Biochemical Characterization of the Soluble Form of Tumor Antigen MUC1 Isolated from Sera and Ascites Fluid of Breast and Pancreatic Cancer Patients

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Abstract
Transmembrane glycoprotein tumor antigen MUC1 that is overexpressed on pancreatic and breast tumor cells can be found in large amounts in soluble form in serum and ascites fluid. MUC1 has been identified as a target of human antitumor antibody and CTL responses that are generated in the absence of helper T cells. The soluble form of MUC1 should support generation of helper T cells, but we have found recently that this form, although effectively endocytosed by dendritic cells, remains trapped in early endosomes and is not trafficked to antigen-processing compartments. The exact biochemical structure of this form of MUC1 has not been elucidated to date, and it is thus not clear what structural characteristics may be responsible for its retention in early endosomes. We have purified soluble MUC1 from ascites fluid of breast/pancreatic cancer patients (ASC-MUC1) and quantitated O-linked carbohydrates. We have altered ASC-MUC1 by enzymatic treatment: trypsin or clostripain digestion, desialylation, and further in vitro glycosylation. We have found that desialylated ASC-MUC1 was further glycosylated by peptidyl N-acetylgalactosamine transferases and was not when sialic acid was present. These alterations created new forms of ASC-MUC1 that might be handled more efficiently by antigen-presenting cells to generate better tumor-specific immunity and used to identify structures that are directly involved in retention of this antigen in early endosomes.

Introduction
MUC1, a member of the mucin family, is a large, rod-like, transmembrane glycoprotein expressed in a polarized fashion on the apical surface of ductal epithelial cells (1). In normal cells, MUC1 has a heavily O-glycosylated central domain, consisting of conserved and tandemly repeated stretches of 20 amino acids (2). Each tandem repeat has five potential sites of O-glycosylation, which can be substituted with polylactosamine-type chains as found on the lactation-associated glycoform of MUC1 (2). In tumor cells that have originated from ductal epithelia, MUC1 is expressed on the entire cell surface. More importantly, O-linked oligosaccharides are dramatically reduced in chain lengths, making this form tumor specific and immunologically distinct (3-5). As such, MUC1 is a potential target for immunotherapy of carcinomas (6). One form of MUC1, a synthetic peptide corresponding to five tandem repeats, is already being tested in clinical trials as a vaccine for pancreatic and breast cancers (7).

Tumor cells release soluble MUC1 (Fig. 1) by an unknown mechanism. This soluble form can be found in large quantities in sera and ascites fluid of cancer patients (8). Because of its abundance, this form is expected to be taken up, processed, and presented by APCs3 to helper T cells. However, this does not appear to be the case, because no MUC1 helper T cell responses have been found in cancer patients. Our recent studies showed that MUC1 purified from ascites fluid (ASC-MUC1) is effectively taken up by dendritic cells but then retained indefinitely in early endosomes instead of being processed in antigen-processing compartments (9). We consider this the primary reason why patients with so much circulating soluble MUC1 do not generate effective immune responses against MUC1+ tumor. Biochemical properties of this MUC1 form are not known; therefore, we undertook detailed characterization of ASC-MUC1 to understand what elements of its structure may influence its processing and presentation to the immune system.

The tumor-specific, underglycosylated integral membrane form of MUC1 is differentiated from the normal, heavily O-glycosylated form by specific monoclonal antibodies directed to peptide epitopes within the tandem repeat (10). Monoclonal antibodies recognizing tumor-specific MUC1 react with their epitopes only when O-linked oligosaccharides are substantially reduced, whereas other mAbs recognize MUC1, regardless of its glycosylation (11, 12). Our initial characterization of ASC-MUC1 was based on reactivity with 56 anti-MUC1 monoclonal antibodies submitted to the ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1. The results from that study showed that ASC-MUC1 was not recognized by mAbs that react only with underglycosylated (tumor-specific) MUC1 (13), suggesting that the tandem repeat region was glycosylated extensively. However, we hypothesized that the

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3 The abbreviations used are: APC, antigen-presenting cell; GalNAc, N-acetylgalactosamine; HPLC, high-performance liquid chromatography; GlcNAc, N-acetylglucosamine; NeuAc, neuraminic acid.
presence of oligosaccharides may be the cause of inefficient processing of this form of MUC1. Because glycosylation could not be determined from those experiments, we have now undertaken further analysis and characterization of ASC-MUC1, including carbohydrate and protein modifications. Quantitative analysis of O-linked carbohydrates on a monosaccharide level showed that ASC-MUC1 contains 5% w/w of O-linked oligosaccharides, indicating a substantially reduced degree of glycosylation compared with forms retained on the surface of normal or tumor cells. To characterize individual domains of this soluble form, we altered ASC-MUC1 by sequential enzymatic treatment. Trypsin and clostripain digestion generated fragments consisting primarily of the tandem repeat region with short segments of NH2- and COOH-terminal domains. Neuraminidase treatment removed α2,3- and α2,6-linked sialic acid residues, which enabled us to further O-glycosylate this form in vitro by adding GalNac residues onto available serine and threonine residues. We have generated several new forms of ASC-MUC1: (a) with truncated NH2- and COOH-terminal domains, thus primarily consisting of the tandem repeat region; (b) enzymatically desialylated; and (c) desialylated and further in vitro O-glycosylated with radiolabeled [3H]GalNac as a marker.

Materials and Methods

Antibodies. Anti-MUC1 monoclonal antibodies used in this study were VU-3C6 and BCP7. Antibody VU-3C6 was obtained from the culture supernatant of a hybridoma generously provided by Dr. Jo Hilgers (Department of Obstetrics and Gynecology, Academisch Ziekenhuis, Vrije Universiteit, Amsterdam, the Netherlands) and grown in our laboratory. BCP7 antibody was from Dr. Ian McKenzie (Austin Research Institute, Medical Center, Heidelberg, Germany). Epitopes for VU-3C6 and BCP7 at 1:1 ratio was used for immunodetection.

ASC-MUC1 Purification. ASC-MUC1 was purified according to a protocol published previously (14). Briefly, cell-free ascites fluid collected from patients with breast or pancreatic tumors was adjusted to pH 4.7 with 2 M acetic acid and stirred overnight in the cold. Precipitated proteins were removed by centrifugation, and the clear supernatant was collected and adjusted to pH 6.0 and concentrated 3-fold with Amicon Ultrafiltration Cell (Amicon, Inc., Beverly, MA) using a PM30 membrane with a Mf 30,000 cutoff for proteins. Concentrated preparation was run through a CM-Sephadex G-25 (Pharmacia Amersham Biotech AB, Uppsala, Sweden) column equilibrated with 50 mM sodium acetate (pH 6.0), and flow-through fractions containing unbound mucin and other proteins were collected. This preparation was subjected to equilibrium density-gradient centrifugation in a CsCl gradient as published previously (15). The final step in the purification was HPLC molecular sieving on a BioSep SEC-4000 column (Phenomenex, Torrance, CA). MUC1-containing fractions were pooled and extensively dialyzed against distilled H2O. The purity of the fractions was monitored by SDS-PAGE using 8% gels stained with Coomassie Brilliant Blue R-250 and immunoblotting.

Electron Microscopy-Molecular Replicas. Lyophilized ASC-MUC1 was solubilized in 0.5 M ammonium acetate, diluted 1:1 in glycerol, and then sprayed onto freshly cleaved mica. Mica was then transferred to a Cressington freeze-fracture machine and outgassed to 1 × 10^-7 Torr at room temperature for 5 h. The sample was shadowed while rotating in a plane oriented at a 5° angle with a platinum vapor stream for 12 s at 80 mA. Samples were overcoated with carbon at a 90° angle for 12 s at 100 mA. Replicas were floated onto double-distilled H2O and then collected onto 200-mesh copper grids. Micrographs were obtained on a JEOL JEM 1210 transmission electron microscope (Peabody, MA) at 80 kV.

SDS-PAGE and Immunoblots. SDS-PAGE was performed as described by Laemmli (16) under nonreducing conditions, using the Mini-Protein III (Bio-Rad Laboratories, Inc., Hercules, CA) electrophoresis system. Usually, polyacrylamide gels were 8%. Typical transfer onto nitrocellulose membrane was overnight at 30 V in Towbin buffer with methanol reduced to 10%. A mixture of anti-MUC1 monoclonal antibodies VU-3C6 and BCP7 at 1:1 ratio was used for immunodetection.

Monosaccharide Analysis. Monosaccharide analysis was performed on ASC-MUC1, which was quantitated by sample weight after drying overnight in dessicator under vacuum in the presence of KOH and P2O5. Known amounts of MUC1 preparations were resuspended in distilled H2O to a concentration of 1 mg/ml and stored frozen. For any given saccharide analysis, aliquots of ASC-MUC1 preparations were placed into glass tubes and dried again in a dessicator under vacuum in the presence of KOH and P2O5.

ASC-MUC1 preparations were hydrolyzed with 2 M trifluoroacetic acid at 121°C for 2 h. The sample was dried under stream of nitrogen with addition of tert-butanol and subjected to reduction in the presence of 1 M ammonium hydroxide containing sodium borohydride (10 mg/ml) at 4°C overnight. After the reduction step, borohydride was removed, and alditols were

Fig. 1 Schematic representation of ASC-MUC1 molecule. Vertical arrows, trypsin and/or clostripain (other than Arg-Pro bonds) cleavage sites that are outside the tandem repeat region (18). Horizontal arrows, the distance from potential cleavage site to the tandem repeat region. *, potential site of N-glycosylation (18).
O-acetylated by the addition of acetic acid anhydride and pyridine in a 1:1 ratio and heated for 20 min at 121°C. The sample was dried under a stream of nitrogen and dissolved in chloroform. Acetylated alditols of monosaccharides were analyzed by gas chromatography-electroionization mass spectrometry analysis (MD800; Fisons, Manchester, United Kingdom) on 15-m DB5 capillary column heated from 50°C to 250°C (7.5°C/min.). The alditol acetates were registered by monitoring the total ion current and single ions at m/z 145, 217, 289, and 361 (hexose and pentose) or at m/z 144, 216, 288, and 360 (N-acetyhexosamine).

O-Linked Glycans Profiling. Oligosaccharide profiles were analyzed after hydrazinolysis of salt-free, dry mucin samples. Glycans were liberated in hydrazine (Glyco, Novato, CA) for 5 h at 60°C, dried, and re-N-acetylated according to instructions from Glyko. The reducing glycans were labeled by reductive amination using a mixture of 2-aminobenzamide (1 M in acetic acid) and sodium cyanoborohydride (2 M in DMSO), 2:3 v/v, and incubation at 60°C for 2 h. The labeled glycans were separated from excess of fluorescent dye by paper chromatography and finally extracted into 500 µl of water. 2-AB-labeled glycans were chromatographed on normal-phase HPLC on polymer-based amino-phase column (astec; 0.45 × 250 mm) using a gradient of 250 mM aqueous formate in acetonitrile from 20% to 60% over a period of 90 min. Eluting oligosaccharides were registered by on-line detection with a fluorescence detector RF-10A-xl (Schimadzu, Kyoto, Japan) at excitation 330 nm (excitation) and 420 nm (emission). The mixture was subjected to Western blot analysis after separation on SDS-PAGE.

Trypsin Digestion. ASC-MUC1 preparations were dissolved in 8 M urea and extensively dialyzed against 10 mM Tris-HCl buffer (pH 8.8). Two µl of trypsin (Promega Corp., Madison, WI) were added per 120 µg of ASC-MUC1 preparation. The mixture was incubated for 24 h at 37°C. The digest was subjected to Western blot analysis after separation on SDS-PAGE.

Clostripain Digestion. Prior to proteolytic digestion, clostripain was activated for 3 h at room temperature in the presence of 5 mM DTT and 0.5 mM calcium acetate. ASC-MUC1 was resuspended in 10 µl of 25 mM sodium phosphate buffer (pH 7.5), containing 5 mM DTT and 0.2 mM calcium acetate, and mixed with activated clostripain. Reaction mixture was incubated for 24 h at 37°C. Digestion was terminated by adding 10 mM EDTA, and the digest was subjected to Western blot analysis after separation on SDS-PAGE.

Neuraminidase Treatment. Neuraminidase from Clostridium perfringens was purchased from Calbiochem-Novobiochem Corp. (La Jolla, CA). Three hundred µg of ASC-MUC1 preparation were treated with 50 milliunits of neuraminidase in 100 mM phosphate buffer (pH 6.0) at room temperature for 4 h in a total volume of 55 µl.

In Vitro Glycosylation. Peptidyl GalNAc transferases were isolated from breast tumor cells according to the protocol published by Stadie et al. (17). Briefly, 0.5 ml of a pellet of MDA-MB-231 cells was resuspended in 2.5 ml of extraction buffer (0.25 M sucrose, 1 mM EDTA) at 4°C for 10 min. The lysate was centrifuged for 20 min at 1000 × g, and the supernatant was mixed with 2.5 ml of 0.1 M imidazole buffer (pH 7.2), supplemented with 10 mM MnCl₂ and 200 mM NaCl. After 10 min of stirring, the whole mixture was centrifuged for 20 min at 5000 × g. The pellet was collected and resuspended in 4 ml of imidazole buffer with 10 µl of Triton X-100. The lysate was cleared by 1-h ultracentrifugation (120,000 × g), and supernatant containing GalNAc transferases was concentrated with a Centrastir (Millipore, Milford, MA).

The reaction mixture consisted of 50 µg of a substrate, 5 µl of [³H]UDP-GalNAc (7.8 Ci/mol) as a cosubstrate, and 25 µl of the preparation of peptidyl GalNAc transferases isolated from MDA-MB-231 cells. The reaction was carried out for 2 h at 37°C and terminated with 20 mM tetraborate buffer (pH 9.2) containing 1 mM EDTA. After the reaction was terminated, samples were passed through small columns filled with 0.5 ml of Dowex 1 × 8 (Fluka Chemie AG Buchs, Switzerland) to remove unincorporated [³H]UDP-GalNAc. One fifth of the eluant was mixed with 3 ml of scintillation fluid (Ecolite +; ICN Biomedicals, Inc., Irvine, CA), and incorporated radioactivity was measured in a 1600TR Liquid Scintillation Analyzer (Packard Instruments Co., Meriden, CT).

Results

ROd-like Structure and Heterogeneity of ASC-MUC1 Visualized by Electron Microscopy. A unique feature of the MUC1 molecule, schematically depicted in Fig. 1, is the rod-like structure of the tandem repeat region, which keeps this transmembrane molecule extended from the cell surface in vivo. Electron micrographs of MUC1 previously, also reported for DuPAN-2 (8), showed elongated, rod-like as well as globular, "relaxed" structures of this glycoprotein. Electron micrographs of a sample of ASC-MUC1 purified by us consistently showed MUC1 molecules in a stretched, rod-like form (Fig. 2) of different lengths, 680 nm (Fig. 2A), 400 nm long (Fig. 2, B–E), and 280 nm long (Fig. 2F). The size heterogeneity is most likely attributable to the fact that this preparation is a mixture of ascites from multiple patients, having molecules that differ in the number of repeats in the tandem repeat region.

Analysis of the Carbohydrates O-Linked to ASC-MUC1. Quantitative analysis of monosaccharides and sialic acid (Table 1) showed that the content of hexoses and hexosamines in ASC-MUC1 was significantly lower than on normal MUC1. It was also much lower than on other forms of MUC1, such as that isolated from tumor cells grown in culture or from tumor tissue (4). The molar ratio of GlcNAc:GalNAc in ASC-MUC1 was 0.23, indicating a very low level of extended oligosaccharides. The w/w ratio of sialic acid:GalNAc in ASC-MUC1 was 1.05, which is lower than the highly sialylated (1.76) and higher than poorly sialylated (0.7) MUC1 forms isolated from T47D breast carcinoma cells (4). The presence of fucose in ASC-MUC1 makes this form similar to one isolated from MDA-MB-231 cells (4). The average level of glycosylation of ASC-MUC1 per tandem repeat cannot be calculated at this time because the average number of repeats in this heterogeneous preparation isolated from several patients is unknown.

Low monosaccharide contents was also reflected in the
Characterization of Soluble MUC1 in Carcinomas

oligosaccharide yields after hydrazinolysis. Normal-phase HPLC of fluorescently labeled glycans revealed the presence of three major sialylated species: NeuAco2-3Galβ1-3GalNAc (~50%), NeuAco2-3Galβ1-3(NeuAco2-6)GalNAc (~25%), and NeuAco2-3Galβ1-4GlcNAcβ1-6(NeuAco2-3Galβ1-3)-GalNAc (~10%; Table 2). Neither neutral nor any fucosylated glycans were detected. An additional NeuAc-containing glycan eluting after the fraction of disialylated glycans was not identified and found to make up ~6% of the total glycans. In summary, the glycosylation profile of ASC-MUC1 appears to be dominated by acidic, sialic acid-containing oligosaccharides. Density estimations of O-glycosylation after deglycosylation/alkylaminylation in ethylamine and matrix-assisted laser desorption ionization mass spectrometry of proteolytic fragments revealed that only unsubstituted and disubstituted tandem repeat peptides were obtained by clostripain cleavage (data not shown). Again, this strongly favors the assumption that ascerts MUC1 is O-glycosylated at low density.

Susceptibility to Trypsin Digestion. Each MUC1 tandem repeat contains one arginine-proline bond (see Fig. 1 for the sequence), which makes this domain trypsin resistant. Trypsin-resistant bonds within each tandem repeat and trypsin-susceptible bonds located 76 amino acids NH2-terminal (Lys-Asn) and 54 amino acids COOH-terminal (Arg-Ser) to the tandem repeat region (18) allow the digestion of ASC-MUC1 with this protease to obtain a truncated form primarily consisting of the tandem repeat region (see Fig. 1). We also expected that such treatment would remove three of five putative sites for N-glycosylation located COOH-terminal to the tandem repeats. ASC-MUC1 preparation subjected to trypsin digestion contained three forms of mucin that are different in their mobility in 8% SDS-PAGE (Fig. 3, Lane A). The major and fastest migrating species shows a molecular weight higher than Mr 185,000 molecular weight standard protein. Two other species migrate more slowly. This preparation was digested for 24 h at 37°C and analyzed by Western blot after separation on 8% SDS-PAGE gel. After trypsin cleavage, the two high molecular weight forms disappeared, major species were partially degraded, and two new species with apparent molecular weights of Mr 160,000 and Mr 110,000, respectively, were generated (Fig. 3, Lane B).

Susceptibility to Clostripain Digestion. Clostripain is an Arg-C protease that cleaves Arg-Pro bonds that are otherwise resistant to trypsin cleavage (see Fig. 1). However, these bonds become resistant to clostripain cleavage if the tandem repeats are O-glycosylated. A sample of ASC-MUC1 (Fig. 3, Lane A) was subjected to 24-h clostripain digestion at 37°C and analyzed by Western blot after separation on 8% SDS-PAGE gel. Again, the two high molecular weight forms disappeared, and major species were partially degraded, although to a lesser extent than after trypsin digestion. Clostripain-digested ASC-MUC1 was reanalyzed by separation on 4–15% SDS-PAGE gradient gel (Fig. 3, Lane D). Several species with molecular weights ranging from Mr 150,000 to Mr 24,000 were visualized. Separation of the clostripain-digested ASC-MUC1 on a gradient gel resulted in further separation of a major species to two closely migrating ones. This result indicates that these smaller species represent fragments of larger molecules that were not glycosylated sufficiently to prevent clostripain digestion.

Enzymatic Desialylation of ASC-MUC1. ASC-MUC1 was subjected to enzymatic desialylation using α2-3,6-neuraminidase from Clostridium perfringens. This enzyme catalyzes the hydrolysis of terminal α2,3 and α2,6 sialic residues from complex carbohydrates and glycoproteins. Neuraminidase-treated and nontreated samples were separated on 8% SDS-PAGE gel, and MUC1 was detected by immunoblotting. The results presented in Fig. 4 show that all neuraminidase-treated species of ASC-MUC1 migrated more slowly in SDS-PAGE compared with the nontreated species. Slower electrophoretic mobility of neuraminidase-treated MUC1 is attributable to removal of negatively charged sialic acid. A similar effect has been observed previously by us for MUC1 immunoprecipitated from neuraminidase-treated cells and by others (19, 20). An observed equal shift of all neuraminidase-treated MUC1 species suggests that the relative difference in electrophoretic mobility between these ASC-MUC1 species is attributable to factors other than sialic acid contents.

ASC-MUC1 Can Be Further Glycosylated in Vitro Only in the Absence of Sialic Acid. To determine whether ASC-MUC1 can be further glycosylated, 100-μg samples of ASC-MUC1 that were untreated, trypsin digested, and enzymatically

Table 1 Monosaccharide composition analysis of glycans on ASC-MUC1

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>ASC-MUC1</th>
<th>Amounts in μg per 100 μg of mucin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuc</td>
<td>1.02</td>
<td>1.19</td>
</tr>
<tr>
<td>Man</td>
<td>1.19</td>
<td>0.984</td>
</tr>
<tr>
<td>Gal</td>
<td>0.984</td>
<td>0.81</td>
</tr>
<tr>
<td>GalNAc</td>
<td>0.81</td>
<td>0.19</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>0.19</td>
<td>0.34</td>
</tr>
<tr>
<td>NeuAc</td>
<td>0.34</td>
<td>0.85</td>
</tr>
</tbody>
</table>

4 J. Magarian-Blander and O. J. Finn, unpublished data.
Table 2  Profiles of O-linked glycans to the tandem repeats of ASC-MUC1

<table>
<thead>
<tr>
<th>Oligosaccharide structure</th>
<th>% of total O-linked glycans</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuAcα2-6GalNAc-Galβ1-3</td>
<td>31</td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-3GalNAc-</td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-6NeuAcα2-3Galβ1-3GalNAc-</td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-6GalNAc-NeuAcα2-3Galβ1-3</td>
<td>10.7</td>
</tr>
<tr>
<td>Non-identified, sialic acid-containing glycan</td>
<td>6.2</td>
</tr>
</tbody>
</table>

The exact position of NeuAc is not known.

Discussion

We report in this study the results of quantitative carbohydrate analysis of ASC-MUC1 and its further enzymatic modifications on the carbohydrate and protein levels. The preparation used in our experiments represents a native, soluble, form that was derived from a mixture of ascites fluids obtained from several patients. One important observation is that the density of O-linked oligosaccharides on the tandem repeats of ASC-MUC1 is lower than expected. Muller et al. (21) previously reported that soluble form of MUC1 isolated from culture supernatant of T47D breast cancer cells is glycosylated at high density with an average 4.8 GalNAc residues per one repeat and O-linked saccharide chains are short. This further means that almost all potential sites of O-glycosylation were substituted, yet the w/w ratio of saccharides:protein is low in this form. The authors postulate that high density O-glycosylation of MUC1 with short oligosaccharide chains is a characteristic of other tumor cell lines.

In contrary, normal MUC1, which by w/w ratio of saccharides to protein is considered highly glycosylated, has substituted only half of potential sites of glycosylation and O-linked oligosaccharides are long and branched. Our preparation of MUC1 contained only nonglycosylated or diglycosylated tandem repeats (data not shown). One possible explanation of this fact is that tumor cells in vivo produce much higher levels of

Fig. 3  Western blot analysis of proteolytically altered ASC-MUC1. ASC-MUC1 before (Lane A), after 24 h trypsin digestion (Lane B), and after 24 h clostripain digestion (Lane C). Samples were separated by SDS-PAGE in 8% gel under nonreducing conditions. Clostripain digested ASC-MUC1 re-analyzed by SDS-PAGE in 4–15% gradient gel under nonreducing conditions (Lane D). The membranes were blotted using a mixture of 3C6 and BCP7 anti-MUC1 monoclonal antibodies in 1:1 ratio.

Fig. 4  Western blot analysis of neuraminidase-treated ASC-MUC1. ASC-MUC1 before (Lane A) and after 4 h treatment with neuraminidase (Lane B). Samples were separated by SDS-PAGE in 8% gel under nonreducing conditions. The membranes were blotted using a mixture of 3C6 and BCP7 anti-MUC1 monoclonal antibodies in 1:1 ratio.

5 S. Müller and F-G. Hanisch, Recombinant MUC1 VNTR fusion protein expressed in human breast cancer cells reveals strongly fluctuating individual profiles of mainly core 2-based O-linked glycans, submitted for publication.
Characterization of Soluble MUC1 in Carcinomas of MUC1 isolated from MCF-7 cells. Muller bond, His-Gly to Val-Arg, has been found in the tandem repeats by trypsin but not by clostripain digestion are most likely tandem repeat regions. Observed smaller fragments generated molecular weights around M_r 200,000, M_r 160,000, and M_r 200,000 species is a truncated form containing O-glycosylated

Fig. 5 In vitro glycosylation of ASC-MUC1 using GalNAc transferases isolated from MDA-MB-231 breast tumor cells and [3H]GalNAc as a cosubstrate. Three forms of ASC-MUC1 were used as a substrate: desialylated with neuraminidase (ASC-Ne); trypsin digested (ASC-Tr); and nontreated (ASC-MUC1). Synthetic MUC1, a 100-mer peptide, was concurrently used as a positive control. Glycosylation is expressed as a number of cpm representing incorporated [3H]GalNAc.

of sialyltransferases than those in in vitro culture, which is reflected by the low density O-glycosylation and short oligosaccharide chains that are highly sialylated. This agrees with our observation discussed below, that the presence of sialic acid on the already O-linked glycans interferes with initial step of glycosylation of the neighboring sites.

Quantitative analysis of monosaccharides showed that ASC-MUC1 contains fucose residues, although we have not found fucosylated oligosaccharide species in our preparation. There are two possible explanations of this discrepancy. Either the level of oligosaccharide species containing fucose was under detection threshold of HPLC detector, or fucose detected in the monosaccharide analysis originated from N-linked glycans.

To further modify ASC-MUC1, we took advantage of several already known structural features of the MUC1 glycoprotein: (a) the repeats have an Arg-Pro bond that is trypsin resistant, whereas the NH_2- and COOH-terminal domains have trypsin-susceptible cleavage sites. Trypsin digestion also removes three of five potential sites of N-glycosylation (Fig. 1); (b) this Arg-Pro bond, although normally susceptible to clostripain digestion, becomes resistant when the tandem repeat of MUC1 is glycosylated beyond Tn antigen (GalNAc-Ser/Thr).

Trypsin digestion generated three MUC1 species with molecular weights around M_r 200,000, M_r 160,000, and M_r 100,000, respectively. The M_r 200,000 species was also generated by clostripain digestion, and in both cases, species migrating slower in SDS-PAGE disappeared. We conclude that this M_r 200,000 species is a truncated form containing O-glycosylated tandem repeat regions. Observed smaller fragments generated by trypsin but not by clostripain digestion are most likely attributable to amino acid replacement in the tandem repeats. One such replacement introducing a new trypsin-susceptible bond, His-Gly to Val-Arg, has been found in the tandem repeats of MUC1 isolated from MCF-7 cells. Muller et al. (21) reported that frequency of sequence variants in the amino acid sequence of the polypeptide tandem repeat region can be as high as 50%. We conclude from our data, however, that substitution of Pro residues located COOH-terminally to Arg residue is much less frequent than other reported errors. In addition to the major M_r 200,000 species, clostripain digestion generated several other fragments with smaller molecular weight. These smaller fragments were generated by the cleavage of Arg-Pro bonds within the tandem repeat region, which were not protected by O-glycosylation.

Neuraminidase treatment of ASC-MUC1 removed α2,3 and α2,6 sialic residues, reflected by a slower electrophoretic mobility (less negative charge) in SDS-PAGE. The neuraminidase-treated form of ASC-MUC1 could be successfully further glycosylated by peptidyl GalNAc transferases isolated from MDA-MB-231 breast tumor cells, whereas forms that still carry sialic acid could not. This result indicates that ASC-MUC1 has multiple serine and threonine residues that have not been glycosylated with GalNAc. Thus, the presence of sialic acid on glycosylated serines and threonines prevents even the first step of glycosylation of other serine and threonine residues. This observation is in agreement with relatively low w/w ratio of saccharides:glycoprotein in this preparation.

In summary, we have found that the soluble form of MUC1 in serum and/or ascites fluid is glycosylated at lower levels than suggested previously, but this amount of glycosylation still prevents efficient processing of this antigen by APCs. Furthermore, our in vitro glycosylation experiments showed that ASC-MUC1 has serine/threonine residues still available for the initial step of O-glycosylation, transfer of GalNAc residues, which can occur only in the absence of sialic acid on already glycosylated residues. Enzymatic modifications have generated additional forms of soluble MUC1 that might be taken up and processed more efficiently by APCs. This will allow us to study the positive and negative effects of more extensive glycosylation of this form on antigen processing.

References


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