Biochemical and Biophysical Research Communications **269,** 247–253 (2000)
doi:10.1006/bbrc.2000.2276, available online at http://www.idealibrary.com on IDEAL®

Helicobacter pylori Induces Formation of Stress Fibers and Membrane Ruffles in AGS Cells by rac Activation

Riitta Palovuori,*,† Annina Perttu,* Ying Yan,‡ Riitta Karttunen,‡ Sinikka Eskelinen,*,† and Tuomo J. Karttunen*,[1]

**Department of Pathology, ‡Department of Microbiology, and †Biocenter Oulu, University of Oulu, PL 5000, FIN 90401 Oulu, Finland*

Received January 31, 2000

***Helicobacter pylori* induces signaling cascades leading to changes in cytoskeleton and an inflammatory response. Information on the morphological changes and cytoskeletal rearrangements induced by attachment of the bacterium is contradictory and signal transduction pathways are not well known. Since rho family of small GTPases is known to mediate cytoskeletal response to various extracellular stimuli, and is also involved in several other important signal transduction pathways, we have investigated the role of rac and cdc42 in *H. pylori*-induced cytoskeletal changes in cultured carcinoma AGS cells. AGS cells grown with serum expressed actin filaments in the form of short stress fibers and thin network at the edges, which were depolymerized by removal of serum. In serum-starved cells both type I and type II strains of *H. pylori* induced formation of actin filaments and lamellipodia-like structures. Microinjection of active rac induced similar changes, but injection of inactive rac prevented the effects of *H. pylori,* while active or inactive cdc42 did not have any significant effect. Cytoskeletal effects of *H. pylori* were inhibited by actinomycin D, but not completely by cycloheximide. These results indicate that rac activation is involved in signal transduction cascade leading to cytoskeletal reorganization induced by *H. pylori* and that gene activation and synthesis of new proteins is necessary in this process.**

Helicobacter pylori is gram-negative curved bacillus, which is a human pathogen causing chronic gastritis, peptic ulcer and gastric neoplasia including both lymphoid and epithelial malignancies (1).

H. pylori is able to adhere to gastric epithelial cells. Several molecules have been proposed as receptors for *H. pylori* adhesion, of which the Lewis B antigen seems to be most important *in vivo* (2). Experiments with gastric epithelial cell lines (AGS, KatoIII) have shown that bacterial attachment results in rearrangement of the actin beneath the adhered bacterium, formation of pedestal and effacement of the microvilli at the site of adhesion (3–5). However, other investigators have not been able to demonstrate rearrangement of the cytoskeleton after *H. pylori* attachment (6, 7).

Attachment of *H. pylori* is the first step in the pathogenesis of *H. pylori* induced tissue damage. Attachment is followed by secretion of IL-8 by epithelial cell and changes in its cytoskeleton (8, 9). These effects are important in the development of tissue damage both by induction of the inflammatory cell recruitment by IL-8 secretion and by decreasing the stability of cytoskeleton and cell membrane.

There is not much information of signal transduction pathways activated by *H. pylori* attachment. Segal *et al.* (4) found that cytoskeletal changes were associated with tyrosine phosphorylation of 145-kDa and 40-kDa proteins, which have not been characterized. Induction of IL-8 secretion by *H. pylori* is regulated by a transcription factor NFκB (9).

Rho family of small guanosine triphosphatases is involved in the reorganization of actin cytoskeleton in response to various extracellular stimuli (10). Similarly with other members of the ras superfamily, these proteins act as molecular switches to control cellular processes. Cytoskeletal effects include formation of contractile actomyosin filaments by rho, actin polymerization into lamellipodial structures by rac and formation of filopodial membrane protrusions by cdc42 (11, 12). These GTPases have been shown to be important in several other processes including cell cycle regulation, NFκB activation, activation of response to inflammatory stimuli and malignant transformation (10). Recently it has been shown that small GTPases are targets for various bacterial toxins including cytotoxic necrotizing factor of *E. coli*, which causes activation of rac and cdc42 (13).

[1] To whom correspondence should be addressed. Fax: +358-8-537 5943. E-mail: tuomo.karttunen@oulu.fi.

0006-291X/00 $35.00

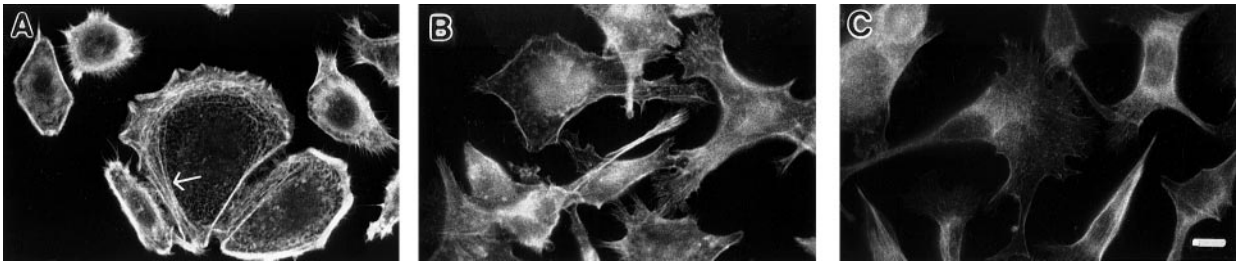

FIG. 1. Effect of fetal calf serum and serum starvation on the actin cytoskeleton of AGS cells. The cells grown in the presence of fetal calf serum (A) and in the absence of serum for 30 minutes (B) and 1 hour (C). Arrows show thin rhodamine phalloidin-labeled actin filaments. Bar: 10 μm.

We have studied the role of serum and two small GTPases rac and cdc42 in production of cytoskeletal changes caused by *H. pylori*. We report here that *H. pylori* induces formation of stress fibers and lamellipodia in serum-starved AGS cells. Furthermore, by injecting active and inactive GTPases we show, that rac but not cdc42 is involved in signal transduction pathway of the cytoskeletal changes.

MATERIALS AND METHODS

Cell culture. Human gastric adenocarcinoma (AGS) cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). They were cultured for fluorescence microscopy on glass coverslips or for microinjection studies on etched grid coverslips marked by numbers and letters (Bellco, Vineland, NJ) in Ham's F12 medium (Gibco BRL, Gaithersburg MD) supplemented with L-glutamine, with 10% fetal calf serum (FCS, HyClone, Logan, UT), antibiotics and antimycotics in a humidified 5% CO_2 atmosphere.

Helicobacter pylori culture. H. pylori strain CCUG17874 (Culture Collection, University of Goteborg, Sweden), and two local strains (581/96 and 523/96) isolated from dyspeptic children were cultured on Mueller-Hinton plates supplemented with sheep blood (5%) and antibiotics (vancomycin 10 mg/L, trimethoprim 5 mg/L, cefsulodin 5 mg/L, amphotericin-B 5 mg/L, Oxoid, Basingstoke, Hampshire, UK). The strain CCUG17874 is cagA+vacAs1, and the strains 581/96 and 523/96 cagA-vacAs2m2iceA1, cagA-vacAs2m2iceA2, respectively as tested with PCR using published primers (14).

Infection assays. The bacteria were washed with PBS (0.01 M NaH_2PO_4/Na_2HPO_4, 145 mM NaCl, pH 7.2) and harvested in F12 medium without FCS and antibiotics/antimycotics. Bacterial concentration was adjusted to optical density 0.5 using wave length of 565 nm. AGS cells were transferred to medium without FCS, antibiotics and antimycotics about 15 minutes before bacteria were added (1 ml bacteria/2 ml media) and the mixture was incubated at +37°C for time periods indicated in Results, usually two hours. After that the cells were fixed and stained for actin as described below.

Microinjection experiments. Active and inactive forms of recombinant human cdc42 and rac proteins, purchased from Cytoskeleton (Denver, CO), were used for microinjections at a concentration of 200 μg/ml in the injection buffer. For identification of the injected cells in inactive rac or cdc42 injection, Texas Red goat anti-rabbit antibody (Molecular Probes, Eugene, OR) was used as a marker at a concentration of 0.7 mg/ml. Microinjections were carried out using an Eppendorf micromanipulator 5171 and microinjector 5246 (Hamburg, Germany) installed on an Axiovert 405 M inverted microscope with a heating stage (Zeiss, Oberkochen, Germany) as described earlier (15). All the cells within one square of the etched coverslips were injected within a time period of 10 to 20 minutes. The cells were injected in normal growth medium and transferred to medium without FCS and antibiotics/antimycotics 15 minutes before adding *H. pylori* bacteria.

Actinomycin D and cycloheximide treatment. Actinomycin D and cycloheximide were purchased from Sigma Chemical Co (St. Louis, MO). Stock solutions of 1 mg/ml, made in F12 medium without FCS and antibiotics/antimycotics, were diluted to concentration of 0.2, 1, 2 μg/ml for actinomycin D and 0.1, 10 μg/ml for cycloheximide. Actinomycin D and cycloheximide were added to cells 15 minutes before *H. pylori* infection.

Fluorescence microscopy. For actin staining, the cells were fixed in 4% formaldehyde, 0.2% Triton X-100 in cytoskeleton-stabilizing buffer, PEM (100 mM Pipes, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8) for 10 minutes at room temperature and post-fixed in ethanol for few seconds at −20°C. After washing in PBS, unspecific protein binding sites were blocked with 10% FCS in PBS-glycine (0.02 M glycine in PBS) and thereafter the cells were labeled with rhodamine phalloidin or bodipy phallacidin (Molecular Probes, Eugene, OR) in 5% FCS in PBS-glycine. After washing with PBS-glycine and water, the specimens were mounted in Shandon mounting liquid (Immu-Mount, Pittsburgh, PA) and viewed by Zeiss Axiovert 405 microscope and 100× objective. The fluorescence and phase contrast images were photographed using KODAK p3200 Tmax film.

RESULTS

Depolymerization of Actin in Serum-Starved AGS Cells

It has been shown that actin filaments may be depolymerized when the cells are serum-starved (16, 17). Therefore we wished to investigate the behavior of AGS cells in serum-starved conditions before starting experiments with Helicobacteria. We studied the actin structures in AGS cells, by incubating them without serum for different time periods and thereafter returning them back to serum-containing medium. In medium with FCS, AGS cells had stress fibers and some lamellipodia-like structure although not as well developed as in non-transformed fibroblasts (Fig. 1A). In serum-starved cells, the actin filaments began to disrupt within 30 minutes (Fig. 1B) and were totally destroyed after one hour (Fig. 1C). The phenomenon was reversible when the cells were returned back to serum-containing medium (data not shown).

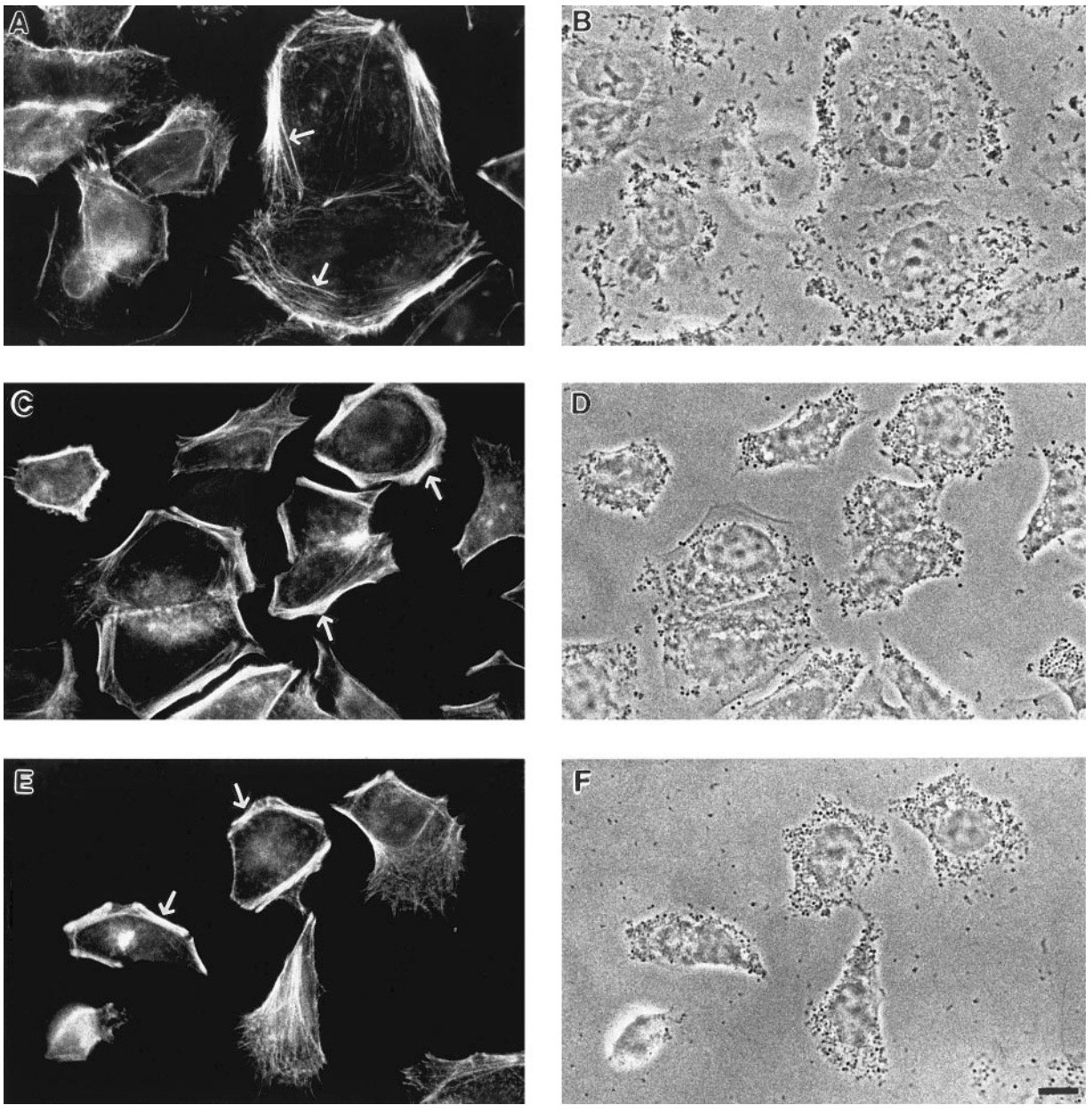

FIG. 2. *Helicobacter pylori*-induced actin polymerization in serum-starved AGS cells 2 hours after infection. Both type I (strain CCUG17874: A, B) and type II strains (strain 523 C, D; strain 481: E, F) induce similar changes In fluorescence images arrows show rhodamine phalloidin-labeled lamellipodia-like structures (A, C, E). Phase-contrast micrographs show the accumulation of bacteria around the cells (B, D, F). Bar: 10 μm.

Polymerization of Actin in H. pylori-Infected Cells

We analyzed the effect of *H. pylori* on the cell morphology and cytoskeleton in serum-free medium to avoid the interference of serum on the signaling pathways activated by *H. pylori.* Both cagA and toxin positive strain (CCUG17874) and cagA and toxin negative strains (481/96 and 523/96) induced formation of large actin ruffles and belts (lamellipodia-like structure) especially into the edges of spread cells where the clusters of bacteria were seen (Figs. 2A–2F). The phenomenon seemed to be time dependent and the best ruffles and lamellipodia-like structure were seen within 2 hours after infection.

Inactive rac Prevents Formation of Ruffles and Actin Belts in H. pylori-Infected Cells

In Swiss 3T3 cells, it has been shown that activation of cdc42 induces formation of filopodia and activation of rac formation of lamellipodia and membrane ruffling, whereas the assembly of stress fibers is induced

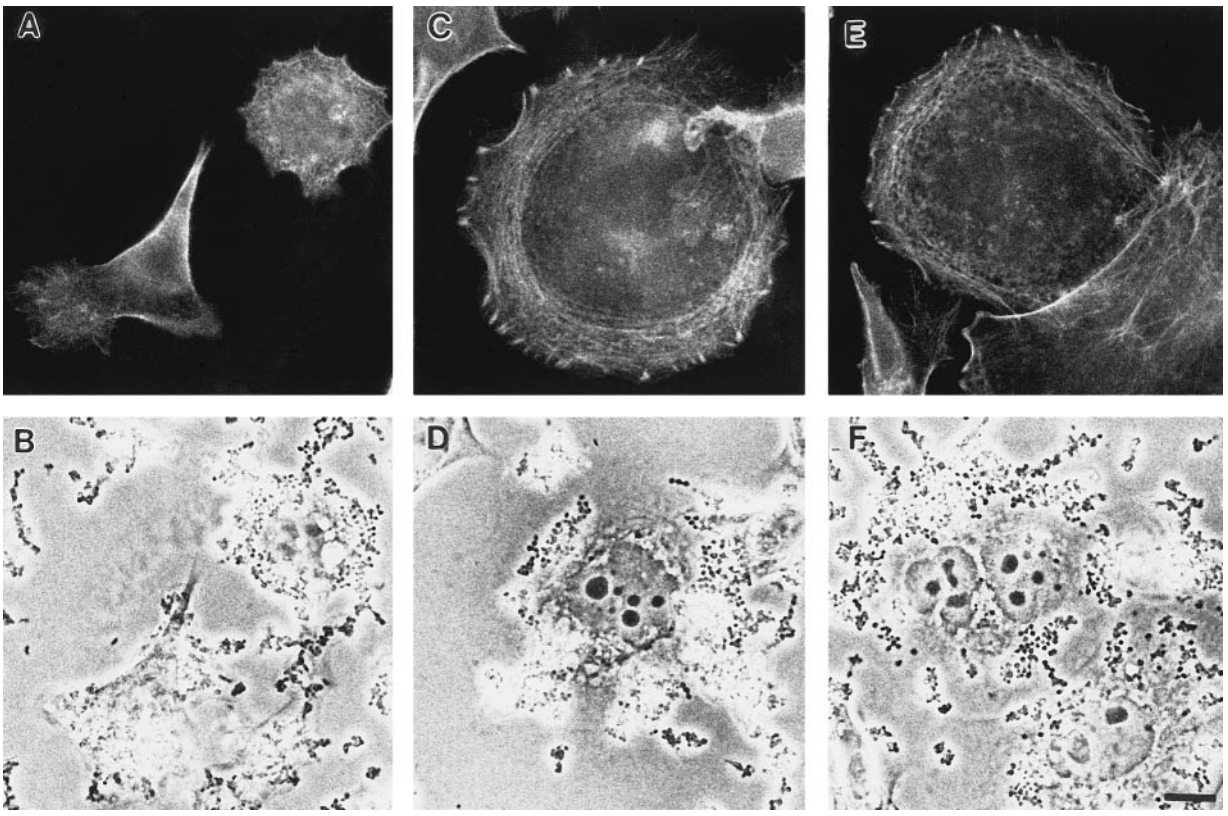

FIG. 3. Inhibition of actin polymerization by inactive rac in *Helicobacter pylori*-infected AGS cells. Fluorescence images showing bodipy phallasidin-labeled actin after injection of inactive rac (A) or inactive cdc42 (C) and after treatment with *H. pylori*. Texas Red goat anti-rabbit antibody was used as a marker for the identification of injected cells. Fluorescence image of uninjected control is shown in E. Phase-contrast micrographs show the corresponding distribution of bacteria around the cells (B, D, F). Bar: 10 μm.

through rho activation (11, 17). To find out the mechanism of formation of actin fibers and membrane ruffles in *H. pylori* infected cells, we decided to microinject inactive rac and cdc42 protein constructs into the cells before bacteria infection. Formation of *H. pylori* induced actin ruffles and lamellipodia-like network were prevented by inactive rac (Figs. 3A and 3B). In addition in some cells microvilli were seen as aggregates (data not shown). In contrast, inactive cdc42 could not prevent the formation of membrane ruffles (Figs. 3C and 3D) and the cells were similar to controls (Figs. 3E and 3F)

Active rac Induces Similar Actin Structures as H. pylori

To compare the effect of *H. pylori* to pure activation of rac and cdc42 in AGS cells, we injected active forms of rac and cdc42 to serum-starved and antibiotics-depleted cells. Active rac seemed to induce very thin network of actin filaments and ruffles at the edges of the cells (Fig. 4A) within 1 hour after microinjection but the induction was not as strong as with *H. pylori*. Conversely, active cdc42 did not induce formation of ruffles but small microspikes were formed (Fig. 4B) and the cells were spread. Thus, it is evident that *H. pylori* induces a signaling cascade leading to activation of rac and formation of lamellipodia-like network in serum-starved cells.

Inhibition of Protein Synthesis by Actinomycin D or Cycloheximide

To find out the role of RNA and protein synthesis in actin polymerization process of *H. pylori*-treated cells, we used different concentration of actinomycin D to inhibit RNA synthesis and cycloheximide to inhibit protein synthesis. Actinomycin D at a concentration of 2 μg/ml seemed to prevent Helicobacteria-induced actin polymerization in AGS cells (Figs. 5A and 5B). Only some microvilli were seen. By using more diluted solutions of actinomycin D (1 and 0.2 μg/ml) some focal adhesions and stress fibers became visible (data not shown). Instead, actin polymerization was not completely prevented with cycloheximide at a concentration of 10 μg/ml (Figs. 5C and 5D).

DISCUSSION

There is a link between *H. pylori* infection and the development of chronic gastric inflammation, peptic

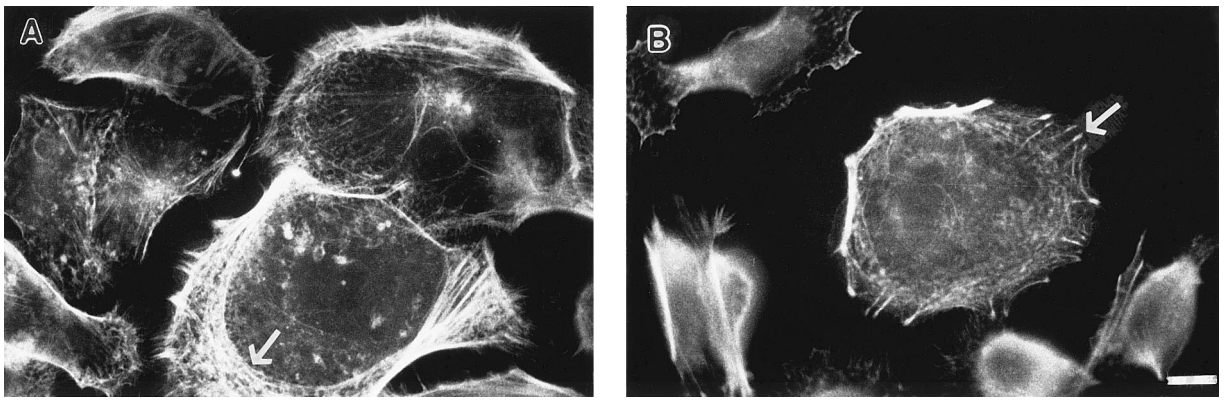

FIG. 4. Polymerization of actin in serum-starved AGS cells by active rac and cdc42 proteins. Arrows show lamellipodia-like structures in rac-injected cells (A) and filopodia-like structures in cdc42-injected cells (B). Bar: 10 μm.

ulcer and gastric malignancy. The steps from the infection during early childhood to the development of these diseases has been in the focus of extensive research. It has been shown that *H. pylori* has genetic virulence factors which may modify the grade and nature of inflammation. The best characterized factors are a cag pathogenicity island (PAI) and *vacA* gene coding for the vacuolating cytotoxin. The presence of PAI associates with increased IL-8 secretion response of the epithelial cells (18). As *cagA* gene is a marker for PAI, the CagA+ strains (type I strains; 18) are more virulent than CagA− strains (type II strains) causing IL-8 secretion and neutrophil recruitment. It is not known which molecules coded by the cag PAI actually cause IL-8 secretion, and how is this mediated. On the other hand it is known that NFκB is a transcription

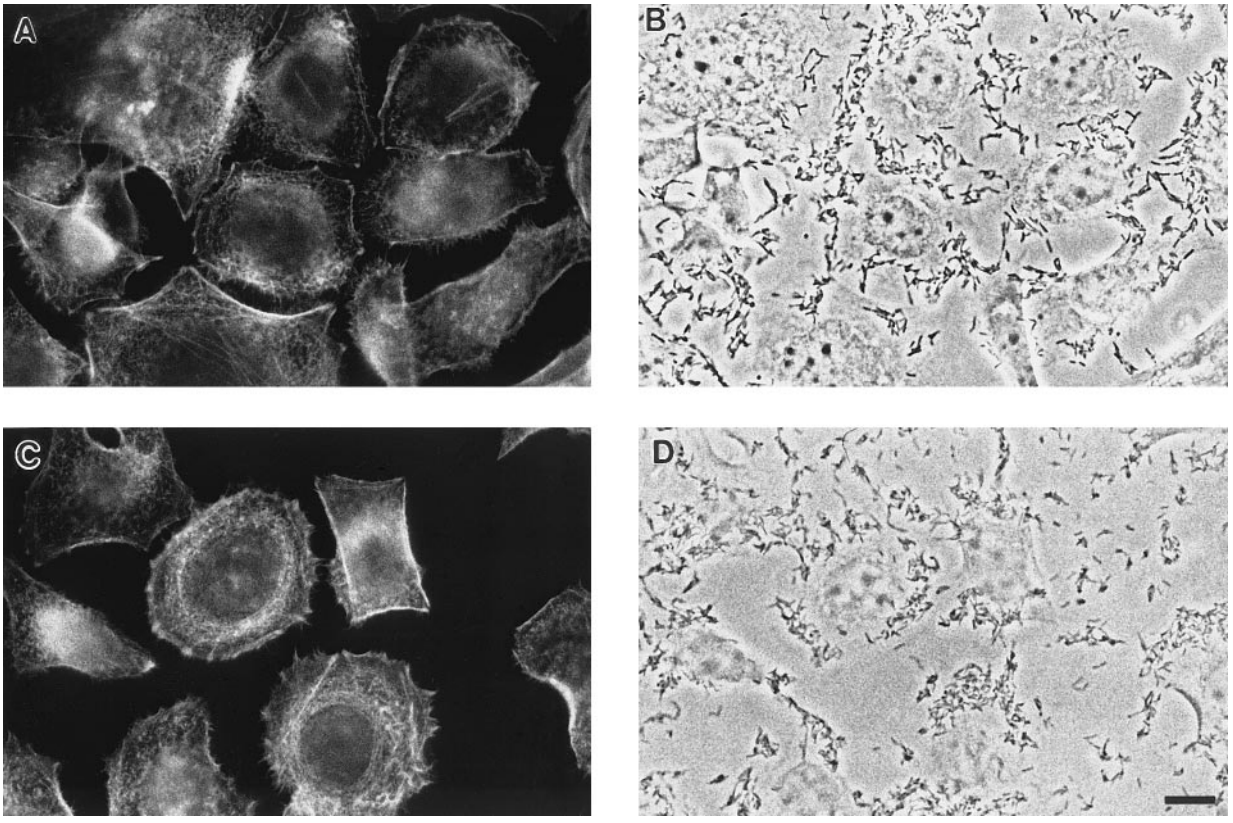

FIG. 5. Effect of actinomycin D (A) and cycloheximide (C) on actin in *Helicobacter pylori*-infected AGS cells labeled with rhodamine phalloidin. The lack of lamellipodia-like structures in actinomycin D-treated cells is evident, whereas the cells treated with cycloheximide had short filaments. Corresponding phase-contrast images are shown in B and D. Bar: 10 μm.

factor in this process (8, 9). The information on the morphological changes and cytoskeleton rearrangements induced by the bacterium are contradictory (4–6, 19, 20). There is still need for a careful study on the effects of an attachment of *H. pylori* to the gastric epithelial cells and subsequent events triggered by it.

In the present work we investigated the signaling pathways induced by the attachment of *H. pylori* to the surface of cultured gastric adenocarcinoma AGS cells and monitored the reorganization of the actin cytoskeleton and cell morphology. First we analyzed the actin structures in the non-treated AGS cells which seemed to be reasonably well-developed taking into the account that these are transformed cells. In serum containing media these consisted of stress fibers and some lamellipodia quite comparable to those in non-transformed fibroblasts (12, 21). Second, we analyzed the effect of serum starvation on these actin structures since it has been shown that lysophosphatidic acid in serum is able to activate a small GTPase rho and induce formation of stress fibers (17). AGS cells seemed to be extremely sensitive to serum. Actin filaments were very rapidly depolymerized due to serum depletion and appeared again by adding serum to the medium. We analyzed the effect of *H. pylori* on serum-starved AGS cells to be sure that the effects are solely due to the adherence of bacteria and not due to a combined effect of bacteria and some components in the fetal calf serum. The effect of bacteria was very clear and both cagA and toxin positive and cagA and toxin negative strains of *H. pylori* induced a strong polymerization of actin including development of stress fibers and formation of a meshwork of actin around the cell edges resembling lamellipodia in fibroblasts (21).

The earlier reports on the effect of *H. pylori* on actin filaments in gastric epithelial cells are contradictory either finding no effect (19, 20), or describing polymerization of actin at the adhesion site (3, 5). The effect of serum in these experiments has not been taken into account which hampers the comparison of the results. In the previous adhesion experiments with *H. pylori* carried out in the presence of serum, the stress fibers were already present in the cells and the additional effects of *H. pylori* might have been hidden. In our hands, in carefully controlled circumstances, the formation of lamellipodia in AGS cells by *H. pylori* was very reproducible and occurred both with type I and type II strains.

It has been shown in many cell types that activation of small GTPases of rho family is crucial for the polymerization of actin filaments and more specifically, activation of rac is crucial for the formation of lamellipodia (11). Therefore we expected that the adherence of *H. pylori* on AGS cells has to activate rac or another GTPase, cdc42, which has been reported in some circumstances to activate rac (12), although its main documented effect is the formation of filopodia in motile cells (11, 21). To test this hypothesis we microinjected into the AGS cells inactive forms of cdc42 and rac before infection with *H. pylori*. As we assumed, inactive rac prevented the action of bacteria on the cytoskeleton and actin remained depolymerized, although bacteria were attached to the cells. To be sure that the *H. pylori*-induced effect led to the activation of rac we wished to compare it to direct activation of rac GTPase. Therefore we microinjected into AGS cells active forms of cdc42 and rac. Since AGS cells are not motile, cdc42 did not induce filopodia as in fibroblasts. On the other hand, active rac induced formation of similar meshwork of actin filaments along the cell edges as different strains of *H. pylori*. Thus, we feel confident that *H. pylori* infection leads to activation of the rac pathway in AGS cells. According to our observations this holds true for both type I and type II strains of the bacterium.

The GTPase rac links ligand–receptor interactions to a number of biological responses via several effector proteins finally leading to actin organization, apoptosis, JNK activation, NFκB activation and in NADPH oxidase regulation (reviewed by Aspenström (22)). To better understand the signaling pathway downstream from rac after its activation by *H. pylori* we analyzed the effect of two inhibitors, actinomycin which inhibits the RNA synthesis and cycloheximide which inhibits the protein synthesis, on the polymerization of actin by *H. pylori*. From these inhibitors, actinomycin prevented the formation of lamellipodia by *H. pylori*. Thus, the gene expression and synthesis of novel proteins seem to be a prerequisite for the reorganization of actin filaments after the adherence of *H. pylori* bacteria and subsequent activation of rac.

The upstream and downstream effectors of rac in AGS cells are unknown. One of the cell surface receptors leading to rac activation is the platelet-derived growth factor receptor (22). On the other hand, one of the best known receptors on epithelial cell for *H. pylori* is Lewis^b which binds adhesion molecule BabA2 (23, 24). The relationship between rac activation, BabA-Le^b and molecules of PAI needs further studies. However, since our results suggest that rac mediated cytoskeletal effects are induced both by type I and type II strains, while mainly type I strains induce IL-8 production (18), it is likely that rac independent mechanisms are mainly involved in IL-8 induction. Interestingly, however, one of the targets of rac is POSH (plenty of SH3 domains; 25), which triggers multiple cellular responses including nuclear apoptosis and nuclear translocation of NFκB. Thus it is possible, that while induction of IL-8 secretion by *H. pylori* is known to be regulated by NFκB (9), rac is part of this signal transduction pathway. Compared with the downstream effectors of rac those of rho GTPase are far better characterized. Recently, beautiful results have been obtained using an inhibitor of rho kinase, a down-

stream effector of rho, in the fields of tumor invasion and hypertension by Narumiya and his co-workers (26, 27). If a similar inhibitor could be found for rac, that could have a great potential in the treatment of gastric inflammation induced by *H. pylori* and other mucosal bacterial pathogens. In the present work, as a first step, the identification of rac as a component in the signaling pathway activated by *H. pylori* is made.

ACKNOWLEDGMENTS

We thank Professor V. P. Lehto for valuable advice, Ms. Marja-Leena Hannus, Ms. Marja-Liisa Martti, Ms. Tarja Piispanen, and Ms. Marja Tolppanen for technical assistance, and Mr. Tapio Leinonen and Hannu Wäänänen for preparing the photographs.

REFERENCES

1. Dunn, B. E., Cohen, H., and Blaser, M. J. (1997) *Clin. Microb. Rev.* **10,** 720–741.
2. Boren, T., Falk, P., Roth, K. A., Larson, G., and Normark, S. (1993) *Science* **262,** 1892–1895.
3. Segal, E. D., Falkow, S., and Tompkins, L. S. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1259–1264.
4. Segal, E. D., Lange, C., Covacci, A., Tompkins, L. S., and Falkow, S. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 7595–7599.
5. Smoot, D. T., Resau, J. H., Naab, T., Desbordes, B. C., Gilliam, T., Bull-Henry, K., Curry, S. B., Nidiry, J., Sewchand, J., and Mills-Robertson, K. (1993) *Infect. Immun.* **61,** 350–355.
6. Ismaili, A., Philpott, D. J., Dytoc, M. T., and Sherman, P. M. (1995) *Infect. Immun.* **63,** 3316–3326.
7. Pucciarelli, M. G., Ruschkowski, S., Trust, T. J., and Finlay, B. B. (1995) *FEMS Microbiol. Lett.* **129,** 293–299.
8. Aihara, M., Tsuchimoto, D., Takizawa, H., Azuma, A., Wakebe, H., Ohmoto, Y., Imagawa, K., Kikuchi, M., Mukaida, N., and Matsushima, K. (1997) *Infect. Immun.* **65,** 3218–3224.
9. Sharma, S. A., Tummuru, M. K., Blaser, M. J., and Kerr, L. D. (1998) *J. Immunol.* **160,** 2401–2407.
10. Mackay, D. J., and Hall, A. (1998) *J. Biol. Chem.* **273,** 20685–20688.
11. Nobes, C. D., and Hall, A. (1995) *Cell* **81,** 53–62.
12. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) *Cell* **70,** 401–410.
13. Lerm, M., Selzer, J., Hoffmeyer, A., Rapp, U. R., Aktories, K., and Schmidt, G. (1999) *Infect. Immun.* **67,** 496–503.
14. Yamaoka, Y., Kodama, T., Gutierrez, O., Kim, J. G., Kashima, K., and Graham, D. Y. (1999) *J. Clin. Microbiol.* **37,** 2274–2279.
15. Eskelinen, S., and Lehto, V. P. (1994) *FEBS Lett.* **339,** 129–133.
16. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) *J. Cell Biol.* **111,** 1001–1007.
17. Ridley, A. J., and Hall, A. (1992) *Cell* **70,** 389–399.
18. Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R., and Covacci, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14648–14653.
19. Dytoc, M., Gold, B., Louie, M., Huesca, M., Fedorko, L., Crowe, S., Lingwood, C., Brunton, J., and Sherman, P. (1993) *Infect. Immun.* **61,** 448–456.
20. Ismaili, A., Philpott, D. J., Dytoc, M. T., Soni, R., Ratnam, S., and Sherman, P. M. (1995) *J. Infect. Dis.* **172,** 1393–1396.
21. Nobes, C. D., and Hall, A. (1999) *J. Cell Biol.* **144,** 1235–1244.
22. Aspenstrom, P. (1999) *Curr. Opin. Cell Biol.* **11,** 95–102.
23. Ilver, D., Arnqvist, A., Ogren, J., Frick, I. M., Kersulyte, D., Incecik, E. T., Berg, D. E., Covacci, A., Engstrand, L., and Boren, T. (1998) *Science* **279,** 373–377.
24. Su, B., Hellstrom, P. M., Rubio, C., Celik, J., Granstrom, M., and Normark, S. (1998) *J. Infect. Dis.* **178,** 1379–1390.
25. Tapon, N., Nagata, K., Lamarche, N., and Hall, A. (1998) *EMBO J.* **17,** 1395–1404.
26. Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T., and Narumiya, S. (1999) *Nat. Med.* **5,** 221–225.
27. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) *Nature* **389,** 990–994.