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Helicobacter pylori Activates Mitogen-activated Protein Kinase Cascades and Induces Expression of the Proto-oncogenes c-*fos* and c-*jun**

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***Helicobacter pylori* is an etiological agent in the development of mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma. Patients infected with *H. pylori* carry a 3–6-fold increased risk of developing cancer compared with uninfected individuals. *H. pylori* strains expressing the cytotoxin-associated antigen A (CagA) are more frequently associated with the development of neoplasia than cagA-negative strains. However, the molecular mechanism by which *H. pylori* causes neoplastic transformation remains unclear. Here we report that exposure of gastric epithelial cells to *H. pylori* induces activation of the transcription factor activator protein 1. Activation of the proto-oncogenes c-*fos* and c-*jun* is strongly induced. We show that *H. pylori* activates the ERK/MAP kinase cascade, resulting in Elk-1 phosphorylation and increased c-*fos* transcription. *H. pylori* strains that do not express CagA or that are mutated in *cag* genes encoded by the CagI pathogenicity island do not induce activator protein 1, MAP kinase activity, or c-*fos* or c-*jun* activation. Proto-oncogene activation may represent a crucial step in the pathomechanism of *H. pylori* induced neoplasia.**

Although the rate of gastric cancer has recently declined, it remains the second most prevalent cancer in the world today (1, 2). The bacterium *Helicobacter pylori* has recently been recognized as an etiological agent in the development of mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma (3–6). Epidemiological studies have demonstrated an up to 6-fold increased risk of developing adenocarcinoma in patients infected with *H. pylori*. (4, 5). Therefore, in 1994, *H. pylori* was classified as a type I carcinogen for humans by the IARC/WHO (7). Using an animal model, it has recently been demonstrated directly that long term infection with *H. pylori* induces gastric adenocarcinoma (8–10). 37% of Mongolian gerbils infected with *H. pylori* for 62 weeks developed gastric cancer, whereas none of the control animals succumbed ($n = 27$ infected animals, 30 control animals). Moreover, the topographic location and the histology of these tumors were similar to those observed in humans (8). However, the molecular mechanism by which the bacterium causes neoplastic transformation remains unknown. Several studies have demonstrated that *H. pylori* stimulates gastric epithelial cell proliferation (11–13). It has been suggested that transformation is not mediated directly by *H. pylori* but rather occurs nonspecifically due to increased mutations following rapid cell proliferation.

H. pylori infection can cause a broad range of diseases. Although most infected individuals only develop a superficial gastritis, some patients progress to chronic gastritis, duodenal ulceration, or, rarely, cancer (14, 15). This variability in clinical manifestation may arise because individual *H. pylori* strains differ in virulence. Several virulence factors have been described and include the presence of a vacuolating cytotoxin, VacA, and a cytotoxin-associated antigen A, CagA (16, 17). Various studies have reported an increased risk of developing distal gastric cancer for patients infected with CagA-positive strains compared with cagA-negative individuals (18–20).

In order to study *H. pylori* pathogenesis *in vitro*, the bacterium was co-cultured with gastric epithelial cell lines. In this model, *H. pylori* induces secretion of the chemokine IL-8[1] (21, 22). These data confirm the observation that mucosal biopsies from patients with *H. pylori* infections contain significantly elevated levels of IL-8, compared with specimens from uninfected individuals (23–25). *In vitro,* CagA-positive strains elicit a significantly higher IL-8 response than CagA-negative strains (21, 24, 26). However, isogenic mutants, altered only in the *cagA* gene, induce as much IL-8 production as the parental strain (27). Therefore, although *cagA* represents a marker of enhanced pathogenicity, the protein itself is not required.

The recent identification of a *H. pylori* "pathogenicity island" represents a major advance in our understanding of bacterial pathogenesis (28). The pathogenicity island, a 40-kilobase segment of DNA, harbors over 40 genes that encode pathogenicity factors, including *cagA* (28). Isogenic mutants, altered in one of six *cag* genes (*cagE, cagG, cagH, cagI, cagL,* and *cagM)* fail to elicit IL-8 secretion from gastric epithelial cells (29). These Cag proteins show similarity to bacterial secretion systems, particularly the type IV secretion system described in *Bordetella pertussis* (30). It has therefore been proposed that these Cag proteins form a membrane associated complex on the *H. pylori* surface, possibly functioning as a secretion system (31).

The AP-1 family of transcription factors plays a pivotal role in cell proliferation and neoplastic transformation (32). AP-1 complexes consist of homo- and heterodimers of the proto-

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[1] The abbreviations used are: IL, interleukin; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; ERK extracellular-regulated kinase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CDK, cyclin-dependent kinase; MOPS, 3-*N*-morpholinopropanesulfonic acid.

oncogene families Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and ATF (ATF2, ATF3/LRF1, and B-ATF), all members of the bZIP (basic region leucine zipper) family of DNA-binding proteins. The proteins are inactive in quiescent cells but are rapidly activated by a variety of extracellular stimuli, including growth factors, cytokines, and cellular stress signals (33, 34). The transforming counterparts of these cellular proteins, the activated oncogenes v-*fos* and v-*jun,* are transduced by the FBJ and FBR murine osteogenic sarcoma viruses (35, 36). However, aberrant, deregulated expression or overexpression of the cellular Fos and Jun proteins also causes neoplastic transformation (37, 38). Transgenic mice that overexpress c-Fos develop osteo- and chondrosarcomas (39). Simultaneous expression of both c-*fos* and c-*jun* increases the rate of tumor formation. Saez *et al.* (40) have shown that c-Fos is required for the malignant progression of skin tumors. c-Fos has been called a "master switch" of cell proliferation and differentiation. Treatment of cultured fibroblasts with c-*fos* antisense RNA or micro-injection of anti-c-Fos antibodies inhibits cell proliferation (41, 42). The generation of c-*jun*–/– knockout mice has demonstrated an absolute requirement of c-Jun for the proliferation of fibroblasts, as cells isolated from c-*jun*–/– mice are defective in proliferation (43).

Because of their central role in controlling proliferation, the expression and activity of Fos and Jun proteins is tightly controlled. Much of this control is exerted by three related kinase cascades collectively called MAP kinase cascades (see Fig. 11) (34, 44). These signal transduction pathways consist of three levels of interacting kinases: a MAP kinase kinase kinase phosphorylates a MAP kinase kinase, which in turn phosphorylates a MAP kinase. The MAP kinase, finally, transduces to the nucleus, where it phosphorylates a transcription factor, thereby activating it. Three distinct but interacting MAP kinase pathways have been described in detail: the ERK1/2 pathway, the SAP kinase/JNK pathway, and the p38 pathway (see Fig. 11) (reviewed in Ref. 34). A variety of extracellular signals induce MAP kinase activity, but individual stimuli may selectively activate one of the other pathways. The ERK1/2 pathway is the main effector of growth factor receptor signaling.

AP-1 activity is regulated by all three MAP kinase pathways mentioned (34). Regulation occurs both at the transcriptional level and at the posttranscriptional level. Expression of c-*fos*, which is nearly absent in quiescent cells, is controlled at the level of transcription. All three MAP kinases, ERK1/2, SAPK/JNK and p38, can phosphorylate the transcription factor Elk-1, a member of the ternary complex family (45, 46). Elk-1 binds the serum response element motif in the c-*fos* promoter, thereby inducing c-*fos* transcription (47). In contrast, c-*jun* is regulated both transcriptionally and posttranscriptionally. A few c-Jun homodimers preexist in resting cells. In addition, c-*jun* transcription is up-regulated by activated MAP kinases (48). Subsequently, c-Jun can increase its own transcription by binding to the TRE motif in its promoter (34). Novel c-Fos synthesis leads to the formation of Jun/Fos heterodimers, which have a 10-fold higher DNA binding affinity than Jun/Jun homodimers, resulting in increased AP-1 activity (49). Posttranscriptionally, c-Jun activity is potentiated through phosphorylation of the transcriptional activator domain by SAPK/JNK (50).

Because AP-1 plays such a crucial role in cell proliferation and transformation and *H. pylori* induces a hyperproliferation of gastric epithelial cells and sometimes neoplasia, we investigated whether *H. pylori* is able to induce AP-1 activity in gastric epithelial cells. We show here that exposure of gastric epithelial cells to various *H. pylori* strains strongly induces AP-1 DNA binding. *H. pylori* selectively activates the ERK/MAP kinase cascade; p38 is not induced. The stimulation of ERK leads to phosphorylation of the transcription factor Elk-1 and markedly increases c-*fos* transcription. At the protein level, expression of c-Fos and phosphorylation of c-Jun is strongly induced. We show that *H. pylori* strains that do not express CagA or that are mutated in *cag* genes encoded by the CagI pathogenicity island do not induce AP-1, MAP kinase activity, c-Fos, or c-Jun expression. Our observations provide a molecular mechanism for the observed stimulation of gastric epithelial cell proliferation during *H. pylori* infection. Moreover, activation of the proto-oncogenes c-*fos* and c-*jun* may represent one crucial step in the development of *H. pylori* induced gastric neoplasia.

EXPERIMENTAL PROCEDURES

Cell Culture—AGS cells (ATCC CRL 1739) were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum and 50 μg/ml penicillin-streptomycin (all from Life Technologies, Inc.). Cells were maintained in the logarithmic growth phase for stimulation experiments.

H. pylori Strains and Culture—H. pylori strains 151 and 2012 are clinical isolates and have been previously described (51, 52). Strains NTCT 11638 and ATCC 43405 were isolated from patients with active chronic gastritis and are reference strains (53). Strain G27, as well as 12 isogenic strains, each mutated in a single gene encoded within the pathogenicity island, have also been described (28). Stock cultures were maintained at −70 °C in brucella broth supplemented with 30% glycerol. The strains were cultured in brucella broth supplemented either with 10% fetal calf serum or with 1% cyclodextrin in a microaerobic atmosphere at 37 °C. For co-culture AGS cells were serum deprived for 12 h to reduce serum-induced AP-1 activity. The *H. pylori* strains were cultured to an A_{695} of 1.0 (a density of 10^8 bacteria per ml) and assayed microscopicaly for viability. Bacterial cultures were washed three times in Ham's F-12 medium and then co-cultured with 10^6 AGS cells (2×10^5/ml) for 1 h. Cells were washed two times in phosphate-buffered saline and harvested by centrifugation. Protein extracts were prepared as described below. These were subsequently analyzed in electrophoretic mobility shift assays, in Western blots, or in immunoprecipitations.

Electrophoretic Mobility Shift Assays—Total cell extracts were prepared using a high salt detergent buffer (Totex) (20 mM Hepes, pH 7.9, 350 mM NaCl, 20% (w/v) glycerol, 1% (w/v) Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5 mM Na_3VO_4, 50 mM NaF, 50 mM $NaPP_i$, 0.1% phenylmethylsulfonyl fluoride, 1% aprotinin). Adherent cells were washed two times with phosphate-buffered saline, scraped in 1 ml of phosphate-buffered saline, harvested by centrifugation, and resuspended in 4 cell volumes (50 μl) of Totex buffer. The cell lysate was incubated on ice for 30 min and then centrifuged for 5 min at 13000 × *g* at 4 °C. The protein content of the supernatant was determined (Bio-Rad), and equal amounts of protein (10–20 μg) added to a reaction mixture containing 20 μg of bovine serum albumin (Sigma), 2 μg of poly(dI-dC) (Roche Molecular Biochemicals), 2 μl of Buffer D+ (20 mM Hepes, pH 7.9, 20% glycerin, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride), 4 μl of Buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride), 5 mM $MgCl_2$ and 100,000 cpm (Cerenkov) of a ^{32}P-labeled oligonucleotide (Promega) in a final volume of 20 μl. Samples were incubated at room temperature for 25 min. For the supershift assays, protein extracts were diluted 3-fold, and 2 μl of antibody were added to the reaction simultaneously with the protein and incubated as described above. Anti-c-Fos-all, anti-c-Fos, anti-Fra1, anti-Fra2, anti-c-Jun-all, anti-c-Jun, anti-JunB, and anti-JunD antibodies were purchased from Santa Cruz Biotechnology. The AP-1 oligonucleotide (Promega) was labeled using [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Promega).

MAP Kinase Inhibitors—The MAP kinase inhibitors PD 98059 and SB 202190 were purchased from Calbiochem. U0126 was obtained from Promega. Stock solutions of 10 or 20 mM respectively were prepared in Me_2SO (DMSO). The inhibitors were added directly to the culture medium at the indicated final concentrations 30 min prior to co-culture.

MAP Kinase Assays—MAP kinase assay kits (New England Biolabs) were used per the manufacturer's recommendations. Briefly, 200 μg of total cell extracts were incubated with Sepharose A-immobilized anti-phospho-MEK1/2- or anti-phospho-ERK1/2 antibodies overnight. Immunocomplexes were collected by centrifugation and washed twice in

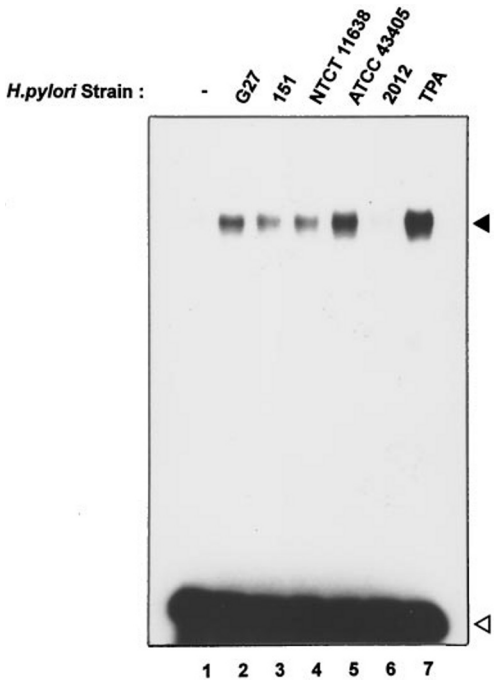


FIG. 1. **The effect of various *H. pylori* strains on AP-1 DNA binding in the gastric epithelial cell line AGS.** AGS cells were co-cultured with 1 ml of bacterial culture of various *H. pylori* strains as indicated (*lanes 2–6*). Strains G27, 151, NTCT 11638, and ATCC 43405 contain the cag pathogenicity island, whereas strain 2012 is cag-negative. Control cells were left untreated (*lane 1*). As a positive control, cells were stimulated with 50 ng/ml (*lane 7*). After 1 h of co-culture, total cell extracts were prepared and assayed in an EMSA using a high affinity AP-1-binding site as a probe. The *filled arrowhead* indicates specific AP-1 complexes, and the *open arrowhead* shows unbound oligonucleotide. This experiment was performed 10 times with similar results.

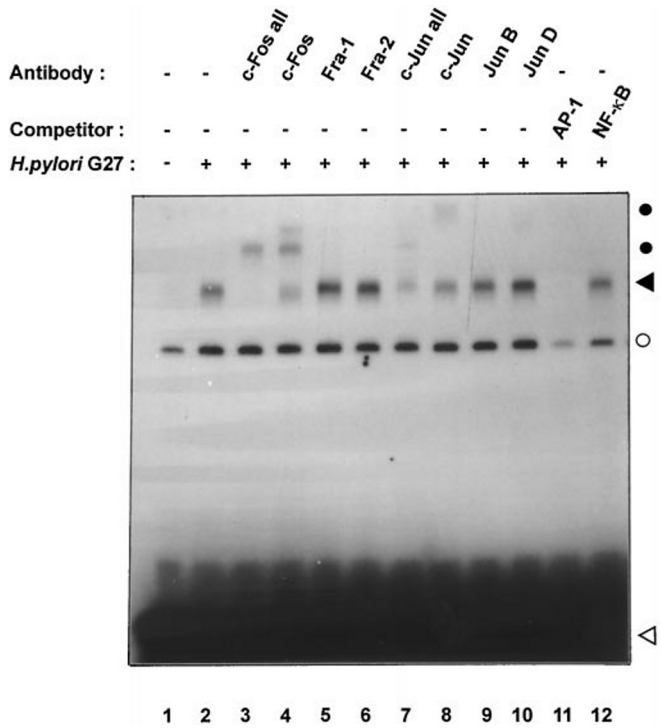


FIG. 2. **Supershift and competition analysis of *H. pylori*-activated AP-1.** EMSA supershift and competition assays of total cell extracts from AGS cells co-cultured with *H. pylori* strain G27. *Lane 1,* untreated AGS cells; *lanes 2–12,* AGS cells co-cultured with *H. pylori* strain G27. *Lanes 3–10,* extracts were incubated with the antibodies indicated. *Lanes 10* and *11,* extracts were incubated with a 100-fold excess of the nonradioactive oligonucleotide indicated. The *filled arrowhead* points to the specific AP-1 complex. The *filled circles* denote the supershifted complexes. The *open circle* denotes nonspecific binding to the probe, and the *open arrowhead* shows unbound oligonucleotide. This experiment was performed three times with similar results.

lysis buffer (20 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $NaPP_i$, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4) and then twice in kinase buffer (25 mM Tris, 10 mM $MgCl_2$, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4). Kinase assays were performed by resuspending the samples in 50 μl of kinase buffer and incubating them for 30 min at 37 °C with 200 μM ATP and 2 μg of either recombinant ERK-2- or an Elk-1-glutathione *S*-transferase fusion protein as a substrate. The reactions were separated by SDS-polyacrylamide gel electrophoresis, and the phosphorylated substrates were detected by phospho-specific anti-ERK1/2 or anti-Elk-1 antibodies (New England Biolabs). For the detection of ERK-2 a secondary anti-mouse IgG, which was preadsorbed to multiple species (Dianova), was substituted for the supplied secondary antibody.

Western Blots—Total cell extracts (30 μg) were boiled in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred at 0.8 mA/cm^2 for 1 h onto Immobilon P membranes (Millipore) using a semidry blotting apparatus (Owl). Nonspecific binding sites were blocked by immersing the membrane in blocking solution (TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 (v/v)) containing 5% skim milk powder; the anti-Ekl-1 antibody was incubated in TBST containing 5% bovine serum albumin) overnight at 4 °C. After a short wash in TBST, the membranes were incubated in a 1:1000 dilution of primary antibody (anti-Elk-1 and phospho-specific anti-c-Jun-Ser-73, New England Biolabs; anti-c-Fos, Santa Cruz Biotechnologies) in blocking solution for 1 h at room temperature, followed by 30 min of washing with TBST. Bound antibody was decorated with peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Amersham Pharmacia Biotech; diluted 1:1000 in blocking solution) for 1 h at room temperature. After washing for 30 min in TBST, the immunocomplexes were detected using ECL Western blotting reagents (Amersham Pharmacia Biotech). Exposure to Kodak XAR-5 films was performed for 5–10 s.

Northern Blots—Total cellular RNA was harvested using an acidic phenol extraction (Trizol, Life Technologies, Inc.).10 μg of RNA were loaded onto a 1% agarose gel cast in 1× MOPS buffer (20 mM MOPS, pH 7.0, 5 mM NaOAc, 1 mM EDTA) containing 0.6% formaldehyde. The RNA was transferred onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) by capillary blotting and fixed by UV cross-linking. The blot was hybridized in ExpressHyb solution (CLONTECH) at 68 °C. A 500-base pair fragment of the c-*fos* cDNA was labeled using the Prime-It-II labeling kit (Stratagene) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech). The blots were washed three times for 10 min in 2× SSC, 0.05% SDS at room temperature, and twice at 50 °C for 15 min in 0.1× SSC, 0.1% SDS.

After the first hybridization, the membrane was reprobed with a 1.2-kilobase pair *Pst*I fragment of the human β-actin gene to control for equal loading of the RNA.

RESULTS

Exposure of Gastric Epithelial Cells to H. pylori Activates the Transcription Factor AP-1—In order to investigate whether exposure of gastric epithelial cells to *H. pylori* activates the transcription factor AP-1, AGS cells were co-cultured with various *H. pylori* strains for 1 h. Subsequently, cell extracts were prepared and assayed for AP-1 DNA binding in an electrophoretic mobility shift assay (EMSA). Co-culture of AGS cells with several *H. pylori* strains caused the appearance of a novel DNA-protein complex (Fig. 1, *lanes 2–5*), which was subsequently identified as AP-1 by competition assay (Fig. 2, *lanes 11* and *12*). The five strains differed in their ability to induce AP-1 DNA binding: strains ATCC 43405 and G27 strongly induced the transcription factor, strains 151 and NTCT 11638 showed a moderate effect, and strain 2012 did not activate AP-1 (Fig. 1).

Because the AP-1 complex can consist of various subunits, we wished to determine the composition of *H. pylori*-induced AP-1. Antibody supershift assays revealed that *H. pylori* activates a heterodimer composed mainly of c-Fos and c-Jun pro-

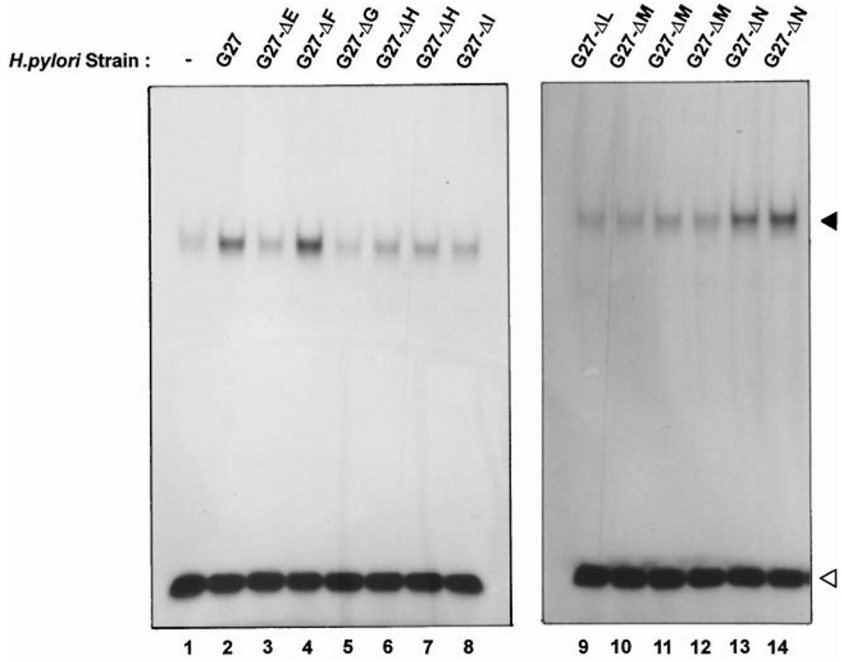


FIG. 3. **The effect of mutations in the *cag* pathogenicity island on *H. pylori*-mediated AP-1 activation.** AGS cells were co-cultured with 1 ml of bacterial culture of *H. pylori* strain G27 (*lane 2*) or its isogenic mutants (*lanes 3–14*). Control cells were left untreated (*lane 1*). After 1 h of co-culture, total cell extracts were prepared and assayed in an EMSA using a high affinity AP-1-binding site as a probe. The *filled arrowhead* indicates specific AP-1 complexes, and the *open arrowhead* shows unbound oligonucleotide. This experiment was performed two times with similar results.

teins. An antibody that reacts with all Fos proteins, as well as an antibody that exclusively recognizes c-Fos, supershifted the complex (Fig. 2, *lanes 3* and *4*). In contrast, antibodies to Fra-1 or Fra-2 had no effect, indicating that these proteins are not present in *H. pylori*-induced AP-1 (Fig. 2, *lanes 5* and *6*). Similarly, an antibody that recognizes all Jun family members and an antibody directed solely against c-Jun both reacted with the complex (*lanes 7* and *8*). Antibodies to JunB and JunD showed no effect, again suggesting that these proteins are not contained in this AP-1 complex (*lanes 9* and *10*).

Proteins Encoded by the cag Pathogenicity Island Are Required for AP-1 Activation—We have previously reported that the ability of *H. pylori* strain G27 to induce the activity of a different transcription factor, NF-κB, requires the presence of several genes located in the pathogenicity island *cagI* (54, 55). It has been suggested that the products of these genes form a structure on the *H. pylori* surface and that this may represent the NF-κB inducing agent. We investigated whether the same gene products are required for AP-1 activation. Twelve isogenic mutants of strain G27, each altered in a single locus within the pathogenicity island, were tested for their ability to induce AP-1. The various strains were co-cultured with AGS cells for 1 h, after which cell extracts were analyzed for AP-1 DNA binding by EMSA. Mutations in *cagF* and *cag N* had no effect on the ability of strain G27 to induce AP-1 (Fig. 3, *lanes 4, 13,* and *14*). In contrast, mutations in six pathogenicity island genes, *cagE*, *cagG*, *cagH*, *cagI*, *cagL,* and *cagM*, reduced AP-1 activation by *H. pylori* strain G27 to background levels (Fig. 3, *lanes 3, 5–12*). Interestingly, exactly the same gene products are also required for NF-κB activation (54, 55). These results suggest that the same *H. pylori* protein complex may be responsible for inducing both NF-κB and AP-1 activity. However, activation of NF-κB and AP-1 occurs through different signal transduction pathways within the eukaryotic cell. Moreover, AP-1 can be activated by a variety of signaling cascades. We therefore investigated which signaling molecules are activated by *H. pylori* in gastric epithelial cells.

H. pylori Activates the ERK/MAP Kinase Cascade—All three MAP kinase cascades, the ERK, the SAPK/JNK, and the p38 pathways, have been shown to mediate AP-1 induction in response to extracellular signals (34). We asked whether one of these pathways is activated in gastric epithelial cells following exposure to *H. pylori*. Several specific inhibitors of the MAP kinases cascades have been described. PD 98059 and U0126 specifically inhibit MEK1 and MEK2, the kinases that phosphorylate ERK, but have no effect on other kinases including SAPK/JNK (56, 57). SB 202190, in contrast, selectively inhibits p38 and has no effect on MEK1/2 (58). To assess the role of the various MAP kinases in AP-1 activation by *H. pylori*, AGS cells were pretreated with various concentrations of the MAP kinase inhibitors for 30 min, after which they were co-cultured with *H. pylori* strain G27 for 1 h. Subsequently, cell extracts were analyzed for AP-1 DNA binding in an EMSA. As a control for inhibitor activity, pretreated cells were also stimulated with 100 ng/ml TPA or 200 IU/ml tumor necrosis factor-α, potent inducers of the MEK1/2 and p38 cascades, respectively (59, 60). Both inhibitors of MEK1/2, PD98059 and U0126, completely inhibited AP-1 activation by *H. pylori* (Fig. 4, *A* and *B*) at concentrations similar to those required to inhibit TPA-stimulated AP-1 induction (Fig. 4*B* and data not shown). In contrast, the p38 inhibitor SB202190, which inhibited tumor necrosis factor-α-mediated AP-1 activation had no effect on the ability of *H. pylori* to induce the transcription factor (Fig. 4*C*). These results suggest that *H. pylori* selectively activates the ERK/MAP kinase but not the p38 kinase cascade in AGS cells.

However, the use of inhibitors provides only indirect evidence for the involvement of MAP kinases. A more direct proof of kinase activation can be obtained by immunoprecipitating the protein and measuring its ability to phosphorylate a target *in vitro*. We therefore co-cultured AGS cells with *H. pylori* strain G27 for 1 h or stimulated the cells with TPA and prepared cell extracts. MEK1/2 were immunoprecipitated using a phospho-specific monoclonal antibody directed against phospho-Ser-217 and phospho-Ser-221. The immunoprecipitates were exposed to purified ERK in an *in vitro* kinase reaction. The products were separated by SDS-polyacrylamide gel electrophoresis, Western blotted and detected with an antibody that exclusively recognizes ERK phosphorylated on Thr-202 and Tyr-204. Exposure of AGS cells to *H. pylori* clearly resulted in elevated levels of ERK phosphorylation, indicating an activation of the MEK1/2 kinases (Fig. 5, *top, lanes 4* and *5*). To verify that equal amounts of ERK were present in all reactions, the blot was stripped and redecorated with an antibody that recognizes both phosphorylated and unphosphorylated ERK

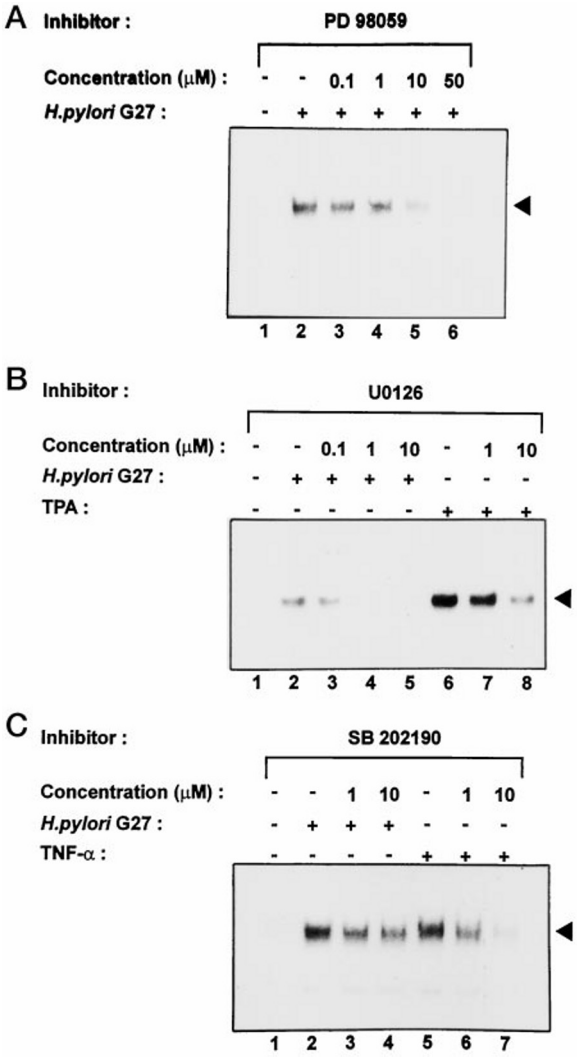


FIG. 4. **The effect of MAP kinase inhibitors on *H. pylori*-mediated AP-1 activation.** AGS cells were pretreated for 30 min with either PD98059 (*A*), U0126 (*B*), or SB202190 (*C*) at the indicated concentrations. Subsequently, cells were co-cultured with 1 ml of *H. pylori* strain G27 or stimulated with 100 ng/ml TPA or 200 IU/ml tumor necrosis factor-α as indicated. Control cells were left untreated (*lane 1*). After 1 h of co-culture, total cell extracts were prepared and assayed in an EMSA using a high affinity AP-1-binding site as a probe. *Filled arrowheads* indicate specific AP-1 complexes. Sections of fluorograms are shown. This experiment was performed two times with similar results.

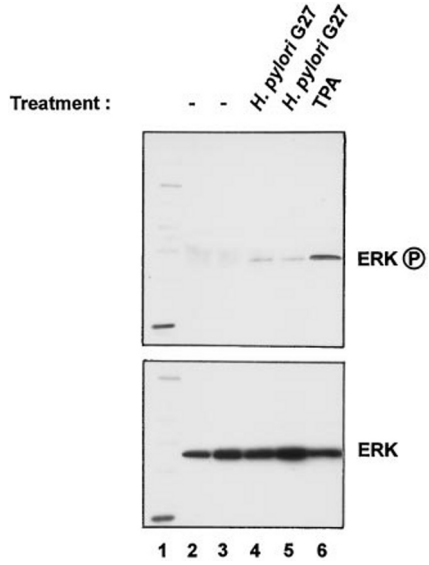


FIG. 5. **The effect of *H. pylori* on MEK1/2 activity in AGS cells.** AGS cells were left untreated (*lanes 2* and *3*), co-cultured with 1 ml of *H. pylori* strain G27 (*lanes 4* and *5*), or stimulated with 100 ng/ml TPA for 1 h (*lane 6*). Subsequently, cell extracts were prepared and subjected to immunoprecipitation with an antibody directed against phosphorylated MEK1/2. *Lane 1,* size standard. *Top,* immunoprecipitates were added to an *in vitro* kinase assay using purified ERK-2 as a substrate and analyzed by Western blot using an antibody that exclusively recognizes the phosphorylated form of ERK (ERK Ⓟ). *Bottom,* the Western blot was stripped and redecorated with an antibody that recognizes both phosphorylated and unphosphorylated ERK. This experiment was performed two times with similar results.

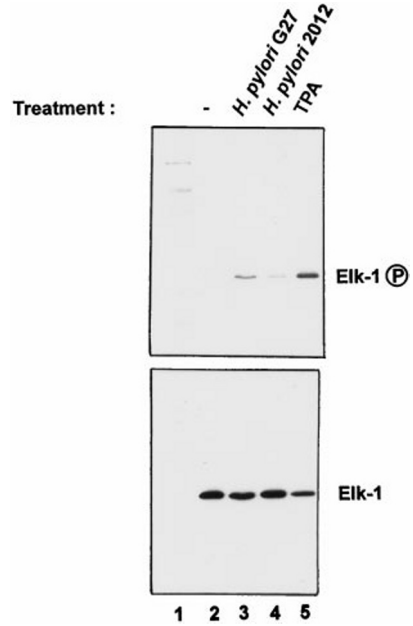


FIG. 6. **The effect of *H. pylori* on ERK activity in AGS cells.** AGS cells were left untreated (*lane 2*), co-cultured with 1 ml of *H. pylori* strain G27 (*lane 3*) or strain 2021 (*lane 4*), or stimulated with 100 ng/ml TPA for 1 h (*lane 5*). Subsequently, cell extracts were prepared and subjected to immunoprecipitation with an antibody directed against phosphorylated ERK1/2. *Lane 1,* size standard. *Top,* immunoprecipitates were added to an *in vitro* kinase assay using purified Elk-1 as a substrate and analyzed by Western blot using an antibody that exclusively recognizes the phosphorylated form of Elk-1 (Elk-1 Ⓟ). *Bottom,* the Western blot was stripped and redecorated with an antibody that recognizes both phosphorylated and unphosphorylated Elk-1.

(Fig. 5, *bottom*). Similar amounts of ERK were present in all lanes. Therefore, exposure of AGS cells to *H. pylori* strain G27 activates MEK1/2 activity.

Phosphorylation of ERK by MEK1/2 in turn activates this kinase, resulting in the phosphorylation and activation of the transcription factor Elk-1 (61). We therefore determined ERK activity in AGS cells co-cultured with *H. pylori.* In this experiment, two different *H. pylori* strains were used, strain G27, which induces AP-1 activity (Fig. 1, *lane 2*), and strain 2012, which induces no detectable AP-1 DNA binding (Fig. 1, *lane 6*). Again, AGS cells were co-cultured with the bacteria for 1 h, after which cell extracts were prepared and subjected to immunoprecipitation. An antibody directed against phosphorylated ERK (residues Thr-202 and Tyr-204) was used. The immunoprecipitated proteins were subjected to an *in vitro* kinase assay using purified glutathione *S*-transferase-Elk-1 as a substrate. Whereas *H. pylori* strain G27 strongly induced Elk-1 phosphorylation, strain 2012 had only a weak effect (Fig. 6, *top, lanes 3* and *4*). Again, the blot was stripped and redecorated with an antibody that recognizes both phosphorylated and unphosphorylated Elk-1 (Fig. 6, *bottom*). Equal amounts of Elk-1 were

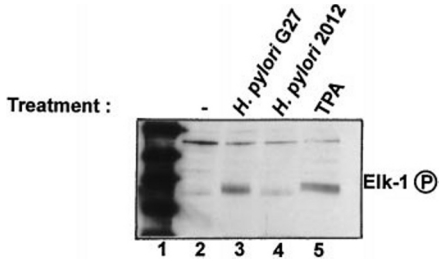


FIG. 7. **The effect of *H. pylori* on Elk-1 phosphorylation.** AGS cells were left untreated (*lane 2*), co-cultured with 1 ml of *H. pylori* strain G27 (*lane 3*) or strain 2012 (*lane 4*), or stimulated with 100 ng/ml TPA for 1 h (*lane 5*). Subsequently, cell extracts were prepared and analyzed in a Western blot using an antibody directed specifically against phosphorylated Elk-1. *Lane 1,* size standard.

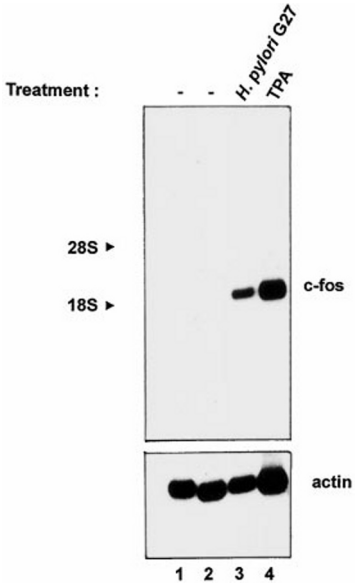
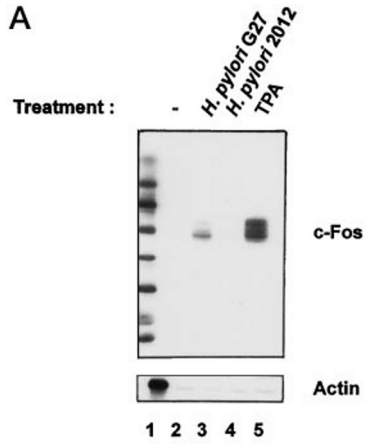


FIG. 8. **The effect of *H. pylori* on c-*fos* mRNA levels in AGS cells.** AGS cells were left untreated (*lanes 1* and *2*), co-cultured with 1 ml of *H. pylori* strain G27 (*lane 3*), or stimulated with 100 ng/ml TPA for 1 h (*lane 4*). Subsequently, total RNA was prepared, and 10 μg was analyzed in a Northern blot using a 500-base pair fragment of the c-*fos* cDNA as a probe (*top*). The blot was stripped and rehybridized with a 1.2-kilobase pair *Pst*I fragment of the human β-actin cDNA (*bottom*). A fluorogram of the filter is shown. The positions of the 28 S and 18 S ribosomal RNAs are indicated.



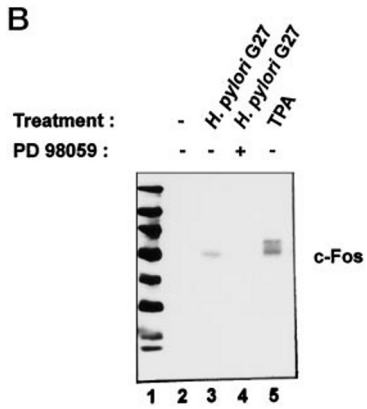


FIG. 9. **The effect of *H. pylori* on c-Fos protein levels in AGS cells.** *A,* AGS cells were left untreated (*lane 2*), co-cultured with 1 ml of *H. pylori* strain G27 (*lane 3*) or strain 2012 (*lane 4*), or stimulated with 100 ng/ml TPA for 1 h (*lane 5*). Subsequently, cell extracts were prepared and analyzed in a Western blot using an antibody directed against the N terminus of the c-Fos protein (*top*). The blot was stripped and redecorated with an antibody to human β-actin (*bottom*). *Lane 1,* size standard. *B,* AGS cells were left untreated (*lane 2*), co-cultured with 1 ml of *H. pylori* strain G27 (*lanes 3* and *4*), or stimulated with 100 ng/ml TPA for 1 h (*lane 5*). Cells in *lane 4* were pretreated with 50 μM PD98059 for 30 min prior to co-culture. Subsequently, cell extracts were prepared and analyzed in a Western blot using an antibody directed against the N terminus of the c-Fos protein. *Lane 1,* size standard. This experiment was performed two times with similar results.

present in all reactions. Therefore, in AGS cells, ERK activity is induced strongly by a cagA-positive *H. pylori* strain but only weakly by a cagA-negative *H. pylori* strain. ERK activation leads to the phosphorylation and activation of the transcription factor Elk-1 (61).

H. pylori Induces Elk-1 Phophorylation—In order to test directly whether *H. pylori* exposure leads to Elk-1 phosphorylation in AGS cells, we assayed the extracts shown in Fig. 6 in a Western blot. The membrane was probed with an antibody that exclusively recognizes Elk-1 phosphorylated on serine 383. AGS cell co-cultured with *H. pylori* strain G27 but not with strain 2012 contained phosphorylated Elk-1 (Fig. 7, *lanes 3* and *4*). *H. pylori* induced Elk-1 phosphorylation as strongly as the tumor promoter TPA (Fig. 7, compare *lanes 3* and *5*).

H. pylori Induces c-fos Transcription—The c-*fos* promoter is regulated by several control elements, which respond to extracellular signals. Activation of the MAP kinase cascades results in phosphorylation of the transcription factor Elk-1 (61), which, together with the serum response factor, binds to the serum response element in the c-*fos* promoter (62). This results in the activation of c-*fos* transcription and a subsequent increase in the amount of c-*fos* mRNA (63). We therefore investigated

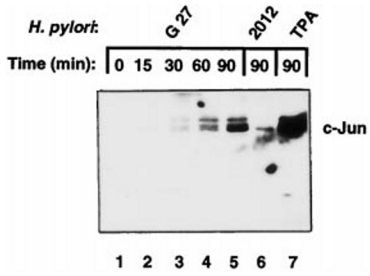


FIG. 10. **The effect of *H. pylori* on c-Jun phosphorylation in AGS cells.** AGS cells were left untreated (*lane 1*), co-cultured with 1 ml of *H. pylori* strain G27 (*lanes 2*-5) or strain 2012 (*lane 6*), or stimulated with 100 ng/ml TPA (*lane 7*) for various times as indicated. Subsequently, cell extracts were prepared and analyzed in a Western blot using an antibody directed against c-Jun phosphorylated on Ser-73. This experiment was performed two times with similar results.

whether exposure of gastric epithelial cells to *H. pylori* induces c-*fos* transcription. AGS cells were co-cultured with *H. pylori* strain G27 or stimulated with 100 ng/ml TPA for 1 h. Total RNA was prepared, and 10 μg were analyzed on a Northern blot by hybridization to the human c-*fos* cDNA (Fig. 8). Co-culture with *H. pylori* markedly increased the level of c-*fos*

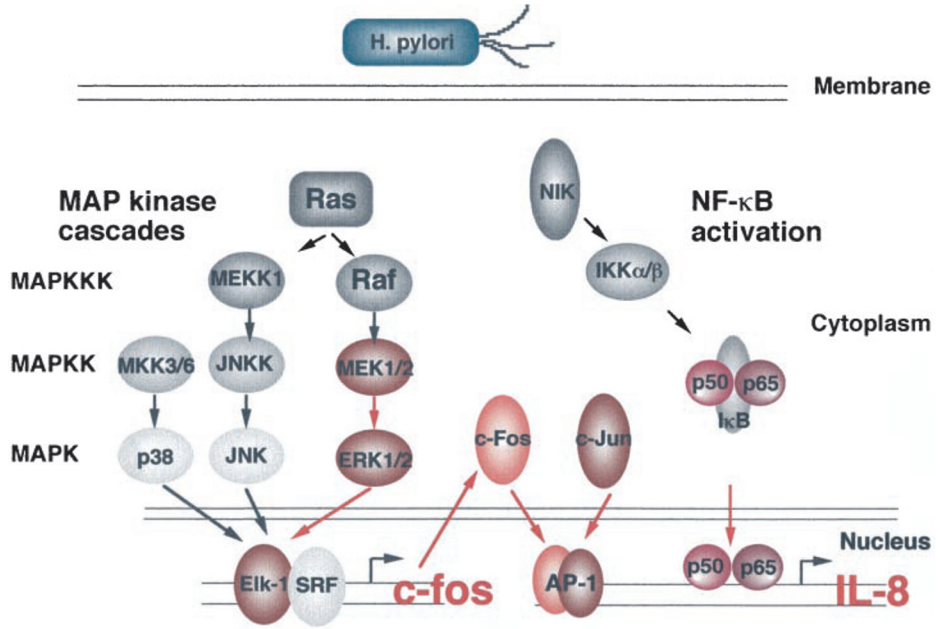


FIG. 11. **Signal transduction cascades activated by *H. pylori* in gastric epithelial cells.** Proteins depicted in *red* were shown either previously (55) or in this paper to be activated by *H. pylori* in AGS cells. Proteins drawn in *gray* are not known to be activated by *H. pylori*. The three MAP kinase cascades (the ERK, JNK, and p38 cascades) are shown on the *left*. *MAPKK,* MAP kinase kinase; *MAPKKK,* MAP kinase kinase kinase; *JNKK,* JNK kinase. Activation of MEK1/2 leads to the phosphorylation of ERK1/2 (indicated by an *arrow*), which in turn translocates to the nucleus and phosphorylates the transcription factor Elk-1. Elk-1 binds the c-*fos* promoter together with the serum response factor (*SRF*) at the serum response element. Phosphorlyation activates Elk-1, leading to increased c-*fos* transcription. c-Fos, together with c-Jun, forms the transcription factor AP-1, which binds the IL-8 promoter, inducing transcription of the gene. The activation pathway of the transcription factor NF-κB is seen on the right. Co-culture with *H. pylori* leads to NF-κB activation via degradation of the inhibitory subunit IκB. NF-κB activity is required for IL-8 transcription (55).

mRNA in AGS cells. The induction was almost as strong as that caused by the tumor promoter TPA (compare Fig. 8, *lanes 3* and *4*).

In order to determine whether the observed increase in c-*fos* mRNA leads to a concomitant increase in the level of c-Fos protein in these cells, AGS cells were again co-cultured for 1 h with *H. pylori*. As in Fig. 6, two different *H. pylori* strains were used, one that induces AP-1 (G27) and one that does not (2012). Cell extracts were prepared and analyzed for c-Fos protein by Western blot. The antibody used is directed against the N terminus of the c-Fos protein. Exposure of AGS cells to *H. pylori* strain G27 led to a strong increase in c-Fos protein levels (Fig. 9*A, lane 3*). In contrast, strain 2012, which does not induce AP-1 DNA binding, did not induce c-Fos protein levels (Fig. 9*A, lane 4*). The Western blot was stripped and redecorated with an antibody to actin to assure equal loading of protein in each lane (Fig. 9*A, bottom*).

The previous experiment was repeated, but this time, one sample was preincubated for 30 min with 50 μM PD 98059 prior to co-culture with *H. pylori* strain G27. Pretreatment with the MEK1/2 inhibitor completely abrogated the ability of *H. pylori* to induce c-Fos protein levels (Fig. 9*B, lane 4*), confirming our previous observation that the induction of this MAP kinase cascade is required for *H. pylori*-mediated AP-1 activation (Fig. 4).

H. pylori Induces c-Jun Phosphorylation—The *H. pylori*-induced AP-1 complex is composed of both c-Fos and c-Jun (Fig. 2). c-Jun activity is regulated by phosphorylation (50). We therefore investigated whether c-Jun phosphorylation is also stimulated by *H. pylori*. AGS cells were co-cultivated with *H. pylori* strain G27, strain 2012, or stimulated with 100 ng/ml TPA for various times as indicated (Fig. 10). Cell extracts were prepared and analyzed in a Western blot using an antibody that specifically recognizes c-Jun phosphorylated on Ser-73. Exposure of AGS cells to *H. pylori* strain G27 but not to strain 2012 induced phosphorylation of the c-Jun protein (Fig. 10, *lanes 3–6*). The Western blot was stripped and redecorated with an antibody to c-Jun. All samples contained an equal amount of c-Jun protein (data not shown), arguing that c-Jun activity is not modulated by novel synthesis of the protein.

DISCUSSION

The molecular mechanism by which *H. pylori* infection increases the risk of gastric cancer remains unknown. It has been shown that *H. pylori* induces hyperproliferation of the gastric epithelium (11–13). However, whether neoplastic transformation results from an unspecific accumulation of mutations following increased proliferation or whether it can be specifically induced by *H. pylori* remains unclear. Interestingly, infection with a *H. pylori* strain carrying a pathogenicity island (type I strain) is associated with a higher risk of developing neoplasia, suggesting that these strains may trigger specific oncogenic events (18–20).

Here, we show that strains carrying an intact pathogenicity island induce activation of the MAP kinases MEK1/2 and ERK1/2 (Figs. 5 and 6). Moreover, these strains induce expression of the proto-oncogene c-*fos* and phosphorylation of c-Jun and activate the transcription factor AP-1 (Figs. 1 and 8–10, summarized in Fig. 11). Strains that do not carry a pathogenicity island or that are mutated in individual *cag* genes do not induce these responses or have only a weak effect (Figs. 1, 3, 9, and 10).

Deregulated expression of MAP kinases or AP-1 proteins has been shown to induce neoplastic transformation. For example, expression of a constitutively active form of MEK-1 induces focus formation and growth in soft agar and causes tumor formation in nude mice (64). Activation of MAP kinases is both necessary and sufficient for the transformation of NIH3T3 cells (65). Likewise, aberrant c-*fos* and c-*jun* activation can promote neoplastic transformation. Overexpression of c-*fos* in trans-

genic mice leads to the development of osteosarcomas and chondrosarcomas in these animals (39). Deregulated c-*fos* and c-*jun* expression transforms rat fibroblasts (36, 66, 67). Our results suggest that aberrant MAP kinase and c-*fos* and c-*jun* activation by *H. pylori* may contribute to the neoplastic transformation of gastric epithelial cells, promoting the development of adenocarcinoma.

How could deregulated MAP kinase or AP-1 activation promote neoplastic transformation? Mitogenic stimulation of cells causes them to enter the cell cycle and commit to DNA synthesis. Cell division proceeds in four distinct phases called G_1, S, G_2, and M. Mitogenic stimulation by growth factors or cytokines is only effective during the G_1 phase and induces transcription of the D-type cyclin genes (D1, D2, and D3) (68). Their concentration is rate-limiting for progression through the G_1 phase. Thus, overexpression of cyclin D1 in fibroblasts accelerates the G_1 phase, whereas microinjection of anti-cyclin D1 antibodies blocks these cells in G_1 (69). D-type cyclins form complexes with the cyclin-dependent kinases (CDKs) CDK4 and CDK6. The cyclin D-CDK complex phosphorylates the Rb protein, thereby releasing the Rb-tethered transcription factor E2F. E2F induces the transcription of genes required for DNA synthesis, moving cells into S phase (70).

Interestingly, constitutive activation of the ERK/MAP kinase pathway leads to G_1/S transition. Moreover, cyclin D1 expression is up-regulated by ERK1/2 (71). The cyclin D1 promoter contains an AP-1-binding site (72). It is therefore intriguing to speculate that MAP kinase activation induces AP-1, which in turn increases cyclin D1 transcription. In support of this hypothesis, c-*jun*–/– cells are resistant to transformation by the proto-oncogene *ras,* an inducer of the ERK/MAP kinase cascade (73). However, this model remains to be proven experimentally. If this model is correct, co-culture of gastric epithelial cells with *H. pylori* should induce cyclin D expression. Furthermore, p53 should become hyperphosphorylated. We are currently investigating these hypotheses. Watanabe *et al.* (8) reported that, in the Mongolian gerbil model, chronic *H. pylori* infection causes intranuclear p53 accumulation in 40% of the adenocarcinomas investigated. Likewise, Ramljak *et al.* (74) have recently shown that chronic *H. hepaticus* infection in mice, which leads to the development of hepatocellular tumors, increases expression of cyclin D1, Cdk4, and c-Myc and leads to a hyperphosphorylation of Rb.

We have demonstrated that type I *H. pylori* strains activate MEK1/2. During growth factor-induced mitogenesis, MEK1/2 are activated by the GTP binding protein Ras and its substrate, the MEK kinase Raf-1. We are currently investigating whether *H. pylori* also activates these proteins or whether it bypasses these steps and directly activates MEK1/2, perhaps using other signal transducers.

We report here that *H. pylori* strains carrying a pathogenicity island induce mitogenic signals and proto-oncogene expression in gastric epithelial cells. Our observations suggest that the epithelial hyperproliferation observed in chronic *H. pylori* infection is specifically stimulated by the bacterium. The data argue that the development of cancer is not due to a nonspecific accumulation of random mutations but may be triggered by the constitutive and prolonged activation of mitogenic signal transduction pathways by *H. pylori*.

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