

# The glycoprotein 71 of ecotropic Friend murine leukemia virus

## Structure of the oligosaccharides linked to asparagine-12

Michael Schlüter, Dietmar Linder, Rudolf Geyer, Gerhard Hunsmann<sup>+</sup>, Josef Schneider<sup>+</sup> and Stephan Stirm\*

*Biochemisches Institut am Klinikum der Universität, Friedrichstrasse 24, D-6300 Giessen and <sup>+</sup>Forschergruppe Tumorimmunologie, Stefan Meier-Str. 8, D-7800 Freiburg, FRG*

Received 9 February 1984

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.

<i>Glycoprotein</i>	<i>Glycosylation site</i>	<i>Leukemia</i>	<i>Methylation analysis</i>
	<i>Oligosaccharide structure</i>	<i>Retrovirus</i>	

### 1. INTRODUCTION

The MuLVs are enveloped RNA viruses (type C retroviruses), the surface of which is studded with 'spikes' consisting mainly of one glycopolyptide [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 com-

prise 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.

\* To whom correspondence should be addressed

**Abbreviations:** endo H, endo- $\beta$ -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

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<sub>3</sub>).

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<sub>r</sub>* 2500–3500), Thr-148 ··· Asn-168 ··· Asn-266 ··· Asn-302 ··· Glu-311 (*M<sub>r</sub>* 21000–24000), Gly-324 ··· Asn-334 ··· Asn-341 ··· Glu-354 (*M<sub>r</sub>* 5000–7000), and Val-355 ··· Asn-374 ··· Asn-410 ··· Glu-420 (*M<sub>r</sub>* 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<sub>r</sub>* 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<sub>4</sub>, and per-

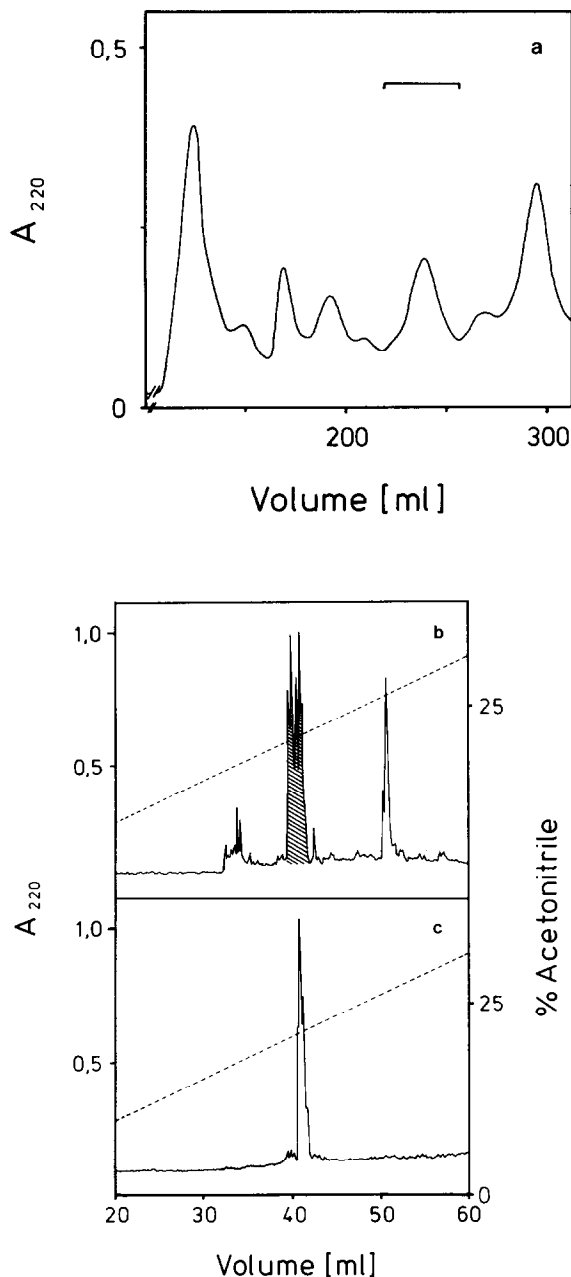


Fig.1. Isolation of N-terminal glycopeptide from F-MuLV gp71. (a) Biogel P-30 chromatography of F-MuLV gp71/gp69 after digestion with protease from *S. aureus* V8. (b) Subfractionation of gp fragments with  $M_r$  around 2000–4000 (bracket in a) by rHPLC; only the microheterogeneous, hatched fraction 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) <sup>a</sup>	— <sup>b</sup>
9	Q	1200
10	V	720
11	Y	510
12	(N) <sup>a</sup>	— <sup>b</sup>
13	I	140
14	T	180
15	W	110
16	(E) <sup>a</sup>	— <sup>b</sup>

<sup>a</sup> Assigned on the basis of the known sequence [5,6,10]

<sup>b</sup> 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<sub>III</sub>' and 'M<sub>IV</sub>' [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 <sup>a</sup>	Peak ratio <sup>b</sup> 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	<u>1.0<sup>b</sup></u>	<u>1.0<sup>b</sup></u>
2,4,6-ManOH	0.15	0.2
2,4-ManOH	1.45 <sup>c</sup>	1.3 <sup>c</sup>
2-ManOH	0.1 <sup>c</sup>	0.1 <sup>c</sup>
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

<sup>a</sup> 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.

<sup>b</sup> As detected by flame ionization; based on 3,4,6-ManOH = 1.0

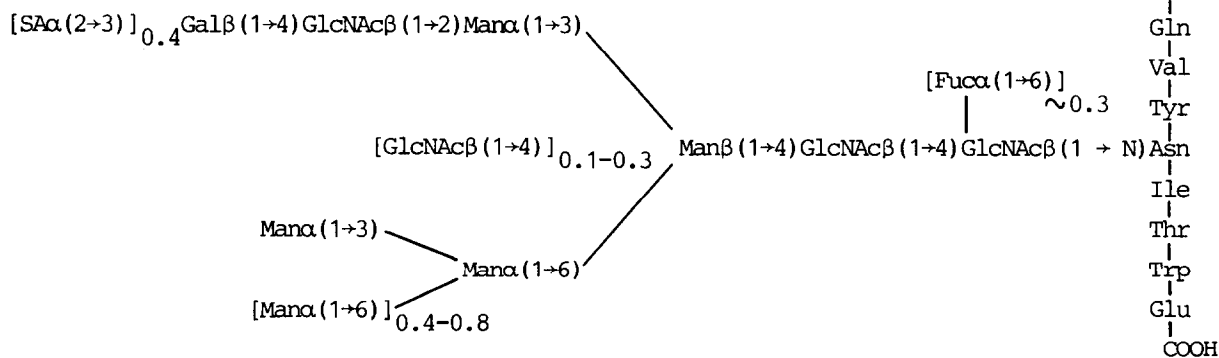
<sup>c</sup> Corrected for loss during hydrolysis [19]

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.

## ACKNOWLEDGEMENTS

We wish to express our gratitude to Michael Dreisbach for the amino acid, and to Werner Mink and Siegfried Kühnhardt for the methylation analyses. This project was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbe-  
reich 47 and Hu 244/6) and by Fonds der Chemischen Industrie (341).



Scheme 1

## REFERENCES

- [1] Dickson, C., Eisenmann, R., Fan, H., Hunter, E. and Teich, N. (1982) in: RNA Tumor Viruses (Weiss, R. et al. eds) pp.513–648, Cold Spring Harbor Laboratory, Cold Spring Harbor.
- [2] Famulari, N.G. (1983) *Curr. Top. Microbiol. Immunol.* 103, 75–108.
- [3] Koch, W., Zimmermann, W., Oliff, A. and Friedrich, R. (1984) *J. Virol.* 49, 828–840.
- [4] Troxler, D.H., Ruscetti, S.K. and Scolnick, E.M. (1980) *Biochim. Biophys. Acta* 605, 305–325.
- [5] Koch, W., Hunsmann, G. and Friedrich, R. (1983) *J. Virol.* 45, 1–9.
- [6] Chen, R. (1982) *Proc. Natl. Acad. Sci. USA* 79, 5788–5792.
- [7] Geyer, R., Geyer, H., Stirm, S., Hunsmann, G., Schneider, J., Dabrowski, U. and Dabrowski, J. (1984) *Biochemistry*, submitted.
- [8] Geyer, R., Geyer, H., Egge, H., Schott, H.-H. and Stirm, S. (1984) *Eur. J. Biochem.*, submitted.
- [9] Murray, M.J. and Kabat, D. (1979) *J. Biol. Chem.* 254, 1340–1348.
- [10] Linder, D., Stirm, S., Schneider, J., Hunsmann, G., Smythers, G. and Oroszlan, S. (1982) *J. Virol.* 42, 352–355.
- [11] Seifert, E., Claviez, M., Frank, H., Hunsmann, G., Schwarz, H. and Schäfer, W. (1975) *Z. Naturforsch.* 30c, 698–700.
- [12] Kratzin, H., Yang, C., Krusche, J.U. and Hilschmann, N. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* 361, 1591–1598.
- [13] Geyer, R., Geyer, H., Hunsmann, G., Schneider, J. and Stirm, S. (1982) *Biochim. Biophys. Acta* 717, 491–501.
- [14] Laursen, R.A. and Machleidt, W. (1980) *Methods Biochem. Anal.* 26, 201–284.
- [15] Lottspeich, F. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* 361, 1829–1834.
- [16] Hakomori, S. (1964) *J. Biochem. (Tokyo)* 55, 205–207.
- [17] Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B. and Lönnegren, J. (1976) *Chem. Commun. Univ. Stockholm* 8, 1–75.
- [18] Geyer, R., Geyer, H., Kühnhardt, S., Mink, W. and Stirm, S. (1983) *Anal. Biochem.* 133, 197–207.
- [19] Conchie, J., Hay, A.J. and Lomax, J.A. (1982) *Carbohydr. Res.* 103, 129–132.
- [20] Kobata, A. (1979) *Anal. Biochem.* 100, 1–14.
- [21] Berger, E.G., Buddecke, E., Kamerling, J.P., Kobata, A., Paulson, J.C. and Vliegenthart, J.F.G. (1982) *Experientia* 38, 1129–1162.