDA.

In this study, an important role for NFκB in *H. pylori*-induced PLD1 induction was demonstrated using inhibitors, siRNAs, chromatin immunoprecipitation assay and site-directed mutagenesis. We have identified two functional *H. pylori*-responsible NFκB-binding sites on the PLD1 promoter. *H. pylori* also increases PLD2 expression in a CagA-dependent mechanism. However, CagA-induced PLD2 expression is independent of NFκB as NFκB response elements are not in the PLD2 promoter and PLD2 expression was not affected by overexpression of NFκB.

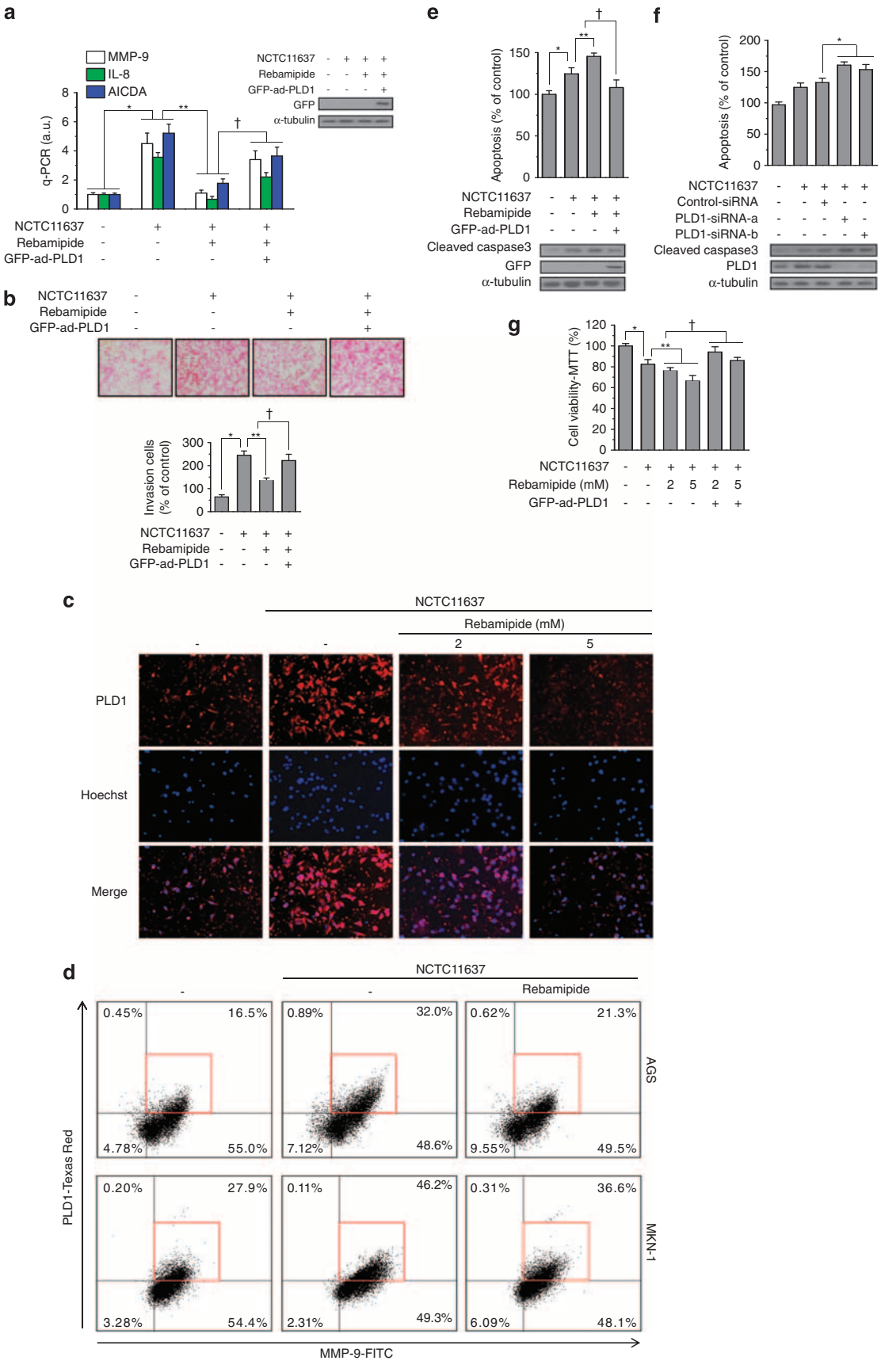
We reported that PLD isozymes, PLD1 and PLD2, act as novel targets and positive regulators of the Wnt/β-catenin/TCF pathway.[38–40] CagA interacts with E-cadherin and destabilizes E-cadherin/β-catenin complexes, leading to the activation of β-catenin, and induces intestinal transdifferentiation in gastric epithelial cells.[41] Thus, it ispossible that *H. pylori cagA*-induced PLD expression might be mediated via the β-catenin/TCF pathway.

CagA also associates with and activates the cytoplasmic protein tyrosine phosphatase SHP-2 or the tyrosine kinase c-Met receptor, resulting in cytoskeletal reorganization, cell elongation, scattering and induction of motogenic responses.[42,43] Moreover, *H. pylori cagA* leads to enhanced degradation of p53, a tumor suppressor, and thereby downregulates its activity in gastric epithelial cells.[44,45] Therefore, CagA through its association with various host proteins might be actively involved in *H. pylori*-mediated gastric carcinogenesis. Despite accumulating *in vitro* evidence for the transforming potential of CagA, the exact role of CagA in *in vivo* tumorigenesis had remained obscure. Infection of mice with *H. pylori* does not result in the development of gastric carcinoma, probably due to poor host adaptation. Whereas long-term infection with *H. pylori* can induce gastric carcinoma in Mongolian gerbils, it remains uncertain whether CagA has an active role in carcinogenesis in gerbils.[46,47] Accordingly, rodent models have so far failed to demonstrate a causal link between CagA and the development of neoplasms *in vivo*. To address this important question, transgenic mice systemically expressing CagA were generated.[14] Mice expressing CagA showed gastric epithelial hyperplasia, and some of them developed gastric polyps as well as adenocarcinomas of the stomach and small intestine.[14]

Interestingly, expression of PLD1 and p-IκBα is aberrantly upregulated in *cagA*-positive *H. pylori*-infected GC patient tissues, compared with those in *H. pylori*-uninfected GC. Considering a direct link between CagA and the PLD1 signaling pathway, it is suggested that *cagA*-positive *H. pylori*-induced NFκB activation might lead to risk of GC via upregulation of PLD1 expression. The mechanism might be attributed to the direct action of *H. pylori* infection by the introduction of bacterial macromolecules through the type IV secretion system encoded by *cag*PAI.[2,4]

Rebamipide, a mucosal-protective agent, has been used clinically for treatment of gastritis and peptic ulcers.[21] Clinical and experimental data demonstrate that rebamipide accelerates gastric ulcer healing, prevents ulcer relapse and protects GM against acute injury caused by various noxious agents.[24,48] One

Figure 6. Rebamipide abolishes *H. pylori*-induced invasion of GC cells through downregulation of PLD1. (**a**) AGS cells were infected with adenovirus for empty vector or PLD1 (green fluorescent protein (GFP)-ad-PLD1) and pretreated with rebamipide (5 mM) for 30 min and then infected with NCTC11637 for 20 h. The expression of MMP-9, IL-8 and AICDA were analyzed by q-PCR. $^*P<0.01$, $^{**}P<0.05$ and $^\dagger P<0.05$. Expression of GFP-PLD1 was analyzed by immunoblot. (**b**) AGS cells were infected with GFP-adenovirus for empty vector or PLD1 and seeded in Matrigel-coated invasion chambers (BD Biosciences, San Jose, CA, USA), and pretreated with rebamipide and then infected with NCTC11637. The cells invaded from the Matrigel-coated membranes to the other side were fixed, stained (left) and counted (right) under a microscope in five random fields at × 200. (**c**) AGS cells were seeded in Matrigel-coated invasion chambers and pretreated with rebamipide and then infected with NCTC11637. Staining of invaded cells was performed directly on the Matrigel membrane. The invaded cells on the membrane were incubated with anti-PLD1 antibody and Texas Red-labeled secondary antibody (Jackson Laboratory, Sacramento, CA, USA), and nuclei were stained with Hoechst. (**d**) The cells were pretreated with rebamipide (5 μM) and then infected with NCTC11637 for 20 h. Fluorescence-activated cell sorting analysis of PLD1 and MMP-9 expression was performed by staining of MMP-9-fluorescein isothiocyanate (FITC) and PLD1-Texas Red antibodies. (**e**) AGS cells were infected with adenovirus for empty vector or PLD1 and pretreated with rebamipide (5 mM) for 30 min and infected with NCTC11637 for 20 h, and then analyzed by caspase 3 activity assay and immunoblotting using the antibody to cleaved caspase 3. (**f**) AGS cells were transfected with siRNAs for PLD1 and infected with NCTC11637 for 24 h, and then analyzed by caspase 3 activity assay and immunoblotting using the cleaved caspase 3 antibody. (**g**) AGS cells were infected with adenovirus for empty vector or PLD1 and pretreated with rebamipide for 30 min and infected with NCTC11637 for 24 h, and then analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based cell viability assay. $^*P<0.05$, $^{**}P<0.05$ and $^\dagger P<0.05$. Data are presented as the mean ± s.d. of three independent experiments.


a
MMP-9
IL-8
AICDA
q-PCR (a.u.)
NCTC11637
Rebamipide
GFP-ad-PLD1
GFP
α-tubulin
b
Invasion cells (% of control)
c
NCTC11637
Rebamipide (mM)
PLD1
Hoechst
Merge
d
NCTC11637
Rebamipide
AGS
MKN-1
PLD1-Texas Red
MMP-9-FITC
0.45%
16.5%
4.78%
55.0%
0.89%
32.0%
7.12%
48.6%
0.62%
21.3%
9.55%
49.5%
0.20%
27.9%
3.28%
54.4%
0.11%
46.2%
2.31%
49.3%
0.31%
36.6%
6.09%
48.1%
e
Apoptosis (% of control)
Cleaved caspase3
f
Control-siRNA
PLD1-siRNA-a
PLD1-siRNA-b
PLD1
g
Cell viability-MTT (%)

study indicates that rebamipide may suppress the incidence and number of duodenal cancers induced by *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine in mice.[49] However, the effect of rebamipide on GC remains largely unknown.

In this study, we demonstrate that rebamipide suppresses *cagA*-positive *H. pylori*- or CagA-induced PLD1 expression via inhibition of NFκB. *H. pylori* may induce invasiveness of GC cells through induction of genes related to invasion, such as MMP-9 and IL-8.[50] It is suggested that rebamipide-induced PLD1 repression is involved in downregulation of MMP-9 and IL-8 genes, which is increased during infection of *H. pylori*. Infection with *cagA*-positive *H. pylori* has been reported to increase the expression of AICDA, a key enzyme for somatic hypermutation in human gastric epithelial cells, leading to multiple mutations in the *p53* tumor suppressor gene, which is associated with GC.[32] Here, we showed that rebamipide-induced PLD1 downregulation causes suppression of the AICDA expression increased by infection with *H. pylori*. Thus, it is tempting to speculate that *cagA*-positive *H. pylori*-induced PLD1 expression might lead to the generation of somatic mutations in the host genome, such as p53 mutations, in gastric epithelium via upregulation of AICDA expression.

H. pylori has been associated with both increased and reduced levels of apoptosis in the gastric epithelium, depending on the human population studied.[33] *H. pylori*-stimulated apoptosis of gastric epithelial cells.[51,52] It is known that *H. pylori* stimulates apoptosis by increasing the expression of cell-surface receptor Fas, Fas ligand and proapoptotic protein Bak.[52–54] A previous study also showed that apoptotic inducer was increased within *cagA*-positive *H. pylori*-colonized mucosa.[51] Here, we showed that rebamipide stimulated the apoptosis induced by infection with *H. pylori*, and ectopically expressed PLD1 suppressed *H. pylori*/rebamipide-induced apoptosis. Moreover, depletion of PLD1 promoted *H. pylori*-induced apoptotic activity, suggesting that *H. pylori*-induced PLD1 expression might have a role in the modulation of *H. pylori*-mediated apoptotic activity.

In summary, we demonstrate PLD1 as a new target of *H. pylori cagA* in GC, and rebamipide as a potential drug for GC theraphy via inhibition of *H. pylori*-mediated PLD1 induction and invasion. Overall, this study provides a rationale for further testing of the anticancer properties of rebamipide.

MATERIALS AND METHODS

Cell lines and compounds

Human GC cells (AGS, MKN-1) were purchased from Korea Cell Line Bank (Seoul, Korea) and maintained in RPMI-1640 with 10% heat-inactivated fetal bovine serum with 1% antibiotic-antimycotic (Gibco BRL, Grand Island, NY, USA) and 15 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). Rebamipide was kindly provided by Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan).

Bacterial strains

The *cagA*-positive *H. pylori* strains (NCTC11637, 60190-*cagA*$^+$, G27, 26695-*cagA*$^+$), *cagA*-negative isogenic mutants (60190-*cagA*$^-$,26695-*cagA*$^-$) and *cag*PAI-negative *H. pylori* strains (Tx30A) have been described previously.[55–57] All *H. pylori* strains were cultured according to standard procedures[58] and kindly provided by Dr Kwang-Ho Rhee (HpKTCC, Gyeongsang National University, Jin-ju, Korea).

Plasmids and siRNA

A dominant-negative -IκBα (S32A, S36A IκBα) and pGL2-3X NFκB containing a triple tandem repeat of an NFκB-binding motif were used. NCTC11637-*H. pylori* strain *CagA* gene was cloned into HA-tagged pCMV vector. The full-length of CagA, ΔCRPIA (R952/986A) and ΔPY (deletion of CRPIA motif, residues 871–1026) mutant constructs from 26695-*cagA*$^+$ *H. pylori* strain were kindly provided by Dr Masato Suzuki (The University of Tokyo, Tokyo, Japan). The human PLD1 and PLD2 promoter luciferase reporter genes (*pGL4-PLD Luc*) were described previously.[18] SiRNAs for control and NFκB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two kinds of siRNA sequences for PLDs were described previously.[27] Recombinant adenovirus of empty vector and PLD1 were kindly provided by Dr In-Kyu Lee (Kyungbuk National University, Daegu, Korea). Infections were performed at a multiplicity of infection of 50 in complete medium. After 18 h of incubation in the presence of viral particles, the medium was changed and cells were cultured for 24 h. Under these conditions, $>80\%$ of the cells expressed green fluorescent protein.

RNA isolation and quantitative reverse transcription–PCR

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using AMV-RTase (Promega, Madison, WI, USA), and PCR reactions were performed. Real-time q-PCR reactions were performed in triplicate, and final results were found by using a relative standard curve. For qPCR primer sequences, see Supplementary Table I.

Tissue samples

Specimens of human GM were endoscopically obtained from *H. pylori*-infected patients with GC ($n=14$) and *H. pylori*-uninfected patients with GC ($n=20$) at the Gyeongsang National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. All samples were obtained with informed consent under Institutional Review Board-approved protocols. All pathological samples from patients with GC were evaluated by two experienced pathologist according to the updated sydney system.[59] The biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin stain.

Statistical analysis

Data are mean ± s.d. Data were analyzed by the Student's *t*-test and $P<0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)