The O-linked glycosylation of secretory/shed MUC1 from advanced breast cancer patient serum

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Running title: Glycosylation of breast cancer patient serum MUC1

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Abstract

MUC1 is a high molecular weight glycoprotein that is over-expressed in breast cancer.

Aberrant O-linked glycosylation of MUC1 in cancer has been implicated in disease

progression. We investigated the O-linked glycosylation of MUC1 purified from the

serum of an advanced breast cancer patient. O-glycans were released by hydrazinolysis

and analyzed by liquid chromatography-electrospray ionization-mass spectrometry (LC-

ESI-MS) and by high performance liquid chromatography (HPLC) coupled with

sequential exoglycosidase digestions. Core 1 type glycans (83%) dominated the profile

which also confirmed high levels of sialylation: 80% of the glycans were mono-, di- or

tri-sialylated. Core 2 type structures contributed approximately 17% of the assigned

glycans and the oncofoetal Thomsen-Friedenreich (TF) antigen (Galβ1-3GalNAc)

accounted for 14% of the total glycans. Interestingly, two core 1 type glycans were

identified that had sialic acid α2-8 linked to sialylated core 1 type structures (9% of the

total glycan pool). This is the first O-glycan analysis of MUC1 from the serum of a breast

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cancer patient; the results suggest that amongst the cell lines commonly used to express recombinant MUC1 the T47D cell line processes glycans that are most similar to patient derived material.

Introduction

MUC1 is a high molecular weight transmembrane glycoprotein found on numerous epithelia. During breast cancer an atypical expression pattern is exhibited, changing from the apical border of epithelial cells to apolar cell surface distribution (Heyderman *et al*., 1979). In addition to altered cell surface location MUC1 can be found in the cytoplasm; and at higher levels in the circulation of patients (Robertson *et al*., 1999). The majority of the extracellular domain of MUC1 consists of a variable number (30-100) of tandem repeats (VNTR) (Gendler *et al*., 1990). Each repeat consists of 20 amino acids with five serine and threonine residues, all of which can act as sites for O-linked glycosylation (Müller *et al*., 1999).

MUC1 is initially expressed as a precursor protein and is auto-proteolytically cleaved in the endoplasmic reticulum (Levitin *et al*., 2005; Macao *et al*., 2006; Hilkens and Buijs, 1988). The extracellular subunit remains associated with the transmembrane and cytoplasmic domain throughout intracellular processing and is transported to the cell surface as a stable heterodimeric complex (Ligtenberg *et al*., 1992; Baruch *et al*., 1999; Julian *et al*., 2002). The extracellular domain of the MUC1 heterodimeric complex can be shed into bodily fluids and in addition, a secreted isoform of MUC1 (MUC1/sec) that lacks the transmembrane domain and cytosolic components of MUC1 is also present in the circulation (Boshell *et al*., 1992; Smorodinsky *et al*., 1996; Wreschner *et al*., 1990).

The sialylation of MUC1 is incomplete when the glycoprotein is first observed on the cell surface and the addition of sialic acid only takes place following trafficking and successive rounds of recycling through the trans-golgi (Hilkens and Buijs, 1988). The sialic acid content of secreted MUC1 differs from that of the transmembrane bound MUC1 as the former only goes through the golgi once and is not subjected to further rounds of sialylation (Engelmann *et al*., 2005).

Previous studies comparing the O-linked glycans of MUC1 from breast cancer cell lines and non-malignant breast epithelial cell lines demonstrated a variation in the O-glycan population. These studies have mainly focused on various cancer cell lines due to limiting sample amounts for glycan analysis. A shift from branched di-sialylated core 2 type O-glycans on epithelial cell line MUC1 to shorter mono-sialylated core 1 type O-glycans on MUC1 from the T47D tumour cell line has been observed (Lloyd *et al*., 1996). However, MUC1 from the MCF-7 cell line has been shown to consist principally of neutral core 2 type O-glycans (Müller and Hanisch, 2002). Interestingly, Engelmannn *et al*. (2005) demonstrated that a secreted MUC1 probe in MCF-7 cells contained predominantly neutral core 2 type O-glycans in contrast to a membrane bound MUC1 probe in the same cell line, which contained mainly sialylated core 1 glycans. Thus the glycosylation varies depending on the location of the mucin.

In addition to altered glycan processing, cancer-associated MUC1 may also be more extensively glycosylated in comparison to MUC1 from healthy tissue. Müller *et al*. (1999) demonstrated that T47D MUC1 contained an average of 4.8 glycans of a possible 5 per 20 amino acid repeat. This indicated a significantly higher level of glycosylation

than the 2.7 glycans per repeat that was observed on lactation-associated MUC1 (Muller *et al*., 1997).

In this study we analyzed the O-linked glycosylation of MUC1 purified from the serum of an advanced breast cancer patient using HPLC, sequential exoglycosidase digestion and LC-ESI-MS. This combination of methods enabled us to identify the O-linked glycans present on the secretory/shed isoform of MUC1 in breast cancer.

Results

Isolation of MUC1 - MUC1 was isolated from the serum of an advanced breast cancer patient using a double antibody affinity matrix (C595/NCRC-11) followed by size exclusion chromatography. The murine monoclonal antibodies, C595 and NCRC-11, were used for the purification of MUC1 because they are directed against epitopes within the VNTR, and C595 binds MUC1 irrespective of the number of repeats or its glycosylation (Karsten *et al*., 2004). The efficiency of purification was monitored by ELISA and the CA15.3 assay values of the serum pre- and post-affinity chromatography. Following reduction and alkylation the sample was subject to separation by size exclusion chromatography to remove co-purified antibodies, albumin and other protein contaminants. The purity of purified MUC1 was analyzed by SDS-PAGE and is shown in Figure 1A. MUC1 could not be visualized by silver staining which is possibly due to high levels of glycosylation. Western blot analysis of the purified ABC serum MUC1 using C595 monoclonal antibody confirms the presence of high molecular weight MUC1 (Figure 1B).

Analysis of O-linked glycans by HPLC - O-glycans were cleaved and labelled with 2-AB from serum MUC1 isolated from a breast cancer patient who had been diagnosed with metastatic disease. The glycan pool was analyzed by NP-HPLC, in combination with sequential digestion using an array of exoglycosidases, and WAX-HPLC. Calibration of the NP-HPLC was performed using an external standard of 2-AB-labeled glucose oligomers to create a dextran ladder which was used to convert the retention times of glycans to glucose units (GU). Preliminary structures were assigned by comparing the glucose unit (GU) values of the experimental data with our 'in-house' structural database for O-glycans and confirmed from the GU values of digestion products and the known specificities of the enzymes (Royle *et al.*, 2002) (Figure 2). The data were consistent with glycan compositions obtained from the MS analysis. Hydrazinolysis, predictably, gave rise to base catalysed β elimination (peeling) resulting in the presence of NeuNAcα2-3Gal (25% of all glycans). This fragment remains after the elimination of terminal GalNAc from susceptible O-glycans (both core 1 and core 2 type glycans) and was therefore excluded from determining the percentage of individual glycans, calculating the ratios of core 1 type to core 2 type glycans and calculating the ratio of sialylated to neutral glycans. A list of the assigned O-linked glycans attached to MUC1 and their behavior following exoglycosidase digestion together the masses detected by LC-ESI-MS are detailed in Table 1. In addition, Table 1 details the percentages of the whole undigested glycan pool and the percentages once the base catalysed β elimination (peeling) glycan was removed from the analysis. Charged structures present on ABC serum MUC1 were also investigated by WAX-HPLC fractionation of the total glycan pool based on total charge (Figure 3).

Identification of core type glycans - The O-glycans present on ABC serum MUC1 revealed a major population of core 1 type glycans, core 2 type glycans accounted for only around 17% of the patient derived glycans. The percentage of core 1 and core 2 type glycans was estimated using the peak areas in Figure 2. The exact contribution of core 1 and core 2 type glycans could not be calculated due to the co-elution of peaks 8 (di-sialylated core 1) and 9 (mono-sialylated core 2) on NP-HPLC (Figure 2). These two glycans were successfully separated by WAX-HPLC indicating that the major component was the di-sialylated core 1 type glycan (Figure 3). However, the percentage of core 2 type glycans could still not be determined accurately as peak 9 co-eluted with peaks 10 and 11 in WAX-HPLC (Figure 3).

Identification of sialylated glycans - NP-HPLC was used to identify sialylated O-linked glycans by comparing the undigested total glycan pool with its ABS digested counterpart. Provisional structures were assigned using the O-glycan database, then confirmed by sequential exoglycosidase digestion and comparison of the resultant digestion products against a structural database and known digestion pathways (Royle *et al*., 2002). WAX-HPLC fractionation was also used to confirm the identity of sialylated glycans. LC-ESI-MS was used to confirm the mass and hence composition of each structure. LC separation prior to mass spectral analysis helped to separate the glycans from the background contamination that would interfere with MS analysis. In addition, the LC column allowed the masses to be cross checked with elution time (GU) to confirm that individual masses correlated with the glycans identified by NP-HPLC for the 2-AB labeled glycans.

Exoglycosidase digestion with the sialidase ABS, revealed that the majority of O-linked glycans present on ABC serum MUC1 were sialylated (Figure 2). When quantified as a percentage of the total undigested glycan pool, 80.5% of the O-linked glycans were found to be either mono-, di- or tri-sialylated structures. The principal O-glycan species on ABC serum MUC1 were sialylated core 1 type glycans. NeuNAcα2-3Galβ1-3GalNAc (peak 4, Table 1) was the most abundant glycan. In addition, the oncofoetal Thomsen-Friedenreich (TF) antigen (Galβ1-3GalNAc) accounted for 14.3% of the glycans.

Two new O-glycan structures were identified that were not in the structural database (Royle *et al.*, 2002) and have not previously been reported on MUC1. These were peaks 12 and 15 which we have identified as core 1 type glycans containing 2 and 3 sialic acids, respectively. The tri-sialylated peak 15 eluted in the tri-sialylated region on WAX-HPLC, eluted last by NP-HPLC (GU 5.7) and also last by LC-ESI-MS where a clear peak of m/z 1279.3 indicated a composition of three sialic acids, one Hex and one HexNAc. The sialidase used to investigate the sialic acid linkage of these structures has specificity for both α2-3 and α2-8 linkages. We propose an α2-8 sialic acid linkage to the structure as it is well documented that sialic acids add on to each other in the α2-8 position and have been identified previously on both human (Fukuda *et al.*, 1987) serum glycoproteins. In addition, it is known from measuring the GU values of glycans released from glycosphingolipids that the addition of a sialic acid α2-8 to a sialic acid increases the GU by 1.3 (Wing *et al.*, 2001). The difference between peak 8 and peak 15 is 1.3 GU. There is a higher GU difference between peaks 12 and 4 (2 GU) but the evidence that α2-8 linked sialic acid adds more GU than α2-6 linked sialic

acid and that this peak elutes after peak 8, as well as eluting in the di-sialylated fraction of WAX-HPLC suggests that it is also a α2-8 linked sialic acid containing structure.

Identification of fucosylated glycans - The fucosylation of O-linked glycans on ABC MUC1 was investigated by NP-HPLC and exoglycosidase digestion with the fucosidases AMF and BKF. No change in the NP-HPLC profiles was observed and demonstrated the absence of fucosylated glycans (Figure 2).

Discussion

The glycosylation status of breast cancer associated MUC1 has long been of interest because of the altered immunogenicity that the MUC1 VNTR exhibits in response to glycosylation. Many monoclonal antibodies have demonstrated alternate binding characteristics in response to the glycosylation status of MUC1 (Karsten *et al.*, 2004). A shift from core 2 to smaller core 1 type glycans on MUC1 is proposed to make the protein backbone more accessible to immune surveillance.

The O-linked glycosylation profiles of MUC1 purified from a number of breast cancer cell lines differ significantly from one another, indicating a cell specific rather than a cancer defined pattern of O-glycans. MUC1 purified from the T47D breast cancer cell line contains predominantly core 1 type glycans, with less than 5% of core 2 type structures. In contrast, MUC1 purified from the MCF-7 breast cancer cell line contains primarily core 2 type glycans (83%) (Müller and Hanisch 2002).

The O-linked glycans attached to ABC serum MUC1 consist of core 1 type glycans (83%), sialic acid α2-8 linked to sialylated core 1 type glycans (8.8%), the TF

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antigen (Galβ1-3GalNAc) (14.3%) and core 2 type glycans (17%). The principal glycans present on ABC serum MUC1 are similar to those present on MUC1 secreted from the T47D breast cancer cell line, being sialylated core 1 type structures, having high levels of Galβ1-3GalNAc (peak 1), and lacking fucosylated structures. In addition, although ABC serum MUC1 has 17% core 2 type structures, it resembles MUC1 from the T47D breast cancer cell line more closely in its level of core 2 type glycans (less than 5%) than MUC1 purified from other cell lines analyzed by Müller and Hanisch (2002). ABC serum MUC1 does differ from T47D MUC1 in an increased diversity of structures, including the sialic acid α2-8 linked to sialylated core 1 type structures identified here.

The O-glycan pool of ABC serum MUC1 was heavily sialylated; 80.5% of the total assigned structures were sialylated. This level of sialylation is similar to that observed on MUC1 from the breast cancer cell lines T47D, MDA-231 and ZR-75-1 (93.2%, 70.3%, and 88.4% respectively). MCF-7 shows a dramatically different level of sialylation, as only 5.2% of its O-linked glycans contain sialic acid (Müller and Hanisch 2002).

The oncofoetal antigen TF (Galβ1-3GalNAc) (14.3% of the total O-glycan pool) was released from ABC serum MUC1. An interaction between the TF antigen on MUC1 and galectin-3 has recently been demonstrated. This interaction has been proposed to increase the adhesion between cancer and endothelial cells, suggesting a role in cancer progression (Yu *et al.*, 2007). Sialic acid α2-8 linked to sialylated O-glycan structures on serum glycoproteins have not been described in detail previously, although N-linked poly-sialylation is a feature of glycoproteins of the brain, such as neural cell adhesion molecule (N-CAM) (Hoffman *et al.*, 1982) and the sodium channel α subunit (Zuber *et*

al., 1992). The N-linked poly-sialylation of N-CAM has been shown to modulate cell adhesion (Fujimoto *et al.*, 2001). O-linked poly-sialylation has been identified on CD36 from human milk (Yabe *et al.*, 2003) and on unidentified proteins in MCF-7 and RBL basophilic leukemia cells (Martersteck *et al.*, 1996). In addition a Neu5Gcα2-8Neu5Gc2→ reactive monoclonal antibody has identified disialic acid on carbonic anhydrase II, an immunoglobulin light chain, vitronectin, and plasminogen in mouse serum, which has been confirmed by fluorometric analysis and mild acid hydrolysates-fluorometric anion exchange chromatography (Yasukawa *et al.*, 2006). It is unclear as to the effects that these sialic acid α2-8 linked to sialylated structures may have on the functions or properties of MUC1. In addition, it would be of interest to know if these structures are present during the early stages of disease and if they have diagnostic/prognostic significance, however this would be difficult due to low amounts of MUC1 and the glycan structure.

We have analyzed the O-glycans attached to MUC1 circulating in the serum of an advanced breast cancer patient. High levels (14.3%) of the oncofoetal antigen TF were detected. This antigen has been implicated in a number of interactions; such as increased cancer cell endothelial adhesion by interaction with galectin-3 (Yu *et al.*, 2007). Interestingly, 8.8% of ABC MUC1 O-linked glycans had sialic acid α2-8 linked to sialylated core 1 type structures. The glycan pool was dominated by core 1 based structures most of which were sialylated. This is similar to the O-linked glycosylation present on the T47D cell line MUC1 which may therefore be the most appropriate cell line for the study of 'cancer state' O-linked glycosylation of MUC1.

Materials and Methods

Materials - The monoclonal antibody hybridoma cell lines NCRC-11 and C595 were kindly provided by Professor Alan Perkins at the University of Nottingham, UK. Serum samples were taken from patients under informed consent at the Breast Institute, University of Nottingham. MUC1 from an individual with advanced breast cancer (ABC) was investigated.

Purification of NCRC-11 and C595 mouse monoclonal antibodies - NCRC-11 and C595 hybridoma cell lines were grown at 37°C in the presence of 5% CO_2. RPMI 1640 containing 100U/ml Penecillin, 100µg/ml Steptomycin, 10% foetal calf serum, 1µg/ml fungizone and 3.6ng/ml β-mercaptoethanol (all Sigma-Aldrich, Missouri, USA) was used for both C595 and NCRC-11 hybridoma cell line culture. Cell culture supernatant was isolated by centrifugation at 400 x g and was 0.45µm filtered (Minisart, Sartorius, Goettingen, Germany) prior to circulation through activated CNBr sepharose 4B (GE Healthcare, Uppsala, Sweden) coupled to a 9-mer VNTR peptide; APDTRP (Peptide Protein Research Ltd., Fareham UK) at 4°C over 17-24 hours. Unbound protein was washed from the column, with 10 column volumes of phosphate buffered saline (PBS) containing 0.2M sodium chloride. Bound antibody was eluted from the column using 2 column volumes of 3M sodium thiocyanate, and fractions were immediately desalted using a PD10 column (GE Healthcare, Uppsala, Sweden). The reactivity of purified antibody was confirmed in enzyme linked immunosorbant assays (ELISA).

Purification of MUC1 - C595 and NCRC-11 were bound to activated CNBr Sepharose 4B (GE Healthcare, Uppsala, Sweden) at 1.7mg of protein per ml of matrix. 5ml of antibody matrix was used for MUC1 purification. Serum was diluted 1:10 in PBS with 0.2M sodium chloride, 0.45μm Minisart filtered and circulated through the C595/NCRC-11 affinity matrix for 17-24 hours at 4°C prior to elution. MUC1 was eluted from the columns using three elution buffers (0.25M glycine/HCl pH2.5; 25mM diethylamine pH11; 100mM diethylamine pH11) and eluted column fractions were assayed against C595 mouse monoclonal antibody and peroxidase conjugated affinipure rabbit anti-human IgG and IgM (H+L) (Jackson Immunoresearch, Pennsylvania, USA) in an ELISA. The affinity purification was repeated five times. Positive fractions were pooled and concentrated using Amicon Ultra-15 centrifugal filter units (30,000 MWCO) (Millipore, Massachusetts, USA).

Concentrated serum MUC1 was reduced using 50mM *threo*-1,4-dimercapto-2,3-butanediol (DTT) for one hour rolling at room temperature. The reduced sample was then alkylated using 100mM iodoacetamide (IAA) for 30 minutes protected from light at room temperature prior to size exclusion chromatography using an HiPrep 26/60 Sephacryl S-300 (GE Healthcare, Uppsala, Sweden) with a flow rate of 0.5ml/min. Following size exclusion chromatography fractions were assayed by ELISA and fractions positive for MUC1 were again pooled and concentrated using Amicon Ultra-15 centrifugal filter units (30,000 MWCO). Purity was assessed by SDS polyacrylamide gel electrophoresis (PAGE) by resolution in a 10% gel using a Mini-Protean 3 Cell (BioRad Laboratories, Hercules, USA) for 50 minutes at a constant voltage of 150V. Gels were stained with silver using a Silver Stain Kit (BioRad Laboratories, Hercules, USA). CA15.3 levels

were determined using the Bayer Centaur automated CA15.3 immunoassay and glycoprotein was stored at -20°C until analysis.

Western blot analysis - Purified MUC1 was resolved in a 10% SDS polyacrylamide gel for 50 minutes at a constant voltage of 150V, followed by gel equilibration in Tris-glycine buffer. Protein transfer from gels to 0.2μm nitrocellulose membrane (BioRad Laboratories, Hercules, USA) was performed with a BioRad Trans blot system (using a constant voltage of 40V over 5 hours). Membranes were blocked with 5% Marvel in Tris buffered saline containing Tween-20 (TBS-t) for 15-20 hours at 4°C prior to incubation with C595 mouse monoclonal antibody in the same buffer for 15-20 hours at 4°C. Immunocomplexes were detected with horseradish peroxidase conjugated anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark) for two hours, prior to detection with enhanced chemiluminesence (ECL) detection reagents and developed on Hyperfilm (GE Healthcare, Uppsala, Sweden).

Hydrazinolysis of MUC1 O-glycans - MUC1 was dialysed against 0.1% trifluoroacetic acid (TFA) at 4°C for over 36 hours in a microdialysis block and lyophilised. Hydrazinolysis was performed as Patel *et al*. (1993) and Merry *et al*. (2002). Briefly, lyophilized MUC1 was cryogenically dried for 24 hours and O-glycans were released by incubation at 60°C for 6 hours with anhydrous hydrazine. Excess hydrazine was removed by evaporation and the released O-linked glycans were re-*N*-acetylated by incubation with acetic anhydride in a saturated solution of sodium bicarbonate; and sodium salts were removed using Dowex AG50 X12(H+) 200-400 mesh (BioRad Laboratories,

Hercules, CA). O-glycans were separated from peptide components by descending paper chromatography on pre-washed Whatmann 3M chromatography paper in butanol:ethanol:water (8:2:1v/v) for 48 hours. Glycans were recovered from their chromatography paper origin by 4 x 1.5ml washes and concentrated using a rotary evaporator.

2-AB labelling of MUC1 O-glycans - 2-AB labeling was performed as Bigge *et al*. (1995), using the Ludger Tag™ 2-AB kit (Ludger Ltd, Oxford, UK). 2-AB labeled glycans were separated from excess fluorophore by ascending paper chromatography with acetonitrile (Royle *et al*., 2006). Glycans were eluted from their paper origin with water.

Normal Phase (NP)-HPLC of MUC1 2-AB labeled O-glycans - 2-AB labeled glycans were separated on a 2695 Alliance Separation Module, fitted with a temperature control module and a 474 fluorescence detector (all Waters, Massachusetts, USA). Fluorescence was measured at 420nm with excitation at 330nm. Separation was performed as Royle *et al*. (2002) using a TSK Amide-80 250 x 4.6mm column (Anachem, Luton, UK).

Exoglycosidase digestion of 2-AB labeled O-glycans - 2-AB labeled glycans were subject to a range of exoglycosidase digestions followed by NP-HPLC. Digestions were preformed in 50mM sodium acetate buffer, pH5.5, for 16 hours at 37°C. Exoglycosidases used were: *Arthrobacter ureafaciens* sialidase (ABS EC 3.2.1.18), 1-2 units/ml; *S. pneumonia* sialidase recombinant from *Escherichia coli* (NAN1 EC 3.2.1.18), 1 unit/ml;

bovine kidney α-fucosidase (BKF EC 3.2.1.51) 1 unit/ml; almond meal α-fucosidase (AMF EC 3.2.1.111), 3 milliunits/ml; bovine testes β-galactosidase (BTG EC 3.2.1.23), 2 units/ml; Jack bean β-*N*-acetyl-hexosaminidase (JBH EC 3.2.1.30), 10 milliunits/ml, purchased from Prozyme, San Leandro, CA, USA.

Weak anion exchange (WAX)-HPLC fractionation of MUC1 2-AB labeled O-glycans - The fluorescently labeled glycans were separated on a 2695 Alliance Separation Module, fitted with a temperature control module and a 474 fluorescence detector (all Waters, Milford, USA). Fluorescence was measured at 420nm with excitation at 330nm. Separation was performed as Royle *et al*. (2002) using a Vydac 301VHP575 7.5 x 50mm column. Both the undigested and ABS digested O-glycans of MUC1 were subject to WAX-HPLC and fractions were collected and subjected to further analysis by NP-HPLC.

Glycan analysis by liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) - Glycans were analysed using an LC Packings Ultimate HPLC equipped with a Famos autosampler (Dionex Ltd, Leeds, UK) interfaced with a Q-Tof Ultima Global mass spectrometer (Waters-Micromass, Manchester, UK). Chromatographic separation was achieved using a 2 x 250mm microbore NP-HPLC TSK gel Amide-80 column (Hichrom) with the same gradient and solvents as used with the standard NP-HPLC but at a lower flow rate of 40μL/min (Royle *et al*., 2002). The mass spectrometer was operated in positive ion mode with 3 kV capillary voltage; RF lens 60; source temp $100^{o}C$; desolvation temp $150^{o}C$; cone gas flow 50 lL/Hr; desolvation gas flow 450 L/Hr.

Acknowledgements

Abbreviations

The abbreviations used are: ABC, advanced breast cancer; 2-AB, 2-aminobenzamide; GU, glucose units; HPLC, high performance liquid chromatography; LC-ESI-MS, liquid chromatography-electrospray ionization mass spectrometry; NP, normal phase; TF, Thomsen-Friedenreich antigen; WAX, weak anion exchange.

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Legends to Figures

Figure 1: Analysis of advanced breast cancer serum (ABC) MUC1 on a reducing 10% SDS polyacrylamide gel. (A) 10% SDS polyacrylamide gel stained with silver; track 1: Sigma wide range MWt markers; track 2: ABC serum MUC1 contains no detectable contaminating proteins. MUC1 was not visualized by silver staining which is possibly due to high levels of glycosylation. (B) Western blot of ABC serum MUC1 with murine C595 monoclonal antibody indicating the presence of high molecular weight MUC1 compared against Amersham full-range MWt Rainbow markers.

Figure 2: Structural analysis of advanced breast cancer (ABC) patient MUC1. O-glycans were released from MUC1 by hydrazinolysis, fluorescently labeled with 2-aminobenzamide (2-AB) and analyzed by normal phase (NP)-HPLC. Glycans were determined by their elution position measured in glucose units (GU) in comparison to a database of GU values of glycans, their GU value following exoglycosidase digestion and their weak anion exchange (WAX)-HPLC elution time. Peaks are identified by numbers defined in Table 1. The control was 2-AB labeled glycans from fetuin. ABS removes α2-3, α2-6 and α2-8 linked sialic acid, BTG removes β1-3 and β1-4 linked galactose, JBH removes GalNAc and AMF removes α1-3 or 4 linked fucose.

Table 1: Aliquots of the 2-aminobenzamide (2-AB) labeled glycan pool from advanced breast cancer (ABC) serum MUC1 were incubated with different exoglycosidases and the products analyzed by normal phase (NP)-HPLC. Liquid chromatography-electrospray

ionization mass spectrometry (LC-ESI-MS) data is also displayed and was performed on the total glycan pool from ABC MUC1. NAN1 and ABS, ABS/BTG, ABS/BTG/BKF, ABS/BTG/AMF and ABS/BTG/JBH exoglycosidase digestions are compared against the whole glycan pool where ABS removes α2-3, α2-6 and α2-8 linked sialic acid, NAN1 removes α2-3 linked sialic acid, BTG removes β1-3 and β1-4 linked galactose, JBH removes GalNAc, AMF removes α1-3 or 4 linked fucose and BKF removes α1-2 and α1-6 linked fucose. ↑ denotes increased % peak area; ↓ denotes decreased % peak area and = shows % peak area remained the same following digestion; nd: not detected. Adjusted against peak 2 denotes the % contribution of individual glycans, when the base catalyzed β elimination (peeling) glycan (peak 2) is removed from analysis; *Proposed structure. The α2-8 sialic acid is allocated as such because sialic acids have previously been reported to be linked to each other in this way. However, due to the ABS sialidase having specificity for both α2-3 and α2-8 sialic acids, it is possible that the sialic acid could have either linkage.

Figure 3: Structural analysis of advanced breast cancer (ABC) patient MUC1 by weak anion exchange (WAX)-HPLC. The fluorescently 2-aminobenzamide (2-AB) labeled O-glycan pool of ABC serum MUC1 was subjected to charge-based separation by WAX-HPLC. Peaks are identified by numbers defined in Table 1. (A) The O-linked profile of a fetuin O-glycan control. (B) The undigested O-glycan profile of ABC serum MUC1; the major charged structures are visible. (C) The α2-3, α2-6, and α2-8 linked sialidase (ABS) digested O-glycan profile of ABC serum MUC.
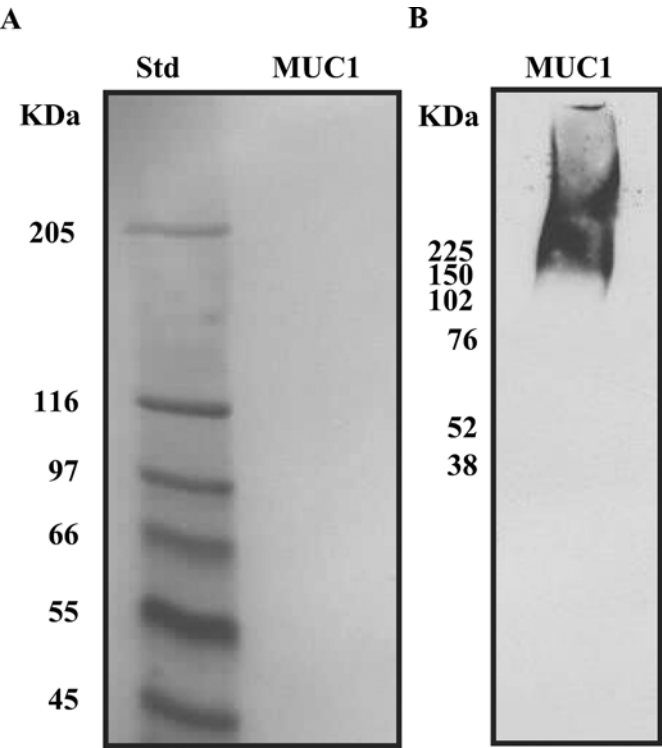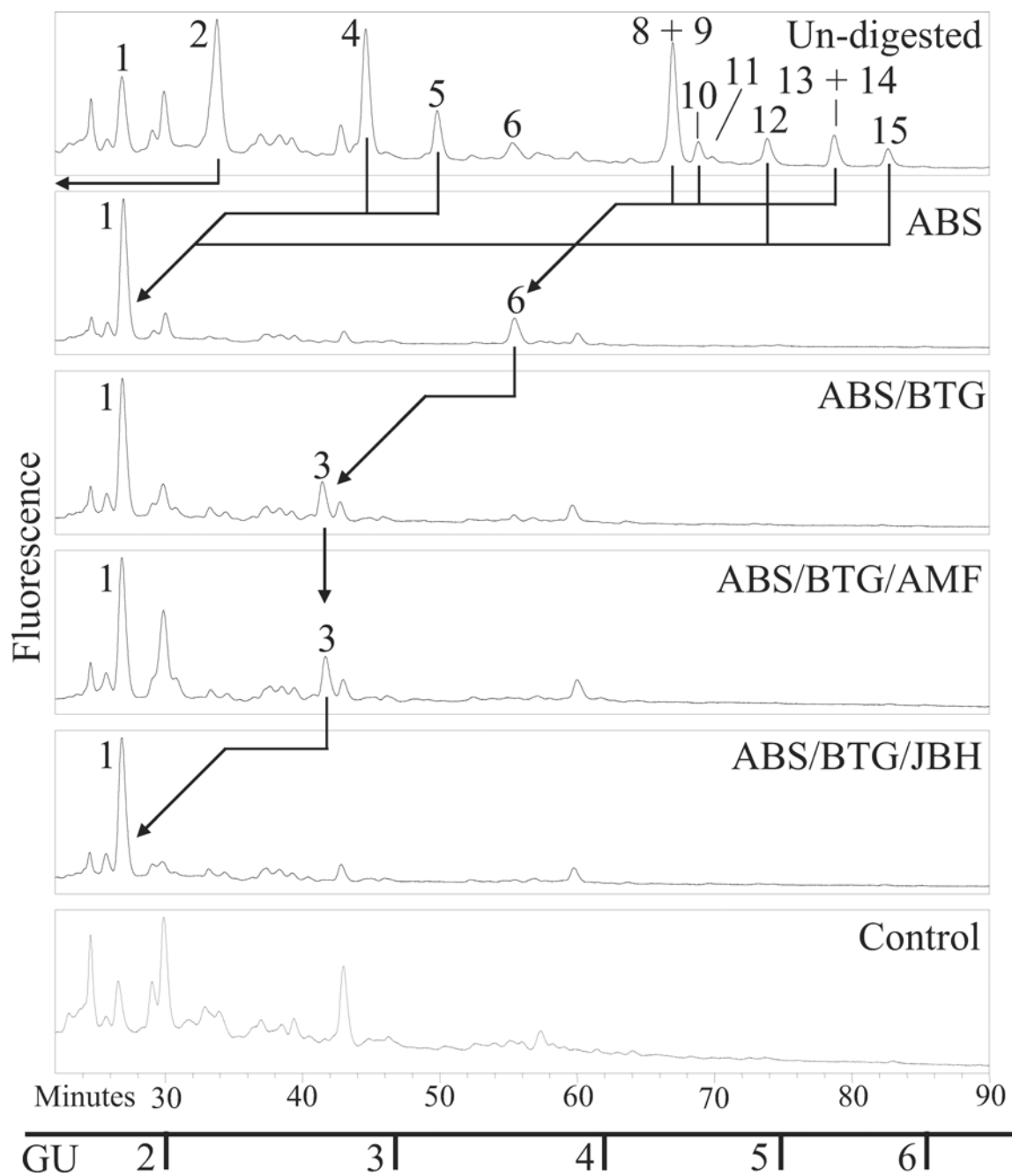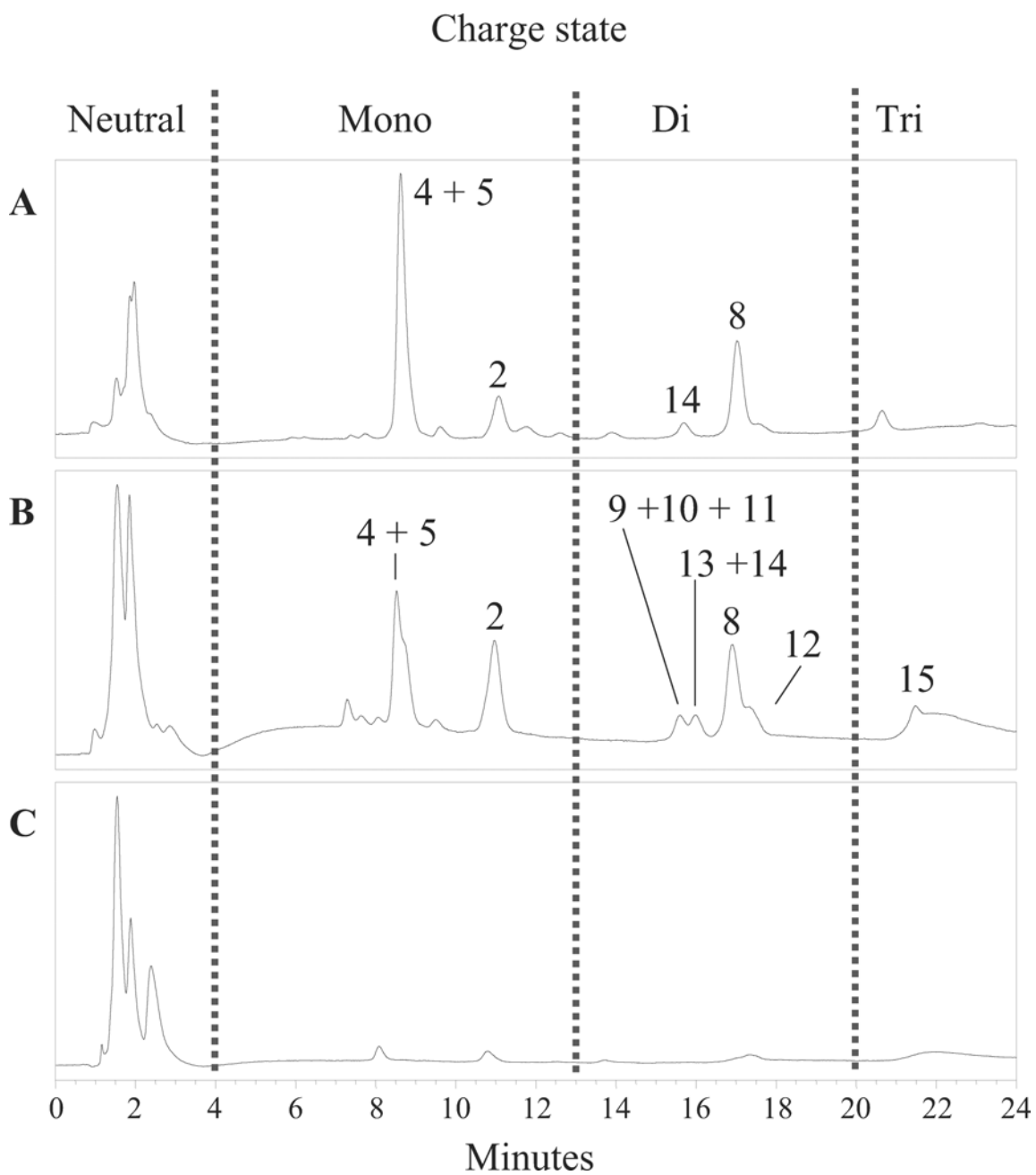
Figure 1

Figure 2

Figure 3

Table 1

| | | Mass [M+Na]+ | | Composition | | | | | | | Exoglycosidase Digestions | | | | | | |
Glycan	Peak No.	Detected	Calculated	Hex	HexNAc	NeuNAc	Retention time LC-ESI-MS	GU	Undigested whole glycan pool (%)	Adjusted against peak 2 (%)	NAN1 BTG	BTG	ABS	ABS BTG	ABS BTG BKF	ABS BTG BKF AMF	ABS BTG JBH
Galβ1-3GalNAc	1	406.1	406.1	1	1	0	76.1	1.8	10.7	14.3	=	↑	↑	↑	↑	↑	↑
NeuNAcα2-3Gal	2	494.1	494.2	1	0	1	80.7	2.2	25.0	0.0	=	=	↓	↓	↓	↓	↓
Galβ1-3[GlcNAcβ1-6]GalNAc	3	nd	609.2	1	2	0	nd	2.7	0.0	0.0	=	=	=	↑	↑	↑	↓
NeuNAcα2-3Galβ1-3GalNAc	4	697.1	697.2	1	1	1	83.4	2.9	19.7	26.3	=	↓	↓	↓	↓	↓	↓
Galβ1-3[NeuNAcα2-6]GalNAc	5	697.1	697.2	1	1	1	88.2	3.3	7.2	9.6	=	↑	↓	↓	↓	↓	↓
Galβ1-4GlcNAcβ1-6[Galβ1-3]GalNAc	6	771.2	771.3	2	2	0	91.5	3.6	3.8	5.1	↓	↑	↑	↓	↓	↓	↓
NeuNAcα2-3Galβ1-3[GlcNAcβ1-6]GalNAc	7	nd	900.4	1	2	1	nd	3.7	0.0	0.0	↑	=	=	=	=	=	=
NeuNAcα2-3Galβ1-3[NeuNAcα2-6]GalNAc	8	988.2	988.3	1	1	2	90.9	4.4	18.4	24.5	=	↓	↓	↓	↓	↓	↓
NeuNAcα2-3Galβ1-4GlcNAcβ1-6[Galβ1-3]GalNAc	9	1062.2	1062.4	2	2	1	93.4										
Galβ1-4GlcNAcβ1-6[NeuNAcα2-3Galβ1-3]GalNAc	10	1062.2	1062.4	2	2	1	93.4	4.5	3.0	4.0	↓	↓	↓	↓	↓	↓	↓
NeuNAcα2-6Galβ1-4GlcNAcβ1-6[Galβ1-3]GalNAc	11	1062.2	1062.4	2	2	1	95.1	4.6	1.0	1.3	=	↑	↓	↓	↓	↓	↓
NeuNAcα2-8NeuNAcα2-3Galβ1-3GalNAc*	12	988.2	988.3	1	1	2	92.2	4.9	4.2	5.6	=	↓	↓	↓	↓	↓	↓
NeuNAcα2-6Galβ1-4GlcNAcβ1-6[NeuNAcα2-3Galβ1-3]GalNAc	13	1353.3	1353.5	2	2	2	95.1	5.3	4.5	6.0	=	↓	↓	↓	↓	↓	↓
NeuNAcα2-3Galβ1-4GlcNAcβ1-6[NeuNAcα2-3Galβ1-3]GalNAc	14	1353.3	1353.5	2	2	2	95.1										
NeuNAcα2-8NeuNAcα2-3Galβ1-3[NeuNAcα2-6]GalNAc*	15	1279.3	1279.4	1	1	3	96.7	5.7	2.4	3.2	=	↓	↓	↓	↓	↓	↓