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ISOLATION OF GLYCOPEPTIDES CONTAINING INDIVIDUAL GLYCOSYLATION SITES OF FRIEND MURINE LEUKEMIA VIRUS GLYCOPROTEIN: STUDIES OF GLYCOSYLATION BY METHYLATION ANALYSIS*

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ABSTRACT

Glycopeptides containing individual *N*-glycosylation sites of the glycoprotein from Friend murine leukemia virus were isolated by digestion of the viral glycoprotein with protease of *S. aureus* (V8) or with trypsin followed by fractionation of the resulting (glyco)peptides by gel filtration and reversed-phase, high-performance liquid chromatography at pH 6. Isolated glycopeptides were assigned to the known amino acid sequence of the protein by amino acid analysis and by determination of the NH_2-termini. The carbohydrate moieties of each glycosylation site were analysed by methylation analysis. A high selectivity of the glycoprotein glycosylation was found with regard to the distribution of oligomannosidic, mixed, and *N*-acetyl-lactosaminic oligosaccharides.

INTRODUCTION

The surface of murine leukemia viruses (MuLV's) is studded with "knob"-like projections consisting mainly of one glycoprotein. This viral constituent mediates the first contact with the host cell receptors[1–3], thus determining viral tropism[4], and carries antigenic determinants essential in active and passive immunisation against virus-induced leukemia[5,6]. Furthermore, an implication of these molecules in leukemogenesis has been suggested[7–11].

Because of the multitude of biological functions associated with this viral component, amino acid sequences of a number of glycoproteins derived from eco- and polytropic MuLV-strains have been established[4,12–21]. As a prerequisite for studies of the correlation of biological functions with glycosylation, we have initiated a structural analysis of the carbohydrate moieties of glycoproteins from the Friend-virus (F-MuLV) complex as produced by Eveline suspension cells[22], *i.e.*,

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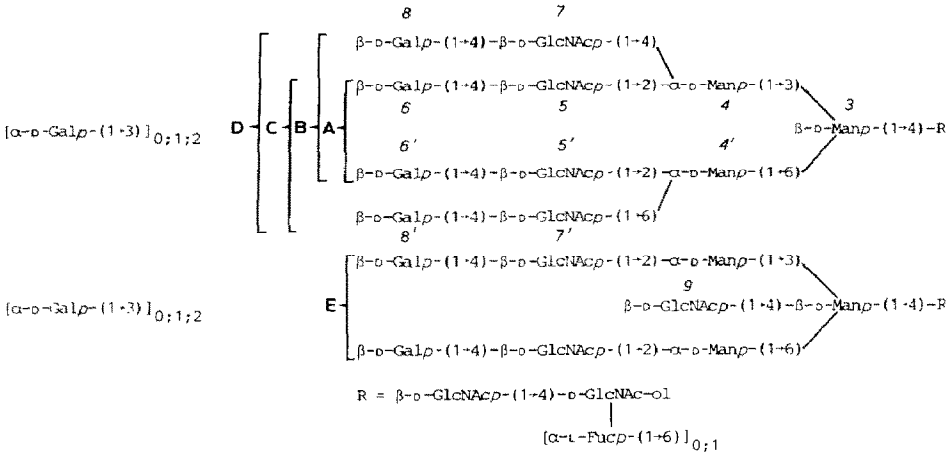
a virus-cell system which allows the isolation of glycoprotein[23] in amounts sufficient for carbohydrate and peptide analysis. As also described[20,24,25] for other strains of MuLV, Eveline suspension cells produce ecotropic as well as recombinant polytropic Friend-viruses. The isoglycoproteins of the individual virus species are closely related, but vary especially in the amino-terminal domains of the molecules. Therefore, isolation of viral glycoprotein from this source leads to a mixture of (at least) three viral glycoprotein species[4,16,18,26].

In preceding studies[27,28], we analysed the structures of the major (desialylated) *N*-linked glycans from the mixture of F-MuLV isoglycoproteins. Oligosaccharides of the oligomannosidic (OM), the mixed (M), and the *N*-acetyl-lactosaminic (AL) type were found, the majority of which comprise structures A, B, D, and E (Scheme 1). Characteristically, some of these glycans are substituted by terminal α-D-Gal*p*-(1→3) residues.



Scheme 1. Compilation of structures of *N*-acetyl-lactosaminic (AL) asialoglycans (numbering of sugar residues according to Vliegenthart *et al.*[29,30]).

We now describe the isolation of glycopeptides containing individual glycosylation sites from the major species of the F-MuLV isoglycoproteins, namely, glycoprotein (gp) 71, which has been shown to comprise eight potential sites for *N*-glycosylation (*i.e.*, Asn-X-Ser/Thr sequences)[14,18]. The glycopeptides obtained were characterised by amino acid analysis and by identification of the NH_2-termini, as well as by methylation analysis, thus allowing the assignment of the oligosaccharides mentioned to distinct glycosylation sites of gp71.

MATERIALS AND METHODS

Virus. — The helper-independent component of the Friend murine leukemia virus (F-MuLV) complex[31], as produced by Eveline cells[22], was used throughout. The cells were propagated in Dulbecco's modified Eagle's medium (Gibco, Paisley,

Great Britain), supplemented with 10% (v/v) complement-inactivated (30 min, 56°) fetal bovine serum; the virus particles were harvested by differential centrifugation[32].

Isolation and purification of viral glycoprotein. — The isoglycoproteins were released from the virus particles by freezing and thawing, and were purified by chromatography on phosphocellulose P-11 (Whatman) as detailed previously[16].

Preparation of viral glycopeptides. — Samples (~5 mg) of reduced and carboxymethylated glycoprotein were digested (1:100; w/w) with trypsin (TPCK treated; Serva, Heidelberg, GFR) for 5 h at 37° in 0.2M ammonium hydrogencarbonate buffer (pH 8.2). Digestion with *S. aureus* protease was performed as described earlier[33]. The glycoprotein digests were fractionated on a column (1.6 × 170 cm) of Biogel P-10 or P-30 (100–200 mesh; BioRad) at 25°, using 10mM ammonium acetate buffer (pH 6) containing NaN_3 (0.2 g/L) at a flow rate of 2.5 cm/h. A Waters system (Model 6000A pumps, Model 660 solvent programmer, and Model UK6 injector) combined with a Gynkotek (München, GFR) photometer (Model SP4) served for the sub-fractionation by reversed-phase h.p.l.c. Essentially, the conditions described by Kratzin *et al.*[34] were employed, *i.e.*, a column (0.46 × 25 cm) of ODS-Hypersil (3 μm; Shandon) and a linear gradient of acetonitrile in 25mM ammonium acetate buffer (pH 6). Peak fractions were collected as recorded by continuous flow-monitoring of absorbance at 220 nm.

Protein analytical methods. — The amino acid (or 2-amino-2-deoxyhexose) composition of (glyco)peptides was determined with a Biotronik (Frankfurt, FRG) Model 6001 analyser after 24 h (or 2 h) of hydrolysis at 110° (or 100°) in 6M (or 4M) aqueous HCl, using *o*-phthaldialdehyde as the colouring reagent.

Dansylation was carried out as described by Gray[35]. Identification of dansylamino acids[36,37] was performed on polyamide sheets (Schleicher and Schüll).

Methylation analysis. — Isolated glycopeptides were permethylated by the Hakomori method[38], and the partially methylated alditol acetates obtained after hydrolysis, *etc.*, were analysed by capillary g.l.c.–m.s. as detailed previously[39,40].

RESULTS AND DISCUSSION

Isolation of glycopeptides. — For the isolation of glycopeptides containing individual glycosylation sites of F-MuLV gp71, the following strategy was adopted. The mixture of isoglycoproteins was digested with trypsin and/or with protease from *S. aureus* (V8) which, under appropriate conditions, cleaves almost exclusively at the CO_2H-termini of Glu residues. The cleavage products were prefractionated by gel filtration. An example of a tryptic digest is shown in Fig. 1. Fractions were analysed for glycopeptides by determination of 2-amino-2-deoxyhexoses after acid hydrolysis, combined as indicated, and subjected to reversed-phase h.p.l.c. (Fig. 2a–c). Glycopeptides were monitored as described above and each peak fraction was subjected to amino acid analysis. Glycopeptide fractions having identical amino acid composition were combined (Fig. 2, hatched

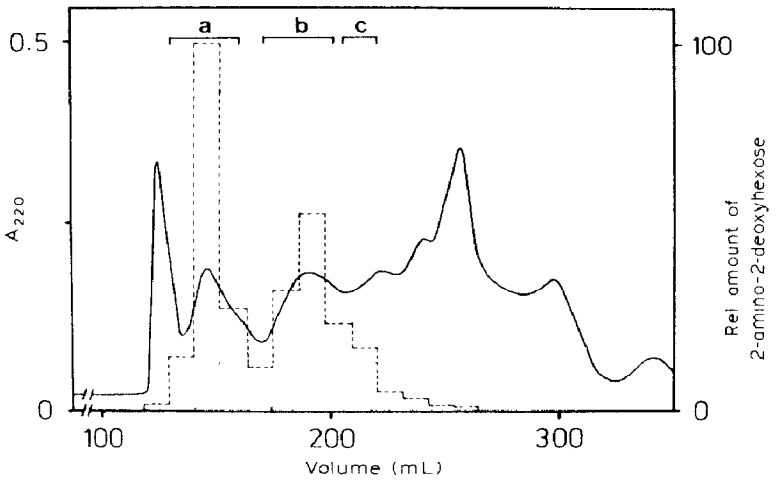


Fig. 1. Biogel P-30 chromatogram of carboxymethylated F-MuLV glycoproteins after digestion with trypsin. The elution profile was recorded by continuous flow-monitoring of absorbance at 220 nm (——), and by determination of 2-amino-2-deoxy-D-glucose (-----) and 2-amino-2-deoxy-D-galactose (· · ·). Glycopeptides were combined as indicated (fractions a,b,c,)

fractions) and further characterised by end-group determination. Comparison of these data with the known amino acid sequence of F-MuLV gp71[14,18] allowed the identification and assignment of gp71-derived glycopeptides.

Using this approach, the following results were obtained. The peptide containing glycosylation site 1 (PGS-1) at Asn-12, *i.e.*, the NH_2-terminal glycopeptide of gp71, was isolated after digestion with *S. aureus* protease as described previously[33].

PGS-3, PGS-7, and PGS-8 (comprising Asn-266, Asn-374, and Asn-410, respectively) were directly obtained by h.p.l.c. of tryptic glycopeptide fractions a and b (Figs. 1, 2a, and 2b). PGS-2 (with Asn-168) was isolated by h.p.l.c. (Fig. 2c) and further purified by gel filtration using Biogel P-10 (data not shown). Despite a potential trypsin-cleavage site at Lys-308[14,18], PGS-4,5,6 (with Asn-302, Asn-334, and Asn-341, respectively) were isolated "en bloc" (Fig. 2a). This glycopeptide was further digested with protease from *S. aureus*, and the products were again subjected to h.p.l.c. (Fig. 3). In this manner, PGS-4 and PGS-5,6 could be separated. Attempts to split the latter glycopeptide at Pro-339 by use of the proline-specific endopeptidase from *Flavobacterium meningosepticum* failed. Possibly, this was due to steric hindrance, since residues Asn-334 and Asn-341 are only six amino acids apart (see Fig. 4).

Peptide analyses. — The glycopeptides obtained were subjected to amino acid analysis, and the NH_2-terminal amino acids were identified by dansylation (Table I). Although considerable heterogeneity is displayed by the corresponding h.p.l.c. profiles (Figs. 2 and 3), these peptide analyses demonstrated that glycopeptides comprising homogeneous peptide moieties had been isolated. The heterogeneity

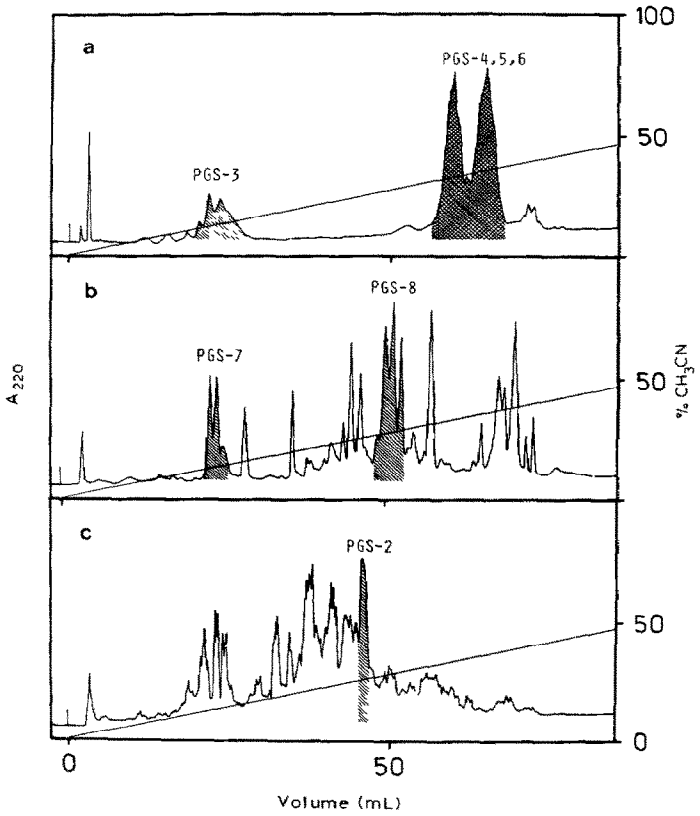


Fig. 2. Isolation of F-MuLV glycopeptides by reversed-phase h.p.l.c. of fractions a–c in Fig. 1, using a linear gradient of acetonitrile in ammonium acetate buffer (pH 6). Glycopeptide fractions were monitored as in Fig. 1. Hatched fractions represent gp71-derived glycopeptides as identified by amino acid analysis of individual peak fractions and determination of NH_2-terminal amino acids of combined (hatched) fractions comprising identical amino acid composition; PGS-3, peptide comprising glycosylation site 3, *etc.*

observed was obviously due to variations in glycosylation as pointed out previously[33].

In the case of PGS-4 and PGS-5,6 (with Asn-302 and Asn-334/Asn-341, respectively), exclusive attribution to the gp71-species is not possible, because the isoglycoproteins also obtained from the F-MuLV-complex as minor components have been shown to comprise identical amino acid sequences in the corresponding domains of the molecules[4]. Observed amino acid exchanges are in accordance with Chen[14].

The assignment of the isolated glycopeptides to the complete amino acid sequence of F-MuLV gp71 is summarised in Fig. 4.

Carbohydrate analysis. — Separation of glycopeptides by reversed-phase h.p.l.c. at pH 6 was found to be essential for carbohydrate analysis, because acidic chromatographic conditions (*e.g.*, separation at pH 2) resulted in the loss of fucose

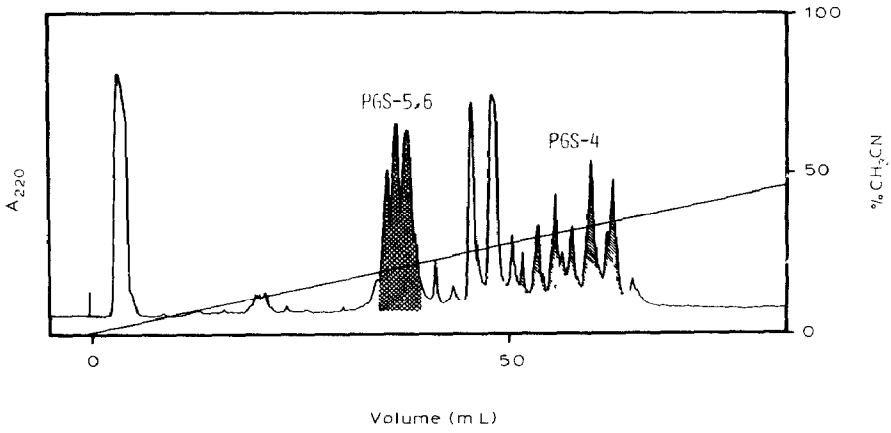


Fig. 3. Separation of PGS-4 and PGS-5,6. PGS-4,5,6 (Fig. 2a) was digested with *S. aureus* protease, and the products were subjected to reversed-phase h.p.l.c., as in Fig. 2. Glycopeptide fractions were monitored as in Fig. 1. Hatched fractions represent gp71-derived glycopeptides, as identified by amino acid analysis of individual peak fractions and determination of NH_2-terminal amino acids of combined (hatched) fractions comprising identical amino acid composition; PGS-5,6, *etc.* as in Fig. 2.

TABLE I

AMINO ACID ANALYSIS OF GLYCOPEPTIDES FROM F-MuLV gp71[a]

Amino acid	*Composition*[b]						
	PGS-1[c] *(Ala)*[e]	*PGS-2 (Ala)*[e]	*PGS-3 (Ser)*[e]	*PGS-4*[d] *(Leu)*[e]	*PGS-5,6*[d] *(Gly)*[e]	*PGS-7 (Thr)*[e]	*PGS-8 (Gly)*[e]
Cys[f]	(–) —	(1) 1.1	(–) —	(–) —	(1) 1.0	(1) 1.1	(2) 2.3
Asx	(1) 1.3	(4) 5.3	(2) 2.0	(4) 4.1	(2) 2.1	(1) 1.1	(2) 2.3
Thr[g]	(1) 1.0	(3) 2.8	(5) 3.9	(2) 1.8	(2) 1.8	(3) 3 1	(6) 4.5
Ser[g]	(2) 1.8	(4) 2.9	(5) 4.5	(–) —	(4) 3.3	(–) —	(2) 2.3
Glx	(2) 2.0[h]	(2) 2 7	(1) 1.3	(4) 3.4	(1) 1.0	(1) 1 3	(–) —
Gly	(1) 1.7	(–) —	(2) 1.3	(1) 1.8	(2) 1.9	(–) —	(3) 3.3
Ala	(2) 1.8	(1) 1.9	(2) 1.2	(2) 2.2	(4) 3.6	(1) 1.0	(3) 3.8
Val	(1) 1.1	(4) 3.1	(–) 0.9	(1) 1.0	(3) 2.7	(–) —	(2) 2.2
Met	(–) —	(–) —	(–) 0.8	(–) —	(–) —	(–) —	(–) —
Ile	(1) 1.0	(1) 1.1	(1) 1 3	(–) —	(–) —	(–) —	(–) —
Leu	(–) —	(1) 1.2	(1) —	(5) 5.3	(1) 1.0	(2) 2.1	(4) 4.3
Tyr	(1) 1 0	(2) 1.9	(–) —	(1) 1.0	(1) 0.9	(–) —	(2) 1 9
Phe	(–) —	(–) —	(–) —	(–) —	(–) —	(–) —	(–) —
His	(1) 1.1	(–) —	(–) —	(–) —	(2) 1.8	(1) 1.0	(–) —
Lys	(–) —	(2) 2.0[i]	(–) —	(1) 1.0[i]	(1) 1.0[i]	(1) 1.0[i]	(–) —
Arg	(–) —	(–) —	(1) 1.0[k]	(–) —	(–) —	(–) —	(1) 1.0[k]
Trp[l]	(1)	(2)	(–)	(–)	(–)	(–)	(1)
Pro[l]	(2)	(1)	(9)	(1)	(1)	(–)	(2)

[a]Peptides containing individual glycosylation sites (PGS's) were obtained by digestion with trypsin and/or *S. aureus* protease, and separated by gel filtration and by reversed-phase h.p.l.c. [b]Molar ratios of amino acids; data expected from the sequence of F-MuLV gp71, as determined by Koch *et al.*[18], are given in parenthesis; observed amino acid exchanges are in accordance with Chen[14]. [c]See Schluter *et al.*[33]. [d]Due to sequence homologies with other, minor F-MuLV isoglycoproteins, exclusive assignment to the gp71-species is not possible. [e]NH_2-terminal acid, determined by dansylation [f]Determined as carboxymethylcysteine. [g]Data not corrected for losses during hydrolysis. [h]Based on Glx = 2.0. [i]Based on Lys = 2.0. [j]Based on Lys = 1.0. [k]Based on Arg = 1 0. [l]Not determined

TABLE II

METHYLATION ANALYSIS OF GLYCOPEPTIDES FROM F-MuLV gp71[a]

Alditol[b]	*Peak ratio as obtained from oligosaccharides linked to:*						
	Asn-12[c]	*Asn-168*	*Asn-266*	*Asn-302*[d]	*Asn-334*[d] *Asn-341*[d]	*Asn-374*	*Asn-410*
2,3,4-FucOH	0.3	—	1.1	0.1	0.45	0.6	0.65
2,3,4,6-ManOH	1.4	3.0[f]	—	—	0.75[g]	—	—
3,4,6-ManOH	1.0[e]	0.3	1.8[g]	1.5[g]	1.25[g]	1.9[g]	2.0[g]
2,4,6-ManOH	0.15	—	—	—	—	—	—
3,6-ManOH	—	—	0.1[g]	0.15[g]	—	—	—
3,4-ManOH	—	—	0.1[g]	0.35[g]	—	0.1[g]	—
2,4-ManOH[h]	1.45	2.05	0.6	1.0	0.95	0.6	0.4
2-ManOH[h]	0.1	—	0.3	0.05	0 25	0.2	0.45
2,3,4,6-GalOH	0.6	—	1.1	0.9	1.15	0.9	1 1
2,4,6-GalOH	0.4	—	7.2	1.4	0.6	1.2	1 2
2,3,4-GalOH	—	—	0.45	0.3	—	0.4	0 35
3,4,6-GlcN(Me)AcOH	0.15	—	0 9	0.15	0.6	0.55	0.9
3,6-GlcN(Me)AcOH	2.6	1.35	4.6	4.15	2.8	4.25	3.7
3-GlcN(Me)AcOH	0.1	—	0.25	0.1	0.25	0.5	0.4
4,6-GalN(Me)AcOH	—	—	0.55	—	—	—	—
Conclusions[i]	M	OM	AL-A AL-E (AL-D) (*O*-glycans)[j]	AL-A AL-C AL-D	AL-A AL-E (incomplete)	AL-A AL-E (AL-C)	AL-A AL-E

[a]Peptides containing distinct glycosylation sites (PGS's) of gp71 were separated by gel filtration and by h.p.l.c., permethylated, and hydrolysed, and the methylated sugar components were reduced and per-*O*-acetylated. The partially methylated alditol acetates were analysed by capillary g.l.c.–m.s. [b]2,3,4-FucOH, 2,3,4-tri-*O*-methylfucitol; 3,4,6-GlcN(Me)AcOH, 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol; *etc.* [c]Data taken from Schlüter *et al.*[33]. [d]Due to sequence homologies with other, minor F-MuLV isoglycoproteins, exclusive assignment to the gp71-species is not possible. [e]Based on 3,4,6-ManOH = 1.0. [f]Based on 2,3,4,6-ManOH = 3.0. [g]Based on sum of 2,3,4,6-, 3,4,6-, 3,6-, and 3,4-ManOH = 2.0 (Man-residues *4* and *4'* in Scheme 1). [h]Corrected for losses during acetolysis[41]. [i]M, mixed-type glycans; OM, oligomannosidic glycans; AL, *N*-acetyl-lactosaminic glycans; for AL-A to AL-E, compare Scheme 1. [j]Structures of gp71-derived *O*-glycans were not established.

(~30%) and sialic acid residues (~15%), as indicated by decreasing amounts of terminal fucose and by increasing amounts of terminal galactose. Therefore, the h.p.l.c. separations were carried out at pH 6 throughout.

The results obtained after methylation of the isolated glycopeptides are presented in Table II.

Using Man-residues *3*, *4*, and *4'* (Scheme 1) as diagnostic criteria, a comparison of the methylation results with the structures of the desialylated *N*-glycans, as obtained from the total F-MuLV glycoproteins[27,28], allowed the following conclusions about the *N*-glycosylation of F-MuLV gp71: (*a*) as described previously[33], glycosylation site 1 at Asn-12 is substituted solely by mixed (M)-type oligosaccharides; (*b*) Asn-168 carries exclusively oligomannosidic (OM) glycans; (*c*) Asn-

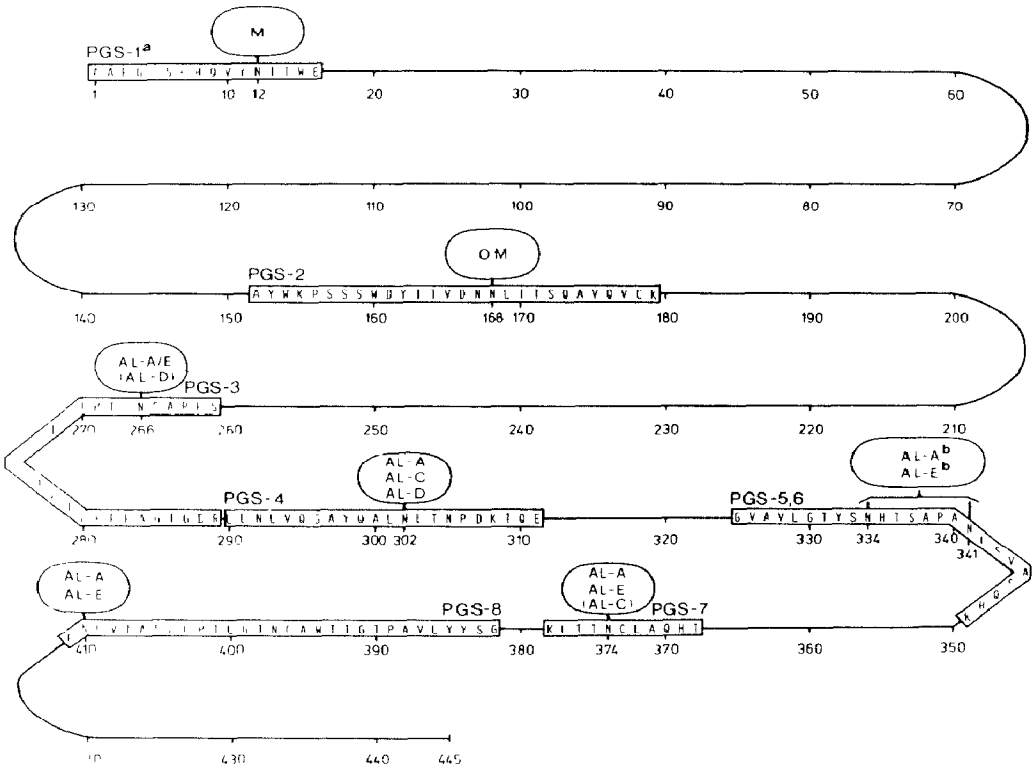


Fig. 4. Schematic representation of F-MuLV gp71. The amino acid sequences of the isolated glycopeptides are delineated in one-letter symbols, and the types of *N*-glycans attached to the individual asparagine residues are given. Amino acid sequences are based on data obtained from Chen[14] and Koch *et al.*[18]. M, mixed-type glycans; OM, oligomannosidic glycans; AL, *N*-acetyl-lactosaminic glycans (for AL-A to AL-E, see Scheme 1) [a]From Schlüter *et al.*[33]. [b]Oligosaccharides comprising incomplete lactosamine antennae

266 is substituted by *N*-acetyl-lactosaminic (AL) oligosaccharides (mainly AL-A and AL-E). As a unique feature, the glycopeptide comprising this glycosylation site also contains 3-substituted 2-acetamido-2-deoxy-D-galactose residues, indicating the presence of additional *O*-linked glycans, the structures of which have not yet been analysed; (*d*) Asn-302 bears biantennary (AL-A) and, significantly, most of the tri- and tetra-antennary *N*-acetyl-lactosaminic glycans (AL-C and AL-D) present in gp71; (*e*) Asn-334/Asn-341 is substituted with biantennary oligosaccharides (AL-A, AL-E), which characteristically comprise species with incomplete lactosamine antennae; (*f*) Asn-374 and Asn-410 bear almost exclusively biantennary glycans (AL-A, AL-E).

The methylation data demonstrated the presence of triantennary oligosaccharides of type AL-C at Asn-302 and Asn-374 (Table II), although such species have not been identified during our studies of the major *N*-glycans in the mixture of F-MuLV isoglycoproteins[28]. Possibly, these oligosaccharides represent minor

species which, when attached to a few, distinct glycosylation sites, can only be detected when the corresponding purified glycopeptides are analysed. Asn-266 and Asn-302 might also carry mixtures of oligosaccharides AL-B and AL-C instead of AL-D.

As summarised in Fig. 4, our studies thus revealed a high specificity in the glycosylation of F-MuLV gp71 with regard to the distribution of oligomannosidic, mixed, and bi- or multi-antennary *N*-acetyl-lactosaminic oligosaccharides. However, the glycans at each glycosylation site still display considerable (micro)heterogeneity, as also observed, for instance, in the case of human IgD[42], the murine major histocompatibility antigen[43], and the Sindbis virus glycoproteins[44].

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