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

FERRY NAMAVAR, 1, 8 MARION SPARRIUS, 1 ENNO C. I. VEERMAN, 2 BEN J. APPELMELK, 1 AND CHRISTINA M. J. E. VANDENBROUCKE-GRAULS 1

Departments of Medical Microbiology 1 and Oral Biochemistry, 2 Medical School, Vrije Universiteit, 1081 BT Amsterdam, The Netherlands

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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 adhesin 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-acetylmuraminylactose (7, 8), H type 2 antigens, and blood group-related Lewis b (1, 5). We have previously demonstrated that H. pylori binds to sulfated glycosans present 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 adhesin proved to bind 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%; Nz, 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 as described previously (40), and the extracted membrane pellets were suspended in 10 mM Tris-HCl (pH 7.2) and stored at −80°C. To prepare bacterial extracts, the bacterial cells from two confluent agar plates were suspended in 0.15 M NaCl, vortexed for 1 min, and centrifuged for 30 min at 5,000 × g (13). The supernatant containing the bacterial extracts was stored at −80°C. After column chromatography, these materials were solubilized in sodium dodecyl sulfate (SDS) for SDS-polyacrylamide gel electrophoresis (PAGE).

Isolation of high-molecular-weight mucin and coupling to epoxy-activated Sepharose 6B. Unstimulated human whole saliva from one donor (nonsecretor, blood group A, lacking the secretor gene, encoding [α-2,3]-fucosyltransferase) was collected in an ice-cooled vessel. Isolation and purification of high-molecular-weight mucins (MG1; molecular mass, >106 kDa) from this saliva were performed, as previously described (37), by ultracentrifugation followed by filtration over Sepharose HR 500.

Purified mucin was coupled to an epoxy-activated Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden) column (8.5 cm by 1.8 cm) as specified by the manufacturer (26). The coupling efficiency was 80 to 90%; this was calculated by an enzyme-linked immunosorbent assay (ELISA) measurement of the amount of residual mucin left in the supernatant when monoclonal antibody (MAb) F2, raised against high-molecular-weight salivary mucin, was used (38). This MAb recognizes the epitope SO3-Galβ1-3GalNAc- moiety of the sulfo-Lewis a antigen. The presence of direct binding of proteins to the Sepharose matrix alone was verified by using the matrix material after incubation with 1 M ethanolamine to hydrolyze activated groups.

Affinity chromatography and SDS-PAGE. Affinity chromatography was performed by loading 0.5 mg of bacterial extracts diluted in 1 column volume (5 ml) of washing buffer (50 mM sodium acetate, 50 mM NaCl [pH 5.0]) on the Sepharose-mucin column. After 60 min at room temperature, the column was washed extensively with 6 column volumes of washing buffer—0.1% Tween 20 to remove unbound proteins. Proteins bound to mucin were eluted stepwise (flow rate, 1 ml/min) with 1.5 and 3 M guanidine-HCl (Sigma). The fractions obtained after elution were dialyzed against distilled water for 24 h at 4°C. Protein was precipitated by mixing 1 ml of cold acetone (−20°C) with 200 μl of ethanolamine fractions. After incubation for 10 min at −20°C, the precipitated proteins were centrifuged for 5 min at 19,000 × g. The pellet was air dried and solubilized in 50 μl of sample buffer (0.06 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol). Protein profiles of each solubilized fraction were analyzed by SDS-PAGE in 12.5% discontinuous Tricine-based polyacrylamide gels (29) followed by silver staining.

Binding assays. The binding of H. pylori components to purified mucin and a panel of biotinylated synthetic oligosaccharides (Table 1) was studied by an ELISA. Microtiter plates (Immulon II; Greiner) were coated with 100 μl of bacterial extract or affinity-purified proteins from the 1.5 and 3 M guanidine-HCl fractions. After incubation for 2 h at 37°C, the plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST), mucin (20 μg/ml), or synthetic oligosaccharide (1 μg/ml) dissolved in PBS or 150 mM NaCl. Tw20 (pH 5.0) was added, and the mixture was incubated for 2 h at 37°C. After being washed, the plates were probed with MAb F2 (1 μg/ml in PBST) for detection of mucin or with streptavidin-conjugated peroxidase for detection of biotinylated oligosaccharides. The plates were washed, and bound antibodies were detected using luminol and peroxidase substrate (31) as visualizing reagents. Additionally, we tested the binding of MAb F2 to mucin coated on microtiter plates in the same manner with biotin-conjugated MAb and streptavidin-conjugated peroxidase for detection of biotinylated carbohydrates.
TABLE 1. Synthetic oligosaccharides, multivalently bound to polyacrylamide carriers, used in this study

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Structure</th>
</tr>
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<tbody>
<tr>
<td>Lewis x</td>
<td>Galβ1-4[Fucβ1-3]GlcNAcβ-R</td>
</tr>
<tr>
<td>Lewis y</td>
<td>Fucβ1-2Galβ1-4[Fucβ1-3]GlcNAcβ-R</td>
</tr>
<tr>
<td>Lewis a</td>
<td>Galβ1-3[Fucβ1-4]GlcNAcβ-R</td>
</tr>
<tr>
<td>Lewis b</td>
<td>Fucβ1-2Galβ1-3[Fucβ1-4]GlcNAcβ-R</td>
</tr>
<tr>
<td>Sialyl-Lewis a</td>
<td>Neu5Acα2-3Galβ1-3[Fucβ1-4]GlcNAcβ-R</td>
</tr>
<tr>
<td>Sialyl-Lewis x</td>
<td>Neu5Acα2-3Galβ1-4[Fucβ1-3]GlcNAcβ-R</td>
</tr>
<tr>
<td>Sulfo-Lewis a</td>
<td>SO3-3Galβ1-3[Fucβ1-4]GlcNAcβ-R</td>
</tr>
<tr>
<td>Sulfo-Lewis x</td>
<td>SO3-3Galβ1-4[Fucβ1-3]GlcNAcβ-R</td>
</tr>
<tr>
<td>Sulfo-Lewis y</td>
<td>SO3-3Galβ1-2Fucβ1-3GlcNAcβ-R</td>
</tr>
<tr>
<td>H type 1</td>
<td>Fucβ1-2Galβ1-3GlcNAcβ-R</td>
</tr>
<tr>
<td>H type 2</td>
<td>Fucβ1-2Galβ1-4GlcNAcβ-R</td>
</tr>
<tr>
<td>Mannose-6-phosphate</td>
<td></td>
</tr>
</tbody>
</table>

* Gal, galactose; GlcNAc, N-acetylglucosamine; Fuc, fucose; R, synthetic polyacrylamide carrier.

Results

Binding of \( H.\ pylori \) 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 2). Occasionally, a very small quantity of this protein could be found in the 3 M guanidine-HCl fraction. The same 16-kDa protein was also isolated from the OMP with 1.5 M guanidine-HCl (Fig. 2, lane 3). Sometimes a very small trace of 15- and 67-kDa protein bands was found as the background. None of the 16-kDa protein fractions bound to a Sepharose matrix control column. These results showed that a 16-kDa adhesin was present in the bacterial extract and OMP and that it bound specifically to MG1.

Binding of bacterial extract and 16-kDa adhesin to mucin and oligosaccharides. We previously found that whole cells of \( H.\ pylori \) possess a receptor for sulfated oligosaccharides (36). Therefore, the chemical nature of the oligosaccharides involved in the binding of the 16-kDa protein isolated from \( H.\ pylori \) adhesin was further investigated by using ELISA to screen a series of synthetic oligosaccharides (Table 1), multivalently attached to a polysaccharide carrier. The results showed that the bacterial extract and 16-kDa adhesin bound most avidly to polymeric sulfated carbohydrates, including sulfogalactose, sulfo-Lewis a, and sulfo-N-acetylgalactosamine. Intermediate binding was observed with Lewis x, sulfo-Lewis x, Lewis y, H type 1, and mannose-6-phosphate. Very little to no binding was observed with H type 2, Lewis b, sialyl-Lewis x, and Lewis a (Fig. 3). For comparison, a number of other

FIG. 1. SDS-PAGE (12.5% polyacrylamide) of protein fractions of \( H.\ pylori \) ATCC 4350 (A) and 3B3 (B) after affinity chromatography. The affinity column was eluted with 3 M guanidine-HCl (lanes 1) and 1.5 M guanidine-HCl (lanes 2). Unbound proteins after washing are shown in lanes 3. The total bacterial extract profiles are shown in lanes 4. The arrow indicates the position of the 16-kDa mucin-binding adhesin. Molecular size markers (lane M) in kilodaltons are shown on the left.

FIG. 2. SDS-PAGE (12.5% polyacrylamide) of OMPs of \( H.\ pylori \) ATCC 43504 after affinity chromatography. Lanes: 1, total OMP profiles; 2 and 3, fractions eluted with 3 M and 1.5 M guanidine-HCl, respectively. The arrow indicates the 16-kDa mucin binding adhesin eluted with 1.5 M guanidine-HCl. Molecular size markers (lane M) in kilodaltons are shown on the left.

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 a 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 sulfated carbohydrate structures such as sulfo-Lewis a on MG1. In an inhibition experiment, the effect of polyanions such as dextran sulfate and DNA, at the same concentration of oligosaccharides as was used in this study, on the binding of NAP to mucin was tested. Dextran sulfate and DNA reduced the binding to 30 and 70% respectively (data not shown), suggesting that the binding has some specificity for chain geometry and distribution of negative charges. Recently, it has been reported that NAP is homologous to the Escherichia coli DNA binding protein Dps (20).

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-acetylneuraminylactose 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-acetylneuraminylactose 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 a 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

FIG. 3. Binding of synthetic biotinylated oligosaccharides to bacterial extracts, 16-kDa adhesin of H. pylori, and BSA by as determined ELISA. Bound oligosaccharides were detected with streptavidin conjugated to horseradish peroxidase. OD, optical density.