Balance between Polyoma Enhancing Activator 3 and Activator Protein 1 Regulates *Helicobacter pylori*–Stimulated Matrix Metalloproteinase 1 Expression

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Abstract

*Helicobacter pylori* infection and elevated expression of tissue matrix metalloproteinase 1 (MMP-1) are both associated with gastric cancer. We investigated the regulation of MMP-1 expression during *H. pylori* infection. Real-time reverse transcription-PCR was used to examine mucosal MMP-1 mRNA levels in 55 patients with gastric cancers and 61 control patients. Increased MMP-1 mRNA levels in the gastric mucosa and epithelial cells were observed in *H. pylori* infections in which both the cag pathogenicity island (PAI) and outer inflammatory protein A (OipA) were expressed. The combined induction of c-fos, c-jun, and polyoma enhancing activator-3 (pea-3) by *H. pylori* caused maximal increase in MMP-1 expression. Activation of the MMP-1 promoter by *H. pylori* involved occupation of the activator protein 1 (AP-1) sites at −72 and −181 and, surprisingly, vacancy of the −88 PEA-3 site. Electrophoretic mobility shift, supershift, and chromatin immunoprecipitation assays showed increased binding of c-Fos and c-Jun to the AP-1 site and decreased PEA-3 binding to the −88 site during *H. pylori* infection. Importantly, during wild-type *H. pylori* infection, we detected increased PEA-3 binding to the −72AP-1 site and decreased PEA-3 binding to the −88 PEA-3 site. However, during infection with the cag PAI and OipA mutants, PEA-3 binding to the −88 site was detected. MMP-1 and pea-3 activities are increased in gastric cancers. Maximal activation of MMP-1 transcription requires the cag PAI and OipA, which regulate AP-1 and PEA-3 binding. Thus, cag PAI and OipA provide a possible link between bacterial virulence factors and important host factors related to disease pathogenesis. (Cancer Res 2006; 66(10): 5111-20)

Introduction

Approximately 20% of all *Helicobacter pylori* infections lead to peptic ulcer disease or gastric cancer. The risk of these clinically significant outcomes is thought to vary depending on interactions between the host, the environment, and the bacterial factors. Two *H. pylori* virulence factors proven to be associated with increased mucosal inflammation and increased risk of peptic ulcer disease and gastric cancer are the cag pathogenicity island (PAI) and the outer inflammatory protein A (OipA; refs. 1, 2). The cag PAI is a 40-kb genome segment that encodes for ~30 genes (1). OipA is an outer membrane protein of which the expression is transcriptionally regulated by a slipped-strand mispairing of the CT dinucleotide repeats in the 5′ region of the gene (2, 3). The majority of cag PAI-positive bacterial isolates are also OipA positive (4).

Matrix metalloproteinases (MMP) are zinc-containing endopeptidases that degrade extracellular matrix components and are involved in morphogenesis and the remodeling of organs (5, 6). MMP-1 is an interstitial collagenase able to degrade type I collagen, the main extracellular matrix component of gastric mucosa (7). MMP-1 is present in gastric epithelial cells of human gastric mucosa (8) and its expression is elevated in patients with gastric cancers (9–12). A negative correlation between the expression of MMP-1 and survival and/or metastatic potential of gastric cancer has been described (9–12). Whereas several studies have confirmed the relationship between *H. pylori* infection and increased gastric mucosal MMP-1 (13–15), the relationship between gastric mucosal MMP-1 expression and the cag PAI and/or OipA virulence factors has not been examined. Enhanced MMP-1 expression has also been reported in *H. pylori* infection of AGS cells, a gastric epithelial cancer cell line (7, 16), as well as in human gastric wall fibroblasts (14).

The mechanism(s) by which *H. pylori* induces MMP-1 expression in gastric cells was examined in a recent study focused on the roles of mitogen-activated protein kinase (MAPK) on MMP-1 expression in AGS cells (15). The expression of MMP-1 was shown to require the co-operation of two oncogenic transcription factors, activator protein 1 (AP-1), and polyoma enhancing activator 3 (PEA-3; refs. 17–20). However, the extent of MMP-1 expression was stimulus dependent (21). AP-1 transcription factors comprise a ubiquitously expressed family of proteins including the Jun and Fos proto-oncoproteins (22). Previous studies have shown that *H. pylori* induces the expression of c-jun and c-fos (23–26) and causes the transactivation of AP-1 binding sites in several promoters, including interleukin (IL)-6 and IL-8 (27, 28). Therefore, we hypothesize that *H. pylori* infection also leads to the transactivation of AP-1 sites in the MMP-1 promoter. Whereas the function(s) of PEA-3 is still unclear, the overexpression of PEA-3 has been reported in various cancer cells including gastric cancer (29). The role of PEA-3 in *H. pylori* infection has not previously been investigated.

In the present study, we examined the transcriptional regulation of MMP-1 in response to *H. pylori* infection. Furthermore, the role of the *H. pylori* virulence factors, the cag PAI and OipA, on this regulation was examined. We also investigated possible associations between *H. pylori* infection and MMP-1 expression levels in human gastric mucosa.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Yoshio Yamaoka, Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center (111D), Room 3A-320, 2002 Holcombe Boulevard, Houston, TX 77030. Phone: 713-794-7597; Fax: 713-795-4471; E-mail: yysamaoka@bcm.tmc.edu.

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Gastric epithelial cells. Three human gastric epithelial cancer cell lines were used. MKN45 cells were obtained from Riken Cell Bank (Tsukuba, Japan). AGS cells were obtained from American Type Culture Collection (Manassas, VA) and SNU638 cells were a gift from Dr. Antonia R. Sepulveda (Department of Pathology, University of Pittsburgh, Pittsburgh, PA). Cells were routinely maintained as previously described (27). In addition, primary gastric epithelial cells were isolated enzymatically from non-cancer-containing biopsies of adult human stomachs as previously described (27).

H. pylori preparation. H. pylori 272G7F4 (a gift from Dr. Masafumi Nakao, Takeda Chemical Industries, Ltd., Osaka, Japan), its isogenic oipA mutant, and the whole cag PAI-deleted mutant were used (2, 30). We used a multiplicity of infection (MOI) of 100 to eliminate possible confounding effects of reduced adherence by the oipA mutants (28, 29). To avoid the influence of serum, epithelial cells were serum starved for 16 hours before and during treatment with H. pylori, without H. pylori (negative control), or with phospholipid 12-myristate 13-acetate (PMA; 10 ng/mL; positive control).

In some experiments, heat-killed H. pylori were used at the same MOI (27) or the same concentrations of live bacteria were added to the upper well of a transwell plate ( Falcon, Lincoln Park, NJ) whereas the lower well contained subconfluent epithelial cells. In some experiments, gastric cells were pretreated (30 minutes before treatment) with U0126 [a specific inhibitor of MAPK/extracellular signal-regulated kinase (ERK)] (1/2 MEK1/2), SB203580 (a specific inhibitor of p38), or SP600125 [a specific inhibitor of c-Jun NH2-terminal kinase (JNK)]. All of these inhibitors were purchased from Calbiochem (San Diego, CA). In some experiments, gastric cells were transfected with 50 pmol/L of validated SMARTpool for ERK, JNK, or p38 small interfering RNA (siRNA; Dharmacon Research, Denver, CO), c-Jun, c-Fos, or p53 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), or a scrambled siRNA negative control (Dharmacon Research) using Lipoctetamine 2000 reagent (Invitrogen, San Diego, CA).

cDNA array analyses. Total RNA was extracted from infected and uninfected MKN45 cells by using Trizol reagent (Invitrogen) and 5 μg of RNA were used to convert total RNA into 32P-labeled first-strand cDNA. Labeled cDNA samples were hybridized according to the recommendations of the manufacturer to the Human Cytokine Array (GA001), which consists of 375 different cloned cDNAs (R&D Systems, Minneapolis, MN).

Quantitation of mRNA by reverse transcription-PCR. Total RNA was extracted from treated and untreated gastric cells by using Trizol reagent (Invitrogen) and converted cDNA that was then used for real-time reverse transcription-PCR (RT-PCR). The oligonucleotide primers and probes for MMP-1, PAI, and GAPDH mRNA were as previously described (31, 32). The PCR products were cloned into the plasmid pBluescript (Novagen, Madison, WI) and used as a standard. Absolute quantitative real-time RT-PCR using TaqMan probe was done using ABI Prism 7300 Sequence-Detection System (Applied Biosystems). The expression levels were expressed as 1,000 × target mRNA / GAPDH mRNA. The abundances of c-fos mRNA and c-jun mRNA were measured by SYBR green I-based quantitative real-time RT-PCR as previously described (33) and the expression levels normalized to the levels of GAPDH mRNA were expressed as fold induction relative to the uninfected control.

Plasmids. The full-size human MMP-1 promoter reporter gene construct −437 to 299 MmP1Luc contains the firefly luciferase gene. Various 5’ deletion constructs and site-directed mutant plasmids with mutations in three sites [AP-1 at −72 bp (−72AP-1), PAI-3 at −88 bp (−88PAI-3), and AP-1 at −181 bp (−181AP-1)] have previously been described (21). The plasmid pCMV-RacN19, which expresses a dominant-negative mutant form of the Raf protein, was purchased from Clontech (Palo Alto, CA). The pCMV-RhoA/19, pCMV-RacN17, and pCMV-RasN17 expression plasmids, which express the dominant-negative mutant forms of RhoA, Rac, and Ras proteins, respectively, were previously described (21).

Transfection. Each luciferase reporter vector was transiently transfected into logarithmically growing gastric cancer cells using Lipofectamine 2000 reagent (Invitrogen). The luciferase assays were done using the Dual-Luciferase reporter assay system according to the instructions of the manufacturer (Promega, Madison, WI). Five, 10, and 18 hours after stimulation with H. pylori or PMA, the cells were harvested and lysed using passive lysis buffer (Promega), and the lysates were assayed for luciferase activity. Normalized luciferase activity is presented as firefly luciferase activity/Renilla luciferase activity. We also present the luciferase activity as fold increase of luciferase activity in treated cells relative to uninfected, or mock-treated, controls.

Electrophoretic mobility shift assay. Nuclear extracts of treated and untreated gastric cancer cells were prepared using hypotonic/nonionic detergent lysis (34). After extraction, equal amounts of nuclear proteins were allowed to bind to duplex oligonucleotides corresponding to the −181AP-1, −72AP-1, and −88PAI-3 sites. Electrophoretic mobility shift assay (EMSA) was done using standard methods as previously described (34). The sequences of the oligonucleotides used for the MMP-1 promoter-specific gel shift were as follows: 5’-CTTCTTTGGAATTTATGCAGTGCACAG-3’ for −181AP-1 site, 5’-GATCATATAACAGTATTGACAGACACTT-3’ for −72AP-1 site, and 5’-TAGCTTAACAGGAGTGTTATAGAAGAGGCA-3’ for −88PAI-3 site. The sequences of the mutated oligonucleotides used for the MMP-1 promoter-specific gel shifts were as follows: 5’-CTTGTGTAGATTACATTACATTGCAGTGCACAG-3’ for −181AP-1 site (TTAATCA to TTAATGA), 5’-GATCATATAACAGTATTGACAGACACTT-3’ for −72AP-1 site (TTAATCA to TTAATGA), and 5’-TAGCTTAACAGGAGTGTTATAGAAGAGGCA-3’ for −88PAI-3 site (TTAATCA to TTAATGA); only forward primers are presented. For semi-quantitation of the binding, the image was digitized and we compared the amount of radioactive probe in the protein-DNA complexes after standardization with free probe from three different experiments using a Nucleo Vision System (Nucleo Tech Co., San Carlos, CA).

For competition assays, extracts were incubated with 100-fold excess of unlabeled competitors. For supershift assays, antibodies against specific transcriptional factors (i.e., anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2, anti-c-Jun, anti-Jun-B, anti-Jun-D, anti-PEA-3, anti-ER, anti-ERB1, anti-Ets-1, and anti-PU.1 antibodies; Santa Cruz Biotechnology) were added to the extracts 15 minutes before addition of the probe.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation analyses were done using an assay kit following the instructions of the manufacturer (Upstate Biotechnology, Lake Placid, NY). The input and immunoprecipitated DNA were amplified across the MMP-1 promoter region using the primers 5’-CTTGTGTAGATTACATTACATTGCAGTGCACAG-3’ and 5’-AGCTTCCTGTGCCTCCAAATATGCT-3’. PCR products were then resolved on a 1.5% agarose gel. For quantitation, we also did SYBR green I-based quantitative PCR.

In vivo studies for quantitation of mRNA by RT-PCR. Biopsy specimens from Japanese patients with histologically proven primary early gastric cancer were obtained. H. pylori infection status was defined as positive if all three tests (serology, culture, and histology) gave positive results and negative if all three tests gave negative results. Two biopsy specimens from cancer tissues and two from adjacent apparently normal mucosa were used to examine the mucosal MMP-1 and pea-3 mRNA levels. The cag PAI and the oipA status was determined by cultured H. pylori as previously described (2, 4). In addition, antral biopsy samples were selected from our tissue bank consisting of specimens from Colombian patients with chronic gastritis and whose H. pylori status as well as the cag PAI and the oipA status had been characterized (28). Informed consent was obtained from all patients and samples were taken under protocols approved by the institutional ethics committees.

Extraction of total RNA and reverse transcription was done as previously described (35). Mucosal MMP-1 and pea-3 mRNA levels were evaluated as described above.

Statistical analysis. Each in vitro experiment was done at least three times. Statistical significance was assessed by ANOVA and differences identified were pinpointed by Student-Newman-Keuls multiple-range test (in vitro experiments) or Mann-Whitney rank sum test (in vivo experiments).

Results

H. pylori induces MMP-1 mRNA expression in gastric epithelial cells. To identify H. pylori–induced genes, we did cDNA array analysis of cells infected with wild-type, cag PAI mutant, or oipA mutant H. pylori strains. We found that expression of 33/375 genes was increased in response...
genes in MKN45 cells was up-regulated 4 hours postinfection with wild-type H. pylori; the fold increase cutoff used was 2 (see Supplementary Table S1). Expression of 19 of these 33 genes was both cag PAI and OipA dependent. This set of 19 genes includes 6 of the 10 examined MMPs (MMP-1, 3, 7, 9, 12, and 14). To confirm these results, we did real-time RT-PCR analysis of MMP-1 mRNA levels in the three gastric cell lines. Wild-type H. pylori induced MMP-1 mRNA levels in a time-dependent manner with maximal levels observed at 6 hours postinfection (Fig. 1A).

Importantly, this induction was restricted to cag PAI-positive/oipA-'on' wild-type strains (Fig. 1B). We also examined the protein levels of MMP-1 and confirmed that only cag PAI-positive/oipA-on H. pylori induced MMP-1 activity (see Supplementary Fig. S1). The pattern of MMP-1 expression in primary gastric epithelial cells was similar to that observed using stable cell lines. However, MMP-1 expression levels differed among the patients from which these primary cells were generated (Fig. 1C). MMP-1 mRNA levels were lower in noncancer primary cells than in cancer cell lines. Such changes are likely a result of altered signaling in the cancer tissues.

H. pylori induces expression of MMP-1 mRNA via Ras/Raf/RhoA→MAPK→c-Fos/c-Jun/PEA-3 pathways. The expression of MMP-1 is regulated by the activities of AP-1 and PEA-3. c-Fos/c-Jun and PEA-3 are well-known transcription factors of AP-1 and PEA-3, respectively. We measured the mRNA levels of c-fos, c-jun, and pea-3 following H. pylori infection. Real-time RT-PCR analysis showed that either infection with wild-type H. pylori or stimulation with PMA resulted in significant up-regulation of c-fos, c-jun, and pea-3 mRNA expression in MKN45 cells (Fig. 2A). The cag PAI mutant and the oipA mutant strains induced the expression of three mRNAs to a lesser extent than wild-type H. pylori did (Fig. 2B).

The inhibition of MAPK pathways by either chemical inhibitors (SB203580, U0126, and SP600125) or siRNA knockdown decreased H. pylori–induced c-fos and c-jun mRNA expression in MKN45 cells (Fig. 2C). Importantly, the inhibition of ERK and JNK also decreased the H. pylori–induced expression of pea-3 mRNA whereas the inhibition of p38 did not. AGS and SNU638 cells also showed similar results (data not shown). Therefore, unless otherwise noted, all subsequent experiments were done using MKN45 cells.

siRNA-mediated inhibition of all three MAPK pathways significantly suppresses the H. pylori–mediated induction of MMP-1 mRNA expression (Fig. 3A). Inhibition of p38 had no effect on PMA-induced MMP-1 mRNA expression. Additionally, the inhibition of c-fos and or c-jun by siRNA knockdown significantly suppressed the H. pylori–mediated induction of MMP-1 mRNA expression whereas the knockdown of pea-3 had no effect (Fig. 3A). However, the combined inhibition of pea-3, c-fos, and c-jun more dramatically suppressed MMP-1 mRNA expression than the combined inhibition of c-fos and c-jun, suggesting that PEA-3 co-operates with c-Fos/c-Jun to achieve Figure 1. MMP-1 mRNA levels in gastric epithelial cancer cell lines (A and B) and in primary non-cancer epithelial cells (C) detected by real-time RT-PCR. Four independent coculturings were done and each was measured in triplicate for (A and B) and one coculturing was done and measured in triplicate for (C). Data expressed as 1,000 × MMP-1 mRNA / GAPDH mRNA. Points and columns, mean; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with the wild-type strain (B and C). ND, not done.

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maximal MMP-1 induction. In contrast, the inhibition of pea-3 had no effect on PMA-induced MMP-1 mRNA expression, suggesting that PEA-3 has a unique function in the H. pylori–mediated induction of MMP-1 expression.

The MAPK pathway upstream signaling factors include monomeric GTP-binding proteins, such as Ras, Raf, Rac1, and RhoA. Transfection of the dominant-negative Rho (pCMV-RhoN19), Raf (pCMV-RafS621A), and Ras (pCMV-RasN17) mutants suppressed the induction of MMP-1 mRNA expression mediated by H. pylori and PMA (Fig. 3A). In contrast, the transfection of the dominant-negative Rac1 mutant (pCMV-RacN17) had no effect on the induction of MMP-1 mRNA expression.

H. pylori induces MMP-1 expression via promoter activation at AP-1 sites and inhibition at PEA-3 site. The results described above suggest that AP-1 and PEA-3 binding sites are involved in the H. pylori–mediated induction of MMP-1 expression. To better define the role(s) of these binding sites, MKN45 cells were transiently transfected with the reporter plasmid −4372hMMP1luci, which contains the full-length human MMP-1 promoter driving the expression of the luciferase gene. Luciferase activity reached maximal levels 10 hours postinfection (8.9 ± 0.4-fold increase over untreated control) and plateaued by 18 hours. In subsequent experiments, luciferase activity was assessed at 10 hours postinfection. The fold increase in MMP-1 promoter activity was similar in cells infected with the cag PAI or oipA mutant strains and in untreated controls (data not shown).

MKN45 cells were then transfected with plasmids containing serial 5′ to 3′ deletions of the MMP-1 promoter. Basal activity was decreased for the plasmid −192hMMP1luci and was restored and perhaps even slightly increased by deletion to −104 bp (Fig. 4A). This result suggests that the sequences containing the −181AP-1 site may be involved in suppressing the basal activity of the MMP-1 promoter. Basal activity was also markedly decreased for the plasmid −61hMMP1luci, suggesting that the sequences containing the −72AP-1 site have a role in increasing basal activity of the MMP-1 promoter. Wild-type H. pylori infection of cells containing these promoter deletion plasmids (down to −192 bp) resulted in a 7- to 9-fold induction of MMP-1 promoter activity (Fig. 4A), suggesting that the sequence between −192 and −61 bp is critical for the H. pylori–mediated induction of the MMP-1 promoter. Deletion to −104 bp significantly reduced the promoter induction, suggesting that the −181AP-1 site is involved in the H. pylori–mediated induction of the MMP-1 promoter. Deletion to −83 partially, but significantly, restores the induction whereas deletion to −61 bp abolishes the induction. These data suggest that the −72AP-1 site is involved in the H. pylori–mediated induction of the MMP-1 promoter and that the binding of transcription factor(s) to the −88PEA-3 site may interfere with the binding of the AP-1 transcription factor to the −72AP-1 site. Consistent with previous

**Figure 2.** c-fos, c-jun, and pea-3 mRNA levels in MKN45 cells detected by real-time RT-PCR. Three or four independent coculturings were done and each was measured in triplicate. No inhibitors altered mRNA levels in untreated MKN45 cells. No inhibitors altered GAPDH mRNA levels in MKN45 cells regardless of the treatment used. Expression levels normalized to the GAPDH mRNA levels are expressed as fold induction relative to untreated controls. **Points and columns:** mean; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with the wild-type strain. A, time course experiments. MKN45 cells were incubated for 0 to 6 hours with wild-type H. pylori, without H. pylori (negative control), or with PMA (10 ng/mL). B, effects of cag PAI and OIPa on c-fos/c-jun/pea-3 mRNA levels. MKN45 cells were incubated with wild-type H. pylori (column 2) or with mutant strains (columns 2 and 3) for 1 (c-fos and c-jun) or 4 (pea-3) hours. C, effect of inhibition of MAPK pathways on H. pylori–induced c-fos/c-jun/pea-3 mRNA levels. Each MAPK inhibitor (SB203580, U0126, or SP600125; 10 μmol/L) was added to MKN45 cells 30 minutes before infection with wild-type H. pylori, and mRNA levels were measured after 1 (c-fos and c-jun) or 4 (pea-3) hours. MKN45 cells were also transfected with 50 nmol/L of validated SMARTpool siRNA for ERK, JNK, or p38, or a scrambled siRNA negative control (Dharmacon Research). Forty hours posttransfection, the medium was changed and mRNA levels were measured following incubation with wild-type H. pylori.
and PEA-3 are the main transcription factors competing for binding in infection. Studies (21, 37), the sequence between −192 and −61 bp is also critical for the PMA-mediated induction of the MMP-1 promoter (Fig. 4B).

To further examine the relative contributions of these three sites, the activity of a series of mutated MMP-1 promoters was examined (Fig. 4C). Mutation of the −181AP-1 site increased the basal promoter activity and mutation of the −72AP-1 site abrogated the basal promoter activity. These results confirm that the −181AP-1 site is involved in suppression of the basal promoter activity and the −72AP-1 site is involved in increasing the basal promoter activity.

Mutation of the −181AP-1 or the −72AP-1 site abrogated the promoter activity induced by wild-type H. pylori infection (Fig. 4C). This finding is consistent with both AP-1 sites playing important roles in the H. pylori–mediated induction of the MMP-1 promoter. Mutation of the −88PEA-3 site resulted in an increase in the H. pylori–mediated induction of the −192hMMP1luci plasmid, confirming that the PEA-3 site is involved in suppression of the MMP-1 promoter. In addition, mutation of the −88PEA-3 site resulted in an increased H. pylori–mediated induction of the −104hMMP1luci and −192M181AP1luci plasmids. These results are consistent with the −88PEA-3 site having a suppressing effect on the −72AP1 site, which is capable of mediating the induction of the MMP-1 promoter.

The cag PAI and the oipA mutant H. pylori strains induced luciferase activity to very low levels regardless of the reporter plasmids used (maximum, 1.1 ± 0.1-fold). It is clear that the mechanism of the transcriptional induction of the MMP-1 promoter by H. pylori is different from that used by PMA (Fig. 4C). For example, single mutations in both AP-1 sites only slightly suppress the MMP-1 promoter activity and mutation of the −88PEA-3 site has no effect.

**Activation of transcription factors binding sites in H. pylori infection.** The experiments described above show that c-Jun, c-Fos, and PEA-3 are the main transcription factors competing for binding to the AP-1 and PEA-3 sites of the MMP-1 promoter. The data also suggest that the −88PEA-3 site plays a role in suppressing MMP-1 promoter activity, although H. pylori induces p-3 mRNA expression. One possible explanation for this discrepancy is that the PEA-3 transcription factor does not bind to the −88PEA-3 site and/or binds to a site other than the PEA-3 site. To examine the DNA binding proteins bound to the MMP-1 promoter, we did EMSA and supershift assays using the −71AP-1, −181AP-1, and −88PEA-3 sites. At each site, induction of binding was evident 1 hour after H. pylori infection; it peaked at 3 hours postinfection; and it decreased at 6 hours postinfection (data not shown).

One protein complex at −181AP-1 (C1) was weakly detected in uninfected MKN45 cells. Its presence was increased following wild-type H. pylori infection (Fig. 5A, lane 2). Infection with the cag PAI or the oipA mutant strains did not induce binding to the AP-1 site (lanes 3 and 4). Supershift assays showed that c-Jun and c-Fos were components of the −181AP-1 complexes (lanes 8-10). The other factors examined were not components of the −181AP-1 complex (data not shown).

At least three −72AP-1 binding complexes (C1, C2, and C3) were detected in uninfected MKN45 cells. Their presence was increased following infection with wild-type H. pylori (Fig. 5B, lane 2). Infection with the cag PAI or the oipA mutant strains reduced binding to the AP-1 site. Supershift assays showed that C1 contained c-Jun, C2 contained c-Fos, and C3 contained PEA-3 (lanes 8-10). The other factors examined were not components of these −72AP-1 complexes (data not shown).

Three −88PEA-3 binding complexes (C1, C2, and C3) were detected in uninfected MKN45 cells. The presence of the C1 and C3 complexes was significantly reduced following infection with wild-type H. pylori (lane 2). The complexes were not reduced following infection with either the cag PAI or the oipA mutant strains, suggesting that cag PAI and OipA play unique roles in the regulation of PEA-3 binding to the MMP-1 promoter. Supershift assays showed that PEA-3 was the sole component of the −88PEA-3 complexes.
the C3 complex and one of the components of the C2 complex. No Fos, Jun, or other Ets families were found to be component of any of these complexes (data not shown). We were unable to identify any components of the C1 complex. In contrast to findings following H. pylori infection, complex formation was not altered by PMA treatment. Overall, it is interesting that in wild-type H. pylori infections, the binding of PEA-3 to the −72AP-1 site is induced whereas the binding of PEA-3 to the −88PEA-3 site is repressed. Such selective binding of PEA-3 during H. pylori infection should result in maximal MMP-1 induction.

To further confirm these in vitro findings, the in vivo recruitment of c-Fos, c-Jun, and PEA-3 to the MMP-1 promoter following H. pylori infection was examined by chromatin immunoprecipitation assays. As expected, an increase in the binding of c-Fos and c-Jun to the promoter region −188/+18 was detected (Fig. 5D). Importantly, the binding of PEA-3 to this region was initially decreased but then increased.

**H. pylori induces in vivo MMP-1 mRNA levels in the gastric mucosa.** To further study the relationship between the expression of MMP-1 and PEA-3, we examined MMP-1 and pea-3 mRNA levels in early gastric cancers (all distal type) and adjacent normal-appearing mucosa (noncancer regions) from 44 H. pylori–positive patients and 11 patients without evidence of active H. pylori infection. All H. pylori cultured were cag PAI positive and oipA-on. Gastric mucosal MMP-1 mRNA levels were significantly higher in gastric cancer tissues than the in noncancer tissues (Fig. 6A; P < 0.001), a result consistent with the in vitro results described above (Fig. 1). MMP-1 mRNA levels were significantly higher in tissues from H. pylori–infected patients compared with noninfected patients regardless of biopsy location (Fig. 6A). MMP-1 mRNA levels in cancer tissues were higher in intestinal than in diffuse type gastric cancer (P = 0.01), a finding which is in agreement with previous immunohistochemistry or in situ hybridization studies on the localization of MMP-1 mRNA (9, 10, 38).

Interestingly, only 5 of the 55 samples had measurable levels of pea-3 mRNA in the noncancer tissues whereas pea-3 mRNA levels were significantly higher in the cancer tissues, especially in tissues from patients with an H. pylori infection (Fig. 6A). These data clearly suggest that PEA-3 plays an important role in gastric cancer and its expression is H. pylori dependent. In addition, pea-3 mRNA levels correlated with MMP-1 mRNA levels (r = 0.78; Fig. 6A).

To investigate the in vivo effect of the cag PAI and OipA, samples from 61 patients with gastritis (including 41 with an H. pylori infection) were also studied (Fig. 6B). Importantly, the H. pylori–related up-regulation of MMP-1 mRNA was restricted to infections with strains that were both cag PAI and oipA-on.

![Figure 4. MMP-1 promoter activation following H. pylori infection or PMA treatment. Four independent transfections, each done in triplicate, were done. Columns, mean; bars, SE. A and B, effect of 5’ to 3’ MMP-1 promoter deletions on activity. MKN45 cells were transiently transfected with 2 µg of the plasmids containing serial 5’ to 3’ deletions of the MMP-1 promoter and 10 ng of Renilla plasmid as an internal control. The cells were then either infected with H. pylori or stimulated with PMA for 10 hours. Untreated cells served as negative controls. For each sample, luciferase activity was normalized to the activity of the Renilla luciferase vector. Bars, fold increase in H. pylori–infected or PMA-treated cells relative to untreated controls. ***, P < 0.001, compared with −437/93MMP1luci under each condition. The presence of nonsuperseded DNA was not the cause of the reduction in basal expression for −192/92MMP1luci and −61/36MMP1luci because the five independent plasmids for each promoter construct behaved similarly. C, effect of MMP-1 promoter mutations on activity. MKN45 cells were transiently transfected with 2 µg of plasmids containing mutated MMP-1 promoters and 10 ng of Renilla plasmid as an internal control. The cells were then either infected with H. pylori or stimulated with PMA for 10 hours. Untreated cells served as controls. Basal luciferase activity is present as activity normalized to Renilla luciferase vector. Luciferase activity induced by H. pylori infection or PMA treatment is presented as normalized luciferase activity expressed as fold increase in H. pylori–infected or PMA-treated cells relative to uninfected controls. ***, P < 0.001, compared with −192/92MMP1luci.](cancerres.aacrjournals.org/article/66/10/5116/DC1/Figure4.jpg)
These data confirm the results of our in vitro studies described above (Fig. 1). None of the gastric biopsy samples had measurable levels of pea-3 mRNA.

**Discussion**

Our study has shown that *H. pylori* infection mediates an increased expression of MMP-1 in gastric mucosa. Additionally, we show that this increase in MMP-1 expression is associated with two *H. pylori* virulence factors, the cag PAI and OipA. The presence of only one of these factors is not enough to induce an increase in MMP-1 expression. This effect is different from that observed for the *H. pylori*–mediated increase in proinflammatory cytokines (e.g., IL-6 and IL-8) in which only one of these virulence factors is needed to elicit the induction of cytokines in mucosa (4, 27, 28).

We also showed that *H. pylori*–induced MMP-1 expression is associated with the AP-1 and PEA-3 transcription factors. Both the −181 and −72 AP-1 sites play important roles in the *H. pylori*–associated induction of the MMP-1 promoter, although these two sites have different effects. Specifically, mutation of the −181 AP-1 site dramatically suppresses basal promoter activity and mutation of the −181AP-1 site increases basal promoter activity. Although co-operation between PEA-3 and AP-1 transcription factors is known to be required for MMP-1 promoter activity (17–19), the role(s) of PEA-3 on *H. pylori*–mediated MMP-1 promoter activity is unique. Luciferase reporter assays and EMSA supershift assays clearly show that wild-type *H. pylori* infection causes PEA-3 to bind...
to the −88PEA-3 site with reduced affinity and to the −72AP-1 site with increased affinity. This selective binding of PEA-3 in response to H. pylori infection is required for maximal induction of MMP-1 expression. Interestingly, the −181AP-1 site was not involved in PEA-3 binding, suggesting possible co-operation between adjacent binding sites in the promoter. This prediction was confirmed using chromatin immunoprecipitation assays that showed the binding of PEA-3 to the MMP-1 promoter initially decreased and then subsequently increased. We speculate that this initial reduction in binding could represent dissociation of PEA-3 from the −88PEA-3 site and the subsequent increase in PEA-3 binding represents the association of H. pylori–induced, newly synthesized PEA-3 with the −72AP-1 site. Our data showing that H. pylori induces pea-3 mRNA expression in the gastric cancer cells supports this prediction. Alternatively, H. pylori may induce the translocation of PEA-3 from the −88PEA-3 site to the adjacent −72AP-1 site.

Importantly, the reduction in PEA-3 binding to the −88PEA-3 site was not observed following infection with cag PAI and oipA mutant strains. This finding suggests that the cag PAI and OipA are involved in inhibiting PEA-3 binding. We also found that the H. pylori–mediated induction of pea-3 mRNA was dependent on both the cag PAI and OipA virulence factors. Therefore, it is likely that the cag PAI and OipA play major roles in pea-3 expression and both virulent factors influence the balance between inhibiting PEA-3 binding and inducing AP-1 binding.

We also investigated the upstream signaling regulating MMP-1 promoter activity. We showed that H. pylori induces c-fos, c-jun, and pea-3 mRNA expression and that these three factors cooperate to induce maximal MMP-1 expression. This finding is consistent with these three factors being the main AP-1 and PEA-3 transcription factors at the MMP-1 promoter. We confirmed that p38, ERK, and JNK mediated H. pylori–induced c-jun and c-fos expression (Fig. 2C). Inhibition of these three MAPK pathways also suppressed H. pylori–induced MMP-1 mRNA expression (Fig. 3A). However, inhibition of ERK and JNK, but not p38, affected PMA-induced MMP-1 mRNA expression. Recent studies have reported that SB203580 enhances MMP-1 secretion in AGS cells stimulated by epidermal growth factor, tumor necrosis factor, or IL-1β (39). These findings show that the mechanism involved in MMP-1 induction is stimulus dependent.

We also examined the role of the monomeric GTP-binding proteins, Ras, Raf, Rac1, and RhoA, which function upstream in the MAPK pathways. Ras was of particular interest because it can stimulate multiple signaling pathways, including the sequential activation of Raf→MEK1/2→ERK1/2 (40–42). We found that H. pylori–induced MMP-1 promoter activity was primarily through the Ras/Raf- and Rho-dependent pathways. The findings are consistent with recent studies showing that stimulation of MMP-7 by H. pylori in gastric epithelial cells is dependent on RhoA-mediated activation of AP-1 (43). Interestingly, expression of a dominant-negative Rac1 mutant did not affect on H. pylori–induced MMP-1 expression (Fig. 3B). This finding contradicts previous reports showing that H. pylori enhances the activation of Rac1 in AGS cells, a gastric epithelial cell line (44, 45). Other recent data have shown that a dominant-negative Rac1 mutant decreases the H. pylori–induced activation of the IL-6 promoter in MKN28 cells (32). Although H. pylori might enhance the activation of Rac1, the downstream signaling mediated by this Rac1 is likely to be promoter dependent.

Finally, we confirmed our in vitro results by examining the expression MMP-1 and pea-3 in vivo (i.e., in gastric cancer tissues). Gastric mucosal MMP-1 has been shown to be elevated in gastric cancer, and this elevated expression is related to the metastatic potential of the cancer (9–12). We also found that mucosal MMP-1 mRNA levels were significantly higher in gastric cancers with active H. pylori infections than in those cancers without an active infection. In addition, the H. pylori–associated up-regulation of
MMP-1 was restricted to those infections that were both cag PAI positive and oipA+, suggesting that the combined functions of cag PAI and OipA may play an important role(s) in the course of gastric cancer. Additionally, mucosal MMP-1 mRNA levels correlated with mucosal pea-3 mRNA levels and the up-regulation of pea-3 expression was largely restricted to H. pylori+ positive cancer tissues. pea-3 expression in gastric cancer is not well characterized and only one previous study has reported that pea-3 mRNA is up-regulated in gastric cancer tissues (29). In that study, they did not find the correlation between pea-3 expression and MMP-1 expression. This could be a result of the experimental methods they used (nonquantitative RT-PCR). In addition, they did not take account of H. pylori infection. Overall, MMP-1 and pea-3 expression is elevated in gastric cancers via H. pylori infection.

Our study provides a possible link between bacterial virulence factors and important host factors involved in the pathogenesis of gastric disease.

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References


Balance between Polyoma Enhancing Activator 3 and Activator Protein 1 Regulates *Helicobacter pylori*–Stimulated Matrix Metalloproteinase 1 Expression

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