Neutrophil-Activating Protein Mediates Adhesion of *Helicobacter pylori* to Sulfated Carbohydrates on High-Molecular-Weight Salivary Mucin

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In the in vitro binding of surface-exposed material and outer membrane proteins of *Helicobacter pylori* to high-molecular-weight salivary mucin was studied. We identified a 16-kDa surface protein which adhered to high-molecular-weight salivary mucin. This protein binds specifically to sulfated oligosaccharide structures such as sulfo-Lewis a, sulfogalactose and sulfo-N-acetyl-glucosamine on mucin. Sequence analysis of the protein proved that it was identical to the N-terminal amino acid sequence of neutrophil-activating protein.

Moreover, this adhesion was able to bind to Lewis x blood group antigen.

*Helicobacter pylori* is a causative agent in chronic active gastritis, duodenal ulcer, and gastric malignancies (4, 19, 25). This bacterium colonizes the mucus layer as well as the cell surface of the gastric epithelium, especially at intracellular junctions (14, 15). The main component of mucus is a highly glycosylated protein (mucin) that covers and protects the underlying mucosa. It has been reported that *H. pylori* binds to gastric and nongastric epithelial cells in vivo and in vitro (16, 17, 23) and that the binding involves surface structures, namely, phosphatidyl-ethanolamine (18), GM3 ganglioside and lactosylceramide sulfate (28, 30), N-acetylneuraminylactose (7, 8), H type 2 antigens, and blood group-related Lewis b (1, 5). We have previously demonstrated that *H. pylori* binds to sulfated glycosaminoglycans on high-molecular-weight salivary mucins and that the binding is enhanced at lower pHs (36).

The aim of the present study was to identify the *H. pylori* adhesin that binds to specific structures on salivary mucin. We identified a 16-kDa surface protein that adhered specifically to high-molecular-weight salivary mucin. This adhesion proved to be *H. pylori* neutrophil-activating protein (NAP). It mediates adhesion to sulfated carbohydrates on mucin and also binds to Lewis x blood group antigen.

**MATERIALS AND METHODS**

*H. pylori* strains. Two strains of bacteria were used: *H. pylori* ATCC 43504 and *H. pylori* 3B3, which was isolated from the subgingival plaque of a patient with a duodenal ulcer at the University Hospital, Vrije Universiteit, Amsterdam, The Netherlands (22). The bacteria were grown for 4 days under microaerophilic conditions (CO2: 10%; O2: 5%; N2: 85%) at 37°C on Dent agar plates (6) supplemented with 40 mg of 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, Mo.) per ml. They were stored in brain heart infusion medium (Oxoid-Unipath, Basingstoke, United Kingdom) with 20% glycerol at −80°C.

**Protein isolation.** Outer membrane proteins (OMPs) were isolated with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-Sarkosyl (Oxoid-Unipath, Basingstoke, United Kingdom) with 20% glycerol at −80°C. To prepare bacterial extracts, the bacterial cells from two confluent agar plates were suspended in 0.15 M NaCl, which was isolated from the subgingival plaque of a patient with a *H. pylori* infection and immunity (40), and the extracted membrane pellets were suspended in chola-midopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-Sarkosyl as (Oxoid-Unipath, Basingstoke, United Kingdom) with 20% glycerol at −80°C. To prepare bacterial extracts, the bacterial cells from two confluent agar plates were suspended in 0.15 M NaCl, which was isolated from the subgingival plaque of a patient with a *H. pylori* infection and immunity (40), and the extracted membrane pellets were suspended in chola-midopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-Sarkosyl as (Oxoid-Unipath, Basingstoke, United Kingdom) with 20% glycerol at −80°C.}


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were detected with peroxidase-conjugated goat anti-mouse immunoglobulin (American Qualex, La Mirada, Calif.) with o-phenylenediamine (0.4 mg/ml) and H2O2 (0.012%, vol/vol) as substrates. Binding of biotinylated synthetic oligosaccharides (1 mg/ml) to H. pylori components and other proteins was studied by enzymatic detection with streptavidin conjugated to peroxidase. After the color reaction was stopped with 0.1 M sulfuric acid, the optical density in the wells was read at 492 nm. Neuraminidase and sodium metaperiodate (Sigma) mucin treatments were performed as described previously (34).

**Amino acid sequence.** The N-terminal sequence of affinity-purified adhesin was determined after SDS-PAGE, transfer to a polyvinylidene difluoride membrane, and staining with Coomassie blue to localize the protein. The band was excised and analyzed with an automated Edman degradation protein sequencer (Applied Biosystems, Foster City, Calif.) for 12 degradation cycles.

## RESULTS

**Binding of H. pylori proteins to MG1.** To determine whether the surface-exposed structures and OMPs of H. pylori are involved in binding to MG1, affinity chromatography was used. The bacterial extract of H. pylori ATCC 43504 and 3B3 and OMP of strain ATCC 43504 were subjected to affinity chromatography on a mucin-Sepharose column. In several separate experiments, we consistently observed binding of a protein with molecular mass of approximately 16 kDa to chromatographic media, which could be eluted from the column with 1.5 M guanidine-HCl (Fig. 1, lanes 1 and 2). Occasionally, a very small trace of 15- and 67-kDa protein bands was observed. The same 16-kDa protein was also isolated from the OMP with 1.5 M guanidine-HCl (Fig. 2, lane 3). Some- times a very small trace of 15- and 67-kDa protein bands was found as the background. None of the 16-kDa protein fractions eluted with 3 M guanidine-HCl, respectively. The arrow indicates the position of the 16-kDa mucin-binding adhesin. Molecular size markers (lane M) in kilodaltons are shown on the left.

**H. pylori proteins including native and denaturated HSPA, HSPB, mannose binding protein (MBP), and Hpn, as well as BSA, were tested for binding to mucin and synthetic oligosaccharides. Only HSPA bound moderately to Lewis x, sulfo-Lewis a, and mucin, while no binding was observed with the other proteins, again showing the specificity of the 16-kDa adhesin (data not shown).**

Treatment of mucin with sodium metaperiodate and neuraminidase did not affect its binding to the 16-kDa adhesin. **Amino acid sequence of the 16-kDa protein.** The 16-kDa protein contained an N-terminal methionine residue (MKTFEI LKHL GADAIVL). This sequence is identical to the N-terminal amino acid sequence of NAP, a previously recognized mucin-binding adhesin (11).

## DISCUSSION

We isolated from the bacterial extract and the OMPs of H. pylori a 16-kDa protein that binds specifically to the MG1 fraction of salivary mucins. Sequence analysis of the protein proved that it was identical to NAP, a previously recognized protein of H. pylori. Mucins are a family of highly glycosylated proteins which cover epithelial tissues throughout the human body. These proteins are involved in host mucosal defense, but they may also function as recognition and binding sites for...
microorganism, due to the high diversity of carbohydrate structures that they contain (39). Binding by mucin retards the access of microorganisms to the surface of the epithelial cells of the mucosa and favors their removal (21). On the other hand, some motile microorganisms such as *H. pylori* may use their temporary attachment to mucin as a means of “tracking” toward the epithelium, where they colonize and secrete virulence factors such as cytotoxins (10).

*H. pylori* binds to human gastric mucin (30, 34). We used salivary mucins because they are easily obtained and provide an interesting and valuable model system to study the structural and functional aspects of mucins in general (31). Salivary mucins, as constituents of mucous pellicles on epithelial and dental tissues, have a number of functions analogous to those of mucins elsewhere in the body (24). Moreover, *H. pylori* has been detected in various sites of the oral cavity by culture as well as by PCR (22). MG1 has characteristics in common with mucins in other mucous fluids and contains a wide spectrum of structurally different oligosaccharide side chains, some of which carry blood group antigens and function as receptors for bacterial adhesins (24, 35).

In a previous study (35), we reported that *H. pylori* bound most avidly to sulfated mucins and that the binding was enhanced at lower pHs (6.0 to 5.0). In experiments with synthetic polyacrylamide-coupled oligosaccharides, it was found that SO_3^-3-Gal and the SO_3^-3-Lewis x blood group antigen bound to *H. pylori*. In contrast, the binding of sialylated Lewis a and Lewis b antigens was much weaker. In this study, we have identified a 16-kDa adhesin which bound specifically to sulfo-Lewis a antigens. In a previous study (35), we reported that *H. pylori* binds to human gastric mucin (30, 34). We used salivary mucins because they are easily obtained and provide an interesting and valuable model system to study the structural and functional aspects of mucins in general (31). Salivary mucins, as constituents of mucous pellicles on epithelial and dental tissues, have a number of functions analogous to those of mucins elsewhere in the body (24). Moreover, *H. pylori* has been detected in various sites of the oral cavity by culture as well as by PCR (22). MG1 has characteristics in common with mucins in other mucous fluids and contains a wide spectrum of structurally different oligosaccharide side chains, some of which carry blood group antigens and function as receptors for bacterial adhesins (24, 35).

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Several candidate molecules on gastric epithelial cells have been proposed as receptors for *H. pylori* adhesin. In particular, heparan sulfate and heparin bind specifically to *H. pylori* at low pH (2, 3). A 20-kDa hemagglutinin has been identified as a putative colonization factor on *H. pylori* (7). This antigen binds to N-acetyleneuraminylactose on mammalian cells in tissue culture. The amino acid sequence and the gene (*hpaA*) sequence of this adhesin are similar to those of the sialic acid-binding motif of *E. coli* SfaS, K99, and CFA/I (9) but are essentially different from those of the 16-kDa adhesin found in this study. Faucher and Blaser (13) described a 15-kDa antigen of *H. pylori* in bacterial extract that adheres to HeLa cells. Neuraminidase treatment of the HeLa cells had no effect on binding, suggesting that the bacterial extract of *H. pylori* contains a receptor different from the N-acetyleneuraminylactose binding hemagglutinin identified by Evans et al. (7). This adhesin was not further characterized.

*H. pylori* is able to bind to human gastric mucin and sialic acid. Carbohydrate structures other than sialic acid are responsible for this interaction (34). However, the mucin-binding adhesins were not identified. Recently, a 20-kDa membrane-associated protein has been isolated from *H. pylori* (27). Details of this study have not been published yet, but the preliminary results indicate that this protein, like the 16-kDa adhesin isolated in the present study, binds to Lewis x but not to Lewis b antigens.

Analysis of the N-terminal amino acid sequence of the 16-kDa adhesin revealed sequence homology to *H. pylori* NAP (11). NAP is a bacterioferritin-type protein, and the gene (*napA*) that encodes it is detected in all strains tested; however, there is considerable strain variation in the level of expression of NAP activity in vitro (12). The N-terminal amino acid sequence of the 16-kDa protein was compared with the translation product of the genomic sequence of *H. pylori* published recently (33). There were no other genes whose products had similar N-terminal sequences. Furthermore, Yoshida et al. (41) demonstrated that a water extract of *H. pylori* promotes neutrophil adhesion to endothelial cells via CD11a/CD18- and CD11b/CD18-dependent interaction with ICAM-1. Later, it was shown that this proadhesive activity is associated with NAP (12). Recently, it was reported that NAP binds selectively to four compounds of the acid glycosphingolipid fraction of neutrophils (32). It would be interesting to study whether these glycosphingolipids possess sulfated structures similar to those found in this study. These observations suggest that NAP is able to display different functions: binding to nonglycolipid, sulfated carbohydrate structures such as the sulfo-Lewis x fraction of high-molecular-weight salivary mucin and binding to the glycolipid fraction of neutrophils. Moreover, the binding of the 16-kDa adhesin to Lewis x suggests the possibility that this adhesin regulates neutrophil function through cross-linking of Lewis x on CD11/CD18.

## REFERENCES


