Presence of terminal \(N\)-acetylgalactosamine\(\beta1-4N\)-acetylglucosamine residues on \(O\)-linked oligosaccharides from gastric MUC5AC: Involvement in *Helicobacter pylori* colonization?

**Diarmuid T Kenny**\(^2,3\), **Emma C Skoog**\(^3,4\), **Sara K Lindén**\(^3,4\), **Weston B Struwe**\(^5\), **Pauline M Rudd**\(^5\), and **Niclas G Karlsson**\(^1,3\)

\(^2\)School of Chemistry, National University of Ireland, Galway, Ireland; \(^3\)Medical Biochemistry, University of Gothenburg, PO Box 440, 405 30 Gothenburg, Sweden; \(^4\)Mucosal Immunobiology and Vaccine Center, Sahlgrenska Academy, University of Gothenburg, 405 30 Gothenburg, Sweden; and \(^5\)National Institute for Bioprocessing Research and Training, University College Dublin, Dublin, Ireland

Received on October 21, 2011; revised on March 5, 2012; accepted on April 21, 2012

Isolation of MUC5AC mucins from the gastric mucosa from two secretor individuals (one from normal mucosa from a patient with gastric cancer and one from a control) showed different abilities to bind and induce the proliferation of the *Helicobacter pylori* strain J99. Analysis of the released \(O\)-linked oligosaccharides by LC-MS from these individuals showed a very heterogeneous mixture of species from the cancer patient containing both neutral and sialylated structures, whereas the normal sample showed dominating neutral blood group H terminating structures as well as neutral structures containing the di-\(N\)-acetyllactosamine (lacdiNAc) unit GalNAc\(\beta1-4\)GlcNAc\(\beta1\)- on the C-6 branch of the reducing end GalNAc. The linkage configuration of these epitopes were determined using C-4-specific fragmentation for the GalNAc\(\beta1-4\)GlcNAc\(\beta1\)- glycosidic linkage, comparison of the MS\(^3\) fragmentation with standards for linkage configuration and \(N\)-acetylhexasosamine type as well as exoglycosidase treatment. It was also shown that the lacdiNAc epitope is present in both human and porcine gastric mucins, indicating that this is an epitope preserved between species. We hypothesize that the termination on gastric MUC5AC with lacdiNAc is in competition with complex glycosylation such as the \(Le^b\) and H type 1 as well as complex sialylated structures. These are epitopes known to bind the *H. pylori* BabA and SabA adhesins.

**Keywords:** BabA / gastric cancer / *Helicobacter* / lacdiNAc / mucin / SabA

**Introduction**

The gastro intestinal tract is covered in a semi-permeable mucus layer that primarily consists of secreted mucins. This layer serves to protect the gastric epithelial cells from potential damage that could occur due to mechanical and chemical stress or enzymatic activity within the tract. The mucus layer is composed of oligomeric mucin glycoproteins that serve as a scaffold for an abundance of anti-microbial epitopes (McGuckin et al. 2011). The mucin layer can also act as a functional binding site for certain pathogens such as *H. pylori*. *H. pylori* are a spiral-shaped gram-negative bacteria that colonizes the mucus layer covering the gastric epithelium. The bacteria were first isolated and cultured from the gastric mucosa by Marshall and Warren in 1983 and their discovery revealed the *H. pylori*’s association with gastritis, peptic ulcers and several forms of gastric cancer (Marshall and Warren 1984). *H. pylori* infection is the most common bacterial infection worldwide and it is estimated to affect over 50% of the world’s population (Kobayashi et al. 2009). Infection usually begins in early childhood and infection and its associated symptoms such as chronic gastritis and peptic ulcers can persist throughout an individual’s life. *H. pylori* infection can dramatically increase the risk of gastric cancer and has been attributed as a key mitigating factor for up to 65% of gastric cancers, equivalent to 5.5% of all cancers globally (Menaker et al. 2004).

Gastric mucins contain highly diverse carbohydrates that can be utilized by *H. pylori* for adhesion. The attachment of *H. pylori* is mediated by outer membrane proteins (OMPs) with lectin-like properties. The adhesion of *H. pylori* to the gastric surface is mediated by both the presence of these adhesion OMPs on the surface of the *H. pylori* cell and the presence of particular glyco-epitopes on the mucin. Of the OMPs that are involved in attachment, two have been identified as particularly important for the attachment of *H. pylori* to the mucosal layer: blood group antigen-binding adhesin (BabA) and the sialic acid-binding adhesin (SabA). BabA recognizes
the Le\(^b\) and H type-1 epitopes (Ilver et al. 1998) and SabA recognizes sialylated oligosaccharides (Mahdavi et al. 2002).

In the adult stomach, the primary secreted mucins on the gastric wall are MUC5AC and MUC6, which harbor a vast number of different glyco-structures which are predominantly neutral in healthy tissue (Nordman et al. 2002; McGuckin et al. 2011). Therefore, BabA-mediated binding to MUC5AC via Le\(^b\) containing mucin structures is the dominating mode of adhesion (Lindén et al. 2002; Styer et al. 2010). During the course of \textit{H. pylori} infection, inflammation and cancer development, the mucin glycosylation can change, displaying more sialylated and sulfated structures on the mucins (Sakamoto et al. 1989; Cooke et al. 2009). Binding through SabA, therefore, also occur after colonization and subsequent inflammation (Mahdavi et al. 2002). The glands of the gastric mucosa have also been shown to express the oligosaccharide epitope GlcNAco1-4Gal. The presence of this epitope has been indicated to suppress the growth of \textit{H. pylori} (Kawakubo et al. 2004). In contrast, binding to MUC5AC increase \textit{H. pylori} proliferation (Skoog et al. 2011).

We have previously used MUC5AC and MUC6 isolated from the stomachs with and without cancer to investigate the relationship between the mucin glycosylation and \textit{H. pylori} binding and proliferation (Skoog et al. 2011). Binding assays using a wild-type J99 strain of \textit{H. pylori} was used to assess BabA mediated for binding to mucins from the healthy and cancerous stomachs. From this sample set, we identified one interesting characteristic secretor sample that neither bound \textit{H. pylori} nor promoted the proliferation of the bacteria. We were interested in identifying key glyco-epitopes that were present on this healthy tissue differentiating it from the normal tissue from patients with gastric cancer. By identifying key glycosylation characteristics, it may be possible to use this information to predict an individual susceptibility to \textit{H. pylori} infection and thus develop preventative measures to avoid chronic infection.

### Results

We compared the glycosylation of MUC5AC from two secretor individuals, which interacted differently with \textit{H. pylori}. One human gastric mucin (HGM) sample (HGM-1, MUC5AC isolated from the normal mucosa of a patient with gastric cancer) was found to bind to, and promote, the proliferation of the \textit{H. pylori} strain J99 in a BabA/Le\(^b\)-dependent manner, whereas the other sample (HGM-2, MUC5AC isolated from a healthy individual) was negative for both characteristics and did not have any apparent Le\(^b\) activity, despite expressing blood group H (Figure 1 and Tables I and II). We aimed to identify particular features of the glycosylation of HGM-2 that could explain this negative behavior.

#### LC-MS analysis of mucin oligosaccharides

To look at the structural differences between both samples, the O-linked oligosaccharides from MUC5AC isolated from stomach biopsies were released by reductive β-elimination analyzed by LC-MS. The two MS spectra were shown to be very different (Figure 2). The MS spectrum showed that the glycosylation of HGM-1 (Figure 2A) was very heterogeneous, dominated by neutral and sialylated structures containing both Lewis-type fucosylation and blood group A, B and H epitopes (Supplementary data, Table SI). This is contrasted to the HGM-2 glycosylation (Figure 2B) that was dominated by a small number of neutral structures and low levels of sialylation. The LC-MS\(^2\) analysis of this samples verified that this was indeed a secretor individual, i.e. the [M-H]- ions of m/z 530, 733 and 1041 gave LC-MS\(^2\) spectra identical to the MS\(^2\) spectra of blood group H containing structures reported in the MS\(^2\) database UniCarb-DB (Hayes et al. 2011; Table II). The spectra showed confirmative A-fragment ions for blood group H type 2 and H type 3 (Karlsson et al. 2004) and LC-MS retention times (RTs) consistent with the biological standards of the assigned blood group H structures. In addition to these structure, we also found a prominent signal from an [M–H]- ion of m/z 936 corresponding to a composition of three HexNAc residues, one Hex residue and one Fuc residue. In HGM-2, this composition was mainly made up of one dominating isomer as shown by the selected ion chromatogram (SIC) showing a peak at RT 24.1 min corresponding to 95% of the signal from the m/z 936 positive peaks throughout the SIC. This isomer was also present in the HGM-1 sample but it only corresponded to 30% of the isomers. With a

Fig. 1. Immunodetection of Le\(^b\) and terminal GalNAc on gastric MUC5AC. MUC5AC containing HGM-1 and HGM-2 was blotted onto PVDF membranes and probed with biotinylated lectin from \textit{W. floribunda} and antibody against Le\(^b\). Bovine fetuin used as a negative control showed no staining with either of the probes. Table I shows the characteristics of the mucins and their effect on \textit{H. pylori} strain J99 as published (Skoog et al. 2011).
differential expression of these isomers between the samples and with the high overall abundance of the RT 24.1 min isomer in the HGM-2 sample that did not bind to *H. pylori* J99 or promote its proliferation, we decided to further investigate the m/z 936 isomer corresponding to this composition, considering that it was unusually high in HexNAc residues compared with most O-linked oligosaccharides.

**Identification of the di-N-acetyllactosamine epitope on human gastric MUC5AC**

In order to further investigate the features of the HGM-2 glycosylation and in particular the abundant isomer found to be differentially expressed, LC-MS² was performed and the sequences interpreted. The LC-MS² spectrum of the RT 24.1 min m/z 936 isomer is shown in Figure 3A and the MS² of this structure showed the dominating Z fragment of m/z 610 and a lower intense ion corresponding to the Y fragment of m/z 628. An intense m/z 610 fragment ion in the MS² spectrum was also detected for the less intense [M-H]⁻ ion of m/z 790 in the MS spectrum of oligosaccharides released from HGM-2 (data not shown). It has previously been shown that the loss of the C-3 extension of the reducing end GalNAc of provides the most prominent fragmentation ion (Karlsson et al. 2004). In the case of the m/z 936 isomer shown in Figure 3, this loss to the fragment ion m/z 610 corresponds to the mass of a Fuc (m/z 146) and a Hex (m/z 162) residue. The low intense Y fragment ion of m/z 790 in Figure 3A showed that the C-3 branch of the GalNAc was arranged in a sequence corresponding to the blood group H sequence Fucα1-2Galβ1-3. The remaining oligosaccharide after the loss of blood group H type provided a composition corresponding to two HexNAcs and the reducing end GalNAc. In conclusion, the analysis of the MS² spectrum indicated that the overall structure has a core 2 type sequence Galβ1-3(GlcNAcβ1-6)GalNAc that is terminating on the C-3 branch with a Fuc residue giving a blood group H type 3 sequence (Fucα1-2Galβ1-3GlcNAc) and the C-6 branch terminating with the addition of a HexNAc residue on the core 2 GlcNAc. Further insight into the nature of this terminating residue was provided by cross ring O-A fragments of the core 2 GlcNAc residue, where extension to the C-4 provides a diagnostic ion of m/z 304 after loss of water, whereas the extension of C-3 does not give this fragment (Karlsson et al. 2004). This shows that the structure is Fucα1-2Galβ1-3 (HexNAc1-4GlcNAβ1-6)GalNAc. The 1-4 linkage was also

### Table I. Characteristics of isolated HGMs

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Tissue status</th>
<th>Mucin type</th>
<th>Le⁺</th>
<th>Blood group</th>
<th>Helicobacter pylori</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGM-1</td>
<td>Normal tissue, cancer patient</td>
<td>MUC5AC</td>
<td>Trace</td>
<td>AB</td>
<td>+</td>
</tr>
<tr>
<td>HGM-2</td>
<td>Normal tissue, healthy patient</td>
<td>Le⁺</td>
<td>Trace</td>
<td>O,H</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table II. Structures identified on the HGM-2 samples neither binding to nor inducing the proliferation of the *H. pylori* strain J99

<table>
<thead>
<tr>
<th>Structure</th>
<th>masel⁻</th>
</tr>
</thead>
</table>
| Fucα1-2Galβ1-3 | 530 | 530-
| Fucα1-2Galβ1-3 | 733 | 733-
| Fucα1-2Galβ1-3 | 790 | 790-
| Fucα1-2Galβ1-3 | 895 | 895-
| Fucα1-2Galβ1-3 | 936 | 936-
| Fucα1-2Galβ1-3 | 1041 | 1041-
| Fucα1-2Galβ1-3 | 1186 | 1186-
| Fucα1-2Galβ1-3 | 1406 | 1406-
| Fucα1-2Galβ1-3 | 1552 | 1552-

---

"Sequences identified by comparison of MS² spectra from UniCarb-DB (www.unicarb-DB.org). Linkage configuration and positions are based on core 1, 2 and 3 series of extension. Type 2 configuration Galβ1-4GlcNAcβ1-3 is based on the detection of the C-4 fragment. Terminal epitope GlcNAcβ1-4GlcNAcβ1-3 is assumed based on evidence in this report and the presence of the α1-3 fragment ion of m/z 304 in HexNAc-HexNAc sequences confirming the linkage position. Fuc configuration is assumed to be α1-2 if present in a blood group H sequence and α1-3/4 if linked to GlcNAc. GalNAcβ1-3 and Galα1-3 is assumed in blood group AB sequences. Hex always assumed to be Gal and HexNAc to be GlcNAc except in blood group A. Reducing end HexNAc-ol assumed to be GalNAc-ol. Bolded and underlined sequences correspond to C-6 terminating lacdiNAc."
Fig. 2. LC-MS of oligosaccharides released from human gastric Muc5AC. Oligosaccharides detected as [M–H]− ions between 10 and 30 min from HGM-1 (non-tumor tissue from gastric cancer patient) (A) and HGM-2 (normal tissue) (B), from two secretor individuals. Oligosaccharide composition is labeled as [HexNAc, Hex, Fuc, NeuAc]. Inserted is the SIC of m/z 936, where the lacdiNAc containing component Fucα1-2Galβ1-3(GalNAcβ1-4GlcNAcβ1-6)GalNAcol eluting at 24.1 min is indicated (black). The confirmation of the lacdiNAc epitope as described in the text and in Figures 3 and 4. Other isomers detected in the SIC are labeled with RT. These peaks are also numbered, with the number corresponding to their respective structures from Supplementary data, Table SI. Unlabelled peaks correspond to signals from non-isomeric oligosaccharides.
Confirmed using MS² fragmentation after permethylation (data not shown).

Having identified that HGM can be terminated by a di-HexNAc epitope, we set out to uncover the identity of the terminal 4-linked HexNAc of the structure. Further investigations, using both structural databases and literature, showed that certain structural motifs with two HexNACs have been identified in the gastric system. These included the GlcNAcβ1-4GlcNAc chitobiose (Newman and Kabat 1976) and the GalNAcβ1-4GlcNAc di-N-acetyllactosamine (lacdiNAc) motifs (Ikehara et al. 2006). An MS³ approach was adopted, whereby the fragmentation pattern of the known standards containing

![Diagram](image-url)

**Fig. 3.** Identification of the lacdiNAc sequence by MS³. The LC-MS² fragmentation of the component Fucα1-2Galβ1-3(GalNAcβ1-4GlcNAcβ1-6)GalNAcβ in Figure 1 to identify the C-6 branch of the GalNAcol (Z₁β fragment ion of m/z 610) and the linkage position of the GalNAcβ1-4GlcNAcβ1- moiety (α₂A₉c-H₂O C-4-specific fragment ion of m/z 304) (A). (B) The MS³ fragmentation of the α₂A₉c-H₂O ion is seen containing the GalNAcβ1-4 moiety plus part of the cleaved GlcNAc. Inserts show the MS³ fragmentation of the α₂A₉c-H₂O fragment ion of m/z 304 isolated from GalNAcβ1-4Gal (left) and GlcNAcβ1-4GlcNAcβ1-4GlcNAc, showing with the R² values that the linkage of configuration of the sample correspond to the GalNAcβ1-4 linkage of the standard. Confirmation of the β-linkage of the lacdiNAc moiety as described in Figure 4. Key for symbols, see Figure 2.
GlcNAcβ1-4 and GalNAcβ1-4 was compared with the fragmentation of the di-HexNAc structure identified in the dominating m/z 936 isomer. The cross ring 0.2A fragment with an m/z 304 characteristic for the C-4 extension of the core 2 GlcNAc (Figure 3B) was selected for MS3 fragmentation for both the sample and the standards. This particular fragment retained the full terminal HexNAc as well as the linkage position to the second outermost monosaccharide residue on the reducing end. Figure 3B shows the spectra for the MS3 of HGM-2 with the m/z 936 parent and also the subsequent collision of the daughter ion m/z 304 and the MS3 spectra of the daughter ion m/z 304 after the collision of the [M–H]⁻ ion for both standards. Correlation of the MS3 fragments and their intensities from the gastric sample with the standard showed that the standard oligosaccharide with the β1-4 GlcNAc had an R² value of 0.49, whereas the β1-4 GalNAc had an R² value of 0.95. These data confirm that the di-HexNAc terminal epitope is terminating in a terminal 4-linked GalNAc. However, due to the loss of the anomic configuration in the 0.2A–H₂O fragment ion (Doohan et al. 2011), the lacdiNAc β-configuration needed to be confirmed using exoglycosidase digestion (see below). We could also verify that both HGM-1 and HGM-2 contained terminal GalNAc residues, using the lectin from Wisteria floribunda (Table I), previously used to detect lacdiNAc epitopes in HGMs. The amount of lectin binding to the HGM-2 was higher compared with HGM-1 as indicated also in the LC-MS chromatogram (Figure 2) of the lacdiNAc type oligosaccharides.

In order to confirm that the linkage configuration of the terminal GalNAc, we used exo-N-acetyhexosaminidase digestion. With limitations on the amount of HGM available for analysis, we resorted using porcine gastric mucins (PGMs) as an alternative as we could identify the identical structure with an [M–H]⁻ ion of m/z 936 and RT 24.1 min (Supplementary data, Figures S1 and S2). Extensive investigation of exoglycosidase digestions showed that the structure was unaffected by β-hexosaminidases that remove terminal β2,4- and 6-linked GlcNAc (Saccharomyces cerevisiae) and α-N-acetylgalactosaminidase (Figure 4). Treatment with β-N-acetyhexosaminidase from jack bean (JBH), which removes both β-linked GlcNAc and GalNAc residues, showed that a structure with a terminal β1-6-linked GlcNAc was significantly digested within hours after initiating the digestion, whereas the [M–H]⁻ ion of m/z 936 isomer containing the 4-linked GalNAc required extended digestion, consistent with a β1-4 configuration (Figure 4). The preferred removal of 6-linked over to 4-linked N-acetylhexosamine has been reported for this enzyme (Peracaula et al. 2003) and confirms, together with the evidence above, that the m/z 936 isomer contains a lacdiNAc moiety. Increased amount of the product Fucα1-2Galβ1-3GalNAcol was detected in the LC-MS after...
the treatment having removed both N-acetylhexasamines of the C-6 branch on the GalNAcol (data not shown). The oligosaccharides from HGM-2 was also subjected to extended JBH treatment, and the lacdiNAc containing structure from HGM-2 with the [M–H]− ion of m/z 936 was also digested completely (Figure 4), as was its unfucosylated structure [M–H]− ion of m/z 790. These data confirm that the structure described in Figure 3 is indeed Fucε1-2Galβ1-3(GalNAcβ1-4GlcNAcβ1-6)GalNAcol and is present in both human and pig gastric mucins.

**Discussion**

It has been shown that a GalNAc transferase (β1,4-N-acetylgalactosaminytransfase III) is present in human stomach capable of making the lacdiNAc epitope, with a specificity toward the core 2 type structures of O-linked oligosaccharides. The data from staining with the lectin from *W. floribunda*, binding to terminal GalNAc, indicated in the previous report that lacdiNAc is found on a secreted mucin present on the gastric mucosal lining (Ikehara et al. 2006). We believe that the identified lacdiNAc structures on MUC5AC detected in this report are the biological products from this enzyme. LacdiNAc as a structure is a rare modification in mammals and is most frequently described in N-linked oligosaccharides (Green et al. 1985), but more recently it has also been identified on O-linked oligosaccharides from zona pellucida 3 (Dell et al. 2003). The only structural difference between lacdiNAc and lacNAc (Galβ1-4GlcNAcβ1-) is the exchange of a C-2–OH to C-2–NHAc on the outermost Gal residue. Although this looks like a minor difference, the consequences in the biosynthesis are dramatic. Whereas the lacNAc motif is used for the elongation of the oligosaccharides as well as decoration with fucose, sialic acid and sulfate residues, the lacdiNAc epitope appears to be a terminal motif resulting in fewer complex structures with limited decoration, possibly only with sulfate or sialic acid (Green et al. 1985; Marino et al. 2011). This novel terminal epitope may provide additional functionality into the interplay between gastric mucosa and gastric bacteria, where other terminal gastric epitopes such as the Leb structure and sialyl Lea being the target for *H. pylori* binding via BabA (Ilver et al. 1998) or SabA (Mahdavi et al. 2002) and the GlcNAcα1- epitope (Kawakubo et al. 2004) that has been shown to have antibacterial properties. Additional lectin adhesins have been indicated to be present in the *H. pylori* genome, in the phylogenetic tree of OMPs containing BabA, BabB, BabC and SabA (Alm et al. 2000). Additional function and specificity of members of this family is unknown. Interaction with an epitope such as lacdiNAc is complementary to the Leb epitope, since it would not be secretor-dependent and is present in the normal gastric tissue, while binding to SabA is dependent on the induction of sialylation due to *H. pylori*-induced gastritis. The significance of the presence of the lacdiNAc epitope on the gastric mucosal layer will need further investigation. The data in this report indicate that it may be involved in suppressing the binding to and the proliferation of *H. pylori*. This could either be via an active interaction of the epitope and the bacteria or an indirect effect, where the termination of lacdiNAc influence the expression of Leb and complex sialylated structures that are known to influence *H. pylori* bacterium’s ability to establish and thrive in the gastric area.

**Materials and methods**

All materials were obtained from Sigma Aldrich (St Louis, MO) unless otherwise stated. The 18-mΩ water was produced using the MilliQ water purification system (Millipore, Billerica, MA).

**Biological sample preparation**

Oligosaccharides from PGM for hexosaminidase digestion and LC-MS were obtained from Sigma Aldrich or prepared from the antrum of the pig stomach as per the method described earlier (Nordman et al. 1998). Human gastric specimens were obtained after informed consent and the approval of local ethics committee (Lund University Hospital, Lund, Sweden). The mucin sample HGM-1 was isolated from the antrum of the normal (normal as determined by a clinical pathologist) part of the stomach of a patient with gastric cancer, and HGM-2 was isolated from the junction between antrum and corpus from a patient undergoing obesity surgery. Frozen specimens were thawed in the presence of di-isopropyl phosphorofluoridate. Briefly, mucins were extracted in 6 M guanidinium chloride/5 mM Na2EDTA/5 mM N-ethylmaleimide/10 mM sodium phosphate buffer, pH 6.5, and purified by density gradient centrifugation in CsCl/4 M guanidinium chloride as per the method described previously (Lindén et al. 2004). Samples were confirmed to contain MUC5AC with only small contribution of MUC6 (Skoog et al. 2011).

**Binding and proliferation of *H. pylori* J99 to HGM**

Binding of the *H. pylori* strain J99 to mucins was investigated by a microtiter-based assay. The 96-well polysorb plates (NUNC A/S, Roskilde, Denmark) were coated with the isolated HGM diluted in 4 M guanidinium hydrochloride. The microtiter plates were washed three times in washing buffer between all ensuing steps. Unbound sites were blocked with a blocking reagent (Roche, Basel, Switzerland) containing 0.05% Tween. Biotinylated *H. pylori* J99 was added to the mucins and incubated in a bacterial shaker at 37°C for 2 h. The plates were washed three times and incubated for 1 h at room temperature with horseradish peroxidase-conjugated streptavidin. After further washings, the tetramethylbenzidine substrate was added. The reaction was stopped after 25 min with H2SO4 and the plates were read using a microplate reader at 450 nm.

The proliferation of *H. pylori* J99 when cultured on HGM-1 and HGM-2 was tested over a 60-h period of time. J99 was cultured in 96-well plates with either HGM-1 or HGM-2 in brain heart infusion broth supplemented with 10% fetal bovine serum. OD560 measurements were recorded at regular intervals over a 60-h period. Any significant differences in the proliferation level compared with the J99 proliferation in the absence of mucins after the 60-h incubation period were recorded. Significantly lower proliferation compared with the control was considered as a negative result.
Release of O-linked oligosaccharides for LC-MS and N-acetylhexosaminidase digestion

The glyco-variant of MUC5AC isolated as HGM-1, HGM-2 and PGMs was treated with 0.1 U of a JBH (Prozyme, Hayward, CA), β-N-acetylgalactosaminidase (Prozyme from S. pneumoniae) or α-L-acetylgalactosaminidase from Clostridium perfringens (R&D Systems, Minneapolis, MN) in 10 μL of the enzymes buffer. The reaction was incubated at 37°C for 1 h (or 24 h for JBH) and stopped by the addition of 1.0 M HCl. Digested and non-digested oligosaccharides were cleaned with 0.6 mL of HyperCarb® PGC (ThermoFisher Scientific, Waltham, MA) packed in C18 zip tips (Millipore). Borate complexes were removed by repeated exchange beads (Bio-Rad, Hercules, CA) packed in C18 zip tips (Millipore). The released oligosaccharides were dissolved in water for introduction to the LC-MS.

LC-MS and LC-MS\textsuperscript{n} by CID of released oligosaccharides from HGM and PGM

The isolated MUC5AC oligosaccharides were analyzed by LC-MS and LC-MS\textsuperscript{n} using a 10 cm × 250 μm I.D. column containing 5-μm porous graphitized carbon (PGC) particles (Thermo Scientific, Waltham, MA) prepared in-house (Kenny et al. 2011). Oligosaccharides were eluted using a linear gradient from 0-40% acetonitrile over 40 min at a flow rate of 10 μL/min. The eluted oligosaccharides were detected in an ESI-IT MS (LTQ, Thermo Electron Corp., San Jose, CA) in the negative ion mode with a spray voltage of 3.5 kV. Air was used as a sheath gas and mass ranges were defined as per the specific structure to be analyzed. Specific ions were isolated for MS\textsuperscript{2} fragmentation by CID with the collision energy set to 30%. The data were manually interpreted following the guidelines as described (Karlsson et al. 2004) for the interpretation of O-linked oligosaccharides. Structural assignment was performed by the comparison of MS\textsuperscript{2} spectra from isolated chromatographic peaks to structures identified in the UniCarb-DB glycomic database (Hayes et al. 2011). Identified structures in Table II and Supplementary data, Table SI have been submitted to UniCarb-DB (www.unicarb.org).

A GlcNAcβ1-4GlcNAc β1-4GlcNAc standard (Sigma Aldrich) and a GalNAcβ1-4-Gal standard (DextraUK, Reading, UK) were used to obtain the fragmentation spectra of a terminal β1-4-linked GalNAc and a β1-4-linked GlcNAc. The standards were prepared in water to a concentration of 10 μg/μL and analyzed by LC-MS with an in-source fragmentation of 30%. The \textsuperscript{0,2}A\textsubscript{2} fragment from chitotriose and \textsuperscript{0,2}A\textsubscript{2} fragment from GalNAcβ1-4-Gal were isolated with an isolation width of ±3 mass units for MS\textsuperscript{3} fragmentation with collision energy of 30%. Specific structures from HGM-2, identified as having an unknown terminal HexNAc structure by the presence of the diagnostic fragment ion \textit{m/z} 610, were also isolated for MS\textsuperscript{3} fragmentation.

Hexosaminidase treatment of PGM and HGM-2

The LC-MS of oligosaccharides from PGM (Nordman et al. 1998) after reductive β-elimination showed a selective reaction monitoring chromatogram of three parent ions that fragmented into the specific Z-fragment of \textit{m/z} 610 (Supplementary data, Figure S1). This fragment ion is indicative for a HexNAc-HexNAc-HexNAc species and the nature is described in Figure 3A. These parent ions also showed the diagnostic \textsuperscript{0,2}A fragment of \textit{m/z} 304 from 4-linked HexNAc (Supplementary data, Figure S2 for the [M–H]\textsuperscript{−} ion of \textit{m/z} 936). The [M–H]\textsuperscript{−} ion of the \textit{m/z} 936 structure was shown by RT and LC-MS\textsuperscript{2} to be identical to the human laciDNac containing species in HGM-2 and was used for exoglycosidase digestion and LC-MS to confirm the linkage of the terminal HexNAc.

O-Linked oligosaccharides (10 μg) released from PGM and HGM-2 were treated with 0.1 U of a JBH (Prozyme, Hayward, CA), β-N-acetylgalactosaminidase (Prozyme from S. pneumoniae) or α-L-acetylgalactosaminidase from Clostridium perfringens (R&D Systems, Minneapolis, MN) in 10 μL of the enzymes buffer. The reaction was incubated at 37°C for 1 h (or 24 h for JBH) and stopped by the addition of 1.0 M HCl. Digested and non-digested oligosaccharides were cleaned with 0.6 mL of HyperCarb® PGC (ThermoFisher Scientific, Waltham, MA) packed in C18 zip tips and analyzed by LC-MS and LC-MS\textsuperscript{n}. The relative intensities of oligosaccharides corresponding to a GlcNAcβ1-6(Fuc0-1Galβ1-3)GalNAc-ol (IM–H\textsuperscript{−} ion of \textit{m/z} 733) and GlcNAcβ1-GlcNAcβ1-6(Fuc0-1Galβ1-3)GalNAc-ol (\textit{m/z} 936) were compared with the relative intensity of an internal standard of Fuc0-1Galβ1-4GlcNAcβ1-6(Fuc0-1Galβ1-3)GalNAc-ol (\textit{m/z} 1041). The intensity of this particular oligosaccharide remains unchanged after enzymatic digestion.

Lectin and antibody probing of HGM-1, HGM-2 and PGM

HGM and PGM were transferred to PVDF membrane with the amount of mucin corresponding to 5 μg of carbohydrate content. Bovine fetuin was also transferred and used as a negative control. The membranes were blocked with 1% bovine albumin in Tris-buffered saline with 0.05% Tween-20 (TBST). Blots were probed with a biotinylated lectin from W. floribunda (Vector Laboratories, Burlingame, CA) diluted to 1:10,000 in TBST or mouse anti-Leb mono-clonal antibodies (2-5LF, Abcam, Cambridge) diluted to 1:1000 in TBST for 3 h incubation at room temperature. After probing, the blot was washed five times with TBST for 5 min. The blots were incubated in horseradish peroxidase conjugated to streptavidin diluted to 1:8000 in blocking solution for blots probed with lectin from W. floribunda and with horseradish peroxidase conjugated to goat anti-mouse IgG (P0161, Dako, Glostrup, Denmark) diluted to 1:20,000 in blocking solution for blots probed with the Le\textsuperscript{b} antibody for 60 min at room temperature. The blots were further washed for five times with TBST and once with TBS. The blot was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Signal intensity was measured using ImageJ software (National Institute of Health, Bethesda, MA).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/

Acknowledgements

The antral mucin from porcine stomach was a gift from Prof. Ingemar Carlstedt and Dr Henrik Nordman, Lunds University.
Dr Catherine A Hayes is acknowledged for her help in uploading the MS data to UniCarb-DB.

**Funding**

This work was supported by the Swedish Research Council (621-2010-5322 and K20008-58X-20693-01-4), The Swedish Foundation for International Cooperation in Research and Higher Education, Åke Vibergs Foundation and the Swedish Cancer Foundation. The mass spectrometer was obtained by a grant from the Swedish Research Council (342-2004-4434).

**Conflict of interest**

None declared.

**Abbreviation**

BabA, blood group antigen binding adhesin; HMG, human gastric mucin; JBI, jack bean β-N-acetylgalactosaminidase; lacdiNAc, di-N-acetyllactosamine; OMP, outer membrane protein; PGC, porous graphitized carbon; PGD, porcine gastric mucin; RT, retention time; SabA, sialic acid-binding adhesin; SIC, selected ion chromatogram; TBST, Tris-buffered saline with Tween-20; CID, collision induced dissociation; Fuc, Fucose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Hex, hexose; HexNAc, N-acetylatedhexosamine; LC-MS, liquid chromatography-mass spectrometry; PVDF, polyvinylidene fluoride

**References**


