Identification of a new sialic acid-binding protein in *Helicobacter pylori*

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Abstract

A novel sialic acid-specific lectin has been isolated from *Helicobacter pylori* lysate using fetuin–agarose affinity chromatography followed by cleavage of the α(2,3) and α(2,6) linkages of sialic acids using neuraminidase. The protein had a molecular weight of 17.5 kDa on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and was identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry to be protein of unknown function with gene number HP0721. Recombinant HP0721 was shown to bind to fetuin–agarose and sialic acid-containing glycosphingolipids on thin-layer plates suggesting this protein may represent another sialic acid-specific adhesin of *H. pylori*. A *H. pylori* mutant defective for HP0721 was generated and its ability to bind to human AGS cells assayed.

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1. Introduction

*Helicobacter pylori* is a microaerophilic, Gram-negative bacterium, the causative agent in active chronic gastritis [1,2]. Persistent *H. pylori* infection is associated with the development of gastric and duodenal ulcer disease [3–5] and gastric carcinoma [6]. A prerequisite for *H. pylori* infection is an ability to adhere to host gastric epithelium. A number of putative adhesins have been identified in *H. pylori* and it appears that adhesion may well be a multi-factorial process. One receptor that has been identified as a critical adherence ligand for *H. pylori* are sialylated glycoconjugates or more specifically, α(2,3)-sialyllactose [7–10].

In the case of *H. pylori* strain J99, a sialic acid-binding adhesin, SabA has already been identified [11]. In addition, a sialyllactose-binding haemaglutinin precursor (Hpaa) was identified as an immuno-reactive antigen using antisera from infected patients [12]. Whilst sialic acid is not ubiquitous in healthy stomach tissue, it is detectable in inflamed sites, indicating that interaction with sialic acids in the host may be important in the longer term pathology of *H. pylori* infections [11]. The aims of this study were to identify the candidate sialic acid-binding proteins of *H. pylori* strain 26695. In this study, we describe using fetuin–agarose affinity chromatography followed by neuraminidase treatment to identify a new sialic acid-binding adhesin of *H. pylori*. The protein, HP0721, has previously been shown to be cell surfaced localised using selective biotinylation [13] and to be present in the supernatant fraction [14] both of which are in keeping for a role for HP0721 in mediating bacterial/host interactions.
2. Materials and methods

2.1. Bacterial strains and growth conditions

*Helicobacter* strain 26695 was cultured at 37 °C in a micro-aerobic atmosphere (5% O2, 10% CO2 and 85% N2) on 5% horse blood-columbia agar plates for three days, and grown for one additional day on fresh plates.

2.2. Fetuin–agarose affinity chromatography

*H. pylori* were harvested by centrifugation at 4000g then lysed using 1 ml BugBuster Protein Extraction Reagent (Merck Biosciences, Nottingham, UK) plus 200 μg ml−1 lysozyme at room temperature for 2 h. Nucleic acids were degraded using 1 μg ml−1 DNase and 1 μg ml−1 RNase with incubation for 15 min at room temperature. After removal of intact bacteria by two centrifugations at 6000g for 10 min, the lysate was incubated with 100 μl fetuin–agarose beads (Sigma–Aldrich, Poole, Dorset, UK) overnight at 4 °C. The beads were washed five times with 1 ml Dulbecco’s phosphate-buffered saline (Sigma–Aldrich, Poole, Dorset, UK). To identify the total proteins binding to fetuin, sodium dodecyl sulfate (SDS)-loading buffer (4% SDS, 0.1 M Tris, pH 8.8, 2 mM EDTA, 0.1% bromophenol blue, 20% glycerol and 2% β-mercaptoethanol) was added, and the samples were boiled to release any proteins bound to the fetuin and analysed on SDS–polyacrylamide gel electrophoresis (SDS–PAGE). To identify sialic acid-specific binding proteins, the washed beads were incubated with 1 U neuraminidase type III from *Vibrio cholerae* (Sigma–Aldrich, Poole, Dorset, UK) in 0.1 M NaOAc, pH 5.5 and 164 mM NaCl was added to a final concentration of 700 mM and 1% CTAB with incubation at 65 °C for 10 min. The DNA was extracted twice using phenol:chloroform:isoamyl alcohol (IAA) (25:24:1), once with chloroform:IAA (24:1). The DNA was precipitated with 0.7 volumes of isopropanol–acetate and resuspended in water.

The HP0721 gene was PCR amplified using primers HP0721F 5′AGAATTCATATGAAAAACGGTTGG AAAATAC containing a NdeI site and HP0721R 5′GGACTCGAGTTAGTGCTTATCGGTTGTG containing a XhoI site or HP0721HIR 5′GTACTC GAGGTGCTTATCGGTTGTG with the native stop codon removed and XhoI site. Amplification was performed in a volume of 50 μl containing 1× Taq DNA polymerase buffer, 125 μM each dNTPs, 20 pmol each primer, 2 U Taq DNA polymerase (Roche Molecular Biochemicals, Lewes, East Sussex, UK) and 0.1 μg of chromosomal DNA. The amplification conditions were as follows; initial denaturation of DNA at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 10 min. The generated PCR fragments (460 bp) were digested with NdeI/XhoI and ligated into pET24b/NdeI/XhoI (Merck Biosciences, Nottingham, UK). The resulting plasmids were designated pET0721 and pET0721His, the inserts were confirmed by sequencing.

*Escherichia coli* BL21 (DE3) cells were transformed with the recombinant plasmids. The cells were grown at 37 °C in LB medium containing 50 μg ml−1 kanamycin to an OD600 of 0.6 and HP0721 expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside for 2 h. The cells were harvested by centrifugation at 4000g and then lysed using 5 ml BugBuster Protein Extraction Reagent (Merck Biosciences, Nottingham, UK) per gram of pellet plus 200 μg ml−1 lysozyme at room temperature for 15 min. Nucleic acids were degraded using 1 μg ml−1 DNase and 1 μg ml−1 RNase and incubating for 15 min at room temperature. After removal of intact...
bacteria by two centrifugations at 6000 g for 10 min, the lysate was incubated with 10 μl fetuin–agarose beads (Sigma–Aldrich, Poole, Dorset, UK) overnight at 4 °C. The beads were washed five times with 1 ml Dulbecco’s phosphate-buffered saline. SDS-loading buffer is added and the beads boiled to release any bound proteins and analysed on 15% SDS–PAGE.

Following gel electrophoresis, the proteins were transferred to PVDF membrane and stained with Coo-massie blue. The HP0721 bands were excised from the gel and N-terminal sequencing analysis was performed at the University of Cambridge (UK) under the supervision of Dr. Weldon.

2.5. Site-directed mutagenesis

The oligonucleotides used for site-directed mutagenesis of Leu 18 of HP0721 into Ala are L18AF 5′-GCTATTTGTGGCTGCGAACGCCAAAGATTTC and L18AR 5′-GAATCTTTGGCGTTCGCACCACAAATAGC. Mutagenesis was performed using the Quick-Change™ site-directed mutagenesis kit from Stratagene (La Jolla, CA), according to the manufacturer’s instructions. The presence of the correct mutation was verified by DNA sequencing.

2.6. Thin-layer chromatography overlay assay

TLC was performed essentially as described earlier [17] with a solvent system involving chloroform–methanol–water (60:35:8). Briefly, 2.5 μg pure glycolipids (Merck Biosciences, Nottingham, UK) were separated on high-performance TLC (HPTLC) aluminium-backed silica gel 60 plates (Merck Biosciences, Nottingham, UK). Glycolipids were stained with orcinol spray (Sigma–Aldrich, Poole, Dorset, UK). After 5 min at 37 °C, the trypsin was neutralised by washing in medium. For binding studies, cells were plated in flat-bottom 96-well microtitre plates in RPMI 1640 medium supplemented with 10% FBS. For splitting, monolayers were detached by the use of 0.05% trypsin and 0.53 mM EDTA (Gibco–Invitrogen, Paisley, UK). After 5 min at 37 °C, the trypsin was neutralised by washing in medium. For binding studies, cells were plated in flat-bottom 96-well microtitre plates in 200 μl of culture medium at 5 × 10⁴ cells ml⁻¹. They reached confluence in 2 days. The cells were fixed in phosphate-buffered paraformaldehyde (3.5%) at room temperature for 20 min, followed by incubation with 100 mM glycine in PBS for 10 min and finally washing twice in PBS.

2.7. Construction of a mutant strain of H. pylori

For construction of a mutant strain, the HP0721 gene was amplified by PCR from a chromosomal DNA preparation of H. pylori strain 26695 and the amplified fragment was inserted into pGEM®-T Easy Vector (Promega, Southampton, UK). A kanamycin-resistance gene cassette with transcription termination signal (kan) was inserted into a genetically engineered BamHI site in the centre of the HP0721 gene. The plasmid was used for inactivation of chromosomal genes by natural transformation as previously described [18]. Inactivation of the gene was confirmed by PCR amplification and a protein lysate preparation of the mutant was prepared and analysed on SDS–PAGE to confirm loss of protein.

2.8. Epithelial cells

AGS cell line was derived from a human gastric adenocarcinoma and was obtained from the American Type Culture Collection (ATCC). The cells were maintained in RPMI 1640 medium supplemented with 10% FBS. For splitting, monolayers were detached by the use of 0.05% trypsin and 0.53 mM EDTA (Gibco–Invitrogen, Paisley, UK). After 5 min at 37 °C, the trypsin was neutralised by washing in medium. For binding studies, cells were plated in flat-bottom 96-well microtitre plates in 200 μl of culture medium at 5 × 10⁴ cells ml⁻¹. They reached confluence in 2 days. The cells were fixed in phosphate-buffered paraformaldehyde (3.5%) at room temperature for 20 min, followed by incubation with 100 mM glycine in PBS for 10 min and finally washing twice in PBS.

2.9. Bacterial binding experiments

H. pylori from a one-day-old plate was resuspended in PBS, 1 mM MgCl₂ and 1 mM CaCl₂. The bacteria were adjusted to an OD₆₀₀ of 2.0 and labelled with fluorescein isothiocyanate (FITC) (Sigma–Aldrich, Poole, Dorset, UK) according to the previously published protocol [19]. 1 × 10⁷ bacteria ml⁻¹ were added to fixed confluent monolayers of AGS cells in microtitre wells in 50 μl. The bacteria were incubated with the cell monolayer overnight at 4 °C. Microtitre wells were washed three times with 100 μl PBS, 1 mM MgCl₂ and 1 mM CaCl₂ to remove unbound bacteria. The number of bacteria was quantified using a BIO-TEK Synergy HT plate reader and excitation of the FITC at 485 nm and detection of the FITC fluorescent emission at 525. Inhibition of bacterial binding was measured by pre-incubating the bacteria with 1 mg ml⁻¹ fetuin, 1 mg ml⁻¹ asialofetuin or 2 mg ml⁻¹ α(2,3)-sialyllectose for 30 min at room
temperature with gentle rotation, before addition of the mixture to the fixed epithelial cell monolayers. All experiments were performed in triplicate.

3. Results

3.1. Isolation of a novel sialic acid-binding protein

Sialylated glycolipids have been shown to mediate important pathogen–host interactions in *H. pylori* colonisation [7–12]. To identify sialic acid-specific proteins, we have used fetuin–agarose affinity chromatography followed by the cleavage of α(2,3) and α(2,6) sialic acid linkages by neuraminidase to elute any proteins bound to the terminal sialic acid residues of fetuin. Using this method, a protein with molecular weight of 17.5 kDa was eluted (Fig. 1). This protein was also evident in total proteins that bind to fetuin–agarose (Fig. 2). The protein was identified by MALDI-TOF to be HP0721, a protein of unknown function.

3.2. Production of recombinant HP0721

The next stage was to establish that the HP0721 gene was expressed in *E. coli* and that the HP0721 protein made showed biological properties similar to those of native HP0721 isolated from *H. pylori*. The HP0721 gene was successfully cloned into pET24b vector, to allow the expression of an untagged and C-terminal His-tagged HP0721 in *E. coli* BL21 (DE3). Lysates were made from *E. coli* expressing recombinant untagged and his-tagged HP0721. The recombinant proteins both bound to fetuin–agarose beads but appear to have an increased aberrant molecule weight when analysed by SDS–PAGE as compared to native HP0721 purified from *H. pylori* (Fig. 3). HP0721 protein has a classic signal sequence, which will direct the protein to the outer membrane in *H. pylori*. N-terminal sequencing revealed that the recombinant proteins were not being processed by signal peptidase I in *E. coli*. The sequence MKKALKI was obtained. This may be due to a subtle difference in the amino acid requirement for cleavage between *H. pylori* and *E. coli*. Both pathways require a short, positively charged amino-terminal region (n-region); a central hydrophobic region (h-region); and a more polar carboxy-terminal region (c-region) containing the site that is cleaved by the signal peptidase [20]. In *H. pylori* around 60% of outer-membrane proteins have a leucine residue at position −3 relative to cleavage (including HP0721); this is in contrast to most other Gram-negative bacterial signal sequences, where alanine is found 10 times more often than leucine [21]. With this information, a HP0721 construct was prepared that has the leucine in position −3 mutated to alanine. The L18A
mutation appeared to increase overall yield of uncleaved protein and also appeared to generate some cleaved product (Fig. 4), this product runs at a similar molecular weight to the *H. pylori* protein.

3.3. Glycolipid-binding specificity of His-tagged recombinant HP0721 protein

The ability of His-tagged recombinant HP0721 to bind to purified gangliosides on TLC plates was assessed (Table 1). Recombinant His-tagged HP0721 bound strongly to GD1α, and GM3 and showed moderate binding to GM1. There was only weak binding to asialo-GM1 (Fig. 5). These results indicate that carbohydrate sequences linked to GM1, GM3 and GD1α carry the epitope required as a receptor for HP0721 and that HP0721 may have preference for terminal sialic acid residues as opposed to branched sialic acid residues. These data along with the fact that recombinant HP0721 both untagged and His-tagged proteins bound to fetuin–agarose may imply that HP0721 is a sialic acid-specific adhesin.

| Glycolipids screened for the binding of His-tagged HP0721 protein on thin-layer chromatograms |
|-----------------------------------------------|-----------------------------------------------|
| Name                          | Structure                                      | Binding |
| Asiago-GM1                    | Galβ(1→3)GalNAcβ(1→4)Galβ(1→4Glcβ(1→1Cer     | +       |
| GM1                           | Galβ(1→3)GalNAcβ(1→4 NeuAcα2-3)Galβ(1→4Glcβ(1→1Cer | +++     |
| GD1α                          | NeuAcα2→3Galβ(1→3 GalNAcβ(1→4 NeuAcα2-3)Galβ(1→4Glcβ(1→1Cer | ++++    |
| GM3                           | NeuAcα2→3Galβ(1→4Glcβ(1→1Cer                  | ++++    |

*a* Binding to glycolipids separated on HPTLC is graded as follows: ++++, strong binding; +++, good binding; ++, moderate binding and +, weak binding.

3.4. Inhibition of *H. pylori* binding to epithelial cells

Inhibition of wild-type *H. pylori* strain 26695 and a ΔHP0721 mutant binding to AGS epithelial cells was measured by pre-incubating the bacteria with test compounds including α(2,3)-sialyllactose and fetuin (Sigma–Aldrich, Poole, Dorset, UK). The wild-type 26695 strain bound to AGS cells and this binding was shown to be inhibited by both fetuin and to a greater degree to α(2,3)-sialyllactose (Fig. 6). The HP0721 mutant also bound to AGS cells and was inhibited by fetuin and α(2,3)-sialyllactose in the same manner as wild-type 26695.

4. Discussion

In this study, we have identified a potential sialic acid-binding protein using fetuin–agarose affinity chromatography followed by cleavage of the α(2,3) and
(2,6) linkages of sialic acids using neuraminidase. This protein was identified by MALDI-TOF as a protein with unknown function with gene number HP0721. The protein has a typical signal sequence but has no obvious transmembrane β-sheet domains although it has previously been shown to be cell surface localised using selective biotinylation [12]. The protein does possess an amphipathic α-helix, which it could use to anchor itself on the outer membrane, or to form stable interactions with other surface localised proteins. In the primary amino acid sequence of the protein, a hexapeptide motif can be found that is shared by other well-characterised bacterial sialic acid-binding protein, including K99, CT-B and CFA-I. This motif consists of a region of at least three basic residues within a seven amino acid stretch (KKxxxK), which previous reports have shown that if these residues were mutated in K99 to neutral residues, binding to sialic acid was abolished [22–24]. This region could potentially form part of the receptor-binding domain in the HP0721 protein. However, analysis of outer-membrane proteins of H. pylori shows that there is a high propensity for this sequence (Bennett and Roberts, unpublished results) and this probably reflects the basic nature of many H. pylori proteins. As such, one must be cautious in interpreting this observation.

The recombinant HP0721 protein has been expressed in E. coli, to produce untagged and His-tagged forms. The proteins migrated aberrantly on SDS–PAGE when compared to native HP0721 from H. pylori. N-terminal sequencing has revealed that the signal sequence of recombinant HP0721 is not being processed and this contributes to its aberrant mobility on SDS–PAGE. Site-directed mutagenesis of the leucine residue in position -3 to the predicted cleavage site to an alanine has been successful and expression of this mutated gene resulted in greater production of unprocessed HP0721 protein and a band at a molecular weight of processed product was visualised. This may suggest that in order for H. pylori proteins that possess a leucine in the -3 position to be successfully expressed, correctly processed and be directed to the outer membrane in E. coli, the leucine should be mutated to an alanine residue.

Both His-tagged and untagged recombinant HP0721 bind to fetuin–agarose, and His-tagged HP0721 appeared to bind preferentially to sialylated structures on TLC overlay experiments. This suggests that HP0721 is able to bind to sialic acid residues, and based on the increased binding to GD1a ganglioside, preferentially to terminal sialic acid residues. The binding of strain 26695 was inhibited by the addition of fetuin and α(2,3)-sialyllactose (Fig. 6) indicating that interactions involving sialic residues on the surface of AGS cells and receptors on the surface of H. pylori are important in mediating this interaction. The lack of a functional
SabA adhesin in strain 26695 [11] would indicate that there must be other sialic acid-binding proteins on the surface of strain 26695. The observation that the ΔHP0721 mutant showed a slight reduction in binding to AGS cells as compared to the wild-type strain 26695 in keeping with HP0721 functioning as an adhesin. However, the binding of the ΔHP0721 mutant was still inhibited by fetuin and α(2,3)-sialyllactose to the same extent as wild-type 26695 indicating that other sialic acid-binding proteins are likely to exist on the surface of strain 26695 in addition to HP0721. As such, it is likely that interactions between H. pylori and host cells are likely to be multifactorial with a number of cell surface proteins capable of mediating microbe/host interactions. The fact that sialic acid residues are scant in healthy stomach tissue but increase during inflammation [11], would suggest that interactions with sialic acid may be important in the longer term survival and the maintenance of a chronic state. Indeed, it is possible that sialic acid-binding proteins such as HP0721 may have less to do with mediating high affinity adhesion, but more to do with sequestering free sialic acid residues that will build up at sites of inflammation. The binding of sialic acid may be the first step in the use of sialic acid as a source of nutrients or as it may be to moderate the local inflammatory response. Experiments are in progress to further establish the role of HP0721 in the pathology of H. pylori infections.

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