Acidic pH Changes Receptor Binding Specificity of *Helicobacter pylori*: a Binary Adhesion Model in which Surface Heat Shock (Stress) Proteins Mediate Sulfatide Recognition in Gastric Colonization

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The gastric pathogen *Helicobacter pylori* is one of a number of bacteria which bind specifically to gangliosylceramide, gangliosylceramide, and phosphatidylethanolamine in vitro at neutral pH. Since this organism encounters an acid pH during initial infection of the stomach, we have monitored the effect of pH on receptor binding specificity and found induction of specific binding to sulfoglycolipids (sulfatide) following brief treatment at low pH. We have previously shown that heat shock proteins (hsps) bind to sulfatide, and the suspicion that this was a stress-induced response is supported by the fact that a similar change in *H. pylori* binding specificity was observed if the organisms were briefly exposed to heat shock treatment. Following the stress stimulus, the change in glycolipid binding specificity was prevented by the inclusion of inhibitors of protein synthesis or by incubation with anti-hsp antibodies. Expression of hsps in the surface extract and surface reactivity with anti-hsp antibodies correlated with the change in glycolipid binding specificity. Despite the presence of high levels of *H. pylori* cell surface urease activity, which may neutralize the microenvironmental pH, the acid-induced change in binding specificity was enhanced in the presence of urea. These studies suggest that cell surface hsps mediate sulfatide recognition by this organism under stress conditions. A binary receptor model is proposed for gastric colonization by *H. pylori*.

Helicobacter pylori is a microaerophilic human gastric pathogen responsible for gastritis and peptic ulcer disease (40), which may subsequently develop to gastric carcinoma (45, 47). Carbohydrate, and particularly glycolipid (26, 32), recognition has been found to be a common mechanism for bacterium-host cell adhesion and as such is thought to play a crucial role in infection. While several receptor-ligand interactions have been described for *H. pylori* (3, 14, 16, 35, 53), indicating that *H. pylori* adhesion may be multifactorial, we have found *H. pylori* to specifically bind to the glycolipids gangliosylceramide (Gal β 1-3GalNac β 1-4Gal β 1-4Glu cer [Gg₄]) and gangliosylceramide (GalNac β 1-4Gal β 1-4Glu cer [Gg₃]) and the phospholipid phosphatidylethanolamine (PE) in vitro (21, 34, 38), as monitored by thin-layer chromatogram (TLC) overlay binding and receptor enzyme-linked immunosorbent assays (21, 38). This receptor binding specificity is shared by a variety of microorganisms (27–29, 48). *H. pylori* is spiral and flagellate (55) to facilitate penetration of the mucous barrier covering the gastric epithelium. The pH of this epithelium is neutral (1); thus, bacteria which have penetrated the mucous layer likely bind to host cells at neutral pH. However, during initial infection and in binding to gastric tissue which has been denuded of mucous protection, helicobacters are exposed to the acid pH of the stomach. It has been proposed that the highly active surface urease (11) of this organism protects it from pH-induced damage (5, 17). We have therefore investigated the effect of low pH on the glycolipid receptor binding specificity of intact

viable *H. pylori* organisms under microaerobic conditions. Such adverse conditions might be considered to elicit a stress response, and since our recent studies have shown that various stress-induced or heat shock proteins (hsps) specifically bind sulfoglycolipids in vitro and can function as mycoplasma adhesins (4), we also determined the effect of heat shock on the glycolipid receptor binding specificity of *H. pylori*.

MATERIALS AND METHODS

Materials. Plastic-backed silica thin-layer plates (Polygram SilG) were from Macherly-Nagel (Duren, Germany). *Escherichia coli* PE (the most efficient commercially available form of PE for *H. pylori* binding [34]) and sulfogalactosyl ceramide (SGC) from bovine brain tissue were from Sigma (St. Louis, Mo.). Gg₃ and Gg₄ were prepared by acetic acid hydrolysis of bovine brain gangliosides GM2 and GM1, respectively, as described elsewhere (36). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins were purchased from Bio-Rad (Richmond, Calif.). Gold-conjugated (particle size, 10 nm) goat anti-rabbit immunoglobulins were from Sigma. Skirrow's campylobacter selective supplement was from Oxoid-Unipath (Basingstoke, United Kingdom). Polyclonal rabbit anti-*H. pylori* GroEl-related hsp (10, 15) and anti-*Mycoplasma hyopneumoniae* hsp 70 antibodies (4) (which cross-react with *H. pylori* hsp 70) were generously supplied by G. Perez-Perez (Vanderbilt University) and D. Faulds (Berlex Biosciences), respectively.

Bacterial cultures and culture conditions. *H. pylori* NCTC 11637 (55) was a gift from S. Goodwin (United Arab Emirates University). *H. pylori* LC11 and anti-flagellar antibodies were supplied by P. Sherman (Hospital for Sick Children). Fresh clinical *H. pylori* isolates from pediatric antral gastric biopsy specimens from children with primary gastritis and associated duodenal ulcer (9) were kindly provided by M. Roscoe (Department of Microbiology, Hospital for Sick Children).

H. pylori strains were maintained on Skirrow's medium blood agar plates under reduced oxygen conditions (CO₂, 10%; O₂, 5%; N₂, 85%) at 37°C. After bacteriological identification as gram-negative organisms and determination of positive activity for oxidase, catalase, and urease, *H. pylori* strains were stored at –70°C as described previously (9) until used in these studies.

Assay of *H. pylori*-glycolipid binding by TLC overlay. *H. pylori* organisms were overlaid (without using polybutylmethacrylate [57]) as previously described (34, 35, 38). A 5- μ g amount of purified lipids was separated by TLC on plastic-backed

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silica plates by using chloroform-methanol-water (5:4:1, vol/vol). Plates were blocked by incubation with 2% gelatin for 2 h at 37°C and washed. *H. pylori* suspensions were incubated with lipids on the plastic plates, with or without 5 mM urea, for 2 h at 37°C under conditions of reduced oxygen. Overlay assays were also performed with *H. pylori* previously exposed for 5 min to heat shock (42°C) or pH shock (pH 2.0 and then neutralized) and then incubated for 30 min at neutral pH at 37°C prior to assay. Bound organisms were detected by using anti-*H. pylori* rabbit antiserum and a peroxidase conjugate detection system (34).

Stress treatment. The effect of low pH or increased temperature on *H. pylori* glycolipid binding specificity was monitored under two conditions: firstly with stress maintained throughout the binding assay and secondly following a brief 5-min stress stimulus only.

H. pylori cells from Skirrow's medium agar plates were resuspended in 100 mM Tricine-buffered saline (TBS). The organisms were diluted 10-fold into 0.1 M sodium acetate or sodium citrate buffer (10^7 cells per ml) at the pH indicated in Fig. 1 and assayed for glycolipid binding by TLC overlay for 90 min under microaerobic conditions at room temperature (34). For brief treatment, organisms were treated at pH 2.5, which was neutralized with 10 N NaOH after 5 min.

Separate plates were incubated with organisms at pH 7.0 at 37°C or at 42°C. No difference in binding specificity was observed when the assay was done at room temperature or 37°C.

Inhibition of stress-induced change in receptor binding by anti-hsp antibodies or erythromycin. Polyclonal rabbit anti-*H. pylori* hsp 60, anti-*M. hyopneumoniae* hsp 70 antibodies, and nonimmune rabbit sera were compared for their ability to inhibit the binding of *H. pylori* to SGC after pH shock. *H. pylori* organisms (strain LC11) were subjected to pH 2.5 for 5 min and neutralized. The organisms were incubated with antibody for 30 min under microaerobic conditions and assayed for binding to SGC and PE previously separated by TLC. Similar experiments were performed by adding 0.06 µg of erythromycin per ml instead of antibodies to the bacterial suspension immediately following stress treatment.

Western blotting (immunoblotting). *H. pylori* broth culture (strain LC11) was centrifuged and heat shocked at 42°C for 5 min. After washing of the cells with brucella broth, envelope proteins were extracted with water (38) at increasing lengths of time (0, 5, 30, 60, and 90 min) following return to 37°C. A 1-µg amount of envelope proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting (4) with anti-*H. pylori* GroEL hsp antibodies.

Immunoelectron microscopy. *H. pylori* broth culture (strain LC11) was centrifuged and heat shocked at 42°C for 5 min. After being washed with brucella broth, cells were incubated at 37°C for 30 min prior to being processed for immunogold electron microscopy at 4°C as described elsewhere (2) by using anti-hsp 60 antibodies followed by colloidal-gold (particle size, 10 nm)-conjugated goat anti-rabbit immunoglobulin.

Immunofluorescence. *H. pylori* plate cultures (strain LC11) were suspended in 10 mM TBS and treated at pH 2.0 with sodium citrate containing 5 mM urea for 5 min, the pH was neutralized, and cells were stained for hsp 70 expression by indirect immunofluorescence. Bacteria were sonicated for 30 s and incubated in anti-mycoplasma hsp 70 diluted in 1% bovine serum albumin (BSA) for 30 min at 4°C. Bacteria were washed, and bound antibody was visualized with fluorescein-conjugated antispecies antibodies by using a Richert-Jung Polyvar fluorescent microscope under incident UV illumination with filters at 450 to 495 nm.

RESULTS

Effect of pH on *H. pylori* glycolipid binding specificity monitored by TLC overlay. The specificity of *H. pylori* binding based on a TLC overlay assay under standard conditions was observed to conform to that previously described (38), viz., Gg₄ > PE >> GM₃, and there was no binding to SGC. *H. pylori* NCTC 11637 bound PE better than the strain LC11 we have previously studied (Fig. 1). Strain LC11 did not bind to SGC under control conditions (Fig. 1b, panel A), while strain 11637 bound SGC minimally under nonstress conditions (Fig. 1a, panel A). Both strains demonstrated markedly increased binding to SGC under conditions of decreasing pH (particularly at pH 2.5 to 2.0, corresponding to stomach pH) (Fig. 1). A similar effect was seen for six freshly isolated clinical *H. pylori* strains, indicating that this is a general phenomenon, likely of in vivo significance.

An alteration in binding specificity similar to that observed at acid pH was also seen when the assay was performed at neutral pH at 42°C (Fig. 1, panels B), which is the temperature usually employed to induce the heat shock response (31).

Prolonged in vitro passage gradually increased background binding to SGC by TLC overlay, likely because of the subop-

FIG. 1. Effect of stress on *H. pylori* glycolipid binding. Exponentially growing *H. pylori* organisms were assayed for glycolipid receptor binding as described elsewhere (35) or following heat or low-pH shock. (a) Strain 11637. Panels: A, control (pH 7.0); B, 42°C (pH 7.0) (increased binding to SGC is indicated by the arrow); C, pH 5.0; D, pH 4.5; E, pH 4.0. (b) Strain LC11. Panels: A, control (pH 7.0); B, 42°C (pH 7.0); C, pH 4.0; D, pH 3.0; E, pH 2.5. For all panels, 5 µg of each glycolipid was used. Lanes 1, GM₃; lanes 2, Gg₄; lanes 3, SGC; lanes 4, PE from *E. coli*.

timal conditions of routine *H. pylori* culture. Fresh clinical isolates routinely demonstrate minimal SGC binding.

While prolonged exposure to acid pH may be physiological for *H. pylori*, classical induction of the stress response is rapid, requiring only a few minutes to initiate hsp transcription (31). These experiments were therefore repeated following a 5-min exposure of *H. pylori* to either acid pH (followed by neutralization) or 42°C followed by a 30-min incubation at 37°C prior to assay of binding specificity by TLC overlay. A similar induction of sulfatide binding was observed (Fig. 2) for both *Helicobacter* strains (and many other clinical isolates [data not shown]) following either stress response (though sulfatide binding was, in general, more marked following low-pH stress).

Blocking of stress-induced change in receptor binding specificity. (i) Inhibition of protein synthesis. Inclusion of erythromycin (or chloramphenicol [data not shown]) to inhibit protein synthesis after a 5-min stress induction by either heat shock or low pH blocked the change in binding specificity, and binding to SGC remained at background levels (Fig. 2).

(ii) Anti-hsp antibodies. Blocking experiments using specific antibodies raised against hsp 60 (GroEL related [58]) from *H. pylori* (10, 15) or anti (mycoplasma)-hsp 70 antibodies (4) showed that binding to SGC following pH stress was specifically inhibited by either antibody (Fig. 3). Binding to PE was unaffected. Nonimmune serum or unrelated antibodies were not inhibitory.

Surface-expressed hsps. Although there was no marked difference in protein content, Western blots showed an increase in hsp 60 content in the water extract after exposure of organisms to brief heat shock (Fig. 4a) (hsp 70 was also detected in the water extract but its content did not change after heat shock [data not shown]). hsp 60 was maximally detected in the

FIG. 2. Rapid induction of stress response and prevention by addition of erythromycin. *H. pylori* organisms (strain 11637) were briefly exposed (5 min) to 42°C or pH 2.5 and neutralized and assayed for binding by TLC overlay. Erythromycin (0.06 µg/ml) was added to some cultures prior to assay of binding. (a) Panels: A, control *H. pylori* binding; B, binding following heat shock (42°C); C, binding following heat shock plus erythromycin; D, stress (pH 2.5)-induced *H. pylori* binding. A 5-µg amount of lipid was used in each lane. Lanes 1, SGC (heat shock-induced binding is arrowed in panel B); lanes 2, PE (the lower band is deacyl PE). (b) Lanes: 1, control *H. pylori* binding; 2, binding following pH 2.5 shock; 3, binding following pH 2.5 shock plus erythromycin. Bands indicate *H. pylori* binding. A 5-µg amount of Gg₄ (bound in each lane) and SGC (pH shock-induced *H. pylori* binding is arrowed in lane 2) was added in each lane.

water extract 30 min after heat shock, but it was maintained over the next 60 min. The surface expression of *H. pylori* hsp 60 post-heat shock was confirmed by immunoelectron microscopy (Fig. 4b). Organisms treated with nonimmune serum showed no labelling. Grain counting showed a threefold increase in the number of surface gold particles per organism following heat shock, but this was completely prevented for heat-shocked organisms maintained in the presence of 50 µg of chloramphenicol per ml. After pH shock, protein which can be extracted by water treatment of *H. pylori* is greatly diminished (data not shown). However, immunofluorescence showed markedly increased binding of anti-hsp 70 antibodies to *H. pylori* following brief pH shock from a background of virtually zero (Fig. 4c).

FIG. 3. Anti-hsp inhibition of *H. pylori* binding to sulfatide. *H. pylori* organisms (strain LC11) were subjected to pH 2.5 for 5 min and neutralized. The organisms were incubated with antibody for 30 min and assayed for binding to 5 µg of SGC (upper band) and PE (lower band) by TLC overlay. a, anti-*H. pylori* GroEl-related hsp; b, anti-mycoplasma hsp 70; c, nonimmune rabbit serum.

FIG. 4. Increased hsp expression on the surface of *H. pylori* following stress. (a) hsp 60 in *H. pylori* water extract following heat shock. *H. pylori* plate cultures (strain LC11) under conditions of reduced oxygen were subjected to brief heat shock (42°C for 5 min), and a water extract of surface proteins (38) was prepared at increasing times following return to 37°C. Lanes: B, immediately after pH shock; C, after 5 min; D, after 30 min; E, after 60 min; F, after 90 min. The extracts were separated by SDS-PAGE and stained after transfer to nitrocellulose (38) by using anti-*H. pylori* GroEl hsp antibodies (10). Lane A, prestained molecular mass standards: from the top, phosphorylase b, 130 kDa; BSA, 75 kDa, ovalbumin, 50 kDa; carbonic anhydrase, 35 kDa; soybean trypsin inhibitor, 29 kDa; lysozyme, 21 kDa. (b) Surface expression of *H. pylori* monitored by immunoelectron microscopy. Shown is anti-hsp 60 staining of *H. pylori* broth culture (strain LC11) processed for immunogold electron microscopy after brief heat shock. Magnification, ca. $\times 35,630$. (c) Surface expression of hsp 70 following low-pH treatment of *H. pylori* detected by indirect immunofluorescence. Shown is anti-hsp 70 staining of (A) control organisms at pH 7.0 and (B) low-pH-shocked organisms. Magnification, ca. $\times 3,300$.

Effect of urea on pH-induced change in receptor binding. Since urease activity is thought to protect *H. pylori* from stomach acidity, we determined whether the presence of urea during acid shock would prevent the low-pH-induced change in glycolipid binding specificity. In the presence of 5 mM urea, low-pH-induced binding to SGC was maintained (Fig. 5).

DISCUSSION

Induction of heat shock proteins following a variety of stress stimuli is a phylogenetically conserved response (23, 31) to

FIG. 5. Effect of urea on pH-induced change in *H. pylori* receptor binding specificity. TLC overlay was performed by using untreated *H. pylori* (strain LC11) (a) or organisms which were exposed to pH 2.0 for 5 min and then neutralized (b) or maintained at pH 2.0 throughout the assay (c). The organisms were maintained in the presence of 5 mM urea throughout the experiment. Lanes: 1, PE (plus deacyl PE); 2, SGG; 3, SGC.

minimize denaturing effects on protein tertiary structure. Constitutive (or cognate) hsp ensure correct folding of macromolecular proteins within the endoplasmic reticulum (6, 7). They may also act as chaperones in subcellular organelle protein targeting (8). There are several reports of the presence of surface hsp in other bacteria (4, 51, 54) and other cells (4, 18, 43). Only a few studies (4, 51) have suggested a role in cellular adhesion. The mechanism by which such proteins can be located at the cell surface when an appropriate signal-leader sequence is lacking, without invoking cell leakage (49), may relate to the finding that several proteins have been found to contain the signal for such transport in a C-terminal sequence (24, 56). The significant heterogeneity of the C-terminal domains of hsp might allow the expression of such sequences in a subset of hsp.

Our studies indicate that increased levels of surface hsp 70 may be responsible for SGC binding following pH shock and increased levels of GroEl may be responsible for SGC binding following heat shock. hsp 70 and hsp 60 cooperate in their chaperone functions (50), and this may be reflected in the adhesin function of hsp 70 also. We have shown that members of the 70-kDa hsp family specifically bind to sulfoglycolipids in vitro (4) but that this property has not yet been demonstrated for hsp 60. It is possible that increased levels of surface hsp 70 on *H. pylori* following low-pH treatment mediate the induced sulfatide recognition, whereas surface hsp 60 may play a chaperone function, similar to that of PapD in *E. coli* pili (22), to facilitate the surface transport or conformation of hsp 70 to effect SGC binding after heat. Alternatively, since hsp 60 and hsp 70 share chaperone functions, heat-induced elevation of surface hsp 60 may “free up” hsp 70 for SGC binding.

The change in glycolipid binding specificity is only really significant when pHs as low as that in the stomach are used (Fig. 1). This implies that this is not a mechanism that itself is pH dependent; rather, there is a physiological mechanism of regulation which becomes operative under the specific environmental conditions of this organism's biological niche.

Extensive in vitro passage of *H. pylori* results in increased SGC background binding at pH 7.0, which likely explains why the 60-kDa GroEl-related hsp has been previously detected on the *H. pylori* cell surface (13) and in the surface extract of *H. pylori* (10) in association with urease (15). This cell surface extract also contains an adhesin responsible for the Gg₃, Gg₄, and PE binding specificity of this organism (38).

It has been postulated that the cell surface urease protects *H. pylori* from the extreme acidity of the stomach by local production of ammonia (11, 20), and it has been found that the presence of urea increases *H. pylori* survival time at acid pH

(39, 41). It was therefore possible that the change in binding specificity we observed after pH stress might be neutralized and not occur in vivo. We found, however, that the binding to SGC (and SGG-sulfogalactosyl glycerolipid [37]) induced by exposure of *H. pylori* to low pH was maintained, even enhanced (not shown), in the presence of urea (Fig. 5). This indicates that the change in binding specificity is of physiological relevance and, moreover, that urease does not protect *H. pylori* from an acid-pH stress response.

While the viability of *H. pylori* under acid conditions in the absence of urea is low (39), we have successfully cultured receptor-bound organisms after TLC overlay under such conditions, suggesting that receptor-bound organisms may be more pH resistant. It should be pointed out that in addition to the surface hsp being induced by low pH, the binding of these organisms to sulfated glycolipids is maintained at pH 2 (Fig. 1). Within this time frame (90 min), SGC was not hydrolyzed (data not shown). It is unusual that a ligand-receptor interaction should be maintained at this pH, and it is therefore likely that the observed change in the binding specificity following low-pH induction plays a role in initial infection, especially since sulfated glycolipids are highly represented in the stomach (44, 53).

Several receptor-ligand interactions may be involved in *H. pylori* adhesion (33). A sialyl lactose binding hemagglutinin has been identified (14), but a role in adhesion has yet to be demonstrated, although ganglioside binding has been shown in vitro (25, 52, 53). Recent studies, however, have shown that this “adhesin” is in fact a cytosolic protein not involved in cell adhesion (46). Studies of fluorochrome-coupled *H. pylori* attachment to human gastric sections have implicated fucose-containing blood group antigens (Le^b) in the binding (3, 16) of up to 40% of *H. pylori* strains. However, the extensive fixation and dehydration used in paraffin embedding and deparaffinization used in these studies will remove glycolipids and ablate any PE binding. Despite the epidemiological association of gastritis and duodenal ulcer with blood group status (30), *H. pylori* colonization does not correlate with blood group (42). *H. pylori* binding to blood group glycolipids from erythrocytes or human stomach tissues by TLC overlay is not significant in comparison with binding to PE (35). It may be that interaction with blood group antigens, if a property shared by viable organisms, is a virulence property subsequent to gastric mucosal adhesion. Moreover, most investigations of *H. pylori* receptor binding specificity (3, 16, 25, 52, 53) have not been performed under microaerobic conditions required to maintain organism viability and motility necessary for virulence (12) nor have they considered the possible influence of low pH.

We would speculate that hsp-mediated SGC adhesion may be important in initial infection. Various lipids are associated with mucus, and it may be that binding to gastric mucus-associated sulfoglycolipid facilitates gastric retention. We have found low-pH-dependent binding of *H. pylori* to glycolipids extracted from human gastric mucus (data not shown). *H. pylori* is a highly motile organism whose spiral progression is hypothesized to allow penetration of the mucus barrier to facilitate the intimate attachment at the gastric mucosal cell surface (12). The pH at the gastric mucosal surface is, however, neutral (1). It is therefore likely that once this location is achieved (except at erosion sites?), cell surface hsp adhesins are no longer expressed. Thus, penetration of the mucus layer should be followed by binding to the epithelial cells via the Gg₃, Gg₄-PE receptor (19, 34, 35, 38) and adhesin (38) we have described. Our studies indicate an environmentally regulated binary receptor binding specificity for *H. pylori* which may play a sequential role in infection and colonization.

The induction of cell surface hsp's following a stress response in *H. pylori*, resulting in a change in glycolipid receptor binding specificity, may relate to the differences between our TLC overlay receptor specificity studies (19, 34, 35, 38) and those of others (25, 52, 53) who have reported that sulfatides were the major receptors for *H. pylori*.

It will be of interest to determine whether stress-induced surface hsp-mediated sulfated glycolipid recognition is an important factor in other bacterial diseases, for example, during fever.

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