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Receptor Affinity Purification of a Lipid-Binding Adhesin from *Helicobacter pylori*

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Our previous work has shown that *Helicobacter pylori* specifically recognizes gangliotetraosylceramide, gangliotriaosylceramide, and phosphatidylethanolamine in vitro. This binding specificity is shared by exoenzyme S from *Pseudomonas aeruginosa*, and monoclonal antibodies against this adhesin prevent the attachment of *H. pylori* to its lipid receptors. We now report the use of a novel, versatile affinity matrix to purify a 63-kDa exoenzyme S-like adhesin from *H. pylori* which is responsible for the lipid-binding specificity of this organism.

Helicobacter pylori is a gastric pathogen associated with gastritis, duodenal ulcer (5, 6), and possibly subsequent development of gastric carcinoma (22–24). We have shown by thin-layer chromatography (TLC) overlay that this organism shows the same lipid-binding specificity as exoenzyme S from *Pseudomonas aeruginosa* (21) in that phosphatidylethanolamine (PE), gangliotriaosylceramide (galNAcβ1-4galβ1-4glc cer [Gg_3]), and gangliotetraosylceramide (galβ1-4galNAcβ1-4galβ1-4glc cer [Gg_4]) are recognized (18, 19). This binding specificity is typical of many microorganisms (16), and we have proposed (20) that such organisms contain exoenzyme S-related adhesins.

This report describes the purification of the first such adhesin from *H. pylori*, by affinity chromatography with a novel immobilized PE matrix. This method is suitable for facile purification of any lipid-binding ligand.

MATERIALS AND METHODS

PE from *Escherichia coli*, egg lecithin, and cholesterol were purchased from Sigma. Gg_4 and Gg_3 were prepared from bovine and human brain tissue, respectively. A mixture of glycolipid standards was kindly provided by MicroCarb Inc., Gaithersburg, Md. Plastic-backed silica gel (SILG) TLC plates were from Brinkmann. Goat anti-rabbit horseradish peroxidase conjugate was from Bio-Rad. Monoclonal anti-exoenzyme S antibodies and purified exoenzyme S from *P. aeruginosa* were generous gifts from D. Woods, University of Calgary, Calgary, Alberta, Canada.

TLC overlay. A mixture of glycolipid standards (ceramide monohexoside, ceramide dihexoside, globotriaosylceramide, globotetraosylceramide, and gangliosides GM_3, GM_2, and GM_1) and purified preparations (5 μg) of Gg_4, Gg_3, or PE were separated by TLC with chloroform–methanol–0.1% aqueous KCl (5:4:1, vol/vol). In some experiments, the plates were pretreated with 1% polyisobutylmethacrylate (29) prior to assay of *H. pylori* binding. Plates were blocked by incubation with 3% gelatin for 2 h at 37°C. Plates were incubated with *H. pylori* under microaerophilic conditions (or protein extracts under air) for 2 h at room temperature, and binding was detected immunologically (19, 21).

Preparation of affinity matrix. Glycolipid-ligand interactions are most simply demonstrated by the TLC overlay procedure (29). This procedure is widely used in many different fields. Essentially, it involves detection of a ligand bound to a carbohydrate (glycolipid) receptor immobilized on a silica gel TLC plate. We therefore used this principle to develop a facile means of generating any glycolipid (or lipid) affinity matrix by merely absorbing the lipid receptor onto silica.

Briefly, 10 mg of PE (from *E. coli*) in 1 ml of chloroform was mixed and vortexed with 1 g of dried Celite. The chloroform was evaporated under nitrogen at 37°C. The PE matrix was blocked by shaking with 5 ml of 1% glycine at room temperature overnight. The gel was washed with 100 mM Tricine-buffered saline (TBS) by centrifugation prior to use. No PE was found in the washing or 5 mM EDTA elution buffer.

Purification of *H. pylori* adhesin. *H. pylori* cultures grown under microaerophilic conditions were centrifuged, and the pellet was extracted with water as previously described (11, 25). An aliquot of the water extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting (immunoblotting) with monoclonal antibodies against exoenzyme S (28). The water extract was also subjected to TLC overlay binding to standard glycolipids, using monoclonal anti-exoenzyme S antibodies to detect bound material (17).

The PE affinity matrix was incubated with 5 ml of water extract (1 mg/ml) in TBS containing phenylmethylsulfonyl fluoride (1 mM) for 1 h at room temperature with a rotating shaker. The unbound proteins were washed out with 4 × 20 volumes of TBS, and the bound protein was eluted by incubation for 30 min with 5 mM EDTA with a rotating shaker. The eluted protein obtained in the supernatant after centrifugation was dialyzed at 4°C and lyophilized. The eluted protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. A section of the transfer was stained with monoclonal anti-exoenzyme S antibodies, and the remainder was stained with Coomassie blue. The single eluted protein was sequenced from the transfer via automated N-terminal Edman degradation with the Picotag high-performance liquid chromatography system (Waters).

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Microtiter binding assay. PE from *E. coli* was mixed with cholesterol and phosphatidylcholine in a ratio 10:1:1 by weight in methanol (15). Wells of flat-bottom microtiter plates were coated with receptor lipid and dried at room temperature. A pilot experiment established that 1 μg of receptor per well was optimal for *H. pylori* binding. Nonspecific binding sites were blocked with 50 μl of 2% bovine serum albumin (BSA) in 100 mM TBS for 1 h at room temperature. After being washed, wells were incubated under reduced oxygen at 37°C for 90 min with a 50-μl suspension of *H. pylori*. The plates were then washed and incubated for 2 h with a 1:500 dilution of rabbit anti-*H. pylori* serum (19) containing 2% BSA in TBS. The plates were washed again and incubated with goat antibody to rabbit immunoglobulin conjugated with horseradish peroxidase for 1 h at room temperature. After washing, bound horseradish peroxidase was detected colorimetrically with *O*-phenylenediamine dihydrochloride. The enzyme reaction was stopped with 0.2 M sulfuric acid, and the optical density at 492 nm was measured with a microplate enzyme-linked immunosorbent assay reader.

RESULTS

Binding specificity. The glycolipid-binding specificity of *H. pylori* is shown in Fig. 1. None of the standard neutral glycolipids or gangliosides were bound. Gg_4, Gg_3, and PE were recognized. At high concentrations, GM_3, but not sulfatide, was also bound (Fig. 1c). However, this binding was lost if the plate was pretreated with polyisobutylmethacrylate (Fig. 1b) and the binding was far less than for PE.

Extraction of adhesin. The *H. pylori* surface protein (water) extract was found to contain two proteins cross-reactive with an anti-exoenzyme S antibody (Fig. 2), one corresponding in molecular mass to *P. aeruginosa* exoenzyme S and a species with a higher molecular mass of about 60 kDa. TLC overlay of this extract indicated that one or both of these species specifically binds to Gg_3, Gg_4, and PE (Fig. 3) in a manner similar to the intact organism (Fig. 1).

Affinity purification. The water extract containing the exoenzyme S-like proteins was applied on a novel PE matrix. We had previously shown that binding of exoenzyme S from *P. aeruginosa* to PE was inhibited in the presence of EDTA (17), therefore, after the column was washed to remove unbound material, the column was batch eluted with 5 mM EDTA. This resulted in elution of a single protein with a molecular mass of 63 kDa, which was reactive with a monoclonal anti-exoenzyme S antibody (Fig. 2). TLC overlay confirmed the binding specificity of this PE-affinity purified adhesin for Gg_3, Gg_4, and PE (Fig. 4). The binding specificity was dependent on the cationic environment, since Gg_3 and Gg_4, but not PE, were more strongly bound in the presence of Ca^{2+} and Mg^{2+} (Fig. 5).

Preliminary experiments indicate that the affinity-purified adhesin is a potent inhibitor of *H. pylori* receptor binding to PE in vitro (Fig. 6).

Amino acid sequence. The N-terminal sequence of this affinity-purified adhesin was determined after SDS-PAGE and transfer to a polyvinylidene difluoride membrane. The sequence contained an N-terminal methionine residue (Met-Val-Asn-Lys-Asp-Val-Lys-X-Thr-X-Ala-Phe [X = failure in high-performance liquid chromatography injection after cycle]). The subsequent sequence showed no homology to previously described proteins.

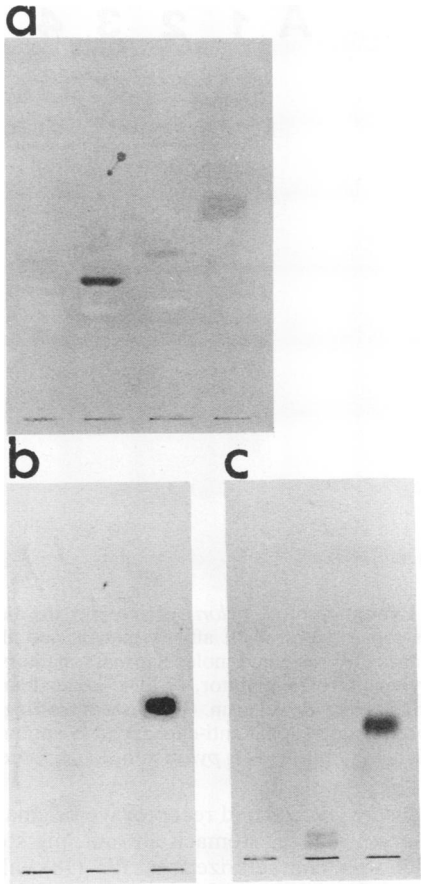

FIG. 1. TLC overlay binding specificity of *H. pylori* organisms. Standard glycolipids were separated by TLC and tested for *H. pylori* binding as described in Materials and Methods. Panel a (5 μg of lipids) (for detection by orcinol spray, see Fig. 3a): lane 1, separated mixture of standards; lane 2, Gg_4; lane 3, Gg_3; lane 4, PE. Panels b and c (15 μg of lipids): lane 1, sulfogalactosyl ceramide; lane 2, GM_3; lane 3, PE (the solvent was chloroform-methanol-water, 65:25:4). Panel b was treated with polyisobutylmethacrylate, as described in Materials and Methods, prior to the binding assay.

DISCUSSION

Several candidate receptors for attachment of *H. pylori* to target cells have been proposed (9), including the glycolipids GM_3 and sulfogalactosylceramide (sulfatide) (26, 27). Recently, the gene for an adhesin with binding specificity for sialyllactose has been cloned (10). The 20-kDa product of this gene is entirely distinct from the species we have purified, and the relative roles of these adhesins must be addressed in future studies.

Glycolipid receptor studies using TLC overlay have involved pretreatment of the TLC plate with polyisobutylmethacrylate, which we have shown can result in spurious binding results (29). At high concentrations, we have detected weak binding to GM_3 (only in the absence of polyisobutylmethacrylate treatment) but not sulfatide (Fig. 1). PE is more strongly bound. At the level at which it was present in the glycolipid standards (~ 2 μg), GM_3 was not bound (Fig. 1a, lane 1). Since the binding of *H. pylori* to GM_3 was eliminated after polyisobutylmethacrylate treatment, it is likely that the discrepancy between our results and those of Saitoh et al. (26, 27) is due to *H. pylori* strain differences.

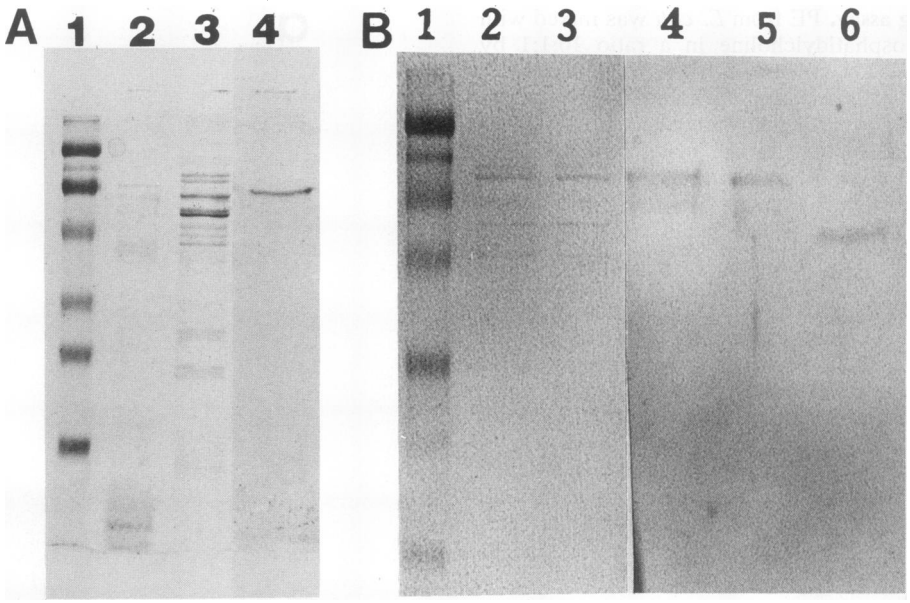

FIG. 2. Extraction of *H. pylori* surface proteins and affinity purification of *H. pylori* adhesin. (A) Protein staining. The *H. pylori* water extract was separated on a PE affinity matrix, and aliquots of fractions were separated by SDS-PAGE and detected by Coomassie blue staining. Lanes: 1, prestained molecular mass markers (phosphorylase *b*, 130 kDa; BSA, 75 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 39 kDa; soybean trypsin inhibitor, 27 kDa; α-lactalbumen, 17 kDa); 2, exoenzyme S from *P. aeruginosa*; 3, *H. pylori* water extract; 4, PE affinity-purified *H. pylori* adhesin. (B) Western blotting. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained for reactivity with a monoclonal anti-exoenzyme S antibody. Lanes: 1, prestained molecular mass standards; 2 and 3, *H. pylori* water extract; 4 and 5, PE affinity-purified *H. pylori* adhesin; 6, exoenzyme S from *P. aeruginosa*.

The *H. pylori* glycerolipid receptor we originally detected in human erythrocytes, stomach antrum, pig stomach, and HEp2 cells was characterized as PE (18), although the binding to different commercial forms of PE was varied. This was the major lipid species from stomach tissue recognized. We have found that binding of *H. pylori* to cultured cells is barely above the background for cells lacking PE (8). Thus, this ligand-receptor interaction is a major mechanism for attachment of *H. pylori* to host cells in vitro, as well as in vivo (19). Bismuth treatment of *H. pylori* results in immediate inhibition of PE binding in vitro (14), concomitant with the observed inhibition of mucosal attachment in vivo (1). *H. mustelae*, considered to be a ferret model for *H. pylori* infection, also binds to PE and Gg_4 in vitro, and this binding is also inhibited in the presence of anti-exoenzyme S antibodies or the exoenzyme S protein (12).

Water extraction of *H. pylori* has proven to be an effective method of solubilizing *H. pylori* surface proteins (11), including urease (7). This extract has been implicated in the adhesion of *H. pylori*, and it was questioned whether the cell

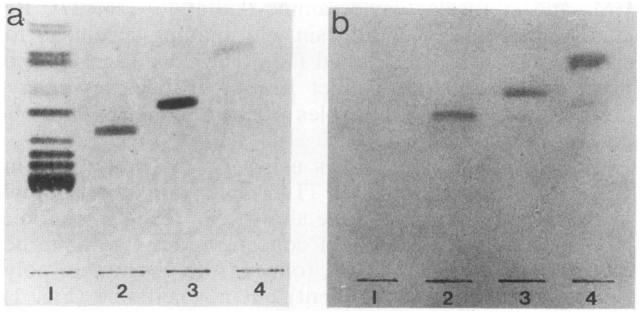

FIG. 3. TLC overlay of *H. pylori* surface proteins. Lanes: 1, standard glycolipids (from the top, ceramide monohexoside, ceramide dihexoside, globotriaosylceramide, globotetraosylceramide, GM_3, GM_2, GM_1, and 2 μg of lipids) 2, Gg_4; 3, Gg_3; 4, PE. Panel a was visualized by orcinol spray, and panel b (binding of *H. pylori* water extract) was visualized with anti-exoenzyme S antibodies.

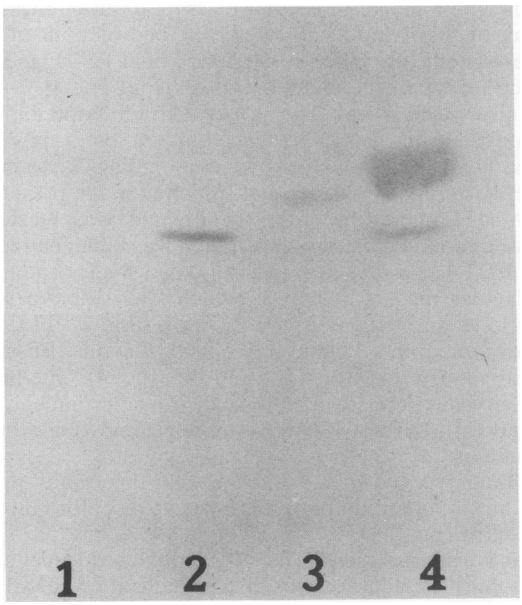

FIG. 4. TLC overlay of affinity-purified *H. pylori* adhesin. The affinity-purified *H. pylori* adhesin (5 μg/ml) was tested for binding of glycolipids (2 μg) separated by TLC as described in the legend to Fig. 3. Bound adhesin was detected with a monoclonal anti-exoenzyme S antibody. Lanes: 1, standard glycolipids, as in Fig. 3a; 2, Gg_4; 3, Gg_3; 4, PE.

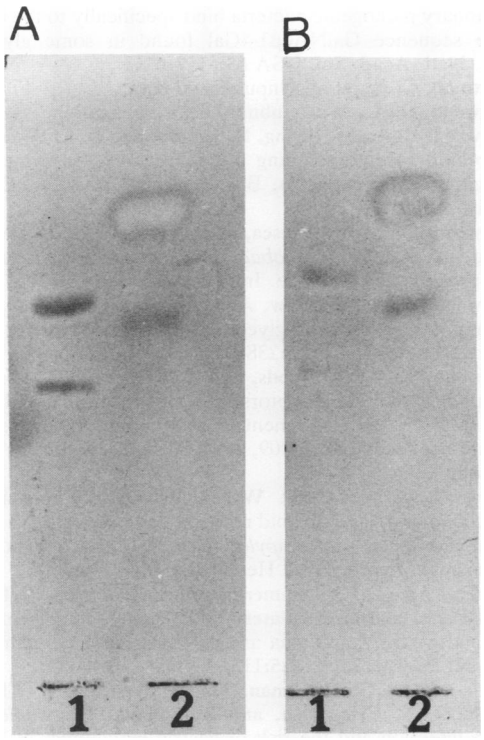

FIG. 5. Effects of cations on purified adhesin binding specificity. Panels: A, binding in the presence of 5 mM Ca^{2+} and 5 mM Mg^{2+}; B, binding in TBS alone. Lanes: 1, Gg_3 and Gg_4; 2, PE.

surface urease was involved (11). It is likely that the adhesin we purified, which is similar in molecular weight to one urease subunit (7), was responsible for the inhibition of adhesion observed for partially purified urease preparations (11). This inhibition was not sialyllactose dependent (11). Our adhesin may also be related to the non-sialyllactose-dependent 59-kDa erythrocyte-binding antigen recently isolated from *H. pylori* (13).

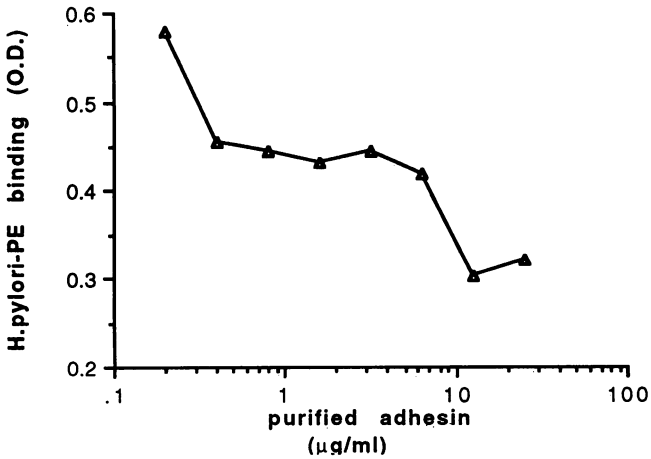

FIG. 6. Inhibition of *H. pylori* receptor binding by purified adhesin. PE-containing microtiter plates were preincubated with increasing concentrations of the affinity-purified adhesin for 30 min at room temperature. Wells were washed and incubated with *H. pylori*. Binding was determined with anti-*H. pylori* serum as described in Materials and Methods. O.D., optical density.

We isolated one of two anti-exoenzyme S antibody-reactive proteins found in the surface extract of *H. pylori* organisms. Occasionally, both proteins were bound and eluted from the affinity matrix, and it may be that the lower-molecular-weight species has the binding specificity expected, but the conditions for purification of this species have yet to be optimized. The 63-kDa surface protein was affinity purified on a novel phospholipid matrix and eluted with EDTA. The binding specificity of exoenzyme S from *P. aeruginosa* was dependent on the cation concentration (17). An effect was also observed in the TLC overlay procedure when binding of the purified *H. pylori* adhesin to Gg_3 and Gg_4, but not that to PE, was enhanced when the assay was performed in the presence of divalent cations (Fig. 5). Similarly, the carbohydrate binding of selectins has been found to be Ca^{2+} dependent (2).

The purified adhesin was an effective inhibitor of *H. pylori* binding to PE in vitro (Fig. 6), supporting the contention that this adhesin is responsible for the *H. pylori* receptor-binding specificity we have reported and is therefore an appropriate focus for future studies on the role of adhesion in the pathogenesis of this organism.

The procedure we used to generate the PE affinity matrix is simple and widely applicable for any lipid receptor. We used the PE matrix to affinity purify a similar species from *Haemophilus influenzae* (16a). TLC overlay is the most commonly used procedure for detection of glycolipid (or neoglycolipid)-binding ligands. Our affinity matrix can be prepared with any glycolipid (or lipid) for which a ligand is detected by such overlays. The matrix requires no chemistry and takes less than 5 min to prepare, and the glycolipid receptor can be recovered without loss. We have used this procedure to purify several other glycolipid-binding ligands (16b).

We have proposed (20) that the many organisms which show a common binding specificity for Gg_3 and Gg_4 in vitro also bind PE and contain adhesins related to exoenzyme S (from *P. aeruginosa*) which mediate this binding specificity. We have found that such organisms contain exoenzyme S-like proteins as monitored by Western blotting with polyclonal and monoclonal antibodies against *P. aeruginosa* exoenzyme S. Solid-phase glycolipid receptor binding by these organisms is inhibited by such antibodies and by exoenzyme S itself (20).

These adhesins, which, because of their cation-dependent, lectin-like activity, we propose to call M (microbial)-selectins (4), may contain a consensus amino acid sequence responsible for the common binding specificity, which is expressed in a background of various different proteins. Exoenzyme S has been confirmed as an adhesin for *P. aeruginosa* (3). The M-selectin we isolated was also able to inhibit the binding of *H. pylori* in our in vitro microtiter assay. These studies indicate that the exoenzyme S-related 63-kDa M-selectin is the adhesin responsible for the lipid-binding specificity of *H. pylori* we have observed in vitro and is a member of a newly recognized, widely distributed family of bacterial glycolipid-binding adhesins.

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