**Helicobacter pylori** attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins

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**ABSTRACT** The consequences of *Helicobacter pylori* attachment to human gastric cells were examined by transmission electron microscopy and immunofluorescence microscopy. *H. pylori* attachment resulted in (i) effacement of microvilli at the site of attachment, (ii) cytoskeletal rearrangement directly beneath the bacterium, and (iii) cup/pedestal formation at the site of attachment. Double-immunofluorescence studies revealed that the cytoskeletal components actin, α-actinin, and talin are involved in the process. Immunoblot analysis showed that binding of *H. pylori* to AGS cells induced tyrosine phosphorylation of two host cell proteins of 145 and 105 kDa. These results indicate that attachment of *H. pylori* to gastric epithelial cells resembles that of enteropathogenic *Escherichia coli*. Coccoid *H. pylori*, which are thought to be terminally differentiated bacterial forms, are capable of binding and inducing cellular changes of the same sort as spiral *H. pylori*, including tyrosine phosphorylation of host proteins.

*Helicobacter pylori* has succeeded where many other pathogens have failed. It can establish itself in an environment where there is little competition from other microorganisms and can remain in its niche for decades before its host exhibits any serious effects. Although spiral bacteria were observed in gastric biopsies for years, *H. pylori* was not cultivated or identified until just 12 years ago, when it was discovered that this bacterium was the causal agent of type B gastritis, peptic ulcers, and gastric cancer (for a review, see ref. 1).

*H. pylori* is a spiral, Gram-negative rod that attaches specifically to gastric epithelial cells lining the antrum of the stomach. It is highly motile by means of one or more polar-sheathed flagella, each containing a terminal bulb. Its shape and motility permit the microbe to maneuver easily through the gastric mucus layer. Gastric biopsies show *H. pylori* within and beneath the mucus layer, in close proximity to the surface of the gastric epithelial cells, attached to the gastric cells, and, occasionally, within mucus-secreting gastric cells. Coccoid forms of *H. pylori* have been observed both in vivo and in vitro (2–4). The coccoid form is considered to be nonviable by culture; it is not clear whether it serves any function in the pathogenesis of infection.

The nature of *H. pylori* attachment to cells has been controversial. Some investigators have reported that *H. pylori* binding, which causes microvilli effacement, actin rearrangement, and pedestal formation (5–9), is similar to that observed for "attaching and effacing" *Escherichia coli* (EPEC) (10). Other workers assert that *H. pylori* attachment does not result in pedestal formation or actin rearrangement (11).

We report here that *H. pylori* attachment to AGS cells clearly is associated with cytoskeletal rearrangement. We further demonstrate that phosphorylation of host cell proteins occurs in the immediate vicinity of bacterial attachment. Immunoblot analysis shows that binding of *H. pylori* to AGS cells induces tyrosine phosphorylation of two host cell proteins of 145 and 105 kDa.

**MATERIALS AND METHODS**

**Bacterial Strains and Cell Lines.** *H. pylori* strain 87A300, which is a human clinical isolate that produces the vacuolating cytotoxin (vacA) and cytotoxin-associated protein (cagA), was obtained from the State of California Department of Health Services, Berkeley, CA. It was grown as described (5). Briefly, *H. pylori* was passaged on either 5% sheep blood plates (TSA II; BBL) or on plates of brucella agar (Difco) to which had been added 5% fetal bovine serum (FBS; Gibco). The plates were incubated in a BBL GasPak jar containing an anaerobic gas pack (without a catalyst) or in a 5% CO2/95% air incubator. Liquid cultures of *H. pylori* were grown by suspending at least one-quarter of a 2- to 4-day-old plate of *H. pylori* into 30 ml of brucella broth/5% FBS. The flask was placed into a GasPak jar that contained a GasPak anaerobic system envelope (without a catalyst) and was grown with agitation (80 rpm) at 37°C. To obtain *H. pylori* cultures that were coccoid in morphology, but were still cultivable, an overnight liquid culture was grown as described above, placed in a standard 37°C incubator, and grown for an additional 2 days with shaking. AGS cells (ATCC CRL 1739, a human gastric adenocarcinoma epithelial cell line) were grown in Dulbecco’s modified Eagle’s medium (DMEM)/10% FBS.

**Antibodies.** Rabbit polyclonal anti-*H. pylori* antibodies were produced against whole heat-killed *H. pylori* strain 87A300. Monoclonal anti-talin and monoclonal anti-α-actinin were obtained from Sigma Immuno Chemicals (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and Transduction Laboratories (Lexington, KY). Anti-mouse IgG crystalline tetramethylrhodamine isothiocyanate conjugate and anti-rabbit IgG fluorescein isothiocyanate conjugate were obtained from Sigma Immuno Chemicals (St. Louis, MO). Working dilution of the polyclonal anti-*H. pylori* antibody was 1:100. Working dilutions of all other antibodies were determined by the manufacturer.

**Immunofluorescence (IF).** For IF studies, 5 × 104 AGS cells were grown on 12-mm glass coverslips (Belleco, Vineland, NJ). Monolayers were washed two times with PBS, and 1 × 105 bacteria were added per well in a final vol of 200 μl of DMEM (multiplicity of infection, 2). The plates were incubated. At appropriate time points, the wells were aspirated, washed five times with PBS (pH 7.4) to remove nonadherent bacteria, and...
were made by pelleting the bacteria and suspending processed for IF. Fluorescence microscopy was performed on a Nikon Optiphot. Laser scanning confocal microscopy was performed with a Bio-Rad MRC 600.

**Actin Staining.** Actin condensation was revealed by using rhodamine phalloidin, which labels filamentous actin (6).

**Transmission Electron Microscopy.** *H. pylori*-infected AGS monolayers were washed two times in PBS and fixed for 15-35 min at 4°C in 2% glutaraldehyde. The monolayer was then rinsed two times with PBS for 2 min each and incubated in 1% OsO₄ for 30 min at room temperature. The cells were washed two times with double-distilled H₂O for 10 min each time and postfixed in 1% aqueous uranyl acetate for 15 min. Upon washing the cells two times with double-distilled H₂O, the samples were dehydrated as described (5). The samples were infiltrated in ethanol/poly/bed812 (1:1) for 5 min. For embedding, a gelatin capsule was filled with a plastic embedding medium and the monolayer was drained of 100% resin. The capsule was filled with a plastic embedding medium and the monolayer was drained of 100% resin. The capsule was inverted on top of the area over the cells. The coverslip connecting the inverted gelatin capsule was placed in a 60°C oven for 24 hr to polymerize. Appropriate sections were obtained with an Ultra Cut microtome and examined in a Philips 201 transmission electron microscope.

**Immunoblot Analysis.** AGS cells were cultured overnight in DMEM. The cells were washed once with PBS (pH 7.4) and 4 ml of fresh medium was added to each dish. *H. pylori* (5 × 10⁷) were removed from a liquid overnight culture and added to 5 × 10⁵ AGS cells. After incubation in a 5% CO₂/95% air incubator for 2 hr, cell lysates were made with RIPA buffer and stored at -80°C until needed. Whole-cell lysates of *H. pylori* were made by pelleting the bacteria and suspending the pellet in an equal amount of PBS and 2X SDS lysis buffer (250 mM Tris-HCl, pH 6.8/4% SDS/20% glycerol/0.002% bromophenol blue/10% 2-mercaptoethanol). SDS/PAGE and electrotransfer were performed as described (5). Anti-phosphotyrosine binding and detection were performed with the ECL system (Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

**Immunogold Labeling.** Grids were floated, section side down, on PBS (pH 7.4) for 10 min and then washed successively with 0.1 M glycine for 10 min, 2% bovine serum albumin/PBS for 10 min, and washed four times with PBS for 5 min each before fixation with 2% glutaraldehyde for 1 min. Finally, the grids were washed four times for 1 min each with double-distilled H₂O and then the sections were stained with 1% uranyl acetate and lead citrate.

**RESULTS**

**Binding of *H. pylori* to Human Gastric Cells Results in Actin Rearrangement and Pedestal Formation.** AGS cells, grown to a subconfluent monolayer on a glass coverslip, were placed in a perfusion chamber mounted on the stage of a Nikon Diaphot 200 microscope. Motile, spiral-shaped *H. pylori* were added at a multiplicity of infection of 1. Observed by Nomarski video microscopy, initial contact and attachment occurred very quickly (within a few minutes). Attachment invariably occurred at the aflagellated end or nose of the bacterium. Subsequently, the entire length of the bacterium was seen in intimate contact with the AGS cell. After attachment, many bacteria were observed to be internalized by the host cell (data not shown).

The attachment of *H. pylori* to cultured human gastric adenocarcinoma cells (AGS; ATCC CRL 1739) was studied further by transmission electron microscopy. Figs. 1 and 2 illustrate the interaction of *H. pylori* strain (87A300) to AGS cells after 2 and 24 hr of incubation, respectively. Attachment of *H. pylori* resulted in (i) effacement of microvilli at the site of attachment, (ii) actin condensation directly beneath the bacterium, and (iii) pedestal formation at the site of attachment. After attachment, we also commonly observed the bacterium to make a transition from the spiral form to the coccoid form. This transition did not occur in bacterial cultures incubated alone, suggesting that the conversion might be induced in some way by cell contact. Coccoid forms could be readily distinguished from spiral forms cut transversely.

Intracellular bacteria were frequently observed (Fig. 2). Numerous steps of internalization of the coccoid form by the cells also could be seen, including some bacteria that were totally engulfed and appeared to be enclosed within a cytoplasmic vacuole. Intracellular spiral forms were occasionally observed and were usually located within defined membrane-bound vacuoles (data not shown). In general, intracellular *H. pylori* did not appear to replicate, and many degenerate

**FIG. 1.** Transmission electron microscopy of *H. pylori* 87A300 attached to AGS cells. Attachment was for 2 hr. (×12,000.)

**FIG. 2.** Transmission electron microscopy of *H. pylori* 87A300 attached to AGS cells. Attachment was for 24 hr. (×1800.)
bacterial forms were observed intracellularly within hours of uptake (12).

The attachment process between *H. pylori* and gastric cells was examined at a more intimate level by double immunofluorescence. AGS cells infected with either spiral or coccoid *H. pylori* cultures were fixed and stained with polyclonal anti-*H. pylori* antibody to identify *Helicobacter* associated with the cells and with the fluorochrome dye rhodamine phallolidin, which binds to actin filaments. As demonstrated in Fig. 3, bacteria (stained green) colocalized with actin filaments (stained red), which produced a yellow color. Attachment of *H. pylori* resulted in distinct actin foci surrounding each attached bacterium. Similar results were obtained for attachment of spiral *H. pylori* (data not shown).

Actin rearrangement within the host cell occurred directly beneath the site of attachment of *H. pylori*, forming a very fine condensed structure concentric to the bacterium (Figs. 1 and 3). A transverse section of the host cell revealed that rearrangement can take on the appearance of a wave (data not shown). Viewed from above, the bacterium is observed to be within a circle of actin. The cytoskeletal rearrangements induced by attachment with *H. pylori* became apparent within 20 min of attachment, although the changes progressed with additional passage of time (2 hr postattachment). The temporal factor may be related to the observations that (i) coccoid *H. pylori* appeared to induce a stronger cytoskeletal response than spiral *H. pylori*, and (ii) spiral *H. pylori*, when attached to a gastric cell surface, tended to become coccoid after a few hours.

**Involvement of Additional Cytoskeleton Elements in Binding of *H. pylori* to Gastric Cells.** To determine whether additional host cytoskeletal elements were affected by the binding of *H. pylori* to gastric cells, AGS cells, which had been exposed to *H. pylori*, were probed with anti-α-actinin or anti-talin monoclonal antibodies. α-Actinin functions as a cross-linker for actin filaments, while talin, a component of focal adhesions, has been postulated to play a role in signal transduction by linking actin filaments to transmembrane receptors. Both of these proteins have been shown to be involved in cytoskeletal rearrangement resulting from the attachment of EPEC (13) and *Yersinia* invasion-mediated uptake (14). Double IF showed that both α-actinin and talin were concentrated at the site of *H. pylori* to gastric epithelial cells.

**Induction of Tyrosine Phosphorylation of Host Cell Proteins upon Attachment of *H. pylori*.** To investigate whether tyrosine phosphorylation of host cell proteins is associated with attachment of *H. pylori*, AGS cells were exposed to *H. pylori* and stained with a monoclonal antibody against phosphotyrosine and polyclonal anti-*H. pylori* antibodies. As shown in Fig. 4, we were clearly able to demonstrate colocalization of bacteria with phosphotyrosine, as evidenced by a localized increase in tyrosine-phosphorylated proteins at the site of *H. pylori* attachment.

To determine which host cell proteins were phosphorylated upon *H. pylori* attachment, extracts were made of AGS cells alone and after *H. pylori* binding. These extracts were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal antibody against phosphotyrosine (Fig. 5). The anti-phosphotyrosine antibody identified two host proteins, a major one of 145 kDa and a minor of 105 kDa (not seen in this photograph but seen in a darker exposure), as being induced in AGS cells postattachment of *H. pylori*. Phosphorylated proteins of these molecular masses were not seen in an extract of *H. pylori* alone, indicating that the induced tyrosine-phosphorylated proteins were of host cell origin. Colocalization of phosphorylated proteins and attached bacteria was confirmed by immunogold labeling. Fig. 6 shows that 2 hr postinfection, attachment of *H. pylori* resulted in the juxtaposition of phosphotyrosine that contained proteins and *H. pylori*.

**DISCUSSION**

We have investigated the mechanism of adhesion and the consequences of attachment by *H. pylori* to human gastric cells. *H. pylori* attachment to AGS cells is strikingly similar to EPEC (10) and is characterized by effacement of microvilli, pedestal formation, cytoskeletal rearrangement, and phosphorylation of host proteins at the site of adhesion.

Transmission electron microscopy confirms the finding that attachment of *H. pylori* to gastric cells can induce pedestal formation (Figs. 1 and 2) and that *H. pylori* attachment to the cell is correlated with the loss of microvilli at that site (Fig. 1). The effacement observed here is consistent with the pathology seen in gastric biopsies of patients and previous *in vitro* studies (9, 11, 15). Pedestal formation and actin filament rearrangement have been noted by others using primary and established gastric epithelial cells (9) but not by those using nongastric cells (11).

Pedestal formation describes the creation of an upright support, constructed of host cell material, beneath an attached bacterium and is considered to be one of the hallmarks of EPEC attachment. Not all bound *Helicobacter* were observed to be associated with these structures, nor is it known at this time...
time why only a portion of the attached bacteria induced the change. We observed, however, the *H. pylori* that had become coccoid in shape were more likely to induce pedestal formation than spiral *H. pylori*, which when attached appeared to become fused with the epithelial cell membrane. The heterogeneity in pedestal formation induction may be related to the mechanism of attachment of *H. pylori* to gastric cells. Several candidate adhesins have been identified in *H. pylori* (16–18). Thus, the attachment and effacement phenotype may be the result of several factors acting cooperatively. Genes homologous to *E. coli* *eae* have been detected in *H. pylori* (11).

Attachment of *H. pylori* to AGS cells induced tyrosine phosphorylation of two host cellular proteins (Fig. 5). The amount of a 145-kDa protein was greatly increased, while a second protein of 105 kDa was moderately increased. The identities of the 145- and 105-kDa proteins are not currently known. Confocal immunofluorescent microscopy (Fig. 4) and immunogold labeling (Fig. 6) illustrated that attached bacteria are in intimate contact with tyrosine-phosphorylated proteins clearly distinct from focal adhesion sites. This suggests that *H. pylori* adhesion triggers signal transduction. Cytoskeletal components, responding to transduction signals induced by extracellular signals, can be affected in both structure and function by tyrosine phosphorylation. Fischer *et al.* (19) showed that overexpression of a tyrosine phosphatase in BHK cells caused actin filaments to become resistant to cytochalasin-induced disassembly, suggesting that the tyrosine phosphatase might act on the membrane-associated structures (focal adhesions) where actin bundles terminate. Talin and vinculin, part of focal adhesions, are phosphorylated on tyrosyl residues and are thought to be associated with the cytoplasmic domain of integrin receptors, which also contain phosphotyrosine [for a review see Burridge *et al.* (20)].

Although we do not know the role of tyrosine phosphorylation of host cell proteins in the pathogenesis of gastric diseases associated with *H. pylori* infection, we speculate that this event is involved in the inflammatory response, which is an invariable component of gastritis, ulcers, and, presumably, gastric cancer. Signal transduction stimulated by adherence might generate a cytokine response in the acute phase or initiate oncogenic transformation as a long-term effect.

The piracy of eukaryotic protein tyrosine kinases by bacterial pathogens has been identified previously. EPEC (21) and *Salmonella typhimurium* (22) both encode genes (*cfn* and *invA*, respectively) that modify host phosphorylation, leading to invasion of the bacterium into the host cell. *Yersinia pseudotuberculosis* contains a similar gene, *inv* (23), and an additional gene, *yoph*, which through dephosphorylation provide an antiphagocytic phenotype (24, 25). EPEC induces tyrosine phosphorylation of three eukaryotic proteins (21), all apparently cytoskeletal associated. The major protein is a 90-kDa protein, and the two minor proteins are 39 and 72 kDa. The identity of these proteins is currently not known. *Listeria monocytogenes* has been shown to induce the tyrosine phosphorylation of two isoforms (42 and 44 kDa) of the mitogen-activated protein kinase (26).

Our use of an established cell line (albeit a human gastric epithelial cell line) to study *H. pylori* attachment is not ideal. However, a comparison of adhesion of *H. pylori* to surface mucus cells from different origins shows that adhesion of *H. pylori* with human cells of gastric origin in vitro resembles that seen in vivo (27). This was true whether the gastric cells were...
a primary culture derived from stomach biopsies or cells from a cell line of human adenocarcinoma (AGS).

\textit{H. pylori} is generally considered to be an extracellular pathogen, although there have been reports of intracellular \textit{H. pylori} both \textit{in vivo} and \textit{in vitro} (2, 12, 28). We and others have observed intact viable bacteria within a host cell along with degraded forms (29, 30). The mechanism of uptake has been postulated to be receptor-mediated endocytosis (12) following adherence to a coated pit. The relevance and fate of intracellular \textit{H. pylori} is still unknown, although a study has shown a correlation between high bacterial density of colonization, intracellular bacteria, and severe epithelial damage (ulceration) (28).

\textit{H. pylori} is known to convert from its spiral, motile form to a cocccoid form that is nonmotile and nonculturable. \textit{In vitro}, this conversion generally occurs when a bacterial culture is old, but cocccoid forms are also seen in gastric biopsies. Induction of a shape change from spiral to coccoid upon binding to the gastric cell line HG1T1 has been observed by Neiman-Simha and Megraud (8). A recent study from Eaton et al. (31) stated that the cocccoid form of an \textit{H. pylori} strain was unable to establish infection in the gnotobiotic piglet model. However, cocccoid forms are aflagellate and it has been shown previously that a nonmotile strain was not able to cause infection in this model (32). Using a BALB/c mouse model with intragastric inoculation of fresh and laboratory passaged strains with spiral or cocccoid morphology, Cellini et al. (33) demonstrated that while neither form of the passaged strain was able to colonize, both forms of the fresh strain colonized and produced gastric alterations. All measured responses produced by the cocccoid inoculum were the same as those produced by the spiral forms but with a delayed time course. Additionally, Cellini and colleagues further observed spiral organisms in gastric biopsies of mice infected with cocccoid cultures and obtained cultivable \textit{H. pylori} from the mouse stomach, indicating that the cocccoid forms reverted to the spiral form during infection.

The results of our studies indicate that the cocccoid form of \textit{H. pylori} is fully capable of attaching to gastric cells and induces the same cytoskeletal changes seen upon attachment of spiral \textit{H. pylori}, including induction of host cell phosphotyrosine activity. Thus, the cocccoid form might serve as the infectious form in environmental sources such as water, whereas the spiral form appears as the predominant type in the gastric mucus. Therefore, the potential pathogenicity of the cocccoid form should be addressed.

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