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Monoglucosylated glycans in the secreted human complement component C3: implications for protein biosynthesis and structure

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Abstract The monoglucosylated oligomannose *N*-linked oligosaccharide ($Glc_1Man_9GlcNAc_2$) is a retention signal for the calnexin–calreticulin quality control pathway in the endoplasmic reticulum. We report here the presence of such monoglucosylated *N*-glycans on the human complement serum glycoprotein C3. This finding represents the first report of monoglucosylated glycans on a human serum glycoprotein from non-diseased individuals. The presence of the glucose moiety in 5% of the human C3 glycoprotein suggests that this glycosylation site is sequestered within the protein and is consistent with previous studies identifying a cryptic conglutinin binding site on C3 that becomes exposed upon its conversion to iC3b.

Keywords: Complement component C3; Glycosylation; Monoglucosylation; Calnexin; Calreticulin

1. Introduction

The endoplasmic reticulum (ER) folding pathway is an ancient and highly regulated quality control system for protein biosynthesis [1]. It is notable for both its degree of conservation in eukaryotes and its efficiency in preventing the exit of misfolded proteins from the ER. Glycosylation has a critical role in protein folding and is consequently required for the efficient secretion of serum glycoproteins [1]. *N*-linked glycosylation occurs co-translationally by the transfer of $Glc_3Man_9GlcNAc_2$ to the side chain of asparagine found within the glycosylation sequon N-X-S/T (where X is not proline) of the nascent polypeptide [2]. The Glcα1-2Glcα1-3Glcα1-3 moiety is fully hydrolysed from proteins that exit the ER [1]. Monoglucosylation is the recognition signal for the chaperones calnexin and calreticulin and is maintained on misfolded glycoproteins by UDP–glucose glycoprotein:glucosyltransferase [3]. Misfolded, monoglucosylated glycoproteins are retained within the ER and undergo further folding events. α-glucosidase II activity permits secretion by removing the terminal α1-3-linked glucose (G3); correctly folded glycoproteins are not reglucosylated by the glucosyltransferase and thereby exit the ER and traffic to the Golgi for further carbohydrate processing [1,3]. Previously, the secretion of glucosylated glycoproteins in human sera has only been observed in rare pathological states such as glucosidase I deficiency (CDG type IIb) [4].

C3 is a central component of the innate immune system that, with the other complement proteins, forms a major host mechanism for the detection and clearance of potential pathogens [5,6]. C3 is the most abundant complement protein present in human plasma at 1.0–1.5 mg/ml [7]. Activation of the complement pathway by, for example, bacteria, results in the deposition of thousands of activated C3 (C3b) molecules on the microbe surface. C3b deposition facilitates phagocytosis by opsonising the target surface. In addition, C3b can associate with the C3 activating proteases (C3bBb and C4b2a, activating the alternative or classical pathway, respectively) to form the corresponding C5 convertases (C3bBb3b and C4b2a3b). The C5 convertases catalyse the formation of the 'Membrane Attack Complex', C5b-9, which inserts into lipid bilayers, forming pores that lead to cell lysis. Cell surface glycoproteins, such as CD46, CD55 and CD59, protect host cells by negatively regulating the complement cascade.

The majority of complement glycoproteins are synthesised in the liver [7]. However, plasma C3 has a small contribution (4.5%) from the kidney [8]. C3 is synthesised as a single polypeptide chain precursor, which is cleaved before secretion into a 70-kDa β-chain disulfide-linked to a 116-kDa α-chain; each chain has a single glycosylation site [9]. The structures of the carbohydrates found on mature secreted proteins are heavily influenced by the tissue-specific expression of glycan processing enzymes and also the molecular environment of individual glycosylation sites [2].

In this study, the chemical structures of the *N*-linked glycan species on a panel of liver-derived complement components are presented. Despite sharing a similar biosynthetic pathway, the glycosylation of C3 differs entirely from the other glycoproteins studied. Moreover, we present here the presence of a population of monoglucosylated glycans on the C3 α-subunit. This is the first report of such structures on mature glycoproteins in non-diseased human sera.

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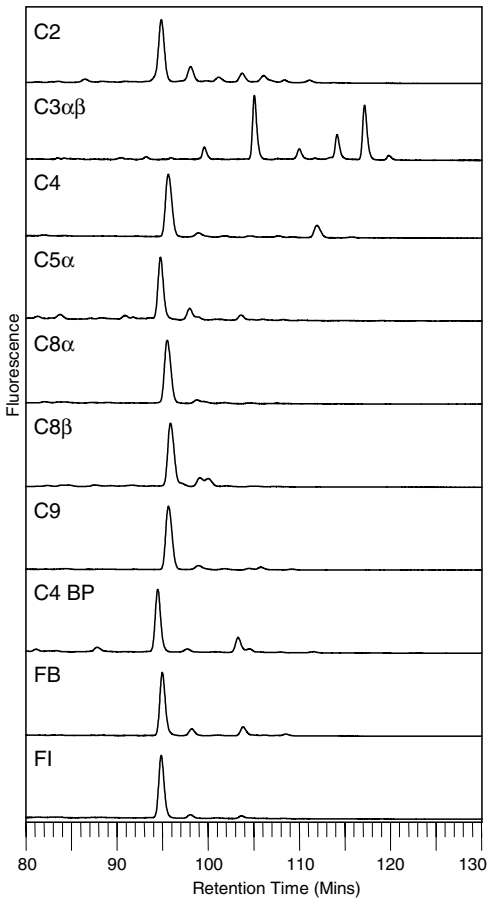

Fig. 1. NP-HPLC of desialylated 2AB-labelled *N*-glycans from a panel of human complement glycoproteins; C2, C3αβ, C4, C5α, C8α, C8β, C9, C4 BP, FB, and FI.

2. Materials and methods

2.1. Enzymes

Peptide *N*-glycanase F (PNGase F, EC 3.5.1.52) and exoglycosidase enzymes were obtained from Glyko Inc. (Upper Heyford, UK). Exoglycosidase digestions were performed as described by Rudd et al. [10] at the following concentrations with the buffers supplied by the manufacturer: Jack bean α-mannosidase (EC 3.2.1.24), 100 mU/ml; *Arthrobacter ureafaciens* sialidase, ABS (EC 3.2.1.18), 1–2 U/ml; almond meal α-fucosidase (EC 3.2.1.111), 3 mU/ml; bovine testes β-galactosidase, BTG (EC 3.2.1.23), 1–2 U/ml; *Streptococcus pneumoniae* β-hexosaminidase (EC 3.2.1.30), 120 U/ml; and *Aspergillus saitoi* α-mannosidase, ASM (EC 3.2.1.24), 1 mU/ml.

2.2. Purification of complement glycoproteins from human sera

Purification of complement proteins was performed according to the following: C3 and C4 [11]; Factor I (FI) [12]; Factor B (FB) [13]; C5 [14]; C4 binding protein (C4 BP) [15]; C2 [16]; C8 [17]; C9 [18].

2.3. Release of N-glycans

In-gel PNGase F digestion of purified glycoproteins. Oligosaccharides were purified for normal phase high-performance liquid chromatography (NP-HPLC) analysis from Coomassie-stained SDS–PAGE gels containing target glycoprotein following PNGase F digestion as described by Kuster et al. [19]. Briefly, relevant Coomassie blue-stained bands containing ~10 μg of glycoprotein were excised from reducing SDS–PAGE gels and washed with 20 mM $NaHCO_3$, pH 7.0. The washed gel bands were dried in a vacuum centrifuge before rehydration with 30 μl of 20 mM $NaHCO_3$, pH 7.0, containing 100 U/ml of PNGase F. After incubation for 12 h at 37 °C, the enzymatically released *N*-glycans were eluted with water extractions. Salts were removed with a 5-min incubation at room temperature with 50 μl of an acid-activated AG-50W X12 slurry (BioRad, 200–400 mesh), which was removed by filtration with a 0.45-μm pore-size filter (Millex-LH, hydrophobic polytetrafluoroethylene).

Release of serum protein N-glycans by hydrazinolysis. Pooled human serum was dialysed into 0.1% trifluoroacetic acid and 50 μl was lyophilized. *N*-Glycans were released by hydrazinolysis, re-*N*-acetylated, and purified as described by Butler et al. [20].

2.4. Fluorescent labelling and NP-HPLC

The released *N*-glycan samples were then dried and fluorescently labelled with 2-aminobenzamide (2AB) by reductive amination [21]. Dried aliquots of 2AB-labelled oligosaccharides were dissolved in a solution of 20 μl distilled water and 80 μl acetonitrile and analysed by NP-HPLC using a 4.6×250-mm Glycosep-*N* column (Glyko Inc.). The gradient was formed over 152 min with the percentage of solvent A in acetonitrile rising from 20% to 58% (where solvent A is 50 mM formic acid, adjusted to pH 4.4 with ammonia). The elution of the oligosaccharides was measured using a Jasco FP-260 fluorescence detector ($E_{ex} = 330$ nm, $E_{em} = 420$ nm) and calibrated in glucose units (GU) using a 2AB-labelled dextran hydrolysate. The elution peaks of each NP-HPLC trace were assigned GU values by comparison with the calibration ladder and preliminary structural assignments were made by reference to a database of known standards [22]. Each released *N*-glycan pool was subjected to exoglycosidase digestions to further sequence the oligosaccharide chains [10,22].

3. Results

3.1. Oligosaccharide analysis highlights the glycosylation of C3

Glycan analysis was performed on a panel of complement glycoproteins known to be synthesised mainly by hepatocytes [7,8]. Fig. 1 shows a stacked plot of the NP-HPLC traces of released, desialylated 2AB-labelled *N*-glycans. Comparison of these profiles shows that C3 is unusual among hepatocyte-derived glycoproteins in containing large oligomannose glycans (>6 GU).

3.2. C3 contains glucosylated oligomannose glycans

NP-HPLC of the 2AB-labelled glycan pool of whole C3 demonstrated the presence of six glycan species at 6.20, 7.05, 7.95, 8.82, 9.52, and 10.2 GU (Fig. 2). Reference to an extensive database of glycan standards [10,22], together with GU values reported in previous studies [23,24], identified the first five peaks as the oligomannose series $Man_{5\text{-}9}GlcNAc_2$ and the additional peak at 10.2 GU as the monoglucosylated glycan $Glc_1Man_9GlcNAc_2$. Glycan analysis of separated C3α and C3β subunits revealed that $Man_{8\text{-}9}GlcNAc_2$ and the putative $Glc_1Man_9GlcNAc_2$ were located on the C3 α subunit, whilst the C3β subunit contained the smaller $Man_{5\text{-}7}GlcNAc_2$ glycans (Fig. 2).

3.3. Exoglycosidase digestions confirm the presence of $Glc_1Man_9GlcNAc_2$ on C3

The entire pool of 2AB-labelled C3 glycans was susceptible to digestion by Jack bean α-mannosidase but resistant to digestion with sialidase, β-galactosidase, β-hexosaminidase, and α-fucosidase (data not shown). *N*-linked oligosaccharides from the C3α and C3β chains were further analysed by digestion of the 2AB-labelled glycans with ASM (Fig. 3). This exoglycosidase specifically hydrolyses terminal α1-2-linked mannoses of oligomannose structures [24]. However, because the Glcα1-3Man linkage of $Glc_1Man_9GlcNAc_2$ cannot be hydrolysed by ASM, the digestion of the D1 and C α1-2 mannose residues of the capped α1,3 arm is prevented (Fig. 4) [24]. Digestion of the

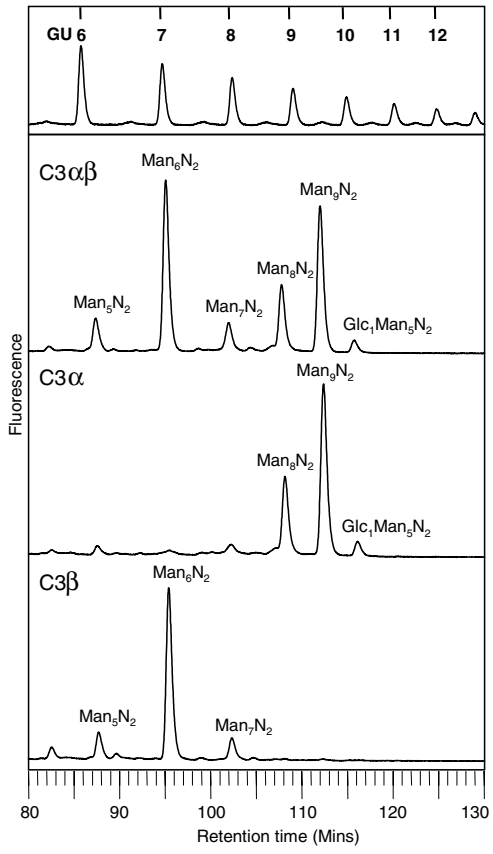

Fig. 2. NP-HPLC of 2AB-labelled *N*-glycans from C3. C3α and C3β were separated by reductive SDS–PAGE and the *N*-glycans released in-gel by PNGase F. The column was calibrated, and GU values assigned, using a dextran hydrolysate ladder. Man, mannose; N, GlcNAc.

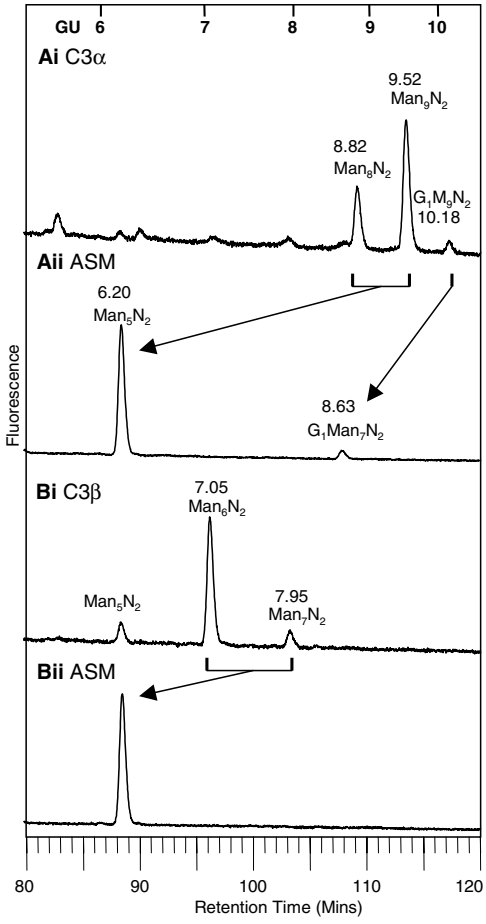

Fig. 3. NP-HPLC of 2AB-labelled C3α and C3β *N*-glycans with and without digestion with ASM, with GU values. (Ai) Undigested oligosaccharides from C3α; (Aii) and following digestion with ASM. (Bi) Undigested oligosaccharides from C3β; (Bii) and following digestion with ASM. G, glucose; Man, mannose; N, GlcNAc.

C3α glycans with ASM generated two peaks at 6.20 and 8.63 GU (Fig. 3A), whereas the same digest of the C3β glycans resulted in the generation of a single peak at GU 6.20 (Fig. 3B). The ASM digestion of the C3β glycans hydrolysed the terminal Manα1-2Man bonds corresponding to the complete digestion of $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ to $Man_5GlcNAc_2$, consistent with previous studies which identified a $Man_{5\text{-}7}GlcNAc_2$ series on the C3β subunit [25]. However, the peak at 10.2 GU in the undigested C3α profile and its transformation to 8.63 GU following the ASM digest demonstrate the presence of a small population of monoglucosylated *N*-glycans. The GU transformation of monoglucosylated glycans following ASM digestion was in agreement with previous studies [24]. Importantly, the transformation is consistent with the glucose cap being on the D1 arm, as only two mannose residues were cleaved with ASM [24]. If the glucose cap was located on either the D2 or D3 arm, then three mannoses would be released.

3.4. *Human sera do not contain detectable levels of* $Glc_1Man_9GlcNAc_2$

N-linked oligosaccharides were released from pooled human sera by hydrazinolysis. No neutral glycans were detected over 10 GU, demonstrating that the absolute abundance of monoglucosylated glycans in human sera is extremely low (Fig. 5).

4. Discussion

In this study, the identification of oligomannose *N*-glycan structures on both the α- and β-subunits of human C3 is confirmed and extended to include a population of monoglucosylated *N*-glycans.

The majority of complement proteins (C1r, C1s, C2, C3, C4, C5, C6, C8, C9, FB, FH, FI, and C4 BP) are synthesised in the liver, and a common tissue origin influences the carbohydrate structures observed on the mature secreted glycoproteins [2]. However, oligomannose *N*-glycans were identified on both C3 and C4, whereas the remaining members of the panel displayed broadly similar, complex glycans. Since the panel selected was based on common tissue origin, the most likely explanation for this variation in glycosylation is the effect of local protein structure on glycosylation rather than a distinct biosynthetic mechanism [2]. One caveat is that a small amount (4.5%) of C3 is synthesised in the kidney [8]. However, this small contribution from a different tissue source cannot account for the entirely different glycosylation of C3 compared with other complement glycoproteins.

Secretion of glycoproteins with glucosylated *N*-glycans has been reported in disease [4,26] and in non-mammalian systems [24,27–34]. Recently, $Glc_1Man_9GlcNAc_2$ was identified on the

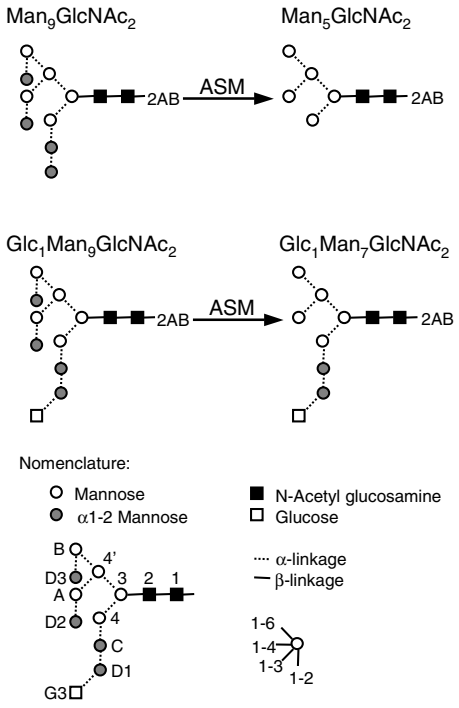

Fig. 4. Schematic diagram of $Glc_1Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ and the resulting structures following digestion with ASM.

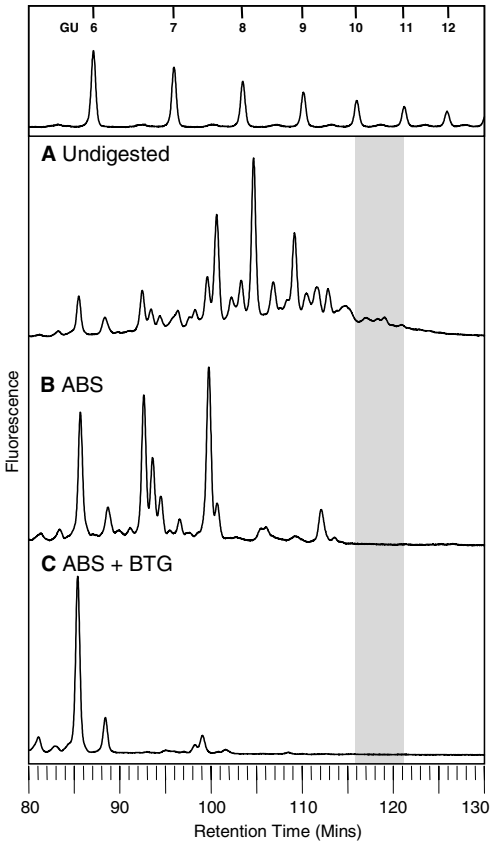

Fig. 5. NP-HPLC of 2AB-labelled *N*-glycans from human sera. (A) Undigested oligosaccharides; (B) following digestion with ABS; (C) following digestion with ABS and BTG. The grey box highlights the region where glucosylated $Man_9GlcNAc_2$ would be expected.

acid hydrolase α-mannosidase from Jack bean [32]. This structure and other oligomannose glycans on α-mannosidase could only be removed by Endo H under denaturing conditions. Additionally, monoglucosylated *N*-glycans on the lepidopteran hemolymph arylphorin storage protein could not be released under native conditions by PNGase F, again suggesting that the protein moiety protects the core of the glycan from enzymatic cleavage [24].

Buried glycosylation sites have been observed in the crystal structures of several antibodies [35]. Crystal structures of IgG Fc reveal that the glycan at Asn-297 is located between the two adjacent heavy chains and forms many contacts with the protein surface [36]. Interstitial glycans are present across all antibody isotypes [35–37], with the notable exception of IgA [38,39]. The glycans at these interstitial sites exhibit underprocessed glycosylation. For example, the glycans on the Fc of serum IgA are highly sialylated in contrast to the simpler neutral glycans of IgG Fc [38]. These structurally buried *N*-glycans are generally critical for correct folding and biosynthesis [2]. Interestingly, avian IgY, which is orthologous to human IgG, has been reported to contain significant levels of $Glc_1Man_9GlcNAc_2$ [28,29,33,34]. The only previous report of monoglucosylated *N*-glycans on a mature human protein of which the authors are aware is the site-specific identification of the $Glc_1Man_9GlcNAc_2$ structure on the IgD:WAH myeloma protein at the interstitial glycosylation site [26]. Furthermore, it has recently been shown that the glucosylated structures of IgY are also located at the interstitial Asn-297 Fc site, where they remain unavailable for calnexin, calreticulin, or α-glucosidase II interactions [34]. In contrast to the two other glycosylation sites in IgD, only the interstitial glycan is essential for folding and secretion and is unable to bind concanavalin-A [40].

The calnexin and calreticulin recognition site has been explored through inhibition studies which demonstrated that the entire 1,3 arm tetrasaccharide, Glcα1-3Manα1-2Manα1-2Man, forms part of the recognition epitope [41]. The presence of monoglucosylated *N*-glycans in secreted glycoproteins suggests that the sugar is in a region that folds independently of the calnexin–calreticulin mechanism and renders the α-glucosidase II cleavage site inaccessible.

The proteolytic state of C3, which changes during the complement cascade, dramatically influences the presentation of a lectin binding site at Asn-917 on the C3 α-subunit [42–44]. The bovine lectin conglutinin binds specifically with this oligomannose *N*-glycan on the α-subunit, but can only do so when C3 has been activated and cleaved (at three sites in the α-chain) to a form known as iC3b. Conglutinin does not bind C3 or the intermediate product C3b [42–44]. These lectin studies, together with the identification of monoglucosylated *N*-glycans on the C3α-subunit reported here, strongly suggest that the *N*-glycosylation site at Asn-917 is protected by the protein.

5. Conclusions and perspectives

Secretion of glycoproteins bearing the ER retention signal demonstrates that the calnexin–calreticulin quality control pathway is not 100% efficient. The secretion of the

monoglucosylated retention signal is shown here to occur on complement component C3. Previously, it was reported that a concealed conglutinin binding site on C3 was revealed upon conversion to iC3b. Together with the identification of secreted monoglucosylated *N*-glycans presented here, these data indicate that the glycosylation site on C3α is sterically hidden by the protein. In conclusion, the presence of monoglucosylated *N*-glycans in mature glycoproteins is emerging as a marker of structurally integral glycosylation sites.

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