Differential Activation of Mitogen-Activated Protein Kinases in AGS Gastric Epithelial Cells by *cag*$^+$ and *cag*$^-$ *Helicobacter pylori*[1]

Sarah Keates,[2]* Andrew C. Keates,* Michel Warny,* Richard M. Peek, Jr.,† Paul G. Murray,‡ and Ciarán P. Kelly*

The aim of this study was to determine whether *Helicobacter pylori* activates mitogen-activated protein (MAP) kinases in gastric epithelial cells. Infection of AGS cells with an *H. pylori cag*$^+$ strain rapidly (5 min) induced a dose-dependent activation of extracellular signal-regulated kinases (ERK), p38, and c-Jun N-terminal kinase (JNK) MAP kinases, as determined by Western blot analysis and in vitro kinase assay. Compared with *cag*$^+$ strains, *cag*$^-$ clinical isolates were less potent in inducing MAP kinase, particularly JNK and p38, activation. Isogenic inactivation of the *picB* region of the *cag* pathogenicity island resulted in a similar loss of JNK and p38 MAP kinase activation. The specific MAP kinase inhibitors, PD98059 (25 μM; MAP kinase kinase (MEK-1) inhibitor) and SB203580 (10 μM; p38 inhibitor), reduced *H. pylori*-induced IL-8 production in AGS cells by 78 and 82%, respectively ($p < 0.01$ for each). Both inhibitors together completely blocked IL-8 production ($p < 0.001$). However, the MAP kinase inhibitors did not prevent *H. pylori*-induced IκBα degradation or NF-κB activation. Thus, *H. pylori* rapidly activates ERK, p38, and JNK MAP kinases in gastric epithelial cells; *cag*$^+$ isolates are more potent than *cag*$^-$ strains in inducing MAP kinase phosphorylation and gene products of the *cag* pathogenicity island are required for maximal MAP kinase activation. p38 and MEK-1 activity are required for *H. pylori*-induced IL-8 production, but do not appear to be essential for *H. pylori*-induced NF-κB activation. Since MAP kinases regulate cell proliferation, differentiation, programmed death, stress, and inflammatory responses, activation of gastric epithelial cell MAP kinases by *H. pylori cag*$^+$ strains may be instrumental in inducing gastroduodenal inflammation, ulceration, and neoplasia. ***The Journal of Immunology*, 1999, 163: 5552–5559.**

Helicobacter pylori chronically infects over half the world's population, but in the majority of cases, infection results only in asymptomatic chronic active gastritis (1). Symptomatic gastroduodenal disease, specifically peptic ulceration or gastric neoplasia, develops in only approximately 10% of infected individuals (2–4). The host's immune and inflammatory response to bacterial virulence factors is likely to play a critical role in determining the clinical outcome of *H. pylori* infection (5, 6). In this study we examine the ability of *H. pylori* to activate mitogen-activated protein (MAP)[3] kinases, key elements in the regulation of cellular responses to external inflammatory and proliferative signals. We find that different strains of *H. pylori* vary in their ability to activate MAP kinase pathways in AGS gastric epithelial cells. The differential activation of MAP kinase and other host cellular signaling pathways is a possible mechanism for strain-specific variations in the outcome of gastric *H. pylori* infection.

MAP kinases are a family of ubiquitous, highly conserved, cell signaling molecules (7–9). Upon activation by upstream kinases, MAP kinases phosphorylate downstream kinases and/or mediators, including transcription factors. MAP kinases can be activated by a wide variety of extracellular stimuli and transmit signals from the cell surface to the nucleus to regulate gene expression. Key cellular functions that are regulated at least in part by MAP kinase signaling include cell proliferation, cell survival, and cytokine production. Three main groups of MAP kinases have been characterized to date: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 MAP kinases (7–9). These MAP kinase subfamilies form three parallel cascades that can be activated simultaneously or independently. ERK MAP kinases are strongly activated by growth factors and phorbol ester, but weakly by inflammatory stimuli. In contrast, JNK and p38 MAP kinases are stimulated by inflammatory cytokines and stress stimuli, but minimally by growth factors.

We and others have shown previously that *H. pylori* infection activates IL-8 gene expression in gastric epithelial cells in vitro and in vivo (10–16). IL-8 mRNA and protein levels are increased in the gastric mucosa of patients with *H. pylori* gastritis, and immunohistochemical studies demonstrate increased IL-8 protein in gastric epithelial cells from infected individuals (11, 13, 16, 17). IL-8 is a potent neutrophil-activating chemotactic cytokine or chemokine. Thus, IL-8 release by infected gastric epithelial cells may be instrumental in regulating neutrophil infiltration of the gastric mucosa in *H. pylori* gastritis. *H. pylori* also increases IL-8 mRNA

*Gastroenterology Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; †Division of Gastroenterology, Vanderbilt University Medical Center, Nashville, TN 37232; and ‡School of Health Sciences, University of Wolverhampton, Wolverhampton, United Kingdom

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[2] Address correspondence and reprint requests to Sarah Keates, Dana 601, Gastroenterology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. E-mail address: ciaran_kelly@caregroup.harvard.edu

[3] Abbreviations used in this paper: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; MEKK1, MAP kinase kinase kinase; NIK, NF-κB-inducing kinase; EGF, epidermal growth factor; MEK-1, MAP kinase kinase.

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levels and protein production in cultured monolayers of AGS and other gastric epithelial cell lines (10, 12, 13, 15, 18). Contact with the bacterium initiates epithelial cell signaling events that result in activation of the transcription factor NF-κB (10, 14, 15, 19–22). Activated NF-κB then translocates to the nucleus where it upregulates IL-8 gene transcription. NF-κB activation occurs within minutes of *H. pylori* infection of AGS gastric epithelial cells in vitro, and activated NF-κB is also evident in gastric epithelial cells from patients with *H. pylori* gastritis (10, 14, 15, 19, 22).

The *cag* pathogenicity island of *H. pylori* is a 40-kb region immediately upstream to the *cagA* gene that encodes over 40 putative bacterial proteins (12). Based on sequence homology, *cag* region gene products appear to constitute a bacterial secretion system that may be involved in the export or surface expression of bacterial virulence factors. In the developed world, approximately 70% of clinical *H. pylori* isolates carry the *cag* pathogenicity island. Carriage of cag^+ strains has been associated in most published studies with more severe gastritis and a greater risk for peptic ulceration and gastric cancer than *H. pylori* cag^- infection (6, 12, 23–26). Gene products of the *cag* pathogenicity island are also known to participate in epithelial cell activation by *H. pylori*. *H. pylori* cag^+ strains are more potent in activating epithelial cell IL-8 production than cag^- bacteria (16, 27). Furthermore, disruption of specific *cag* region genes markedly reduces *H. pylori*-mediated tyrosine phosphorylation of gastric epithelial cell proteins, NF-κB activation, and IL-8 gene transcription (12, 14, 19, 21, 22, 28).

A number of recent studies implicate MAP kinases as upstream mediators of NF-κB activation and cytokine gene expression (9, 29–34). This led us to examine whether MAP kinases participate in regulating *H. pylori*-induced IL-8 production by gastric epithelial cells. The aims of this study were to determine whether *H. pylori* activates MAP kinases in AGS gastric epithelial cells, whether MAP kinase activation is required for *H. pylori*-mediated NF-κB activation and IL-8 production, and whether cag^+ and cag^- strains of *H. pylori* differ in their ability to activate epithelial cell MAP kinases.

Materials and Methods

AGS and MKN-28 gastric epithelial cell lines

AGS gastric epithelial cells (American Type Culture Collection, Manassas, VA) were grown in Ham's F-12 medium (pH 7.4; Sigma, St. Louis, MO) supplemented with 10% FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (15). MKN-28 cells (provided by R. Peek, Jr., Nashville TN) were grown in DMEM with 10% FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell culture experiments were conducted in 6-, 12-, or 96-well polypropylene tissue culture plates (Corning Costar, Cambridge, MA).

H. pylori *strains, clinical isolates, and isogenic mutants*

H. pylori were plated onto *Brucella* agar supplemented with 5% horse blood (BBL, Becton Dickinson Microbiology, Cockeysville, MD) and incubated at 37°C in a microaerophilic environment (15, 35). After 3–7 days the bacteria were harvested into pyrogen-free Dulbecco's PBS (Cellgro, Mediatech, Herndon,VA). The bacteria were pelleted by centrifugation at 4000 × *g* for 10 min, and bacterial numbers were determined by resuspension in PBS to an $OD_{600\ nm}$ of 1.5, corresponding to 3.6×10^8 CFU/ml as described previously (15). Defined numbers of bacteria were then resuspended in antibiotic-free Ham's F-12 medium. Unless otherwise stated experiments were performed using the $cagA^+$ and $vacA^+$ *H. pylori* strain 43504 (American Type Culture Collection) (15).

H. pylori clinical strains were isolated from gastric mucosal biopsies obtained during upper gastrointestinal endoscopy as previously described (16). The presence of *cagA* was determined by PCR of bacterial genomic DNA and vacuolating cytotoxin activity was determined by neutral red assay (16, 36). All of the cag^+ clinical isolates used in this study were toxigenic, while the cag^- strains did not produce a functional cytotoxin. Isogenic *H. pylori* mutants lacking the *picB* or *cagA* genes were also studied together with their parental cag^+, toxigenic, wild-type strain (no. 60190) (28). The *H. pylori* clinical isolates, strain 60190, and the $picB^-$ and $cagA^-$ mutants were obtained from the culture collection of the Vanderbilt University Campylobacter and Helicobacter Laboratory (Nashville, TN) and have been described previously (16, 28).

H. pylori filtrate was prepared by suspending the bacteria in antibiotic-free medium for 30 min at room temperature, pelleting the bacteria at 4000 × *g* for 10 min and then filtering the medium through a 0.2-μm pore size filter (Acrodisc, Gelman, Ann Arbor, MI). In some experiments *H. pylori* were heat treated for 30 min by boiling or were treated with 2 or 20 μg/ml chloroamphenicol (Amersham, Arlington Heights, IL) for 60 min at 37°C.

Western blot analysis of phospho-specific MAP kinase activation

AGS cells were grown to confluence on twelve-well plates and maintained in serum-free medium for 24 h before the experiment. Cells were fed with fresh serum-free medium 2 h before stimulation. At the end of the experiment the monolayers were washed three times with PBS and lysed with SDS buffer (containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.1% (w/v) bromophenol blue). Samples were then sonicated, heated to 100°C for 5 min, and loaded onto a 10% SDS-PAGE gel. After running the gel, the proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked for 3 h at room temperature with a 5% (w/v) solution of dried milk in Tris-buffered saline (pH 7.4). This was followed by an overnight incubation at 4°C with the phospho-specific MAP kinase Abs diluted 1/1000 in blocking buffer. The membranes were then washed three times with Tris-buffered saline and incubated at room temperature for 1 h with peroxidase-conjugated goat anti-rabbit IgG (1/2000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). A SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) was used for detection.

Phospho-specific p44/p42 MAP kinase Ab was used to detect ERK1/2. This Ab detects p44 and p42 MAP kinase (ERK1 and ERK2) only when they are catalytically activated by phosphorylation at Thr^{202} and Tyr^{204}. Phospho-specific p38 MAP kinase Ab was used to detect p38 activated by phosphorylation at Thr^{180} and Tyr^{182}. Phospho-specific p54/p46 MAP kinase Ab was used to detect JNK. This Ab detects p54 and p46 MAP kinase only when they are activated by phosphorylation at Thr^{183} and Tyr^{185}. All three Abs were obtained from New England Biolabs (Beverly, MA). Non-phosphospecific Abs to ERK, p38, and JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Controls for these Western blot experiments consisted of AGS cells harvested at the zero time point for kinetic experiments or cultured in the absence of *H. pylori* and harvested at the same time as the test cells (usually after 1 h of incubation) for all other experiments.

In vitro kinase assay

Confluent monolayers of AGS cells were lysed in 1 ml of lysis buffer (150 mmol NaCl, 1% Nonidet P-40, 0.8 mmol $MgCl_2$, 5 mmol EGTA, 1 mmol Na_3VO_4, 15 μg/ml leupeptin, 1 mmol PMSF, and 50 mmol HEPES, pH 7.5). ERK2 (p42), p38, or JNK1 (p46) MAP kinases were precipitated with 2 μg of rabbit specific IgG (Santa Cruz Biotechnology). After incubation for 2 h at 4°C, 20 μl of protein G-Sepharose (Santa Cruz Biotechnology) were added, and samples were further incubated for 1 h. Immunopellets were washed twice in lysis buffer, twice in kinase buffer (30 mM NaCl, 0.1 mM Na_3VO_4, 2 mM DTT, 20 mM $MgCl_2$, and 30 mmol HEPES, pH 7.5.) and then resuspended in 40 μl of kinase buffer. The kinase reaction was started by addition of 20 μM ATP, 100 μCi/ml [γ-^{32}P]ATP (DuPont-NEN, Boston, MA), and 10 μg myelin basic protein (Sigma) as substrate for ERK and p38, and 2 μg of GST c-Jun_{1-79} (Stratagene, La Jolla, CA) as substrate for JNK1. Samples were subjected to SDS-PAGE (12%) and analyzed by autoradiography.

Treatment of AGS cells with specific MAP kinase inhibitors

In some experiments AGS cells were treated with the specific p38 MAP kinase inhibitor SB203580 (10 μM; Calbiochem, La Jolla, CA) for 30 min before exposure to *H. pylori* and during the incubation period of the experiment (37). A similar protocol was used for experiments using the MAP kinase inhibitor PD98059 (25 μM; Calbiochem). PD98059 blocks ERK1/2 activation by specifically inhibiting MEK1, the kinase that catalyzes ERK1/2 phosphorylation (38).

IL-8 protein levels in AGS cell-conditioned medium were measured by ELISA as previously described (15, 35, 39). Statistical analyses were performed using SigmaStat for Windows version 2.0 (Jandel Scientific Software, San Rafael, CA). Unless stated otherwise, ANOVA followed by protected *t* tests were used for intergroup comparisons.

Western blot analysis of IκBα degradation

AGS cells were pretreated with the specific MAP kinase inhibitors PD98059 (25 μM) and/or SB203580 (10 μM) for 30 min preceding infection with *H. pylori* (10^8 bacteria/ml). Incubation of AGS cells with *H. pylori* was maintained for 1 h in the presence of the inhibitors, after which time the cells were washed three times with PBS and lysed. Equal amounts of protein were loaded onto a 10% SDS-PAGE, the gel was run, and the proteins were then transferred to nitrocellulose membranes. The blots were blocked for 1 h in a 5% (w/v) solution of dried milk in Tris-buffered saline solution containing 0.1% Tween-20, followed by immunoblotting with a rabbit polyclonal Ab against IκBα (Santa Cruz Biotechnology) at a 1/1000 dilution. After washing the blot three times with Tris-buffered saline, a peroxidase-conjugated goat anti-rabbit Ab (Jackson ImmunoResearch Laboratories) at a 1/2000 dilution was applied. Immunoreactive bands of the 36-kDa IκBα protein were detected using SuperSignal chemiluminescent substrate.

EMSA for NF-κB activation

AGS cells were pretreated for 30 min with the MAP kinase inhibitors PD98059 (25 μM) and/or SB203580 (10 μM). The AGS monolayers were then infected with *H. pylori* for 1 h. To prepare nuclear extracts AGS cells were washed three times with ice-cold PBS and scraped in ice-cold TNE buffer (40 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, and 1 mmol/L EDTA). Cells were pelleted by centrifugation and then resuspended in 400 μL of buffer A (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol-bis(B-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid, and 0.5 mmol/L PMSF), and incubated on ice for 10 min. Nonidet P-40 (23 μl of a 10% solution) was added, and the cells were incubated for an additional 2 min on ice. After centrifugation at 12,000 × *g* for 5 s, the nuclear pellet was resuspended in 85 μl of buffer B (20 mmol/L HEPES (pH 7.9), 0.42 mol/L KCl, 1 mmol/L EDTA, 1 mmol/L ethylene glycol-bis(B-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid, and 0.1 mmol/L PMSF) and incubated on ice for 30 min. After a further centrifugation at 12,000 × *g*, for 2 min, the supernatants were recovered as nuclear extracts that were immediately frozen on dry ice and stored at −80°C.

Single-stranded complementary oligonucleotides bearing the human IL-8 gene NF-κB site were prepared by custom oligonucleotide synthesis (Genosys Biotechnologies, The Woodlands, TX). After annealing, 100 ng of the double-stranded oligonucleotide was labeled in a Klenow fill-in reaction in the presence of [α-^{32}P]dCTP. The probe was then purified on a Sephadex G-25 spin column (Boehringer Mannheim, Indianapolis, IN) and diluted to yield approximately 15,000 cpm and 0.1 ng of DNA/μL. Binding reactions (20 μl) contained 0.1 ng (~15,000 cpm) of double-stranded probe, 5–12 μg of extracted protein (the protein concentration of the samples were adjusted to ensure equal loading), 2 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ), 10 mmol/L 2-ME, and 1% Ficoll. After first incubating the protein extracts for 10 min at room temperature, the radiolabeled probe was added. After an additional 30 min at room temperature, the reaction mixtures were then loaded on a nondenaturing 6% polyacrylamide gel in 0.2 mol/L glycine, 25 mmol/L Tris-HCl, and 1 mmol/L EDTA. The gel was run, dried, and exposed to autoradiography film for 6–18 h at −80°C with an intensifying screen.

Supershift assays with anti-NF-κB p50 (sc-114), and anti-NF-κB p65 (sc-109; both from Santa Cruz Biotechnology) were performed to confirm the identity of the complexes binding the IL-8 promoter κB probe. Each Ab (2 μg) was added to the DNA probe at the start of the 30-min incubation.

Results

H. pylori *activates MAP kinases in AGS gastric epithelial cells*

Confluent monolayers of AGS gastric epithelial cells were infected with *H. pylori* strain 43504 at bacterial densities ranging from 10^6-10^8/ml. After 1 h cell lysates were prepared and examined by Western blot analysis using phospho-specific Abs to ERK1/2, p38, and JNK isoforms p54 and p46. Control cells showed low or undetectable levels of activated MAP kinases (Fig. 1*A*). Contact with *H. pylori* resulted in a marked and dose-dependent increase in the phosphorylation of all three MAP kinases. In the case of ERK, two immunoreactive bands are evident representing phosphorylated p44 (ERK1, *upper band*) and p42 (ERK2, *lower band*) MAP kinases. For JNK two immunoreactive bands are again evident representing phosphorylated p54 (*upper band*) and p46 (*lower band*) MAP kinases.

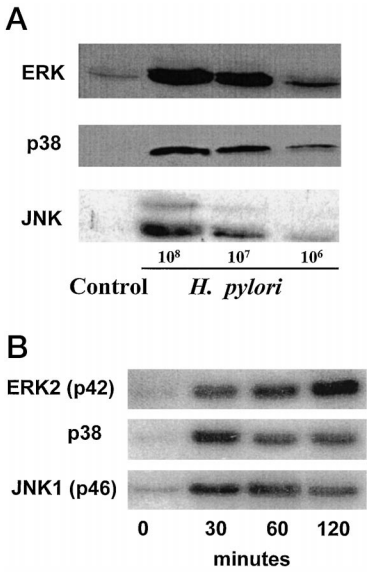

FIGURE 1. *H. pylori* activates the MAP Kinases ERK, p38, and JNK in AGS gastric epithelial cells. *A*, AGS monolayers were cultured alone (Control) or with *H. pylori* (10^8-10^6 bacteria/ml). After 1 h cell lysates were prepared and subjected to Western blot analyses using phospho-specific Abs against ERK1 (p44, *upper band*) and ERK2 (p42, *lower band*), p38 and JNK p54 (*upper band*), and p46 (*lower band*) MAP kinases. *B*, AGS monolayers were infected with *H. pylori* (10^8 bacteria/ml). At varying time points cell lysates were prepared, and ERK2 (p42), p38, and JNK1 (p46) kinase activity was measured by immunoprecipitation and in vitro kinase assay.

Activation of AGS cell MAP kinases by *H. pylori* was confirmed using in vitro kinase assays. As shown in Fig. 1*B* the kinase activities of ERK2, p38, and JNK1 in cell extracts were each markedly increased within 30 min of *H. pylori* inoculation of AGS monolayers.

Intact H. pylori *are required to activate MAP kinases in AGS cells*

We and others have reported that contact with intact bacteria is required for *H. pylori*-mediated activation of the transcription factor NF-κB in gastric epithelial cells (10, 15, 18, 20, 21). We next examined whether contact with *H. pylori* was also required for epithelial cell MAP kinase activation. As shown in Fig. 2 *H. pylori* that were killed by heat treatment for 30 min at 100°C were no longer capable of MAP kinase induction. Soluble factors contained in a cell-free *H. pylori* filtrate also failed to activate epithelial cell MAP kinases. Pretreatment of *H. pylori* with chloramphenicol, a bacteriostatic agent that inhibits bacterial protein synthesis, had no apparent inhibitory effect on MAP kinase phosphorylation. These data demonstrate that MAP kinase activation requires live, intact bacteria and does not result from soluble factors present in bacterial culture filtrate. However, de novo bacterial protein synthesis is not required.

H. pylori *cag*$^+$ *and cag*$^-$ *strains induce different patterns of MAP kinase activation in AGS gastric epithelial cells*

Fig. 3*A* illustrates the time course of MAP kinase activation following gastric epithelial cell contact with *H. pylori*. Phosphorylation of ERK1/2 and p38 are clearly evident within 5 min of *H. pylori* inoculation. Both JNK isoforms are activated within 30 min. Phosphorylation of ERK1/2, p38, and JNK appears to be maximal at 30–60 min.

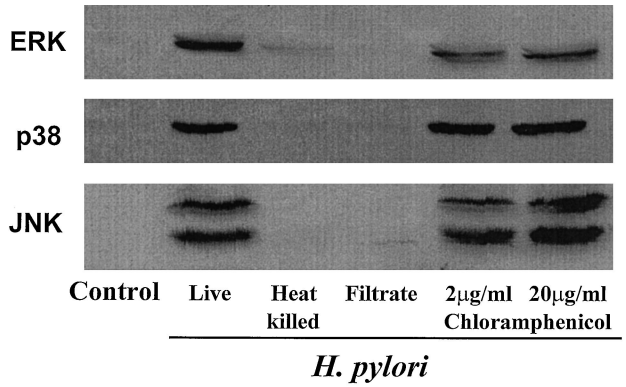

FIGURE 2. Contact with intact bacteria is required for *H. pylori*-mediated MAP kinase activation in gastric epithelial cells. AGS monolayers were incubated alone (Control) or with live *H. pylori* (10^8 bacterial/ml), heat-killed *H. pylori* (100°C for 30 min), a sterile *H. pylori* filtrate, or bacteria that had been pretreated for 60 min with chloramphenicol (2 or 20 μg/ml). Epithelial cell lysates were prepared after 1 h of incubation and analyzed by Western blot using phospho-specific MAP kinase Abs. These experiments were performed on three occasions, and a representative blot is shown.

H. pylori strains that carry the *cag* pathogenicity island (*cag*$^+$) show greater potency in activating gastric epithelial cell inflammatory responses (12, 14, 16, 18, 21, 22, 27, 28, 40). Infection with *cag*$^+$ strains may also be associated with a greater risk for symptomatic gastroduodenal disease (6, 12, 23–26). This led us to determine whether *cag*$^+$ and *cag*$^-$ strains of *H. pylori* differ in their ability to activate epithelial cell MAP kinases. As shown in Fig. 3*A* incubation of AGS cells with the *cag*$^+$ *H. pylori* strain 43504 resulted in marked activation of ERK1/2, p38, and JNK. Incubation with the same concentration of a *cag*$^-$ *H. pylori* (strain J68) again resulted in the rapid activation of ERK1/2 (Fig. 3*B*). However, the intensity of ERK1/2 phosphorylation was less than that observed with the *cag*$^+$ strain. The *cag*$^-$ isolate also induced far less phosphorylation of p38 and very little phosphorylation of JNK compared with the *cag*$^+$ strain.

The differences in MAP kinase activation induced by strains 43504 and J68 led us to study a panel of *cag*$^+$ and *cag*$^-$ *H. pylori* clinical isolates. As illustrated in Fig. 4 all three of the *cag*$^+$ *H. pylori* isolates induced a marked activation of the MAP kinases ERK1/2, p38 and JNK. Each of the three *cag*$^-$ *H. pylori* isolates was less potent in inducing MAP kinase activation. Furthermore, a consistent pattern of differential MAP kinase phosphorylation was observed with the three *cag*$^-$ strains; ERK1/2 phosphorylation was somewhat less intense than with the *cag*$^+$ strains, p38 phosphorylation was substantially less, and there was minimal phosphorylation of JNK.

The product of the picB region of the cag pathogenicity island of H. pylori *participates in MAP kinase activation in AGS gastric epithelial cells*

Having found distinct patterns of MAP kinase activation by *cag*$^+$ and *cag*$^-$ *H. pylori* clinical isolates, we next examined the participation of specific *cag* pathogenicity island gene products in this process. The *picB* region of the *cag* pathogenicity island is known to be involved in gastric epithelial cell NF-κB activation and IL-8 up-regulation (12, 14, 22, 28). As shown in Fig. 5 the *cag*$^+$ reference strain 60190 (wild type) induced the phosphorylation of ERK1/2, p38, and JNK in AGS gastric epithelial cells as observed with other *cag*$^+$ *H. pylori*. The *cagA*$^-$ isogenic mutant was similar to the wild type in terms of its ability to activate epithelial cell MAP kinases apart from a possible reduction in ERK1/2 phosphorylation. However, the *picB*$^-$ isogenic mutant was less potent in inducing epithelial cell MAP kinase activation. Compared with the parental wild-type strain, ERK1/2 phosphorylation was only slightly reduced, p38 phosphorylation was moderately reduced, and JNK phosphorylation was markedly reduced. Thus, disruption of *picB* in this *cag*$^+$ strain resulted in a pattern of MAP kinase activation similar to that seen with *cag*$^-$ strains (see Fig. 4).

MAP kinase activation is required for IL-8 up-regulation in H. pylori*-infected AGS gastric epithelial cells*

H. pylori infection is known to stimulate gastric epithelial cell IL-8 production, and MAP kinases have been reported to regulate the upstream signaling events that control cytokine transcription. We asked, therefore, whether MAP kinase activation might be involved in *H. pylori*-induced IL-8 production by gastric epithelial cells. In these experiments we used the specific MAP kinase inhibitors SB203580, which blocks p38 kinase activity, and PD98059, which blocks MEK1 kinase activity, thereby preventing ERK1/2 phosphorylation. In control experiments, SB203580 (10 μM) inhibited p38 MAP kinase activity in *H. pylori*-infected (strain 43504; 10^8 bacteria/ml) AGS cells as demonstrated using the in vitro p38 kinase assay (data not shown). Similarly, the ability of PD98059 (25 μM) to block MEK1 kinase activity in *H. pylori*-infected AGS cells was confirmed by Western blotting using the phosphospecific ERK1/2 Ab (data not shown).

AGS cells were pretreated for 30 min with the MAP kinase inhibitors used singly or in combination. The gastric epithelial cell monolayers were then inoculated with *H. pylori* (strain 43504; 10^8

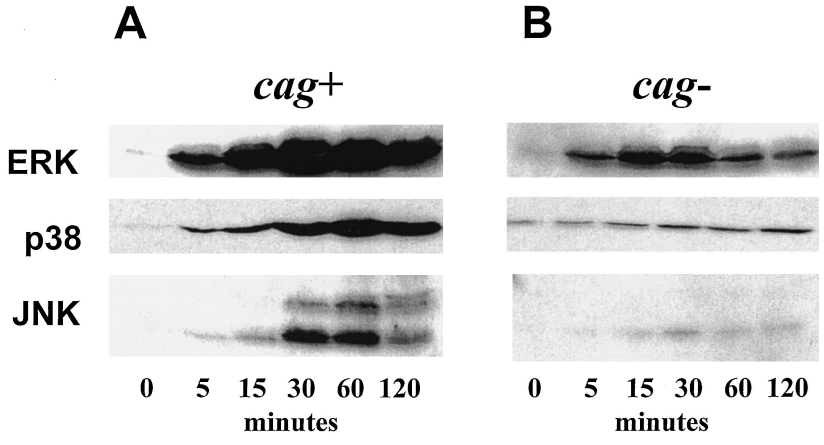

FIGURE 3. cag$^+$ and *cag*$^-$ *H. pylori* strains differ in their ability to activate MAP kinases. *A*, AGS monolayers were infected with *H. pylori cag*$^+$ strain 43504 (10^8 bacteria/ml). Cell lysates were prepared after varying incubation times and subjected to Western blot analyses using phospho-specific MAP kinase Abs. *B*, AGS monolayers were infected with *H. pylori cag*$^-$ strain J68 (10^8 bacteria/ml). Cell lysates were prepared after varying incubation times and subjected to Western blot analyses using phospho-specific MAP kinase Abs. These experiments were performed on three occasions, and representative blots are shown.

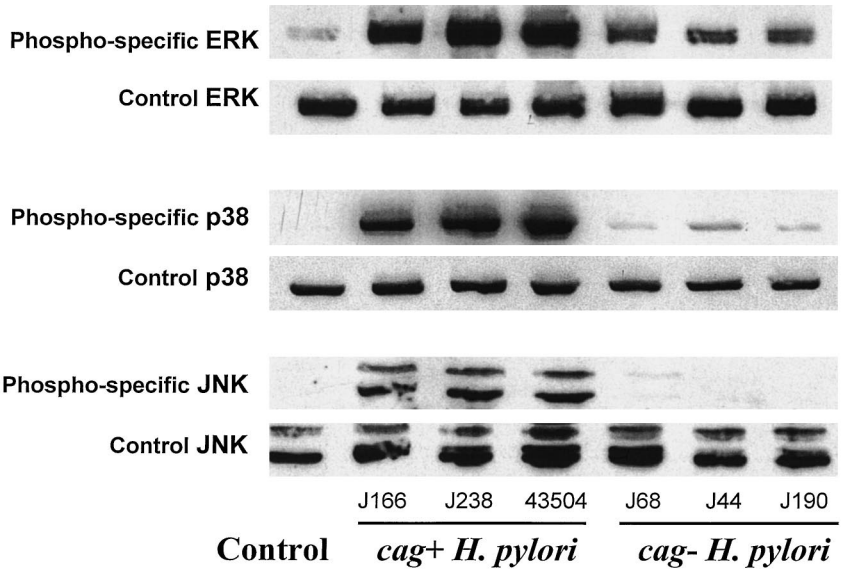

FIGURE 4. cag$^+$ and *cag*$^-$ *H. pylori* strains demonstrate a consistent pattern of differential MAP kinase activation. AGS monolayers were cultured alone (Control) or were infected with one of three *H. pylori cag*$^+$ strains (10^8 bacteria/ml; *lane 2*, J166; *lane 3*, J238; *lane 4*, 43504) or with one of three *H. pylori cag*$^-$ clinical isolates (10^8 bacteria/ml; *lane 6*, J68; *lane 7*, J44; *lane 8*, J190). After 1 h of incubation, cell lysates were prepared and then subjected to Western blot analyses using phospho-specific MAP kinase Abs (*upper panels*). Western blots performed using control Abs that recognize ERK, p38, or JNK regardless of their phosphorylation states are shown (*lower panels*). These experiments were performed on three occasions, and a representative blot is shown.

bacteria/ml). IL-8 levels were measured in the conditioned medium harvested 7 h later.

H. pylori induced a 36-fold increase in AGS cell IL-8 production (Fig. 6). The p38 inhibitor SB303580 reduced epithelial cell IL-8 production by 82% ($p < 0.001$), the MEK1 inhibitor PD98059 reduced IL-8 production by 78% ($p < 0.001$), and a combination of the two inhibitors completely abrogated *H. pylori* stimulation of IL-8 production (42% of control IL-8 level; $p < 0.0001$).

Using the MKN-28 gastric epithelial cell line, the p38 inhibitor reduced *H. pylori*-stimulated IL-8 production by 39% (from 251 ± 7 to 152 ± 5 pg/ml, mean ± SD; $n = 4$; $p < 0.001$), the MEK1 inhibitor by 60% (from 251 ± 7 to 100 ± 14 pg/ml; $n = 4$; $p < 0.001$), and a combination of the two inhibitors by 100% (from 251 ± 7 to 0 ± 8 pg/ml; $n = 4$; $p < 0.001$).

IκBα degradation and NF-κB activation are not altered by the MAP kinase inhibitors PD98059 and SB203580 in H. pylori*-infected AGS gastric epithelial cells*

Previous studies have demonstrated that *cag*$^+$ *H. pylori* activate gastric epithelial cell NF-κB, leading to up-regulation of IL-8 mRNA transcription and protein production (12, 14, 15, 22, 28). We now find that *cag*$^+$ bacteria also activate MAP kinases, leading to an increase in IL-8 production by gastric epithelial cell lines. In view of these parallel findings we next asked whether MAP kinase activation was part of the upstream signaling pathway leading to IκB kinase activation, IκB degradation, and the subsequent activation and nuclear translocation of NF-κB.

As shown in Fig. 7*A* detectable levels of IκBα were evident in control AGS gastric epithelial cells by Western blot analysis. Exposure of the gastric epithelial cell to *H. pylori* resulted in a marked reduction in IκBα levels consistent with IκBα kinase activation leading to IκBα phosphorylation and degradation. This marked reduction in IκBα levels was not prevented by pretreatment of epithelial cells with the MAP kinase inhibitors PD98059 and/or SB203580.

Fig. 7*B* shows an EMSA of nuclear extracts from AGS gastric epithelial cells examining NF-κB binding to an oligonucleotide probe containing the IL-8 promoter κB binding sequence. Nuclear extracts from control cells contained little activated NF-κB (*lane*

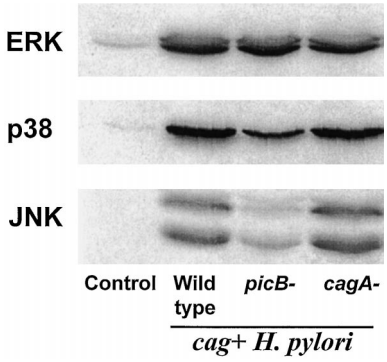

FIGURE 5. The *picB* region of the *cag* pathogenicity island participates in MAP kinase activation. AGS monolayers were cultured alone (Control) or were infected with the *H. pylori cag*$^+$ strain 60190 (wild type; 10^8 bacteria/ml), or with isogenic mutants lacking the *cag* pathogenicity island genes *picB* (*picB*$^-$) or *cagA* (*cagA*$^-$). After 1 h of incubation cell lysates were prepared and then subjected to Western blot analyses using phospho-specific MAP kinase Abs. These experiments were performed on three occasions, and a representative blot is shown.

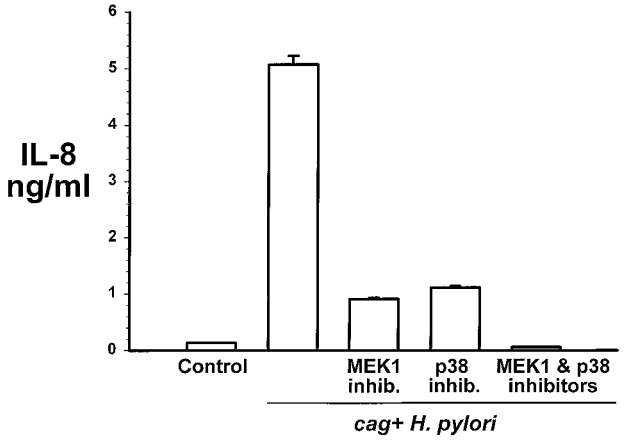

FIGURE 6. MAP kinase activation is required for IL-8 up-regulation in *H. pylori*-infected AGS gastric epithelial cells. AGS monolayers were cultured alone (Control) or were infected with the *H. pylori cag*$^+$ strain 43504 (10^8 bacteria/ml) with or without pretreatment with the p38 inhibitor SB203580 (10 μM) and/or the MEK1 inhibitor PD98059 (25 μM). IL-8 levels were measured in conditioned medium harvested after 7 h. Data are expressed as the mean ± SD ($n = 4$).

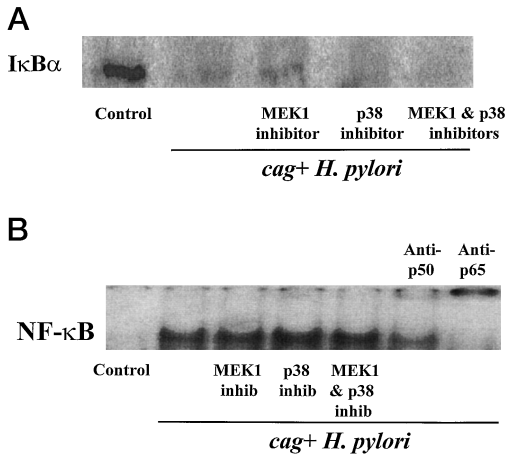

FIGURE 7. The MAP kinase inhibitors PD98059 and SB203580 do not influence IκBα degradation and NF-κB activation in *H. pylori*-infected AGS gastric epithelial cells. AGS monolayers were cultured alone (Control) or were infected with the *H. pylori cag*$^+$ strain 43504 (10^8 bacteria/ml) with or without 30 min of pretreatment with the p38 inhibitor SB203580 (10 μM) and/or the MEK1 inhibitor PD98059 (25 μM). *A*, After 1 h cell lysates were prepared and subjected to Western blot analysis for evaluation of IκBα levels. *B*, Nuclear extracts were also prepared (at 1 h) and evaluated by EMSA for binding activity to an IL-8-specific NF-κB oligonucleotide probe. EMSA supershift studies were performed using Abs against p50 and p65 NF-κB proteins. These experiments were performed on two occasions with identical results.

1). NF-κB binding to the IL-8 promoter site was markedly increased following *H. pylori* infection (*lane 2*). Activation and nuclear translocation of NF-κB did not appear to be influenced by the MAP kinase inhibitors PD98059 and/or SB203580 (*lanes 3–5*). EMSA supershift studies using Abs to p50 and p65 κB proteins confirmed that p65-containing NF-κB is the predominant form binding to the IL-8 promoter site.

Discussion

We have shown that contact with *H. pylori* rapidly activates ERK1, ERK2, p38, JNK p46, and JNK p54 MAP kinases in AGS gastric epithelial cells. We also find that *H. pylori cag*$^+$ strains are more potent than *cag*$^-$ strains in inducing MAP kinase activation. Differential MAP kinase activation by *cag*$^+$ and *cag*$^-$ *H. pylori* strains is particularly evident for JNK phosphorylation and appears to be dependent upon genes within the *cag* pathogenicity island. Inhibitors of p38 and MEK1 MAP kinases prevent *H. pylori*-induced IL-8 production. However, p38 and MEK1 MAP kinase activity do not appear to be essential for *H. pylori*-induced NF-κB activation.

H. pylori has adapted to interact specifically with gastric-type epithelial cells. *H. pylori* infection is limited to areas of the gastrointestinal tract that are lined by gastric epithelium, and the bacterium is known to activate several gastric epithelial cell signaling events. Previous studies have shown that adherence of *H. pylori* to AGS gastric epithelial cells induces tyrosine phosphorylation of host proteins, cytoskeletal reorganization, NF-κB activation, and up-regulation of expression of a variety of inflammatory response genes including IL-8 (10, 13, 15, 21, 41). We now report that *H. pylori* also induces the phosphorylation of ERK, JNK, and p38 MAP kinase family members. Threonine and tyrosine phosphorylation of MAP kinases was evident within 5 min of *H. pylori* inoculation. Contact with intact bacteria appears to be required for MAP kinase activation. A similar requirement for bacterial contact or adherence was described previously for gastric epithelial cell NF-κB activation by *H. pylori* (15). Our experiments using the bacterial protein synthesis inhibitor chloramphenicol indicate that MAP kinase activation does not require de novo bacterial protein production. Instead, epithelial cell activation appears to result from contact with preformed bacterial factors, which is consistent with the observed rapid onset of MAP kinase activation.

We did not examine specifically whether *H. pylori* LPS activates MAP kinases in AGS cells. However, in a previous study we were unable to demonstrate AGS cell NF-κB activation (or IL-8 production) in response to LPS (15). Furthermore, soluble factors present in bacterial culture filtrate did not activate AGS cell MAP kinases. These findings suggest that *H. pylori* LPS is not responsible for MAP kinase activation in gastric epithelial cells. This is in contrast to human monocytic cells, which are activated by *H. pylori* culture filtrate and by purified *H. pylori* LPS as shown by other investigators and by us (35, 42–44).

We find that *H. pylori cag*$^+$ strains are more potent than *cag*$^-$ strains in inducing gastric epithelial cell MAP kinase phosphorylation. A consistent pattern of differential MAP kinase activation was observed with the panel of *cag*$^+$ and *cag*$^-$ isolates examined in this study. The most striking differences between the *cag*$^+$ and *cag*$^-$ strains were seen for JNK phosphorylation, which was minimal after exposure to the *cag*$^-$ bacteria. p38 MAP kinase activation was also far less evident with the *cag*$^-$ strains. In previous studies, disruption of the *picB* region of the *cag* pathogenicity island resulted in a marked reduction in the ability of *H. pylori* to activate epithelial cell NF-κB and up-regulate IL-8 protein production (22, 28). We now find that a *picB*$^-$ mutant also shows reduced potency in MAP kinase activation compared with its *cag*$^+$ parental strain. Again, an almost complete loss of JNK activation and a markedly reduced potency of p38 activation were evident. Thus, the pattern of MAP kinase activation induced by the *picB*$^-$ mutant recapitulates the pattern observed using the *cag*$^-$ clinical isolates. These findings indicate that gene products of the *cag* pathogenicity island are necessary for the observed differences in MAP kinase induction by *H. pylori cag*$^+$ and *cag*$^-$ strains.

H. pylori vacuolating cytotoxin has been reported to inhibit EGF-mediated signal transduction and ERK2 activation in Kato III gastric epithelial cells (45, 46). Broth culture supernatants from a *vacA*$^+$ *H. pylori* strain, but not its isogenic *vacA*$^-$ mutant, inhibited epithelial cell EGF receptor activation, and ERK2 phosphorylation in response to EGF. We now find that *H. pylori* bacteria, but not culture filtrate, activate ERK1 and ERK2 in gastric epithelial cells. Furthermore, ERK activation is more pronounced following exposure to *cag*$^+$ strains compared with *cag*$^-$ strains that secrete less vacuolating cytotoxin. Thus, vacuolating cytotoxin produced by *cag*$^+$ bacterial strains in our experimental system is unable to prevent *H. pylori*-induced ERK activation. The interplay between bacterial activation of gastric epithelial cell MAP kinases and the blockade of related signaling pathways by vacuolating cytotoxin requires further investigation.

MAP kinase signaling regulates the expression of many proinflammatory cytokines, including IL-8. This led to our experiments using the specific MAP kinase inhibitors SB203580, which blocks p38 kinase activity, and PD98059, which blocks MEK1 activity, thereby preventing ERK1/2 phosphorylation. Unfortunately, a suitable JNK inhibitor was not available for use in our studies. Both SB203580 and PD98059 substantially reduced IL-8 production by *H. pylori*-stimulated AGS cells. Thus, activation of both the p38 and MEK1-ERK1/2 pathways are required for a maximal gastric epithelial cell IL-8 response to *H. pylori*. When both pathways were blocked by combining the two inhibitors, *H. pylori*-induced IL-8 production was abolished.

Cross-talk between the MAP kinase and NF-κB pathways has been demonstrated in a number of recent studies. For example, the MAP kinase family members MAP kinase kinase kinase (MEKK1) and NF-κB-inducing kinase (NIK) can each directly activate the IκB kinase signalsome, resulting in IκB phosphorylation and release of activated NF-κB (29–31, 47–49). Thus, we examined whether MAP kinase activation by *H. pylori* was an upstream signaling event in the pathway leading to NF-κB activation and, hence, IL-8 gene transcription. Although the MAP kinase inhibitors SB203580 and PD98059 were both effective in blocking IL-8 production, neither had any apparent effect on *H. pylori*-induced IκBα degradation or on NF-κB activation and binding to the IL-8 promoter κB site. These data suggest that p38 and MEK1 MAP kinases are not required for *H. pylori*-mediated IκBα degradation or NF-κB activation. Thus, the MAP kinase and NF-κB pathways may exert independent regulatory effects on gastric epithelial cell IL-8 production following *H. pylori* infection. In other systems, p38 MAP kinases and NF-κB have been shown to regulate cytokine gene expression by independent pathways (32, 50, 51). However, the mechanism by which p38 regulates cytokine production without altering NF-κB activation and DNA binding is not known.

Bacterial adherence to or at least contact with the gastric epithelial cell appears to be necessary for *H. pylori*-induced MAP kinase activation. However, the adhesins or other bacterial factors, host receptors, and intermediary host signaling molecules that are engaged to activate MAP kinase family members are unknown. Further studies are needed to elucidation these upstream events and define the specific bacterial and host factors that interact to activate the epithelial cell signaling pathways. The potential downstream effects of epithelial cell MAP kinase activation are multiple and varied. In this study we examined MAP kinase-mediated activation of IL-8 production. MAP kinases regulate cell proliferation, differentiation, and programmed death, in addition to stress and inflammatory responses. Hence, activation of gastric epithelial cell MAP kinases by *H. pylori* may be instrumental in inducing gastroduodenal inflammation, ulceration, and neoplasia. The differential activation of MAP kinases by cag^+ and cag^- strains may be an important determinant of strain-specific differences in the host response to *H. pylori* infection of the gastric mucosa.

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