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Branching and elongation with lactosaminoglycan chains of N-linked oligosaccharides result in a shift toward termination with α2→3-linked rather than with α2→6-linked sialic acid residues

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Abstract The activity of bovine colostrum CMP-NeuAc: Galβ1→4GlcNAcβ-R α2→6-sialyltransferase (α6-NeuAcT) toward oligosaccharides that form part of complex-type, N-linked glycans appears significantly reduced when a bisecting GlcNAc residue or additional branches are present, or when core GlcNAc residues are absent. By contrast human placenta CMP-NeuAc:Galβ1→4GlcNAcβ-R α2→3-sialyltransferase (α3-NeuAcT) is much less sensitive to structural variations in these acceptors. Furthermore the α3-NeuAcT shows a much higher activity than the α6-NeuAcT with oligosaccharides that form part of linear and branched lactosaminoglycan extensions. These results indicate that, in tissues that express both enzymes, branching and lactosaminoglycan formation of N-linked glycans will cause a shift from termination with α2→6-linked sialic acid to termination with α2→3-linked sialic acid residues. These findings provide an enzymatic basis for the sialic acid linkage-type patterns found on the oligosaccharide chains of N-glycoproteins.

Key words: N-glycan branching; Oligosaccharide; Lactosaminoglycan; Sialylation; Sialyltransferase

1. Introduction

Many complex, *N*-acetyllactosamine-type oligosaccharides occurring on N-glycoproteins contain sialic acid in α2→3- or α2→6-glycosidic linkage at their non-reducing termini [1,2]. It has appeared that α2→3-linked sialic acid is more frequently present on tri- and tetra-branched glycans, whereas α2→6-linked sialic acid is predominant on di-antennary oligosaccharides (reviewed in [3]). A minimum of two different sialyltransferases is required for the synthesis of these linkages. So far only one sialyltransferase has been described that catalyses the transfer of sialic acid in α2→6-linkage to Gal of the *N*-acetyllactosamine units of glycoprotein glycans. This enzyme, regardless of its species or tissue origin, is very specific for Galβ1→4GlcNAc and acts with very low activity on Galβ1→3GlcNAc and Galβ1→3GalNAc, if at all [4,5]. This sialyltransferase is highly expressed in human [6,7] and rat [8–11] liver. On the other hand two α3-NeuAcTs have been described that are capable to catalize the synthesis of a NeuAcα2→3Galβ1→4GlcNAc sequence. In human placenta a α3-NeuAcT was identified [12] that was later shown to have a high preference for Galβ1→4GlcNAc [13]. Recently a sialyltransferase has been cloned that probably corresponds to this placenta enzyme [14,15]. Interestingly, the recombinant form of this enzyme is also capable of acting on Galβ1→3GalNAc [15] and (to a much lesser extent) on Galβ1→3GlcNAc [14,15]. Northern analysis revealed high mRNA levels for this enzyme in human placenta [7]. Another α3-NeuAcT acting on Galβ1→4GlcNAc was first purified from rat liver [9]. The natural as well as the recombinant form of this enzyme, however, has a high preference for acceptor substrates containing a terminal Galβ1→3GlcNAc unit [16,17].

So far oligosaccharide acceptor specificities for these enzymes have been mainly determined with small, linear structures. The results obtained in these studies, therefore, did not provide an explanation for the specific α2→3/α2→6-sialylation patterns occurring on N-linked oligosaccharide chains. We have recently determined the acceptor specificity of the α3-NeuAcT from placenta with di-, tri- and tetra-antennary glycan structures [13]. In this study these data are complemented with the acceptor properties of these branched substrates for the α6-NeuAcT isolated from bovine colostrum. Comparison of the activities of both sialyltransferases indicates that branching of N-linked glycans as well as lactosaminoglycan formation, which are controlled by the action of distinct *N*-acetylglucosaminyltransferases, result in a shift from termination with α2→6-linked sialic acid to termination with α2→3-linked sialic acid residues.

2. Experimental

2.1. Materials

CMP-[^{14}C]NeuAc (specific radioactivity 4.1 Ci/mol) was obtained from New England Nuclear (Boston, MA) and was diluted with unlabelled CMP-NeuAc [18] to the desired specific activity. The various oligosaccharides, which were used to examine the acceptor specificities of the sialyltransferases, were kindly donated by the following persons: compounds 1, 15, 16 and 17 by Drs. S. David, J. Alais and A. Veyrières (Université Paris-Sud, Orsay, France); compounds 2, 3, 5, 7, 9, 11 and 13 by Dr. J. Lönngren (University of Stockholm, Stockholm, Sweden); compound 18 by Dr. H. Lönn (Biocarb, Lund, Sweden) and compounds 4, 6, 8, 10, 12 and 14 by Drs. G. Strecker and J.C. Michalski (Université de Lille, Villeneuve d'Ascq, France).

2.2. Enzymes and activity assays

α6-NeuAcT was partially purified from bovine colostrum as described previously [19]. α3-NeuAcT was prepared from human placenta

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Abbreviations: CMP-NeuAc; Cytidine 5′-monophospho-*N*-acetylneuraminic acid; NeuAc, *N*-acetylneuraminic acid; GalT, galactosyltransferase; GlcNAcT, *N*-acetylglucosaminyltransferase; α6-NeuAcT, CMP-NeuAc:Galβ1→4GlcNAcβ-R α2→6-sialyltransferase (EC 2.4.99.1); α3-NeuAcT, CMP-NeuAc:Galβ1→4GlcNAcβ-R α2→3-sialyltransferase (EC 2.4.99.6).

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as reported earlier [13]. This enzyme was stored as a microsomal pellet in aliquots at −20°C, and was resuspended directly before use.

The incubation mixtures for the assay of α6-NeuAcT activity toward the various acceptor substrates contained in a volume of 50 μl: an amount of oligosaccharide having 50 nmol terminal Gal residues, 25.5 nmol CMP-[^{14}C]NeuAc (1.05 Ci/mol), 5.0 μmol of sodium cacodylate pH 6.7, 0.043 mU α6-NeuAcT (16 μg of protein) and 0.4 μl glycerol. Incubation mixtures for α3-NeuAcT contained in a volume of 50 μl:

Table 1
Acceptor specificity of bovine colostrum α6-NeuAcT and human placenta α3-NeuAcT toward oligosaccharide acceptors that form part of N-linked glycans

	Oligosaccharide	Relative activity (%)	
		α6-NeuAcT	α3-NeuAcT
1.	Galβ1→4GlcNAc	29	135
2.	Galβ1→4GlcNAcβ1→2Man	69	157
3.	Galβ1→4GlcNAcβ1→6Man	44	153
4.	Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc	18	127
5.	Galβ1→4GlcNAcβ1→2Manα1↘6 Man Galβ1→4GlcNAcβ1→2Manα1↗3	39	116
6.	Galβ1→4GlcNAcβ1→2Manα1↘6 Manβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1↗3	100	100
7.	Galβ1→4GlcNAcβ1→2Manα1↘6 GlcNAcβ1→4Man Galβ1→4GlcNAcβ1→2Manα1↗3	46	66
8.	Galβ1→4GlcNAcβ1→2Manα1↘6 GlcNAcβ1→4Manβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1↗3	66	48
9.	Galβ1→4GlcNAcβ1→2Manα1↘6 Man Galβ1→4GlcNAcβ1→2Manα1↗3 Galβ1→4GlcNAcβ1↗4	29	122
10.	Galβ1→4GlcNAcβ1→2Manα1↘6 Manβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1↗3 Galβ1→4GlcNAcβ1↗4	61	132
11.	Galβ1→4GlcNAcβ1↘6 Galβ1→4GlcNAcβ1→2Manα1↘6 Man Galβ1→4GlcNAcβ1→2Manα1↗3	3	75
12.	Galβ1→4GlcNAcβ1↘6 Galβ1→4GlcNAcβ1→2Manα1↘6 Manβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1↗3	35	89

Table 1 (*continued*).

Oligosaccharide	Relative activity (%)	
	α6-NeuAcT	α3-NeuAcT
13. Galβ1→4GlcNAcβ1↘6 Galβ1→4GlcNAcβ1→2Manα1↘6 Man Galβ1→4GlcNAcβ1→2Manα1↗3 Galβ1→4GlcNAcβ1↗4 Galβ1→4GlcNAcβ1	7	68
14. Galβ1→4GlcNAcβ1↘6 Galβ1→4GlcNAcβ1→2Manα1↘6 Manβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1↗3 Galβ1→4GlcNAcβ1↗4 Galβ1→4GlcNAcβ1	23	83

Acceptors were present at an amount as to give a concentration of terminal Gal residues of 1 mM. The activities were assayed as described in section 2. The relative activities with each acceptor are expressed as a percentage of the incorporation obtained with the diantennary oligosaccharide **6**, which amounted 2.61 nmol·mg^{-1}protein·min^{-1} for α6-NeuAcT and 25.3 pmol·mg^{-1}protein·min^{-1} for α3-NeuAcT.

oligosaccharide (50 nmol terminal Gal), 25.5 nmol CMP-[^{14}C]NeuAc (1.05 Ci/mol), 5.0 μmol of sodium cacodylate pH 6.7, Triton X-100 (final concentration 0.1% by vol) and placenta microsomes (0.051 mU α3-NeuAcT, 2 mg of protein). The mixtures were incubated at 37°C for 30–45 min whereafter the incubations were stopped by cooling on ice and dilution with 100 μl of ice cold H_2O. When present, microsomal material was removed by centrifugation. Incorporation of [^{14}C]NeuAc into the acceptors was assayed by applying the mixtures to a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 37°C, at a flow rate of 8 ml/h. The incorporation of [^{14}C]NeuAc was calculated from the radioactivity in the fractions containing the product. Under the incubation conditions employed CMP-NeuAc and oligosaccharide substrates were present at saturating concentrations [3,13,20]. Because of the limited availability of the acceptors it was, however, not possible to estimate K_m and V_{max} values for the individual oligosaccharides. Therefore only relative rates (kinetic efficiencies) are given.

3. Results and discussion

A number of oligosaccharides, that form part of complex, *N*-acetyllactosamine-type N-linked glycans, were tested as acceptors for bovine colostrum α6-NeuAcT and human placenta α3-NeuAcT. All structures possessed at least one terminal Galβ→4GlcNAc unit as a minimum acceptor requirement for these enzymes. They differed, however, in the underlying sequence as well as in the degree of branching. It appears that the α6-NeuAcT is much more sensitive to changes in the structure of the acceptors than the α3-NeuAcT (Table 1). The activity of the latter enzyme varies by a factor of 3, whereas this variation amounts up to a factor of 30 for the α6-NeuAcT.

The best acceptor substrate for the α6-NeuAcT is the diantennary oligosaccharide **6**. It has been described that the α6-NeuAcT highly prefers the Gal at the Manα1→3Man branch for attachment of a sialic acid residue and that the rate of sialic acid attachment in α2→6-linkage to the Gal at the Manα1→6Man branch is comparatively low [3,20]. This might explain why oligosaccharide **4**, which represents the Manα1→6Man branch of N-linked glycans, is a relatively poor acceptor. The α3-NeuAcT does not show any preference for either branch of the di-antennary substrate [13], which is consistent with the good acceptor properties of oligosaccharide **4** for the latter enzyme.

Bisecting GlcNAc residues are the result of action of GlcNAcT III [21]. When, as in compound **8**, such a residue is present, both the α6-NeuAcT and the α3-NeuAcT show a lower activity (Table 1). Similarly the presence of a bisecting GlcNAc residue has previously been demonstrated to block the reactions catalyzed by mannosidase II, GlcNAcT II and IV, and α6-fucosyltransferase [21,22] and to partially inhibit those catalyzed by GlcNAcT V [23], β4-GalT [24,25], α3-fucosyltransferase (D.H. van den Eijnden and W.E.C.M. Schiphorst, unpublished results), α3-GalT [23] and β3-GlcNAcT (i-enzyme) [26]. This suggests that a bisecting GlcNAc residue has a profound effect on the conformation of the oligosaccharide structure resulting in an interference with most of the subsequent processing steps of protein N-glycosylation.

Action of GlcNAcT IV results in the formation of oligosaccharides with a GlcNAcβ1→2(GlcNAcβ1→4)Manα1→3Man branching point [21,22]. The tri-antennary oligosaccharide **10**, containing such a branching point, shows a reduced acceptor property for the α6-NeuAcT as compared to that of the diantennary oligosaccharide **6** (Table 1). By contrast, the presence of this GlcNAcβ1→4-branch improves the acceptor property for the α3-NeuAcT. The isomer of this structure, the tri'-antennary oligosaccharide **12**, possesses a GlcNAcβ1→2(GlcNAcβ1→6)Manα1→6Man branching point which results from the action of GlcNAcT V [22,27]. The presence of this branch has an even stronger negative effect on the acceptor property for the α6-NeuAcT, whereas the activities of the α3-NeuAcT with this substrate and the di-antennary oligosac-

Table 2
Acceptor specificity of bovine colostrum α6-NeuAcT and human placenta α3-NeuAcT toward oligosaccharide acceptors that are structurally related to linear (blood group i-active) and branched (blood group I-active) polylactosaminoglycans

Oligosaccharide		Relative activity (%)	
		α6-NeuAcT	α3-NeuAcT
15.	Galβ1→4GlcNAcβ1→3Gal	55	161
16.	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc	33	133
17.	Galβ1→4GlcNAcβ1 ↘6 Gal ↗3 Galβ1→4GlcNAcβ1	21	78
18.	Galβ1→4GlcNAcβ1 ↘6 Galβ1→4Glc ↗3 Galβ1→4GlcNAcβ1	25	109

Acceptors were present in an amount as to yield a concentration of terminal Gal residues of 1 mM. The activities were assayed as described in section 2. The relative activities with each acceptor are expressed as a percentage of the incorporation obtained with the diantennary oligosaccharide **6** (Table 1).

charide **6** are comparable. The results obtained with tetra-antennary oligosaccharide **14**, which contains both additional branches, are in line with those of oligosaccharides **10** and **12** (Table 1). These data indicate that in tissues that express both sialyltransferases, such as human liver [6,7], the sialic acid linkage-type pattern of α2→3- and α2→6-linked sialic acid on the glycoprotein products is not only dependent on the relative expression levels of these enzymes, but also on whether or not the oligosaccharide structures have been acted upon by GlcNAcT IV and V earlier in the glycosylation process. Branching due to the action of these GlcNAcTs appears to result in a shift from termination with α2→6-linked sialic acid to termination with α2→3-linked sialic acid residues. This mechanism thus provides an enzymatic basis for the sialic acid linkage-type patterns found on the oligosaccharides on many N-glycoproteins [3] as well as on sialo-oligosaccharides isolated from urine [28] and placenta [29] of galactosialidosis patients. Glycoproteins containing sialic acid in both linkage types carry most of the α2→3-linked sialic acid residues on tri-, tri'-, and tetra-antennary oligosaccharides, whereas the di-antennary glycans predominantly contain sialic acid in α2→6-linkage [30–33].

An interesting property of the α6-NeuAcT is observed when activities are compared toward oligosaccharides with or without a GlcNAc residue at their reducing end. Comparison of such oligosaccharide pairs (**5/6**, **7/8**, **9/10**, **11/12**, **13/14**) shows that the absence of a reducing GlcNAc results in a strongly decreased activity of the α6-NeuAcT (Table 1). By contrast the activity of the α3-NeuAcT is essentially unaffected by the absence of such a residue. It is possible that the better acceptor property for the α6-NeuAcT of oligosaccharides, in which a reducing GlcNAc is present, is simply due to the fact that this residue keeps the subterminal Man in β-anomeric configuration. However, this does not explain why the magnitude of the effect is not the same for all oligosaccharide pairs. Previously it has been shown that a minimum of one core GlcNAc is absolutely required to preserve the preference of the α6-NeuAcT for the Gal at the Manα1→3Man branch [20]. It was proposed that the core GlcNAc residue, such as present in oligosaccharide **6**, **8**, **10**, **12** and **14** forms an essential part of a sequence that is recognized by the α6-NeuAcT in a lectin-like way. This would imply that this enzyme has either a second carbohydrate recognizing domain in addition to the one that interacts directly with the Galβ1→4GlcNAc unit of the glycans, or that it has a large, extended binding site that recognizes the entire Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4-GlcNAc sequence of these glycans. Alternatively, a minimum of one core GlcNAc residue might be essential for keeping the Manα1→3Man branch in an orientation that allows optimal binding to the α6-NeuAcT. Comparison of the results obtained with the α6-NeuAcT and the α3-NeuAcT suggests that these enzymes recognize their acceptor substrates in a fundamentally different way. This is consistent with the fact that, apart from the 'sialyl motif', that is involved in binding of the CMP-NeuAc donor-substrate [34], and a second small region of sequence similarity [35], these enzymes are non-homologous [14,15].

As discussed above, action of GlcNAcT V yields branched structures that, after β4-galactosylation, are acted upon by the α6-NeuAcT at a much lower rate than by the α3-NeuAcT. On the other hand, such branched structures have been demonstrated to be preferred acceptor substrates for β3-GlcNAcT, which enzyme is involved in the initiation as well as the elongation of N-linked lactosaminoglycan chains [26]. Action of GlcNAcT V thus promotes lactosaminoglycan chain formation of such chains, while it leads to inhibition of α6-sialylation leaving α3-sialylation essentially unimpaired. In Table 2 it is shown that several representative, partial structures of linear and branched lactosaminoglycan chains are better substrates for the α3-NeuAcT than for the α6-NeuAcT. Lactosaminoglycan formation thus reinforces the effect that branching by GlcNAcT V has on the sialic acid linkage type patterns of N-glycoproteins.

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