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Identification of the *N*-Acetylneuraminyllactose-Specific Laminin-Binding Protein of *Helicobacter pylori*

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The interaction of the gastroduodenal pathogen *Helicobacter pylori* with the glycoprotein laminin was investigated. Binding of ^{125}I-radiolabelled laminin in a liquid-phase assay by both hemagglutinating and poorly hemagglutinating strains was rapid, saturable, specific, partially reversible, of high affinity, and insensitive to pH. Inhibition of laminin binding by fetuin, but not asialofetuin, and reduced bacterial binding to periodate- or sialidase-treated laminin indicated that glycosylation, particularly sialylation, was important for laminin binding by *H. pylori*. Inhibition experiments with monosaccharides, disaccharides, and trisaccharides showed that the strains bound to a region spanning a trisaccharide. In particular, inhibition and displacement studies showed that binding to the trisaccharide *N*-acetylneuraminyl-α(2-3)-lactose [NeuAc(2-3)Lac] was preferential to that to the NeuAc(2-6)Lac isomer. Complete inhibition of laminin binding by both hemagglutinating and poorly hemagglutinating strains was achieved only when isolated lipopolysaccharide (LPS) was used as an inhibitor in combination with heat or protease treatment of *H. pylori* cells, thereby confirming the involvement of both LPS and a protein adhesin in laminin binding. Further inhibition experiments indicated that the protein receptor, rather than LPS, on *H. pylori* bound NeuAc(2-3)Lac. By using a Western blotting procedure, a 25-kDa outer membrane protein was identified as mediating laminin binding by both hemagglutinating and poorly hemagglutinating *H. pylori* strains. The specificity of binding was confirmed by complete inhibition of laminin binding by the 25-kDa protein with NeuAc(2-3)Lac. The data collectively suggest that a 25-kDa outer membrane protein acts in a lectin-like manner with LPS to mediate attachment of *H. pylori* to laminin.

Since its initial isolation (67), *Helicobacter pylori* has become recognized as one of the most common bacterial pathogens of humans (60). *H. pylori* is the primary cause of active chronic gastritis and plays a pivotal role in the development of peptic ulcer disease (3, 20, 37, 50, 67). Epidemiological data have also suggested that persistent infection with this bacterium is a risk factor for the development of adenocarcinoma and lymphoma of the stomach (3, 16, 43, 47, 70).

The gastric mucosae of patients infected with *H. pylori* show a massive infiltration with plasma cells, T lymphocytes, mononuclear phagocytes, and neutrophils, and the bacteria appear to undermine the mucosal integrity by penetrating the tight junctions of epithelial cells (3, 26, 57). The maintenance of the integrity of the gastric epithelium involves specific interaction of epithelial cell surface receptors, termed integrins, with distinct extracellular matrix adhesive proteins, one of which is laminin (19, 51). Laminin is also important for the structure of the basement membrane by its formation of networks with type IV collagen, entactin/nidogen, and heparan sulfate proteoglycans (71). The laminin molecule is a 900-kDa glycoprotein consisting of multiple domains, arranged in an extended four-armed cruciform shape (2). In addition to domains binding collagen and heparan sulfate in the basement membrane, domains include a cell-signalling site with mitogenic action, a region involved in calcium-dependent aggregation, and a receptor-mediated cell attachment site (2, 19, 31). Laminin is composed of three polypeptide chains: an A chain (400 kDa), a B1 chain (220 kDa), and a B2 chain (220 kDa) (2, 61). A characteristic feature of the laminin molecule is its high carbohydrate content of 12 to 27%, most of which is present in complex-type oligosaccharides (1, 17, 27) and plays a role in cellular adhesion processes (5, 6, 72).

Strains of *H. pylori* bind laminin, although to differing extents (40, 62, 63, 65). Hemagglutinating *H. pylori* strains bind larger amounts than poorly hemagglutinating *H. pylori* strains (65). Although the precise contribution of attachment in *H. pylori* pathogenesis has not been unequivocally established, the bacterium has been observed adhering to gastric mucosal cells in vivo (22, 29). The capacity to bind laminin is unlikely to be involved in the initial colonization of gastric mucosal cells, since putative primary adhesins of *H. pylori* that recognize receptors in the mucus layer and on the epithelial cell surface have been described (for a review, see reference 38). Nevertheless, the ability to bind laminin may explain the association of the bacterium with intercellular junctions (21). Furthermore, *H. pylori* interferes with the interaction between laminin and a laminin receptor on gastric epithelial cells (54), which may explain the disruption of tight junctions observed in *H. pylori*-infected tissue and the development of gastric epithelium leakiness (21, 30, 54, 57).

The interaction of *H. pylori* with laminin is complex. A phosphorylated structure in the core oligosaccharide of lipopolysaccharide (LPS) mediates the interaction of a hemagglutinating *H. pylori* strain with laminin, whereas a conserved nonphosphorylated structure in the core oligosaccharide mediates the interaction of a poorly hemagglutinating strain (65). Also, a second adhesin is involved in laminin binding by *H. pylori*. It has been suggested that the initial recognition and binding of laminin by *H. pylori* may occur through LPS and that

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subsequently another interaction with a lectin-like protein adhesin occurs (38, 63, 65).

To date, the laminin-binding protein of *H. pylori* has not been identified, and the interaction of poorly hemagglutinating strains with laminin has not been characterized completely. This prompted the present investigation. We compared the interactions of hemagglutinating and poorly hemagglutinating *H. pylori* strains with laminin. The binding site of *H. pylori* in the carbohydrate moiety of laminin was determined, and a 25-kDa protein that mediates attachment of the bacterium was identified by Western blot (immunoblot)-type analysis. The evidence presented indicates that this protein mediates laminin binding by both hemagglutinating and poorly hemagglutinating *H. pylori* strains.

MATERIALS AND METHODS

Chemicals and reagents. Laminin, purified from Engelbrett-Holm-Swarm transplantable mouse tumors, was obtained from AMS Biotechnology (Stockholm, Sweden). Recombinant human laminin B1 chain (provided by T. Pikkarainen, University of Oulu, Oulu, Finland) was produced in Sf9 insect cells with the baculovirus expression system (48). Gamma globulin was obtained from KABI AB (Stockholm, Sweden). Human type I and IV collagens, as well as fetuin and asialofetuin from fetal calf serum, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fibronectin was purified from human plasma by the method of Vuento and Vaheri (66). Bovine serum albumin (BSA) fraction V was obtained commercially (Boehringer GmbH, Mannheim, Germany). Iodobeads were purchased from Pierce Chemical Co. (Rockford, Ill.). Trypsin (EC 3.4.21.4), proteinase K from *Tritirachium album* (EC 3.4.21.14), pronase E from *Streptomyces griseus* (bacterial protease type XIV), *Vibrio cholerae* sialidase (EC 3.2.1.18), and sugars used in inhibition experiments (L-fucose, D-glucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, lactose from milk sugar, and *N*-acetylneuraminyllactose from bovine colostrum) were obtained from Sigma. The trisaccharides *N*-acetylneuraminyl-α(2-3)-galactopyranosyl-β(1-4)-glucopyranose [NeuAc(2-3)Lac] and *N*-acetylneuraminyl-α(2-6)-galactopyranosyl-β(1-4)-glucopyranose [NeuAc(2-6)Lac] were purchased from Oxford GlycoSystems (Abingdon, England). LPSs from *H. pylori* were prepared as in a previous study (63) by hot phenol-water extraction and purified by treatment with RNase A (Sigma), DNase II (Sigma), and proteinase K (Sigma) and ultracentrifugation. The purity of LPS preparations was verified by chemical analyses as described previously (39, 65).

Bacterial strains and growth conditions. *H. pylori* strains with differing hemagglutinating activities and differing abilities to bind laminin were used in binding studies. Four hemagglutinating strains which bound large to intermediate amounts of laminin (CCUG 17874, 1139, 51, and 52) and two poorly hemagglutinating strains which bound small amounts of laminin (CCUG 17875 and 66) were selected for this study (63, 65). *H. pylori* CCUG 17874 and CCUG 17875 (same as NCTC 11637 and NCTC 11638, respectively [National Collection of Type Cultures, London, England]) were obtained from the Culture Collection of the University of Göteborg, Göteborg, Sweden. The other strains were isolates from endoscopic biopsy specimens obtained from the University Hospital, Lund, Sweden. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). *H. pylori* strains were grown on blood agar under microaerobic conditions at 37°C for 48 h (63).

Radioiodination of laminin. Laminin was labelled with ^{125}I (sodium salt; specific activity, 14.5 mCi/μg; Amersham International, Amersham, England) by a modified chloramine-T method involving Iodobeads (35). The specific activity of the labelled laminin was about 2×10^6 cpm/μg. The protein concentration was determined by the method of Lowry et al. (34).

Binding assays. Liquid-phase binding assays were performed as described previously (65). Briefly, bacterial growth was harvested from cultures, washed once with phosphate-buffered saline (PBS), and finally adjusted to an A_{595} of 1.0 (10^8 cells per ml). The bacterial suspension (100 μl, 10^7 cells) was mixed with 0.55 pmol of ^{125}I-labelled laminin in 0.1 M PBS containing 0.1% (wt/vol) BSA (PBS-BSA) and incubated at 20°C for 1 h, unless otherwise stated. Subsequently, bacteria were pelleted by centrifugation (2,000 × *g* at 4°C for 15 min) and washed with PBS. The total radioactivity and the radioactivity of the pellet were determined by conventional scintillation counting in a gamma counter (Wallac, Turku, Finland). Assays were performed in triplicate, and the radioactivity associated with the pellet was expressed as a percentage of the total radioactivity added to the sample.

To assess the influence of glycosylation of laminin on bacterial binding, 0.55 pmol of ^{125}I-laminin was treated at 20°C for 16 h with 0.1 M sodium *meta*-periodate in 0.05 M sodium acetate buffer (pH 5.0) and then tested for bacterial binding. Similarly, the influence of sialylation of laminin on bacterial binding was assessed after 0.55 pmol of ^{125}I-laminin was incubated with sialidase (2 U) in 10 mM sodium acetate buffer (pH 5.0) at 37°C for 12 h. ^{125}I-laminin (0.55 pmol) incubated under the same conditions, but in the absence of periodate and sialidase, was used as a control.

To assess the effect of temperature on the ability of bacteria to bind laminin, bacterial suspensions (10^7 cells in 100 μl of PBS) were heated in a water bath at either 20°C for 60 min or 80°C for 10 min before use in binding assays. The involvement of surface proteins in the binding of laminin was assessed by treating different bacterial suspensions with trypsin (50 μg/ml), proteinase K (5 μg/ml), and pronase E (5 μg/ml) at 37°C for 1 h as described previously (63). After enzymatic treatments, the bacterial cells were washed twice, resuspended in PBS, and tested for laminin binding as described above. For control experiments, laminin binding was determined with untreated bacterial cells.

Inhibition assays. The method used was essentially the same as that described previously (63, 65). To test the ability of unlabelled laminin, other extracellular matrix proteins, and glycoproteins to inhibit ^{125}I-laminin binding, bacterial suspensions (100 μl, 10^7 cells) were incubated with 100 μg of the different proteins at 20°C for 1 h unless otherwise stated. Subsequently, bacteria were pelleted by centrifugation (2,000 × *g* at 4°C for 15 min) and washed with PBS, 0.55 pmol of ^{125}I-laminin in PBS-BSA was added, and the mixture was incubated at 20°C for a further 1 h. The extent of bacterial binding to ^{125}I-laminin was determined as described above in triplicate assays.

In a similar manner, monosaccharides, disaccharides, and trisaccharides, which are constituents of the sugar chain of laminin, were tested for their ability to inhibit laminin binding. Untreated, heat-treated, or pronase E-treated bacteria in suspension were first incubated with the respective sugar (300 μg, unless otherwise stated) before determination of bacterial binding to ^{125}I-laminin. The binding assays were performed as described above.

To assess the role of LPS in laminin binding, 0.55 pmol of ^{125}I-labelled laminin in PBS-BSA was preincubated with *H. pylori* LPS (100 μg) at 20°C for 1 h before incubation with bacteria (untreated or heat or enzyme treated) under the same conditions as in binding assays. The resulting cell suspensions were centrifuged (2,000 × *g* at 4°C for 15 min), the pellets were washed with PBS, and the amount of bound radioactivity was determined.

Displacement assays. To determine the reversibility of laminin binding, cell suspensions (100 μl, 10^7 cells) were first incubated with ^{125}I-laminin at 20°C for 1 h as in binding assays and then incubated with excess unlabelled laminin (100 μg) or 3-sialyllactose (100 μg) for up to a further 1 h. Subsequently, the extent of bacterial binding to ^{125}I-laminin was determined in triplicate assays.

Production of polyclonal antisera. Specific antiserum to laminin was produced as described previously (64). Briefly, lambs were given intradermal injections at several sites with purified laminin (1 mg) in 10 mM Tris-buffered (pH 7.4) saline mixed with an equal volume of complete Freund's adjuvant. Booster injections were given every third week with the same antigen concentration without adjuvant. When an adequate antibody titer was obtained, the lambs were exsanguinated, and the serum was collected and stored at −20°C. Preimmune sera from the same animals were used for control experiments.

Isolation of outer membranes. Cells were washed with 10 mM Tris-HCl (pH 7.5), suspended in the same buffer, and sonicated 30 times for 30-s intervals on ice at 30 W. The resulting suspension was treated with DNase II (Sigma) and RNase A (Sigma) as described previously (64), and the cell debris was removed by centrifugation (1,000 × *g* for 15 min at 4°C). Phenylmethylsulfonyl fluoride (1 mM) was added to the suspension, and the whole envelope fraction was collected by centrifugation (100,000 × *g* for 1 h at 4°C). Inner and outer membranes were separated in sucrose gradients by the method of Osborn et al. (44). Fractions were removed, washed with 10 mM Tris-HCl (pH 7.5) three times, and assayed for protein content (34) and NADH oxidase activity (44). The fraction containing the least NADH oxidase activity was the crude outer membrane preparation. Subsequently, this preparation was treated with 0.5% (wt/vol) sodium lauryl sarcosine as described previously (52) to solubilize any contaminating cytoplasmic proteins. The sarcosyl-insoluble membrane fraction (outer membrane) was collected by centrifugation (40,000 × *g* for 30 min at 4°C), washed three times with Milli Q water, aliquoted, and stored at −20°C until use. The purity of outer membrane preparations was confirmed by the absence of NADH oxidase activity.

Electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the discontinuous buffer system described by Laemmli (28). Electrophoresis was conducted with a constant current of 35 mA, a stacking gel of 4% acrylamide, and a separating gel of 10% acrylamide. Separated outer membrane proteins were stained with Coomassie blue R-250. Alternatively, separated proteins were electrotransferred from gels to nitrocellulose membranes by the method of Sankaran et al. (52). After the transfer, unreacted sites on the membranes were blocked with Tris-HCl-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) containing 1% (wt/vol) BSA (64). To detect laminin-binding proteins, nitrocellulose sheets were incubated at 20°C for 3 h with 300 μg of laminin in TBS containing 0.03% (vol/vol) Tween 20 (TBS-Tween) (33). After incubation, nitrocellulose membranes were washed with TBS-Tween and reactive bands were visualized with lamb antiserum to laminin as the first antibody (1:1,000 in TBS) and anti-lamb immunoglobulin-horseradish peroxidase conjugate (Dakopatts, Copenhagen, Denmark) as the second antibody (1:3,000 in TBS) (63). Preimmune sera from the same animals were used for control experiments.

To assess the influence of glycosylation and sialylation of laminin on protein binding, periodate-treated laminin and sialidase-treated laminin, respectively,

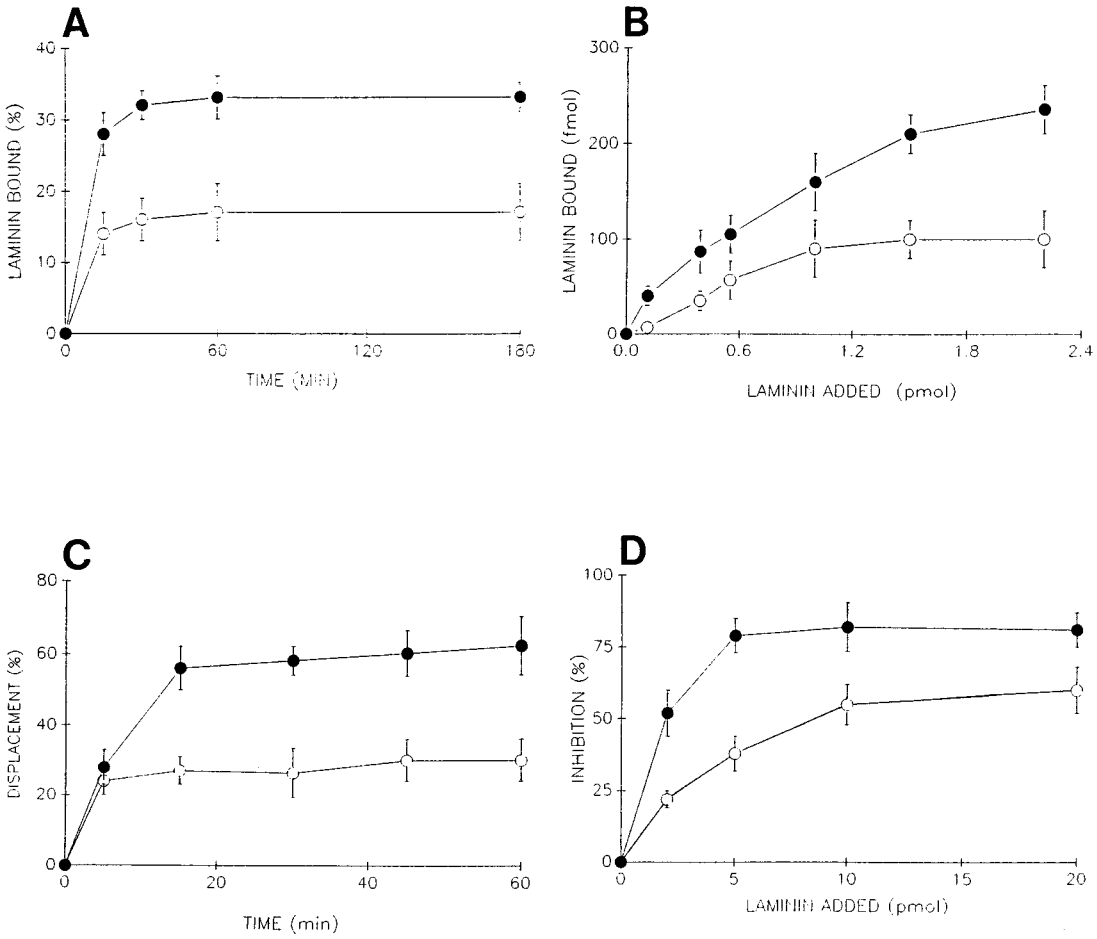

FIG. 1. Interaction of ^{125}I-laminin with *H. pylori* CCUG 17874 (●) and CCUG 17875 (○). (A) Kinetics of bacterial binding of ^{125}I-laminin. (B) Saturation of bacterial binding of ^{125}I-laminin. (C) Displacement of bound ^{125}I-laminin with unlabelled laminin. (D) Inhibition of bacterial binding of ^{125}I-laminin with unlabelled laminin. Data points represent the means of three determinations, and error bars indicate standard deviations.

were incubated with the electrotransferred outer membrane proteins and reactive bands were detected on the nitrocellulose sheets by the procedure described above. Furthermore, monosaccharides, disaccharides, and trisaccharides, which are constituents of the sugar chain of laminin, were tested for their ability to inhibit laminin binding by first incubating the respective sugar (300 μg) with the electrotransferred proteins before determination of laminin binding.

Statistical analysis. Student's *t* test was used to assess the significance of differences between means in binding and inhibition assays.

RESULTS

Characterization of laminin binding by hemagglutinating and poorly hemagglutinating strains. Both hemagglutinating *H. pylori* CCUG 17874 and poorly hemagglutinating *H. pylori* CCUG 17875 bound ^{125}I-laminin, although to different extents, in a time-dependent manner (Fig. 1A). Identical results were obtained when the assays were performed in air or under microaerobic conditions. The kinetics of laminin binding by CCUG 17874 was in agreement with previous investigations (62, 63). The binding of ^{125}I-laminin by both strains was rapid and saturable, and maximal binding was attained at 60 min (31% for CCUG 17874; 17% for CCUG 17875). Continuation of incubation for up to 3 h did not significantly ($P > 0.1$) affect the amount of ^{125}I-labelled protein bound (Fig. 1A). Therefore, for further experiments, an incubation time of 1 h was chosen to ensure maximum binding.

Binding of ^{125}I-laminin by cells of CCUG 17874 and CCUG 17875 was unaffected and remained constant over a pH range of 4.0 to 9.0 (data not shown). Quantitation of bound ^{125}I-laminin as a function of increasing concentration of radiolabelled protein showed that the binding of both strains was saturable (Fig. 1B) and indicated that the bacterial strains contain a limited number of laminin receptors. Scatchard plot analysis (53) of the binding data yielded a binding constant (K_d) of 8.5 pM for CCUG 17874 in the range of those reported previously (62, 63) and a K_d of 4.1 pM for CCUG 17875. Assuming that the molecular mass of laminin is about 900 kDa, there are about 2,000 binding sites on CCUG 17874 and 1,200 sites on CCUG 17875 with the aforementioned binding constants.

Valid Scatchard analysis requires the binding reaction to be at equilibrium, and hence the reversibility of laminin binding to both strains was determined in a displacement assay. Addition of an excess of unlabelled laminin (100 μg) to cells of CCUG 17874 and CCUG 17875 with previously bound ^{125}I-laminin partially displaced the bound laminin in a time-dependent manner (Fig. 1C). Maximal displacement of 62 and 30% for CCUG 17874 and CCUG 17875, respectively, occurred at 60 min. Continuation of incubation did not significantly ($P > 0.10$) increase the extent of displacement. Since laminin binding by both strains is in part irreversible, the predicted binding constants for the hemagglutinating and poorly hemagglutinat-

TABLE 1. Effect of various proteins as inhibitors of binding of ^{125}I-laminin to *H. pylori* CCUG 17874 and CCUG 17875

Inhibitor	% Inhibition of binding to *H. pylori* strain[a]:	
	CCUG 17874	CCUG 17875
None	0 ± 2	0 ± 3
Gamma globulin	0 ± 3	0 ± 2
Collagen type I	10 ± 8	11 ± 9
Collagen type IV	10 ± 8	11 ± 8
Fibronectin	25 ± 10*	18 ± 8*
Fetuin	56 ± 7**	31 ± 5**
Asialofetuin	25 ± 12	16 ± 10
Laminin	82 ± 7***	60 ± 6***

[a] Mean of three determinations ± standard deviation. Values include the significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$) of the difference between inhibition in the presence and absence of inhibitor as determined by Student's *t* test.

TABLE 2. Effect of modification of laminin before determination of binding to *H. pylori* CCUG 17874 and CCUG 17875

Compound	Comparative decrease (%) in binding to *H. pylori* strain[a]:	
	CCUG 17874	CCUG 17875
Control (native laminin)	0 ± 3	0 ± 2
Periodate-treated laminin	65 ± 8***	49 ± 10**
Sialidase-treated laminin	50 ± 9**	34 ± 9*
Recombinant B1 chain	75 ± 5***	56 ± 8**

[a] Mean of three determinations ± standard deviation. Values include the significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$) of the comparative decrease in laminin binding as determined by Student's *t* test.

ing *H. pylori* strains must be regarded as a minimum estimate of the affinity of binding of laminin.

Inhibition experiments to evaluate the specificity of ^{125}I-laminin binding to the hemagglutinating and poorly hemagglutinating strains were performed. Preincubation of bacterial cells with unlabelled laminin resulted in inhibition of ^{125}I-laminin binding to CCUG 17874 and CCUG 17875 (Fig. 1D). The observed inhibition was dose dependent and saturable, with maximal inhibition of about 80 and 60% for CCUG 17874 and CCUG 17875, respectively, at 20 pmol of laminin.

Inhibition of laminin binding by *H. pylori* with other proteins. The inhibitory effects of various proteins on binding of ^{125}I-laminin by CCUG 17874 and CCUG 17875 are shown in Table 1. All assays were performed in the presence of 0.1% BSA to minimize any possible contribution of nonspecific protein-protein interactions to quantitation of binding. Preincubation of gamma globulin, collagen type I, and collagen type IV resulted in no significant inhibition ($P > 0.05$) of binding. Fibronectin did induce some inhibition ($P < 0.05$) of binding of both strains. Since the only common chemical property of fibronectin and laminin is that they are glycoproteins, this partial inhibition of laminin binding by fibronectin may reflect the importance of protein glycosylation in binding. Supporting this, highly glycosylated fetuin inhibited ($P < 0.01$) the binding of CCUG 17874 and CCUG 17875 whereas asialofetuin did not significantly inhibit binding ($P > 0.05$). The latter result also indicated that terminal sialic acid is involved in laminin binding in the hemagglutinating and poorly hemagglutinating *H. pylori* strains. However, of the proteins tested, unlabelled laminin produced the greatest and most significant inhibition ($P < 0.001$) of bacterial binding by both strains, thereby confirming the specificity of bacterial binding.

Influence of protein glycosylation on laminin binding by *H. pylori*. Both CCUG 17874 and CCUG 17875 exhibited a significant decrease in binding to modified laminin (Table 2). Bacterial binding was reduced after periodate or sialidase treatment of laminin and supports the importance of carbohydrates, particularly sialic acid residues, in binding to laminin. Consistent with this, binding of CCUG 17874 and CCUG 17875 to the recombinant B1 chain of human laminin, which contains only high-mannose-type oligosaccharides, was greatly decreased (75 and 56% reduction, respectively).

Furthermore, to identify the region in the carbohydrate chain of laminin involved in bacterial binding, constituents of the sugar chains of laminin were tested for their ability to inhibit binding. The monosaccharides L-fucose, D-glucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic (sialic) acid and the disaccharide lactose [galactopyranosyl-β(1-4)-glucopyranose] did not significantly inhibit ($P > 0.05$) the binding of CCUG 17874 and CCUG 17875 (data not shown). In contrast, the trisaccharide *N*-acetylneuraminyllactose (sialyllactose) from bovine colostrum [85% NeuAc(2-3)Lac, 15% NeuAc(2-6)Lac] significantly inhibited laminin binding by CCUG 17874 (80% ± 7%; $P < 0.001$) and by CCUG 17875 (60% ± 6%; $P < 0.01$) compared to controls (0% ± 3% and 0% ± 2%, respectively).

To fully characterize the specificity of carbohydrate recognition in laminin binding, we compared the inhibitory effects of two isomers of sialyllactose, 3-sialyllactose [NeuAc(2-3)Lac] and 6-sialyllactose [NeuAc(2-6)Lac]. 3-Sialyllactose inhibited the binding of laminin by CCUG 17874 and CCUG 17875 in a dose-dependent and saturable manner, with maximal inhibition of 80 and 62%, respectively, at 16 nmol (Fig. 2A). However, 6-sialyllactose did not inhibit laminin binding by either strain (data not shown). Addition of 3-sialyllactose (100 μg) to cells of CCUG 17874 and CCUG 17875 with previously bound ^{125}I-laminin partially displaced the bound laminin in a time-dependent manner (Fig. 2B). The observed maximal displacement of 63 and 32% for CCUG 17874 and CCUG 17875, respectively, was comparable to and not significantly different from ($P > 0.05$) that obtained with unlabelled laminin (Fig. 1C). In contrast, 6-sialyllactose did not induce the displacement of bound ^{125}I-laminin from either strain (data not shown). As shown in Fig. 3, 3-sialyllactose also inhibited the binding of laminin by other hemagglutinating and poorly hemagglutinating *H. pylori* strains (strains 1139, 51, and 52 and strain 66, respectively).

Involvement of a protein adhesin in laminin binding. The binding of laminin was greatly but not completely inhibited by heating cells of CCUG 17874 and CCUG 17875 and also by treating cells with proteolytic enzymes (Table 3). The amounts of inhibition after heating of cells of CCUG 17874 and CCUG 17875 at 80°C for 10 min or treatment with pronase E were similar to those reported previously (63, 65). Although heat and protease treatments induced a lower inhibition of laminin binding by CCUG 17875, the results indicated the involvement of a protein receptor on both hemagglutinating and poorly hemagglutinating strains of *H. pylori* in the binding of laminin.

Preincubation of ^{125}I-laminin with LPS before incubation with heat-treated or enzyme-treated bacteria resulted in complete inhibition of laminin binding to CCUG 17874 and CCUG 17875 (data not shown), thereby confirming the involvement of *H. pylori* LPS with a protein receptor in binding to laminin (65). Furthermore, preincubation of heat-treated or pronase-treated cells with 3-sialyllactose significantly ($P < 0.001$) inhibited bacterial binding (Table 3), but the inhibition was similar

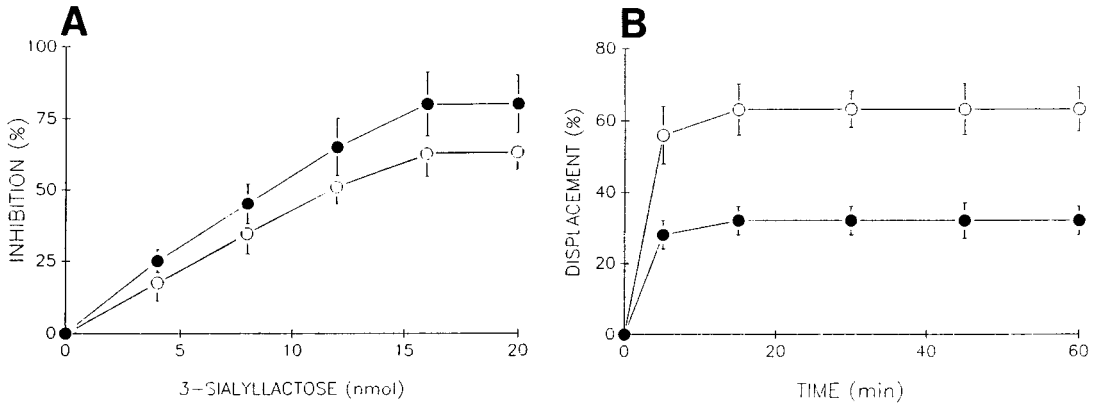

FIG. 2. Inhibition of binding of ^{125}I-laminin (A) and displacement of ^{125}I-laminin bound (B) to *H. pylori* CCUG 17874 (●) and CCUG 17875 (○) with 3-sialyllactose. Data points represent the means of three determinations, and error bars indicate standard deviations.

to but not significantly ($P > 0.1$) different from that induced by heat-treated or pronase-treated cells alone. In contrast, preincubation of ^{125}I-laminin with LPS and preincubation of CCUG 17874 and CCUG 17875 with 3-sialyllactose, both before assessment of bacterial binding, completely inhibited the binding of ^{125}I-laminin by CCUG 17874 and CCUG 17875 (data not shown). Collectively, these results suggest that a protein receptor, rather than LPS, on *H. pylori* binds 3-sialyllactose.

Identification of the protein adhesin by Western blotting. Coomassie blue staining of SDS-PAGE gels of outer membrane protein preparations of *H. pylori* revealed a complex mixture of proteins (Fig. 4A). The availability of antiserum to laminin allowed us to detect the glycoprotein bound to the electrophoretically separated outer membrane proteins of *H. pylori*. Binding assays were performed in the presence of 0.03% Tween 20 to minimize any possible contribution of nonspecific hydrophobic interactions to laminin binding. As shown in Fig. 4B, Western blotting of the outer membrane proteins of the *H. pylori* hemagglutinating strain CCUG 17874 (lane 1) and poorly hemagglutinating strain CCUG 17875 (lane 2) showed laminin binding to a 25-kDa protein. Also, ^{125}I-laminin, identical to that used in liquid-phase binding assays, bound to the 25-kDa protein on nitrocellulose membranes, thereby confirming that radioiodination of the glycoprotein did not cause steric or conformational changes affecting binding. No reactive bands were observed in immunoblots when preimmune sera were used in control experiments (results not shown) or when periodate-treated (Fig. 4C) or sialidase-treated (Fig. 4D) laminin, instead of native laminin, was incubated with the outer membrane proteins. The 25-kDa protein also mediated laminin binding by the other hemagglutinating and poorly hemagglutinating *H. pylori* strains (1139, 51, 52, and 66). Furthermore, laminin binding by the 25-kDa protein in immunoblots of outer membrane proteins of CCUG 17874 and CCUG 17875 was inhibited with 3-sialyllactose (Fig. 4E). However, no inhibition of binding to the 25-kDa protein was observed with 6-sialyllactose, LPS, or any of the other monosaccharides or disaccharides tested above (data not shown), thus confirming the specificity of binding.

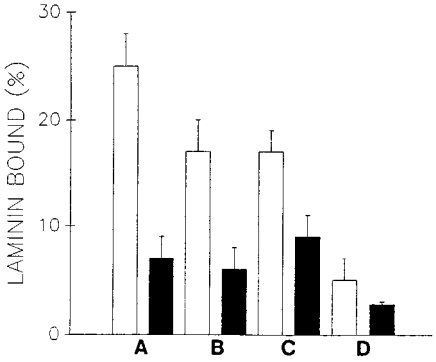

FIG. 3. Effects of sialyllactose on binding of ^{125}I-laminin to *H. pylori* 1139 (A), 51 (B), 52 (C), and 66 (D). Binding of laminin without preincubation with 3-sialyllactose (□) and after incubation with 100 μg of 3-sialyllactose (■) is shown. Data represent the means of three determinations, and error bars indicate standard deviations.

DISCUSSION

The interaction of hemagglutinating *H. pylori* strains with laminin has been investigated previously (62, 63, 65), but characterization of the interaction of poorly hemagglutinating strains has been incomplete. The results of the present study show that binding of laminin by both hemagglutinating and

TABLE 3. Effect of treatment of *H. pylori* CCUG 17874 and CCUG 17875 cells on binding of ^{125}I-laminin

Treatment	% Inhibition of binding of *H. pylori* strain[a]:	
	CCUG 17874	CCUG 17875
None	0 ± 3	0 ± 4
Heat		
20°C for 60 min	0 ± 2	0 ± 3
80°C for 10 min	85 ± 4***	64 ± 8***
Enzymes		
Trypsin	77 ± 5***	54 ± 6***
Proteinase K	58 ± 7**	44 ± 5***
Pronase E	80 ± 6***	58 ± 6***
With 3-sialyllactose		
80°C for 10 min	80 ± 7***	58 ± 4***
Pronase E	85 ± 3***	60 ± 4***

[a] Mean of three determinations ± standard deviation. Values include the significance (**, $P < 0.01$; ***, $P < 0.01$) of the difference between inhibition before and after treatment of bacterial cells as determined by Student's *t* test.

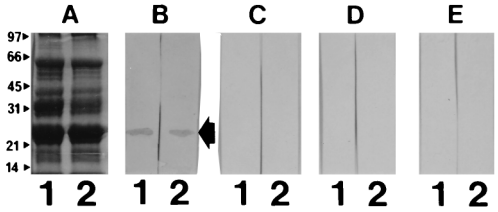

FIG. 4. Analysis of binding of laminin to outer membrane proteins of *H. pylori* CCUG 17874 (lane 1) and CCUG 17875 (lane 2). (A) Coomassie blue-stained SDS-PAGE gel of outer membrane proteins. (B to D) Binding of laminin (B), lack of binding of periodate-treated laminin (C), and lack of binding of sialidase-treated laminin (D) to a 25-kDa outer membrane protein (arrow) as determined by Western blot analysis. (E) Inhibition of binding of laminin to the 25-kDa outer membrane protein after preincubation with 3-sialyllactose. The arrow indicates the position of the protein of 25 kDa that bound laminin. The relative positions of molecular mass markers (in kilodaltons) are indicated on the left. Identical results were obtained with the other hemagglutinating and poorly hemagglutinating *H. pylori* strains, 1139, 51, 52, and 66.

poorly hemagglutinating *H. pylori* strains is rapid, saturable, specific, of high affinity, and insensitive to pH. Although the calculated binding constant of 8.5 pM for the hemagglutinating *H. pylori* CCUG 17874 was consistent with previous reports (62, 63), laminin binding by the poorly hemagglutinating strain CCUG 17875 was of higher affinity (K_d = 4.1 pM). In contrast, however, the available number of sites for laminin per cell is about 2,000 on CCUG 17874 and 1,200 on CCUG 17875, thereby explaining the lesser extent of laminin binding by the latter strain. Despite these differences in affinities, both values were less than the laminin binding constant of 0.5 pM for enteroinvasive *Escherichia coli* (64) but higher than the 2.9 nM reported for *Staphylococcus aureus* (33) and the 40 to 80 nM reported for species of streptococci (58, 59). Nevertheless, the partial irreversibility of binding precluded a completely accurate assessment of binding constants, and the affinities may be higher than the estimates obtained here. Other than the high affinity of the binding interaction, the reasons for the partial irreversibility of laminin binding by *H. pylori* have yet to be defined. Such a property may indicate that more than one interaction is occurring during glycoprotein binding (62) and is consistent with the involvement of both a protein adhesin and LPS in laminin binding by *H. pylori* (65). Furthermore, this conclusion is supported by the incomplete inhibition of bacterial binding with unlabelled laminin, which reflects the involvement of two adhesins with differing affinities and mechanisms of interaction with laminin.

Consistent with previous reports on laminin binding by hemagglutinating *H. pylori* strains (62, 63), we observed that gamma globulin, collagen type I, and collagen type IV did not inhibit laminin binding by hemagglutinating or poorly hemagglutinating *H. pylori* strains. Likewise, these proteins do not displace bound ^{125}I-laminin (41). Laminin possesses about 40 amide-linked oligosaccharides with repeating units of poly-*N*-acetyllactosaminyl side chains attached to the trimannosyl core portion of bi-, tri-, and tetra-antennary complex-type oligosaccharides (1, 17, 27). Inhibition of laminin binding by fibronectin and fetuin but not asialofetuin indicated that glycosylation, particularly sialylation, was important for binding by hemagglutinating and poorly hemagglutinating *H. pylori* strains. This was supported by reduced bacterial binding to modified laminin (periodate or sialidase treated) and to the recombinant B1 chain of human laminin which contains only high-mannose-type oligosaccharides because of the inefficiency of carbohydrate processing by the baculovirus expression system in insect cells (48). Inhibition experiments with monosaccharides, disaccharides, and trisaccharides that are constituents of the sugar chains of laminin showed that the strains bind to a region spanning a trisaccharide. Binding is specific for 3-sialyllactose, not the 6-sialyllactose isomer, as confirmed by inhibition and displacement studies.

Likewise, sialyllactose from bovine colostrum [85% NeuAc(2-3)Lac] but not from human milk [85% NeuAc(2-3)Lac] has been reported to inhibit *H. pylori* hemagglutination (12, 23, 24). Furthermore, hemagglutinins that are specific for sialic acid and inhibitable by fetuin have been demonstrated on *H. pylori* (10, 11). Similarly, *H. pylori* adherence to Y-1 mouse adrenal cells has been reported to be rapid, sialidase sensitive, and blocked by a sialoglycoprotein (11). Although Clyne and Drumm (4) reported no correlation between hemagglutination titers of *H. pylori* strains and their ability to adhere to isolated human gastric epithelial cells, collagenase digestion was used during epithelial cell preparation in their investigation. This would disrupt the extracellular matrix and basement membrane, with loss of laminin and the receptor for sialic acid-specific binding.

Surface proteins are involved in laminin binding by both hemagglutinating and poorly hemagglutinating *H. pylori* strains since protease treatments and heating of bacterial cells reduced binding. These results are consistent with the sensitivity of sialic acid-specific hemagglutination to such treatments and previously reported reductions in the laminin-binding ability of hemagglutinating *H. pylori* strains (12, 62, 63). The reduced binding ability exhibited by bacteria treated at 80°C for 10 min, a temperature which will cause perturbation rather than denaturation of proteins, suggests that a particular protein conformation is critical for binding. However, complete inhibition of laminin binding by both hemagglutinating and poorly hemagglutinating *H. pylori* strains was achieved only when LPS was used as an inhibitor in combination with heating or protease treatments, thereby confirming the involvement of both LPS and a protein adhesin in laminin binding as we had suggested previously (65). Furthermore, inhibition experiments indicated that the protein receptor, rather than LPS, on *H. pylori* binds 3-sialyllactose. The site of interaction of LPS with laminin remains to be identified.

Preliminary investigations suggested that adherence of *H. pylori* to laminin was attributable to a conserved afimbrial 19.6-kDa protein of *H. pylori* with weak hemagglutination activity (7). However, further investigations showed that the protein was a cytosolic iron-binding protein whose binding of laminin was due to nonspecific hydrophobic interactions (8). In the present study, a 25-kDa outer membrane protein that binds laminin was detected by Western blotting. During immunoblotting, conditions were employed to minimize any possible nonspecific hydrophobic interactions (8) and the specificity of binding was confirmed by complete inhibition of binding with 3-sialyllactose. The 25-kDa lectin-like adhesin was present in preparations from hemagglutinating and poorly hemagglutinating strains. In contrast, a 25-kDa outer membrane protein has been previously observed in chemically stained SDS-PAGE gels of some but not all *H. pylori* strains (9). This anomaly may be explained by the higher sensitivity of protein detection by Western blot-type analysis. Although a crude preparation of the protein reported here exhibits hemagglutination activity (41), the relationship of this protein to previously described hemagglutinins (12, 23) requires further investigation, particularly since the biological relevance of the latter hemagglutinins is unclear. For example, a putative adhesin of *H. pylori*, HpaA protein, a 20-kDa subunit protein with hemagglutination activity and binding specificity for the same isomeric form of sialyllactose as the 25-kDa laminin-binding pro-

tein, has been reported by Evans et al. (12, 13). However, HpaA was not detected in our stringently purified outer membrane preparations by SDS-PAGE and Coomassie blue staining. Moreover, O'Toole et al. have shown that HpaA is a lipoprotein located in the cytoplasmic fraction of the cell (45).

Nevertheless, enteroinvasive *E. coli* and uropathogenic *E. coli* bind laminin, and the protein adhesins on the bacterial surface have been characterized (64, 68); the binding specificity of the *H. pylori* 25-kDa protein is similar to that of the hemagglutinating sialylgalactoside-binding fimbriae of uropathogenic *E. coli* and enteroinvasive *E. coli* (25, 36, 46). Also, the erythrocyte receptor for the K99 fimbrial adhesins of enterotoxigenic *E. coli* (55) is the same isomeric form of sialyllactose to which *H. pylori* binds. The hemagglutinin of *Streptococcus suis* recognizes sialylated *N*-acetyllactosaminyl glycopeptides on the surface of erythrocytes (32) and thus differs slightly in specificity from that of the *H. pylori* 25-kDa protein.

The specific laminin receptor on *S. aureus* has been identified as a 52-kDa surface protein (33), but unlike *H. pylori*, the binding site on the laminin molecule remains unidentified. Similarly, the laminin receptor on *Streptococcus pyogenes* has been characterized as a protein of >100 kDa (59). Interaction of *Pseudomonas aeruginosa* has been reported to be mediated by 57- and 59-kDa nonpilus outer membrane proteins (49). In general, the laminin-binding proteins of pathogenic microorganisms vary in molecular size from 32 kDa (*Toxoplasma gondii*) to 145 kDa (*Streptococcus gordonii*) (18, 56), possibly reflecting the occurrence of different classes of laminin-binding proteins with different pathogenic roles.

Although the contributions of laminin binding to the pathogenesis of *S. aureus*, *E. coli*, *Treponema pallidum*, and streptococci have been established (14, 33, 56, 58, 59, 64, 68), the role of adherence to laminin in the pathogenicity of *H. pylori* may not at first be apparent. However, histological data indicate that the gastric epithelium is compromised by the apparent penetration of *H. pylori* at intercellular junctions (21, 57), the integrity of which is maintained through the interaction of cell surface receptors with components of the extracellular matrix, particularly laminin (19, 51). *H. pylori* LPS inhibits the interaction between a laminin receptor on gastric epithelial cells and laminin (54). In addition, the lectin-like adhesin of *H. pylori* recognizes 3-sialyllactose in the oligosaccharides of laminin which are important in cellular adhesion processes (5, 6, 72). Therefore, after attachment of *H. pylori* to laminin, the subsequent disruption of epithelial cell-basement membrane interaction would explain the development and continuance of lost mucosal integrity during *H. pylori* infection (40, 54) and hence explain the development of gastric epithelium leakiness (30). Furthermore, the ability to attach to laminin would assume major importance once an initial lesion in the ulceration process exposed the basement membrane (62). Indeed, *H. pylori* produces cytotoxins and enzymes which may degrade the epithelium, potentially causing cellular destruction (29, 42) and exposure of the underlying basement membrane (15). Even in the absence of *H. pylori*-induced disruption of mucosal integrity, a high rate of cell turnover in the gastric epithelium, feeding, and consumption of exfoliating agents such as ethanol, aspirin, and spicy food can cause loss of gastric epithelial cells and expose the basement membrane (69).

In conclusion, laminin binding by both hemagglutinating and poorly hemagglutinating *H. pylori* strains was characterized and found to be mediated by a 25-kDa outer membrane protein in combination with LPS. The 25-kDa lectin-like adhesin is also one of the few bacterial laminin binding proteins to be identified and whose site of attachment is known. Although the lectin-like adhesin was expressed in both groups of strains, fewer laminin-binding sites were present on poorly hemagglutinating strains. Therefore, further studies are required to investigate the possible relationship between hemagglutination and laminin binding by this protein. In addition, the site of interaction of *H. pylori* LPS with laminin remains to be identified.

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