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Received 17 May 2005
Accepted 24 August 2005

Intrabacterial proton-dependent CagA transport system in Helicobacter pylori

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Helicobacter pylori CagA modifies the signalling of host cells and causes gastric diseases. Although CagA is injected into gastric epithelial cells through the type IV secretion machinery, it remains unclear how CagA is transported towards the machinery in the bacterial cytoplasm. In this study, it was determined that the proton-dependent intracytoplasmic transport system correlates with the priming of CagA secretion from H. pylori. The cytotoxicity of neutral-pH- and acidic-pH-treated H. pylori was examined in the AGS cell line. The amount of phosphorylated CagA in AGS cells incubated with acidic-pH- and neutral-pH-treated H. pylori was determined by enzyme immunoassay and Western blot. The production of CagA and adherence of the treated bacteria were examined by enzyme immunoassay and light microscopy, respectively. To clarify how CagA is transported towards the inner membrane of the treated bacteria, the localization of CagA was analysed by immunoelectron microscopy. The proportion of hummingbird cells in the AGS cell line rapidly increased following the inoculation of acidic-pH-treated H. pylori but increased more slowly with neutral-pH-treated H. pylori, and the phenomenon correlated with the amount of phosphorylated CagA in AGS cells. CagA was densely localized near the inner membrane in the acidic-pH-treated bacterial cytoplasm, but this localization was not observed in the neutral-pH-treated bacterial cytoplasm, suggesting that CagA shifts from the centre to the peripheral portion of the cytoplasm as a result of an extracellular decrease in pH. This phenomenon depended on the presence of UreI, a proton-dependent urea channel, but not on the presence of urea. The pH treatments did not enhance CagA production or the adherence of the bacterium to AGS cells. The authors propose that H. pylori possesses a proton-dependent intracytoplasmic transport system that probably accelerates priming for CagA injection.

INTRODUCTION

Recent studies of Helicobacter pylori have shown that the pathogenesis of gastric diseases corresponds to a high production of bacterial urease, the expression of cytotoxins, such as CagA and VacA, and the involvement of host tissue-specific cell receptors for bacterial adherence (Guruge et al., 1998; Covacci et al., 1999). Consistently, molecular epidemiological studies have suggested that cagA-positive H. pylori infection significantly increases the risk of gastric carcinoma (Blaser et al., 1995; Weel et al., 1996; Parsonnet et al., 1997; Covacci et al., 1999). The major genetic difference in H. pylori associated with gastric diseases was found in the cag pathogenicity island (PAI), a locus of about 40 kb containing more than 31 genes (Censini et al., 1996; Akopyants et al., 1998). The PAI sequence was detected frequently in isolates from patients with gastritis, peptic ulcer and MALT lymphoma (Xiang et al., 1995; Peng et al., 1998; Backert et al., 2000).

CagA is injected into gastric epithelial cells through the type IV secretion machinery encoded in PAI, the study of which is considered to reveal the pathogenesis of gastric diseases in gastric epithelial cells (Covacci et al., 1999; Backert et al., 2000, 2001). Backert et al. (2000) reported that the transport of CagA is dependent on the functional cagA gene and virulence (vir) genes of a type IV secretion apparatus encoded in the cag PAI of H. pylori. Two-dimensional gel electrophoresis of proteins isolated from infected AGS cells revealed an H. pylori strain-specific and time-dependent tyrosine phosphorylation and dephosphorylation of several 125–135 kDa and 75–80 kDa proteins. One of the 125–135 kDa proteins represents the H. pylori CagA protein, which is

Abbreviations: EIA, enzyme immunoassay; PAI, pathogenicity island.
translocated into the host cell membrane and the cytoplasm (Backert et al., 2000). In _vivo_, the pathogenicity associated with CagA indicates the cytopathic effect on a cultured gastric epithelial cell line as a unique morphological change, termed the hummingbird phenotype, which is characterized by the elongation of cells (Keates et al., 1999; Segal et al., 1999; Backert et al., 2001; Higashi et al., 2002; Selbach et al., 2002; Tsutsumi et al., 2003).

Because the suppression of gastric acid secretion by medication is often effective in reducing the lesions of gastric diseases, and there have been reports that the suppression is the crucial mechanism by which the activity of _H. pylori_ is eradicated, the acidity of the bacterial environment can be associated with pathological changes in the gastric mucosa (Lamers, 1996; Gschwantler et al., 1997) with _CagA_ associated with pathological changes in the gastric mucosa eradicated, the acidity of the bacterial environment can be associated with pathological changes in the gastric mucosa (Lamers, 1996; Gschwantler et al., 1998). The above-mentioned phenomenon suggests that _H. pylori_ possesses a specific system for colonization and/or active transport of cytotoxins, particularly under acidic-pH conditions.

Recently, the proton-dependent intrabacterial transport of urease molecules has been demonstrated by contrast-enhanced immunoelectron microscopy (Hong et al., 2003). A proton opens the urea channel, UreI, and simultaneously stimulates the transport of the urease molecules towards the cell membrane (Hong et al., 2003). To survive under acidic-pH conditions, _H. pylori_ probably transports urease molecules towards UreI to catalyse the hydrolysis of urea (Hong et al., 2003). Although the mechanism underlying the shift is unclear, _H. pylori_ possesses a proton-dependent intrabacterial transport system for urease protein. If the acidity of the bacterial environment is associated with the pathogenesis of gastric diseases caused by _H. pylori_ in gastric epithelial cells, a similar proton-dependent transport system for other proteins, such as CagA, may exist in the bacterium.

In the present study, we examined whether an acidic-pH environment enhances the cytopathic effect of _H. pylori_ in _vivo_ and whether _H. pylori_ possesses a proton-dependent CagA transport system.

**METHODS**

**Bacteria and cell culture.** The _H. pylori_ strain ATCC 43504, a _urel_-deletion mutant described by Weeks et al. (2000) and a _CagA_-negative clinical strain [negative _CagA_ production confirmed by enzyme immunoassay (EIA)] were used in this study. The bacterial cells were cultured on Pylori agar plates (bioMérieux) in a jar containing an anaerobic gas pack (Becton Dickinson Microbiology Systems) and CampyPack Microaerophilic System Envelopes (Becton Dickinson) at 37 °C for 3 days. The colonies of both strains were collected, suspended and treated in either McIlvaine buffer [containing 100 mM citric acid monohydrate and 200 mM disodium hydrogen phosphate, pH 5.0 or 7.0] at 37 °C for 15 min or Brucella broth supplemented with 100 mM 2-(4-morpholino)ethanesulphonic acid buffer (MES buffer) and 5% horse serum (pH 5.5 or 7.0) at 37 °C under microaerophilic conditions for 2 days.

AGS cells of the CRL 1739 line (Dainippon Pharmaceuticals), a human gastric adenocarcinoma epithelial cell line, were cultivated in Ham’s F12 medium (Dainippon Pharmaceuticals) supplemented with 10% foetal bovine serum at 37 °C in a 5% CO₂ atmosphere in a 25 cm² tissue culture flask and a 10 cm² chamber glass slide for 2 days to obtain a monolayer of ~70% confluence.

For microscopy, AGS cells were seeded on a four-well chamber glass slide for 2 days (1 × 10⁶ AGS cells), and then co-cultured with _H. pylori_ (1 × 10⁶ bacteria, m.o.i. of 100), which were treated with McIlvaine buffer at pH 7 and pH 5. The cells were then co-cultured in Ham’s F12 medium, harvested 15 min to 24 h post-inoculation and washed twice with PBS. The morphology of AGS cells was examined 15 min to 24 h post-inoculation. The pHs of the AGS cell media were measured for the process of incubation. The number of cells with the hummingbird phenotype, which was identified by Giemsa counterstaining, was determined under an Olympus BH-2 microscope. Adhered _H. pylori_ cells were also counted for about 100 AGS cells after 15 min of coculture.

**Antibodies.** Mouse monoclonal and rabbit polyclonal antibodies against _H. pylori_ CagA, a mouse monoclonal antibody against _H. pylori_ Urea (Austral Biologicals) and a rabbit polyclonal anti-UreI antibody (Hong et al., 2003) were used as primary antibodies in this study. Phosphorylated CagA proteins were detected by incubation of the membranes with a mouse monoclonal anti-phosphotyrosine antibody PY99 (Santa Cruz Biotechnology) and a rabbit polyclonal anti-CagA antibody (Austral Biologicals). As secondary antibodies for immunoelectron microscopy, 5-nm-collodial-immunogoldlabelled anti-mouse goat IgG and 10-nm-immunogoldlabelled anti-rabbit goat IgG antibodies (Amersham) were used.

**Enzyme immunoassay and Western blot.** CagA in the lysates of _H. pylori_ cells, which were pre-treated at different pHs, was quantified by EIA and Western blot. First, _H. pylori_ cells that were treated with McIlvaine buffer at pH 7 and pH 5 were lysed by sonication for 30 min (W-208, Masuda) and pelleted by centrifugation at 10,000 g for 5 min. The supernatant was harvested and then subjected to EIA to detect _H. pylori_ CagA. To examine tyrosine phosphorylation of the _H. pylori_ CagA in AGS cells incubated with _H. pylori_ strains, the cells were co-cultured with _H. pylori_. After 1 h to 24 h incubation, infected AGS cells were washed once with PBS (containing 1% phosphate inhibitor cocktail #2; Sigma-Aldrich) to remove non-adherent _H. pylori_. Whole-cell lysates with attached bacteria were made by pelleting the cells at 1000 g at 4 °C. The cell pellets were washed again with pre-cooled PBS and sonicated in the presence of proteinase and phosphatase inhibitors (1% phosphatase inhibitor cocktail #2, 2 mg aprotinin ml⁻¹, protease inhibitor cocktail; Sigma-Aldrich). The resulting protein lysates were prepared for examining the tyrosine phosphorylation of the _H. pylori_ CagA by EIA and Western blot.

We utilized an immunoaffinity-purified polyclonal anti- _H. pylori_ CagA rabbit antibody adsorbed to microwells. The microwells were incubated at 4 °C for 24 h. After washing with TBPS (0.05% Tween-20 in PBS), the diluted lysate samples were added to the wells, which were then incubated for 1 h and then washed with TBPS. The monoclonal anti- _H pylori_ CagA mouse antibody was added to the wells, which were then incubated for 1 h at room temperature. The wells were washed with TBPS to remove any unbound materials, and 2,2'-azino-di-[3-ethylbenz-thiazoline-6-sulfonate] (ABTS) (KRL) was added before another 40 min incubation. A stop solution (10% SDS) was added and the absorbance was measured spectrophotometrically at 405 nm. The tyrosine phosphorylation of the _H. pylori_ CagA was examined in the same manner as described above and a mouse monoclonal anti-phosphotyrosine antibody PY99 was used as a detection antibody. Some samples of the resulting protein lysate were suspended in 1× SDS-PAGE buffer and were examined by Western blot as described by Backert et al. (2001).
Fixation and staining. For the analysis of CagA localization in an *H. pylori* cell, the colonies of *H. pylori* (wild-type and clinical strain) were fixed with 1% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4°C for 60 min. Fixed colonies were washed five times with 0.05 M cacodylate buffer and dehydrated in serially graded ethanol concentrations. The samples were then embedded in Lowicryl K4M resin (Electron Microscopy Science). Polymerization was allowed to occur in a UV irradiator (Dosaka EM) at −30°C for 2 days and then at room temperature for 2 days. Ultrathin sections were cut using a Porter Blum ultramicrotome (Sorvall MT-5000, Du Pont) and mounted on a nickel grid (300 mesh) supported by a carbon-coated collodion film.

The ultrathin sections on the grid were treated with 5% normal goat serum in PBS containing 0.1% skimmed milk to block non-specific reactions. The sections were then labelled with primary and secondary antibodies. The sections were first reacted with drops of the primary antibody at 37°C for 30 min and washed with PBS. The sections were then allowed to react with the secondary antibody at 37°C for 60 min and subsequently washed with PBS.

The immunostained sections were fixed with 1% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 37°C for 120 min and washed with PBS. The sections were then allowed to react with the primary antibody at 37°C for 60 min and subsequently washed with PBS.

The ultrathin sections were treated with 5% normal goat serum in PBS containing 0.1% skimmed milk to block non-specific reactions. The sections were then labelled with primary and secondary antibodies. The sections were first reacted with drops of the primary antibody at 37°C for 30 min and washed with PBS. The sections were then allowed to react with the secondary antibody at 37°C for 60 min and subsequently washed with PBS.

The immunostained sections were fixed with 1% glutaraldehyde in 50 mM cacodylate buffer for 15 min and washed five times with distilled water. Finally, the sections were subjected to contrast-enhanced staining with 50 mM cacodylate buffer for 15 min and washed five times with distilled water. The sections were then allowed to react with the primary antibody at 37°C for 60 min and subsequently washed with PBS.

The ultrathin sections were treated with 5% normal goat serum in PBS containing 0.1% skimmed milk to block non-specific reactions. The sections were then labelled with primary and secondary antibodies. The sections were first reacted with drops of the primary antibody at 37°C for 30 min and washed with PBS. The sections were then allowed to react with the secondary antibody at 37°C for 60 min and subsequently washed with PBS.

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All the sections were observed under a transmission electron microscope (H-7100 and H-7600 types, Hitachi). Electron micrographs were taken at a magnification of 30 000 as final magnification.

Morphometric and statistical analyses. The number of immunogold particles associated with *H. pylori* cells was determined on the basis of the immunoelectron photomicrographs. The area of bacterial cells in the photomicrographs was measured using an image analyser (MCID system, Imaging Res.) and the number of immunogold particles per unit area was counted.

To quantify the distribution of immunogold particles, the densities of the particles per square micrometre in three portions of the bacterial cells were determined. The principle of dividing an *H. pylori* cell into three portions was described previously (Hong et al., 2003).

RESULTS

To clarify whether the acidic-pH environment of *H. pylori* enhances the hummingbird cell formation of the AGS cell line associated with CagA, the bacterium was treated at an acidic pH and then inoculated onto an AGS cell line. Representative light microscope photographs of AGS cells at 1 h and 24 h after inoculation are shown in Fig. 1. Hummingbird cells were frequently observed in the 1 h culture inoculated with the wild-type *H. pylori* treated at an acidic pH (Fig. 1a), and less frequently in a similar culture inoculated with the wild-type *H. pylori* treated at a neutral pH (Fig. 1b). A higher frequency of hummingbird cell appearance was observed in the 24 h culture after inoculation with acidic-pH- (Fig. 1c) and neutral-pH- (Fig. 1d) treated *H. pylori* than in the 1 h cultures. Uninoculated cells are shown in Fig. 1(e).

To confirm the rapid appearance of hummingbird cells quantitatively, approximately 1000 cells of each culture were counted, and the proportions of hummingbird cells were compared between cultures inoculated with acidic-pH- and neutral-pH-treated wild-type *H. pylori*. With the acidic-pH-treated wild-type *H. pylori* the proportion of hummingbird cells rapidly increased within 1 h, gradually increased in the next 4 h and had markedly increased by 24 h after inoculation (Fig. 2). The culture inoculated with neutral-pH-treated wild-type *H. pylori* did not show such a rapid increase in the proportion of hummingbird cells in the first hour after inoculation, and the trend of the increase after the first hour was similar to that after the first hour in the culture inoculated with the acidic-pH-treated wild-type *H. pylori.* The culture inoculated with acidic-pH-treated *ureI*-deletion-mutant *H. pylori* did not show such a rapid increase in the proportion of hummingbird cells within the first hour or a remarkable increase at 24 h post-inoculation as observed in the wild-type *H. pylori*.

To confirm that the rapid appearance of hummingbird cells is associated with CagA tyrosine phosphorylation within the host AGS cell, as reported elsewhere (Asahi et al., 2000; Backert et al., 2001), phosphorylated CagA in the cell was measured by EIA and Western blot. In EIA (Fig. 3a), a greater amount of phosphorylated CagA was detected in the early stage of infection with acidic-pH-treated *H. pylori* than with neutral-pH-treated *H. pylori* (*P < 0.01; Student's *t*-test). The rapid increase in phosphorylated CagA was similar to the rapid appearance of hummingbird cells. Furthermore, by Western blot, we confirmed that the acidic-pH-treated *H. pylori* rapidly induced the phosphorylation of CagA in the culture (Fig. 3b). Since Western blot is less sensitive than EIA in general, phosphorylated CagA could not be detected after a 1 h incubation of AGS cell inoculated with the acidic-pH-treated *H. pylori*.

The rapid appearance of hummingbird cells and the rapid increase in the amount of phosphorylated CagA are caused by bacterial and host factors. In this study, we focused on the bacterial factors as follows: (1) acidic-pH treatment of *H. pylori* enhances CagA production, (2) the treatment enhances the attachment of the bacteria to AGS cells and (3) the treatment accelerates transport of CagA.

To examine whether CagA production is enhanced in *H. pylori* treated at an acidic pH, CagA production in the bacterial lysate was measured by EIA. The difference in the optical density (OD_{405}) between the lysates inoculated with the acidic-pH- and neutral-pH-treated bacteria was not statistically significant when determined by Student's *t*-test (Fig. 4a; *α* = 0.01). These findings were supported by the immunoelectron microscopy observation, that is, the overall densities of immunogold particles indicating CagA were 19.32 ± 3.61 μm^{-2} and 18.71 ± 4.32 μm^{-2} in the acidic-pH- and neutral-pH-treated bacterial cytoplasm, respectively (Fig. 4b). The difference in these values was not statistically significant (*α* = 0.01, Student’s *t*-test). All these findings indicate that CagA production was not enhanced by the acidic-pH treatment of *H. pylori*.

To examine whether the acidic-pH treatment of *H. pylori* modifies their adherence, the number of bacterial cells adhering to AGS cells was counted. The numbers of bacterial...
cells were 14.69 ± 3.62 and 13.94 ± 3.32 per AGS cell for acidic-pH-treated and neutral-pH-treated bacterial cells, respectively. A statistically significant difference was not found between these values (Fig. 4c). The results indicate that the acidic-pH treatment does not modify the bacterial adherence to AGS cells.

Considering bacterial factors, the above findings may indicate that the acidic-pH treatment of H. pylori accelerates CagA transport. To determine whether CagA exists in a location advantageous for its secretion in an acidic-pH environment, the ultrastructural localization of CagA in the acidic-pH-treated H. pylori was compared with that in the neutral-pH-treated H. pylori. In neutral-pH-treated wild-type H. pylori, immunogold particles, which label CagA, were located uniformly across the entire cytoplasm (Fig. 5a).
Induced by H. pylori treated at different pHs. AGS cells showing the hummingbird phenotype was higher immediately after inoculation with H. pylori cells treated at an acidic pH than in those inoculated with H. pylori treated at a neutral pH $\left( P < 0.01; \text{Student's} \; t\text{-test}\right)$, but no significant difference was found after 24 h inoculation by the Student's $t$-test ($\alpha = 0.01$). Wild-type H. pylori cells were treated at pH 7 (○) and pH 5 (●); △ represents no H. pylori, as a control. (b) The Western blot was probed with an anti-phosphotyrosine antibody (Anti-pTyr) and an anti-CagA polyclonal antibody (Anti-CagA). Acidic-pH-treated H. pylori induced the rapid phosphorylation of CagA in the culture with AGS cells, but the phosphorylated CagA could not be detected at 1 h incubation of AGS cells inoculated with the acidic-pH-treated H. pylori.

In contrast, the immunogold particles were localized in the periphery, near the cytoplasmic membrane, in the acidic-pH-treated wild-type H. pylori (Fig. 5b). Negligible numbers of CagA immunogold particles were localized in the CagA-negative clinical strain (Fig. 5c).

To confirm the eccentric localization of the immunogold particles in the acidic-pH-treated wild-type, the density of immunogold particles per square micrometre was determined in three different portions of the bacterial cytoplasm (Hong et al., 2003) of 10 randomly chosen cells, and a comparison was made between the acidic-pH- and neutral-pH-treated wild-type H. pylori. The densities in the outer, middle and inner portions of the cytoplasm were not significantly different in the neutral-pH-treated H. pylori. In contrast, the differences in the densities between the outer and inner portions, and between the middle and inner portions were statistically significant in the acidic-pH-treated H. pylori (Table 1, $P < 0.01$). Both negative controls for immunoelectron microscopy, CagA-negative bacteria

**Fig. 2.** Acceleration of production of the hummingbird phenotype of AGS cells by acidic-pH-treated H. pylori. CagA-specific cytotoxicities induced by H. pylori treated at different pHs. AGS cells showing the hummingbird phenotype were counted in five different fields in each of the four chambers of the glass slide. About 1000 AGS cells were counted per experiment in each sample. The percentage of AGS cells with the hummingbird phenotype was higher immediately after inoculation for those inoculated with H. pylori cells treated at an acidic pH than for those inoculated with H. pylori cells treated at a neutral pH. At 24 h incubation, more than 20% of the inoculated cells were significantly elongated and exhibited a spindle-shaped morphology, the same as other AGS cells inoculated with H. pylori treated at an acidic pH or a neutral pH. A higher magnification version of part of (a) is given in (b). Wild-type H. pylori cells were treated at pH 7 (●) or pH 5 (○); urea deletion mutant cells were treated at pH 7 (∇) or pH 5 (○); △ represents no H. pylori, as a control. Values plotted are the mean ± SD for 10 replicate tests.
with anti-CagA antibody and CagA-positive bacteria with non-immunized serum, revealed considerably lower numbers of immunogold particles (\( \alpha = 0.01 \), Student's t-test).

As this phenomenon is similar to the shift of urease (Hong et al., 2003), we further examined the association of urea and UreI molecules with this phenomenon. The distribution of CagA in the wild-type \( \text{H. pylori} \) in the absence of urea was similar to that in the presence of urea (Table 1). The immunogold particles, which label CagA, were distributed uniformly across the entire cytoplasm of \( \text{ureI} \)-deletion mutant \( \text{H. pylori} \) cells treated at neutral (Fig. 6a) and acidic pH (Fig. 6b). The densities at the three intracellular portions of the \( \text{ureI} \)-deletion mutant cells at acidic pH were statistically the same as those at neutral pH (Table 1). These results indicate that the pH-dependent difference in CagA localization is independent of the presence of urea but dependent on the presence of the UreI molecule.

As these dependences and independences are similar to those of urease, which shifts towards UreI molecules in the cytoplasmic membrane (Hong et al., 2003), we further examined whether CagA shifts towards UreI molecules by use of double-immunostaining electron microscopy. The specific co-localization of UreI (large particle) and CagA (small particle) was not observed in \( \text{H. pylori} \) treated at an acidic pH (Fig. 6c).
acidic pH (Fig. 7a) or those treated at a neutral pH (Fig. 7b). We further examined whether the route of intrabacterial transport for CagA is the same for urease. The co-localization of CagA and urease was not observed in *H. pylori* treated at an acidic pH (Fig. 8a), as well as in *H. pylori* treated at a neutral pH (Fig. 8b).

**DISCUSSION**

The cytopathogenicity of *H. pylori* CagA in vitro has been reported as a unique morphological change, termed the hummingbird phenotype, in the AGS cell line (Keates *et al.*, 1999; Segal *et al.*, 1999; Backert *et al.*, 2001; Higashi *et al.*, 2002; Selbach *et al.*, 2002; Tsutsumi *et al.*, 2003). In this study, we found that acidic-pH-treated wild-type *H. pylori* rapidly induced hummingbird cell formation in correlation with a rapid increase of phosphorylated CagA in host cells. Since we could not find an increase in CagA production in the acid-treated *H. pylori*, the rapid appearance of hummingbird cells therefore was not due to the mechanism of increasing of CagA production. These findings are supported by a recent report on micro- and macroarray examination of gene regulation in *H. pylori* under acidic-pH conditions (Ang *et al.*, 2001). Although there are several reports showing that acidic-pH treated wild-type *H. pylori* rapidly induced hummingbird cell formation in correlation with a rapid increase of phosphorylated CagA in host cells.

<table>
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<tr>
<th>Bacteria</th>
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Table 1. CagA immunoreactivity in *H. pylori* treated at different pHs

The density of immunogold particles of CagA in the CagA-negative clinical strain was 3.05 ± 0.92 μm⁻², and in the control, a sample of primary antibody, was 2.11 ± 0.42 μm⁻². A statistically significant difference was not found as determined by the Student’s t-test (α = 0.01). Double indirect immunolabelling was performed using mouse monoclonal anti-CagA antiserum and 10-nm-immunogold-particle-labelled goat anti-mouse IgG. The visible areas of bacterial cells were measured using photomicrographs taken at a magnification of ×30 000 with an optical image analyser. The number of immunogold particles per square micrometre was counted in each portion of 10 bacterial cells. Outer, the area extending from a depth of one-sixth of the diameter to the surface of the cell; middle, the area between the outer and inner portions extending from one- to two-sixths of the diameter; inner, the central area of the cell extending beyond two-sixths of the diameter to the centre of the cell (Hong *et al.*, 2003).

**Fig. 7.** Relationships in the localizations of CagA and Urel with treatment at different pHs. Large immunogold particles are associated with Urel and small particles with CagA. It was revealed that the localizations of CagA-immunogold particles in the acidic-pH- (a) and neutral-pH- (b) treated *H. pylori* were not close to the Urel-immunogold particles after a 2 day incubation. The CagA-immunogold particles were distributed similarly to those in the *H. pylori* treated for 15 min at an acidic pH. Bars, 0.1 μm.
molecules towards the type IV secretion system in a similar manner to the urease shift in an acidic environment (Hong et al., 2003), and we found that CagA shifts from the centre to the peripheral portion of the bacterial cytoplasm as a result of an extracellular decrease in pH. These findings suggest that the shift may accelerate the priming for the injection of CagA by a proton-dependent intracytoplasmic transport system of H. pylori, and consequently hummingbird cells are formed rapidly.

Since Urel plays a critical role in urease transport in an acidic extracellular environment (Hong et al., 2003), we examined the role of Urel in hummingbird cell formation and the intracytoplasmic shift of CagA. Acidic-pH- and neutral-pH-treated ureI-deletion mutant cells also induced a medium level of hummingbird cell formation at 24 h of incubation. At 1–5 h of incubation, the mutant and neutral-pH-treated wild-type H. pylori induced a relatively low level of hummingbird cell formation. Immunoelectron microscopy revealed that the intracytoplasmic shift of CagA occurs only in the presence of Urel. CagA is produced in the cytoplasm of H. pylori, transported to the type IV secretory machinery, primed and injected into a target cell via this machinery (Covacci et al., 1999; Asahi et al., 2000; Backert et al., 2000, 2001). CagA may be transported to the type IV secretory machinery by simple diffusion. Our results indicate that acidic-pH treatment probably accelerates intracytoplasmic transport of CagA in the presence of Urel.

Twenty-four hours after the inoculation of H. pylori, both acidic-pH- and neutral-pH-treated bacteria induced the same level of hummingbird cell formation. This phenomenon may be explained simply by the decrease in the pH in the culture medium during incubation. The pH of the co-cultured medium was decreased by the metabolic organic acid produced by the AGS and bacterial cells in the culture (pH = 6.70 ± 0.06). The acidic-pH condition of the culture medium may accelerate CagA injection into the cells; consequently, a high level of hummingbird cell formation occurred after 24 h of incubation.

We previously demonstrated that the H. pylori urease molecule shifts from the inner portion of the cytoplasm to the urea channel of the Urel molecule in the inner membrane as a result of an extracellular decrease in pH. The shift is urea-independent, proton-dependent and Urel-dependent, suggesting an additional role of the Urel molecule in acidic resistance (Hong et al., 2003). The CagA shift discovered in this study is similar to the urease shift from the inner portion towards the cell membrane of H. pylori. Regarding the shift of urease, it is assumed that a signal from proton-stimulated Urel molecule probably reaches the urease transport system and urease migrates to Urel (Hong et al., 2003). Since the CagA shift is also Urel-dependent, the migration of CagA may be initiated by the same mechanism. CagA is injected through the type IV secretion machinery (Backert et al., 2000). Although we determined that CagA was not co-localized with Urel, the destination of CagA in the cytoplasm is probably the secretion machinery. Computer-aided methods of predicting the subcellular localization of bacterial proteins (Gardy et al., 2003; Perrière & Thioulouse, 2003) have predicted that CagA possesses a periplasmic affinity, which is not the case for the urease. The prediction also supports the hypothesis that the destinations of CagA and urease are probably different.

Concerning the route of transport, as urease and CagA were not co-localized in the immuno-electron microscopic study, it is possible that H. pylori transports CagA independently from urease. Recent reports have indicated that Gram-negative bacteria have the MreB protein, which forms the bacterial cytoskeleton fibre (van den Ent et al., 2001). H. pylori also has a gene homologous to mreB and is supposed to have such a cytoskeleton fibre (Errington, 2003). The fibre localizes in a peptidoglycan factory and is considered to support cell shape, similar to the eukaryotic actin fibre, which closely corresponds to eukaryotic intracytoplasmic transport of proteins.

In conclusion, we found that acidic-pH-treated H. pylori rapidly induce hummingbird cell formation in correlation with a rapid increase of phosphorylated CagA in host cells, suggesting that the priming of CagA for injection is acceler-
ated. The phenomenon corresponds to the intracytoplasmic shift of CagA depending on extracellular protons and the presence of UreI. We propose that *H. pylori* possesses a proton-regulated CagA transport system, which may be a new target for the eradication of *H. pylori*.

**ACKNOWLEDGEMENTS**

We thank Drs G. Sachs, D. R. Scott and D. L. Weeks for providing a stock of ureI-deletion mutant. We also thank Mr Yoshihiko Fujioka of the Department of Preventive and Social Medicine, Osaka Medical College, for his technical help. We also thank Dr Eiko Nakazawa of the Department of Application Technology, Naka Customer Center, Hitachi Science Systems, for her technical help.

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