Biosynthesis of a low-molecular-mass rat submandibular gland mucin glycoprotein in COS7 cells

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We have examined the biosynthesis of a low-molecular-mass mucin from rat submandibular gland (RSMG) expressed recombinantly in COS7 tissue culture cells, focusing primarily on the addition of carbohydrate to the protein core of the mucin. We find evidence for N-linked glycosylation, but this modification is not required for secretion of the mucin. Similarly, although the recombinant RSMG mucin, like its native counterpart, contains large amounts of O-linked carbohydrate, chain extension beyond the initial O-linked GalNAc moiety is not required for secretion. We have identified partially glycosylated mucin by a combination of metabolic pulse–chase and lectin precipitations of the biosynthetic intermediates. Our results suggest that the addition of GalNAc to threonine and serine in the RSMG mucin does not occur simultaneously, as has been described for other O-glycosylated proteins.

INTRODUCTION

Mucin glycoproteins are a principal component of mucus, the slimy, visco-elastic coat that protects all mucosal surfaces of the body [1]. The unique physicochemical properties of mucins are attributed, in part, to the presence of multiple oligosaccharides that are attached via O-glycosidic linkage between GalNAc and serine and threonine residues of the apomucin. Interactions between clusters of O-linked GalNAc and the core protein stiffen the molecule, leading to a characteristic extended 'rod-like' mucin structure [2]. Since O-glycosylation proceeds in a stepwise manner, the addition of the initial GalNAc represents both the initial regulatory step in mucin glycosylation and a key structural determinant of mucin function.

Mucin suprastructure is stabilized by both non-covalent and covalent forces. The marked expansion of mucin shape leads to significant molecular 'overlap' and interweaving among carbohydrate side chains, and non-covalent interactions among these oligosaccharides plays a significant role in imparting a high viscosity to mucins in solution [3,4]. The suprastructure of higher-molecular-mass mucins is further stabilized through the formation of disulphide-bond-mediated oligomerization [5,6].

The biosynthesis of several high-molecular-mass mucins has been examined [7–10], and each of these studies underscores the complexity of the process. For example, in some but not all cases, N-linked glycosylation must precede appropriate formation of disulphide-bonded subunits [8]. In contrast, no studies have been reported that have examined the biosynthetic events of a low-molecular-mass mucin. Recently, the protein backbone of rat submandibular gland (RSMG) mucin has been cloned [11]. The RSMG apomucin is a low-molecular-mass species, consisting of only 322 amino acids. In common with other secreted mucins, the RSMG mucin is highly glycosylated, with greater than 60% carbohydrate content by mass. In addition to the multiple sites for the acquisition of O-glycans, one potential N-glycosylation site is present. Although there is no sequence similarity between the RSMG apomucin and human low-molecular-mass salivary mucin (MG-2) [12], architecturally these two mucins are quite similar. Each contains an N-terminal region that has relatively few potential O-glycosylation sites arranged in a non-repeating manner. Two non-conserved cysteine residues are found in this region. The central portion of both the RSMG apomucin and MG-2 is dominated by the presence of tandem repeats that are highly enriched in hydroxyamino acids and proline. Thus the majority of potential O-glycosylation sites are located in the tandem repeat region. The C-terminal region of the RSMG apomucin and MG-2 is serine- and threonine-rich, but is relatively low in proline content. No obvious repeat motif is observed in the C-terminus; rather, long stretches of serines and threonines are observed [13].

Unfortunately, there is no suitably differentiated cell line in which to study the biosynthesis of RSMG mucin. Therefore we have transiently expressed RSMG apomucin in a well-defined heterologous system (COS7 cell) as an initial step in defining the biosynthetic pathway of this class of mucin. We find that the recombinant mucin is heavily glycosylated and is secreted. Neither N-glycosylation nor disulphide-mediated cross-linking events are necessary for secretion to occur. Through the identification of partially glycosylated intermediates we have determined that the addition of GalNAc occurs sequentially rather than simultaneously, as has been described for other O-glycosylated proteins.

MATERIALS AND METHODS

Vectors, primers and reagents

To facilitate identification and purification of recombinantly expressed RSMG mucin, we engineered both a FLAG™- epitope tag and a heart muscle kinase site at the carboxy-terminus of the apomucin by reverse transcriptase PCR amplification of the apomucin coding region from RSMG cDNA using the primers apo1, 5′-CCG CTG CAG ATG AAA AGG GAA ACT TTC ATC TTG GGC-3′, and apo3, 5′-AAC ACC TAG GAG CAT

Abbreviations used: conA, concanavalin A; RSMG, rat submandibular gland; DMEM, Dulbecco's modified Eagle's medium.

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CAT ACC AAA GTA CAT CTG AAG CCA CTG AAG-3′ [14]. The resulting PCR product was cut with *Pst*I and *Avr*II and cloned into the complementary sites in pKN4 [15], which is based on the expression vector pcDL-SRα296 [16] (DNAX Research Institute of Molecular and Cellular Biology Inc.), to create pKN20, the apomucin expression vector.

Cell culture

All cell-culture reagents were from Life Technologies. COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (w/v) fetal-bovine serum and passaged twice a week. Cells at approx. 70 % confluency were transfected with Wizard Midiprep (Promega) DNA using lipofectamine and the protocol supplied by the manufacturer. At 24 h post-transfection, the cells were washed once, then incubated for 30 min in methionine-free DMEM. Pulse media contained 200 μCi/ml [^{35}S]methionine (Trans-label Express, NEN). After labelling, the chase was initiated by replacement with DMEM/10 % fetal-bovine serum containing 5 mM methionine. Cells were lysed in ice-cold PBS containing 0.2 % (v/v) SDS, 1 % (v/v) Triton X-100 and 1 mM PMSF (lysis buffer).

Immunoprecipitation, glycosidase digestion and analysis of glycans

Nuclei were removed from the lysed cells by centrifugation at 16000 ***g*** for 15 min and the lysates were immediately immunoprecipitated with anti-FLAG™- agarose (IBI). Following an overnight incubation at 4 °C, the immunoprecipitates were washed twice with lysis buffer and the bound material was eluted from the agarose beads by boiling for 15 min in 100 mM Tris/HCl (pH 7.6)/50 mM NaCl/0.2 % SDS/2 % (v/v) 2-mercaptoethanol. The resulting eluant was diluted 10-fold into PBS/1 % Triton X-100 containing 0.5 mg/ml BSA, and then either re-immunoprecipitated overnight with anti-FLAG™-agarose or precipitated with the lectin VVA (*Vicia villosa*) conjugated to agarose beads (E-Y Laboratories). The bound material was resuspended in 2 × Tricine gel loading buffer, heated to 65 °C for 30 min and run on an 8.25 % (w/v)/4 % (w/v) Tricine gel as described by Schägger and von Jagow [17].

Alternatively, following the first immunoprecipitation, the antibody–agarose-bound mucin was digested in a volume of 25 μl with α-*N*-acetylgalactosaminidase (Oxford Glycosystems), according to the recommendations of the manufacturer, before release from the antibody agarose beads by the addition of 25 μl of 2 × elution buffer. The digested material was then either re-immunoprecipitated with anti-FLAG™-agarose or lectin-precipitated as described above.

For analysis of the secreted mucin, COS7 cells were either metabolically labelled with Trans-label Express (Dupont–NEN) for 4 h or the media were collected 72 h post-transfection and immunoprecipitated, then labelled with heart muscle kinase [15]. Briefly, following immunoprecipitation of the culture media and washing [PBS, 0.5 % (v/v) Tween-20], the antibody agarose-bound material was resuspended in 30 μl of 20 mM Tris/HCl (pH 7.6)/75 mM NaCl/15 mM $MgCl_2$ containing 5 μCi of [γ-^{32}P]ATP/5 μl (5 units) of heart muscle kinase (Sigma), freshly reconstituted, and incubated for 1 h at 37 °C. The labelled immunoprecipitates were washed again and analysed by Tricine gel electrophoresis as described above. Glycosidase digestions were performed on the labelled antibody–agarose-bound immunoprecipitates in a volume of 25 μl, according to the recommendations supplied by the manufacturer (Oxford Glycosystems). Secondary lectin precipitations were performed on substrate that was eluted from a primary anti-FLAG™- immunoprecipitation using agarose-bound lectins concanavalin A (conA), PSA (*Pisum sativum*), and PHA-E (*Phaseolus vulgaris*) (EY Laboratories), as described for intracellular mucin biosynthetic intermediates above.

RESULTS AND DISCUSSION

Expression and characterization of recombinant RSMG mucin in COS7 cells

To facilitate the analysis of RSMG apomucin expression in COS7 cells, the recombinant protein was recovered by immunoprecipitation and radiolabelled with ^{32}P using heart muscle kinase. The predominant radiolabelled immunoprecipitated species displayed an apparent molecular mass of over 100 kDa on denaturing Tricine/SDS/PAGE (Figure 1, lane 1). As expected [18], although there are two potential sites for disulphide bond formation, the RSMG mucin, unlike higher-molecular-mass mucins, did not multimerize and migrated as a monomer under non-reducing conditions (Figure 2). Glycosidase digestion of this material suggested that it is heavily O-glycosylated. Mild-acid hydrolysis or sialidase treatment resulted in a decrease in mobility during SDS/PAGE, presumably due to the removal of charged sialic acid (Figure 1, compare lanes 2 and 4 with lane 1). This suggests that the terminal sialic acid moieties contribute little to the aberrant mobility of the mature mucin compared with the predicted mobility of the protein core. Subsequent treatment of sialidase- or acid-hydrolysed material with *O*-glycanase (Figure 1, lanes 3 and 6) or with a combination of β-galactosidase and α-*N*-acetylgalactosaminidase (Figure 1, lane 7) resulted in an increase in the mobility of the mucin. The glycosidase-digested material (lanes 3, 6 and 7, Figure 1) migrated as a diffuse 'smear', which may have been due to incomplete enzymic removal of the carbohydrate side chains and/or due to the microheterogeneity of the sample; proteolysis of the sample was ruled out by identical digestions with a control protein containing no oligosaccharide (results not shown). In addition, substituents on a sub-fraction of the sialic acid residues may render them resistant

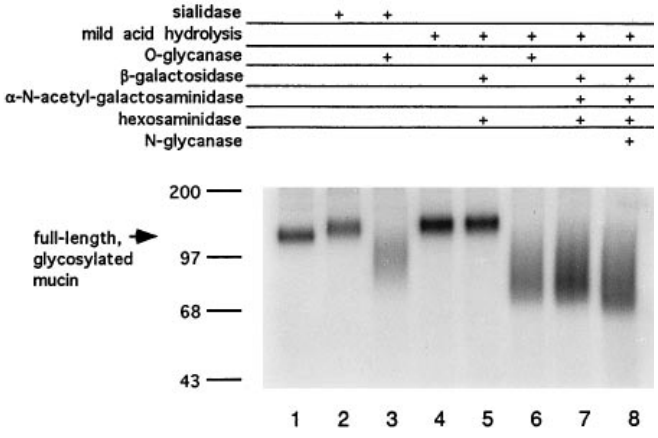

Figure 1 Glycosidase digestion of recombinant RSMG mucin

Mucin, immunoprecipitated with the anti-FLAG™-antibody from the media of COS7 cells transfected with the expression vector pKN20, was radiolabelled and then digested with various glycosidases. Lane 1, no glycosidase; lane 2, sialidase; lane 3, sialidase and *O*-glycanase. Materials run in lanes 4–8 were first subjected to mild acid hydrolysis and then treated as indicated: lane 4, no glycosidase; lane 5, β-galactosidase and hexosaminidase; lane 6, *O*-glycanase; lane 7, β-galactosidase, α-*N*-acetylgalactosaminidase, and hexosaminidase; lane 8, β-galactosidase, α-*N*-acetylgalactosaminidase, hexosaminidase, and *N*-glycanase. All reaction products were separated by Tricine/SDS/PAGE. Molecular-mass markers are given in kDa.

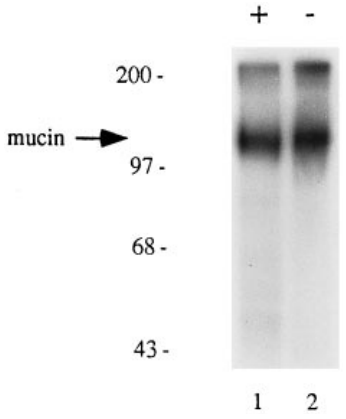

Figure 2 Tricine gel analysis of labelled mucin under reducing (lane 1) or non-reducing (lane 2) conditions

Dithiothreitol was omitted from the heart muscle kinase reconstitution buffer used to label the immunoprecipitated mucin, and 2-mercaptoethanol was omitted from the Tricine gel loading dye mix of the sample in lane 2. Molecular-mass markers are given in kDa.

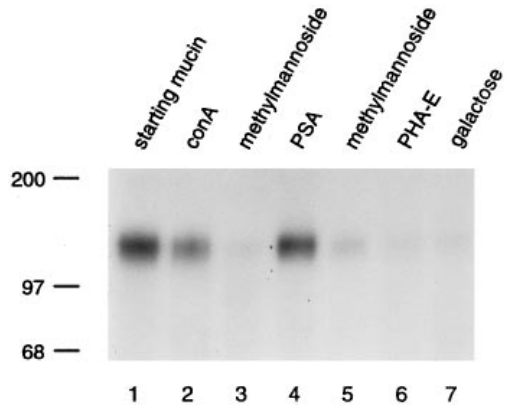

Figure 3 Lectin precipitations of RSMG low-molecular-mass mucin produced in COS7 cells

Mucin was immunoprecipitated with anti-FLAG™-antibody and then radiolabelled with ^{32}P using heart muscle kinase. The mucin was then eluted from the antibody and either re-immunoprecipitated (lane 1), or precipitated with concanavalin A (conA) agarose (lanes 2 and 3), PSA agarose (lanes 4 and 5) or PHA-E agarose (lanes 6 and 7). The saccharides α-methylmannoside (lanes 3 and 5) or galactose (lane 7) were used at 100 mM to inhibit lectin binding and demonstrate specificity. All precipitated materials were then analysed by Tricine/SDS/PAGE. Molecular-mass markers are given in kDa.

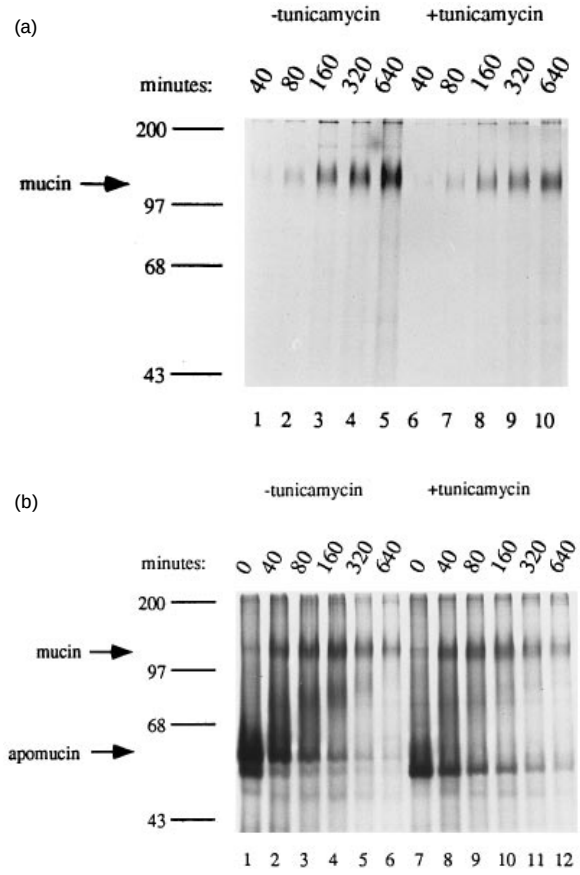

Figure 4 Pulse–chase maturation of RSMG low-molecular-mass mucin in normal and in tunicamycin-treated COS7 cells

Mucin in cell medium (**a**) and lysates (**b**), from untreated COS7 cells or cells treated before and during the pulse–chase with tunicamycin, was immunoprecipitated using anti-FLAG™-antibody and then separated by Tricine/SDS/PAGE. Molecular-mass markers are given in kDa.

to enzymic removal, since *O*-glycanase digestion appeared more complete following mild-acid hydrolysis than sialidase treatment (Figure 1, lane 3 versus lane 6). *N*-Glycanase digestion, used to assess whether the single potential site in the RSMG mucin was occupied, was inconclusive, due to the size heterogeneity of the sample, although the mobility of the resultant product apparently increased following this treatment (Figure 1, compare lanes 7 and 8). However, subsequent results demonstrate that it is likely that the RSMG mucin is N-glycosylated (see below), and the starting material could be precipitated with the lectin PSA (Figure 3, lane 4) and, to a lesser extent, conA (Figure 3, lane 2), although PHA-E lectin was unreactive (Figure 3, lane 6). As expected, inclusion of α-methylmannoside inhibited the precipitation of the mucin with both conA and PSA lectin (Figure 2, lanes 3 and 5).

Time course of RSMG mucin glycosylation and secretion

Glycosylation and secretion of the RSMG mucin was examined using a pulse–chase protocol. Cell lysate derived from transfected (with apomucin reporter) metabolically labelled COS7 cells harvested before the chase period contained a predominant anti-FLAG™-reactive species of approx. 63 kDa (Figure 4b, lane 1). Over the course of several hours, the 63 kDa species disappeared and a new species of greater than 100 kDa appeared (Figure 4b, lanes 2–6). At approx. 80 min the protein began to appear in the cell culture media (Figure 4, lane 2) and by 320 min release of the mature mucin was nearly complete (Figure 4a, lane 4). At intermediate times a 'smear' of material that could represent partially glycosylated mucin was detected (Figure 4b, lanes 2–5).

It should be noted that we observed an approx. 5-fold less recovery of the fully glycosylated mucin compared with the starting unglycosylated apomucin. One possible reason for this is that only a portion (approx. 20 %) of the apomucin is processed to yield secreted mucin. Alternatively, glycosylation may lead to a partial sequestration of the antibody recognition site, thereby diminishing the detectability of the FLAG™-epitope tagged mucin.

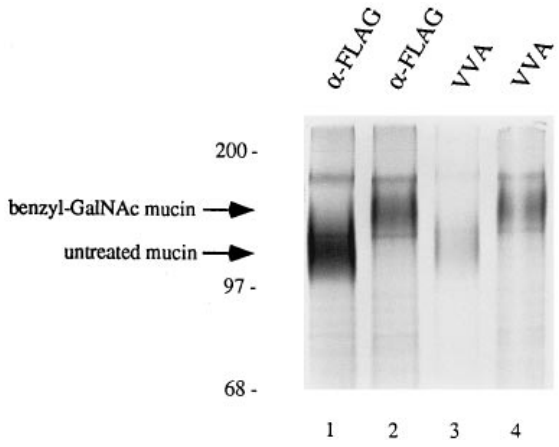

Figure 5 Lectin precipitation of RSMG low-molecular-mass mucin from COS7 cells treated with benzyl-*N*-acetyl-α-galactosaminide, an inhibitor of *O*-glycan chain extension

Metabolically labelled mucin was immunopurified with anti-FLAG™-agarose from the culture media of untreated (lanes 1 and 3) or benzyl-*N*-acetyl-α-galactosaminide-treated COS7 cells (lanes 2 and 4). Following desorption, the immunopure materials were either re-precipitated with anti-FLAG™-agarose (lanes 1 and 2) or precipitated with VVA, a lectin that recognizes unextended GalNAc in α-linkage to Ser/Thr in the protein core. All precipitated materials were then analysed by Tricine/SDS/PAGE. Molecular-mass markers are given in kDa.

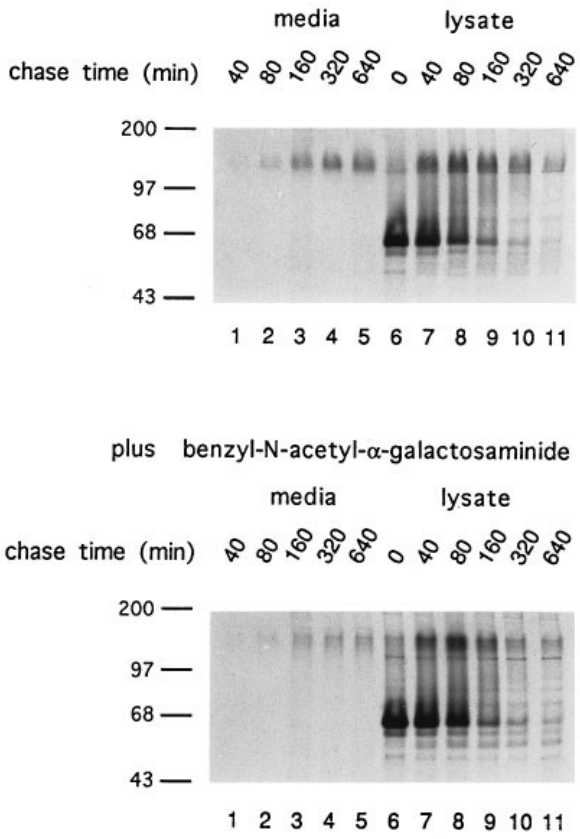

Figure 6 Pulse-chase study of maturation of RSMG low-molecular-mass mucin in untreated and benzyl-*N*-acetyl-α-galactosaminide-treated COS7 cells

Mucin from cell medium and lysates were immunoprecipitated with anti-FLAG™ from untreated COS7 cells or cells treated overnight before and during pulse–chase with benzyl-*N*-acetyl-α-galactosaminide, which inhibits the elongation of *O*-glycans. All precipitated materials were then analysed by Tricine/SDS/PAGE. Molecular-mass markers are given in kDa.

Role of N-linked glycosylation in RSMG mucin processing and secretion

To determine the extent to which N-glycosylation influences the addition of O-glycans, oligosaccharide processing, or secretion, we treated the COS7 cells with tunicamycin, an inhibitor of N-glycosylation, before and during the pulse–chase period. The greater mobility of the pre-chase material from tunicamycin-treated cells suggested that N-glycosylation occurs within the 20 min labelling period before the chase (Figure 4b, compare lanes 1 and 7). However, RSMG mucin processing appeared normal in the absence of N-glycosylation. The rate of sugar addition to the core protein (Figure 4b, lanes 7–12) and the appearance of the mature form of the protein in the media (Figure 4a, lanes 6–10) were essentially unchanged in the presence of tunicamycin.

A mucin that contains underextended oligosaccharides is secreted normally

Benzyl-*N*-acetyl-α-galactosaminide acts as a competitive inhibitor of UDP Gal:GalNAc-β1,3-galactosyltransferase and can prevent the extension of the initial sugar residue added during O-glycosylation [19–21]. Treatment of apomucin-transfected COS7 cells with aryl-*N*-acetyl-α-galactosaminide resulted in the production of a mucin that migrated with decreased mobility (Figure 5, lanes 2 and 4), resembling mucin from which the sialic acid had been removed (Figure 1, lane 4). To ensure the absence of carbohydrate chains that had been extended beyond the initial GalNAc residue, immunopurified recombinant RSMG mucin from untreated COS7 cells and from cells treated with aryl-*N*-acetyl-α-galactosaminide were precipitated a second time with the lectin VVA, which recognizes predominantly unsubstituted GalNAc α-linked to threonine or serine. The mucin purified from aryl-N-acetyl-α-galactosaminide-treated cells was recognized by the lectin VVA, whereas mucin from untreated cells was not (Figure 5, lanes 3 and 4). Recent evidence indicates that aryl-*N*-acetyl-α-galactosaminides may not fully inhibit carbohydrate extension under certain circumstances. Our data would fit a model where either extension or sialic acid addition was inhibited, given that a fraction of the carbohydrate chains were terminated at the initial GalNAc residue.

Although it has not been shown whether aryl-*N*-acetyl-α-galactosaminide-induced intermediate products are transported normally or broken down during secretion, we show here, using pulse-chase analysis, that the aryl-*N*-acetyl-α-galactosaminide-induced RSMG mucin is processed and secreted in the same manner in which mucin from untreated COS7 cells was. Thus mucin appears in the media by 80 min, continues to be produced for at least 5 h, and displays a reduced mobility upon maturation, consistent with a lack of terminal sialic acid (Figure 6). These data suggest that the observed decrease in mobility during RSMG mucin biosynthesis is due to the addition of the core GalNAc moiety and that extension (or sialation) of this core is not required for normal transport and secretion.

Addition of core GalNAc does not occur simultaneously

During pulse–chase analysis of RSMG mucin in untreated COS7 cells, we noted the appearance of intermediate-sized species that migrated between the unmodified and fully glycosylated material

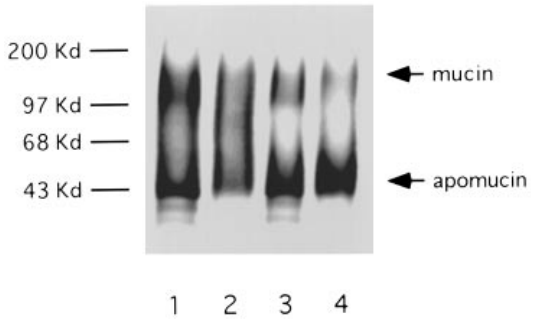

Figure 7 Isolation of partially glycosylated biosynthetic intermediates

RSMG low-molecular-mass mucin was purified from pKN20-transfected COS7 cell lysate following a 1 h pulse/1 h chase in order to examine intracellular intermediates that form during protein maturation. Immunoprecipitation with α-FLAG detected a range of intermediates with molecular masses ranging from that of unglycosylated to that of fully glycosylated mucin (lane 1). Precipitation with the lectin VVA detected only those intermediates containing terminal GalNAcα1-Ser/Thr (lane 2). Following treatment of the starting material with α-*N*-acetylgalactosaminidase, the α-FLAG-agarose (lane 3) and VVA lectin (lane 4) precipitations were repeated. Taken together, these results indicate that the broad-molecular-mass smear of material that is recognized by VVA (lane 2) and disappears upon treatment with α-*N*-acetylgalactosaminidase (lane 3) contains partially glycosylated biosynthetic intermediates where initial GalNAc transfer by UDP-*N*-acetylgalactosamine:polypeptide galactosaminyltransferase has not yet occurred at all of the potential acceptor sites.

on SDS/PAGE (Figure 4b, compare lanes 2 with 3 and 4). Since we have shown that the addition of the chain-initiating GalNAc to the protein core is responsible for the bulk of mobility change upon maturation, it is possible that these intermediates represent mucin containing both substituted and unsubstituted threonine and serine.

VVA lectin is known to react preferentially with GalNAcα1-0 Ser/Thr residues, and we have shown that it reacts specifically with mucin produced from cells treated with the chain-extension inhibitor benzyl-*N*-acetyl-α-galactosaminide [22]. To further explore the nature of the intermediates from untreated cells, we immunoprecipitated metabolically labelled mucin (60 min pulse with ^{35}S methionine followed by a 60 min chase period) using anti-FLAG™– antibody, desorbed the bound material from the immunoadsorbent and re-precipitated it with either anti-FLAG™– antibody a second time or with the lectin VVA. Under these labelling conditions, the potential intermediates were highly represented (Figure 7, lane 1) and could be preferentially brought down with VVA (Figure 7, lane 2). The apomucin protein core was noticeably less reactive (Figure 7, lane 2), and those intermediates where GalNAc addition had apparently reached completion were likewise not well recognized, as would be expected if mucin maturation had proceeded to the point of further carbohydrate chain extension.

When a third aliquot of the FLAG™-precipitated mucin was treated with α-*N*-acetylgalactosaminidase and then analysed by SDS/PAGE (Figure 7, lane 3), much of the intermediate-sized material was shifted to a position of greater mobility, corresponding to the apomucin, suggesting that a significant portion of this material represents partial O-glycosylation where the only sugar present on the protein core is GalNAc. When the α-*N*-acetylgalactosaminidase-treated material was precipitated with VVA, a significant amount of the material in the band co-migrating with the apomucin was brought down (Figure 7, lane 4). This is probably due to incomplete removal of the core GalNAc, as was observed during previous glycosidase digestions (Figures 1 and 2). The fact that VVA also recognizes a small fraction of the more mature, glycosidase-resistant mucin implies that some portion of the carbohydrate side chains on these molecules have not yet acquired substituents that would mask their affinity for VVA.

These results provide evidence for O-glycosylation intermediates that contribute to mucin microheterogeneity. In contrast with other systems, where the addition of the initial O-linked GalNAc has been reported to be simultaneous [23], we find evidence of initiation that is spaced in time. While this raises the interesting possibility that changes in transport time through the Golgi may alter the patterns of glycosylation, it remains to be determined if addition is also spaced in location and occurs in a hierarchical or random manner. The recent demonstration of multiple UDP-*N*-acetylgalactosamine:polypeptide galactosaminyltransferases [24,25] would suggest that the former is a possibility; i.e. each isoform acts on specific hydroxyamino acids of the protein backbone.

Prospects for recombinant mucins as therapeutics

Mucins produced by salivary glands play a significant role in protecting both the hard and soft tissues of the mouth [13]. The sensation of oral dryness (xerostomia) is a prevalent problem, particularly among the elderly, with estimates ranging from 12 to 29 % in different populations [26,27]. Individuals who experience a complete loss of salivary function are at great risk of developing xerostomia and other pathologies [26,28] due, in part, to the loss of the protective qualities provided by the mucins [29,30]. Native animal gastro-intestinal mucins have been used in various formulations of artificial saliva in an attempt to deliver adequate levels of mucins to compromised individuals with varied success [29,30].

While a number of high-molecular-mass apomucins have been cloned, the expression of such recombinant glycoproteins would be expected to be complicated by the diverse range of co- and post-translational processing steps that must occur. In contrast, our studies with the low-molecular-mass RSMG mucin suggest that its biosynthetic pathway is relatively simple and would thus represent a reasonable candidate for expressing in a heterologous system. The cost inherent in the use of mammalian cell host systems makes it unlikely that COS7 cells could be used for such a purpose. However, the successful overexpression of the UDP-*N*-acetylgalactosamine:polypeptide galactosaminyltransferase in Sf9 cells [31] suggests that such cells could be re-engineered to produce the desired recombinant glycoprotein at lower cost.

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