The carbohydrates of mouse hepatitis virus (MHV) A59: structures of the O-glycosidically linked oligosaccharides of glycoprotein E1

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Two size classes of O-glycosidically linked oligosaccharides were liberated from glycoprotein E1 of mouse hepatitis virus (MHV) A59 by reductive \(\beta\)-elimination and separated by h.p.l.c. The structures of the reduced oligosaccharides were determined by successive exoglycosidase digestions and by methylation analyses involving combined capillary gas chromatography-mass spectrometry and mass fragmentography after chemical ionization with ammonia. Oligosaccharide A (Neu5Ac(2→3)Galβ1→3GalNAc) comprised 35% of the total carbohydrate side chains, while the remaining 65% of the oligosaccharides of E1 had the branched structure B: Neu5Ac(2→3)Galβ1→3(Neu5Ac(2→6)GalNAc. Both oligosaccharides were linked to the E1 polypeptide via N-acetylgalactosamine, and 20% of the sialic acids present in E1 glycopeptides were found to consist of N-acetyl-9-mono-O-acetyleneuraminic acid. The reported structures of the O-linked glycans are discussed in the context of the amino acid sequence of E1, which exhibits a cluster of four hydroxyamino acids (Ser-Ser-Thr-Thr) as potential O-glycosylation sites at the amino terminus. Oligosaccharides with identical structures and an identical O-glycosylated tetrapeptide sequence are present in the blood group M-active glycophorin A of the human erythrocyte membrane.

Key words: O-glycosylation/coronaviruses/membrane glycoproteins/glycophorin A

Introduction

Most of the viral glycoproteins studied in detail, such as those of para- and orthomyxoviruses, Semliki Forest virus, Sindbis virus, and vesicular stomatitis virus, contain N-glycosidic carbohydrate protein linkages (for review, see Compan and Klenk, 1979). Like cellular membrane glycoproteins, these viral polypeptides are synthesized on membrane-associated ribosomes and transported through the stacks of the Golgi complex to the plasma membrane, where they are finally sequestered into the budding virus particles. During synthesis and transport these viral polypeptides undergo a number of characteristic modifications involving co-translational N-glycosylation, proteolytic cleavage at the co- and post-translational level, acylation and sulfation. The assembly and post-translational processing of the N-linked glycans have been studied extensively, and a series of enzymatic trimming and elongation reactions, leading to oligomannosidic and complex-type oligosaccharides, respectively, have been characterized at the molecular level (Schachter, 1981). To date, the structures of the N-glycans of >20 viral glycoproteins have been elucidated (Montreuil, 1982).

Much less is known about the synthesis, structures and functions of O-glycosidically linked oligosaccharides, originally recognized in glycoproteins from mucous secretion (Carlson, 1968). In viral glycoproteins this type of carbohydrate side chain was detected only recently, namely in the glycoprotein E1 of bovine and murine coronaviruses (Nieman and Klenk, 1981b; Holmes et al., 1981; Rottier et al., 1981), in the glycoproteins of herpes simplex virus (HSV) (Olofsson et al., 1981; Johnson and Spear, 1983), and in the non-structural hemagglutinin of vaccinia virus (Shida and Dales, 1981). The HSV and vaccinia virus glycoproteins contain both N- and O-linked carbohydrate side chains. However, the matrix glycoprotein E1 of the bovine transmissible gastroenteritis virus and of mouse hepatitis virus (MHV) A59 carries exclusively O-glycosidically linked carbohydrates (Nieman and Klenk, 1981a, 1981b) and in this respect they are unique amongst viral glycoproteins.

The function of glycoprotein E1 in coronavirus maturation has been investigated in detail for MHV A59. Cell fractionation and in vitro translation studies have revealed that E1 is translated on membrane-associated ribosomes, and that the polypeptide is modified by attachment of the O-linked carbohydrates after transport to the Golgi apparatus (Nieman et al., 1982). Since E1 is transmembranal and interacts with the viral nucleocapsid (Sturman et al., 1980), and since it accumulates early in the infectious cycle in perinuclear regions while it is absent from the plasma membrane, it has been suggested that E1 functions as a matrix protein determining the intracellular budding site of coronaviruses (Dubois-Dalq et al., 1982; Tooze et al., 1984). While it is not yet clear which structural features of the E1 molecule direct its transport from its site of synthesis to specific intracellular membranes, where virus budding occurs, the amino acid sequence of E1 has been deduced from cloned cDNA (Armstrong et al., 1983; Nieman et al., 1983). A cluster of four hydroxyamino acids: Ser-Ser-Thr-Thr, each of them providing potential attachment sites for O-glycosidically linked carbohydrate side chains, is located at the amino-terminal end of the E1 molecule, which resides on the outer face of the viral membrane (Sturman et al., 1980; Rottier et al., 1984).

We have isolated the O-glycosidically linked oligosaccharides from glycoprotein E1 of MHV A59 by reductive \(\beta\)-elimination and determined their structures by sequential degradation with exoglycosidases, and by methylation-gas chromatography-mass spectrometry (Hakomori, 1964), employing a recently described version of this latter technique, which allows the analysis of subnanomole quantities of glycans (Geyer et al., 1983).

Results

Isolation of reduced oligosaccharides from glycoprotein E1 of MHV A59

Previous experiments had shown that glycoprotein E1 of
MHV A59 could be metabolically labelled with [3H]galactose and [3H]glucosamine, but not with [3H]fucose or [3H]-mannose, and that it contained exclusively O-glycosidically linked carbohydrate side chains (Niemann and Klenk, 1981a, 1981b). We have now used β-elimination conditions according to Carlson (1968) to isolate preparatively these O-linked side chains from metabolically labelled glycoprotein E1, purified by SDS-polyacrylamide gel electrophoresis. The β-elimination was carried out in the presence of sodium borohydride. An optimum yield of reduced oligosaccharides was obtained after 20 h at 45°C. By this time, 85–95% of the carbohydrate label was released from the protein, as assayed by trichloroacetic acid (TCA) precipitations of aliquots of the reaction mixture. The separation of the individual reduced oligosaccharides was best accomplished by h.p.l.c. (Bergh et al., 1981). Two fractions of reduced oligosaccharides, designated A and B, were eluted from the h.p.l.c.-column using different ratios of acetonitrile-phosphate buffer. As shown in Figure 1, fraction A was eluted with 70% (v/v) and fraction B with 60% (v/v) acetonitrile.

No additional peaks were obtained, when the elution was continued with 50% (v/v) or 40% (v/v) phosphate buffer; solvent systems, which would allow the detection of larger sized oligosaccharides, especially of those with higher degrees of sialic acid substitution (Bergh et al., 1981). A similar elution pattern was obtained when the aggregated 38-K species of glycoprotein E1 was subjected to β-elimination, yielding again oligosaccharide B in about twice the amount of A, as judged by labelling with [3H]galactose (data not shown). After digestion with Vibrio cholerae neuraminidase, both peak fractions A and B were converted into an identical uncharged product, which eluted from the h.p.l.c. column (data not shown) and the Biogel column, in the position of an amino sugar-containing disaccharide alditol (see Figure 3).

**Methylation analyses**

To analyze the composition and the substitution pattern of the purified oligosaccharides, de-salted oligosaccharides A and B, and their common product, obtained by neuraminidase treatment, were subjected to methylation analyses (Hakomori, 1964). The limited amounts available of the individual samples made it necessary to employ a micro-methylation version recently devised for the analysis of N-glycans in the range of 100–500 pmol of oligosaccharide (Geyer et al., 1983). The partially methylated alditol acetates were separated by capillary gas chromatography and characterized on the basis of their retention times as well as by their fragmentation pattern in electron impact mass spectrometry and by their molecular ions, (M + NH₄)⁺ or (M + H)⁺, obtained after chemical ionization with ammonia (Table I).

Both oligosaccharide fractions A and B contained a 3-substituted galactose residue, identified as the peracetal of 2,4,6-GalOH (Figure 2). The mass fragmentographic detection of the ion m/e 369 (Table I) indicated that this derivative had, in both cases, been reduced with sodium borodeuteride. Therefore, this residue could not be involved in the O-glycosidic linkage, since the β-elimination was carried out in the presence of sodium borohydride. The only hydroxynated derivatives identified in oligosaccharides A and B corresponded to a 3-monosubstituted N-acetylgalactosaminitol in structure A and to a 3,6-disubstituted N-acetylgalactosaminitol in B, as revealed by the presence of the (M + H)⁺ ions m/e 336 (in A) and m/e 363 (in B), respectively. Therefore, in both oligosaccharide side chains N-acetylgalactosamine is O-glycosidically linked to the E1 protein backbone. The removal of sialic acid residues from the reduced oligosaccharides A and B, and the subsequent methylation analysis of the product yielded terminal galactose, identified as the peracetate of 2,3,4,6-GalOH, and exclusively 3-monosubstituted N-acetylgalactosaminitol, characterized as the peracetate of 1,4,5,6-GalN(Me)AcOH (Table I). Both compounds were in nearly equal molar amounts.

**Degradation by exoglycosidases**

To provide additional evidence for the sequence and the anomic configuration of the glycosidic linkages, oligosaccharides A and B, radiolabelled with tritiated galactose and glucosamine, were digested sequentially with the V. cholerae neuraminidase and β-galactosidase from bovine testes (Distler and Jourdian, 1973). The reaction products of each step were analyzed by chromatography on a calibrated Biogel P4 column (Figure 3). When oligosaccharide A (panel A, dashed line) and oligosaccharide B (panel A, solid line) were treated with neuraminidase, 1 or 2 mol, respectively, of sialic acid were released. In addition, an identical product was formed, which co-eluted with a reduced disaccharide standard Manβ(1→4)-GlcNAcOH (Figure 3B). This peak fraction was collected and cleaved with β-galactosidase from bovine testes into galactose and N-acetylgalactosaminitol, both of which co-eluted with the corresponding standards (panel C). At this stage no cleavage was obtained with α-galactosidase from green coffee beans and only partial degradation was observed with β-galactosidase from jack bean meal. This latter finding is in accordance with the reported substrate specificity of the jack bean enzyme, which cleaves Gal β(1→4)-linkages in preference to Gal β(1→3)-linkages (Li and Li, 1972).

**Characterization of the sialic acid residues**

More than 20 different kinds of sialic acids have been identified in nature, reflecting species- as well as tissue-specificity (for review see Corfield and Schauer, 1982). In mice three types have been detected in addition to the commonly found N-acetylneuraminic acid Neu5Ac. These include Neu5Gc (N-glycolylnuronamidic acid), Neu5,9Ac₂ (N-acetyl-9-monoo-O-acetylnuronamidic acid), and Neu9Ac₅Gc (N-glycolyln-9, mono-O-acetylnuronamidic acid) (Sarris and Palade, 1979; Reuter et al., 1980).
Table I. Methylation analyses of O-linked carbohydrates from MHV A59 glycoprotein E1. Characterization of the partially methylated alditol acetates obtained

| Peracette of\(^a\) | Peak ratio (relative retention time\(^b\)) of methylalditol acetates obtained from oligosaccharide | Primary mass fragments in electron impact MS\(^d\) | Molecular ions monitored in CI-MS\(^b\) |
|-----------------|-------------------------------------------------------------------------------------------------|---------------------------------|---------------------------------|
|                 | A / B after sialidase | m/e                           | m/e (M + NH\(_4\))\(^+\) m/e (M + H)\(^+\) |
| 2,3,4,6-GalOH   | -                      | 0.8 (1.025)                    | 118, 161, 162, 205              | 340, 341 \(^f\) |
| 2,4,6-GalOH     | 0.4 (1.140)            | 0.4 (1.140)                    | 118, 161, 234                  | 368, 369 |
| 1,4,5,6-GalN(Me)AcOH | 1.0\(^d\) (1.229) | 1.0\(^d\) (1.229)             | 130, 246                       | 336, 337 |
| 1,4,5-GalN(Me)AcOH | -                      | 1.0\(^d\) (1.406)             | 117, 130, 161, 246, 318        | 364, 365 |

\(^a\)2,3,4,6-GalOH: 2,3,4,6-tetra-O-methylgalactitol, etc. 1,4,5,6-GalN(Me)AcOH: 2-deoxy-2-(N-methylacetamido)-1,4,5,6-tetra-O-methylgalactitol, etc.

\(^b\)Peak ratios based on this derivative.

\(^c\)Retention times based on 2,3,4,6-ManOH (Geyer et al., 1983).

\(^d\)Electron impact spectra were obtained at an ionization potential of 70 eV, ionization current: 300 \(\mu\)A, temperature of the ion source: 250°C, multiplier voltage: 1.6 kV.

\(^e\)Chemical ionization mass spectra were obtained with ammonia (0.1 Torr) in the ion source at an ionization potential of 150 eV and an ion source temperature of 120°C.

\(^f\)Detected ions are underlined.

Fig. 2. Methylation analysis of reduced oligosaccharides from glycoprotein E1 of MHV A59. Reconstructed ion chromatographs (RIC) of partially methylated alditol acetates obtained from oligosaccharide A, (A) and oligosaccharide B, (B). The partially methylated alditol acetates were separated by capillary gas chromatography on a fused silica, bonded-phase DB-1 column (0.25 mm inner diameter; 60 m length), using helium (2.8 bar) as a carrier gas. A temperature program was applied from 100°C to 250°C at 2°C/min. Sugar derivatives were detected by mass fragmentography after chemical ionization with ammonia and selective monitoring of the ions as described in Table 1; a and b, identified as peracetylated Cl-deuterated 2,3,4,6-GlcOH and 2,3,6-GlcOH, respectively, probably derived from cellulose contaminants.

Since potential O-substituents are not detectable in oligosaccharides obtained by \(\beta\)-elimination, we used \([\text{H}]\)glucosamine-labelled E1 glycopeptides to assay the types of sialic acids present. Using the method of Shukla and Schauer (1982) and Neu5Ac, Neu5,9Ac\(_2\) and Neu5Gc as internal unlabelled standards, the products released by treatment with \(V.\) cholerae neuraminidase were analyzed by ion-exchange chromatography (Figure 4). As judged by the distribution of radiolabel co-migrating with the various sialic acid standards, 80% of the neuraminic acid residues present in glycoprotein E1 co-eluted with Neu5Ac, while the remaining 20% of the label eluted with the Neu5,9Ac\(_2\) standard (panel B). Both detected sialic acids were specifically degraded by N-acetyll neuraminic acid aldolase from \(Clostridium\) perfringens, yielding unlabelled pyruvic acid and the corresponding radioactive N-acetylmannosamine derivative, which does not bind to the ion-exchange resin (panel C). Since the aldolase cleaves only free sialic acid residues, it can be ruled out that the observed radioactive peak fractions were derived from siaiylated glycopeptides (Comb et al., 1966).
Discussion

Structures of the oligosaccharides

Glycoprotein E1 of MHV A59 contains exclusively O-linked carbohydrate side chains, which can be metabolically labelled with radioactive galactose and glucosamine, and can be liberated by β-elimination under reducing conditions (Niemann and Klenk, 1981b). We have now separated the reduced oligosaccharides by h.p.l.c. into two kinds of side chains designated A and B (Figure 1), and determined their structures by methylation analyses and sequential degradation with exoglycosidases. The results presented above lead to the following conclusions.

Oligoaccharide A comprises ~35% of the side chains of glycoprotein E1 of MHV A59. It is unbranched and contains sialic acid in a terminal non-reducing position, as evidenced by methylation analysis (Table I, Figure 2), and by the susceptibility of the oligosaccharide to V. cholerae neuraminidase (Figure 3). Treatment with this enzyme released 1 mol of sialic acid, as indicated by the altered chromatographic behaviour in h.p.l.c. (not shown) and gel permeation chromatography (Figure 3B). The product, characterized by methylation analyses to be Gal-1→3-GalNAcOH (Table I), was degraded by β-galactosidase from bovine testes to galactose and N-acetylgalactosaminitol (Figure 4). Taken together, these results are in agreement with the following structure for oligosaccharide A:

\[
\text{Neu5Ac} \xrightarrow{2\alpha} 3\text{-D-Gal-1} \xrightarrow{\beta} 3\text{-GalNAcOH}
\]

Neu5,9Ac₂

Oligosaccharide B, comprising the remaining 65% of the O-linked side chains of glycoprotein E1 of MHV A59, has a branched structure, as evidenced by the presence of 1,4,5-GalN(Me)AcOH in methylation analysis (Figure 2, Table I). Oligosaccharide B yielded the same products as A upon neuraminidase treatment. Based on the enzyme-induced shifts in h.p.l.c. and gel permeation chromatography, oligosaccharide B contains 2 mol of sialic acid. As detected with oligosaccharide A, N-acetylgalactosamine was determined to be the innermost sugar residue, being O-glycosidically linked to the E1 polypeptide. Therefore, oligosaccharide B
constitutes a disialylated tetrasaccharide with the following structure:

\[
\begin{align*}
\text{Neu5Ac, Neu5,9Ac_2} & \\
\text{Neu5Ac} & \\
\text{Neu5,9Ac_2} \\
\end{align*}
\]

These are the first structures of O-glycosidically linked oligosaccharides derived from viral glycoproteins. In addition, this is the first report of the presence of O-acetylated neuraminic acid species in viral glycoproteins. 20% of the sialic acid residues present in glycoprotein E1 of MHV A59 were identified as Neu5,9Ac_2. We are currently investigating, whether these O-acetylated derivatives reside in specific positions of either oligosaccharide A or B, or whether they are randomly distributed over all attachment sites.

There are further aspects, which should be discussed in context with the proposed structures. (i) The molar amounts of the partially methylated galactitol acetates, as determined by methylation analyses (Table I, Figure 2), are too low in comparison with the corresponding N-acetylgalactosaminol derivatives. This could be due to too severe hydrolysis conditions during the methylation analysis. In addition, it may reflect the problems, which arise in the analysis of less than nmol amounts of oligosaccharide. (ii) We have reported previously (Niemann and Klenk, 1981b) that glycoprotein E1 of MHV A59 contained N-acetylgalactosamine in addition to the constituent sugars reported here. Since recent amino acid analyses of glycoprotein E1 preparations, obtained by two consecutive runs on polyacrylamide gels, showed the complete absence of this amino sugar, we believe that our previous glycopeptide samples were contaminated with cellular components.

**Attachment sites for the O-linked glycans in glycoprotein E1 of MHV A59**

The amino acid sequence of the E1 polypeptide has been determined recently from cloned cDNA (Armstrong et al., 1983; Niemann et al., 1983). Figure 5 shows the sequence of the amino-terminal domain. This part of the molecule, which resides on the exterior of the viral envelope, carries the carbohydrates (Sturman et al., 1980; Rottier et al., 1984).

It is noteworthy that, in contrast to all other known viral glycoproteins, the E1 polypeptide lacks a hydrophobic leader peptide at the amino-terminal end (Rottier et al., 1984). Instead, a cluster of four hydroxyamino acids, each of them providing potential O-glycosylation sites, is located next to the amino-terminal methionine residue (Figure 5). We do not know yet, which of the hydroxyamino acids are substituted with carbohydrate side chains. Up to three glycosylated E1 species have been resolved by polyacrylamide gel electrophoresis, and it has been suggested that this heterogeneity was caused by differences in glycosylation (Siddell et al., 1981). Therefore, it is likely that up to three of the four hydroxy amino acids residing in this terminal cluster are substituted with O-linked glycans.

**Potential functions of the O-linked carbohydrates**

In Figure 5 we have included the amino-terminal sequence of glycophorin A, the major glycoprotein of the human erythrocyte membrane. Glycophorin A contains oligosaccharide side chains identical to those determined here for glycoprotein E1 of MHV A59 (Thomas and Winzler, 1969). In addition to this relatedness at the carbohydrate level, an identical tetrapeptide sequence — Ser-Ser-Thr-Thr — is present at the amino terminus of glycophorin A (Tomita et al., 1978). This amino acid sequence constitutes, in conjunction with oligosaccharides of structure B, the blood group M determinant (Lisowska and Kordowicz, 1977).

We have evidence that purified glycoprotein E1 of MHV A59 also carries blood group M activity demonstrable with an anti-M rabbit serum, routinely used for blood typing, in an enzyme-linked immunosorbent assay (Niemann and Repp, in preparation). Further experiments are needed to clarify the potential significance of this finding for virus host cell interactions.

It was shown in experiments with the ionophore monensin, that glycosylation of the E1 polypeptide is not essential for intracellular virus particle formation (Niemann et al., 1982). In addition, the matrix protein of the avian infectious bronchitis virus (IBV) contains N-glycosidically linked oligomannosidic side chains (Stern et al., 1982). This raises the question, whether the O-linked carbohydrate side chains detected in the murine and bovine coronaviruses have any unique functions. In this context, it is interesting to note that the amino acid sequence of the IBV matrix glycoprotein, as deduced from cloned cDNA (Boursnell, 1983), contains two sequons for N-glycosylation in series (Marshall and Neuberger, 1970), again adjacent to the terminal methionine residue. It is intriguing to speculate that the amino-termionally located oligosaccharides of IBV and MHV, although attached in different intracellular compartments and of totally different structure, could play an identical role in stabilizing a tertiary structure of the E1 glycoprotein in the viral membrane. We are examining the question using site-directed mutagenesis of the E1 gene.

**Materials and methods**

**Preparation of radiolabelled glycoprotein E1**

MHV A59 was grown in the spontaneously transformed 17Cl1 line of BALB/c 3T3 cells (m.o.i. = 1 p.f.u./cell) in the presence of D6-[H]glactose (40 Ci/mM) or D6-[H]glucosamine (25 Ci/mM) and purified by standard procedures. Glycoprotein E1 was recovered from preparative 12% (w/v) polyacrylamide slab gels as described previously (Niemann and Klenk, 1981b).

**β-Elimination**

Purified viral glycoprotein was incubated in 50 mM NaOH, 1 M NaBH₄, and 0.1% (w/v) SDS for 20 h at 45°C (Carlson, 1968). The reaction was terminated by the addition of 4 M acetic acid at 0°C to pH 5.5. The samples were de-salted by passage over a Biogel P2 column (1 x 23 cm) using distilled water as an eluant. Aliquots were assayed for radioactivity by liquid scintill-
lation counting, the radioactive fractions were pooled and lyophilized.

Preparation of glycopeptides
Purified glycoprotein E1 was degraded with pronase P from Streptomyces griseus in 1 M Tris/HCl, pH 8.0, and de-salted as described above.

H.p.l.c.
The h.p.l.c. procedure of Bergh et al. (1981) was applied using a Waters (Milford, USA) model 6000 pump and a USK injection system. The mixture of reduced oligosaccharides was chromatographed on a Lichrosorb-NH2 column (Merck, Darmstadt, FRG) as detailed in the legend to Figure 1.

Permethylation analyses
Reduced oligosaccharides were permethylated according to Hakomori (1964) using the micromethylation procedure recently devised for the analyses of N-glycans in the picomole range (Geyer et al., 1983). To distinguish intrachain sugar residues from those involved in the O-glycosidic linkage to the polypeptide, partially methylated sugar aldehydes were reduced with sodium borohydride. Sugar derivatives were identified according to their retention times in gas capillary chromatography and by electron impact mass spectrometry and by mass fragmentography after chemical ionization with ammonia based on the characteristic fragments of hexitols and aminohexitols as detailed in Table I.

Digestion with exoglycosidases
For treatment with neuraminidase from V. cholerae (EC 3.2.1.18, Behringwerke Marburg, FRG), radiolabelled glycopeptides or reduced oligosaccharide fractions were dissolved in 300 μl 50 mM sodium acetate buffer, pH 5.5, containing 145 mM NaCl, 1 mM CaCl2 and 0.3 U of the enzyme. The mixtures were incubated at 37°C for 24 h and the reaction was terminated by heating to 100°C for 3 min. The samples were lyophillized, dissolved in 100 μl of distilled water and analyzed by h.p.l.c. or gel permeation chromatography. For treatment with β-galactosidase from bovine testes (EC 3.2.1.23) (kindly supplied by Inka Brockhausen, Hospital for Sick Children, Toronto, Canada), the h.p.l.c. chromatograms from the Biogel P4 column were dissolved in 100 μl 0.15 M sodium phosphate-citrate buffer, pH 4.3, containing 0.02% sodium azide and 26 μU of the enzyme (Distler and Jourdian, 1975). After 12 h incubation at 37°C the same amount of enzyme was added. The reaction was terminated after 24 h by boiling and the products were directly applied onto the Biogel P4 column as described in the legend to Figure 3. β-Galactosidase from jack bean meal and α-galactosidase from green coffee beans (EC 3.2.1.22) were purchased from Sigma (St. Louis, USA).

Identification of sialic acid residues
Sialic acids were analyzed using a modification of the method described by Shulka and Schauer (1982). Chromatographic separation was performed at 50°C on a modified Biotronic (Frankfurt, FRG) model LC 6000 amino acid analyzer, using a column (0.4 x 40 cm) of Aminex A28 (Bio-Rad, Richmond, USA). Elution was performed isocratically with helium de-airated 1 mM aqueous sodium sulfate at a flow rate of 20 μl/h and 50 bar. Neu5Ac (Shulka, St. Louis, USA), Neu5Ac2y and Neu5Gc (kindly provided by G. Reuter, Christian-Albrechts-Universität, Kiel, FRG) were used as internal standards (5 μg each) to the neuraminidase-treated glycopeptide samples. The absorption of sialic acid residues was monitored at 220 nm wavelength, using a Waters (Milford, USA) model 450 variable wavelength detector. In control experiments samples were treated with N-acetylneuraminic acid aldolase (EC 4.1.3.3) from C. perfringens in 20 mM sodium phosphate, pH 7.2, for 16 h at 37°C.

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