Glycobiology of Proteins

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Novel glycosylation routes for glycoproteins: the lacdiNAc pathway
D. H. Van den Eijnden*†, A. P. Neeleman†, W. P. W. Van der Knaap†, H. Bakker*†, M. Agterberg* and I. Van Die*
Departments of *Medical Chemistry and †Medical Microbiology, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

Protein- and lipid-linked complex-type oligosaccharide chains are commonly based on Galβ1→4GlcNAc (N-acetyl-lactosamine, lacNAc) units that serve as backbone structural elements. However, in an increasing number of instances GalNAcβ1→4GlcNAc (N,N'-diacetyl-lactosdiamine, lacdiNAc) units rather than lacNAc units are found on the N- and O-linked oligosaccharide chains of glycoproteins ([1-24]; see Table 1). The group of glycoproteins that carry such chains is quite diverse and comprises inter alia hormones, enzymes, membrane glycoproteins and transport proteins. It appears that lacdiNAc-type chains particularly occur on glycoproteins of lower animal species, but are also present along with lacNAc-type chains on mammalian glycoproteins. Like the lacNAc unit in complex-type oligosaccharide structures, lacdiNAc units may be capped by terminal NeuAc residues in α2-3- or α2-6-linkage [S-12]. They also may contain a Fuc residue in a 1-2-linkage to GlcNAc

Abbreviations used: lacNAc, N-acetyl-lactosamine; lacdiNAc, N,N'-diacetyl-lactosdiamine; Tyv, tyvelose; β4-GalNAcT, β4-N-acetylgalactosaminyltransferase; β4-GalT, β4-galactosyltransferase.
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†To whom correspondence should be addressed.
**Table 1**

Occurrence and terminal substitution of the LacdiNAc unit on N- and O-linked glycoprotein glycans

<table>
<thead>
<tr>
<th>Terminal structure of LacdiNAc-type glycan†</th>
<th>Glycoprotein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalNAcβ1 → 4GlcNAc…</td>
<td>Bovine α-lactalbumin</td>
<td>[1]</td>
</tr>
<tr>
<td></td>
<td>Human urinary kallidinogenase</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>Schistosomal glycoproteins</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>Nematode glycoproteins</td>
<td>[4]</td>
</tr>
<tr>
<td><strong>NeuAca2 → 3GalNAcβ1 → 4GlcNAc…</strong></td>
<td>Pit-viper serine protease</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>Snake-venom batroxobin</td>
<td>[6]</td>
</tr>
<tr>
<td><strong>NeuAca2 → 6GalNAcβ1 → 4GlcNAc…</strong></td>
<td>Pituitary hormones</td>
<td>[7]</td>
</tr>
<tr>
<td></td>
<td>Recombinant tissue plasminogen activator</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>Bovine lactotransferrin</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>Bovine mammary epithelial CD36</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>Recombinant human protein C</td>
<td>[12]</td>
</tr>
<tr>
<td><strong>SO₄²⁻·4GalNAcβ1 → 4GlcNAc…</strong></td>
<td>Pituitary hormones</td>
<td>[13, 14]</td>
</tr>
<tr>
<td></td>
<td>Human Tamm-Horsfall glycoprotein</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Recombinant tissue-factor-pathway inhibitor</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>Murine pro-opiomelanocortin</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Bovine pro-opiomelanocortin*</td>
<td>[18]</td>
</tr>
<tr>
<td><strong>GalNAcβ1 → 4[Fucα1 → 3]GlcNAc…</strong></td>
<td>Human urokinase</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>Schistosomal glycoproteins</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>Honeybee-venom phospholipase A₂</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Bovine pro-opiomelanocortin</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>Recombinant human protein C</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>Sea squirt H-antigen*</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>Axolotl egg glycoproteins*</td>
<td>[22a]</td>
</tr>
<tr>
<td><strong>Tyv1 → 3GalNAcβ1 → 4[Fucα1 → 3]GlcNAc…</strong></td>
<td>Nematode antigen</td>
<td>[23]</td>
</tr>
<tr>
<td><strong>3-OMe-Galβ1 → 3GalNAcβ1 → 4GlcNAc…</strong></td>
<td>Snail haemocyanin</td>
<td>[24]</td>
</tr>
<tr>
<td><strong>Fucα1 → 2Galβ1 → 3GalNAcβ1 → 4GlcNAc…</strong></td>
<td>Snail haemocyanin</td>
<td>[24]</td>
</tr>
</tbody>
</table>

†All glycans are N-linked except those on the glycoproteins indicated with an asterisk, which are O-linked.

Different stages ( cercariae, miracidia and mother sporocysts) of the avian schistosome *Trichobilharzia ocellata* [31]. Characterization of the latter GalNAcTs showed that these enzymes are capable of acting on GlcNAc residues at the non-reducing end of oligosaccharides, whether free or protein-bound, regardless of the underlying structure (Table 2). In this respect and by kinetic analysis with different oligosaccharide and glyco(poly)peptide acceptors these β4-GalNAcTs clearly differ from the pituitary enzyme. Furthermore, by use of specific acceptor substrates, the schistosomal β4-GalNAcT could be readily distinguished from all other β4-GalNAcTs described to date, such as those involved in the synthesis of the Sd superscript 4 blood group determinant, ganglioside GM₁₂ and chondroitin sulphate [31]. In several enzymic properties, however, the β4-GalNAcT rather resembles the β4-galactosyltransferase (β4-GalT) that is common to mammalian species and that is known to control the synthesis of LacNAc units on N- and O-glycoproteins as well as on glycolipids [32]. A mammalian version of the snail and schistosomal (hormone unspecific) β4-GalNAcT’s seems to be present in human embryonic kidney and in several malignant cell lines [33]. In turn a β4-GalT has been found in schistosomes [34]. Because of the similarity in acceptor properties of β4-GalT and β4-GalNAcT it has been proposed that there might be competition for common substrate sites in cells
Table 2
Acceptor specificity of schistosomal UDP-GalNAc:GlcNAcβ-R /β4-GalNAcT

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Relative activity (Km (mM)</th>
<th>Km (mM)</th>
<th>V (m-units·mg of protein⁻¹)</th>
<th>Kinetic efficiency (V·Km⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc</td>
<td>100</td>
<td>36</td>
<td>4.4</td>
<td>0.12</td>
</tr>
<tr>
<td>GlcNAcα-pNP</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAcβ-pNP</td>
<td>596</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAcβ-S-pNP</td>
<td>2031</td>
<td>0.8</td>
<td>4.6</td>
<td>5.83</td>
</tr>
<tr>
<td>GlcNAcβ1→2Man</td>
<td>219</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAcβ1→6Man</td>
<td>378</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galβ1→3GlcNAc</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galβ1→6GlcNAc</td>
<td>&gt; 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycopeptide GP-F2*</td>
<td>263</td>
<td>4.0</td>
<td>4.6</td>
<td>1.15</td>
</tr>
<tr>
<td>Asialo/agalacto-α-subunit of human chorion gonadotrophin</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asialo/agalacto-α, acid glycoprotein</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*GP-F2 is a diantennary glycopeptide having the following structure: GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAcβ1-(Glu)Asn.

Figure 1
Control of the lacNAc and lacdiNAc pathway of oligosaccharide synthesis

β4-Galactosylation, as catalysed by β4-GalT, is the first committed step in the lacNAc pathway leading to the formation of the common Galβ1→4GlcNAc unit occurring on protein- and lipid-linked complex-type oligosaccharides. In the lacdiNAc pathway, that is controlled by a β4-GalNAcT, oligosaccharide structures are formed that are based on GalNAcβ1→4GlcNAc. In cells expressing both β4-GalT and β4-GalNAcT these enzymes act in competition on common substrate sites. Further additions of NeuAc, Fuc, Gal and SO₃⁻ moieties can take place in both pathways.

Addition of β-linked GlcNAc residues to N- and O-protein linked and lipid-linked oligosaccharide structures

That express both these enzymes [31,35] (Figure 1). The expression levels of each of these enzymes would determine whether lacNAc- or lacdiNAc-type chains will prevail on the glycoprotein products of such cells.

With the future goal of studying the control of the lacdiNAc pathway on a molecular biological level, the isolation of a cDNA encoding β4-GalNAcT was attempted. The resemblance in enzymic properties of the β4-GalT and the β4-GalNAcT
prompted us to try this by screening of a *L. stagnalis* genomic library using a cDNA coding for bovine \(\beta 4\text{-GalT}^{[36]}\) as a probe. Under low-stringency conditions hybridizing DNA fragments were obtained that were used to probe snail cDNA libraries. A full-length cDNA clone was isolated from a snail prostate gland library that showed an open reading frame of 1470 bp. In all respects the deduced amino acid sequence, representing a protein of 490 amino acids, showed a domain topology typical for a glycosyltransferase \([37]\). From an albumen gland library a cDNA fragment was isolated that lacked about 350 bp at its 5' end. This partial cDNA showed approx. 70% sequence identity with the prostate cDNA. The deduced amino acid sequences of these two snail cDNAs contained several regions that showed considerable similarity with corresponding exons in the murine \(\beta 4\text{-GalT}\) genome \([38]\). Highest similarity (up to 56%) was found in the regions that constitute the putative catalytic domain. Considering the evolutionary distance between snails and mammals, and the fact that essentially no \(\beta 4\text{-GalT}\) activity can be detected in *L. stagnalis*, this is quite remarkable. It suggests that the glycosyltransferases encoded by the isolated snail cDNAs are evolutionarily related to \(\beta 4\text{-GalT}\) and that the genes of these enzymes and of \(\beta 4\text{-GalT}\) may have evolved from a common ancestor. This would show that these enzymes constitute yet another glycosyltransferase family in addition to those that have been identified to date \([35]\). Studies are in progress to identify the enzymes that are represented by these snail cDNAs. It is expected that one of these cDNAs encodes a \(\beta 4\text{-GalNAcT}\) that is involved in the synthesis of lacdiNAc-based structures.

Further processing of lacdiNAc chains shows analogy with that of lacNAc-based chains. It has been demonstrated that Gal\(\beta 1\rightarrow 4\text{GlcNAc-specific}\ \alpha 6\text{-sialyltransferases} can also act on lacdiNAc to form NeuAc\(\alpha 2\rightarrow 6\text{GalNAc}\beta 1\rightarrow 4\text{GlcNAc}^{[39-41]}\). Similarly it has been found that human milk \(\alpha 3(4)\text{-fucosyltransferase} can act on this disaccharide to yield the lacdiNAc analogue of Lewis\(^X\) (GalNAc\(\beta 1\rightarrow 4\text{Fuc}\alpha 1\rightarrow 3\text{GlcNAc}^{[42]}\). Furthermore a sulphotransferase has been identified in pituitary gland that catalyses the transfer of sulphate to C-4 of GalNAc in lacdiNAc \([43]\), and a \(\beta 3\text{-galactosyltransferase} has been described catalysing the formation of Gal\(\beta 1\rightarrow 3\text{GalNAc}\beta 1\rightarrow 4\text{GlcNAc}^{[44]}\). However, glycosyltransferase-catalysed additions of \(\alpha\text{-NeuAc}^{[41]}\), \(\alpha\text{-Gal} and \(\beta\text{-GlcNAc}, that readily occur to C-3 of Gal residues in lacNAc, have appeared to proceed at very low rates if at all to C-3 of the GalNAc residue in lacdiNAc chains (D. H. Van den Eijnden and W. E. C. M. Schiphorst, unpublished work). This suggests that polyacatos- diaminoglycan \([\text{GalNAc}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 3\text{}]\) sequences will not generally occur in nature.

Little is known about the biological significance of lacdiNAc-based oligosaccharide chains. It has been reported that a specific hepatic reticulo-endothelial receptor for \(\text{SO}_{4}^{2-}\text{4GalNAc}\beta 1\rightarrow 4\text{GlcNAc}\beta-R\) exists that mediates the rapid clearance of the glycoconjugate lutropin \([45]\). It has also been suggested that the lacdiNAc-based Lewis\(^X\) analogue structure present on the oligosaccharide chains of protein C plays a role in the anti-inflammatory effect of this protein \([46]\). In addition it has been proposed \([31]\) that lacdiNAc-type oligosaccharide chains at the surface of schistosomes might create a molecular mimicry that contributes to the evasion of the defence system of the snail host resulting in successful infection \([47,48]\). It will be of interest to investigate whether other terminal structures formed in the lacdiNAc pathway likewise confer specific functions and properties on the glycoconjugates carrying them.

**Note added in proof (received 2 December 1994)**

Recently it has appeared that the prostate cDNA encodes a novel UDP-GlcNAc-GlcNAc\(\beta\text{-R} N\text{-acetylglucosaminyltransferase}. This enzyme might be involved in yet another variant (the ‘chitobio’ pathway) of complex-type glycans processing \([48a]\).

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