Molecular Microbiology (2001) **40**(4), 815–823

Pathogenicity island-dependent activation of Rho GTPases Rac1 and Cdc42 in *Helicobacter pylori* infection

Yuri Churin, Efterpi Kardalinou, Thomas F. Meyer* and Michael Naumann
Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Schumannstrasse 21/22, D-10117 Berlin, Germany.

Summary

Helicobacter pylori **has been identified as the major aetiological agent in the development of chronic gastritis and duodenal ulcer, and it plays a role in the development of gastric carcinoma. Attachment of *H. pylori* to gastric epithelial cells leads to nuclear and cytoskeletal responses in host cells. Here, we show that Rho GTPases Rac1 and Cdc42 were activated during infection of gastric epithelial cells with either the wild-type *H. pylori* or the mutant strain *cagA*. In contrast, no activation of Rho GTPases was observed when *H. pylori* mutant strains (*virB7* and PAI) were used that lack functional type IV secretion apparatus. We demonstrated that *H. pylori*-induced activation of Rac1 and Cdc42 led to the activation of p21-activated kinase 1 (PAK1) mediating nuclear responses, whereas the mutant strain PAI had no effect on PAK1 activity. Activation of Rac1, Cdc42 and PAK1 represented a very early event in colonization of gastric epithelial cells by *H. pylori*. Rac1 and Cdc42 were recruited to the sites of bacterial attachment and are therefore probably involved in the regulation of local and overall cytoskeleton rearrangement in host cells. Finally, actin rearrangement and epithelial cell motility in *H. pylori* infection depended on the presence of a functional type IV secretion system encoded by the *cag* pathogenicity island (PAI).**

Introduction

Helicobacter pylori is a bacterial pathogen that infects the stomach, exhibiting specific tropism for human gastric epithelium (Falk *et al*., 1993). The bacterium has been identified as the major aetiological agent in the development of chronic gastritis and duodenal ulcer, and recent studies suggest that it plays a role in the development of gastric carcinoma (Dunn *et al*., 1997; Covacci *et al*., 1999). *H. pylori* strains are divided into two distinct types: virulent (type I) that contains the *cag* pathogenicity island (PAI); and less virulent (type II) that lacks functional *cag* PAI. The 31 genes of *cag* PAI encode a type IV secretion system (Censini *et al*., 1996). After the attachment of *H. pylori* to cultured gastric epithelial cells, the immunodominant CagA protein encoded by *cag* PAI is translocated into and tyrosine phosphorylated in host cells (Segal *et al*., 1999; Asahi *et al*., 2000; Backert *et al*., 2000; Odenbreit *et al*., 2000; Stein *et al*., 2000). Colonization of epithelial cells by *H. pylori* induces the release of the proinflammatory lymphokine interleukin (IL)-8 (Crabtree *et al*., 1995). Co-ordinate activation of proinflammatory cytokine genes and other genes whose gene products have immunomodulatory functions is mediated by an activatory signalling, leading to post-translational modification and activation of transcription factors such as AP-1 and NF-κB (Baeuerle and Baichwal, 1997; Ip and Davis, 1998). Disruption of specific *cag* PAI genes markedly reduces *H. pylori*-mediated IL-8 gene expression. As expected, these *cag* PAI mutants are also deficient in the ability to activate NF-κB (Keates *et al*., 1997; Münzenmaier *et al*., 1997; Glocker *et al*., 1998; Sharma *et al*., 1998; Naumann *et al*., 1999). *H. pylori* infection also induces the activation of mitogen-activated protein (MAP) kinases and cellular stress response kinases, and this activation is substantially reduced in cells infected with certain *cag* PAI mutant strains (Keates *et al*., 1999; Naumann *et al*., 1999; Wessler *et al*., 2000).

Rac1 and Cdc42 are members of the Rho family of small guanosine triphosphatases (GTPases). Central to their function is the ability to cycle between a GTP-bound active and a GDP-bound inactive conformation, allowing them to function as molecular switches that control a large array of signalling events in a temporal and spatial manner (Mackay and Hall, 1998). Rac1 and Cdc42 control actin polymerization into lamellipodial and filopodial membrane protrusions respectively (Nobes and Hall, 1995). Extension of lamellipodia and filopodia and the formation of new adhesions are key elements in cell migration; thus, these effects on the cytoskeleton reflect the fact that Rac and Cdc42 are important regulators of

Accepted 15 March, 2001. *For correspondence. E-mail meyer@mpiib-berlin.mpg.de; Tel. (+49) 30 28460 400; Fax (+49) 30 28460 401.

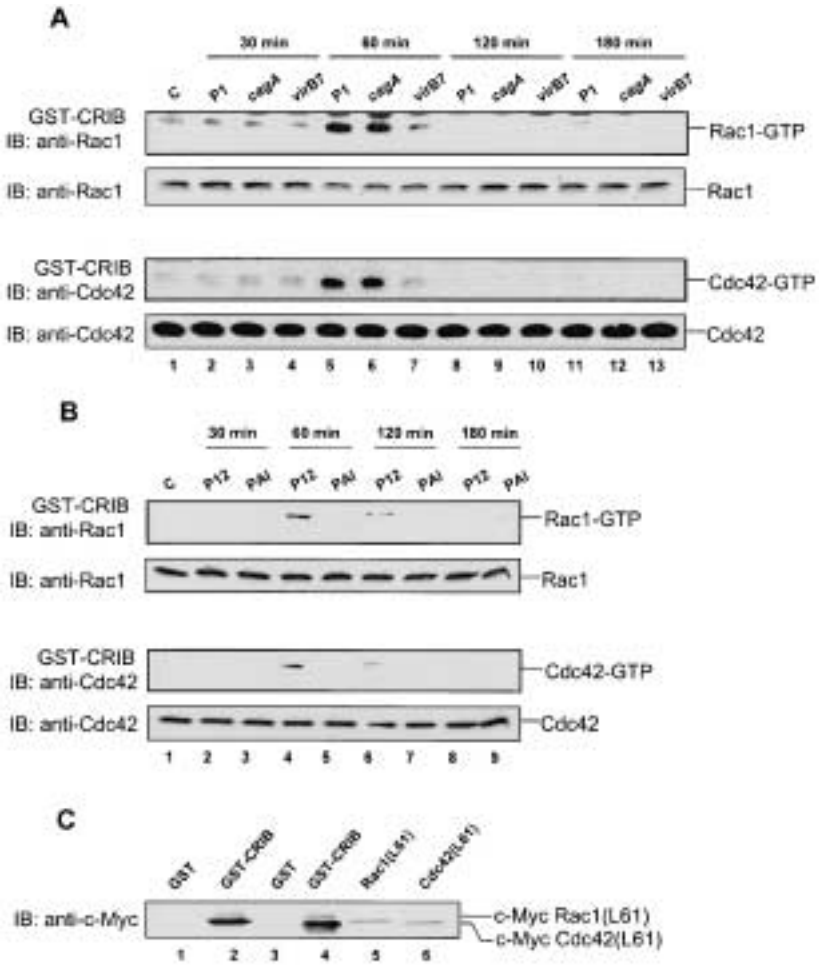

Fig. 1. Infection of AGS cells with *H. pylori* induces activation of Rac1 and Cdc42.
A. AGS cells were infected with the wild-type *H. pylori* strain P1 (lanes 2, 5, 8 and 11) and isogenic mutant strains *cagA* (lanes 3, 6, 9 and 12) and *virB7* (lanes 4, 7, 10 and 13) at a MOI of 50 for the indicated periods of time or were left uninfected as a control (lane 1). Cell lysates were incubated with GST–CRIB fusion protein. The presence of bound, active GTP-Rac1 or GTP-Cdc42 was analysed by Western blotting using anti-Rac1 (1) or anti-Cdc42 (3) antibodies. Similar quantities of Rac1 and CdC42 were confirmed in a Western blot using equivalent volumes of cell lysates (2 and 4).
B. AGS cells were infected with the wild-type *H. pylori* strain P12 (lanes 2, 4, 6 and 8) and the isogenic mutant strain PAI (lanes 3, 5, 7 and 9) at a MOI of 50 for the indicated periods of time or were left uninfected as a control (lane 1). See (A) for the performance of the assay.
C. Rac1 and Cdc42 bind specifically to GST–CRIB fusion protein but not to GST alone. AGS cells were transiently transfected with Rac1 (L61) (lane 2) or Cdc42 (L61) (lane 4) cDNAs. Twenty-four hours after transfection, part of each cell lysate was incubated with either GST–CRIB fusion protein (lanes 2 and 4) or GST alone (lanes 1 and 3). The total amounts of Rac1 (L61) and Cdc42 (L61) are shown in lanes 5 and 6 respectively. The Western blot was probed with an anti-c-Myc antibody.

cell motility (Hall, 1998; Nobes and Hall, 1999). In addition, these GTPases can affect gene transcription through the activation of NF-κB and c-Jun NH_2-terminal kinase (JNK) (Coso *et al*., 1995; Minden *et al*., 1995; Perona *et al*., 1997). Recently, it has been shown that Rac1 and Cdc42 play an essential role in bacteria-induced cytoskeletal and nuclear responses (Chen *et al*., 1996; Naumann *et al*., 1998; 1999; Mounier *et al*., 1999).

Here, we show that colonization of gastric epithelial cells by wild type and the *cagA* mutant of *H. pylori* induces the activation of small GTPases Rac1 and Cdc42. In contrast, the *H. pylori* mutant strain with disrupted *virB7* gene encoding the essential core component of a type IV secretion apparatus and the mutant strain that lacks *cag* PAI failed to activate these GTPases. Further, *H. pylori*-induced Rac1 directs activation of PAK1. GTPases Rac1 and Cdc42 are recruited to the site of bacterial attachment and are probably involved in actin rearrangement observed during bacterial infection. Finally, activation of GTPases Rac1 and Cdc42 leads to changes in epithelial cell phenotype and motility.

Results

H. pylori *activates small Rho GTPases Rac1 and Cdc42*

Small GTPases Rac1 and Cdc42 have been shown to co-ordinate signalling cascades that produce both morphological and nuclear responses to a variety of extracellular signals (Hall, 1998). Here, we examined whether these GTPases contribute to the *H. pylori*-induced cellular responses.

To test directly the activation of Rac1 and Cdc42 during infection with *H. pylori*, pull-down assays were carried out. In detail, Rac1 and Cdc42 GTP loading was determined by specific binding of the active GTPase to the Cdc42–Rac1 interactive binding domain of PAK1 fused to glutathione *S*-transferase (GST–CRIB) (Sander *et al*., 1998). Subconfluent monolayers of AGS cells were infected with wild-type *H. pylori* strain P1 or with the isogenic knock-out mutant strains *cagA* and *virB7*. At different time points after challenge, lysates were prepared, and the amount of Rac1 and Cdc42 precipitated with the GST–CRIB fusion protein was determined by Western blotting. As shown in Fig. 1A, activation of Rac1

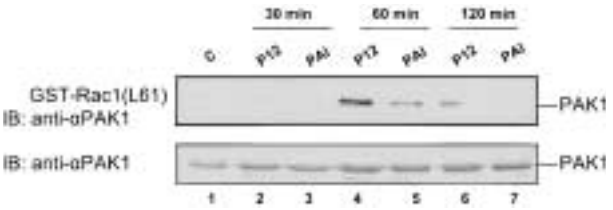

Fig. 2. Rac1 activates PAK1 in H. pylori infection. AGS cells were infected with the wild-type *H. pylori* strain P12 (lanes 2, 4 and 6, top) or the isogenic mutant strain PAI (lanes 3, 5 and 7, top) at a MOI of 50 for the indicated periods of time or were left uninfected as a control (lane 1). Cell lysates were incubated with GST–Rac1 (L61) fusion protein, and bound PAK1 was analysed by Western blotting using anti-αPAK1 antibody. Similar protein amounts of PAK1 were confirmed in Western blot using the equivalent volumes of cell lysates (bottom).

and Cdc42 in AGS cells was detected 1 h after infection with wild-type P1 and mutant *cagA* strains of *H. pylori* and decreased thereafter below the basal level. In contrast, *H. pylori* mutant strain *virB7* failed to activate the GTPases (Fig. 1A). To determine whether GTPase activation depends on the strain used, we infected AGS cells with another wild-type strain of *H. pylori* (P12) and its isogenic mutant lacking the *cag* PAI. Upon infection of the host cells with *H. pylori* P12 but not with the PAI, activated Rac1 and Cdc42 were detected within 1 h after infection (Fig. 1B). As a positive control, we used AGS cells transiently transfected with constructs encoding constitutively active mutants of Rac1 (L61) and Cdc42 (L61). As shown in Fig. 1C, Rac1 (L61) and Cdc42 (L61) bound specifically to GST–CRIB, but not to GST. Taken together, small GTPases Rac1 and Cdc42 are activated in AGS cells in response to *H. pylori* infection. This activation is not strain specific but depends on the presence of *cag* PAI.

H. pylori-*induced Rac1 activates PAK1 in a* cag *PAI-dependent manner*

PAKs have been identified as direct effectors for the active Rho GTPase family members Rac1 and Cdc42 (Manser

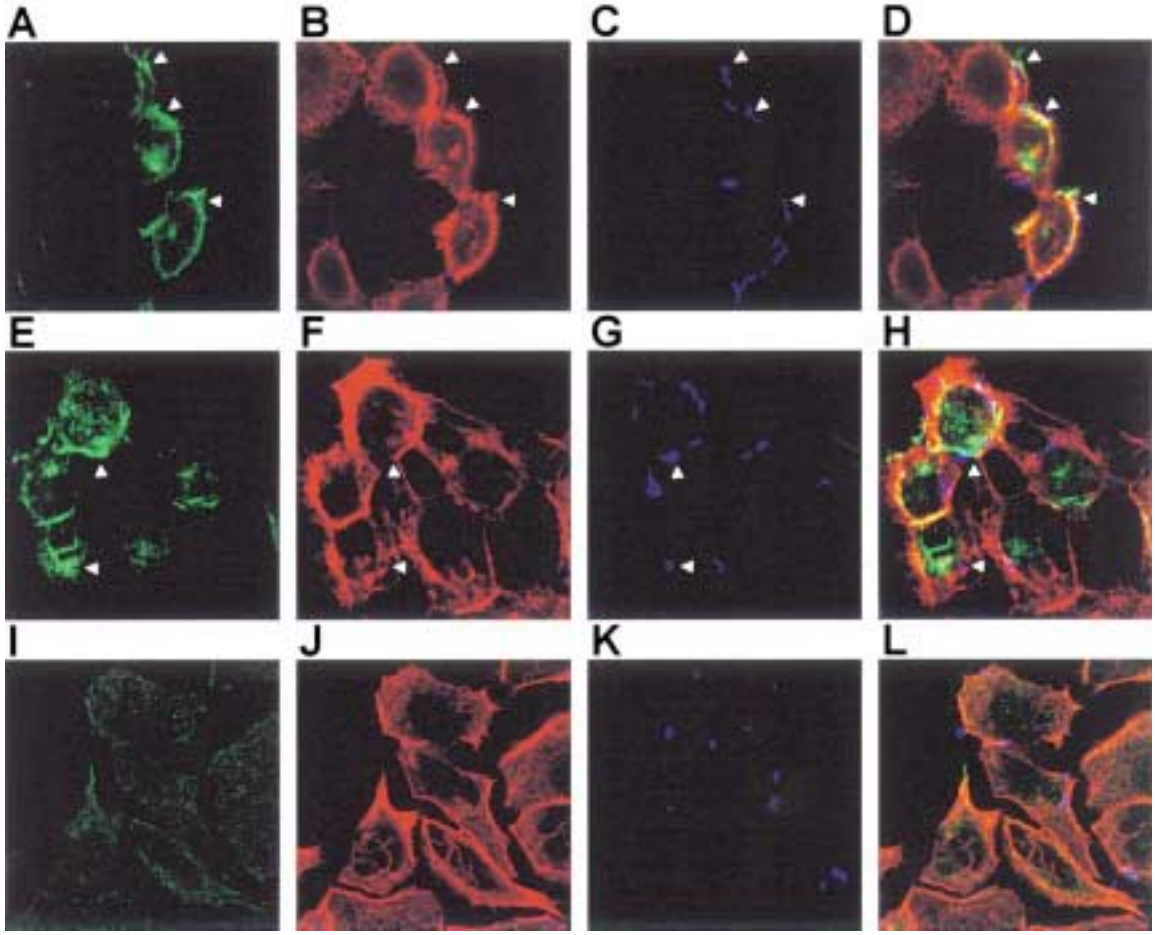

Fig. 3. Subcellular localization of endogenous Rac1 at *H. pylori* attachment sites. AGS cells were infected for 1 h with wild-type *H. pylori* strain P1 (A–D) and isogenic mutant strains *cagA* (E–H) and *virB7* (I–L). Infected cells were stained for Rac1 (shown in green) (A, E and I), F-actin (shown in red) (B, F and J) and *H. pylori* (shown in blue) (C, G and K) and examined by confocal laser scanning microscopy. An overlay of all three channels is shown in (D), (H) and (L). Arrows indicate the positions of representative bacteria associated with increased staining for Rac1.

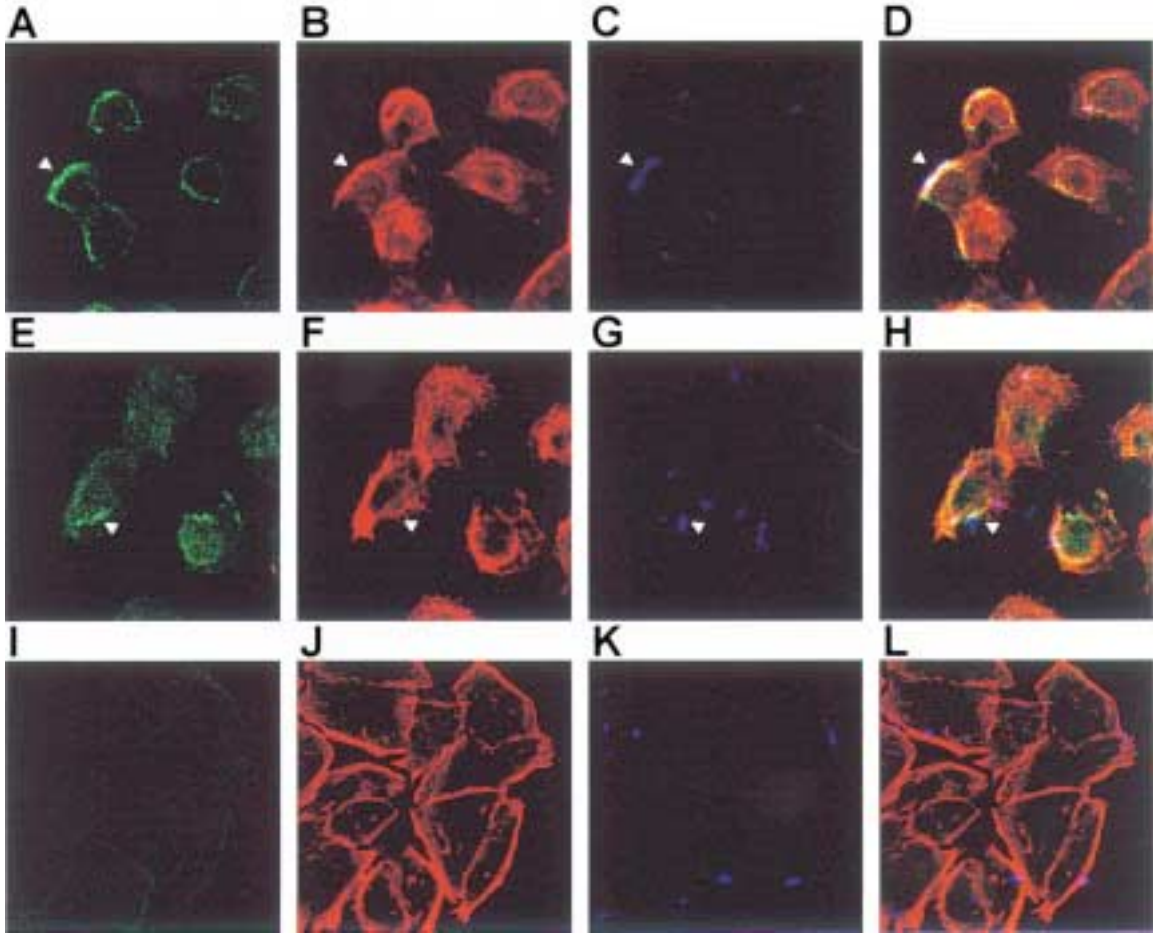

Fig. 4. Subcellular localization of endogenous Cdc42 at *H. pylori* attachment sites. AGS cells were infected for 1 h with wild-type *H. pylori* strain P1 (A–D) and isogenic mutant strains *cagA* (E–H) and *virB7* (I–L). Infected cells were stained for Cdc42 (shown in green) (A, E and I), F-actin (shown in red) (B, F and J) and *H. pylori* (shown in blue) (C, G and K) and examined by confocal laser scanning microscopy. An overlay of all three channels is shown in (D), (H) and (L). Arrows indicate the positions of representative bacteria associated with increased staining for Cdc42.

et al., 1994). Therefore, we investigated whether Rac1 directs activation of PAK1 upon infection of AGS cells with *H. pylori*, and whether its activation depends on *cag* PAI. To examine this, we performed pull-down assays from *H. pylori*-infected AGS cells using the constitutive active form of Rac1 fused to glutathione *S*-transferase (GST–Rac1L61). Lysates were prepared, and the amounts of PAK1 precipitated with GST–Rac1L61 were determined by Western blotting. As shown in Fig. 2, Rac1-dependent activation of PAK1 was observed in AGS cells within 1 h after infection with *H. pylori* P12, whereas *H. pylori* mutant strain PAI had a marginal effect on PAK1 activity. Thus, we have demonstrated that the infection of AGS cells with *H. pylori* activates Rac1 and PAK1 in a *cag* PAI-dependent manner.

Rac1 and Cdc42 proteins are recruited to H. pylori *attachment sites*

In order to analyse the potential recruitment of Rac1 and Cdc42 to bacterial attachment sites, semi-confluent AGS cells were infected with wild-type *H. pylori* strain P1 and mutant strains *cagA* and *virB7*, fixed, stained for bacteria, F-actin and for the respective GTPases and analysed by confocal microscopy (Figs 3 and 4). The observations presented here were made with untransfected cells and reflect the endogenous GTPase recruitment. Rac1 was recruited to the site of bacterial attachment after infection of AGS cells with wild-type *H. pylori* strain P12 (Fig. 3D) and mutant strain *cagA* (Fig. 3H). A similar pattern of staining was observed for Cdc42 (Fig. 4D and H). Infected cells showed profound reorganization of cytoskeleton: actin polymerization and ruffle-like structures were observed at the cell periphery (Figs 3B and F and 4B and F). In contrast, no GTPase recruitment to the site of bacterial adhesion and no dramatic actin cytoskeleton changes were observed in AGS cells infected with *H. pylori* mutant strain *virB7* (Figs 3I–L and 4I–L). These results demonstrate recruitment of Rac1 and Cdc42 to the

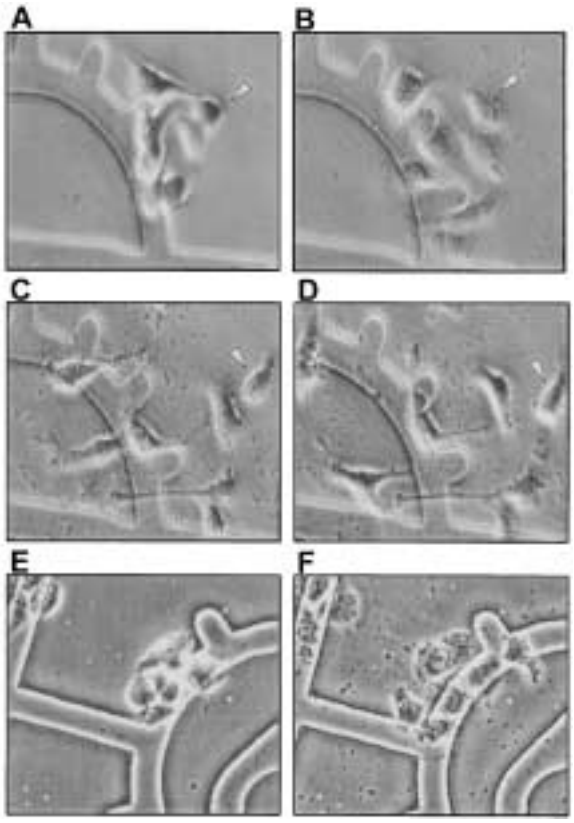

Fig. 5. *H. pylori* stimulate the motility of AGS cells. AGS cells were infected with wild-type *H. pylori* strain P12 and mutant strain PAI at MOI 50. Phase-contrast microscopy was performed at different time points after infection.
A. Uninfected AGS cells.
B, C and D. AGS cells were infected with *H. pylori* P12, and phase-contrast microscopy was performed at 1 h, 2 h and 3 h after infection respectively.
E. Uninfected AGS cells.
F. AGS plus *H. pylori* PAI, 3 h.Arrows indicate the positions of representative bacteria.

site of *H. pylori* attachment to AGS cells and the dependency of cytoskeleton reorganization in host cells on the integrity of the type IV secretion system.

Infection with H. pylori *induces motility of AGS cells*

It has been shown that *H. pylori* can induce cellular spreading and an elongated phenotype upon attachment to cultured gastric epithelial cells (Segal *et al*., 1999). This phenotype mimics that observed for hepatocyte growth factor (HGF) activation of the c-Met receptor tyrosine kinase. HGF stimulates the motility of epithelial cells, initially inducing centrifugal spreading of cell colonies, followed by disruption of cell–cell junctions and subsequent cell scattering (Ridley *et al*., 1995). Therefore, we examined whether *H. pylori* infection stimulates the motility of AGS cells. The cells grown on the CELLocate coverslips were infected with wild-type *H. pylori* strain P12 or with the mutant strain PAI, and phase-contrast microscopy was performed at various time points after infection (Fig. 5). As described previously for HGF (Ridley *et al*., 1995), cell colonies spread centrifugally during the first hour after infection, so that the area covered by each colony increased approximately twofold, but cell–cell contacts were not broken (Fig. 5B). Subsequent to cell spreading, the cells infected with wild-type *H. pylori* strain P12 detached from their neighbouring cells within the colony and migrated in different directions (Fig. 5B and C). The rate of AGS cell migration was $\approx 10\ \mu m\ h^{-1}$. In contrast, *H. pylori* mutant strain PAI could not stimulate the motility of AGS cells (Fig. 5E and F). Thus, the stimulation of host cell motility is *cag* PAI dependent, consistent with Rac1 and Cdc42 activation by wild-type *H. pylori*.

Discussion

In this study, we have investigated early changes in cellular signalling of gastric epithelial cells infected with *H. pylori*. We have demonstrated the activation of members of the Rho subfamily of small GTPases, Rac1 and Cdc42. Interestingly, activation of small GTPases was dependent on the integrity of the *cag* PAI, indicating that the type IV secretion system is involved in the activation process. Moreover, we have shown that infection with wild-type strains caused activation of PAK1, actin reorganization with ruffle-like structures and epithelial cell motility.

The activation of signalling pathways in host cells infected with *H. pylori* could be a result of bacterial protein translocation conferred by the type IV secretion system. CagA is so far the only *H. pylori* protein demonstrated to translocate into host cells (Segal *et al*., 1999; Stein *et al*., 2000; Asahi *et al*., 2000; Backert *et al*., 2000; Odenbreit *et al*., 2000). We have confirmed that the *cagA* mutant was able to elicit the stimulatory effect on the Rho GTPases in host cells. This suggests the existence of other factor(s) than CagA translocated into the host cells by a type IV secretion system. However, we cannot exclude the possibility that the interaction of functional type IV secretion apparatus with an unknown cell surface receptor could stimulate signal transduction pathways leading to Rac1 and Cdc42 activation.

Activated Rac1 and Cdc42 interact with cellular target proteins to trigger a wide variety of cellular responses, including changes in gene transcription and the reorganization of actin cytoskeleton (Mackay and Hall, 1998). 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 of infection (Bodger and Crabtree, 1998). The rapid production of proinflammatory cytokines involves the activation of immediate early transcriptional activators. Recently, it has been shown that *H. pylori* could rapidly activate transcription factors AP-1 and NF-κB

in different epithelial cells, and this activation involves PAK1 kinase (Naumann *et al*., 1999; Foryst-Ludwig and Naumann, 2000). PAK is the best-characterized effector of Rac and Cdc42. PAKs are activated by binding to the GTP-bound form of GTPases (Manser *et al*., 1994; Lim *et al*., 1996). In this study, we observed that Rac1 directs the activation of PAK1 in AGS cells infected with *H. pylori* strain P12. In contrast, *H. pylori* mutant strain PAI had only a modest effect on PAK1 activity (Fig. 2). Thus, Rac1, Cdc42 and PAK1 activation in AGS cells during infection with *H. pylori* depends on virulence factors encoded by *cag* PAI. Activation of Rac1, Cdc42 and PAK1 by *H. pylori* is transient and occurs within 1 h of infection, indicating that this activation is a very early event in *H. pylori* colonization of epithelial cells. Rac1 and Cdc42 have been shown to play a role in host signalling pathways that are activated in response to invasive bacteria (Ireton and Cossart, 1998). *Salmonella typhimurium* induces activation of JNK, and expression of a dominant-negative mutant of Cdc42 prevents JNK activation, suggesting that Cdc42 is required for this nuclear response (Chen *et al*., 1996). Further, it has been shown that infection of human myelomonocytic cells with *Neisseria gonorrhoeae* induced a strong Rac1 activation (Hauck *et al*., 1998). *H. pylori* is an extracellular pathogen, and our data indicate that it also induces the activation of Rac1 and Cdc42 that mediate via PAK1 nuclear responses in infected gastric epithelial cells.

The majority of data concerning cytoskeletal rearrangement during bacterium–host cell interaction and the control of this process by Rho GTPases were obtained from investigation of invasive bacterial pathogens *Shigella flexneri* and *S. typhimurium*. During the initial step in the entry process, *S. flexneri* induces actin polymerization at the site of bacterial contact with the host cell membrane (Adam *et al*., 1995). Actin polymerization forms filopodial structures that give rise to lamellipodial structures. This results in a macropinocytic pocket, which engulfs the microorganism. The Rho GTPases are involved in the formation of the cellular structures that support the formation of the *S. flexneri* entry focus. They are recruited in *S. flexneri* entry structures, and the size of *Shigella*-induced entry foci is reduced in the host cells expressing dominant-negative Rho, Rac1 and Cdc42 proteins (Adam *et al*., 1996; Mounier *et al*., 1999). *S. typhimurium* can also induce actin rearrangements and macropinocytosis in host cells (Francis *et al*., 1993), and expression of the dominant-negative Cdc42 mutant protein in COS1 fibroblasts inhibited this induction and prevented internalization of the bacteria into the host cells (Chen *et al*., 1996). In contrast, actin polymerization during interaction between host cells and enteropathogenic *Escherichia coli* (EPEC) has been shown to be Rho GTPase independent (Ben-Ami *et al*., 1998). Electron micrograph studies have shown that *H. pylori* intimately associates with the cytoplasmic membrane of primary gastric epithelial cells and AGS cells, causing an effacement of microvilli and cellular projections (pedestals) (Smoot *et al*., 1993; Segal *et al*., 1996), whereas other investigators found no pedestal formation (Dytoc *et al*., 1993). We have demonstrated recruitment of the GTPases Rac1 and Cdc42 to the site of *H. pylori* adhesion (Figs 3 and 4). Interestingly, this recruitment is strictly *cag* PAI dependent, consistent with the observed Rac1 and Cdc42 activation (Fig. 1A and B). In our experiments, we observed actin accumulation at the bacterial adhesion sites that was associated with GTPase recruitment. Infection of AGS cells with *H. pylori* wild-type and *cagA* mutant strains disrupted the peripheral bundles and induced the development of small membrane ruffles on the outer membranes of cells (Figs 3B and F and 4B and F). The disappearance of peripheral bundles represents a change in actin reorganization, which was also observed in HGF-treated cells and involves activation of Rac1 and Cdc42 (Ridley *et al*., 1995; Royal *et al*., 2000). In contrast to the infection with *H. pylori* wild-type strains, the *virB7* mutant strain (Figs 3J and 4J) and uninfected cells (data not shown) show that actin is mostly concentrated at the edge of the cells and at cell–cell borders in peripheral bundles associated with the lateral membranes of the cells. Opposing data by Palovuori *et al*. (2000) indicate that the formation of membrane ruffles appears in type I as well as in type II strains of *H. pylori*, which might be attributed to a different experimental approach. Taken together, both Rac1 and Cdc42 are probably involved in the massive actin rearrangement during *H. pylori* infection. Overall changes in GTPase activities in cells, which were monitored using the GTPase pull-down assay, are accompanied by recruitment of GTPases to the sites of bacterial adhesion.

Cell migration is a key aspect of many normal and abnormal biological processes, including embryonic development, defence against infections, wound healing and tumour cell metastasis. The cytoskeletal rearrangements caused by activation of Rho GTPases play a key role in the process of cell motility. It is the adhesion, subsequent loss of attachment and re-adhesion of lamellipodia and filopodia at a cell's leading edge (Hall, 1998; Kaibuchi *et al*., 1999; Schmitz *et al*., 2000). Infection with *H. pylori* stimulates the motility of AGS cells in contrast to mutant strain PAI, which failed to induce motility in host cells (Fig. 5). Thus, the ability to activate GTPases Rac1 and Cdc42 correlates with stimulation of host cell motility, which depends on *cag* PAI.

In conclusion, we have shown that *H. pylori* colonization activates GTPases Rac1 and Cdc42 in AGS cells, and this activation is a very early event in *H. pylori* infection.

Both Rac1 and Cdc42 are probably involved in actin rearrangement observed during bacterial infection. The inability of mutant *H. pylori* strains *virB7* and PAI to induce GTPase activation suggests that a functional type IV secretion system is necessary for Rac1 and Cdc42 activation by *H. pylori* infection in gastric epithelial cells.

Experimental procedures

Cell culture and H. pylori *infection*

Gastric epithelial cells (AGS) were grown in RPMI-1640 containing 4 mM glutamine (Gibco BRL), 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 10% fetal bovine serum (FBS; Gibco BRL) in a humidified 5% CO_2 atmosphere. The cells were seeded in tissue culture plates for 48 h before infection. Sixteen hours before infection, the medium was replaced by fresh RPMI-1640 medium without serum. *H. pylori* strains were cultured on agar plates containing 10% horse serum under microaerophilic conditions (generated by Campy Gen; Oxoid) at 37°C for 48 h. For the infection, bacteria were harvested in phosphate-buffered saline (PBS), pH 7.4, added to the cells at a multiplicity of infection (MOI) of 50 and incubated together with the epithelial cell monolayer for different periods of time. *H. pylori* strains that were used for colonization of AGS cells were wild-type strain P12 and isogenic mutant strain PAI (lacks *cag* PAI; Wessler *et al*., 2000), wild-type strain P1 and isogenic mutants *cagA and virB7* (Ramarao *et al*., 2000). For cultivation, bacteria were resuspended in brain–heart infusion (BHI; Difco) medium, and 10^3 bacteria were seeded per plate.

Fusion proteins and protein interaction assays

A glutathione *S*-transferase (GST)–CRIB (PAK–CRIB domain) fusion protein, containing the Rac1- and Cdc42-binding region of PAK1 was constructed and purified essentially as described previously (Sander *et al*., 1998). To construct a GST–Rac1L61 fusion protein, the coding region of constitutively active Rac1L61 was amplified in a polymerase chain reaction (PCR) and inserted into the vector pGEX2T (Pharmacia Biotech). AGS cells were infected with *H. pylori* for different periods of time. At the indicated time points, cells were washed with cold PBS (containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$) and lysed on ice in buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1% NP-40, 10% glycerol, 100 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mg ml^{-1} aprotinin and 1 mg ml^{-1} pepstatin (1 mM Na_3VO_4 was added to the lysis buffer for PAK assay), and then centrifuged for 10 min at 14 000 *g* at 4°C. The supernatant was incubated with the recombinant GST–CRIB fusion protein, bound to glutathione-coupled Sepharose beads at 4°C for 30 min. The beads and proteins bound to the fusion protein were washed three times in an excess of lysis buffer, eluted in Laemmli sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and then analysed for bound Rac1, Cdc42 or PAK molecules by Western blotting using an antibody against human Rac1 (Transduction Laboratories), Cdc42 (Santa Cruz) and anti-αPAK (Santa Cruz). Aliquots from the supernatants were analysed in a Western blot to confirm the presence of similar quantities of proteins used for precipitation. Transient transfections of constructs containing cDNAs of constitutive active mutants of Rac1L61 and Cdc42L61 (c-Myc epitope at the N-terminus of the proteins) were performed using cationic liposomes (DAC 30; Eurogentec) according to the manufacturer's protocol. Twenty-four hours before transfection, cells were seeded in tissue culture plates and grown to 60–70% confluence.

Phase-contrast microscopy

AGS cells (1×10^2 $well^{-1}$) were grown on CELLocate coverslips in a 12-well tissue culture testplate (TPP) in complete RPMI-1640 medium for 48 h. Sixteen hours before infection, the medium was replaced by fresh RPMI-1640 medium without serum. For infection, bacteria were added to the cells at a MOI of 50. At different time points after infection, phase-contrast microscopy was performed using an Olympus IX50-S8F inverted microscope.

Immunofluorescence labelling and confocal laser scanning microscopy

Immunofluorescence staining of AGS cells infected with *H. pylori* was performed as described elsewhere (Dehio *et al*., 1997). The samples were viewed with a confocal microscope (Leica Lasertechnik) equipped with an argon–krypton mixed gas laser. The corresponding images were processed digitally with PHOTOSHOP 5.0 (Adobe Systems).

Acknowledgements

We thank S. Backert for providing *H. pylori* mutant strains, and A. Sczepek for critical reading of the manuscript.

References

Adam, T., Arpin, M., Prevost, M.C., Gounon, P., and Sansonetti, P.J. (1995) Cytoskeletal rearrangements and the functional role of T-plastin during entry of *Shigella flexneri* into HeLa cells. *J Cell Biol* **129**: 367–381.

Adam, T., Giry, M., Boquet, P., and Sansonetti, P. (1996) Rho-dependent membrane folding causes *Shigella* entry into epithelial cells. *EMBO J* **15**: 3315–3321.

Asahi, M., Azuma, T., Ito, S., Ito, Y., Suto, H., Nagai, Y., *et al.* (2000) *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *J Exp Med* **191**: 593–602.

Backert, S., Ziska, E., Brinkmann, V., Zimny-Arndt, U., Fauconnier, A., Jungblut, P.R., *et al.* (2000) Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* **2**: 155–164.

Baeuerle, P.A., and Baichwal, V.R. (1997) NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules. *Adv Immunol* **65**: 111–137.

Ben-Ami, G., Ozeri, V., Hanski, E., Hofmann, F., Aktories, K.,

Hahn, K.M., *et al.* (1998) Agents that inhibit Rho, Rac, and Cdc42 do not block formation of actin pedestals in HeLa cells infected with enteropathogenic *Escherichia coli*. *Infect Immun* **66**: 1755–1758.

Bodger, K., and Crabtree, J.E. (1998) *Helicobacter pylori* and gastric inflammation. *Br Med Bull* **54**: 139–150.

Censini, S., Lange, C., Xiang, Z., Crabtree, J.E., Ghiara, P., Borodovsky, M., *et al.* (1996) *Cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* **93**: 14648–14653.

Chen, L.-M., Hobbie, S., and Galan, J.E. (1996) Requirement of CDC42 for *Salmonella*-induced cytoskeletal and nuclear responses. *Science* **274**: 2115–2118.

Coso, O.A., Chiariello, M., Yu, J., Teramoto, H., Crespo, P., Xu, N., *et al.* (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**: 1137–1146.

Covacci, A., Telford, J.L., Del Giudice, G., Parsonnet, J., and Rappuoli, R. (1999) *Helicobacter pylori* virulence and genetic geography. *Science* **284**: 1328–1333.

Crabtree, J.E., Covacci, A., Farmery, S.M., Xiang, Z., Tompkins, D.S., Perry, S., *et al.* (1995) *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. *J Clin Pathol* **48**: 41–45.

Dehio, C., Meyer, M., Berger, J., Schwarz, H., and Lanz, C. (1997) Interaction of *Bartonella henselae* with endothelial cells results in bacterial aggregation on the cell surface and the subsequent engulfment and internalisation of the bacterial aggregate by a unique structure, the invasome. *J Cell Sci* **110**: 2141–2154.

Dunn, B.E., Cohen, H., and Blaser, M.J. (1997) Helicobacter pylori. *Clin Microbiol Rev* **10**: 720–741.

Dytoc, M., Gold, B., Louie, M., Huesca, M., Fedorko, L., Crowe, S., *et al.* (1993) Comparison of *Helicobacter pylori* and attaching-effacing *Escherichia coli* adhesion to eukaryotic cells. *Infect Immun* **61**: 448–456.

Falk, P., Roth, K., Boren, T., Westblom, T., Gordon, J., and Normark, S. (1993) An *in vitro* adherence assay reveals that *Helicobacter pylori* exhibits cell lineage-specific tropism in the human gastric epithelium. *Proc Nat Acad Sci USA* **90**: 2035–2039.

Foryst-Ludwig, A., and Naumann, M. (2000) p21-activated kinase 1 activates the nuclear factor kappa B (NF-kappa B)-inducing kinase-ikappa B kinases NF-kappa B pathway and proinflammatory cytokines in *Helicobacter pylori* infection. *J Biol Chem* **275**: 39779–39785.

Francis, C.L., Ryan, T.A., Jones, B.D., Smith, S.J., and Falkow, S. (1993) Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature* **364**: 639–642.

Glocker, E., Lange, C., Covacci, A., Bereswill, S., Kist, M., and Pahl, H.L. (1998) Proteins encoded by the cag pathogenicity island of *Helicobacter pylori* are required for NF-kappaB activation. *Infect Immun* **66**: 2346–2348.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**: 509–514.

Hauck, C.R., Meyer, T.F., Lang, F., and Gulbins, E. (1998) CD66-mediated phagocytosis of Opa_{52} *Neisseria gonorrhoeae* requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway. *EMBO J* **17**: 443–454.

Ip, Y.T., and Davis, R.J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. *Curr Opin Cell Biol* **10**: 205–219.

Ireton, K., and Cossart, P. (1998) Interaction of invasive bacteria with host signaling pathways. *Curr Opin Cell Biol* **10**: 276–283.

Kaibuchi, K., Kuroda, S., and Amano, M. (1999) Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem* **68**: 459–486.

Keates, S., Hitti, Y.S., Upton, M., and Kelly, C.P. (1997) *Helicobacter pylori* infection activates NF-kappa-B in gastric epithelial cells. *Gastroenterology* **113**: 1099–1109.

Keates, S., Keates, A.C., Warny, M., Peek, R.M., Jr, Murray, P.G., and Kelly, C.P. (1999) Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by *cag*+ and *cag*– *Helicobacter pylori*. *J Immunol* **163**: 5552–5559.

Lim, L., Manser, E., Leung, T., and Hall, C. (1996) Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur J Biochem* **242**: 171–185.

Mackay, D.J., and Hall, A. (1998) Rho GTPases. *J Biol Chem* **273**: 20685–20688.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S., and Lim, L. (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**: 40–46.

Minden, A., Lin, A., Claret, F.X., Abo, A., and Karin, M. (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**: 1147–1157.

Mounier, J., Laurent, V., Hall, A., Fort, P., Carlier, M.-F., Sansonetti, P.J., and Egile, C. (1999) Rho family GTPases control entry of *Shigella flexneri* into epithelial cells but not intracellular motility. *J Cell Sci* **112**: 2069–2080.

Münzenmaier, A., Lange, C., Glocker, E., Covacci, A., Moran, A., Bereswill, S., *et al.* (1997) A secreted/shed product of *Helicobacter pylori* activates transcription factor nuclear factor-kappa B. *J Immunol* **159**: 6140–6147.

Naumann, M., Rudel, T., Wieland, B., Bartsch, C., and Meyer, T.F. (1998) Coordinate activation of activator protein 1 and inflammatory cytokines in response *to Neisseria gonorrhoeae* epithelial cell contact involves stress response kinases. *J Exp Med* **188**: 1277–1286.

Naumann, M., Wessler, S., Bartsch, C., Wieland, B., Covacci, A., Haas, R., and Meyer, T.F. (1999) Activation of activator protein 1 and stress response kinases in epithelial cells colonized by *Helicobacter pylori* encoding the *cag* pathogenicity island. *J Biol Chem* **274**: 31655–31662.

Nobes, C.D., and Hall, A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**: 53–62.

Nobes, C.D., and Hall, A. (1999) Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol* **144**: 1235–1244.

Odenbreit, S., Püls, J., Sedlmaier, B., Gerland, E., Fischer, W., and Haas, R. (2000) Translocation of *Helicobacter*

pylori CagA into gastric epithelial cells by type IV secretion. *Science* **287**: 1497–1500.

Palovuori, R., Perttu, A., Yan, Y., Karttunen, R., Eskelinen, S., and Karttunen, T.J. (2000) *Helicobacter pylori* induces formation of stress fibers and membrane ruffles in AGS cells by rac activation. *Biochem Biophys Res Commun* **269**: 247–253.

Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lacal, J.C. (1997) Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev* **11**: 463–475.

Ramarao, N., Gray-Owen, S.D., Backert, S., and Meyer, T.F. (2000) *Helicobacter pylori* inhibits phagocytosis by professional phagocytes involving type IV secretion components. *Mol Microbiol* **37**: 1389–1404.

Ridley, A.J., Comoglio, P.M., and Hall, A. (1995) Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. *Mol Cell Biol* **15**: 1110–1122.

Royal, I., Lamarche-Vane, N., Lamorte, L., Kaibuchi, K., and Park, M. (2000) Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. *Mol Biol Cell* **11**: 1709–1725.

Sander, E.E., van Delft, S., ten Klooster, J.P., Reid, T., van der Kammen, R.A., Michiels, F., and Collard, J.G. (1998) Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J Cell Biol* **143**: 1385–1398.

Schmitz, A.A.P., Govek, E.-E., Böttner, B., and Van Aelst, L. (2000) Rho GTPases: signaling, migration, and invasion. *Exp Cell Res* **261**: 1–12.

Segal, E.D., Falkow, S., and Tompkins, L.S. (1996) *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proc Natl Acad Sci USA* **93**: 1259–1264.

Segal, E.D., Cha, J., Lo, J., Falkow, S., and Tompkins, L.S. (1999) Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc Natl Acad Sci USA* **96**: 14559–14564.

Sharma, S.A., Tummuru, M.K.R., Blaser, M.J., and Kerr, L.D. (1998) Activation of IL-8 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor kappa B in gastric epithelial cells. *J Immunol* **160**: 2401–2407.

Smoot, D.T., Resau, J.H., Naab, T., Desbordes, B.C., Gilliam, T., Bull-Henry, K., *et al.* (1993) Adherence of *Helicobacter pylori* to cultured human gastric epithelial cells. *Infect Immun* **61**: 350–355.

Stein, M., Rappuoli, R., and Covacci, A. (2000) Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*–driven host cell translocation. *Proc Natl Acad Sci USA* **97**: 1263–1268.

Wessler, S., Höcker, M., Fischer, W., Wang, T.C., Rosewicz, S., Haas, R., *et al.* (2000) *Helicobacter pylori* activates the histidine decarboxylase promoter through a mitogen-activated protein kinase pathway independent of pathogenicity island-encoded virulence factors. *J Biol Chem* **275**: 3629–3636.