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Activation of Activator Protein 1 and Stress Response Kinases in Epithelial Cells Colonized by *Helicobacter pylori* Encoding the *cag* Pathogenicity Island*

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Michael Naumann‡§, Silja Wessler‡, Cornelia Bartsch‡, Björn Wieland‡, Antonello Covacci¶, Rainer Haas‖, and Thomas F. Meyer‡**

*From the ‡Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, 10117 Berlin, Germany, ¶IRIS-BIOCINE, Immunological Research Institute, 53100 Siena, Italy, the ‖Max von Pettenkofer Institut für Medizinische Mikrobiologie und Hygiene, Abteilung Bakteriologie, 80336 München, Germany, and the **Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, 72076 Tübingen, Germany*

Helicobacter pylori **interacts with the apical membrane of the gastric epithelium and induces a number of proinflammatory cytokines/chemokines. The subsequent infiltration of macrophages and granulocytes into the mucosa leads to gastric inflammation accompanied by epithelial degeneration. Gastric diseases, *e.g.* peptic ulcer or gastric adenocarcinoma, are more common among people infected with *H. pylori* strains producing VacA (vacuolating cytotoxin A) and possessing a *cag* (cytotoxin-associated antigen A) pathogenicity island. For the induction of the cytokine/chemokine genes in response to *H. pylori*, we studied the signaling leading to the nuclear activation of the early response transcription factor activator protein 1 (AP-1). We found that *H. pylori* strains carrying the pathogenicity island induce activation of AP-1 and nuclear factor κB. In contrast to the wild type or an isogenic strain without the *vacA* gene, isogenic *H. pylori* strains with mutations in certain *cag* genes revealed only weak AP-1 and nuclear factor κB activation. In respect to the molecular components that direct AP-1 activity, our results indicate a cascade of the cellular stress response kinases c-Jun N-terminal kinase, MAP kinase kinase 4, and p21-activated kinase, and small Rho-GTPases including Rac1 and Cdc42, which contributes to the activation of proinflammatory cytokines/chemokines induced by *H. pylori* encoding the *cag* pathogenicity island.**

The immune response to *Helicobacter pylori* infection is initiated by a number of inflammatory mediators including cytokines and chemokines, which are produced from the gastric epithelium. *In vitro* and *in vivo* studies have shown that *H. pylori* induces chemokines IL-8,[1] RANTES, GRO-α, MIP-1α, ENA-78 and MCP-1, and cytokines IL-1, IL-6, and tumor necrosis factor α (1). The epithelial cytokine/chemokine response may be particularly important in the early stages of *H. pylori*-induced inflammation, wherein the epithelium represents the crucial first barrier of defense against pathogen infection. Inflammatory mediators produced from infiltrated polymorphonuclear leukocytes and mononuclear phagocytes could directly damage the surface epithelial layer leading to loss of microvilli, irregularity of the luminal border, and vacuolation (2). The events that commonly follow the infection consist of gastritis, peptic ulcer (3, 4), rarely gastric cancer, and low grade B-cell mucosal-associated lymphoid tissue-associated gastric lymphoma (5, 6).

The inflammatory response and gastric diseases are more common in patients infected with *H. pylori* strains carrying the *cagA* gene (cytotoxin-associated gene A). These strains also produce the toxin VacA (vacuolating toxin A), which is responsible for cytopathic effects (7, 8). The analysis of the genomic region containing the *cagA* gene revealed a 40-kilobase DNA region that is present only in *H. pylori* strains inducing the production of the active form of the toxin and severe gastroduodenal diseases. The 40-kilobase region represents a pathogenicity island and codes for approximately 30 genes (9, 10). Comparison of the genes encoded from the pathogenicity island with genes in other species suggests that the *cag* region encodes a specialized secretion system that exports or allows surface expression of proteins that interact with epithelial cells (10). Knockouts of certain *cag* genes suppressed or reduced the production of IL-8 in epithelial cells (11), affected activation of the immediate early response transcription factor nuclear factor κB (NF-κB) (12), and blocked tyrosine phosphorylation of a 145-kDa host protein (13), suggesting that the integrity of the whole pathogenicity island contributes in chronic inflammation.

The inflammatory reaction requires *de novo* synthesis of defined proteins, which include chemokines attracting macrophages and inflammatory cytokines that serve to amplify and spread the primary pathogenic signal. The mechanism by which these proteins are newly synthesized involves an inducible transcriptional initiation of their respective genes. This is governed by several transcription factors playing a role in regulating immune response genes including the early response transcription factor AP-1 (14). Very little is known about the nature of the *H. pylori*-induced proinflammatory signals and the intracellular signals directing the activation of immediate early response transcription factors. Previous results showed that infection of epithelial cells with *H. pylori* induced the activation of the transcription factor NF-κB (15–17). In line with the known immunostimulatory function of NF-κB and AP-1 (18, 19) epithelial cells infected with *H. pylori* produced increased amounts of numerous proinflammatory cy-

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§ To whom correspondence should be addressed: Max-Planck-Institut für Infektionsbiologie, Abt. Molekulare Biologie, Monbijoustr. 2, 10117 Berlin, Germany. E-mail: naumann@mpiib-berlin.mpg.de.

[1] The abbreviations used are: IL, interleukin; NF-κB, nuclear factor κB; AP-1, activator protein 1; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase; MEKK, MEK kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MKK, MAP kinase kinase; PAK, p21-activated kinase; MOI, multiplicity of infection; PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase.

tokines/chemokines (11). The activation of the early response transcription factor AP-1 and the signaling pathways involved in the production of cytokines/chemokines in *H. pylori*-colonized gastric cells have not been studied so far.

A common mechanism by which eucaryotic cells respond to extracellular signals involves the activation of AP-1 and a family of mitogen-activated protein kinases (MAPK) that consecutively activate their members by phosphorylation. MAPK cascades or modules are composed of a MAPK, a MAPK kinase, or mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK), and a MAPK kinase kinase or MEK kinase (MAPK kinase kinase kinase or MEKK) (20). Subfamiles of MAPKs, the stress-activated protein kinases (SAPK) are activated by multiple environmental stresses and target the transcription factors c-Jun and ATF2, which are components of AP-1 (14). Phosphorylation and activation of c-Jun occur by c-Jun N-terminal kinases (JNKs) in N-terminally located transactivation domains and results in increased transcriptional activity (21, 22). JNKs themselves are activated by MAP kinase kinases 4 and 7 (MKK4 and MKK7) (23–28), and MKK4 could be activated by MEKK1 (29). Numerous MAPK kinase kinases in addition to MEKK1 have recently been identified and are all able to activate the JNK pathway (24, 30–34). The p21-activated kinases (PAKs) have been identified as upstream regulators of the JNK kinase cascade (30, 35, 36) and were the first kinases identified as direct effectors for the active small Rho-GTPases Rac1 and Cdc42 (37). Similar to PAK, the MEK kinases 1 and 4 have also been shown to interact with Rac and Cdc42 (38). Despite the potential for extensive cross-talk, it is generally observed that individual MAPKs are activated in response to distinct sets of environmental stimuli.

Here we show that colonization of gastric cells by *H. pylori* induces the activation of the AP-1 transcription factor by a distinct SAPK cascade involving JNK, MKK4, PAK, and Rho-GTPases but not p38 kinase. Activation of AP-1 and NF-κB is substantially reduced in cells colonized by certain *cag* mutant strains. The identification of *H. pylori*-specific signaling pathways to inflammatory cytokine production casts a light on ways for drug intervention.

EXPERIMENTAL PROCEDURES

Cell Culture and H. pylori Infection—Gastric epithelial cells (AGS) and HeLa cells were grown in RPMI 1640 containing 4 mM glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Life Technologies, Inc.) in a humidified 5% CO_2 atmosphere. The cells were seeded in tissue culture plates for 48 h prior to infection. 16 h before infection, the medium was replaced by fresh RPMI 1640 medium supplemented either with 0.1% fetal calf serum (AGS cells) or 5% fetal calf serum (HeLa cells). *H. pylori* strains were cultured for 48–72 h on agar plates containing 10% horse serum in a microaerophilic atmosphere (generated by Campy Gen, Oxoid, Basingstoke, UK) at 37 °C. For the infection the bacteria were harvested in phosphate-buffered saline, pH 7.4, diluted corresponding to the multiplicity of infection (MOI) as indicated and incubated together with the epithelial cell monolayer for different periods of time. Infection with *H. pylori* was routinely monitored by light microscopy. Stimulation of the cells with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) was performed for the indicated periods of time. In the experiments using 10 ng/ml Toxin B (F. Hofmann and K. Aktories, Freiburg, Germany), the cells were preincubated for 15 min before the bacteria were added.

Bacteria—Different *H. pylori* strains were used for colonization of human epithelial cell lines. The isogenic *P12* strains, *wild type*, *cagA*$^{*-}$ (mutation of *cagA* with a probable polar effect), and *vacA*$^{-}$ (39) and the isogenic G27 strains, *wild type*, *cagF*$^{-}$, and *cagI*$^{-}$ (9) have been described previously. For cultivation the bacteria were resuspended in brain heart infusion (Difco, Detroit, MI) medium, and 10^3 bacteria were seeded per plate. For stock cultures *H. pylori* was resuspended in brain heart infusion and additionally supplemented with 10% fetal calf serum and 20% glycerol and maintained at −70 °C.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared by using a nonionic detergent method as described previously (40). Electrophoretic mobility shift assay for the detection of AP-1 activity in nuclear extracts was performed using oligonucleotides containing the AP-1 binding site: 5′-GATCTTCTAGACCGGATGAGTCATAGCTTG-3′ and 5′-CAAGCTATGACTCATCCGGTCTAGAAGATC-3′. The AP-1 DNA-binding oligonucleotide was labeled using T4 kinase (Roche Molecular Biochemicals) in the presence of [γ-^{32}P]ATP. DNA binding reactions were performed using a binding buffer containing 10 mM Tris, pH 7.5, 2 μg of poly(dI-dC), 1 μg of bovine serum albumin, 10 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 ng of unlabeled double-stranded oligonucleotide, and 10% glycerol. Supershift analysis was performed using c-Jun (sc-45) and ATF2 (sc-187) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) that were preincubated together with nuclear extracts before the ^{32}P-labeled oligonucleotide was added. Electrophoretic mobility shift assay for the detection of NF-κB were performed with an Igκ oligo probe as described previously (41). The oligonucleotide containing the NF-κB recognition site was labeled using the large fragment DNA polymerase (Klenow; Roche Molecular Biochemicals) in the presence of [α-^{32}P]dATP. The DNA binding reactions were performed with 20 μl of binding buffer (2 μg of poly(dI-dC), 1 μg of bovine serum albumin, 5 mM dithiothreitol, 20 mM HEPES, pH 8.4, 60 mM KCl, and 10% glycerol) for 20 min at 30 °C. Supershift analysis was performed using anti-p50, anti-p65, and anti-c-Rel antibodies as described previously (40). For competition experiments cold oligonucleotides were used. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel using 12.5 mM Tris, 12.5 mM boric acid, and 0.25 mM EDTA, pH 8.3. The gels were dried and exposed to Amersham Pharmacia Biotech TM film at −70 °C using an intensifying screen. Quantitation of gel shift assays was performed by scanning the autoradiographs and densitometric analysis using the software program TINA (Raytest, Isotopen Messgeräte GmbH, München, Germany). The fold activity against the control is indicated below the gel shifts.

Enzyme-linked Immunosorbent Assay—IL-8 secretion was assayed from 100-μl supernatants of *H. pylori*-colonized AGS cells or HeLa cells by enzyme-linked immunosorbent assay. The IL-8 enzyme-linked immunosorbent assay was performed as described by the manufacturer's instructions (Pharmingen, Torreyana, CA).

Transient Transfections and Reporter Assays—Transactivating activity of AP-1 was measured in AGS or HeLa cells at 50–70% confluence after cotransfection of the pSV-β-galactosidase construct (Promega, Heidelberg, Germany), 1 μg of a luciferase expression plasmid containing three repeats of the AP-1 binding site as an enhancer element and dominant negative expression constructs: DNPAK2(K278R), DNPAK1(K299R), or DNPAK1(H83L, H86L, K299R), DNMKK4 (K116R, DNJNKK), GFPDNRac1(T17N), and GFPDNCdc42(T17N) using cationic liposomes (DAC-30, Eurogentec, Sart Tilman, Belgium). 16 h after transfection cells were infected with *H. pylori* strains, treated with 100 nM PMA, preincubated with Toxin B, or left untreated. Luciferase assays were performed 3–4 h after treatment as recommended by the manufacturer's instructions (Promega). The results were recorded on a Wallac 409 β-counter (Berthold-Wallac, Bad Wildbad, Germany). The data represent the means ± S.D. calculated from more than three independent experiments as fold induction compared with the control. A portion of the cell lysates that was normalized for equivalent β-galactosidase activity was used for the luciferase assay. Activities varied <10% between transfection experiments. Immunoblots below the graphs indicate the expression of the dominant negative expression constructs DNMKK4, DNPAK1(Myc-tag), GFPDNRac1, and GFPDNCdc42 using the antibodies anti-MKK4 sc-837 (Santa Cruz), anti-Myc 14851A (Pharmingen), and anti-GFP sc-8334 (Santa Cruz), respectively.

Immunoprecipitation and Protein Kinase Assays—To analyze the kinase activity of JNK and p38, cells were lysed in RIPA buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.05% SDS, 5% glycerol, 1 mM EGTA, 10 mM NaF, 10 mM K_2HPO_4, 1 mM Na_3VO_4, 100 μM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, and 4 μM aprotinin. For PAK, cells were lysed in 50 mM Tris, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 1% Nonidet P-40, 2,5% glycerol, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 50 mM NaF, 100 μM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, and 4 μM aprotinin. For immunoprecipitation RIPA buffer lysed cells were disrupted and incubated with anti-JNK1 (sc-474, Santa Cruz) antibody, and anti-αPAK (sc-881, Santa Cruz) antibody detecting PAK1 and partially PAK2 as described previously (42). Immunocomplexes were recovered and washed, and immunoprecipitates were used for *in vitro* kinase reactions using substrates (1 μg of GST-c-Jun (Santa Cruz) for JNK; 1 μg of GST-ATF2 (Santa Cruz) for p38; 2.5 μg of myelin basic protein (Upstate Biotech-

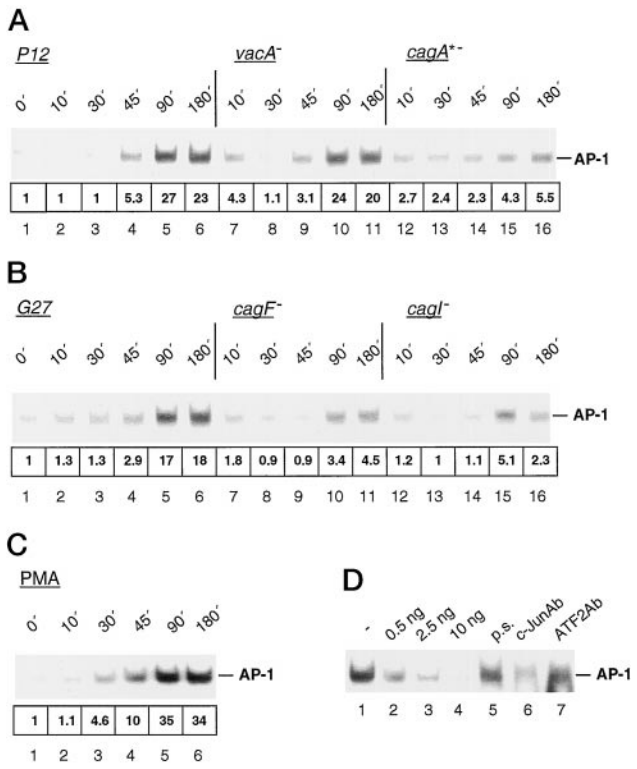

FIG. 1. **Activation of AP-1 in *H. pylori* colonized gastric cells.** AGS cells were analyzed for AP-1 DNA binding activity in response to the colonization by different *H. pylori* strains at a MOI of 100 in a gel retardation assay using a ^{32}P-labeled AP-1 binding site oligonucleotide as a probe. *A* and *B*, nuclear extracts were prepared at different time points postinfection with *H. pylori* strains, *P12* (wild type, *lanes 1–6*), *vacA*$^-$ (*lanes 7–11*), and *cagA*$^{*-}$ (*lanes 12–16*) (*A*) or *G27* (wild type, *lanes 1–6*), *cagF*$^-$ (*lanes 7–11*), and *cagI*$^-$ (*lanes12–16*) (*B*). The DNA binding activity was analyzed. *C*, as a control cells were stimulated with PMA (100 nM). *D*, the specificity of the shifted complexes was analyzed by cold oligonucleotide competition (*lanes 1–4*) and with anti-c-Jun and anti-ATF2 antibodies and preimmune serum (*p.s.*) (*lanes 5–7*). Only sections of the autoradiograms containing the protein-DNA complexes are shown. The position of protein-DNA complexes are indicated by lines labeled AP-1. Quantitation of the gel shifts is indicated as fold activity against the control. The data are representative for at least four independent experiments.

nology Inc.) for PAK). The samples were separated in SDS-polyacrylamide gel electrophoresis and dried, and substrate phosphorylation was visualized by autoradiography (42). Quantitation of the *in vitro* kinase activity was performed by scanning the autoradiographs and densitometric analysis using the software program TINA. The fold activity against the control is indicated below the gels. Equal amounts of each sample were used for immunoblot analysis, as described previously (41) using anti-JNK or anti-PAK antibodies to indicate equivalent protein amounts in all lanes.

RESULTS

H. pylori-induced Activation of the Transcription Factors AP-1 and NF-κB—H. pylori colonization of epithelial cells suggests the induction of intracellular signal transduction pathways that modulate cellular transcription factors. Therefore, we investigated whether *H. pylori* infection induces the transcription factor AP-1, which coordinately induces inflammatory cytokine/chemokine gene expression together with NF-κB.

Subconfluent monolayers of AGS cells or HeLa cells were infected with different *H. pylori* strains. At different time points post-challenge, nuclear protein extracts were prepared and analyzed for the levels of cellular AP-1 DNA binding activity by using a radiolabeled oligonucleotide corresponding to the AP-1 DNA-binding site. As shown in Fig. 1 an enhanced binding of AP-1 was observed in AGS cells within 45 min post infection with the *P12* and *G27* wild type strains (Fig. 1, *A*, *lanes 1–4*, and *B*, *lanes 1–4*). The DNA binding activity induced to similar extents by both *H. pylori* strains was further increased within 180 min (Fig. 1, *A*, *lanes 5* and *6*, and *B*, *lanes 5* and *6*), indicating that members of the c-Jun/c-Fos family were activated. Whether c-Jun or ATF2 represent components that bind to the AP-1 binding site was analyzed in a supershift assay. Nuclear extracts from *H. pylori*-colonized AGS cells, preincubated with an anti-c-Jun antibody before addition of ^{32}P-labeled oligonucleotide, revealed a significantly reduced DNA binding activity of AP-1, whereas the anti-ATF2 antibody did not affect AP-1 DNA binding activity (Fig. 1*D*, *lanes 5–7*). Further, the specificity of the AP-1 DNA-binding capability induced by *H. pylori* was determined using nonlabeled double-stranded oligonucleotide for competition (Fig. 1*D*, *lanes 1–4*). *H. pylori* (*G27*)-induced AP-1 DNA binding activity was slightly weaker than AP-1 activation in response to PMA, which induces AP-1 within 30 min (Fig. 1, *B*, *lanes 3–6 versus C*, *lanes 3–6*). In contrast to wild type strains, AP-1 activity was reduced by isogenic *H. pylori* strains carrying mutations in certain *cag* genes localized in the pathogenicity island (Fig. 1, *A*, *lanes 4–6 versus lanes 14–16*, and *B*, *lanes 4–6 versus lanes 9–11* or *lanes 14–16*). An isogenic mutant of wild type vacuolating cytotoxin-producing *H. pylori* (*P12*) strain carrying a knockout of the *vacA* gene does not affect AP-1 activation (Fig. 1*A*, *lanes 4–6 versus lanes 9–11*). The activation of AP-1 in wild type *H. pylori*-colonized gastric cells was inducible at a MOI of 100, whereas the isogenic *cag* mutant strains showed a strongly reduced AP-1 activity in the following order: *cagA*$^{*-}$, *cagF*$^-$, *cagI*$^-$. This indicates highly specific *H. pylori*-induced signaling, and the critical role of *cag* gene expression in the downstream activation of AP-1. A similar activation of AP-1 was obtained with all *H. pylori* strains used in colonized HeLa cells (data not shown).

Coordinate activation of proinflammatory cytokines involves the activity of the immediate early transcription factors AP-1 and NF-κB. We observed enhanced binding of NF-κB using the Igκ binding site in *H. pylori* (*P12* and *G27*)-colonized AGS cells within 45 min at a MOI of 50 (Fig. 2, *A* and *B*). In contrast to wild type and *vacA*$^-$ strains, NF-κB activation was reduced by isogenic *H. pylori* strains carrying mutations in certain *cag* genes. PMA-treated cells exhibit strong NF-κB activation within 10 min (Fig. 2*C*). The components that bind to the NF-κB binding site were analyzed in a supershift assay, which revealed a supershift and significantly reduced DNA binding activity of NF-κB using anti-p50 or anti-p65 antibodies, whereas the anti-c-Rel antibody did not affect NF-κB DNA binding activity (Fig. 2*D*, *lanes 1–4*). Similar activation of NF-κB was obtained in *H. pylori*-colonized HeLa cells (data not shown). The specificity of the DNA binding activity was examined by adding nonlabeled double-stranded Igκ oligonucleotide for competition (Fig. 2*D*, *lanes 4–6*). Consistent with previous data (15, 17) we observed activation of NF-κB in AGS cells colonized with *H. pylori* wild type and weak NF-κB activation in cells colonized with the isogenic *cag* mutants. This indicates a highly specific *H. pylori*-induced signaling leading to downstream activation of NF-κB. Generally, the integrity of the *cag* pathogenicity island seems to be a prerequiste for an efficient activation of the immediate early response transcription factors NF-κB and AP-1.

To demonstrate that AP-1 and NF-κB activation actually lead to increased proinflammatory cytokine release, we analyzed the IL-8 release by AGS cells and HeLa cells in response to *H. pylori* wild type and the isogenic *cagA*$^{*-}$ strain. *H. pylori* (*P12*) induces IL-8 release in both cell lines within 6 h, whereas IL-8 secretion was reduced by 50% in cells colonized with the *cagA*$^{*-}$ strain (data not shown). The reduced IL-8 release from

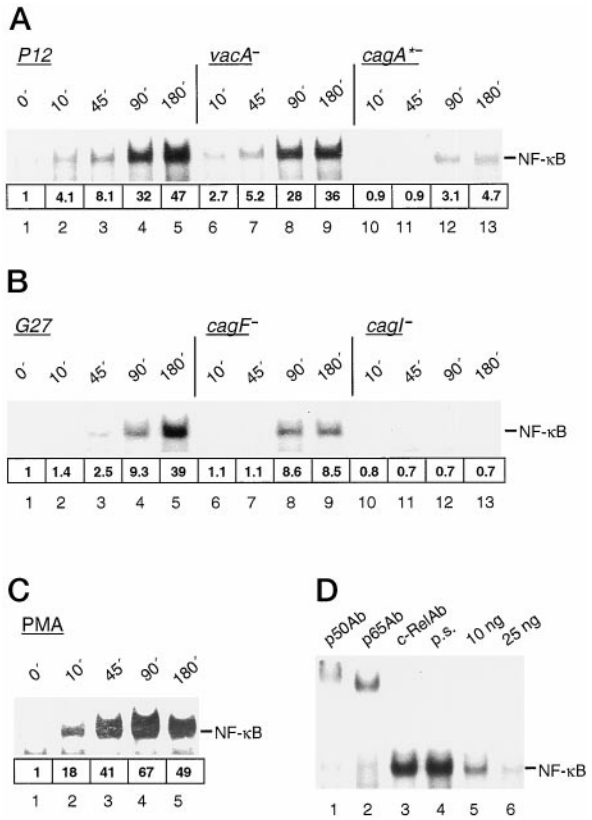

FIG. 2. **The effect of *H. pylori* infection on the activation of NF-κB.** AGS cells were analyzed for NF-κB DNA binding activity in response to the colonization by different *H. pylori* strains at a MOI of 50 in a gel retardation assay using a ^{32}P-labeled Igκ gene NF-κB binding site oligonucleotide. *A* and *B*, nuclear extracts were prepared at different time points postinfection with the *H. pylori* strains *P12* (wild type, *lanes 1–5*), *vacA*$^-$ (*lanes 6–9*), and *cagA*$^{*-}$ (*lanes 10–13*) (*A*) or *G27* (wild type, *lanes 1–5*), *cagF*$^-$ (*lanes 6–9*), and *cagI*$^-$ (*lanes 10–13*) (*B*). The DNA binding activity was analyzed. *C*, as a control cells were stimulated with PMA (100 nM) (*lanes 1–5*). *D*, the specificity of the shifted complexes was analyzed by cold oligonucleotide competition (*lanes 4–6*) and with supershifts using anti-p50, anti-p65, anti-cRel antibodies, and preimmune serum (*p.s.*) (*lanes 1–4*). Only sections of the autoradiograms containing the protein-DNA complexes are shown. The position of protein-DNA complexes are indicated by lines labeled NF-κB. Quantitation of the gel shifts is indicated as fold activity against the control. The data are representative for at least three independent experiments.

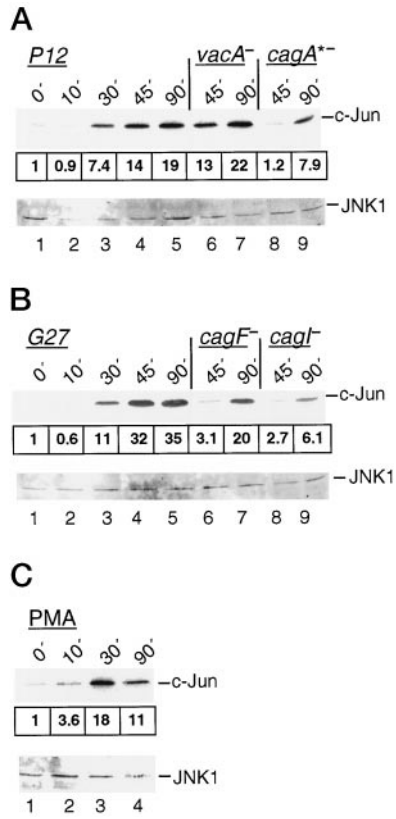

FIG. 3. ***H. pylori*-induced JNK1 activation in epithelial cells.** HeLa cells were infected with different *H. pylori* strains at a MOI of 50 or stimulated with 100 nM PMA. JNK1 was immunoprecipitated with an anti-JNK1 antibody, and immunocomplex kinase activity was determined by phosphorylation of the substrate GST-c-Jun (amino acids 1–79) (c-Jun phosphorylation). Aliquots of the immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted, and the immunoblots were probed with the anti-JNK1 antibody to show similar protein amounts in all lanes (JNK1). *A* and *B*, JNK1 activity at different time points postinfection with the *H. pylori* strains *P12* (wild type), *vacA*$^-$, and *cagA*$^{*-}$ (*A*) and with the *H. pylori* strains *G27* (wild type), *cagF*$^-$, and *cagI*$^-$ (*B*). *C*, as a control, cells were stimulated with PMA (100 nM). Quantitation of the *in vitro* kinase activity is indicated as fold activity against the control. The data are representative for at least three independent experiments.

cagA^{*-} infected AGS cells corroborates the observation that *cag* mutant strains (16) induce weak activation of transcription factors.

Activation of AP-1 Is Mediated by JNK in Response to H. pylori Infection—The dimeric sequence specific enhancer factor AP-1 becomes activated through phosphorylation of c-Jun by members of the stress activated MAPK (SAPK) family in response to environmental stress (14). Therefore, we examined whether the SAPK/JNK and/or p38 are involved in the signaling leading to *H. pylori*-induced AP-1 activation. Cellular extracts from *H. pylori*-colonized epithelial cells were used at different time points post infection for immunoprecipitation of the endogenous kinases, and *in vitro* kinase assays using appropriate substrates were performed.

Wild type *H. pylori* strains at a MOI of 50 induce severalfold JNK1 activation (c-Jun substrate phosphorylation) in subconfluent monolayers of AGS cells (Fig. 3) and HeLa cells (data not shown) within 30 min after infection (Fig. 3, *A*, *lanes 1–3*, and *B*, *lanes 1–3*). The immunoblots, probed with an anti-JNK1 antibody, in the *lower panels* of Fig. 3 is to demonstrate similar JNK protein amounts in all lanes. The immediate JNK1 induction was followed by a sustained JNK1 kinase activation for at least 90 min (Fig. 3, *A*, *lanes 4* and *5*, and *B*, *lanes 4* and *5*). The activation of JNK1 in response to *H. pylori* was delayed compared with the JNK1 induction in PMA-treated cells (Fig. 3, compare *A* and *B*, *lanes 1–5* with *C*, *lanes 1–4*) but showed a similar potential to induce JNK1 activity. To test whether cellular JNK1 activation is at variance in epithelial cells colonized with different *H. pylori* strains, we compared JNK1 kinase activity in AGS cells and HeLa cells colonized either with wild type or isogenic *H. pylori* mutants missing active *cag* genes or deficient for toxin (*vacA*$^-$) expression. The wild type *H. pylori* strains (*P12* and *G27*) induced a strong activation of JNK1 activity, which was not affected in cells colonized with the isogenic *vacA*$^-$ strain (Fig. 3*A*, *lanes 4* and *5* versus *lanes 6* and *7*). Weak JNK1 activation was observed in cells treated with the isogenic strains *cagA*$^{*-}$ and *cagF*$^-$ (Fig. 3, *A*, *lanes 4* and *5 versus lanes 8* and *9*, and *B*, *lanes 4* and *5 versus lanes 6* and *7*). Very weak activation of JNK1 was obtained in cells colonized with the *cagI*$^-$ strain (Fig. 3*B*, *lanes 4* and *5 versus lanes 8* and *9*). In contrast to JNK1, we did not detect any p38 induction paralleled with JNK1 activation (data not shown).

MKK4 Directs H. pylori-induced Activation of AP-1—We studied the signaling pathways contributing to the activation of proinflammatory cytokine genes in response to *H. pylori*-colonized epithelial cells. As upstream activator for JNK/AP-1

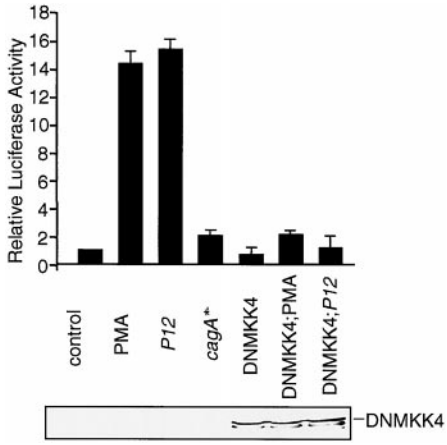

FIG. 4. **Dominant inhibitory MKK4 inhibits AP-1 transactivation activity.** HeLa cells were cotransfected with 1 μg of dominant inhibitory MKK4 (DNMKK4) or empty vector. After overnight incubation the cells were infected with *H. pylori* strains *P12* (wild type) or *cagA*$^{*-}$ at a MOI of 50 or treated with 100 nM PMA for 3 h or left untreated. The data represent the means ± S.D. calculated from more than three independent experiments as fold induction compared with the activity observed in a transfection of the reporter vector and empty vector and in the absence of *H. pylori* strains or PMA. The immunoblot below the graph indicates the expression of the dominant negative MKK4 expression construct recognized by an anti-MKK4 antibody.

activity, MKK4/SEK1 (SAPK/ERK kinase 1) represents an activatory dual kinase, phosphorylating JNK, and p38 (24, 43).

The possible role of MKK4 in *H. pylori*-induced activation of AP-1 was studied by transient transfection of dominant inhibitory MKK4 (DNMKK4). Colonization of HeLa cells with *H. pylori* (*P12*) or PMA-treated cells was induced at least 14 times toward AP-1 transactivation activity compared with untreated cells (Fig. 4). An isogenic *H. pylori cagA*$^{*-}$ strain gave a weak detectable activation of the AP-1 reporter activity. Overexpression of DNMKK4 significantly inhibited *H. pylori* (*P12*)-induced AP-1-dependent reporter gene expression. Similarly, PMA-induced AP-1 activation was blocked by DNMKK4. The experiments were performed after careful titration of the cDNAs to allow specific inhibition of the AP-1 transactivation activity. The strong inhibitory effect of DNMKK4 on *H. pylori*- and PMA-induced AP-1 activation suggests a major role of MKK4 for the JNK kinase signal transduction pathway in which MKK4 is intimately involved by strongly interacting with its upstream activators and/or its downstream elements.

Upstream Activators Directing AP-1 Activity in H. pylori-colonized Epithelial Cells—Upstream kinases involved in stress response signaling are represented by the PAKs (44). PAKs were first demonstrated to interact with small Rho-GTPases Cdc42 and Rac (37), and expression of activated forms of PAK have been shown to activate JNK and p38 pathways (45). To examine whether PAKs are induced in response to *H. pylori* infection, we studied the kinase activity of PAK from *H. pylori* (*P12*)-colonized HeLa cells. *In vitro* kinase reactions with immunoprecipitated PAK1 from *H. pylori* (*P12*)-colonized HeLa cells after the indicated periods of time are shown in Fig. 5*A*. The activity of PAK1 was induced severalfold in HeLa cells by *H. pylori* within 45 min after colonization measuring phosphorylation activity of PAK1 with myelin basic protein as a substrate (*lanes 1–3*). The immediate PAK1 induction further increased within 90 min (*lane 4*). The *lower panel* shows the immunoblot probed with an anti-PAK1 antibody to show similar protein amounts in all lanes. The data suggest that PAKs (*e.g.* PAK1) mediate JNK/AP-1 activation in a *H. pylori*-induced signaling pathway.

To examine whether PAKs are involved in transcriptional

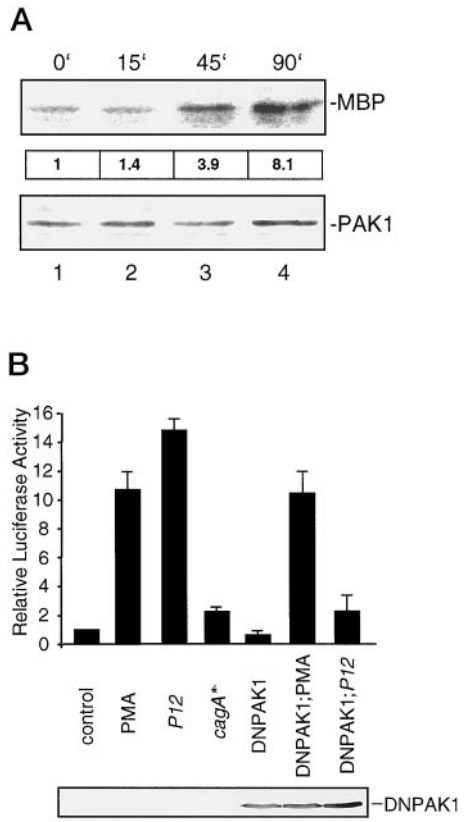

FIG. 5. ***H. pylori*-induced activation of AP-1 transactivation activity by PAK.** *A*, PAK1 activation in response to *H. pylori* epithelial cell contact was studied for the indicated periods of time in HeLa cells treated with *H. pylori* at a MOI of 100 prior to preparation (*lanes 1–4*). PAK1 was immunoprecipitated with an anti-αPAK antibody, and immunocomplex kinase activity was determined by phosphorylation of 2.5 μg of the substrate myelin basic protein (*MBP*) and visualized by autoradiography. Quantitation of the *in vitro* kinase activity is indicated as fold activity against the control. Aliquots of the immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted, and the immunoblots were probed with the anti-αPAK antibody, to show similar protein amounts in all lanes (*PAK1*). The PAK1 protein is indicated. The data are representative for at least three independent experiments. *B*, to study the involvement of PAK in *H. pylori*-induced AP-1-dependent transcription HeLa cells were cotransfected with 0.75 μg of dominant inhibitory PAK1 (*DNPAK1*) or empty vector. After overnight incubation, the cells were infected with *H. pylori* strains *P12* (wild type) or *cagA*$^{*-}$ at a MOI of 50 or treated with 100 nM PMA for 3 h or left untreated. The data represent the means ± S.D. calculated from more than three independent experiments as fold induction compared with the activity observed in a transfection of the reporter vector and empty vector and in the absence of *H. pylori* strains or PMA. The immunoblot below the graph indicates the expression of the dominant negative PAK1 (PAK1Myc) expression construct recognized by an anti-Myc epitope antibody.

responses characteristic of *H. pylori* colonized epithelial cells, we analyzed their capacity to contribute to *H. pylori*-induced AP-1 activation. In transient transfection assays we used an AP-1 reporter construct and PAK1 and PAK2 dominant inhibitory mutant kinases to investigate whether PAKs might be involved upstream in the *H. pylori*-induced signaling cascade leading to AP-1 activation. The PAK1 (K299R) and the PAK2 (K278R) mutants contain mutations that inactivate the catalytic activity of the kinase domains (44). Colonization of HeLa cells with *H. pylori* (*P12*) or PMA-treated cells strongly induced AP-1 transactivation activity compared with untreated cells, whereas the *cagA*$^{*-}$ strain only slightly induced the transactivation activity of AP-1, which was reduced in dominant negative PAK1-transfected cells (Fig. 5*B*). HeLa cells transfected with dominant negative PAK1 and colonized with *H. pylori* (*P12*) strongly reduced the AP-1 transactivation activity, com-

pared with mock transfected cells. In PMA-treated cells transfected with DNPAK1, AP-1 transactivation activity was substantially unaffected, suggesting that PMA induces AP-1 activation via a PAK-independent pathway. The experiments were performed after careful titration of the cDNAs to allow specific inhibition of the AP-1 transactivation activity. Similar to PAK1 we observed reduced transactivation activity of AP-1 in dominant negative transfected PAK2 and *H. pylori*-colonized cells (data not shown). Because mammalian PAKs (PAK1, 2, 3, and 4) exert high similarity in their C-terminal kinase domains (46), they could presumably inhibit downstream signaling of all PAK isoenzymes. To exclude potential effects on the putative upstream components because of titration of Rho-GTPases, we performed experiments with a PAK1 construct that prevents the binding of GTPases. Using this PAK mutant (H83L,H86L,K299R), we received similar results compared with DNPAK1 (K299R) (data not shown). These results suggest that PAK could lie upstream of MKK4 and JNK, leading to AP-1 activation.

Inhibition of AP-1 Transactivation Activity by Toxin B and Rho-GTPases in H. pylori-colonized Cells—PAKs are intermediate in Cdc42/Rac1-mediated activation of JNK (30, 35, 36). We therefore studied whether Rho-GTPases are involved in *H. pylori*-induced signaling pathways leading to AP-1 activation. The activity of the members of Rho-GTPases are specifically inhibited by enterotoxin Toxin B of *Clostridium difficile*, which inactivates Cdc42, Rac1 and Rho (47). Pretreatment of HeLa cells for 15 min with 10 ng/ml Toxin B is sufficient to reduce AP-1 transactivation activity in response to *H. pylori* (*P12*) to base-line levels (Fig. 6*A*, compare *right versus left panels*), indicating the involvement of Rho-GTPases in *H. pylori*-induced AP-1 activation. As a control the PMA-induced AP-1-dependent reporter activity was not affected by Toxin B.

In more detail we analyzed whether the Rho-GTPases Rac1 or Cdc42 are involved in transcriptional responses characteristic of *H. pylori*-colonized epithelial cells. In transient transfection assays we used an AP-1 reporter construct and Rac1 or Cdc42 dominant inhibitory Rho-GTPases to investigate their role in the *H. pylori*-induced signaling cascade leading to AP-1 activation. HeLa cells transfected with dominant negative Rac1 or Cdc42 and colonized with *H. pylori* (*P12*) strongly reduced the AP-1 transactivation activity, compared with mock transfected cells (Fig. 6*B*). In PMA-treated cells transfected with DNRac1 or DNCdc42, AP-1 transactivation activity was substantially unaffected. The experiments were performed after careful titration of the cDNAs to allow specific inhibition of the AP-1 transactivation activity. These results suggest that Rac1 and Cdc42 could lie upstream of PAK1 leading to AP-1 activation.

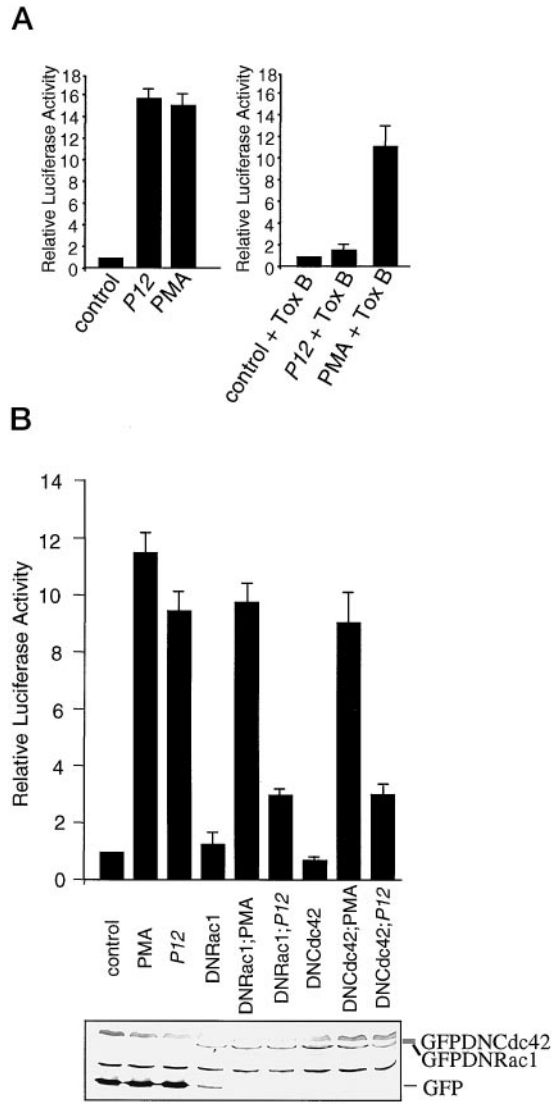

FIG. 6. **Toxin B inhibits *H. pylori*-stimulated AP-1 activation.** *A*, HeLa cells were transfected with the reporter construct, and after overnight incubation, cells were treated with 10 ng/ml Toxin B 15 min prior to the colonization with *H. pylori* strain *P12* at a MOI of 50, or with 50 nM PMA for 3 h, respectively (*right panel*). As a control cells were left without Toxin B (*left panel*). *B*, the involvement of Rac1 and Cdc42 in *H. pylori*-induced AP-1-dependent transcription were studied in HeLa cells cotransfected with 0.75 μg of dominant inhibitory Rac1 (*GFPDNRac1*), Cdc42 (*GFPDNCdc42*), or empty vector. After overnight incubation, the cells were infected with *H. pylori* strains *P12* (wild type) or *cagA*$^{*-}$ at a MOI of 50 or treated with 100 nM PMA for 3 h or left untreated. The data represent the means ± S.D. calculated from more than three independent experiments as fold induction compared with the activity observed in a transfection of the reporter vector and empty vector and in the absence of *H. pylori* strains or PMA. The immunoblot below the graph indicates the expression of the dominant negative GFPRac1 and GFPCdc42 expression constructs and GFP recognized by an anti-GFP antibody.

DISCUSSION

Epithelial cells colonized by *H. pylori* produce immune response mediators, *e.g.* proinflammatory cytokines/chemokines, that lead to a rapid mobilization of phagocytic cells to the sites colonized by the bacteria (1). Because gastric epithelial cells are the first site of contact with *H. pylori*, the activation of cytokine genes would act as an early warning system in the host organism. Proinflammatory cytokines also have activities that are damaging to the integrity of the epithelium, and this can result from an accentuation of their normal protective function. For the induction of proinflammatory cytokines in response to *H. pylori*, it is clear that certain components of *H. pylori* must act to trigger their induction in gastric cells. Candidate *H. pylori* products involved in the induction of the cytokine gene expression are represented by proteins encoded in the pathogenicity island. The Cag proteins form a multimeric structure on the *H. pylori* surface, and this structure seems to be capable of eliciting intracellular signaling in target cells (10). Coordinate activation of proinflammmatory cytokine genes and other gene promoters whose gene products have immunomodulatory functions are mediated by an activatory signaling leading to post-translational modification and activation of transcription factors like AP-1, NF-κB, NF-IL6, etc.

In this report we analyzed the capability of different *H. pylori* strains to induce the transcription factors AP-1 and NF-κB and studied the intracellular signaling leading to AP-1 activation. The rapid production of proinflammatory cytokines involves the activation of immediate early transcriptional ac-

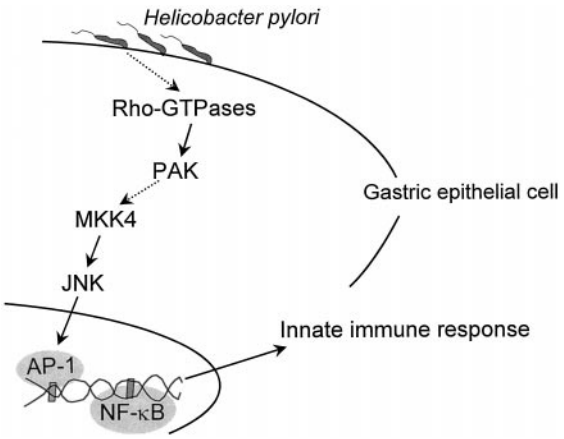

FIG. 7. **Schematic presentation of the signaling leading to AP-1 activation in response to *H. pylori* colonization of epithelial cells.** *H. pylori* induces the activation of the immediate and immediate early response transcription factors AP-1 and NF-κB, which contribute in the activation of immunomodulatory genes. Low molecular weight GTPases and sequential protein kinase pathways controlling AP-1 activation. Downstream of Rho-GTPases, PAK activates unknown MAP kinase kinase kinases, MKK4 and JNK, which directs c-Jun phosphorylation. The *solid arrows* indicate direct activation of downstream targets, and the *dotted arrows* indicate indirect activation through an unknown component.

tivators. The promoters of genes important in the immune response, like IL-8, IL-6, MCP-1, etc., contain binding sites for AP-1 and NF-κB (48). Gel retardation assays using nuclear extracts from *H. pylori*-colonized epithelial cells and a AP-1 consensus motif showed activation of AP-1. The AP-1 activation in AGS cells occurred after *H. pylori* infection with two different strains at a MOI of 100 (Fig. 1, *A* and *B*). Together with AP-1, NF-κB was already induced at a MOI of 50 (Fig. 2, *A* and *B*). In contrast to *H. pylori* wild type strains, isogenic *cag* mutant strains exert a strongly reduced capacity to induce AP-1 or NF-κB, whereas a *H. pylori* strain missing the *vacA* gene does not affect activation of these transcription factors. Previous data indicate that mutations in certain *cag* genes affect *H. pylori*-induced NF-κB activation in AGS and Kato III cells (12, 16, 17). Because certain strains with mutations of *cag* genes affect the activation of AP-1 and NF-κB, we assume complex bacterial stimuli are responsible for triggering the multiple signals in gastric cells. Nevertheless, the biological role of the *H. pylori* cytokine-stimulating *cag* gene products have to be clarified in future studies.

For a better understanding of the crucial outcome of the *H. pylori* infection, we studied the upstream components involved in AP-1 activation, which could target a number of genes involved in immunoinflammatory and proliferative diseases (49). AP-1 activation is regulated at the transcriptional and post-translational level by components of certain kinase cascades in response to a variety of physiological stress stimuli. c-Jun is the central component of all AP-1 dimeric protein complexes, and phosphorylation of c-Jun in the activation domain is exerted only by the SAPK (14). We found in epithelial cells that *H. pylori* stimulates JNK, which phosphorylates c-Jun (Fig. 3). JNK kinase activity in response to *H. pylori* wild type strains and an isogenic strain missing the *vacA* gene was observed rapidly within 30 min after infection at a MOI of 50 and further increased within 90 min, whereas *H. pylori* strains with mutations in defined *cag* genes induced weak JNK activity. Our data indicate that cag^+ *H. pylori* induces an efficient downstream signaling leading to the activation of cytokine genes in gastric epithelial cells and nongastric epithelial cells. The observation that *H. pylori*-induced JNK contributes to the activation of cytokine genes in epithelial cells was also observed in response to *Neisseria gonorrhoeae* epithelial cell contact (42). Stress response kinase p38, which is inducible by osmotic stress (50), was not activated by *H. pylori*. Infection of macrophages by *Yersinia enterocolitica* suppresses activation of JNK as well as p38 kinase activation (51), indicating that pathogens affect different signal transduction pathways in different target cells.

Like physiologic stress inducers (*e.g.* UV light and osmotic shock) (52), human pathogenic *H. pylori* isolates also induce MKK4, and transfection of the dominant negative kinase (DNMKK4) inhibits activation of AP-1 (Fig. 4). The dominant inhibitory effect of MKK4 on *H. pylori*-induced AP-1 activation indicates that JNK is required to mediate full transcriptional activation of AP-1 in response to *H. pylori* and suggests either the formation of a stable JNK/MKK4 complex (53) or sequestration of crucial elements immediately upstream of MKK4. AP-1 activation via JNK/MKK4 could be directed by a number of kinases including the MEKKs 1, 2, 3, and 4 (54–56) and tumor progression locus 2 (Tpl-2) (34), which phosphorylate and activate MKK4. The mixed lineage kinases 2 and 3/SPRK, the DLK/MUK, germinal center kinase, and TAK-1 (tumor growth factor β-activated kinase) show selectivity for the activation of JNK and phosphorylate MKK4 *in vitro* (57). Further, PAKs have been shown to activate the JNK pathway (30, 45, 46, 58), suggesting that PAKs may be involved in MAP kinase signaling pathways. Therefore, the activation of endogenous PAK and the ability of dominant negative PAKs (DNPAK1 and DNPAK2) to block AP-1 activation in response to *H. pylori* colonization of epithelial cells indicates that PAKs may function as components of the signal transduction pathway that leads to MKK4/JNK and AP-1 activation (Fig. 5). Because PAK1, PAK2, and PAK3 exhibiting 92% sequence identity within the kinase domain (44) and the kinase domain of PAK4 shares 53% sequence identity with those of the other PAKs (46), they could putatively inhibit downstream signaling of all PAKs. In the future it will be necessary to identify the *H. pylori*-induced kinase(s) that direct downstream of PAKs MKK4 activation.

The PAKs may also be involved in cytoskeletal organization. PAK1 was reported to induce filopodia and membrane ruffles similar to those induced by Cdc42 and Rac (44). Cdc42 and Rac also play important roles in signal transduction cascades such as those that lead to activation of JNK and p38 activation (30, 36, 45, 58). Activated, GTP-bound forms of Rac and Cdc42 stimulate PAK autophosphorylation and activate its kinase activity (44). By studying the involvement of GTPases in *H. pylori*-induced signaling, we used Toxin B from *C. difficile*, which specifically inhibits the activity of the members of the Rho family (47). AP-1 transcriptional activity is strongly reduced in the presence of Toxin B (Fig. 6*A*), DNRac1, and DNCdc42 (Fig. 6*B*), indicating that GTPases and PAKs may function as components of the same signal transduction pathway that leads to the activation of stress response kinases and the transcription factor AP-1.

From our results, we suggest a pathway through which human pathogenic *H. pylori* expressing *cag*-encoded proteins induce stress response signaling leading to AP-1 activity. AP-1 activation is mediated by JNK, which becomes phosphorylated by MKK4. PAK does not directly phosphorylate MKK4, thus a hitherto unknown MAP kinase kinase kinase contributes to AP-1 activation. Upstream of PAK, activation of AP-1 transcriptional activity, and release of immune response mediators involve the activity of small GTPases of the Rho-family (Fig. 7).

The spectrum of pathological and clinical outcome that follows *H. pylori* infection suggests that *H. pylori* strain-specific and/or host-specific factors are responsible for distinct inflammatory responses. A chronic inflammatory state with the pro-

duction of mediators of inflammation leads to the destruction of the epithelial structure. Because gastric inflammation is a hallmark of *H. pylori* infection, the understanding of the host cell mechanisms involving the local production of cytokines could contribute to treatment of the disease. Cytokines/chemokines and other immune response mediators produced by the host are regulated and controlled at the level of transcription. Halting or reversing the course of the disease could be achieved by disrupting the signal transduction pathways of transcription factors by therapeutic drugs, which has the potential to attenuate the production of immune response mediators.

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Activation of Activator Protein 1 and Stress Response Kinases in Epithelial Cells Colonized by *Helicobacter pylori* Encoding the *cag* Pathogenicity Island

Michael Naumann, Silja Wessler, Cornelia Bartsch, Björn Wieland, Antonello Covacci, Rainer Haas and Thomas F. Meyer

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