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Helicobacter pylori Stimulates Gastric Epithelial Cell MMP-1 Secretion via CagA-dependent and -independent ERK Activation*

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Because the mechanisms of *Helicobacter pylori*-induced gastric injury are incompletely understood, we examined the hypothesis that *H. pylori* induces matrix metalloproteinase-1 (MMP-1) secretion, with potential to disrupt gastric stroma. We further tested the role of CagA, an *H. pylori* virulence factor, in MMP-1 secretion. Co-incubation of AGS cells with Tx30a, an *H. pylori* strain lacking the *cagA* virulence gene, stimulated MMP-1 secretion, confirming *cagA*-independent secretion. Co-incubation with strain 147C (*cagA*$^+$) resulted in CagA translocation into AGS cells and increased MMP-1 secretion relative to Tx30a. Transfection of cells with the recombinant 147C *cagA* gene also induced MMP-1 secretion, indicating that CagA can independently stimulate MMP-1 secretion. Co-incubation with strain 147A, containing a *cagA* gene that lacks an EPIYA tyrosine phosphorylation motif, as well as transfection with 147A *cagA*, yielded an MMP-1 secretion intermediate between no treatment and 147C, indicating that CagA tyrosine phosphorylation regulates cellular signaling in this model system. *H. pylori* induced activation of the MAP kinase ERK, with CagA-independent (early) and dependent (later) components. MEK inhibitors UO126 and PD98059 inhibited both CagA-independent and -dependent MMP-1 secretion, whereas p38 inhibition enhanced MMP-1 secretion and ERK activation, suggesting p38 negative regulation of MMP-1 and ERK. These data indicate *H. pylori* effects on host epithelial MMP-1 expression via ERK, with p38 playing a potential regulatory role.

Helicobacter pylori, highly prevalent in developed nations, and ubiquitous in developing countries (1), is an important risk factor for peptic ulcer disease, gastric adenocarcinoma, and gastric lymphoma (2, 3). However, the mechanisms through which *H.* pylori induces gastric damage are not well elucidated. *H. pylori*, an extracellular bacterium that attaches to gastric epithelial cells via adhesins (4), has evolved to survive within the gastric mucus layer (5). *H. pylori* strains may possess the *cag* pathogenicity island (*cagI*)[3] (6), a 35–40-kb region that contains 25 to 30 open reading frames. Several *cagI* genes encode a type IV secretion system (7), an assembly that permits introduction of CagA, a *cagI*-encoded protein of ~128 kDa (8, 9), into the host gastric epithelial cell. Most CagA isotypes include one or more type C EPIYA motifs that are subject to tyrosine phosphorylation by host cell c-Src/Lyn kinases (10). Phosphorylated CagA interacts with, and activates, the host cell SHP-2 phosphatase, which serves as an important CagA effector (11, 12). The presence of CagA within epithelial cells induces morphologic changes (scattered or hummingbird phenotype), including formation of extensive host cell filopodia (13). Other *cagA* and/or *cagI*-related effects include increased host cell apoptosis (14), nuclear factor-κB activation (15), and secretion of pro-inflammatory chemokines such as interleukin-6 and -8 (16, 17). Compared with *cagA*$^-$ strains, *cagA*$^+$ strains are associated with increased risk of peptic ulcer disease and carcinogenesis (18, 19).

When secreted and activated, matrix metalloproteinases (MMPs) digest extracellular connective tissue (20). In rheumatoid arthritis, MMP secretion by synovial fibroblasts in response to cytokines contributes to cartilage and bone destruction (21, 22). Similarly, pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, stimulate gastric epithelial cells to synthesize and secrete MMPs (23). Moreover, exposure of gastric epithelial cells to *H. pylori* also results in MMP secretion, including MMP-3, -7, and -9 (24–26). Up-regulated MMP expression has been associated with gastric ulceration and neoplasia in humans and in animal models (25–30), suggesting that MMPs participate in gastric tissue

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[3] The abbreviations used are: *cagI*, *cag* pathogenicity island; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; EGF, epidermal growth factor.

erosion and/or tumor invasion. However, the role of CagA in *H. pylori*-induced MMP secretion has not been fully elucidated.

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases including ERK, JNK, and p38 subfamilies (31, 32). Whereas JNK and p38 regulate inflammation (33, 34), ERK is mitogen-responsive, and regulates cell growth and differentiation (31). However, ERK also can regulate inflammatory responses, including neutrophil adhesion and superoxide generation (35). In synovial fibroblasts, ERK activation mediates cytokine- and growth factor-stimulated MMP-1 secretion, enhancing tissue destruction in inflammatory arthritis (36). Although *H. pylori* stimulates ERK activation in gastric epithelial cells (37–41), the kinetics of ERK activation, as well as the role of CagA in this process, are not fully understood. *H. pylori* activation of gastric epithelial cell ERK may regulate both chemokine secretion and nuclear factor-κB activation (42), contributing to proinflammatory responses. In response to *H. pylori* and gastrin, secretion of MMP-7 and MMP-9, respectively, may be regulated by ERK (26, 43). In response to cytokines, ERK mediates, and p38 inhibits, gastric cell MMP-1 secretion (23). Because the primary connective tissue components of gastric stroma are proteins susceptible to MMP-1 degradation (29, 44, 45), MMP-1 secretion may be particularly relevant to the pathogenesis of gastric damage.

In the present studies, we examined the role of *H. pylori* in gastric cell MMP-1 secretion, and the regulation of this process by MAP kinases. We report that *H. pylori* stimulates gastric epithelial cell MMP-1 secretion via both CagA-dependent and -independent mechanisms. Both processes are mediated by ERK, and inhibited by p38. CagA, and particularly the CagA tyrosine phosphorylation site (EPIYA motif), is required for optimal ERK activation and MMP-1 secretion. Moreover, the pathways through which $CagA^+$ and $CagA^-$ strains activate ERK are at least partially distinct. These data indicate that MAPKs are essential regulators of MMP-1 secretion, and provide mechanisms to explain *H. pylori*-mediated tissue injury. Because populations of *H. pylori* cells may contain variants that are both $cagA^-$ and $cagA^+$ (46), and because $cagA^+$ cells may express CagA proteins with variable (0–3) numbers of functional EPIYA motifs (47), variation in *cagA* genotypes within the total population may regulate the signal intensity leading to MMP-1 secretion.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all materials were from Sigma. Anti-phospho-ERK, ERK1, and ERK2 antisera, and horseradish peroxidase-conjugated anti-rabbit antiserum were from Santa Cruz Biotechnology. Anti-MMP-1 and MMP-13 antisera were from Chemicon. 10% Tris glycine/polyacrylamide gels were from Novex/Invitrogen. UO126, PD98059, and SB203580 were from Biomol. SB202190 was from Calbiochem. Centricon™ centrifugal filter devices were from Millipore, and RPMI medium, fetal bovine serum and 2.5% trypsin/EDTA from BioWhittaker. Penicillin G sodium (10,000 units/ml)/streptomycin sulfate (10,000 μg/ml) in 0.85% NaCl was from Invitrogen. The ECL chemiluminescence kit was from Amersham Biosciences. The Ras activity kit was from Active Motif.

Bacterial Strains—To determine the effects of *H. pylori* on AGS cell MMP-1 secretion, we employed three *H. pylori* strains. Strain Tx30a (48), also reported as strain 88-22 (47), lacks the Cag pathogenicity island, and is of low pathogenicity. In contrast, strains 147A and 147C are clonally derived $cagI^+$ strains that were isolated from different gastric regions of the same patient, and are nearly identical except for sequence differences in the 3′ end of *cagA*. 147C possesses a C-type EPIYA motif that is absent in 147A. Because the C-type EPIYA tyrosine in 147C is the target for CagA phosphorylation by the host cell kinase Src, 147A is not susceptible to phosphorylation (47). Strain 7bqs, used to derive the 7bqs *cagA* construct, was obtained from a different host than 147A and 147C (47).

Cell Culture and Cell Treatment—AGS cells (American Type Culture Collection Number CRL-1739)), a human cell line derived from a gastric epithelial tumor (49, 50), were cultured in 6-well plates in RPMI medium containing 10% fetal bovine serum and penicillin/streptomycin. Cells grown to near confluence were serum-starved for 24 h (0% fetal bovine serum), followed by equilibration with fresh medium for 30–60 min prior to co-culture with *H. pylori* and/or other treatments. Unless otherwise specified, the following concentrations of reagents were used: UO126, SB203580, and SB202190 each at 10 μM; PD98059 at 50 μM.

AGS Cell Co-culture and Transfection—Serum-starved AGS cells were co-cultured with *H. pylori* cells of strain Tx30a, 147A, or 147C, at a ratio of 100 bacterial cells/AGS cell. The cultures were incubated for the indicated times at 37 °C, and supernatants collected, concentrated, and analyzed, as described (23). Adherent cells were washed 3 times with cold phosphate-buffered saline, lysed (20 mM Tris, pH 7.4, 1 mM EGTA, 2 mM sodium vanadate, 25 mM sodium fluoride, 0.5% (v/v) Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10^5 kallikrein units/ml aprotinin, and 10 μg/ml each of chymostatin, antipain, and pepstatin) for 20 min at 4 °C, as described (36), and lysates collected and used directly or frozen for further study.

AGS cells grown to ~50% confluence were transfected using the Superfect™ transfection system, according to the manufacturer's instructions (Qiagen, Valencia, CA). Typically, 15 μl of Superfect™ was used/5 μg of DNA/well in 6-well plates. UO126, PD98059, SB203580, or SB202190 was added to cells at the time of transfection. After 2–3 h, the medium was replaced with serum-free medium (still containing the appropriate inhibitor), incubation was continued, and supernatant and/or lysates analyzed for MMP-1, ERK, or CagA.

Immunoblotting and Determination of Raf, MEK, and ERK Activation—For analysis of intracellular proteins from experimentally treated or control AGS cells, equivalent amounts of each lysate were supplemented with Laemmli buffer for SDS-PAGE analysis. For ERK activation, antibodies directed against the phosphorylated active forms of ERK1/2 were employed (1:400). Membranes were subsequently stripped and reblotted for total ERK1/2 (1:800), and ERK activation reported as phosphorylated ERK/total ERK. MEK and Raf activation levels were similarly determined, using antibodies for the phosphorylated and total populations of MEK and Raf. For CagA determinations, lysates were analyzed using anti-CagA antiserum (1:2000). For MMP-1 determinations, supernatants were con-

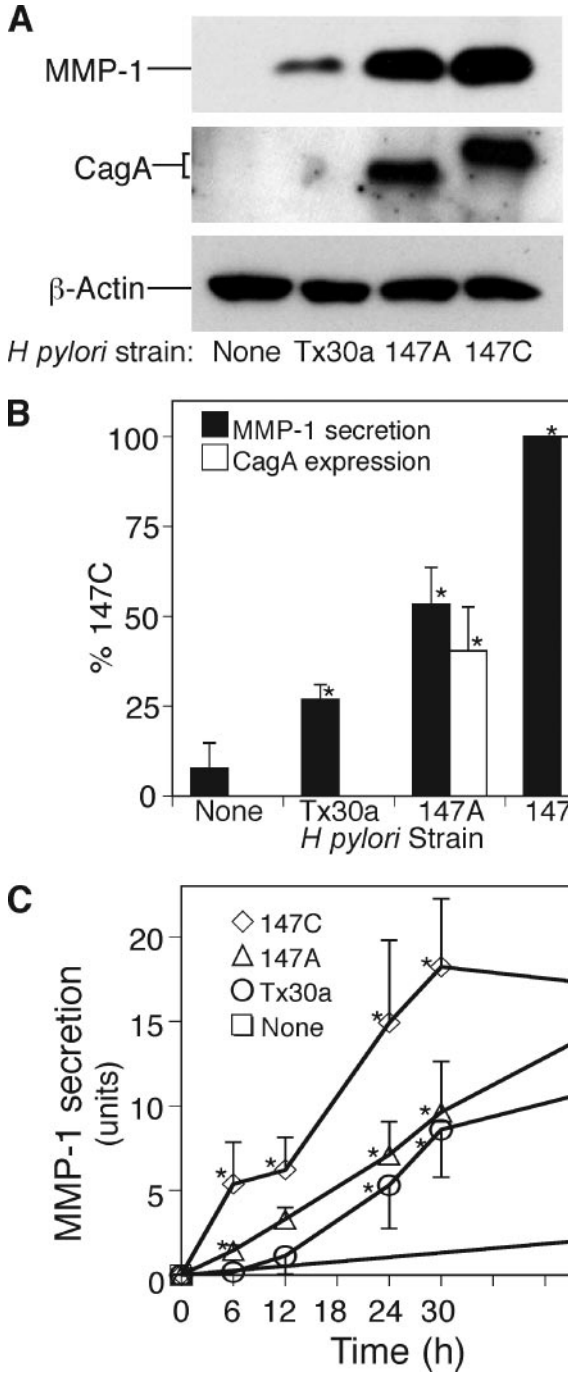

FIGURE 1. ***H. pylori* stimulates MMP-1 secretion from gastric epithelial cells.** *A*, AGS cells were co-incubated overnight with *H. pylori* strains Tx30a, 147A, or 147C, the supernatants were assayed for MMP-1, and the cell lysates examined for CagA and actin by immunoblot. *B*, experiments described in *A* were normalized to actin levels and expressed relative to co-incubation with 147C. *C*, AGS cells were co-incubated with strains Tx30a, 147A, and 147C for the indicated times, and supernatants assayed for MMP-1. *Error bars*, data shown are representative (*A*), or the mean ± S.E. (*B* and *C*) of three to five experiments (*, $p < 0.05$ *versus* no *H. pylori* (*B*) or $T = 0$ (*C*)).

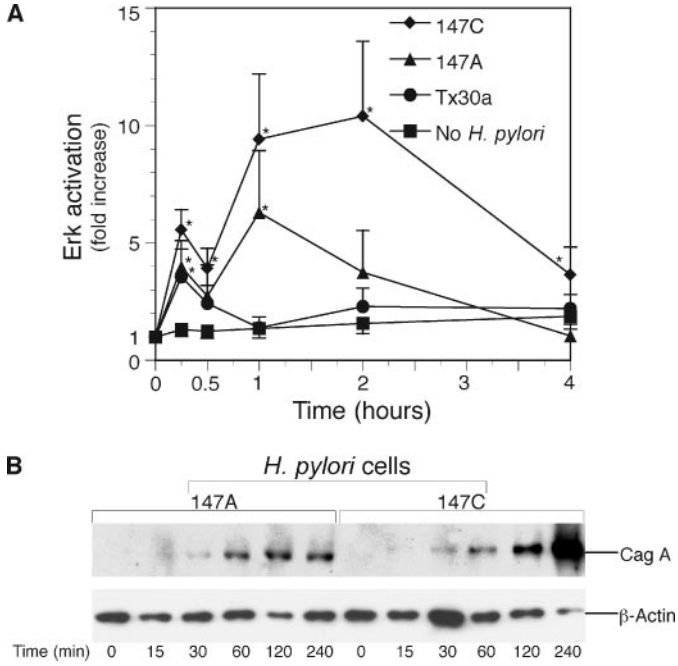

FIGURE 2. ***H. pylori* stimulates ERK activation via CagA-independent and -dependent pathways.** *A*, AGS cells were co-incubated with strains Tx30a, 147A, or 147C for the indicated times and assayed by immunoblotting for ERK activation, determined as phosphorylated ERK normalized to total ERK. Time "0" represents AGS cells not co-incubated with *H. pylori* (*, $p < 0.05$ *versus* $T = 0$). *B*, *AGS* cells were co-incubated with strains 147A or 147C for up the indicated times, washed thoroughly, and assayed for the presence of 147A or 147C CagA, as well as β-actin. *Error bars*, data shown are the mean ± S.E. of eight (*A*) or representative of three (*B*) experiments.

centrated in Centricon™ centrifugal filter devices (M_r cutoff 30,000) for 35 min at 5,000 × *g* at 4 °C. Volume equivalents were assayed for MMP-1 by SDS-PAGE and immunoblotting with anti-MMP-1 antiserum (1:400). In each case, immunoblots were resolved by incubating with horseradish peroxidase-conjugated anti-rabbit antibody, imaged by chemiluminescence and autoradiography according to the manufacturer's instructions, and quantitated by densitometry (36).

Ras Activation—Ras activity was determined as the binding to Ras of the Ras-binding domain (RBD domain) of Raf, determined using an ELISA kit according to the manufacturer's instructions.

Plasmids—The *cagA* open reading frames in *H. pylori* strains 147A, 147C, and 7bqs were generated by PCR amplifying the coding regions from DNA purified from the *H. pylori* cells using forward and reverse primers (EGFPSAC5 (5′-TAAGGAGAGCTCATGACTAACGAAACTATTGAT) and SPKPN3m (5′-TTCCTTGGTACCTTAAGATTTTTGGAAACCACCT) for 147A *cagA* and 147C *cagA*, and SPSAC5 (5′-TAAGGAGAGCTCATGACTAACGAAAC) and SPKPN3 (5′-TTCCTTGGTACCTTAAGATTTTTGGAAAC) for 7bqs-*cagA*), as previously reported (47, 51). The resultant amplicons were inserted in-frame into SacI and KpnI sites in the multiple cloning region of the pSP65SRα mammalian expression vector. All constructs were verified by sequencing. 147A, 147C, and 7bqs have zero, one, and two type C EPIYA tyrosine phosphorylation motifs, respectively (47).

RESULTS

H. pylori Stimulates MMP-1 Secretion from AGS Cells via cagI-dependent and -independent Processes—Co-culture of AGS cells overnight with strain Tx30a, lacking the *cagI*, stimulated MMP-1 secretion (Fig. 1, *A* and *B*). Co-culture with strain 147C (*cagA*$^+$) resulted in significantly increased MMP-1 secretion relative to Tx30a, whereas strain 147A (*cagA*$^+$, but lacking

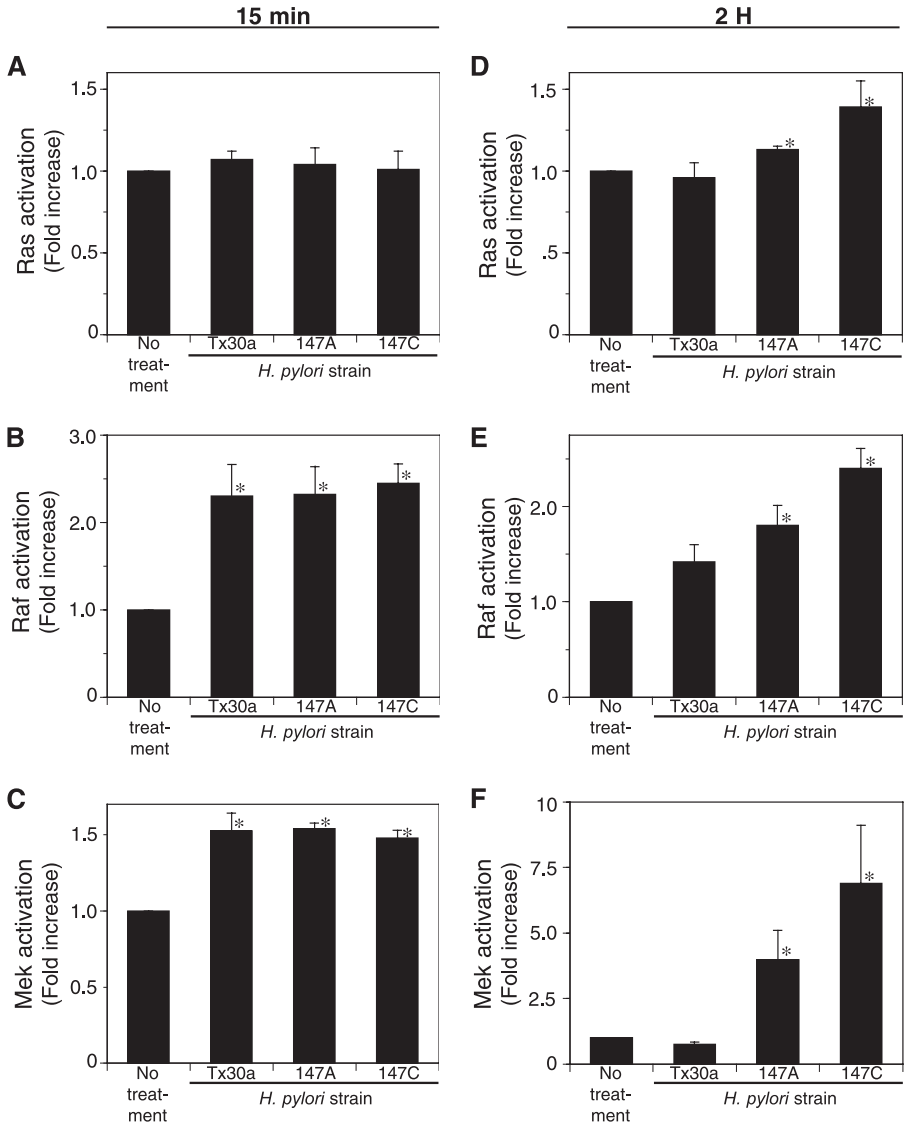

FIGURE 3. **Effects of *H. pylori* on putative upstream elements of ERK pathway signaling.** AGS cells were co-incubated with strains Tx30a, 147A, or 147C for 15 min (*left panels*) or 2 h (*right panels*), and analyzed for Ras (*A* and *D*), Raf (*B* and *E*), or MEK (*C* and *F*) activation as described under "Experimental Procedures." *Error bars*, data shown are the mean ± S.E. of five experiments for each panel. *, $p < 0.05$ *versus* no treatment.

H. pylori Stimulates ERK Activation via cag-dependent and -independent Processes—Because MMP-1 secretion in other cell types is ERK-dependent (36, 52, 53), we examined AGS cell ERK activation in response to *H. pylori*. Co-culture of AGS cells with strains Tx30a, 147A, or 147C resulted in rapid (15 min) ERK activation (Fig. 2*A*). The degree of ERK activation induced after 15 min did not differ significantly between the *cagA*$^+$ and *cagA*$^-$ strains (Tx30a *versus* 147A, $p = 0.8$; Tx30a *versus* 147C, $p = 0.2$; 147A *versus* 147C, $p = 0.3$). The *cagA*$^+$ strains additionally induced a delayed (1–2 h) peak of ERK activation that was not observed with strain Tx30a (Fig. 2, *A* and *B*). This second peak was more prominent, and of longer duration, in cells co-cultured with strain 147C, suggesting that the CagA EPIYA tyrosine phosphorylation site is required for optimal delayed ERK activation. Co-culture of the AGS cells with *H. pylori* did not affect total ERK levels (not shown). CagA protein was not observed in AGS cells after 15 min of *H. pylori* 147A or 147C co-culture, but was present as early as 30 min after co-incubation with either of the strains (Fig. 2*B*). Thus, the kinetics of CagA expression in AGS cells was consistent with a role for CagA in late, but not early phase ERK activation. Despite the continuing, and indeed increasing presence of 147A or 147C proteins in AGS cells exposed to these *H. pylori* strains, ERK activation was transient, indicating that regulatory pathways may limit ERK activation in response to CagA. These data indicate that ERK activation by *H. pylori* is biphasic, with an early phase of CagA-independent ERK activation followed later by a larger, activation of ERK only in *cagA*$^+$ strains.

the EPIYA tyrosine phosphorylation site) induced intermediate levels of MMP-1 secretion. Whereas immunoblotting of AGS cell lysates confirmed that cells co-cultured with strain Tx30a contained no CagA protein, AGS cells co-cultured with strains 147A and 147C had CagA introduced; 147C CagA was slightly greater in molecular weight than 147A CagA, consistent with the presence of an additional indel encoding the EPIYA motif (47). CagA expression was consistently higher in cells co-cultured with strain 147C (Fig. 1, *A* and *B*). The kinetics of MMP-1 secretion varied between strains (Fig. 1*C*). MMP-1 secretion was observed earliest for cells co-cultured with strain 147C, followed by 147A, then Tx30a. These data indicate that *H. pylori* can stimulate MMP-1 secretion in the absence of the *cagI*, but that its presence increases MMP-1 secretion. Moreover, the presence of a tyrosine phosphorylation site on the CagA protein is associated with increased, and more rapid, MMP-1 secretion.

To determine the pathway(s) through which *H. pylori* acts to activate ERK, we tested the effect of *H. pylori* infection on Ras, Raf, and MEK. These proteins constitute the canonical signaling cascade, activated by engagement of epidermal growth factor (EGF) receptors and other protein tyrosine kinase receptors, leading to ERK activation. EGF-stimulated Ras, Raf, and MEK activation after 15 min, with a lesser degree of activation of each after 2 h (data not shown). Incubation of AGS cells with Tx30a, 147A, or 147C *H. pylori* cells for 15 min had no effect on Ras activation (Fig. 3*A*), whereas all three strains induced equivalent levels of both Raf and MEK activation after 15 min (Fig. 3, *B* and *C*). Thus, rapid, CagA-independent ERK activation by *H.*

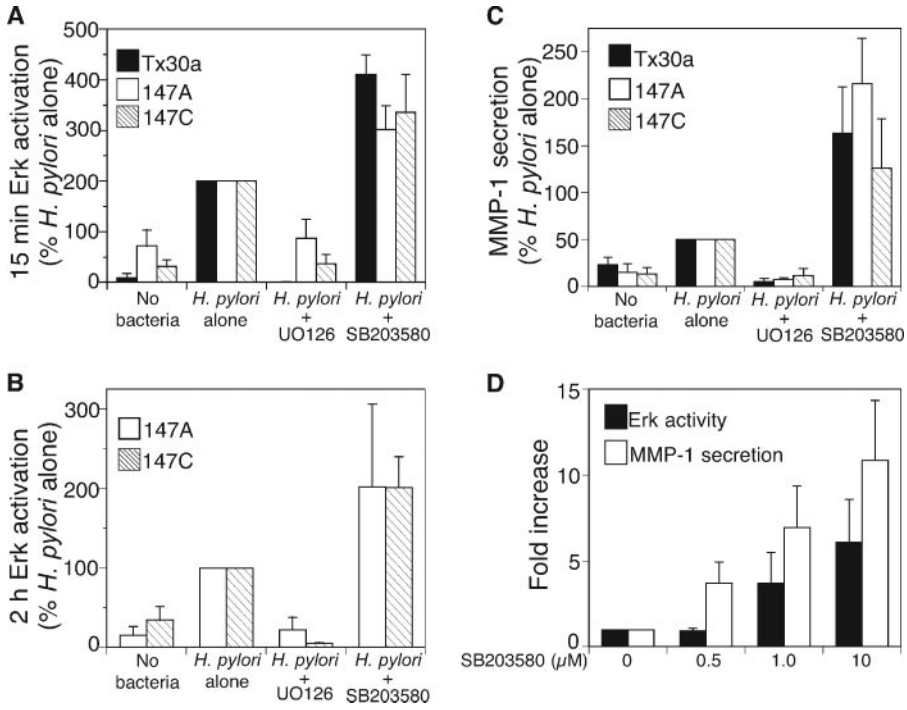

FIGURE 4. **ERK regulates gastric cell MMP-1 secretion.** *A*, *AGS* cells were incubated ± UO126 or SB203580 for 30 min, followed by incubation with strains Tx30a, 147A, or 147C for 15 min, and assay for ERK activation. *B*, cells were incubated ± UO126 or SB203580 for 30 min, followed by incubation with strains 147A or 147C for 2 h, and assay for ERK activation. *C*, AGS cells were incubated ± UO126 or SB203580 for 30 min, followed by overnight co-incubation with strains Tx30a, 147A, or 147C, and assay for MMP-1 secretion. *D*, AGS cells were incubated for 30 min ± the indicated concentrations of SB203580, and analyzed for ERK activation (*black bars*) or MMP-1 secretion (*white bars*) after tumor necrosis factor-α (10 ng/ml) stimulation for 15 min (*ERK*) or overnight (*MMP-1*). *Error bars*, data shown are the mean ± S.E. of three (*A–C*) or six (*D*) experiments.

pylori appears to involve Raf and MEK, but not Ras activation. After 2 h of co-incubation, strain Tx30a did not induce the activation of Ras, Raf, or MEK (Fig. 3, *D* and *E*). In contrast, 2 h of incubation with strains 147A or 147C resulted in stimulation of Ras, as well as Raf and MEK, with 147C providing the more potent stimulus. Thus, early CagA-independent ERK activation, and later CagA-dependent ERK activation in response to *H. pylori*, proceed according to partially divergent signaling pathways.

H. pylori-induced MMP-1 Secretion Is ERK-dependent—To determine whether *H. pylori*-induced MMP-1 secretion was ERK-dependent, we used UO126 (54), a specific inhibitor of MEK, the proximal activator of ERK (55). UO126 inhibited early (15 min) ERK activation in response to co-culture with Tx30a, 147A, or 147C (Fig. 4*A*), as well as later phase (2 h) ERK activation in response to 147A or 147C (Fig. 4*B*). UO126 also inhibited MMP-1 secretion in response to co-culture with each of the three strains (Fig. 4*C*). PD98059, another specific MEK inhibitor (56), also inhibited both ERK activation and MMP-1 secretion (data not shown). These results confirmed that MMP-1 secretion in response to co-culture with each of the three strains was MEK- and ERK-dependent.

Because p38 activity mediates MMP-1 secretion in dermal fibroblasts and several other cell types (57–59), but inhibits MMP-1 secretion in cytokine-stimulated AGS cells (23), we also tested the effects of p38 inhibition on MMP-1 secretion from AGS cells. AGS cell incubation with the specific p38 inhibitor SB203580 (10 μM) (60) enhanced both early and late phase ERK activation (Fig. 4, *A* and *B*), as well as MMP-1 secretion (Fig. 4*C*) in response to Tx30a, 147A, or 147C co-culture. Another specific p38 inhibitor (SB202190) also enhanced ERK activation and MMP-1 secretion stimulated by all three strains (data not shown). Because some reports suggest that SB203580 may have nonspecific effects at concentrations above 1 μM, including possible direct activation of c-Raf (61), we also tested the effects of lower SB203580 concentrations utilizing an established model of SB203580 enhancement of tumor necrosis factor stimulation of AGS cells (23). SB203580 concentrations as low as 1.0 μM enhanced both ERK activation and MMP-1 secretion, indicating that the effect of SB203580 is likely specific and targeted to p38 (Fig. 4*D*). Interestingly, lower concentrations (0.5 μM) of SB203580 enhanced MMP-1 secretion ($p = 0.01$) but not ERK activation. Thus p38 may tonically inhibit AGS cells MMP-1 secretion by both ERK-dependent and -independent effects.

CagA Transfection Stimulates ERK Activation and ERK-dependent MMP-1 Secretion—To determine the effects of CagA *per se* on ERK activation, we transfected AGS cells with the eukaryotic expression vector pSP65SRα, or with pSP65SRα containing inserts of *cagA* from strains 147A or 147C. Transfection with either 147A or 147C *cagA* resulted in expression of CagA protein (Fig. 5, *A* and *E*). Transfection with 147C *cagA* did not affect total ERK levels, but induced ERK activation (Fig. 5, *B*, *C*, and *E*). Transfection with 147A *cagA* induced less ERK activation than 147C *cagA*, confirming that type C EPIYA tyrosine phosphorylation plays a role in maximal direct ERK activation by CagA. ERK activation was transient, peaking at 3–8 h for both 147A and 147C, and returning to baseline by 24 h after transfection (data not shown).

Because co-culture of AGS cells with *H. pylori* stimulated both ERK activation and MMP-1 secretion, we next asked whether *cagA* transfection also stimulates MMP-1 secretion. *cagA* transfection resulted in increased MMP-1, with 147C *cagA* inducing greater MMP-1 secretion than 147A *cagA* (Fig. 5, *D* and *E*). Thus, the presence of CagA in the host cell is sufficient to stimulate MMP-1 secretion as well as ERK activation, and MMP-1 secretion is enhanced by the presence of the type C EPIYA tyrosine phosphorylation motif. In contrast to strain 147C, strain 7bqs contains an additional type C EPIYA region, making it susceptible to two phosphorylation events (47). Transfection of AGS cells with *cagA* 7bqs induced MMP-1 secretion (263% increase *versus* vector control, $p = 0.03$), but no greater than that seen with *cagA* 147C (7bqs *versus* 147C, 85 ±

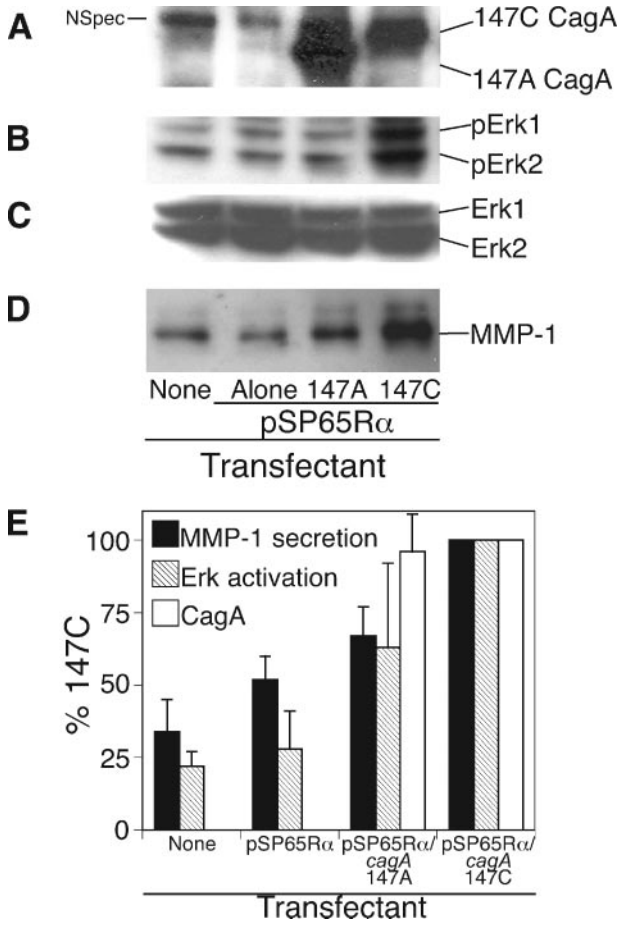

FIGURE 5. **CagA is sufficient to stimulate ERK activation and MMP-1 secretion.** AGS cells were transfected ± pSP65Rα vector, or pSP65Rα encoding 147A or 147C *cagA* for 24 (*A* and *D*) or 3 h (*B* and *C*). Supernatants were analyzed for MMP-1 (*A*), and lysates were analyzed for phosphorylated (*B*) or total (*C*) ERK or CagA (*D*). (*Nspec*, nonspecific band not representing CagA.) *E*, *Error bars*, the mean ± S.E. of multiple experiments such as those shown in *A–D*. ERK activation is reported as phospho-ERK/total ERK, and all conditions are expressed relative to the appropriate transfection with the 147C form of *cagA* ($n = 3$ for pErk/ERK; $n = 4$ for CagA; $n = 10$ for MMP-1).

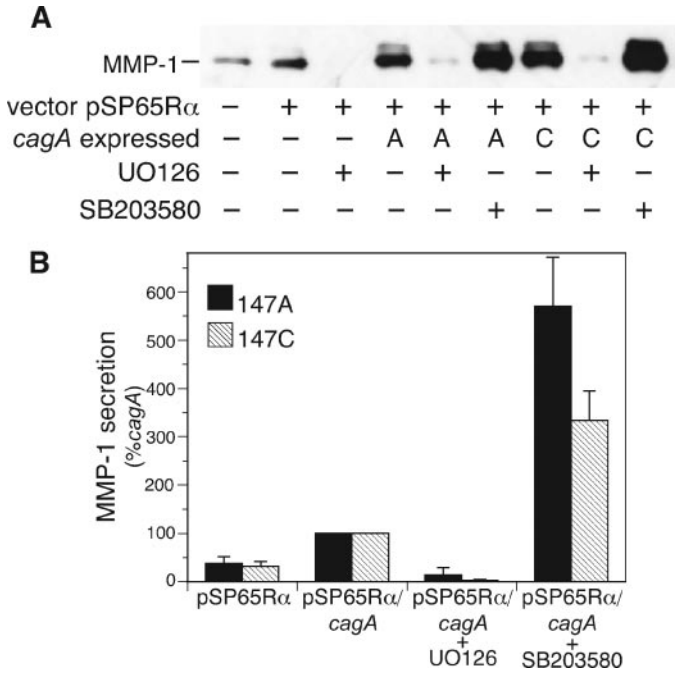

FIGURE 6. **MMP-1 secretion stimulated by *cagA* transfection is ERK-dependent.** *A*, AGS cells were transfected ± pSP65Rα, or pSP65Rα expressing 147A or 147C, in the absence or presence of the MEK inhibitor UO126, or the p38 inhibitor SB203580. After 24 h, supernatants were assayed for MMP-1. *B*, *Error bars*, mean ± S.E. of six experiments showing the effect of UO126 and SB203580 on MMP-1 secretion, relative to transfection with the 147A or 147C forms of *cagA*. Experiments for 147A and 147C were performed separately, and separate vector control results are provided for both sets of experiments.

13% increase, $p = 0.29$). Thus, a single CagA phosphorylation event was sufficient to drive maximal MMP-1 secretion under these experimental conditions.

Finally, we tested whether MMP-1 secretion in response to *cagA* transfection is regulated by MAP kinases. Treatment of AGS cells with MEK inhibitors UO126 (Fig. 6, *A* and *B*) or PD98059 (data not shown) inhibited MMP-1 secretion in response to *cagA* transfection, whether with 147A or 147C. UO126 also inhibited MMP-1 secretion in response to transfection with 7bqs *cagA* (65 ± 15% inhibition *versus* 7bqs alone, $p = 0.006$). Finally, we tested whether, in this system, p38 has a regulatory role in MMP-1 secretion. Consistent with our observations in AGS cells incubated with *H. pylori*, the p38 inhibitor SB203580 enhanced MMP-1 stimulation in response to transfection with *cagA* 147A or 147C (Fig. 6, *A* and *B*), as well as 7bqs (224 ± 72% increase over 7bqs transfection alone, $p = 0.008$). These findings indicate that the presence of CagA within a gastric epithelial cell is sufficient to induce ERK activation and MMP-1 secretion, whereas p38 does not mediate MMP-1 secretion, and may inhibit both CagA-induced ERK activity and MMP-1.

DISCUSSION

H. pylori gastric colonization stimulates inflammatory, erosive, and neoplastic processes (62). That *H. pylori* strains lacking *cagA* induce less inflammation (63), and are less commonly associated with both peptic ulceration, (19, 64) and gastric adenocarcinoma (18, 64), suggests that inflammation and these processes may be linked. Although the mechanisms by which *H. pylori* induces ulceration are not well understood, the ability of *H. pylori* to stimulate gastric cell secretion of various MMPs (24–26, 65–68) suggests that *H. pylori* induces stromal destruction and host cell de-adherence via MMP-mediated degradation of collagens and other connective tissue proteins. These processes may also be important in invasive neoplasia (69). The strong association of *cagA* with both ulcerogenesis and adenocarcinoma led us to hypothesize that the *cagA* product may drive host cell MMP secretion.

We observed that co-culture with *H. pylori* stimulated AGS cell secretion of MMP-1, an important MMP identified within ulcer lesions (28, 70). Because type I and III collagens predominate in gastric mucosa (29, 44), and both are susceptible to MMP-1, (45), these data suggest a mechanism for *H. pylori*-induced gastric stromal destruction. Blood vessels in the gastric lining contain types I and III collagen, as well as type II (also MMP-1-sensitive) (71); MMP-1 secretion may therefore also participate in gastric hemorrhage observed in *H. pylori*-induced ulcers. The ability of microbes to regulate host cell metalloproteinase secretion to promote tissue breakdown is not limited to *H. pylori*. *Borrelia burdorferi*, the pathogen causing Lyme disease, stimulates MMP-1 and -3 secretion from chon-

drocytes, and MMP-2 secretion from dermal fibroblasts and neurons, consistent with a role for MMP secretion in end-organ damage (72–74).

Our data indicate that *H. pylori* stimulates MMP-1 secretion by both *cagI*-independent and -dependent processes. That strain Tx30a, which lacks the *cagI*, nevertheless induced MMP-1 secretion, confirms that *H. pylori* co-culture is sufficient for *cagI*-independent MMP-1 induction. The *cagI* participates to maximize MMP-1 secretion, however, because extracellular MMP-1 concentrations increased significantly when AGS cells were co-cultured with *cagI*$^+$ strains 147A or 147C. Once inside the host cell, most CagA proteins, including that from 147C, are susceptible to c-Src/Lyn kinase-mediated phosphorylation of a tyrosine residue within a C-type EPIYA motif (10, 13). Co-culture of AGS cells with *H. pylori* strain 147C induced MMP-1 secretion greater than that of 147A (expressing CagA lacking the C-type EPIYA motif, but otherwise identical to that of 147C (47)), implicating intracellular CagA phosphorylation in maximal MMP-1 secretion.

Although *H. pylori*-stimulated gastric epithelial cell ERK activation has been reported, studies have differed regarding its CagA dependence (37–40). Our data indicate both CagA-independent and -dependent processes. The early, transient ERK activation following co-culture with either *cagA*$^+$ or *cagA*$^-$ strains is CagA-independent; its rapidity probably indicates outside-in signaling via bacterial/host receptor interactions. *Pseudomonas aeruginosa* rapidly and transiently activates ERK in human cells via contact-dependent ligation of host cell asialo-GM1 receptors and intracellular calcium transients (75); whether a similar mechanism applies to *H. pylori* remains to be determined. A second, delayed ERK activation peak was induced only by *cagA*$^+$ *H. pylori* strains, and greatly increased in the presence of the CagA tyrosine phosphorylation site, indicating that contact with the *cagI*-encoded structures is necessary, and that CagA phosphorylation is required for the maximal effect.

Our data also delineate differences in the signaling pathways through which *H. pylori* strains induce the early and delayed phases of ERK activation. The canonical pathway for ERK activation, elaborated in response to EGFR and other protein-tyrosine kinase receptors, involves protein-tyrosine kinase receptor recruitment of Grb/SOS to the plasma membrane, leading to Ras, Raf, MEK, and finally ERK activation. *H. pylori*-induced ERK activation after 2 h of incubation with CagA-positive strains appears to replicate this process, because a 2-h incubation with 147A and 147C strains resulted in activation of all three upstream signaling elements (Ras, Raf, and MEK). These observations are consistent with a previous suggestion that *H. pylori* may stimulate ERK activation via transactivation of the EGFR (76). Consistent with the inability of strain Tx30a to stimulate late-phase ERK activation, Tx30a had no effect on Ras, Raf, or MEK activation at the 2-h time point. In contrast, a 15-min exposure of AGS cells to Tx30a, as well as 147A and 147C, each resulted in activation of Raf and MEK, consistent with CagA-independent activation of these molecules. Neither Tx30a, 147A, nor 147C induced Ras activation at the 15-min time point, however, indicating that early activation of the ERK signaling pathway depends upon a mechanism that bypasses Ras activation.

To determine whether the additional ERK activation and MMP-1 secretion observed in response to strains 147A and 147C were due strictly to CagA, or required other *cagI*-encoded structures, *cagA* transfection was studied. Transfection with either 147A or 147C *cagA* resulted in MMP-1 secretion, indicating that intracellular CagA alone is sufficient to stimulate MMP-1 secretion. Consistent with the co-culture studies, the greater effect of 147C transfection in stimulating MMP-1 secretion indicated a regulatory role for CagA phosphorylation. Because the presence of an additional C-type EPIYA phosphorylation site in strain 7bqs CagA did not further induce MMP-1 secretion relative to 147C, CagA phosphorylation likely regulates specific interaction(s) between CagA and targets, rather than nonspecific net charge effects. In contrast, morphologic alterations in AGS cells induced by CagA appear proportional to the number of C-type EPIYA sites present (47, 77). Because 7bqs *cagA* has other differences from 147A and 147C (47), the effect on MMP-1 secretion of multiple CagA phosphorylation sites requires further examination. Our data appear to differ from those of a recent report in which the authors concluded that MMP-1 secretion induced by *H. pylori* is CagA-independent (78). However, close review of the data presented in that report suggests that, in fact, a *cagA*-deficient *H. pylori* strain induced less MMP-1 secretion relative to a *cagA*$^+$ strain, consistent with our current observations.

Transfection with *cagA* constructs is a non-physiologic system for studying ERK activation, because in this system CagA accumulates gradually, whereas receptor-mediated ERK activation is a rapid, typically transient process. Physiologic CagA injection by *H. pylori* also is likely to cause more rapid CagA accumulation (79), and therefore stimulate ERK more rapidly, than transfection. Nevertheless, *cagA* transfection induced transient ERK activation (C147A), paralleling MMP-1 secretion, and increasing confidence in the validity of this system.

Both AGS cell ERK activation and MMP-1 secretion, in response to either *H. pylori* co-culture or *cagA* transfection, were inhibited by MEK inhibitors, confirming that MMP-1 secretion in these cells is mediated via MEK/ERK signaling (23). Because MEK inhibitors reduced MMP-1 secretion in response to co-culture with either *cag* negative strain Tx30, or *cag* positive strains 147A or 147C, ERK regulates both CagA-independent and -dependent MMP-1 secretion. Because 1) phosphorylated CagA activates host cell SHP-2 (11), and 2) SHP-2 expression knockdown by small interfering RNA abolishes ERK activation in AGS cells stably expressing an EPIYA-positive CagA (80), both CagA-induced ERK activation and MMP-1 secretion likely are SHP-2-mediated. That MEK inhibition also regulates ERK activity and MMP-1 expression in rheumatoid synovial fibroblasts (36) and osteoarthritis chondrocytes (81) indicates that ERK pathway regulation of MMP-1 secretion is a conserved signaling pathway in cells involved with inflammation and erosion. MEK inhibition also reduces ERK activation, MMP-1 secretion, and collagenolysis, in invasive melanoma cells embedded in Type I collagen matrices (53). The mechanisms by which ERK regulates gastric cell MMP-1 secretion have not been determined, but ERK signaling in other cells reg-

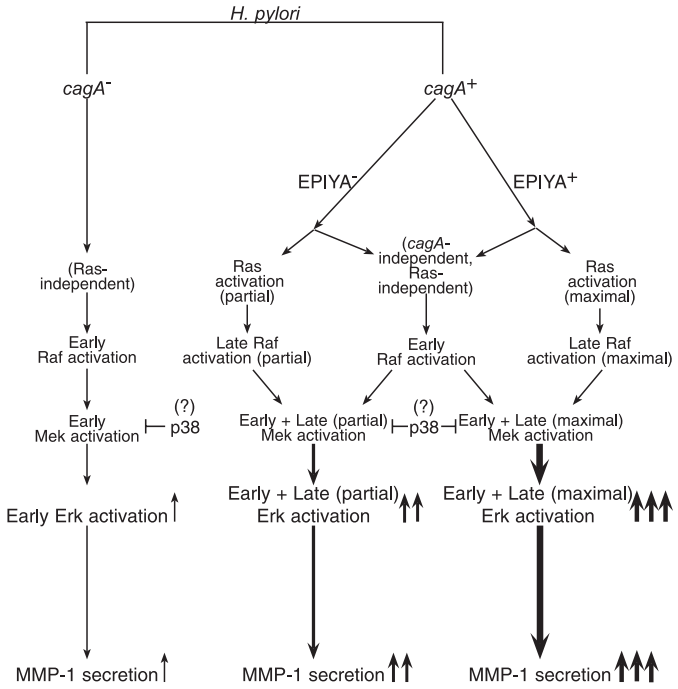

FIGURE 7. **Regulation of gastric cell MMP-1 secretion by *H. pylori*.** *Left*, *CagA*$^-$ *H. pylori* cells stimulate early ERK activation in gastric epithelial cells by a CagA-independent, Ras-independent, Raf- and MEK-dependent mechanism, resulting in MMP-1 secretion. *Right*, *CagA*$^+$ *H. pylori* strains activate the early pathway, but additionally stimulate a later, Ras-, Raf-, and MEK-dependent ERK activation event via CagA injection into host cells. The combination of CagA-independent and -dependent ERK activation results in increased MMP-1 secretion in *cagA*$^+$ strains. The presence of a type C EPIYA tyrosine phosphorylation motif on CagA results in maximal ERK activation in, and maximal MMP-1 secretion from, gastric cells exposed to *cagA*$^+$, EPIYA$^+$ *H. pylori* strains, relative to *cagA*$^+$, EPIYA$^-$ strains. The differing proportions in a population of these three prototypic, *cagA* genotype-distinct *H. pylori* cells, may regulate net MMP-1 secretion in *H. pylori*-infected individuals.

ulates MMP-1 secretion via AP-1 and ETS transcriptional activation (82).

Both ERK activation and MMP-1 secretion were enhanced in response to p38 inhibitors, suggesting that p38 tonically suppresses *H. pylori*-stimulated MMP-1 secretion by inhibiting ERK. Even absent other stimulation, p38 inhibition induces AGS cell ERK activation and MMP-1 secretion, effects that are abrogated by MEK/ERK inhibitors (23). These data differ from reports that p38 mediates MMP-1 secretion in dermal fibroblasts (57–59), but are consistent with the effects of p38 inhibitors on MMP-1 secretion from cytokine-stimulated AGS cells (23), as well as pancreatic cancer cells (83); p38 regulation of ERK and MMP-1 secretion is therefore cell type-specific. The observed ability of p38 inhibitors to enhance ERK activation and MMP-1 secretion must be interpreted with caution, because some investigators have suggested that SB203580 at 10 μM, but not $\leq$1 μM, may nonspecifically activate c-Raf, leading to ERK activation (61, 84). However, we observed SB203580 enhancement of ERK and MMP-1 at concentrations as low as 1.0 μM, suggesting an effect specific to p38, and consistent with a report that SB203580 activates ERK by inhibiting p38 specifically (85). Interestingly, lower concentrations of SB203580 inhibited MMP-1 but not ERK, suggesting that p38 may regulate MMP-1 by both ERK-dependent and -independent effects. Because p38 facilitates gastric and oral mucosal healing (86, 87), enhancement of gastric epithelial cell MMP-1 secretion by p38 inhibitors suggests that these agents, currently under investigation for rheumatoid arthritis (88), may prove ulcerogenic. However, p38 inhibitors also suppress cytokine-stimulated AGS cell secretion of MMP-13 (23, 89), indicating that the overall effects of p38 on gastric stroma will depend upon multiple regulatory effects.

In conclusion, our studies confirm that *H. pylori* cells stimulate gastric epithelial MMP-1 secretion, but vary in their ability to do so according to their particular *cagA* status; variations in *H. pylori* populations within an individual host will determine the net effect on MMP-1 secretion (Fig. 7). Both CagA-dependent and -independent MMP-1 secretion are regulated by ERK (via divergent signaling pathways), and probably inhibited by p38. The ability of *H. pylori* to activate gastric MMP-1 secretion via ERK activation provides a mechanism relevant to ulcerogenesis, and possibly neoplasia, and suggests strategies through which *H. pylori*-induced gastric damage might be ameliorated.

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Mechanisms of Signal Transduction:

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