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Sulfated Glycoconjugate Receptors for the *Bordetella pertussis* Adhesin Filamentous Hemagglutinin (FHA) and Mapping of the Heparin-Binding Domain on FHA

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Filamentous hemagglutinin (FHA) is a major adhesin present on the surface of the gram-negative respiratory pathogen *Bordetella pertussis*. A number of binding mechanisms have been described for the interaction of FHA with eukaryotic cells. We have focused on its function as a sulfated polysaccharide-binding protein and on identifying potential receptors for FHA on the epithelial cell surface. Using a thin-layer overlay technique, we found that FHA binds specifically to sulfated glycolipids but not to gangliosides or other neutral glycolipids. These results suggest that epithelial cell surface sulfated glycolipids function as receptors for FHA. Further studies demonstrated that a Chinese hamster ovary (CHO) cell strain deficient in glycosaminoglycan expression exhibits greatly diminished attachment to FHA. By FHA–Affi-Gel chromatography, a putative receptor for FHA that has characteristics consistent with a heparan sulfate proteoglycan was isolated from epithelial cell extracts. In addition, by using recombinant FHA fusion proteins, a specific glycosaminoglycan-binding domain located near the N terminus of the FHA molecule was identified. Our results indicate that the *B. pertussis* adhesin FHA may utilize sulfated glycolipids and proteoglycans commonly found on the surface of human cells and tissues to initiate infection.

Upon entering the human host, the ability of *Bordetella pertussis* to attach to and infect the epithelium of the upper respiratory tract is an essential first step in the pathogenesis of the disease pertussis. Bacterial surface lectins, which are typically located on fimbriae, have been shown in many systems to be critical for the initiation of infection by mediating bacterial adherence to epithelial cells (14) through lectin-carbohydrate interactions, which play an important role in many biological systems (42). While the nature of the *B. pertussis* fimbrial receptor is not clear, filamentous hemagglutinin (FHA), a large filamentous protein that is both surface associated and secreted from virulent bacteria, has been shown to have lectin-like activity, as demonstrated by its sugar-inhibitable agglutination of erythrocytes (33). The structural gene of FHA has been cloned and sequenced (8, 9), and the role of FHA as a crucial mediator of *Bordetella*-host cell interactions has been well documented (18, 37, 47, 48).

FHA has been shown to promote attachment to a variety of eukaryotic cell types and tissues and appears to interact with complementary eukaryotic receptors through a number of different binding mechanisms. It contains an arginine-glycine-aspartic acid (RGD) sequence (38) which mediates its attachment to macrophages by interacting with the CR3 integrin receptor (37). In addition, previous work has described the interaction of *B. pertussis* with ciliated cells in a manner that can be inhibited by the saccharide galactose, and FHA has been shown to bind to lactose-containing glycolipids (46). Menozzi et al. (33) have shown that this adhesin binds to the highly sulfated polysaccharide heparin and that FHA-mediated agglutination of erythrocytes can be inhibited by sulfated sugars. Brennan et al. (5) have previously demonstrated that virulent *B. pertussis* organisms bind specifically to galactosylceramide-sulfate, or sulfatide. In addition, it was shown that sulfated sugars can partially inhibit the adherence of *B. pertussis* to WiDr cells, a cell line known to express large quantities of sulfatide molecules (20). Also, dextran sulfate, a high-molecular-weight (MW) glucose polymer containing sulfate, but not dextran or galactose, was demonstrated to significantly decrease the attachment of *B. pertussis* to hamster trachea cells. Subsequently, we have found that this adhesion mechanism is at least in part mediated by the heparin-binding activity of FHA (34).

In this paper, we describe the binding of FHA to the epithelial cell surface molecule heparan sulfate glycosaminoglycan, a polysaccharide which is structurally similar to heparin in that they both are based on the disaccharide unit glucuronic acid–*n*-acetylglucosamine that has undergone sulfation modifications and epimerizations of glucuronic acid to iduronic acid (10). In general, heparan sulfate glycosaminoglycans contain fewer sulfate groups and fewer iduronic acid units than does heparin (16). Unlike heparin, heparan sulfate glycosaminoglycans are commonly associated with core proteins to form proteoglycans, which are ubiquitous components of connective tissues and the eukaryotic cell surface. In this paper, we also describe the specific interaction of FHA with sulfatides, indicating that there are at least two potential sulfated and glycosylated receptors for this adhesin on the epithelial cell surface, a glycosaminoglycan and a glycolipid. Lastly, we have mapped the heparin-binding domain on the

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FHA molecule using purified recombinant FHA fusion proteins.

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MATERIALS AND METHODS

Materials. Purified FHA and pertactin were provided by Pasteur Mérieux Serum et Vaccins, Marcy l'Etoile, France. Heparin from porcine intestinal mucosa (MW, 17,000), dextran sulfate (MW, 500,000), dextran (MW, 500,000), and toluidine blue stain were purchased from Sigma (St. Louis, Mo.). Galactose was obtained from Pierce (Rockford, Ill.). [^{35}S] sodium sulfate (546 mCi/mmol) and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, Mass.), and D-[6-^{3}H]glucosamine hydrochloride (33 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, Ill.). Chondroitin ABC lyase, heparitinase, and the protease inhibitor AEBSF [4-(2-aminomethyl)-benzenesulfonyl fluoride hydrochloride] were purchased from ICN Biomedicals (Costa Mesa, Calif.). Chinese hamster ovary (CHO) mutant cell lines *pgs*A 745 and *pgs*B 761 were the generous gift of Jeffrey Esko (University of Alabama, Birmingham). Cell culture reagents were obtained from Biowhittaker (Walkersville, Md.) and ICN Biomedicals. 1-*O*-*n*-Octyl-β-D-glucopyranoside (*n*-octylglucoside) detergent was purchased from Boehringer Mannheim (Indianapolis, Ind.). The glycolipids galactosylceramide, glucosylceramide, ganglioside G_{M1}, asialo G_{M1}, and sulfatide were purchased from Sigma. Plasmid pRIT13202 has been described previously (8), the construction of pMAL2 and pMAL83 is described elsewhere (34), and pMAL-c and *Escherichia coli* TG1 were purchased from New England Biolabs (Beverly, Mass.). This expression system was used as recommended by the supplier. *E. coli* TG1 containing recombinant pMAL-c derivatives were grown in Luria-Bertani medium containing 100 μg of ampicillin per ml (41).

Antibodies. Polyclonal anti-FHA antibodies were generated by immunizing rats with 50 μg of FHA purified by heparin-Sepharose chromatography as described by Menozzi et al. (33). After 3 weeks, two booster doses of 50 μg of purified FHA were given at 3-week intervals. Ten days after the second booster dose, serum was obtained, and nonspecific antibodies were absorbed twice with extracts prepared from *B. pertussis* BPGR4, a strain lacking the FHA structural gene (28). The antipertactin monoclonal antibody (MAb) E4D7 was a gift from Iver Heron, Statens Seruminstitut, Copenhagen, Denmark. The anti-FHA MAbs X3C and X4B have been described previously (24), as has BPE3, which reacts with pertactin (6).

Electron microscopy. *B. pertussis* 18323 was incubated with HeLa cell monolayers and immunogold labeled by using the anti-FHA immunoglobulin M MAb X4B (5-nm gold particles) and the anti-pertactin immunoglobulin G MAb E4D7 (15-nm gold particles) as previously described (23). The samples were embedded and stained as described previously (6) and were viewed in a Hitachi HU 12A transmission electron microscope.

Thin-layer chromatography overlay assay. Latex beads were coated with FHA and pertactin as follows: 50 μl of latex beads (Polysciences, Warrington, Pa.) was sonicated briefly in 400 μl of GBS buffer (0.1 M glycine, pH 9.2, with 0.17 M NaCl) with a microtip and an output control of 4 (Vibracell; Sonics and Materials, Inc.) and then mixed with 50 μg of either antigen at 37°C for 30 min. One hundred microliters of GBS buffer plus 1% bovine serum albumin (BSA) was added to the mixture and incubated for an additional 15 min. The antigen-coated beads were washed by centrifugation three times with a 1/50 dilution of GBS buffer. The pellets were resuspended in GBS buffer plus 1% BSA to a final antigen dilution of 25 μg/ml and a bead concentration of 0.06%. Western blot (immunoblot) analysis indicated that most of the FHA and pertactin bound to the beads, since the washes did not contain significant amounts of either protein. In addition, the FHA-coated beads agglutinated goose erythrocytes, demonstrating that FHA's lectin activity had been retained.

Two micrograms of each glycolipid was separated on aluminum-backed thin-layer silica plates with chloroform–methanol–0.25% KCl (50:40:10) for 45 min, and a sample plate containing representative glycolipids was stained with orcinol reagent. The antigen-binding assay was performed essentially as described previously (5). Briefly, replica chromatograms were dipped in 0.1% polyisobutylmethacrylate, air dried, and blocked with incubation buffer (0.15 M Tris-HCl [pH 7.6] with 0.05 M NaCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$) containing 1% BSA for 2 h. Twenty-five micrograms of pertactin- or FHA-coated beads was mixed in 5 ml of incubation buffer containing 0.2% BSA, added to a plate, and incubated for 2 h. After plates were washed with three changes of incubation buffer plus 0.2% BSA, ascitic fluid from antipertactin MAb BPE3 or anti-FHA MAb X3C was diluted 1:500 in incubation buffer with 0.2% BSA and incubated for 2 h with the appropriate plate. After the plates were washed three times, 10^6 cpm of ^{125}I-goat anti-mouse immunoglobulin (ICN Biomedicals, Irvine, Calif.) was added per plate and incubated for 2 h. The plates were washed again three times and air dried, and bound antigen was detected by autoradiography.

Cell attachment assay. Epithelial cells were attached to FHA-coated plastic plates as previously described (23). Briefly, CHO K1 cells and CHO mutant cell lines were grown in Ham's F-12 medium supplemented with 2 mM glutamine, 20 μg of gentamicin per ml, and 10% fetal calf serum. The cells were labeled overnight with 25 μCi of [^{3}H]thymidine per 75-cm^2 flask to a specific activity of 1 to 2 cpm per cell. Costar 24-well tissue culture plates were coated with 0.5 ml of 20-μg/ml FHA in Dulbecco's phosphate-buffered saline (DPBS) overnight at 4°C. The cells were scraped off the flasks, washed twice in Ham's F-12 medium, and resuspended in Ham's F-12 medium with 0.2% BSA. After the FHA-coated plates were blocked for 1 h in 1% BSA–DPBS at room temperature, 10^5 cells were added to each well in a 1-ml volume. The plates were incubated for either 90 or 120 min at 37°C, at which time the wells were washed three times in DPBS and solubilized in 200 μl of 10% sodium dodecyl sulfate (SDS). Attached cells were quantified by liquid scintillation counting, and each assay was done in triplicate. Binding to BSA-coated plates served as a control.

Metabolic labeling of epithelial cells. HeLa 229 cells were cultured in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 20 μg of gentamicin per ml, and 10% fetal calf serum. CHO cells were cultured in Ham's F-12 medium containing the same supplements. Tissue culture flasks (150 cm^2) containing 70 to 80% confluent monolayers were washed once in medium without fetal calf serum and containing chloride ions substituted for sulfate ions (NIH Media Unit); [^{35}S]sodium sulfate (30 μCi/ml) was then added to the cells in the sulfate-free medium, and the mixture was incubated for 24 h. Cells were also labeled for 24 h in medium without fetal calf serum which contained 10 μCi of D-[6-^{3}H]glucosamine hydrochloride per ml and 1 mM glucose.

FHA–Affi-Gel chromatography. The FHA–Affi-Gel column was made as follows: 3 mg of FHA (1.5 ml) was dialyzed for 2 h against 1 liter of 0.1 M $NaHCO_3$ (pH 8.0) with 0.3 M NaCl and 1 mM protease inhibitor AEBSF (coupling buffer). Affi-Gel 10 (Bio-Rad) gel slurry (1.5 ml) was washed in cold water

followed by coupling buffer. The gel slurry was mixed with FHA overnight at 4°C in the presence of 100 μg of dextran sulfate (MW, 500,000) per ml in order to protect potential sulfated polysaccharide sites on FHA from coupling to the resin. After blocking with an equal volume of 1 M ethanolamine (pH 8.0) for 1 h at room temperature, the column was thoroughly washed with coupling buffer followed by *n*-octylglucoside buffer (0.025 M Tris-HCl [pH 6.9] with 0.15 M NaCl, 1 mM $MnCl_2$, 50 mM *n*-octylglucoside, and 1 mM AEBSF). Two 150-cm^2 tissue culture flasks containing 80 to 90% confluent [^{35}S]sodium sulfate, [^{3}H]glucosamine, or unlabeled HeLa or CHO cells were scraped off, washed three times in DPBS, and resuspended in 1 ml of ice-cold DPBS plus 1 ml of ice-cold *n*-octylglucoside buffer containing 200 mM *n*-octylglucoside. After mixing at 4°C for 30 min, the material was clarified by centrifugation at 17,000 × *g* for 30 min. The supernatant was added to the FHA–Affi-Gel column and incubated in a rotating mixer at 4°C for 4 h or overnight. After washing with 20 column volumes of *n*-octylglucoside buffer, the column was eluted with 500 μg of dextran (MW, 500,000) per ml followed by 500 μg of either heparin or dextran sulfate (MW, 500,000) per ml. In other experiments, the column was eluted with a stepwise NaCl gradient, from 0.25 M to 2 M NaCl in *n*-octylglucoside buffer. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by toluidine blue staining or autoradiography.

Enzymatic digestion of glycosaminoglycans. Glycosaminoglycan material eluted off the FHA–Affi-Gel column with a sulfated polysaccharide was exposed to either glycosidase chondroitin ABC lyase or heparitinase essentially as described by Yamagata et al. (51). Briefly, the chondroitin ABC lyase digestion was performed as follows: acetone-precipitated eluate was resuspended in 10 μl of 0.4 M Tris (pH 8.0) plus 10 μl of 0.4 M sodium acetate, 10 μl of 0.1% BSA, 2 μl of 0.1 M AEBSF, 68 μl of distilled H_2O, and 20 μl of chondroitin ABC lyase (0.1 U in 0.02 M Tris [pH 8.0] with 0.1% BSA). The eluted material was also digested with heparitinase by the above protocol with the following modifications: the pH of the 0.4 M Tris buffer was 7.5, 3 μl of 0.1 M calcium acetate was added, and 0.1 U of the enzyme was resuspended in 0.02 M Tris (pH 7.5) with 0.1% BSA. The samples were digested for 6 h at 37°C, acetone precipitated, and analyzed by SDS-PAGE and toluidine blue staining (40).

Production and purification of recombinant FHA fragments fused to the *E. coli* maltose-binding protein. Various recombinant FHA fragments were produced in *E. coli* TG1 as polypeptides fused to the *E. coli* maltose-binding protein (MalE) by using pMAL-c (15). The construction of pMAL2 and pMAL83 has been previously described (34). To construct the pMAL85 plasmid, the 1,360-bp *Bam*HI-*Pst*I fragment encoding the C-terminal region of the mature FHA was isolated from pRIT13202 and inserted in the correct reading frame into pMAL-c previously digested with the same restriction enzymes. The pMAL80 plasmid was constructed as follows: the self-complementary oligonucleotide 5′-GATCTCGCGGCCG CGA was added to the *Bam*HI site of pMAL-c to make pSS1678, which created a *Not*I site. The 14.7-kb *Bam*HI fragment containing the *vir* locus and 2.85 kb of the *fhaB* gene was cloned into the *Bam*HI fragment of a pBR322 derivative (pSS855) to create pSS1515. A *Not*I-*Hin*dIII fragment from pSS1515 which contained 1,973 bp of the *fhaB* gene (*Not*I to *Bam*HI) and 346 bp of pBR322 (*Bam*HI to *Hin*dIII) was cloned between the *Not*I and *Hin*dIII sites of pSS1678 to create pMAL80. After ligation, *E. coli* TG1 cells were transformed, and the transformed cells were used for production and purification of the recombinant FHA fusion polypeptides.

The fusion proteins, as well as MalE encoded by pMAL-c without any insert, were affinity purified on an amylose resin as recommended by the supplier (New England Biolabs). Briefly, 600 ml of recombinant *E. coli* was grown at 37°C in Luria-Bertani medium containing 100 μg of ampicillin per ml to an optical density at 600 nm of 0.6. The production of the recombinant fusion proteins was then induced for 6 h by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures were then centrifuged, and the pellets were resuspended in 20 ml of phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.06 mM Na_2HPO_4) and frozen at −70°C for 15 min. After thawing, the cells were sonicated with a microtip and an output control adjusted to 4 (Branson sonifier 450). After two sonications of 10 min each at 4°C, the suspensions were centrifuged at 8,000 × *g* for 10 min at 4°C. Analysis of the proteins after centrifugation revealed that, similar to MalE, most of the protein encoded by pMAL2, pMAL83, and pMAL85 was found in the supernatant fraction, while most of the protein encoded by pMAL80 was found in the pellet. Nevertheless, sufficient soluble protein was contained in the supernatant of the *E. coli* TG1(pMAL80) lysate to be purified by affinity chromatography. The supernatants containing the soluble proteins were collected, diluted to 200 ml with PBS, and applied to 5 ml of packed amylose resin previously equilibrated with 100 ml of PBS. Elution of the proteins was performed with PBS containing 10 mM maltose.

Analysis of the heparin-binding activity of purified recombinant FHA fusion polypeptides. The amylose-purified FHA fusion polypeptides, as well as purified FHA and MalE, were applied to 3 ml of packed heparin-Sepharose CL-6B equilibrated with 100 ml of PBS. Forty milliliters of the various protein samples was adjusted to a concentration of 5 μg/ml in PBS plus 5 mM maltose and applied to the heparin matrix at a flow rate of 0.5 ml/min. After being washed with PBS containing 5 mM maltose, bound proteins were eluted with PBS containing 0.5 M NaCl. The protein concentrations of the flowthrough and the elution peak were determined in order to estimate the heparin-binding capacity of each protein. SDS-PAGE and immunoblot analyses using polyclonal anti-FHA antibodies were performed to confirm that the protein material in the different analyzed fractions corresponded to the expected FHA fusion polypeptides.

Analytical methods. Samples were examined by SDS-PAGE essentially as described by Laemmli (21) using 4 to 20% polyacrylamide gradient gels (Integrated Separation Systems, Hyde Park, Md.). In order to visualize anionic polysaccharides, the gels were stained with 0.05% toluidine blue in 0.1 M acetic acid for 1 h and destained with 3% acetic acid followed by distilled H_2O at 4°C to visualize a metachromatic shift. Gels containing radiolabeled bands were dried down, and signal was detected by autoradiography. Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose sheets (Schleicher & Schuell) was performed according to the method of Towbin et al. (45). Immobilized FHA was then probed with the anti-FHA antibodies at a dilution of 1:500 in PBS containing 0.1% Tween and 1% BSA and developed with alkaline phosphatase-linked goat anti-rat immunoglobulin G (ProtoBlot System; Promega, Madison, Wis.). Protein concentrations were determined according to the method of Bradford (4) with BSA as a standard.

RESULTS

Electron micrograph of *B. pertussis* interacting with HeLa cells. Recently, it has been shown that *B. pertussis* can enter

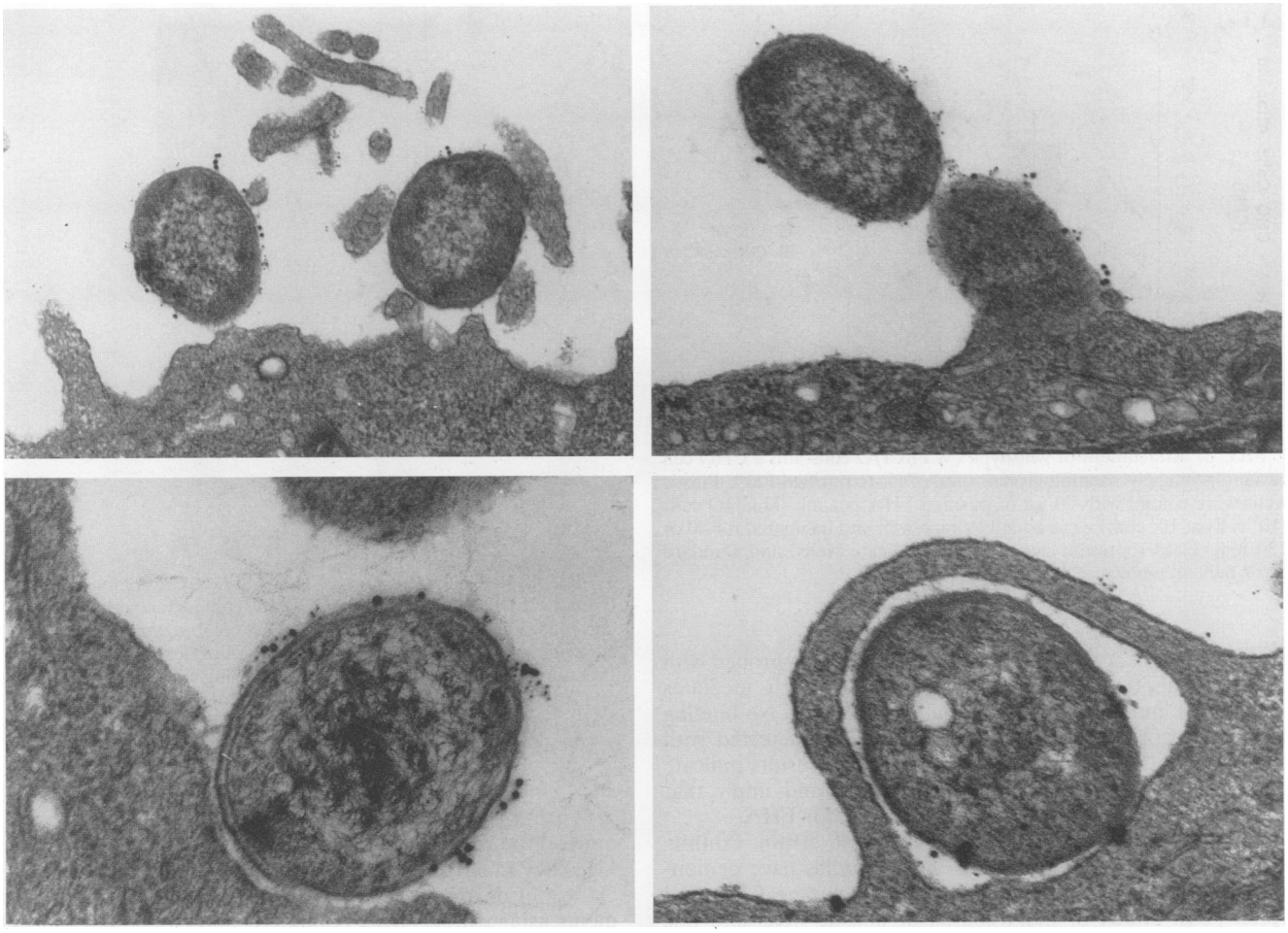

FIG. 1. Electron micrographs of *B. pertussis* 18323 interacting with HeLa cells. *B. pertussis* cells were incubated with HeLa cell monolayers, probed with the anti-FHA MAb X4B and the antipertactin MAb E4D7, and labeled with gold-conjugated anti-mouse immunoglobulin as described in Materials and Methods. FHA was detected with 5-nm gold particles, and pertactin was labeled with 15-nm gold particles. (Top) Magnification, ×60,000; (bottom) magnification, ×90,000.

and survive inside epithelial cells in vitro (13, 23). Electron microscopic examination (Fig. 1) of *B. pertussis* interacting with HeLa cells demonstrates that the bacteria attach to and are actively phagocytosed by the epithelial cells. Simultaneous immunogold labeling of the microorganism with antibodies against FHA (small particles) or pertactin (large particles) shows that these adhesins are distributed over the surface of the organism and often cluster together. As suggested by electron microscopy, FHA is a surface component that actively participates in the adherence of *B. pertussis* to epithelial cells. Little is known, however, about the nature of its receptor on epithelial cells.

FHA binds to glycolipids containing sulfated sugars. Since previous results have indicated that *B. pertussis* can bind to sulfated glycolipids (5), the ability of FHA to interact with sulfatides was investigated. Purified glycolipids were separated by thin-layer chromatography on silica plates. One plate (Fig. 2A) was stained with orcinol and identifies the positions of ganglioside G_{M1} (lane 1), sulfatide (lane 2), galactosylceramide (lane 3), glucosylceramide (lane 4), and asialo G_{M1} (lane 5). A replica plate was probed with latex beads coated with FHA and detected with the anti-FHA MAb X3C followed by ^{125}I-goat anti-mouse immunoglobulin G. FHA bound only to the band corresponding to sulfatide, as detected by autoradiog-

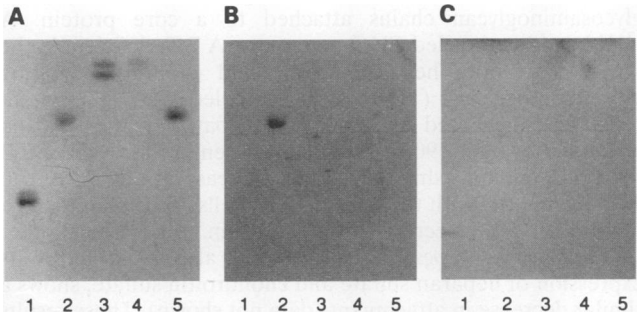

FIG. 2. Specific binding of FHA to sulfated glycolipids on thin-layer chromatograms. Replica chromatograms were either stained with orcinol (plate A) or incubated with FHA-coated beads (plate B) or pertactin-coated beads (plate C). Plate B was incubated with anti-FHA MAb X3C, and plate C was incubated with antipertactin MAb BPE3; each plate was then exposed to 10^6 cpm of ^{125}I-conjugated anti-mouse immunoglobulin. Bound antigen was detected by autoradiography. Lanes contained 2 μg each of ganglioside G_{M1} (lane 1), sulfatide (lane 2), galactosylceramide (lane 3), glucosylceramide (lane 4), and asialo G_{M1} (lane 5). Trivial names for the glycolipids are according to the descriptions of Svennerholm (44).

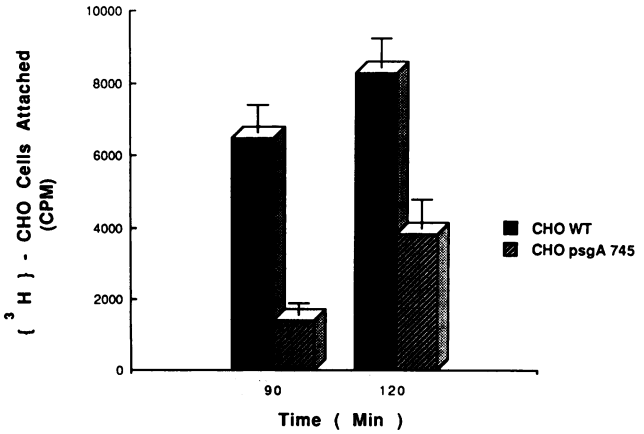

FIG. 3. Attachment of wild-type (WT) CHO cells and a CHO cell variant lacking glycosaminoglycans (*pgs*A 745) to purified FHA. Plastic wells were coated with 20 μg of purified FHA per ml. ^{3}H-CHO cells (10^5 cells at 10^5 cpm) were added to each well and incubated for 90 or 120 min. Data represent averages for triplicate wells, and standard error bars are shown.

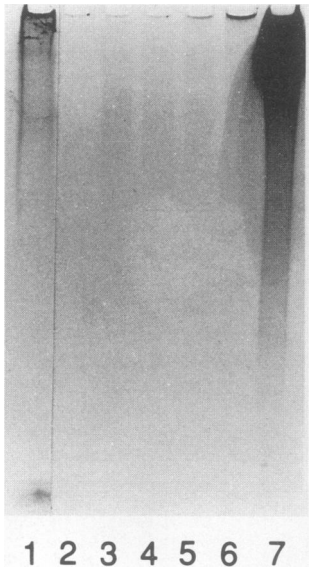

FIG. 4. NaCl elutions from FHA–Affi-Gel chromatography analyzed by SDS-PAGE. Unlabeled CHO cell extracts (lane 1) were chromatographed on an FHA–Affi-Gel column and eluted with a stepwise NaCl gradient, followed by 500 μg of dextran sulfate per ml. Fractions were separated by SDS-PAGE using a 4 to 20% gradient gel and stained with toluidine blue dye. Lane 2, 0.3 M NaCl; lane 3, 0.5 M NaCl; lane 4, 0.7 M NaCl; lane 5, 0.85 M NaCl; lane 6, 1 M NaCl; lane 7, 500 μg of dextran sulfate per ml.

raphy (Fig. 2B). A replica chromatogram was also probed with pertactin, another adhesin of *B. pertussis* which mediates attachment and entry into epithelial cells (23, 25). No binding to sulfatide or the other glycolipids could be detected with pertactin-coated latex beads (Fig. 2C). These results indicate that FHA can bind to sulfated glycolipids and imply that sulfatide may function as a specific receptor for FHA.

A CHO cell glycosaminoglycan-deficient strain exhibits diminished attachment to FHA. Earlier studies have demonstrated that human epithelial cell lines, including CHO and HeLa cells, attach to FHA coated on plastic wells and this interaction can be specifically inhibited by sulfated sugars (23, 34). The finding that FHA can bind to sulfated glycolipids on thin-layer chromatograms prompted us to search for cell surface proteins containing sulfated sugars which might also serve as receptors for FHA. We utilized CHO cell variants which are deficient in the expression of glycosaminoglycans to examine the possibility that FHA interacts with cell surface proteoglycans, macromolecules composed of one or more glycosaminoglycan chains attached to a core protein. A [^{3}H]thymidine-labeled CHO mutant, *pgs*A 745, deficient in the synthesis of both heparan sulfate and chondroitin sulfate glycosaminoglycans (11), and radiolabeled CHO K1 parent cells were incubated in plastic wells coated with FHA and washed after either 90 or 120 min. As seen in Fig. 3, the CHO *pgs*A 745 mutant exhibited a 78% decrease in attachment to FHA compared with the parent CHO cells after 90 min and a more than 50% decrease after 120 min. Likewise, another CHO cell mutant, *pgs*B 761 (12), which also is blocked in its expression of heparan sulfate and chondroitin sulfate, shows a similar decrease in attachment (data not shown). These results implicate cell surface proteoglycans as receptors for FHA.

A high-MW sulfated glycoconjugate binds to FHA. In order to determine whether sulfated epithelial cell glycoconjugates bind to FHA, CHO K1 cells were harvested and extracted in buffer containing *n*-octylglucoside detergent. The extracted cell lysate material (Fig. 4, lane 1) was incubated with an FHA–Affi-Gel resin and then subjected to a stepwise 0.3 to 1 M NaCl gradient. Samples were subjected to SDS-PAGE and incubated with toluidine blue, a stain that allows visualization of anionic polysaccharides. Although the NaCl gradient removes little material from the column (lanes 2 to 6), a diffuse high-MW material is specifically eluted from the column with 500 μg of dextran sulfate per ml (lane 7). Additional experiments using extracts from both CHO and HeLa epithelial cell lines demonstrate that little of the bound material is removed from the column even with 2 M NaCl, indicating that this high-MW glycosylated material binds avidly to FHA. In contrast, when FHA is applied to a heparin or dextran sulfate column, the FHA can be eluted with a relatively low-ionic-strength salt concentration of 300 mM NaCl (33, 34).

In order to further define the epithelial cell components which interact with FHA, HeLa cells were metabolically labeled with [^{35}S]sodium sulfate or [^{3}H]glucosamine, harvested, and extracted in the *n*-octylglucoside buffer. The extracted radiolabeled cell lysate material (Fig. 5, lane 1) was incubated with the FHA–Affi-Gel resin and then eluted with dextran followed by the sulfated polysaccharide heparin. SDS-PAGE of the eluted fractions followed by autoradiography indicates that dextran did not remove any radiolabeled material from the column (Fig. 5, lane 3); however, the heparin eluate is composed of diffuse high-MW material, similar to that stained with toluidine blue, which is strongly labeled with sulfate (Fig. 5, lane 4). In a parallel experiment, [^{3}H]glucosamine also labeled this heparin-eluted material (Fig. 5, lane 5). These results indicate that human epithelial cells produce sulfated and glycosylated proteins or proteoglycans which interact with FHA.

Heparan sulfate glycosaminoglycan is a putative receptor for FHA. Digestion of [^{35}S]sodium sulfate-labeled HeLa or CHO K1 cell extracts with the glycosidases chondroitin ABC lyase and heparitinase indicates that the majority of the sulfate-labeled material that is being applied to the FHA–Affi-Gel column is composed of chondroitin sulfate and heparan sulfate (data not shown). This is consistent with the finding

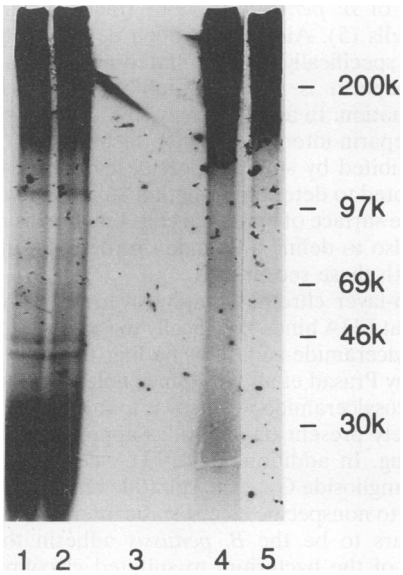

FIG. 5. SDS-PAGE followed by autoradiography analysis of fractions obtained from FHA-Affi-Gel chromatography. An *n*-octylglucoside extract of ^{35}S-sodium sulfate-labeled HeLa cells (lane 1) was chromatographed on an FHA–Affi-Gel column, and the flowthrough (lane 2) was collected. The column was eluted with 500 μg of dextran (MW, 500,000) per ml (lane 3) followed by 500 μg of heparin per ml (lane 4). In a parallel experiment, a ^{3}H-glucosamine-labeled HeLa cell extract was also chromatographed on an FHA–Affi-Gel column and eluted with 500 μg of heparin per ml (lane 5). Fractions were separated by SDS-PAGE using 4 to 20% gradient gels, and labeled material was detected by autoradiography.

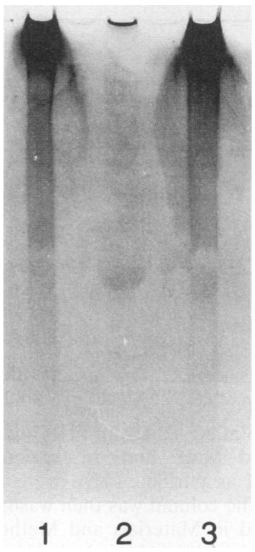

FIG. 6. Enzymatic digestion of the FHA–Affi-Gel eluate. Unlabeled CHO cell extracts were applied to the FHA–Affi-Gel column and eluted with 500 μg of dextran sulfate per ml (lane 1). This material was incubated with heparitinase (lane 2) or chondroitin ABC lyase (lane 3), separated by SDS-PAGE using a 4 to 20% gradient gel, and stained with toluidine blue dye.

of Esko and coworkers, who have shown that in parental CHO K1 cells, the glycosaminoglycan population is composed of approximately 70% heparan sulfate and 30% chondroitin sulfate (12). Therefore, to determine if the high-MW sulfated glycoconjugate which binds to FHA is composed of chondroitin sulfate and/or heparan sulfate glycosaminoglycan, unlabeled CHO cell extracts were mixed with the FHA–Affi-Gel column and eluted with dextran sulfate. The eluate was then incubated with either chondroitin ABC lyase or heparitinase. Figure 6 (lane 1) shows that after separation by SDS-PAGE, the eluted material stains strongly with toluidine blue and turns pink (color not visible in photograph), confirming that the material is an anionic polysaccharide (40). Lane 2 shows the eluted material exposed to heparitinase. Most of the material is digested in the presence of this enzyme. In contrast, the eluted material is not sensitive to chondroitin ABC lyase (lane 3), although control experiments using purified chondroitin sulfate glycosaminoglycan demonstrated that this enzyme is active (data not shown). These results suggest that the predominant material that is binding to the FHA column contains heparan sulfate glycosaminoglycan.

Identification of the heparin-binding domain of FHA. Because our studies suggested that the receptor for FHA on epithelial cells is a heparan sulfate proteoglycan, it was of interest to investigate whether a specific sulfated glycoconjugate-binding domain could be identified on FHA. Several DNA fragments, together covering the entire coding sequence of the mature FHA protein, were subcloned into the pMAL-c expression vector. This expression system allows high recombinant protein production in *E. coli* as fusion proteins with the maltose-binding protein MalE and also convenient one-step purification of the recombinant proteins by affinity chromatography on amylose resin. The DNA fragments that were expressed in this system are depicted in Fig. 7, and the details of their construction and purification are found in Materials and Methods. During purification, the recombinant *E. coli* TG1 strains were harvested and analyzed by SDS-PAGE and Western blotting using polyclonal and monoclonal anti-FHA antibodies. All four clones produced recombinant fusion proteins of the expected size and are recognized by anti-FHA antibodies (data not shown).

After purification, the four FHA-hybrid proteins and MalE were applied to a heparin-Sepharose column, washed, and eluted with a high salt concentration. The quantities of protein

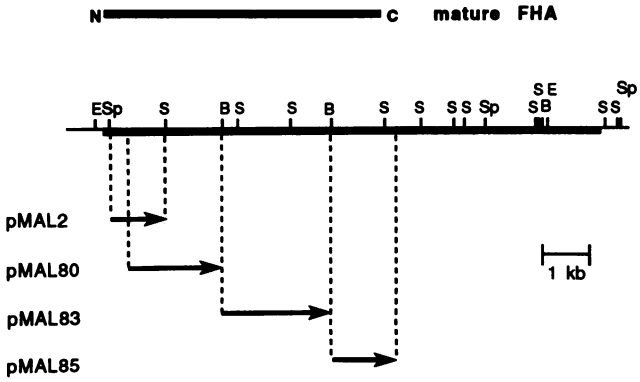

FIG. 7. Restriction map of the *fhaB* gene and recombinant DNA fragments subcloned into pMAL-c. The *fhaB* gene is represented by the horizontal line. The thick line represents the open reading frame. The top line shows the extent of the mature protein. The N terminus and the C terminus are indicated by N and C, respectively. Arrows indicate the length and the direction of the various *fhaB* DNA fragments expressed in the different pMAL-c derivatives. Restriction sites: B, *Bam*HI; E, *Eco*RI; S, *Sal*I; Sp, *Sph*I.

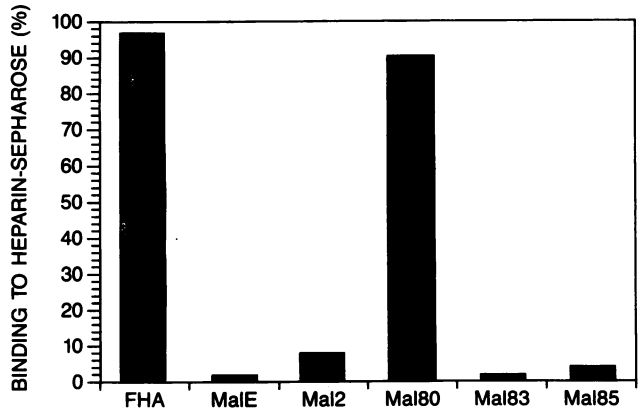

FIG. 8. Heparin-binding activity of FHA and MalE fusion proteins. The amylose-purified MalE and the various MalE-FHA fusion polypeptides, as well as purified FHA, were applied to a heparin-Sepharose column. The column was then washed and eluted with 0.5 M NaCl as described in Materials and Methods. Flowthrough and eluted fractions were collected, and their protein contents were analyzed and compared with the protein concentrations of the samples before chromatography. The indicated values express the relative amounts of the proteins bound to heparin-Sepharose and recovered in the eluted fractions compared with the amounts of proteins present prior to chromatography.

in the flowthrough and in the eluted fractions were compared with that present in the protein sample prior to chromatography. The results shown in Fig. 8 indicate that more than 90% of the recombinant fusion proteins Mal-2, Mal-83, Mal-85, and, as expected, MalE, were recovered in the flowthrough. However, the protein encoded by pMAL80 was found to be almost completely retained by heparin-Sepharose and eluted with 0.5 M NaCl, a condition similar to that used for the elution of full-length *B. pertussis*-produced FHA. Control experiments demonstrated that, like FHA, none of the FHA-hybrid proteins bound to immobilized dextran (data not shown and see reference 34).

These studies indicate that the binding of FHA to heparin is not caused by nonspecific electrostatic interactions of any basic amino acid residue with heparin but that a specific heparin-binding domain is located in the N-terminal domain of the FHA molecule, most likely between amino acid residues 442 and 863. These results are consistent with our previous findings (34) indicating that the heparin-binding domain of FHA is distinct from the previously identified carbohydrate-binding domain (36) and the RGD-containing domain (38).

DISCUSSION

Previous work has described the involvement of the RGD sequence in FHA in the binding to the CR3 integrin receptor on macrophages (37) and the interaction of FHA with lactose-containing glycolipids (46). Our studies have indicated that the RGD sequence of FHA does not play a role in the attachment of epithelial cells to FHA or the entry of *B. pertussis* into HeLa cells (23). However, other evidence suggests that temperature-induced adherence of *B. pertussis* to CHO cells does require the RGD domain of FHA (1). In this study, we show that FHA may also promote *B. pertussis* adhesion to the epithelial cell surface via other mechanisms that involve a lectin-like interaction with a cell surface sulfated glycoconjugate(s). We have previously shown that sulfated polysaccharides can inhibit the attachment of *B. pertussis* to both tracheal cells and other epithelial cells (5). Also, it has been demonstrated (33) that FHA binds specifically to the sulfated polysaccharide heparin and that heparin is a potent inhibitor of FHA-mediated hemagglutination. In addition, only sulfated sugars can disrupt the FHA-heparin interaction, and attachment of CHO cells to FHA is inhibited by sulfated polysaccharides (34). Here, we have attempted to determine whether sulfated glycoconjugates found on the surface of epithelial cells function as receptors for FHA and also to define a domain on the FHA molecule that interacts with these receptors.

By a thin-layer chromatography overlay technique, it was observed that FHA binds specifically to the glycolipid sulfatide, or galactosylceramide-sulfate, a finding that has been shown previously by Prasad et al. (36). Since neither galactosylceramide nor glucosylceramide was shown to interact with FHA, the sulfate moiety present on sulfatides appears to be crucial for FHA binding. In addition, since FHA does not bind to the sialylated ganglioside G_{M1}, the sulfatide binding is not likely to be due only to nonspecific electrostatic interactions. Therefore, FHA appears to be the *B. pertussis* adhesin that mediates attachment of the bacterium to sulfated glycolipids (5). It is known that sulfatides are abundant in the respiratory tract and that like *B. pertussis*, the respiratory pathogen *Mycoplasma pneumoniae* also binds specifically to sulfatides (20). Therefore, sulfatides are suitably positioned to function as initial receptors for *B. pertussis*.

Since FHA binds to heparin as well as to sulfated glycolipids, the role of other sulfated cell surface glycoconjugates as potential receptors for this adhesin was investigated. By utilizing a CHO cell mutant, *pgs*A 745, which is defective in the xylosyltransferase enzyme and consequently fails to link chondroitin sulfate or heparan sulfate glycosaminoglycans to proteoglycan core proteins (11), the presence of glycosaminoglycans was found to be important for FHA binding to CHO cells. The CHO *pgs*A 745 mutant has been characterized by Esko and coworkers (11), and it expresses only about 6% of both heparan sulfate and chondroitin sulfate glycosaminoglycans, compared with the parent cells (17). In our attachment assay, this mutant exhibits a 78% decrease in attachment to FHA after 90 min compared with the parent CHO cell line. This suggests that the CHO cells are attaching to FHA via cell surface glycosaminoglycans. The residual 22% adherence of the CHO mutant to FHA could be due to the small amount of glycosaminoglycan expressed by this mutant line. In addition, the increased binding to FHA observed over time could be accounted for by the presence of other receptors for FHA on the CHO cell surface that are unrelated to glycosaminoglycans, leading to multiple interactions of FHA with cell surface components.

To further examine the FHA interaction with glycosaminoglycans, *n*-octylglucoside extracts from both HeLa cells and CHO cells were chromatographed on an FHA–Affi-Gel affinity column. High-MW material that was both sulfated and glycosylated was specifically eluted with either heparin or dextran sulfate but not with dextran. Digestion with specific glycosidases indicated that this material contains glycosaminoglycans sensitive to the action of heparitinase. The interaction between FHA and heparan sulfate appears to be very specific for this glycosaminoglycan, since enzymatic digestion of the epithelial cell extracts demonstrated that while both chondroitin sulfate and heparan sulfate glycosaminoglycan were present in the material added to the FHA column, only the heparan sulfate glycosaminoglycan bound to FHA. Binding of heparan sulfate to the FHA affinity column is also of high avidity since 2 M NaCl does not efficiently elute the glycosaminoglycan off the

column. In contrast, when FHA is purified by heparin affinity chromatography, FHA can be eluted from heparin-Sepharose with 300 mM NaCl (33). This relatively mild elution condition may indicate that primarily ionic interactions are involved in FHA-heparin interactions. However, both the sulfate moiety and the carbohydrate moiety of heparan sulfate may be involved in binding to FHA. Alternatively, since many biological activities of proteoglycans involve both the glycosaminoglycan chains and the core protein (19), it is possible that FHA interacts with heparan sulfate proteoglycan more avidly than with heparin. Although we have no evidence that the core protein is directly involved in the interaction with FHA, it may play a crucial role in the interaction by providing appropriate spacing of the attached glycosaminoglycan chains for more-efficient interactions with the FHA ligand. Multivalent binding of the heparan sulfate side chains could result in stronger binding of the glycosaminoglycan to FHA.

Because heparin and heparan sulfate are highly related molecules and sulfatide is also a sulfated glycoconjugate, the binding site on FHA for all three of these molecules is probably the same. As shown by the specific binding of only one of four different recombinant FHA peptides to heparin-Sepharose, the FHA-heparin interaction occurs through a specific domain of the FHA molecule. This strongly suggests that simply the presence of positively charged residues distributed throughout the FHA molecule is insufficient for heparin binding. While a strict consensus sequence for protein-heparin interactions has not yet been identified among the various heparin-binding proteins studied, a rather precise spatial arrangement of basic amino acids in these proteins appears to be critical (43). Since the elution profile of the pMAL80-encoded FHA polypeptide from heparin-Sepharose was identical to that of the full-length FHA, the coding sequence of pMAL80 most likely contains the major heparin-binding domain. Although we cannot rule out the possibility that lack of binding of the other fusion proteins to heparin reflects improper folding of these recombinant proteins, immunoblot analyses indicated that anti-FHA antibodies were able to bind to these fusion proteins. The FHA coding sequence in pMAL80 extends from amino acid residues 206 to 863, if one utilizes the amino acid numbering proposed by Delisse-Gathoye et al. (8). The pMAL2-encoded FHA polypeptide, which does not bind to heparin, extends from positions 16 to 441. It contains an approximately 100-amino-acid sequence homologous to the N-terminal regions of *Serratia marcescens* and *Proteus mirabilis* calcium-dependent hemolysins (8). The data presented in this study rule out the direct involvement of this homologous region in sulfated polysaccharide binding. More recent evidence indicates that this region is involved in the export of FHA into the extracellular milieu of *B. pertussis* (49).

Since the pMAL2-encoded polypeptide overlaps with 235 N-terminal residues of the pMAL80-encoded FHA polypeptide, this suggests that the heparin-binding site is most likely located between residues 442 and 863. Interestingly, this region contains most of the A repeats and the first two B repeats of FHA, as defined by Locht et al. (27). Obviously, we cannot exclude the possibility that part of the heparin-binding domain of FHA extends slightly further upstream of residue 442 and also includes the first A repeat, and it is tempting to speculate that the repeats play a role in the sulfated polysaccharide binding of FHA. Repeats of approximately the same length are present in adhesins of other bacterial pathogens that interact with eucaryotic matrix proteins (32). The precise role of individual repeats or residues within these repeats remains to be investigated. The putative heparin-binding region is also the domain of FHA recognized by MAb X3C, which has been shown to inhibit the adherence of epithelial cells to FHA (22, 24). We have previously provided evidence which suggests that the heparin-binding site is located near the amino-terminal end of FHA (34). The results presented here further localize the heparin-binding domain to this region of FHA.

The receptor-ligand interaction of the *B. pertussis* adhesin FHA and sulfated glycoconjugates is reminiscent of other adherence mechanisms. Sulfated glycolipids appear to play a general role in cell adhesion, since a number of eukaryotic adhesion molecules, including laminin, thrombospondin, and von Willebrand factor, bind specifically to sulfatides (39). Proteoglycans are also essential components of the extracellular matrix and the cell surface which participate in many biological activities, such as mechanical support, cell adhesion, cell proliferation, anticoagulation, and binding of growth factors and other ligands (10, 19). Heparan sulfate proteoglycans, which appear to specifically interact with FHA, are produced by virtually all animal cells, with typical concentrations in the range of 10^5 to 10^6 molecules per cell (16, 52). Unlike other glycosaminoglycans, which have a relatively regular repeating structure, heparan sulfate chains contain a great deal of structural diversity (i.e., blocks of sulfated residues, many distinctive regions of sulfated moieties, and variability in carbohydrate modifications), making this ubiquitous molecule ideally suited for specific interactions with a range of ligands (52). This may explain why FHA exhibits a preference for heparan sulfate over other glycosaminoglycans, showing a selectivity for specific carbohydrate epitopes as well as the quantity and distribution of anionic charges.

In addition to *B. pertussis*, a growing list of viruses, bacteria, and parasites utilize glycosaminoglycans and proteoglycans as receptors for attachment. Herpes simplex virus initiates the disease process by binding to cell surface heparan sulfate, a step which is followed by other higher-affinity receptor-ligand interactions (29, 50). Binding to heparan sulfate has also been reported for *Streptococcus pyogenes* (3), and both chondroitin sulfate and heparan sulfate inhibit the binding of a *Streptococcus mutans* protein to heart tissue (7). Also, the heparan sulfate-binding proteins described for *Staphylococcus aureus* may be general glycosaminoglycan binding proteins since dermatan sulfate and chondroitin sulfate interfere with the binding of heparan sulfate to *S. aureus* (26). Zhang and Stephens have provided evidence that *Chlamydia trachomatis* utilizes heparan sulfate glycosaminoglycans as a molecular bridge between the organism and the host cell receptors (53). The gastric pathogen *Helicobacter pylori* has a strong affinity for heparan sulfate, and this binding is not inhibited by other anionic glycosaminoglycans (2). The parasite *Trypanosoma cruzi* expresses a 60-kDa surface protein that binds selectively to heparan sulfate and collagen found in the extracellular matrix (35).

The initiation of *B. pertussis* infection is triggered when the organisms are inhaled into the upper respiratory tract. Presumably, the first site that the bacteria encounter is the mucus covering the epithelial lining of the respiratory tract. The tracheobronchial mucus glycoproteins possess oligosaccharides that are sulfated (31). In addition, sulfated glycolipids are present throughout the respiratory tract (20), and proteoglycans are ubiquitous on the epithelial cell surface and in the extracellular matrix. Attachment to these sulfated glycoconjugates could effectively secure the microorganism to the site where productive infection can be initiated. FHA appears to be the primary *B. pertussis* adhesin responsible for early events in the infectious cycle. Portions of the FHA molecule show a strong resemblance to a number of eukaryotic attachment proteins (27, 37), and this bacterial adhesin appears to have

usurped eukaryotic cell adhesion mechanisms for mediating adherence of *B. pertussis* to mammalian cells. Recently, it has been shown that FHA possesses extended regions of tandem 19-residue repeats that strongly resemble the leucine-rich repeats present in many eukaryotic proteins implicated in intercellular adhesion (30). In this paper, we demonstrate another example of FHA's ability to mimic eukaryotic adhesion mechanisms: its attachment to specific glycolipids and glycosaminoglycans.

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