Glycoconjugate Journal (1996) **13:** 19–26

MINI-REVIEW

O-linked protein glycosylation structure and function

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Received 12 June 1995, revised 28 July 1995

There has been a recent resurgence of interest in the post-translational modification of serine and threonine hydroxyl groups by glycosylation, because the resulting O-linked oligosaccharide chains tend to be clustered over short stretches of peptide and hence they can present multivalent carbohydrate antigenic or functional determinants for antibody recognition, mammalian cell adhesion and microorganism binding. Co-operativity can greatly increase the affinity of interactions with antibodies or carbohydrate binding proteins. Thus, in addition to their known importance in bearing tumour associated antigens in the gastrointestinal and respiratory tracts, glycoproteins with O-linked chains have been implicated as ligands or co-receptors for selectins (mammalian carbohydrate binding proteins). Microorganisms may have adopted similar mechanisms for interactions with mammalian cells in infection, by having relatively low affinity ligands (adhesins) for carbohydrate binding, which may bind with higher affinity due to the multivalency of the host ligand and which are complemented by other virulence factors such as interactions with integrin-type molecules. In addition to specific adhesion signals from O-linked carbohydrate chains, multivalent O-glycosylation is involved in determining protein conformation and forming conjugate oligosaccharide-protein antigenic, and possible functional determinants.

Keywords: O-glycosylation, O-linked protein, structure, function

O-glycosylation: structural and functional diversity

O-glycosylation based on the linkage GalNAcα1-O-Ser/Thr is found on serum and cell membrane glycoproteins and high molecular weight mucins which line the gastrointestinal tract and bronchial airways. Serum glycoproteins usually only have a small number of O-linked chains, the roles of which are largely unknown. However the past difficulty experienced in their characterization suggests that they confer protease resistance to, and form integrated structural motifs with, the underlying protein. In two membrane glycoprotein families, erythrocyte glycophorins and tissue fibronectins, the protein and oligosaccharide together are necessary for recognition of antibodies, designated respectively as blood group M/N (Table 1) and oncodevelopment antigen FDC-6 [1]. Membrane glycoproteins also often have multiple O-glycosylation sites which are proposed to function in a structural way to rigidify areas of the protein to form extended arms forcing functional globular domains away from the membrane. Examples include the T cell membrane antigen CD8 [2] and the low density lipoprotein (LDL) receptor [3]. In the former case the membrane proximal part of the protein bearing the multiple O-linked chains had to be removed before crystals could be obtained for X-ray studies. This could be due to the general hydrodynamic character, the multiple possible conformations, or the heterogeneity of structure of the oligosaccharide chains. The majority of the O-linked chains on serum and membrane glycoproteins are of the sialylated tri- and tetrasaccharide type based on the type 1 core (Table 2) having sialic acid linked α2-3 to Gal and/or α2-6 to GalNAc. However, longer and more diverse oligosaccharides have also been detected on these glycoproteins, for example those on human chorionic gonadotropin [4], glycophorin A [5] and immunoglobulin

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Table 1. Blood Group and related antigens expressed on O-linked cores (Table 2).

FDC-6	—Val-Thr(GalNAc)-His-Pro-Gly-Thr—(Fibronectin)
M	H_2N-Ser-Ser-Thr-Thr-Gly-Gly—(Glycophorin) * * *
N	H_2N-Leu-Ser-Thr-Thr-Glu—(Glycophorin) * * *
	where * is disialotetrasaccharide (disialyl T antigen)
T (Thomsen Friedenreich antigen)	Galβ1-3GalNAcα1-Ser/Thr
Disialyl T	NeuAcα2-3Galβ1-3GalNAcα1-Ser/Thr α2,6 NeuAc
Sialyl T^n	NeuAcα2-6GalNAcα1-Ser/Thr
T^n	GalNAcα1-Ser/Thr
Type I backbone	(Galβ1-3GlcNAcβ1-3/6)$_n$(Galβ1-3)GalNAcα1-Ser/Thr
Type II backbone	(Galβ1-4GlcNAcβ1-3/6)$_n$(Galβ1-3)GalNAcα1-Ser/Thr
Blood group H	Fucα1-2Galβ1-
Blood group A	GalNAcα1-3 Galβ1- Fucα1-2
Blood group B	Galα1-3 Galβ1- Fucα1-2
CAD (SD^a)	GalNAcβ1-4 Galβ1- NeuAcα2-3
Sialyl Le^a	NeuAcα2-3Galβ1-3GlcNAcβ1- \|1,4 Fucα
$Lewis^a$	Galβ1-3GlcNAcβ1- \|1,4 Fucα
$Lewis^b$	Galβ1-3GlcNAcβ1- \|1,2 \|1,4 Fucα Fucα
X antigen (SSEA-1, Le^x)	Galβ1-4GlcNAcβ1- \|1,3 Fucα
Y antigen (Le^y)	Galβ1-4GlcNAcβ1- \|1,2 \|1,3 Fucα Fucα

A [6]. On different cells of the haemopoetic lineage (erythroid, myeloid, lymphoid) there are characteristic sets of O-linked oligosaccharides varying in core structure as well as sialylation [7]. For T cells, activation results in a "dramatic stimulation of the β1-6GlcNAc-transferase" [8] giving oligosaccharides with predominantly core 2 (Table 2) rather than core 1.

The main characterization of the diversity of possible O-linked glycosylation patterns has been carried out on secreted mucin glycoproteins. From the sequence data

Table 2. The Ser/Thr linked oligosaccharide core region sequences of serum, cell membrane and mucin glycoproteins.

Sequence	*Core type*
Galβ1-3GalNAcol	1
GlcNAcβ1 6 GalNAcol	2
3 Galβ1	
GlcNAcβ1-3GalNAcol	3
GlcNAcβ1 6 GalNAcol	4
3 GlcNAcβ1	
GalNAcα1-3GalNAcol	5
GlcNAcβ1-6GalNAcol	6
GalNAcα1-6GalNAcol	7
Galα1-3GalNAcol	8

Cores 1, 2, 3 are common to many glycoproteins [12]. Core 4 was first characterized in sheep gastric mucins [89] and has since been identified in several animal and human mucins [39]. Core 5 [40] was first documented in humans in meconium [90] and in sialylated form in adenocarcinoma [91]. Core 6 has been documented to date in human meconium [92] and bovine κ-casein [93], core 7 in bovine submaxillary mucin [70] and core 8 in human respiratory mucin [94].

available eight different core regions can be defined (Table 2): i.e. those having β-Gal linked at C-3 in the absence (core 1) or presence (core 2) of β-GlcNAc at C-6; β-GlcNAc at C-3 in the absence (core 3) or presence (core 4) of β-GlcNAc at C-6: α-GalNAc at C-3 (core 5); β-GlcNAc at C-6 (core 6); α-GalNAc at C-6 (core 7) or α-Gal at C-3 (core 8). Vacant C-6 hydroxyl groups of cores 1, 3 and 5 and 8 can be glycosylated with one of a family of sialic acids, e.g. NeuAc, NeuGc (absent from normal human tissues but present in human tumours), KDO (2-keto-3-deoxy-octanoic acid [9]) and KDN (2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid). Mucins from the jelly coat of amphibian eggs for example carry mammalian types of O-glycans and in addition have many unusual structures containing KDN which it is suggested are involved in fertilization and are species specific [10, 11].

A large number of glycosyltransferases form chains with the characteristic blood group precursor type backbone and peripheral region antigenic sequences [12–14]. Thus:

(1) similar to the core GalNAc substituents, Gal can be substituted at C-3, C-6 or both C-3 and C-6 by GlcNAc;

(2) Gal can also have chain terminating substituents, such as Fucα1-2, Galα1-3, GalNAcα1-3, GalNAcβ1-4 (blood group H, B, A and Sd^a respectively; Table 1) and GlcNAcα1-4 (unlike in glycolipids, GalNAc has not been found in the backbone, but besides the blood group A sequence, can occur in the core as $\pm$GalNAcα1-3/6GalNAcα-Ser/Thr; GlcNAcα1-4 is so far a sequence unique to O-linked oligosaccharides);

(3) GlcNAcβ1-3 linked to GalNAc can form part of long chains of repeating Galβ1-4GlcNAc or Galβ1-3GlcNAc sequences with the presence of chain terminating blood group substitution;

(4) the GlcNAcβ1-6GalNAc arm has Galβ1-4 attached to GlcNAc with and without Fucα1-3 to GlcNAc but may also be further substituted to give longer chains as discussed above.

Structural and conformational characterization of O-glycosylation

The structural characterization of the carbohydrate chains of mucins has largely been achieved by release of oligosaccharide alditols by alkaline borohydride degradation and analysis by ^{1}H NMR together with mass spectrometry and chromatographic methods. Comprehensive ^{1}H NMR chemical shift assignments have now been obtained on a large number of these closely related oligosaccharide sequences. This data-base [15] can be explored to yield conformational information on selected oligosaccharide sequences, but because of the reduced state of the terminal *N*-acetylgalactosamine (GalNAcol) and the absence of protein these cannot be extrapolated too far to understanding the native conformation. The discussion given in the legend to Table 3 points to the relative levels of steric overcrowding that occurs on substituents near GalNAcol which are expected to be mirrored in the unreduced oligosaccharide. The conformational space explored by residues in the unreduced tri- and tetra-saccharides comprising GalNAc and 1-3 arm substituents has been studied by molecular dynamics simulations [16] and some of the interpretations discussed above are indicated in the resulting low energy conformers shown in Fig. 1. Conformation studies by NMR and molecular mechanics or molecular dynamics studies have been carried out on O-linked glycopeptides and glycoproteins [17–20] and data on oligosaccharide glycosides [21] and synthetic glycopeptides [22] are now accumulating.

Pollex-Krüger *et al.* [21] have extended studies on GalNAc-peptide and substituted or unsubstituted type 1 cores to several of the different core region sequences shown in Table 2. They demonstrate the stereochemical restrictions in the oligosaccharides in the absence of interactions with attached amino acids and the accessibility of C-6 and C-3 hydroxy groups for further addition of monosaccharides. The relative flexibility of the core 2 sequence with additional Gal linked to GlcNAc is also illustrated. Imberty *et al.* [23] have carried out molecular mechanics studies of the disaccharides GlcNAcβ1-3Gal and GlcNAcβ1-6Gal which are added to the core sequences to make up the backbones [12] of the blood

Table 3. The ^{1}H-NMR chemical shift data given in ppm (with acetone standard set at 2.225 ppm at 22 °C) for a series of related neutral oligosaccharide alditols varying in the substituents at Galβ1-3GalNAcol taken from a large database of chemical shifts of related sequences [70, 94–102]. The nomenclature is discussed in Table 4. In columns A–C it can be seen that there is a large difference in chemical shift (>0.03 ppm [15]) for H4 of GalNAcol on addition of Fucα1-2 and GalNAcα-3 to Gal and for the latter substituent a large difference also for GalNAcol H2, H5 and NAc. Similar chemical shifts for GalNAcol are found in B compared to D showing that these are not affected by the substituent at GlcNAcβ1-6, but there are differences in C compared to E presumably due to steric overcrowding. Similarly the chemical shifts for the fucose residue on the 1-3 arm are the same for oligosaccharides B, D and H showing that these are also not affected by substituents on the 1-6 arm. For oligosaccharides F, G and H, a downfield shift for H4 of GalNAcol is seen on addition to Gal at C3 of either GlcNAcα1-4 or Fucα1-2.

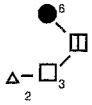
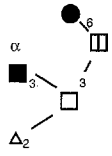
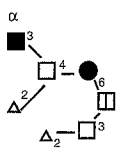
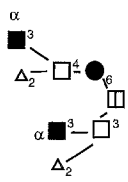
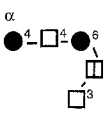
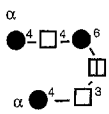
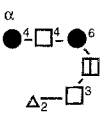
		A	B	C	D	E	F	G	H
									
GalNAcol	H2	4.395	4.403	4.309	4.404	4.270	4.401	4.412	4.408
	H3	4.061	4.084	4.081	4.080	4.081	4.065	4.086	4.086
	H4	3.466	3.503	3.579	3.498	3.584	3.460	3.517	3.500
	H5	4.282	4.257	4.161	4.271	4.220	4.291	4.268	4.274
	NAc	2.067	2.058	2.036	2.053	2.036	2.068	2.054	2.055
Fucα	H1		5.222	5.372	5.221	5.384			5.222
(on 1 → 3 arm)	H5		4.277	4.316	4.277	4.319			4.281
	CH_3		1.244	1.232	1.240	1.228			1.244
Fucα	H1				5.319	5.349			
(on 1 → 6 arm)	H5				4.319	4.318			
	CH_3				1.252	1.247			

Table 4. The symbols suggested for use in representing O-linked oligosaccharide chains.

The following rationalization of symbols used when presenting NMR data for O-linked chains is proposed. From the extensive work of Vliegenthart and colleagues in this field a square (□) is the convention for Gal. We can extrapolate this to related symbols having straight lines (△, ▽, ◇) for monosaccharides related to galactose, i.e. sialic acids (e.g. neuraminic acid; 2-keto-3-deoxy-D-glycero-D-galactononulosonic acid, ▲), fucose (6-deoxy-L-galactose, △) and *N*-acetyl-D-galactosamine (■). On the other hand monosaccharides related to Glc would have round symbols. In addition a proposal is to have filled in symbols for *N*-acetylated sugars and open symbols for neutral sugars. Therefore the nomenclature shown is proposed.

Gal □	GalNAc ■	GalNAcol ◫	GlcNAc ●
Glc/Man ○	Fuc △	NeuAc ▲	NeuGc ◆

group related determinants. The resulting poly-*N*-acetyllactosamine sequences have been studied by NMR [24] and molecular dynamics [16].

Additional conformational information from earlier studies on neutral oligosaccharides is available on the Fucα1-2Galβ1-3/4GlcNAc sequence [25–29] and fucosylated GlcNAc sequences [30–34]. More recently considerable effort has been afforded both by NMR and molecular dynamics studies to characterize the conformational states of the Le^x (Table 1) and sialyl Le^x oligosaccharides which are present on O- as well as N-linked chains [reviewed in 35]. For sialylated O-linked oligosaccharides in general, NMR data are available for reduced alditols [36–44] and conformational studies for sialylated variants of core 1 and 3 have been published [17, 18, 45–47]. Further variation in structure, function and antigenicity can be obtained by sulphation [48–53] which includes sulphate esters at C-6 of GlcNAc in core 2, at C-3 of Gal linked β1-4 to GlcNAc in core 2 and at C-6 of terminal or internal Gal. For a non-mucin glycoprotein, bovine pro-opiomelanocortin, a further sulphated sequence SO_4-4GalNAcβ1-4GlcNAcβ1-, has now been documented on O-linked chains [54].

In addition to NMR, analysis of either sulphated, sialylated or neutral alditols and glycopeptides includes high pH anion exchange chromatography [53, 55–57], PGC-HPLC [58, 59], normal phase-HPLC [60] or mass spectrometry (MS) [51, 54]. Lectin overlay assays have

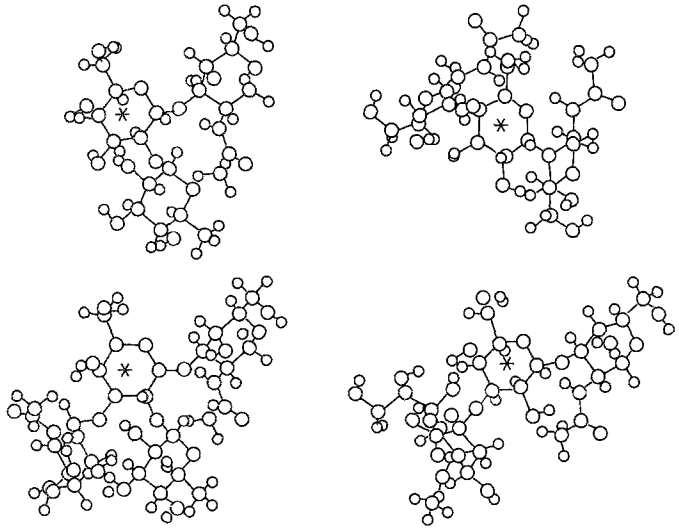


Figure 1. Low energy conformers of substituted core 1 di- and trisaccharides [16]. From top left anti-clockwise Fucα1-2Galβ1-3GalNAc, GalNAcα1-3[Fucα1-2]Galβ1-3GalNAc, NeuAcα2-3Galβ1-3GalNAc and GlcNAcα1-4Galβ1-3GalNAc where * is the Gal in each case and GalNAc is to the right hand side with CH_2 at C-1.

also been extremely useful in charting the distribution of oligosaccharides in the gastrointestinal tract [61, 62]. Gradually monoclonal antibodies [63] and glycosyltransferase probes [64] are becoming commercially available. More recently electrospray-MS has been shown to be a highly sensitive method of analysis for intact O-linked glycosylation [65], which previously had been difficult to analyse. Because of better methods of analysis and purification we are now beginning to discern functions, especially from the success of NMR analysis to characterize the structure and diversity and to predict specific conformational inferences. This information together with molecular graphics can lead to a good overall visualization of O-linked glycosylation and form the basis for further conformational studies in the presence and absence of ligands (antibodies, selectins, etc.).

Molecular recognition of O-linked glycosylation

Mucin oligosaccharides were in the past considered to have non-specific roles in maintaining the viscoelastic, hydrodynamic, protease resistance and pH buffering properties of the lining of the gastrointestinal and respiratory tracts. However the diversity of the oligosaccharides found, their controlled biosynthesis and specific protein skeletons [14] suggested additional recognition or protective roles. The three obvious areas are in tumour surveillance, bacterial colonization and protection of the underlying epithelial cells from mitogenic plant lectins.

Tumour-associated changes in the oligosaccharide chains of mucins have been extensively studied in the past and recently these are being correlated with expression of a family of genes designated *MUC* which code for the mucin protein. Thus, for example, the expression of the sialyl Le^a sequence (Table 1), known as a carcinoma marker in the gastrointestinal tract [34], has been traced to the MUC1 and MUC3 apoproteins [66, 67]. Besides sialyl Le^a, the antigens T, T^n and sialyl T^n are all masked in the normal gastrointestinal mucosa and therefore attempts have been made to boost the naturally occurring anti-Tn antibody titre [67] in colon carcinoma patients by, for example [68], immunizing with differently sialylated ovine submaxillary gland mucin which has almost exclusively short chain $\pm$NeuAcα2-6GalNAc sequences (unlike bovine submaxillary mucin [40–42, 69, 70]). A sequel to this is that the exposed T antigen, which is normally masked either by sialic acid or by additional substitution of the core 1 to form other cores (Table 1), is now available for binding to the peanut lectin [71] which can lead to a hyperplastic response *in vitro*. This interaction, made possible by the protected lectin arriving as far as the large intestine and binding to inflamed or carcinomatous tissue, may damage the mucosa further.

In addition to the potential to bind lectins, mucosal oligosaccharides are being increasingly implicated as ligands for bacterial adhesins. Therefore it can be surmised that one function for the diversity of oligosaccharide chains is to allow many interactions with microorganisms which, in the lung for example, may be an important factor in maintaining the sterilization of the respiratory tract [14]. In the oral cavity, the mosaic of bacterial adhesion sites afforded by the salivary mucins may be involved in the early events of the nonimmune defence of the oral cavity [72] and in the gastrointestinal tract mucins would maintain the rich bacterial flora. In pathogenic infection bacteria and viruses have evolved several different virulence factors, one of which may be to bind O-linked oligosaccharide chain sequences. For example, there is evidence that the Le^b antigen (Table 1) and the sequence Galα1-3Galβ1-4GlcNAc are involved, respectively, in the attachment to human gastrointestinal epithelium of *Helicobacter pylori* [73] and *Clostridium difficile* toxin A [74] which are the suggested causative agents of gastric ulcers and pseudomembranous colitis, respectively. *Bifidobacterium bifidum*, a normal inhabitant of the human colon exerts a beneficial effect on the host's health by colonization via mucosal adherence [75]. The α2-3 sialylated core 1 is the preferred sequence for binding of *Streptococcus sanguis* to human buccal epithelial cells [76] and the polyoma virus to red blood cells *in vitro* [77]. This specificity compares to the interaction of the influenza virus haemagglutinin with sialic acid terminating either O- or N-linked chains in either α2-3 or α2-6 linkage [78].

In addition to having various oligosaccharide binding specificities, microorganism adhesins may further resemble mammalian lectins in having polyvalent binding domains and making use of additional integrin interactions to increase affinity. For example, mimicry of eukaryotic selectins by prokaryotic adhesive ligands has been shown for the S2 and S3 subunits of pertussis toxin [79] which have sequence homology with the lectin domains of the eukaryotic selectin family and also upregulate the leukocyte integrins CD11b/CD18. Further, the adhesin of *Bordetella pertussis* filamentous haemagglutinin (FHA), which binds sulphated oligosaccharides, has the amino acid sequence motif RGD which interacts with integrin receptors [80].

O-glycans linked via GalNAcα1-Ser/Thr of non-mucous glycoproteins are being increasingly implicated in endogenous protein function and regulation. The stable surface expression of the LDL receptor [81, 82] and decay accelerating factor [81] is dependent on intact O-glycosylation. On the other hand interleukin 2 receptors require O-glycosylation for correct intracellular sorting [81]. The variation in O-glycosylation during differentiation has been mentioned above. This has also been documented for the promyelocytic cell line HL60 [83]. Tumour necrosis factor secreted from a B-lymphoblastoid cell line has been shown to have variable O-glycosylation associated with differential proteolytic processing [84]. Similarly the decrease in glycosyl and sialyl transferases of colonic adenoma cell lines correlates with their progression to overt adenocarcinoma [64]. In addition to the eight α-GalNAc cores described above and the Xyl-O-Ser linkage of proteoglycans (not discussed here), several other monosaccharides have been documented as being O-linked to mammalian proteins. Of these the most common appears to be the attachment of GlcNAcβ1 to Ser or Thr in nuclear and cytoplasmic proteins (reviewed in [85]). It has been suggested that the addition of O-GlcNAc is a regulatory modification with a reciprocal relationship to Ser/Thr phosphorylation and the activation of T cells for example is accompanied by rapid changes in nuclear and cytoplasmic O-GlcNAc levels. More recently certain EGF domains of proteins have been shown to be O-glycosylated with Glc or Fuc as the linkage monosaccharides (reviewed in [86]). The di- and trisaccharides Xylα1-3Glcα1-O-Ser and Xylα1-3Xylα1-3Glcα1-O-Ser have been isolated from human factor IX, factor VII, protein Z and bovine thrombospondin. O-linked oligosaccharides linked through fucose have been isolated from several EGF-like domains and in proteins expressed in CHO cells [87], primarily as either a single monosaccharide or a disaccharide of Glcβ1-3Fuc, although a tetrasaccharide of NeuAcα2-6Galβ1-4GlcNAcβ1-3Fuc has also been isolated. As yet functions for O-fucosylation and O-glucosylation have yet to be assigned.

Acknowledgement

The authors are grateful to Mrs G. Evans for secretarial help and to the UK Medical Research Council and European Union for funding.

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