The glycoprotein 71 of ecotropic Friend murine leukemia virus

Structure of the oligosaccharides linked to asparagine-12

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The glycoprotein from Friend murine leukemia virus was digested with protease from *Staphylococcus aureus* V8. A glycopeptide comprising the N-terminal glycosylation site (Asn-12) was isolated from the mixture of fragments and analyzed by amino acid sequencing and methylation-capillary gas chromatography–mass spectrometry before and after treatment with sialidase from *Vibrio cholerae*. Asn-12 was thus found to be substituted by a family of partially sialylated, fucosylated, and intersected glycoprotein *N*-glycans of the hybrid type.

Glycoprotein *Glycosylation site* *Leukemia* *Methylation analysis* *Oligosaccharide structure* *Retrovirus*

1. INTRODUCTION

The MuLVs are enveloped RNA viruses (type C retroviruses), the surface of which is studded with 'spikes' consisting mainly of one glycopolypeptide [1]. Because of the intriguing multitude of biological functions associated with these viral gps, notably because of their possible implication in leukemogenesis [1–3], the amino acid sequences of the gps of several MuLV strains have been established, including that of the gp71 of the ecotropic F-MuLV [4], which was found to comprise 8 potential *N*-glycosylation sites [5,6]. In addition, we [7,8] recently elucidated the structures of the desialylated *N*-glycans from the isoglycoproteins (gp71 and gp69) [3,9,10] of helper-independent Friend virus [4] as produced by Eveline suspension cells [11] in amounts sufficient for carbohydrate analysis. For a complete structure determination of F-MuLV gp71, therefore, the assignment of oligosaccharides to specific Asn-X-Ser/Thr sequences in these isoglycoproteins, as well as their degree of sialylation remain to be established. We report here on the structure of the carbohydrate unit linked to Asn-12, at the N-terminal sequence of F-MuLV gp71.

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Abbreviations: endo H, *endo-β-N*-acetyl-D-glucosaminidase H from *Streptomyces griseus*; GC, gas chromatography; gp, glycoprotein; gp71, gp of apparent M_r 71000; HPLC, high-performance liquid chromatography; rHPLC, reversed-phase HPLC; MS, mass spectrometry; MuLV, murine leukemia virus; F-MuLV, Friend strain of MuLV; PTH amino acids, phenylthiohydantoin amino acids; SA, sialic acid

2. MATERIALS AND METHODS

2.1. *Preparation and proteolysis of viral glycoprotein*

As detailed in [7,10], the mixture of isoglycoproteins gp71 and gp69 was isolated from particles of helper-independent Friend virus [4] as produced by

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Eveline cells [11]. Samples (about 5 mg) of reduced and carboxymethylated gp71/gp69 were digested (24 h at 37°C) with protease from *S. aureus* V8 (60 μg; Miles, Elkhart, IN) in 0.2 M ammonium bicarbonate buffer of pH 8.2 (repeated once).

2.2. *Fractionation of (glyco)peptides*

The digests were prefractionated through a column (1.6 × 170 cm) of Biogel P-30 (100–200 mesh; BioRad, Richmond, CA) at 25°C employing a 10 mM ammonium acetate buffer of pH 6.0 (containing 0.2 g/l NaN_3).

A Waters (Milford, MA) system (model 6000A pumps, model 660 solvent programmer and model U6K injector) combined with a Gynkotek (Munich) photometer (model SP4) served for the subfractionation by rHPLC. Essentially, the conditions described in [12] were employed, i.e., a column (0.46 × 25 cm) of ODS-Hypersil (3μ; Shandon, Runcorn, England) and a linear gradient of acetonitrile in the ammonium acetate buffer.

Peak fractions were collected as recorded by continuous flow-monitoring of absorbance at 220 nm.

2.3. *Digestion with sialidase*

The enzyme from *V. cholerae* (Behringwerke, Marburg) was used; for experimental details see [13].

2.4. *Protein analytical methods*

The amino acid (or hexosamine) composition of (glyco)peptides was determined with a Biotronik (Frankfurt) model LC 6001 analyzer after 24 h (or 2 h) of hydrolysis at 110°C (or 100°C) in 6 N (or 4 N) aqueous HCl, using *o*-phthaldialdehyde as a colouring reagent.

The amino acid sequence analysis of the N-terminal glycopeptide was performed with a Sequemat (Watertown, MA) solid-phase sequencer (model Mini 15) after coupling to aminopropyl glass with 1-ethyl-3-dimethylaminopropylcarbodiimide (both Pierce, Rockford, IL) [14]. The PTH amino acids were identified by rHPLC using the Waters equipment including an absorbance detector (model 440) coupled to a Hewlett Packard (Palo Alto, CA) integrator (model 3380A), and following the experimental details in [15], except that a longer column (0.46 × 40 cm) of LiChrosorb RP-18 (5 μ; Merck, Darmstadt) was employed.

2.5. *Carbohydrate analytical methods*

The N-terminal glycopeptide was permethylated as in [16], and the partially methylated alditol acetates obtained after hydrolysis etc. were analyzed by GC–MS [17], using the micromethylation-capillary GC–MS version of this technique and the instrumentation detailed in [18].

3. RESULTS

3.1. *Isolation of N-terminal glycopeptide of F-MuLV gp71*

Under appropriate conditions, the protease of *S. aureus* V8 cleaves proteins almost exclusively at the C-termini of Glu residues. The fragments obtained by the action of this enzyme on F-MuLV gp71 could thus be expected to comprise glycopeptides with the following *N*-glycosylation sites: Ala-1 ··Asn-12··Glu-16 (M_r 2500–3500), Thr-148··Asn-168··Asn-266··Asn-302··Glu-311 (M_r 21000–24000), Gly-324··Asn-334··Asn-341··Glu-354 (M_r 5000–7000), and Val-355··Asn-374··Asn-410··Glu-420 (M_r 9000–10000) (cf. [5,6]). For the isolation of an N-terminal glycopeptide of F-MuLV gp71 containing only the first *N*-glycosylation site (Asn-12), the following strategy was adopted: The mixture of isoglycoproteins gp71 and gp69 was digested with *S. aureus* protease, and the products were prefractionated by gel filtration; the peak material comprising fragments of M_r about 2000–4000 was collected and subjected to rHPLC. A fraction containing glucosamine was thus isolated (fig.1), the microheterogeneity of which appears to be due to variations in glycosylation, because it is diminished by sialidase treatment.

3.2. *Amino acid sequence of N-terminal glycopeptide*

The glycopeptide thus isolated was found to contain Asx, Thr, Ser, Glx, Gly, Ala, Val, Ile, Tyr, His, and GlcN in molar ratios approaching 1:1:2:2:1:2:1:1:1:1:3 (Pro and Trp not determined), and was sequenced with the results shown in table 1.

3.3. *Analysis of carbohydrate moiety in N-terminal glycopeptide*

The glycopeptide (fig.1b) and its sialidase digestion product (fig.1c) were permethylated, hydrolyzed, reduced with $NaBH_4$, and pera-

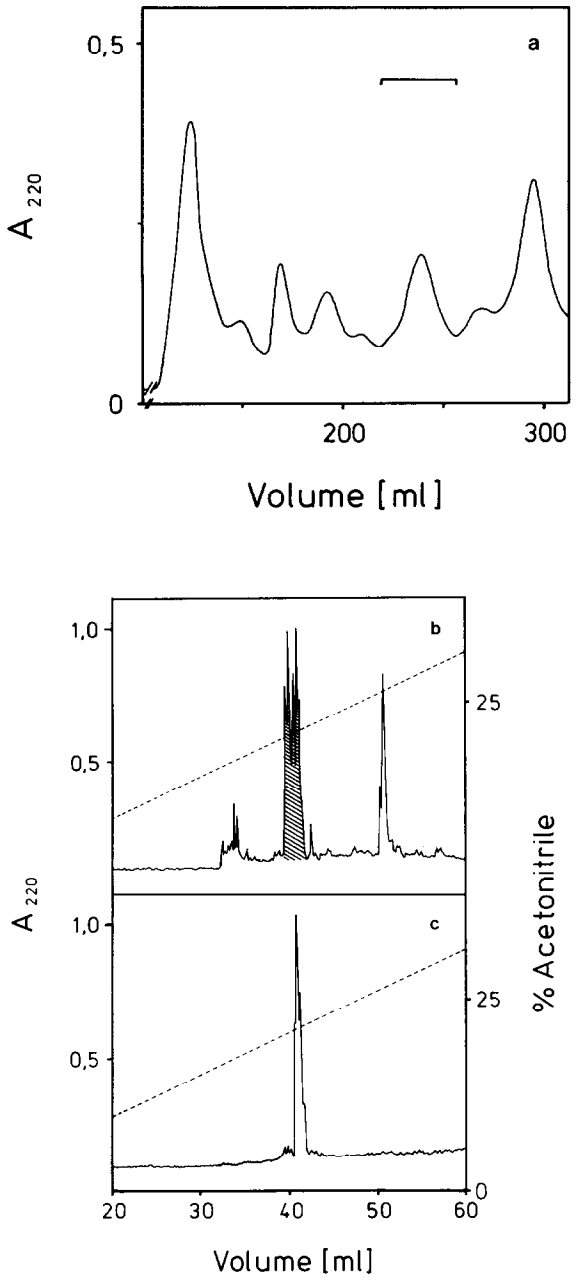

Fig.1. Isolation of N-terminal glycopeptide from F-MuLV gp71. (a) Biogel P-30 chromatography of F-MuLV gp71/gp69 after digestion with protease from *S. aureus* V8. (b) Subfractionation of gp fragments with M_r around 2000–4000 (bracket in a) by rHPLC; only the microheterogeneous, hatched fraction was found to contain glucosamine. (c) rHPLC of glycopeptide fraction (hatched peak in b) after treatment with sialidase from *V. cholerae*.

Table 1

Amino acid sequence of N-terminal glycopeptide of F-MuLV gp71 (data not corrected for background)

Step	Amino acid identified	Yield (pmol)
1	A	2800
2	A	2300
3	P	900
4	G	910
5	S	530
6	S	690
7	P	960
8	(H)[a]	– [b]
9	Q	1200
10	V	720
11	Y	510
12	(N)[a]	– [b]
13	I	140
14	T	180
15	W	110
16	(E)[a]	– [b]

[a] Assigned on the basis of the known sequence [5,6,10]
[b] Not identified

cetylated, and the partially methylated hexitol acetates obtained were analyzed by GC–MS. The results are presented in table 2.

4. DISCUSSION

A comparison of the amino acid sequence data presented in table 1 with those published for gp71 of ecotropic F-MuLV [5,6,10] and for two smaller isoglycoproteins also found in helper-independent Friend virus as produced by Eveline cells [3,10] clearly shows that the glycopeptide isolated here constitutes the N-terminus of the former glycoprotein. In addition, these data confirm that the peptide portion of the glycopeptide is homogeneous.

A comparison of the methylation results shown in table 2, on the other hand, with the structures of the desialylated oligosaccharides as obtained from total gp71/gp69 [7,8], leads to the conclusion that it is mainly the endo H-sensitive [20] glycans of the 'mixed type' [21] previously designated 'M_{III}' and 'M_{IV}' [8] which are linked to Asn-12 of gp71. However, as evidenced by the detection

Table 2

Methylation analysis of N-terminal glycopeptide from F-MuLV gp71

Peracetate of[a]	Peak ratio[b] obtained from glycopeptide	
	Before digestion with sialidase	After
2,3,4-FucOH	0.3	0.3
2,3,4,6-ManOH	1.4	1.2
3,4,6-ManOH	1.0[b]	1.0[b]
2,4,6-ManOH	0.15	0.2
2,4-ManOH	1.45[c]	1.3[c]
2-ManOH	0.1[c]	0.1[c]
2,3,4,6-GalOH	0.6	1.0
2,4,6-GalOH	0.4	–
2,3,4-GalOH	trace	–
3,4,6-GlcN(Me)AcOH	0.15	0.3
3,6-GlcN(Me)AcOH	2.6	2.5
3-GlcN(Me)AcOH	0.1	0.1

[a] 2,3,4,6-ManOH, 2,3,4,6-tetra-*O*-methylmannitol etc.; 3,4,6-GlcN(Me)AcOH, 2-deoxy-2-(*N*-methylacetamido)-3 4,6-tri-*O*-methylglucitol etc.

[b] As detected by flame ionization; based on 3,4,6-ManOH = 1.0

[c] Corrected for loss during hydrolysis [19]

of 2,4,6-GalOH before but not after sialidase treatment, and of 2,3,4-FucOH and 3-GlcN(Me)AcOH, respectively, these oligosaccharides are partially sialylated and fucosylated in the native glycoprotein. In addition, and also in agreement with our earlier results [8], a minority of the hybrid glycans in the N-terminal glycopeptide appear to carry intersecting GlcNAc residues [see values for 2-ManOH and 3,4,6-GlcN(Me)AcOH].

The structure of the N-terminal glycopeptide of F-MuLV gp71 may thus be summarized as shown in scheme 1.

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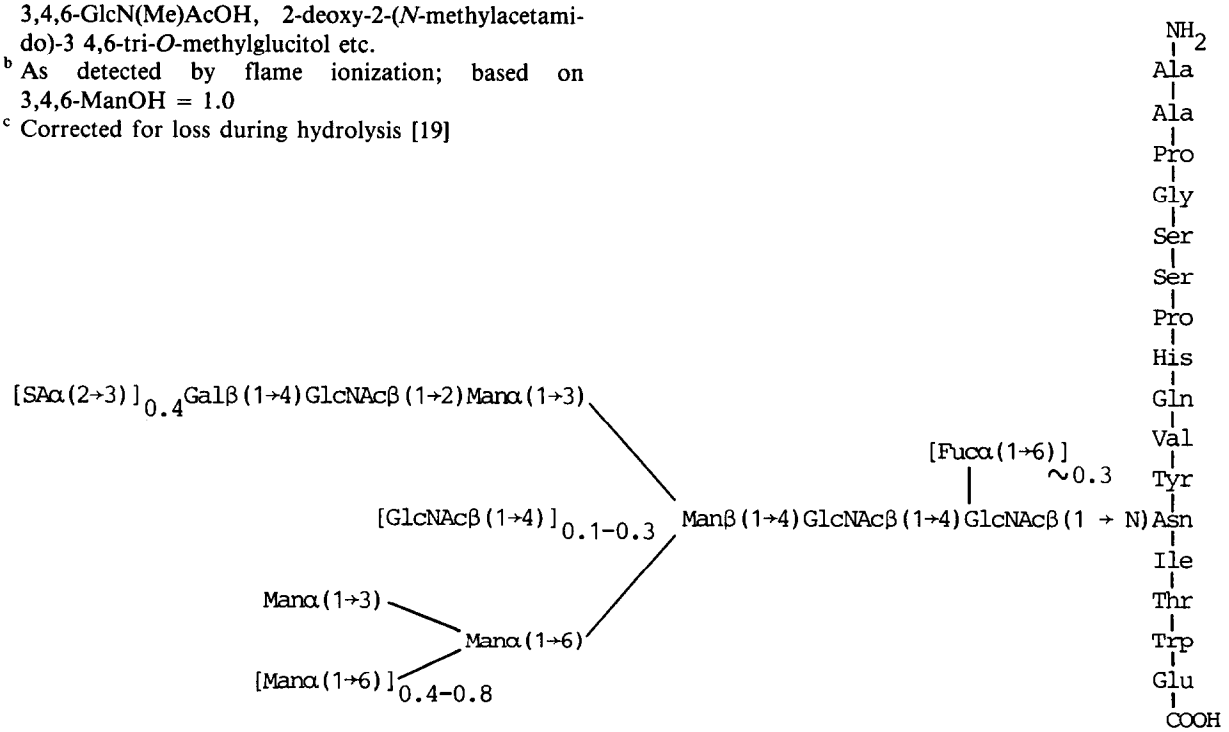

Scheme 1

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