Sequence Analysis of Lactosamine Type Glycans of Individual Membrane Proteins of Semliki Forest Virus

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SUMMARY

$^3$H-fucose and $^{14}$C-glucosamine labelled glycopeptides of the individual membrane proteins E1, E2 and E3 of Semliki Forest virus could be sequentially digested with $\alpha$-neuraminidase, $\beta$-galactosidase, N-acetyl-$\beta$-glucosaminidase, $\alpha$- and $\beta$-mannosidase, N-acetyl-$\beta$-hexosaminidase and finally with $\alpha$-fucosidase. The degradations of the virus glycopeptides proceeded in the same way as stepwise digestions of reference glycopeptides of the lactosamine type obtained from IgG and $\alpha$1-acid glycoprotein. This suggests that all three membrane glycoproteins of Semliki Forest virus contained glycans with a monosaccharide sequence characteristic for lactosamine type oligosaccharides. The number of both distal and proximal N-acetyl-glucosamine residues was estimated to be usually two. According to exo- and endo-glycosidase digestions, fucose seemed to be attached to the innermost N-acetyl-glucosamine unit.

INTRODUCTION

Semliki Forest virus (SFV) is surrounded by a lipid bilayer containing three membrane proteins E1, E2 and E3 (Garoff et al. 1974; Zimiecki & Garoff, 1978). The immediate precursor of E2 and E3 in the infected cell is P-62 (Ranki et al. 1972; Simons et al. 1973; Garoff et al. 1974). P-62 seems to contain mainly high mannose type glycans consisting of only mannose and N-acetyl-glucosamine (Stenvall & Renkonen, 1979). However, E3 has been suggested to contain lactosamine type (type A, complex) glycans, that consist of sialic acid, galactose, mannose, glucosamine and fucose (Garoff et al. 1974; Mattila et al. 1976; Rasilo & Renkonen, 1979). The presence of lactosamine type glycans in E2 has not been ascertained. The monosaccharide composition (Garoff et al. 1974) and affinity chromatography of its glycopeptides (Rasilo & Renkonen, 1979) suggest their presence, but labelling experiments gave inconclusive results (Mattila et al. 1976). However, E2 contains high mannose type glycans (Mattila et al. 1976). If E2 and E3 contain lactosamine type oligosaccharides, the processing of P-62 into E2 and E3 would appear to involve a conversion of high mannose type glycans into lactosamine type glycans. In the present paper the monosaccharide sequence of the radiolabelled glycopeptides of E1, E2 and E3 was studied using stepwise digestion with exo-glycosidases.

METHODS

Preparation of radiolabelled virus glycopeptides. One Falcon bottle (75 cm$^2$) of BHK-21 cells was infected with SFV. The infected cells were labelled from 3 to 10 h p.i. with 250 $\mu$Ci of D-1-$^{14}$C-glucosamine hydrochloride (59 mCi/mmol) or with 1 mCi of L-1-$^3$H-fucose.
(3.3 Ci/mmol; The Radiochemical Centre, Ltd., Amersham, U.K.) in Eagle's minimal essential medium supplemented with 0.2% bovine serum albumin and actinomycin D (1 g/ml). The virus was purified in a discontinuous sucrose gradient (Söderlund et al. 1972). A parallel unlabelled culture was added before purification. The virus proteins were fractionated by discontinuous gel electrophoresis in the presence of SDS (Neville, 1971), eluted from the gels (Mattila et al. 1976) and hydrolysed with pronase (Sefton & Keegstra, 1974).

Radioactive labelling of the reference glycopeptides. The GP-II-5 glycopeptide was obtained from desialylated α1-acid glycoprotein by chymotrypsin and pronase digestion (Schmid et al. 1977). The GC-4 glycopeptide was obtained from bovine colostrum IgG1 by chymotrypsin digestion (Cheron et al. 1976). Both glycopeptides were gifts from Dr J. Montreuil, Villeneuve d'Ascq, France. The glycopeptides were labelled at their free amino groups with 1.97 μCi of 14C-formaldehyde (4.45 mCi/mmol: The Radiochemical Centre, Amersham; Gahmberg & Andersson, 1977).

Glycosidase digestions. The virus glycopeptides were digested with neuraminidase (EC 3.2.1.18, Vibrio cholerae, Behringwerke) as described (Pesonen & Renkonen, 1976). The asialoglycopeptides were desalted on Bio-Gel P-2, dissolved in 150 µl of 0.05 M-sodium citrate buffer, pH 4.0, and digested sequentially with β-D-galactosidase (EC 3.2.1.53, jack bean, Dr Y.-T. Li, New Orleans, U.S.A.), N-acetyl-β-D-hexosaminidase (EC 3.2.1.52, jack bean, Dr Y.-T. Li), α-D-mannosidase (EC 3.2.1.24, jack bean, Boehringer), N-acetyl-β-D-hexosaminidase and finally with α-L-fucosidase (EC 3.2.1.51, beef kidney, Boehringer). The α-D-mannosidase preparation has been found to contain β-D-mannosidase activity (Pesonen & Renkonen, 1976). In the case of GP-II-5, α-D-mannosidase (jack bean, Dr Y.-T. Li) and β-D-mannosidase (EC 3.2.1.54, Aspergillus niger, Dr J. Montreuil) were used. Concentrations and digestion times for all exo-glycosidases have been described (Pesonen & Renkonen, 1976) except for N-acetyl-β-D-hexosaminidase and β-D-mannosidase, that were added twice to the reaction mixtures to the final concentrations of 6 units (U)/ml and 25 U/ml, respectively, and incubated for 48 h. Glycopeptides were dissolved in 150 µl of 0.025 M-citrate-phosphate buffer, pH 6.0, and incubated with 3 mU of endo-β-D-N-acetyl-glucosaminidase D (Diplococcus pneumoniae, Dr T. Muramatsu, Kobe, Japan) for 26 h, after removal of sialic acid, galactose and distal N-acetyl-glucosamine units.

Gel chromatography. A column (0.9 × 58 cm) of Bio-Gel P-6 (100 to 200 mesh, Bio-Rad) was used for apparent mol. wt. estimations as described by Pesonen & Renkonen (1976). The following standards were used to calibrate the column: thyroglobulin glycopeptides (relative mol. wt., Mr, 4100) tritiated by the galactose oxidase technique (Gahmberg & Hakomori, 1973), as well as Galβ1-4GlcNACβ1-2-Manα1-3[Galβ1-4GlcNACβ1-2-Manα1-6]Manβ1-4GlcNACot1 (M, 1434), Galβ1-4GlcNACβ1-2Manβ1-4GlcNACot (M, 908), lacto-N-fuco-pentaol (M, 858) and Galβ1-3Galβ1-4GlcNACot (M, 504), all reduced with NaBH4.

A column (1 × 63 cm) of Bio-Gel P-2 (100 to 200 mesh, Bio-Rad) eluted with 0.02% sodium azide was used for desalting.

Affinity chromatography. A column (0.9 × 5 cm) of concanavalin A-Sepharose 4B (Pharmacia) was equilibrated and washed with 0.01 M-tris-HCl buffer, pH 7.5, containing 0.1 M-NaCl and 0.02% of each NaN3, CaCl2, MgCl2 and MnCl2, and subsequently eluted with the same buffer containing 0.2 M-α-methyl mannoside (Grade II, Sigma).

Identification of monosaccharides. 14C-labelled enzymically released monosaccharides were identified on a Kieselgel 60 F254 plate (Merck) sprayed with 0.05 M-sodium tetraborate and developed twice with n-propanol/H2O 7/2. The tracks were scraped and counted for radioactivity.

Acid hydrolysis of 3H-fucose labelled E3 was performed with 100 µl of 4 N-HCl for 4 h
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Fig. 1. SDS-PAGE pattern of (a) $^3$H-fucose and (b) $^{14}$C-glucosamine labelled SFV. BPB: bromophenol blue. Bars indicate pooling of proteins.

RESULTS

Isolation of the virus glycopeptides

The fractionation of the virus membrane proteins is shown in Fig. 1. The $^3$H-fucose labelled E1 (Mr 49000) and E2 (Mr 51000) were reasonably well resolved and E3 (Mr 10000) migrated as a sharp band (Fig. 1a). No radioactivity migrated in the region of the non-glycosylated core protein (Mr 30000), suggesting that the label was not metabolized into amino acids. No labelling of other monosaccharides was observed: after acid hydrolysis of $^3$H-fucose labelled E3, the radioactivity migrated in paper chromatography like natural fucose (data not shown).

The $^{14}$C-glucosamine labelled E1 and E2 were not well resolved and E3 migrated as a broad band (Fig. 1b). No metabolism of the label into amino acids was evident, since pronase digestion did not release any radioactivity from the polypeptides (see below). Slight metabolism of the label into $^{14}$C-mannose was observed: after complete hydrolysis by exo-glycosidases of the E3 glycopeptides, 90% of the $^{14}$C-label migrated in TLC like N-acetyl-glucosamine and 10% like mannose (data not shown).

The proteins were pooled as indicated in Fig. 1, desalted and hydrolysed with pronase. The glycopeptides were isolated by gel chromatography (Fig. 2). About 25% of the $^{14}$C-label of the E1 glycopeptide preparation eluted as a shoulder (Fig. 2a, fractions 37 to 45)
which probably represented high mannose type glycopeptides originating from E2. The apparent mol. wt. of the intact glycopeptides are presented in Table 1.

**Stepwise degradation of virus glycopeptides**

The glycopeptide peaks were pooled, desalted on Bio-Gel P-2, and subjected to stepwise degradation with exo-glycosidases. The gel chromatography profile of each digestive step is presented in Fig. 3. Stepwise degradation with neuraminidase, β-galactosidase, N-acetyl-β-hexosaminidase, α- and β-mannosidase, N-acetyl-β-hexosaminidase and α-fucosidase proceeded in the same way for glycopeptides of E1, E2 and E3. The digestions resulted in a gradual reduction of the apparent mol. wt. of the glycopeptides, thus indicating loss of monosaccharide residues at each degradative step. The apparent mol. wt. of the glycopeptides at each digestive stage are presented in Table 1. The results suggest that the 3H-fucose labelled glycopeptides of E1, E2 and E3 consisted of antennae composed of sialic acid, galactose and N-acetyl-glucosamine, attached to core glycopeptides composed of α- and β-mannose, N-acetyl-glucosamine and fucose. This sequence of monosaccharides is characteristic for lactosamine type glycans (Fig. 5).

The digestions of glycopeptides of E1 and E2 seem to have proceeded rather quantitatively.

**Table 1. Apparent molecular weights of glycopeptides corresponding to different stages of stepwise degradation with exo-glycosidases**

<table>
<thead>
<tr>
<th>Glycopeptide</th>
<th>Treatment</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
<th>M</th>
<th>GP-1</th>
<th>GC-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) Intact glycopeptides</td>
<td>3700$</td>
<td>3500$</td>
<td>4350$</td>
<td>3800</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>(2) Neuraminidase</td>
<td>2800</td>
<td>2900</td>
<td>3050$</td>
<td>2800</td>
<td>3050$</td>
<td>4100$</td>
</tr>
<tr>
<td></td>
<td>(3) 2, then β-galactosidase</td>
<td>2500</td>
<td>2500</td>
<td>2700</td>
<td>2200</td>
<td>2550$</td>
<td>3750</td>
</tr>
<tr>
<td></td>
<td>(4) 3, then N-acetyl-β-hexosaminidase</td>
<td>1500</td>
<td>1700$</td>
<td>1900$</td>
<td>1350$</td>
<td>1750$</td>
<td>3100</td>
</tr>
<tr>
<td></td>
<td>(5) 4, then α- and β-mannosidase</td>
<td>1050</td>
<td>1250$</td>
<td>1270</td>
<td>1200</td>
<td>1350$</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>(6) 5, then N-acetyl-α-hexosaminidase</td>
<td>1100$</td>
<td>1150$</td>
<td>900</td>
<td>800$</td>
<td>1050</td>
<td>2200$</td>
</tr>
</tbody>
</table>

* Glycopeptides labelled with 3H-fucose and 14C-glucosamine.
† Glycopeptides labelled with 3H-fucose.
‡ Mixture of all lactosamine type glycopeptides (Pesonen & Renkonen, 1976).
§ Two or three determinations.
|| Digested also with α-fucosidase.
¶ No reduction in apparent mol. wt., though liberation of 14C-glucosamine label indicated loss of monosaccharides (see Table 2).
Fig. 3. Bio-Gel P-6 elution profiles of stepwise degraded glycopeptides. (A) E1, (B) E2, (C) E3 and (D) GP-II-5. (a) Neuraminidase; (Da) intact asialo GP-II-5; (b) neuraminidase, then β-galactosidase; (c) neuraminidase, then, in succession, β-galactosidase, N-acetyl-β-hexosaminidase; (d) neuraminidase, then, in succession, β-galactosidase, N-acetyl-β-hexosaminidase, and α- and β-mannosidase; (e) neuraminidase, then in succession β-galactosidase, N-acetyl-β-hexosaminidase, α- and β-mannosidase and N-acetyl-β-hexosaminidase; (f) neuraminidase, then, in succession, β-galactosidase, N-acetyl-β-hexosaminidase α- and β-mannosidase, N-acetyl-β-hexosaminidase and α-fucosidase. After each enzyme digestion a sample was taken for gel chromatography, the digest was heated for 3 min in boiling water and the next enzyme was added. The fractions containing blue dextran (V₀) are placed underneath each other. (A) and (C): • -- •, 14C-glucosamine; • -- •, 2H-fucose. (B) • -- •, 3H-fucose; (D) • -- •, 14C-methylated.
Table 2. Amount of $^{14}$C-glucosamine label in different N-acetyl-glucosamine residues*

<table>
<thead>
<tr>
<th></th>
<th>Distal GlcNAc residues†</th>
<th>Proximal GlcNAc residues‡</th>
<th>Non-releasable GlcNAc residues§</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1$</td>
<td>53 %</td>
<td>47 %</td>
<td>24 %</td>
</tr>
<tr>
<td>$E_3$</td>
<td>55 %</td>
<td>45 %</td>
<td>28 %</td>
</tr>
</tbody>
</table>

* All figures are corrected for the finding that from all enzymically released $^{14}$C-label of $E_3$ glycopeptides, 90% was N-acetyl-$^{14}$C-glucosamine and 10% $^{14}$C-mannose.
† Liberated as free monosaccharides.
‡ Remaining in glycopeptides after removal of distal units.
§ Remaining in glycopeptides after exhaustive digestion with all exo-glycosidases.
‖ Contains exclusively N-acetyl-$^{14}$C-glucosamine.

In the case of the $E_3$ glycopeptides, undigested material appeared from the mannosidase step on (Fig. 3 C, d to f). However, digestion of the $E_3$ glycopeptides with endo-$\beta$-N-acetyl-glucosaminidase D, after removal of sialic acid, galactose and distal N-acetyl-glucosamine residues was nearly complete (Fig. 4a). This enzyme cleaves $NN'$-di-acetyl-chitobiose linkages (Koide & Muramatsu, 1974).

$^3$H-fucose was liberated from the glycopeptides at the final stage of the stepwise degradation scheme. After $\alpha$-fucosidase digestion, treatment of the $E_1$ and $E_3$ glycopeptides once more with N-acetyl-$\beta$-hexosaminidase did not change the elution profiles from those presented in Fig. 3 A (f) and 3 C (f). This suggests that fucose was attached to the innermost N-acetyl-glucosamine residue of these glycopeptides. This is supported by the following finding. When the endo-$\beta$-N-acetyl-glucosaminidase D digest of the $^3$H-fucose labelled $E_3$ glycopeptides (see above) was applied on a column of concanavalin A-Sepharose, 91% of the label could be washed down with buffer and 9% eluted with $\alpha$-methyl mannoside (Fig. 4b).

Digestion of N-acetyl-glucosamine residues liberated $^{14}$C-glucosamine label from glycopeptides of $E_1$ and $E_3$ [Fig. 3 A (c), (e); 3 A (e) 3 C (c), (e)]. Fifty three % of the radioactivity was released as N-acetyl-$^{14}$C-glucosamine from $E_1$ glycopeptides after digestion of the distal residues (Table 2). Digestion of both distal and proximal residues liberated 76% of radioactivity as N-acetyl-glucosamine and 24% remained in a glycopeptide peak of mol. wt. 1100 (Fig. 3 A, e). Fifty five % of $^{14}$C-glucosamine was released from the $E_3$ glycopeptides after digestion of the distal N-acetyl-glucosamine residues (Table 2). Digestion of all N-acetyl-glucosamine residues liberated 72% of the label as N-acetyl-$^{14}$C-glucosamine and 28% remained in a heterogeneous glycopeptide population (Fig. 3 C, e). The results suggest that there were mainly two distal and two proximal N-acetyl-glucosamine residues in the glycopeptides of $E_1$ and $E_3$. $^{14}$C-glucosamine labelled $E_2$ glycopeptides were not analysed because of eventual contamination of the lactosamine type glycopeptides with high mannose type glycopeptides.

Stepwise degradation of reference glycopeptides

The structure of the reference glycopeptide GC-4 is shown in Fig. 5. The reference glycopeptide GP-II-5 has the same basic structure as GC-4, but it has a third N-acetyl-lactosamine antenna attached to an $\alpha$-mannose unit; it contains two amino acids but no fucose (Dr J. Montreuil, personal communication). GC-4 and GP-II-5 behaved in stepwise exo-glycosidase digestions in the same way as the virus glycopeptides. The gel filtration profiles of the successive degradative stages of GP-II-5 are shown in Fig. 3 (D). The apparent mol. wt. of GP-II-5 and GC-4 are shown in Table 1. These are larger than the corresponding theoretical mol. weights of the reference glycopeptides. This is most likely due to the fact, that the standard curve for mol. wt. estimations was composed merely of oligosaccharides.
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Fig. 4. (a) Bio-Gel P-6 and (b) concanavalin A-Sepharose elution profiles of $^3$H-fucose labelled asialoglycopeptides of E3 digested with $\beta$-galactosidase, N-acetyl-$\beta$-hexosaminidase and endo-$\beta$-N-acetyl-glucosaminidase D. (b) Fractions 1 to 11 washed with buffer, fractions 12 to 24 eluted with 0.2 M-$\alpha$-methyl mannoside.

Fig. 5. Structure of reference glycopeptide GC-4. The peptide contains 12 amino acids (Cheron et al. 1976). Gal, galactose; GlcNAc, N-acetyl-glucosamine; Man, mannose; Fuc, fucose; Glc, glucose.

In addition to size, other factors such as charge seem to effect elution behaviour in small pore acrylamide gels (Tabas et al. 1978).

Reductions in the apparent mol. wt. of the reference glycopeptides caused by digestion of the distal N-acetyl-glucosamine residues were exaggerated (Table 1). Analogous exaggerations are probably incorporated into data concerning the virus glycopeptides. This has earlier been interpreted in terms of three N-acetyl-lactosamine antennae and eventual terminal N-acetyl-glucosamine residues in the mixture of the virus glycopeptides (Pesonen & Renkonen, 1976).

**DISCUSSION**

The same linear sequence of monosaccharides was found in the $^3$H-fucose labelled glycopeptides of all membrane proteins of SFV, E1, E2 and E3. This monosaccharide sequence, characteristic for lactosamine type glycans (Fig. 5), shows that the glycopeptides were composed of sialosyl-N-acetyl-lactosamine antennae attached to core glycopeptides consisting of $\alpha$- and $\beta$-mannose, N-acetyl-$\beta$-glucosamine and $\alpha$-fucose residues. Some cross-contamination of E1 and E2 has to be taken into account because of the rather poor separation of these proteins in SDS-PAGE. The virus glycopeptides behaved in stepwise exo-glycosidase digestions in the same way as two reference glycopeptides of lactosamine type. Earlier it was found that the monosaccharide sequence of the mixture of SFV glycopeptides labelled with $^3$H-fucose or $^3$H-galactose was compatible with lactosamine type structures (Pesonen & Renkonen, 1976).
E2 was earlier found to be labelled only marginally with monosaccharides characteristic for the lactosamine type glycans (Mattila et al. 1976; Kääriäinen & Renkonen, 1977). The E2 population isolated then and that studied here seem to have been different. E2 has been suggested to be heterogeneous with regard to its glycans (Mattila, 1979), which leads to diffuse bands in SDS-PAGE. This may explain the different results. The present data show that E2 contains lactosamine type oligosaccharides. This finding is supported by the behaviour of E2 glycopeptides in affinity chromatography on concanavalin A-Sepharose (Mattila & Renkonen, 1979; Rasilo & Renkonen, 1979).

Lactosamine type glycans are frequent in soluble glycoproteins (Montreuil, 1975; Kornfeld & Kornfeld, 1976). They have also been found in some membrane proteins (Thomas & Winzler, 1971; Kawasaki & Ashwell, 1976; Järnefelt et al. 1978). The distribution of $^{14}$C-glucosamine label in the SFV glycans suggests that the number of distal and proximal $N$-acetyl-glucosamine residues was usually two. This is supported by the behaviour of SFV glycopeptides in affinity chromatography on concanavalin A-Sepharose (Rasilo & Renkonen, 1979). Two- and three-antennary lactosamine type glycopeptides have been found in the membrane proteins of Sindbis virus and vesicular stomatitis virus, respectively (Etchison et al. 1977; Reading et al. 1978; Burke & Keegstra, 1979).

Exo- and endo-glycosidase digestions suggest that fucose was attached to the innermost $N$-acetyl-glucosamine residue of the virus glycopeptides. The same location has been reported for the fucose residues of Sindbis and vesicular stomatitis virus membrane proteins (Reading et al. 1978; Burke & Keegstra, 1979). In rat brain glycoproteins fucose may be also attached to the distal $N$-acetyl-glucosamine residues (Krusius & Finne, 1978).

The structural proteins of SFV are formed from polyprotein precursors. The precursor of E2 and E3 is P-62 (Ranki et al. 1972; Simons et al. 1973; Garoff et al. 1974). The oligosaccharides of P-62 found in the infected cells appear to be mainly of the high mannose type (Stenvall & Renkonen, 1979). However, E2 and E3 isolated from the virions appear to contain lactosamine type glycans. This suggests that processing of P-62 into E2 and E3 involves the conversion of high mannose type glycans into lactosamine type glycans. This sequence of events has been proposed to take place also in the glycosylation of Sindbis and vesicular stomatitis virus glycoproteins (Robbins et al. 1977; Hunt et al. 1978; Tabas et al. 1978).

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