Hazelhurst-vesicular-stomatitis-virus G and Sindbis-virus E1 glycoproteins undergo similar host-cell-dependent variation in oligosaccharide processing

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We have examined and compared the host-cell-dependent glycosylation of the G glycoprotein of vesicular-stomatitis virus (Hazelhurst strain) and the E1 and E2 glycoproteins of Sindbis virus replicated by baby-hamster kidney, chicken-embryo fibroblast and mouse L929 monolayer cell cultures. The results of endo- β -N-acetylglucosaminidase H digestion of viral proteins labelled with [3 H]mannose or leucine and Pronase-digested glycopeptides labelled with [3 H]mannose indicated that both the G protein and the E1 protein contained a similar mixture of endoglycosidase-resistant oligosaccharides of the complex acidic type and less extensively processed endoglycosidase-sensitive oligosaccharides of the neutral or hybrid type, with a relatively greater content of the endoglycosidase-sensitive oligosaccharides for virus replicated in the chicken as against hamster or mouse cells. A large fraction of the G protein and the majority of the E₁ proteins from the mammalian host cells contained acidic-type oligosaccharides at both glycosylation sites, whereas most of the G and E1 glycoproteins from the avian host cells and essentially all of the E2 protein from all three host-cell types contained an acidic-type oligosaccharide at one site and neutral- or hybrid-type oligosaccharide at the other site. The relative increase in neutral- and hybrid-type oligosaccharides with five-mannose core structures observed for the G and E1 proteins of virus released from the avian host cells suggested that two specific steps in oligosaccharide processing (mediated by α -mannoside II and N-acetylglucosaminyltransferase I) were less efficient at one of the glycosylation sites of the vesicular-stomatitis-virus G protein and Sindbis-virus E1 protein in the avian as against mammalian host cells.

The G protein of vesicular-stomatitis virus (VS virus) and the E1 and E2 proteins of Sindbis virus are among the best characterized glycoproteins of lipid-enveloped animal viruses. VS virus is a member of the rhabdoviruses and contains a negative-strand RNA genome in a helical nucleocapsid (Wagner, 1975), whereas Sindbis virus is a member of the group-A togaviruses and contains a positive-strand RNA genome in an icosahedral capsid (Strauss & Strauss, 1977). Because viral

glycoproteins are glycosylated by host-cell enzymic machinery, the glycoproteins of these two viruses have been used extensively as experimental models to help elucidate the complex biosynthetic pathway for the addition and processing of the oligosaccharides of membrane glycoproteins (Hubbard & Ivatt, 1981; Kaariainen & Pesonen, 1982).

The amino acid sequences for these three viral glycoproteins (derived from the nucleic acid sequences of the viral mRNA species) each contain two potential asparagine glycosylation sites (Asn-Xaa-Ser/Thr) (Rice & Strauss, 1981; Rose & Gallione, 1981; Gallione & Rose, 1983). Analyses of tryptic or Pronase-digested glycopeptides of the Sindbis-virus glycoproteins and the G protein from both the Indiana serotype and New Jersey serotype (Ogden strain, Concan subtype) indicated that both potential sites were in fact

Abbreviations used: VS virus, vesicular-stomatitis virus; BHK, baby-hamster kidney; CEF, chick-embryo fibroblast; NP40, Noridet P40; SDS, sodium dodecyl sulphate; endo H, endo- β -N-acetylglucosaminidase H; Con A, concanavalin A.

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glycosylated (Burke & Keegstra, 1979; Robertson *et al.*, 1976; Reading *et al.*, 1978; Hsieh *et al.*, 1983a). The mature G proteins of both the Indiana and New Jersey (Ogden strain) serotypes of VS virus contain complex acidic-type oligosaccharides [(NeuNAc $\pm$ Gal - GlcNAc)₂₋₃Man₃GlcNAc₂ - ($\pm$ fucose)-Asn] at both sites (Etchison *et al.*, 1977; Reading *et al.*, 1978), whereas the Sindbis-virus glycoproteins contain both acidic-type and high-mannose, neutral-type (Man₅₋₉GlcNAc₂-Asn) oligosaccharides (Burke & Keegstra, 1976, 1979; Hsieh *et al.*, 1983a), along with minor amounts of hybrid-type oligosaccharides [NeuNAc $\pm$ Gal-GlcNAc-(Man)₄₋₅GlcNAc₂-Asn] (Hunt *et al.*, 1981; Hunt, 1982). Extensive host-cell-dependent 'microheterogeneity' has been demonstrated within the acidic-type oligosaccharide chains of the VS-virus G protein, with variation in the number and linkage of the outer chain units (tri- versus bi-antennary structures), amount of terminal sialic acid, and presence or absence of fucose (Burge & Huang, 1970; Etchison & Holland, 1974; Etchison *et al.*, 1981). Similar microheterogeneity in sialic acid and fucose content has also been observed for the biantennary acidic-type structures of the Sindbis-virus glycoproteins (Burke & Keegstra, 1976, 1979; Hunt, 1982). These and other studies with viral glycoproteins suggest that, although the host cell may have some effect on the size and composition of the asparagine-linked oligosaccharides, the structure and conformation of the viral polypeptide backbone is the major determinant of the mature oligosaccharide structures (Hubbard & Ivatt, 1981). Different extents of processing of a common high-mannose precursor oligosaccharide (Glc₃Man₉GlcNAc₂) by cellular enzymes in the Golgi apparatus result in the different acidic-, neutral- or hybrid-type oligosaccharides on mature viral glycoproteins (Robbins *et al.*, 1977; Hunt *et al.*, 1978; Li *et al.*, 1978; Tabas *et al.*, 1978).

We have recently shown that the previously uncharacterized G protein of the Hazelhurst strain (Hazelhurst subtype) of the New Jersey serotype of VS virus (VS virus_{NJ}) was glycosylated in BHK cells with an unusual array of acidic-, hybrid- and neutral-type oligosaccharides compared with the G proteins of the other subtypes and serotypes of VS virus, with some of the mature G proteins containing acidic-type structures at both glycosylation sites and the remainder containing an acidic-type structure at one site and a hybrid- or neutral-type structure at the other site (Hunt *et al.*, 1983). This major processing heterogeneity was reminiscent of that demonstrated for the Sindbis-virus E1 glycoprotein, where one glycosylation site invariably contains an acidic-type oligosaccharide, and the second site has predominantly high-mannose,

neutral-type oligosaccharides for CEF host cells and predominantly acidic-type oligosaccharides for BHK host cells (Burke & Keegstra, 1976; Rice & Strauss, 1981; Hsieh *et al.*, 1983a). Therefore we were especially interested in determining whether the Hazelhurst VS-virus_{NJ} G protein would exhibit a similar variation in glycosylation when the virus was replicated in different host cells, including CEF and BHK cells. In addition, we wished to compare the oligosaccharides from VS-virus_{NJ} G with those from Sindbis-virus E1 and E2 replicated in the same host-cell types, in order to determine which steps in the oligosaccharide processing pathway for the VS-virus_{NJ} G and Sindbis-virus E1 glycoproteins might be less efficient in the CEF versus BHK host cells.

Materials and methods

Cells and viruses

BHK 21 cells, L929 cells and CEF cells of the C/E phenotype (obtained from Dr. Stephen Wright, University of Utah, Salt Lake City, UT, U.S.A.) were grown in monolayer cultures at 37°C in Eagle's minimal essential medium (MEM; Gibco), supplemented with 5% (v/v) fetal-bovine serum (Gibco). The Indiana serotype of VS virus (VS virus_{IND}), Hazelhurst strain of VS virus_{NJ} (obtained from Dr. Judith Lesnaw, University of Kentucky, Lexington, KY, U.S.A.) and Sindbis virus were replicated in BHK cells, purified, and the titre determined by plaque assay as described previously (Robertson *et al.*, 1978; Hunt, 1981).

Radiolabelling of viruses

Confluent cell cultures in 75 cm² flasks were infected with either VS virus_{NJ}, VS virus_{IND} or Sindbis virus at approx. 10 plaque-forming units/cell in 2.0 ml of MEM lacking serum, and 10 ml of MEM with 5% serum was added after an adsorption period of 30 min at 37°C. At 3 h after infection, the medium was removed and the virus-infected cell cultures were incubated in 5.0 ml of MEM containing 2% serum, one-third the normal amount of glucose (0.33 mg/ml) and either 100 μ Ci of [2-³H]mannose [15.8 Ci/mmol; New England Nuclear Corp. (NEN)]/ml or 20 μ Ci of L-[3,4,5-³H]leucine (146.5 Ci/mmol; NEN)/ml. After radiolabelling for approx. 18–20 h at 37°C, the medium was harvested and clarified, and radiolabelled virus was purified by pelleting through a cushion of 20% (w/w) sucrose as described previously (Davidson & Hunt, 1983).

Preparation and glycosidase treatment of viral glycoproteins and glycopeptides

Radiolabelled glycoproteins were extracted with butan-1-ol from NP40 detergent-treated virus (Hunt & Summers, 1976). These extracted glyco-

proteins, or glycoproteins isolated by preparative SDS/polyacrylamide-gel electrophoresis, were extensively digested with Pronase (Calbiochem-Behring Corp.) and then desalted on a column (120cm $\times$ 1.0cm) of Sephadex G-15/G-50 (Pharmacia Fine Chemicals) (Hunt & Summers, 1976).

For endo H (Miles Laboratories or Health Research, Albany, NY, U.S.A.) digestion of intact proteins, virus was first boiled for 2min in the presence of 1% NP40/1% SDS, and then incubated overnight at 37°C in the presence or absence of endo H (Hunt *et al.*, 1983). The reaction was terminated by boiling for 2min in the polyacrylamide-gel sample buffer containing 2% SDS. Pronase-digested glycopeptides were digested with endo H as described previously (Hunt *et al.*, 1978).

SDS/polyacrylamide-gel electrophoresis

Untreated and endo H-treated virion samples were subjected to electrophoresis in 8%-(w/w)-polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970). After electrophoresis, the slab gels were fixed and stained in a solution consisting of 0.04% (w/w) Coomassie Brilliant Blue R (Sigma), 50% (v/v) methanol and 7% (v/v) acetic acid. After destaining in a solution of 5% (v/v) methanol and 7% (v/v) acetic acid, the gels were processed for fluorography in En³Hance (NEN), dried, and exposed at -70°C to Kodak X-Omat L-film. Densitometer tracings were made from the autoradiographs and integrated using a Flur-Vis Autoscanner (Helena Laboratories, Beaumont, TX, U.S.A.).

Sindbis-virus E1 and E2 glycoproteins were purified by preparative SDS/polyacrylamide-gel electrophoresis of 1%-NP40-treated virions in 8%-(w/v)-polyacrylamide slab gels. We have observed that prior treatment of viral proteins with NP40 (but not Triton X-100) resulted in a relative decrease in the electrophoretic mobility of E1 and a corresponding increase in the separation between E1 and E2 on polyacrylamide slab gels (L. A. Hunt, unpublished work). After electrophoresis, segments of the unfixed gel containing E1 and E2 were cut out and frozen, with the positions determined by prestained M_r standards (Bethesda Research Laboratories) in adjacent lanes of the slab gel. The positions of E1 and E2 were later confirmed by Coomassie Brilliant Blue staining of the remaining gel. Radiolabelled protein was eluted from the appropriate unfixed gel segments by incubation in 1 ml of water before digestion with Pronase.

Gel filtration and lectin affinity chromatography of Pronase-digested glycopeptides

Glycopeptides were analysed before and after digestion with endo H by gel filtration through

columns (120cm $\times$ 1.5cm) of Bio-Gel P-4 (-400 mesh; Bio-Rad Laboratories) along with unlabelled and ¹⁴C-labelled gel-filtration standards (Hunt & Summers, 1976; Hunt *et al.*, 1978). The gel-filtration standards were not used as M_r standards, but were useful in comparing different samples run separately on the same or different columns. Before the unknown ³H-labelled samples were run, each column was calibrated with various ³H-labelled oligosaccharides (Man_nGlcNAc₁, $n = 3-9$) as described previously (Hunt, 1981).

Pronase-digested glycopeptides and endo-H released oligosaccharides were fractionated on columns of Con A-agarose (Sigma) as described in detail elsewhere (Hunt, 1982). Unbound glycopeptides or oligosaccharide were washed through the columns with 10mM-Tris/HCl, pH 7.4, and bound glycopeptides or oligosaccharides were sequentially eluted with the same buffer containing 10, 50 and 100mM- α -methyl mannoside. The 100mM- α -methyl mannoside-containing buffer used for the Con A-agarose columns was warmed to 60°C before addition to the top of the columns. Portions of 1.0ml fractions were assayed for radioactivity, and peak fractions of unbound and bound/eluted radiolabel were concentrated by freeze-drying before further analysis by Bio-Gel P-4 gel filtration.

Results

Endo H analysis of Hazelhurst VS-virus_{NJ} G protein from virus replicated in BHK, CEF and L929 cells

In order to demonstrate possible differences in the relative amounts of the more highly processed, acidic-type oligosaccharides (Endo-H resistant) versus the less extensively processed neutral- and hybrid-type oligosaccharides (sensitive to endo H) for G protein from virus replicated in different host-cell types, [³H]mannose- or [³H]leucine-labelled virion proteins were subjected to digestion with endo H and then compared by SDS/polyacrylamide-gel electrophoresis with untreated virion proteins. As shown in Fig. 1 for VS virus_{NJ} replicated in BHK and CEF cells, the G protein from both host cells was a mixture of two classes of glycoprotein, with the first resistant to endo H and containing acidic-type oligosaccharides at both sites, and the second class partially sensitive to endo H and containing a neutral- or hybrid-type oligosaccharide at one of the two glycosylated sites. The relative amount of the partially endo-H-digested G protein (indicated by G') was significantly higher in VS virus_{NJ} from the CEF host cells, suggesting that the oligosaccharides at one site on the G protein were less extensively processed in the CEF than they were in BHK host cells. The [³H]leucine-labelled non-glycosylated

Fig. 1. SDS/polyacrylamide-gel electrophoresis of Hazelhurst VS virus before and after digestion with endo H. Equivalent amounts of [3 H]mannose- or [3 H]-leucine-labelled VS virus_{NJ} from BHK or CEF host cells were incubated in the absence (-) or presence (+) of endo H, and were then analysed by electrophoresis in an 8%-(w/w)-polyacrylamide slab gel and fluorography of the gel. G indicates the undigested or endo H-resistant forms of the glycoprotein, and G' indicates the endo H-digested form containing only one of the original two oligosaccharides. L, N and M indicate the non-glycosylated virion polypeptides.

virion proteins (L, N, M) were unchanged by the endo H treatment, indicating that proteolytic activity was not responsible for the G' species.

The results from the endo H digestion of [3 H]-leucine-labelled VS virus_{NJ} from L929 host cells were similar to those with virus from the BHK host cells, with similar amounts of the two classes of G protein (Fig. 2). In contrast, the G protein from VS virus_{IND} replicated in the same L929 host-cell type demonstrated an essentially complete resistance to endo H, as expected for viral polypeptides containing only complex acidic-type oligosaccharides (Fig. 2).

The approximate ratios of [3 H]leucine radiolabel in the endo H-resistant (G) to that in partially endo H-sensitive (G') forms of the glycoprotein from virus replicated in the different host cells were determined by integration of densitometer scans: 1.0:0.8 for BHK cells, 1.0:4.0 for CEF cells, and 1.0:1.3 for L929 cells. In calculating the corresponding ratios for [3 H]mannose-labelled VS virus_{NJ} G, the value for the endo H-resistant G was divided by 2, since this protein still contained two acidic-type oligosaccharides with radiolabel in the Man₃ core, whereas the partially endo H-digested G' protein contained only a single residual acidic-type oligosaccharide. With this normalization factor, the approximate ratios of G to G' for [3 H]-mannose-labelled VS virus_{NJ} were 1.0:1.3 for BHK host cells and 1.0:2.7 for CEF host cells. Although

Fig. 2. SDS/polyacrylamide-gel electrophoresis of [3 H]-leucine-labelled VS virus_{NJ} and VS virus_{IND} from L929 cells, before and after digestion with endo H. Equivalent amounts of Hazelhurst VS virus_{NJ} (NJ) or VS virus_{IND} (IND) were incubated in the absence (-) or presence (+) of endo H and then analysed as described in the legend to Fig. 1.

the values derived from [3 H]mannose- and [3 H]-leucine-labelled VS virus_{NJ} were not identical, they supported the following conclusion: approximately half of the G protein from the BHK-cell-derived VS virus_{NJ} and almost all of the G protein from the CEF-cell-derived VS virus_{NJ} contained a less extensively processed neutral- or hybrid-type oligosaccharide at one of the two glycosylated sites, and the remaining G protein contained the more extensively processed acidic-type oligosaccharides at both sites.

Endo H analysis of E1 and E2 proteins from Sindbis virus replicated in BHK, CEF and L929 cells

The analysis of [3 H]mannose-labelled glycoproteins from Sindbis virus replicated in BHK as against CEF cells is shown in Fig. 3. For BHK-cell-derived virus the majority of radiolabel was in the endo H-resistant protein species (E1) that contained acidic-type oligosaccharides at both sites, whereas for CEF-cell-derived virus most of radiolabel was in the partially endo H-digested protein species (E1') that had lost a neutral- or hybrid-type oligosaccharide from one glycosylation site. The relative amounts of the more extensively processed and endo H-resistant E1 compared with the less extensively processed and partially endo H-sensitive E1 (E1') from virus replicated in the different host cells was calculated by using the same normalization factor (division by 2) for E1 that was used for [3 H]mannose-labelled VS-virus_{NJ} G, and the approximate ratios of E1 to E1' were 1.0:1.0 for BHK cells and 1.0:22.4 for CEF cells.

Essentially all of the [3 H]mannose-labelled E2 protein from virus replicated in either BHK or CEF cells was converted into the faster-migrating

Fig. 3. SDS/polyacrylamide-gel electrophoresis of [^3H]-mannose-labelled Sindbis virus before and after digestion with endo H

Virus from BHK or CEF host cells was analysed after incubation in the absence (–) or presence (+) of endo H as described for VS virus_{NJ} in the legend to Fig. 1. E1 and E2 indicate the undigested or endo H-resistant forms of the Sindbis-virus glycoproteins, whereas E1' and E2' indicate the endo H-digested forms containing only one of the original two oligosaccharides.

and less intensely labelled E2' protein species, consistent with the removal of a [^3H]mannose-labelled neutral- or hybrid-type oligosaccharide from one of the two glycosylation sites on each E2 polypeptide (Fig. 3).

The results of similar endo H analysis of [^3H]-mannose- and [^3H]leucine-labelled Sindbis virus replicated in L929 cells is shown in Fig. 4. Almost all of the E1 protein was resistant to endo H, indicative of acidic-type oligosaccharides at both glycosylation sites, whereas essentially all of the E2 protein was converted by endo H into the lower- M_r E2' species, consistent with the removal of a neutral- or hybrid-type oligosaccharide from one of the two glycosylation sites. The approximate ratios of E1 to E1' for Sindbis virus replicated in the L929 cells were 1.0:0.3 for [^3H]mannose-labelled virus (normalized E1 value) and 1.0:0.2 for [^3H]leucine-labelled virus, indicative of even more extensive oligosaccharide processing for E1 than observed in the BHK host cells.

Analysis of [^3H]mannose-labelled G glycopeptides and oligosaccharides from Hazelhurst VS virus_{NJ} replicated in BHK and CEF cells

In order to characterize the composition and structure of the asparaginyl-oligosaccharides that were responsible for the difference in endo-H sensitivity of the Hazelhurst VS virus_{NJ} G protein synthesized in BHK as against CEF host cells, Pronase-digested glycopeptides from [^3H]-mannose-labelled virus were analysed by gel

Fig. 4. SDS/polyacrylamide-gel electrophoresis of [^3H]mannose(Man)- or [^3H]leucine(Leu)-labelled Sindbis virus from L929 cells, before and after digestion with endo H

The conditions of analysis were the same as those described in the legend to Fig. 3. In the [^3H]leucine-labelled samples, C indicates the non-glycosylated viral capsid protein and 43 indicates a contaminating cellular protein of M_r approx. 43000 (presumably cellular actin).

filtration before and after digestion with endo H. As shown in Fig. 5(a) for the endo H-digested samples, the glycopeptides and endo H released oligosaccharides from the mature G protein from both BHK and CEF host cells were a heterogeneous mixture of endo H-resistant acidic-type glycopeptides and endo H-released material with the properties of sialic acid-containing hybrid-type oligosaccharides (Hunt & Wright, 1981) and $\text{Man}_5\text{--}_8\text{GlcNAc}_1$ -size neutral oligosaccharides. The distribution of radiolabel within these three classes was 60% acidic, 9% hybrid and 31% neutral for G from BHK cells, and 56% acidic, 15% hybrid and 29% neutral for G from CEF cells. The predominant peak of radiolabel in the profiles of both samples (S_1 peak) was eluted in the position of glycopeptides with biantennary oligosaccharides containing two terminal sialic acid residues $[(\text{NeuNAc} - \text{Gal} - \text{GlcNAc})_2\text{Man}_3\text{GlcNAc}_2 - (\pm\text{Fuc})\text{Asn-peptide}]$ (Hunt *et al.*, 1983).

The major differences between the G oligosaccharides from virus replicated in the CEF host cells and those from the previously characterized G from virus replicated in the BHK cells (Hunt *et al.*, 1983) were: (i) an increase in the amount of sialic acid-containing hybrid-type oligosaccharides; (ii) an increase in the amount of oligosaccharides with the elution properties of asialo hybrid-type oligo-

Fig. 5. Bio-Gel P-4 filtration of endo H-digested glycopeptides from [^3H]mannose-labelled Hazelhurst VS virus $_{\text{NJ}}$ (a) Profiles of glycopeptides from VS virus $_{\text{NJ}}$ replicated in BHK (○) or CEF cells (●); (b) profiles of Con A-unbound (○) versus Con A-bound and 10mM- α -methyl mannoside-eluted glycopeptides and oligosaccharides (●) from VS virus $_{\text{NJ}}$ replicated in CEF cells. The two profiles in each panel were superimposed by alignment of the peak elution positions of gel-filtration standards (indicated by vertical arrows, from left to right): Blue Dextran, ^{14}C -labelled $\text{Man}_5\text{GlcNAc}_1$ (broken arrow), stachyose and mannose. S_0 , S_1 and S_2 indicate the elution positions of endo H-resistant acidic-type glycopeptides containing 3, 2 and 1 residues of terminal sialic acid respectively. X and X' indicate the elution positions of sialic acid-containing and asialo hybrid-type oligosaccharides released by endo H (Hunt & Wright, 1981). 7, 6 and 5 indicate the elution positions of endo-H-released, $\text{Man}_n\text{GlcNAc}_1$ oligosaccharides, with $n = 7, 6$ or 5.

saccharides ($\text{Man}_{8-9}\text{GlcNAc}_1$ -size, X' peak); and (iii) an increase in the relative amount of $\text{Man}_5\text{GlcNAc}_1$ compared with $\text{Man}_6\text{GlcNAc}_1$ -size oligosaccharides.

The endo-H-digested oligosaccharides from the CEF-cell-derived VS $_{\text{NJ}}$ virus were fractionated by affinity chromatography on Con A-agarose to

analyse the structural heterogeneity in the oligosaccharides (Hunt, 1982): biantennary acidic-type glycopeptides and five-mannose hybrid-type oligosaccharides should be bound to Con A, whereas triantennary acidic-type glycopeptides and four-mannose hybrid-type oligosaccharides should be unbound. In addition, the asialo hybrid oligosaccharide with a five-mannose core should be bound less tightly and be eluted at a lower α -methyl mannoside concentration than would large neutral oligomannosyl core structures with seven to nine mannose units. The gel filtration of the Con A-unbound as against Con A-bound and 10mM- α -methyl mannoside-eluted material from the CEF-cell-derived virus (Fig. 5b) demonstrated the presence of asialo hybrid-type oligosaccharides (X' peak; approx. 6% of total radiolabel) that were not present in significant amounts in the corresponding endo H-released oligosaccharides from virus replicated in BHK cells (Hunt *et al.*, 1983). In addition, a minor peak of Con A-unbound oligosaccharides eluted after the major peak of Con A-bound/eluted hybrid-type oligosaccharides (peak X in Fig. 5b) was indicative of a minor fraction of the hybrid-type structures possessing a four-mannose core, with the predominant species possessing a five-mannose core. Besides demonstrating this structural microheterogeneity within the hybrid-type oligosaccharides, these results indicated an even greater relative increase in total hybrid-type oligosaccharides from the G protein of virus replicated in CEF as against BHK cells (21 versus 9%) than was estimated from the profile of endo H-digested glycoproteins in Fig. 5(a) (15 versus 9%).

The ratio of radiolabel in the Con A-bound and 10mM- α -methyl mannoside-eluted acidic-type glycopeptides (biantennary, predominantly S_1 species) compared with that in the Con A-unbound glycopeptides (triantennary, predominantly S_0 species) was approx. 3.8:1.0 for CEF-cell-derived VS-virus $_{\text{NJ}}$ G (Fig. 5b), a value that was very similar to the ratio previously determined for BHK-cell-derived VS-virus $_{\text{NJ}}$ G (4.2:1.0) (Hunt *et al.*, 1983). The additional shoulder of S_2 glycopeptides was consistent with biantennary acidic-type structures containing only a single terminal sialic acid residue.

Analysis of [^3H]mannose-labelled E1 and E2 glycopeptides and oligosaccharides from Sindbis virus replicated in BHK and CEF cells

The structure and composition of the oligosaccharides of the Sindbis-virus glycoproteins from the two different host-cell types were examined in a manner analogous to that of the oligosaccharides of the VS-virus $_{\text{NJ}}$ G protein, in order to determine whether significant host-cell-dependent alterations

also occurred within hybrid- and neutral-type oligosaccharides of both E1 and E2 proteins, or only the E1 protein. As shown in the profiles of endo H-digested glycopeptides in Fig. 6(a), differences were observed between the oligosaccharides of the E1 proteins derived from the CEF as against BHK host cells, with a relative increase in the amount of the $\text{Man}_5\text{GlcNAc}_1$ -size neutral oligosaccharides and a relative deficiency in sialic acid content for both acidic-type glycopeptides (S_1 , S_2 and S_3) and hybrid-type oligosaccharides (X and X') for the CEF-cell-derived viral sample. The distribution of radiolabel in the three major classes of oligosaccharide structures was: 51% in acidic-type glycopeptides, 12% in sialic-acid containing,

hybrid-type oligosaccharides and 37% in neutral oligosaccharides for E1 from BHK host cells, as against 38% in acidic-type glycopeptides, 7% in sialic acid-containing, hybrid-type oligosaccharides and 55% in neutral oligosaccharides for E1 from CEF host cells.

In contrast, the major differences in the profiles of oligosaccharides and glycopeptides from E2 from the two host-cell types (Fig. 6b) was a relative increase in the amount of the predominant $\text{Man}_7\text{GlcNAc}_1$ -size neutral oligosaccharides and a shift toward less-sialylated forms of the acidic-type glycopeptides in the virus from CEF as against BHK host cells.

Analysis of the endo H-digested glycopeptides from Sindbis-virus glycoproteins after fractionation by Con A affinity chromatography provided further evidence for hybrid-type oligosaccharides on both E1 (Fig. 7) and E2 (results not shown) for

Fig. 6. Bio-Gel P-4 gel filtration of endo H-digested glycopeptides from [^3H]mannose-labelled Sindbis-virus E1 and E2 proteins

(a) Profiles of E1 glycopeptides from virus replicated in BHK (○) or CEF cells (●); (b) profiles of E2 glycopeptides from virus replicated in BHK (○) or CEF cells (●). The conditions of gel filtration and the designations of various glycopeptides and oligosaccharide species in this and the next Figure were the same as described for VS-virus_{NJ} G glycopeptides in the legend to Fig. 5. S_3 indicates the elution position of asialo acidic-type glycopeptides.

Fig. 7. Bio-Gel P-4 gel filtration of Sindbis-virus glycopeptides after Con A affinity chromatography. Endo H-digested and [^3H]mannose-labelled glycopeptides equivalent to those analysed in Fig. 6(a) were fractionated on Con A-agarose before gel filtration, and the profiles of Con A-unbound (○) and 10mM- α -methyl mannoside-eluted (●) glycopeptides/oligosaccharides were superimposed. (a) BHK host cells; (b) CEF host cells.

virus replicated in either BHK or CEF cells. Whereas the biantennary acidic-type glycopeptides (S_1 peak in Fig. 7a) and the endo H-released hybrid-type oligosaccharides (X peak in Fig. 7a) from BHK-cell-derived E1 were essentially all in the fully sialylated forms, the corresponding glycopeptides (S_1 and S_2 peaks in Fig. 7b) and oligosaccharides (X and X' peaks in Fig. 7b) from the CEF-cell-derived E1 were a mixture of fully sialylated and sialic acid-deficient forms, as was also observed from the VS-virus_{NJ} G protein from the same host-cell types (Fig. 5). The asialo hybrid structures represented approx. 8% of the total radiolabel in the CEF-cell-derived E1 protein, so that the total amount of hybrid-type oligosaccharides in E1 was increased in the CEF (15%) compared with the BHK host cells (12%).

Discussion

Endo-H digestion of both intact viral membrane glycoproteins and Pronase-digested glycopeptides prepared from these viral proteins has been used to characterize the oligosaccharide components of the G protein of VS virus_{NJ} (Hazelhurst subtype) and the E1 and E2 proteins of Sindbis virus replicated in BHK, CEF and L929 host cells. We have demonstrated that the host-cell-dependent variation in the glycosylation of the G protein parallels that observed for the Sindbis-virus E1 protein in the present studies and in previously published reports (Burke & Keegstra, 1976, 1979; Hsieh *et al.*, 1983a). A large proportion of the mature G and E1 proteins contained the more extensively processed acidic-type oligosaccharides at both glycosylation sites for virus replicated in BHK and L929 cells, whereas most of the mature G and E1 proteins from virus replicated in the CEF cells contained an acidic-type oligosaccharide at one site and an endo H-sensitive neutral-(or hybrid-)type oligosaccharide at the other glycosylation site. In contrast, essentially all of the Sindbis-virus E2 protein contained one endo H-resistant and one endo H-sensitive oligosaccharide, regardless of the host-cell type. We cannot conclude whether these results were indicative of specific differences in oligosaccharide biosynthesis between mammalian cells and avian cells, or between continuous cell lines and secondary embryo fibroblast cultures, without more extensive surveys of host-cell-dependent variation in the glycosylation of the VS-virus_{NJ} G and Sindbis-virus E1 proteins.

Much of the relative increase in endo H-sensitive oligosaccharides in the VS-virus_{NJ} G and Sindbis-virus E1 from virus replicated in the CEF as against BHK host cells represented increased amounts of neutral- and hybrid-type oligosacchar-

ides containing a five-mannose core (Figs. 5–7). These results indicated that the first enzymic processing step in Golgi membranes [removal of the four α 1,2-linked mannose units from the nine-mannose precursor oligosaccharides by α -mannosidase I (Tulsiani *et al.*, 1982)] was relatively efficient in both the CEF and BHK cells, but that the next two enzymic processing steps for one of the oligosaccharide chains on the VS-virus_{NJ} G and Sindbis-virus E1 proteins were less efficient in the CEF than in the BHK host cells: (i) addition of the first branch *N*-acetylglucosamine by *N*-acetylglucosaminyltransferase I (Tabas & Kornfeld, 1978; Harpaz & Schachter, 1980); and (ii) the removal of the final two mannose units by α -mannosidase II after the addition of the branch *N*-acetylglucosamine to the five-mannose intermediate (Tabas & Kornfeld, 1978). A blockage in this second step would explain the increased amounts of hybrid-type oligosaccharides in the mature G and E1 proteins of virus released from the CEF as against the BHK cells. Hybrid-type oligosaccharides with a structure equivalent to those observed for the VS-virus_{NJ} G protein (Hunt *et al.*, 1983) and the Sindbis-virus E1 and E2 proteins have been shown to accumulate in the place of complex acidic-type oligosaccharides on viral and cellular glycoproteins synthesized in cells treated with swainsonine, a specific inhibitor of α -mannosidase II (Tulsiani & Touster, 1983; Kang & Elbein, 1983).

The factors that determine the extent of processing of asparagine-linked oligosaccharides on viral and cellular glycoproteins are not well understood, but a number of likely factors have been suggested (Hubbard & Ivatt, 1981; Kornfeld, 1982; Pollack & Atkinson, 1983; Hsieh *et al.*, 1983a,b): conformation of the protein and location of the oligosaccharide along the polypeptide, the relative accessibility of the precursor oligosaccharides to specific glycosidases and glycosyltransferases in the Golgi, and the transit time through the Golgi. The relative accessibility and processing of the oligosaccharides may also be affected by protein-protein interactions within intracellular E1–E2 (or E1–PE2) dimers or possible G oligomers. Specific glycosidase digestions of intact Sindbis virions have suggested that the mature neutral-type oligosaccharides are less accessible and less exposed at the virion surface than the complex acidic-type oligosaccharides, but this selectivity in glycosidase digestion could not be detected after solubilization of the glycoproteins with non-ionic detergent (McCarthy & Harrison, 1977; Hsieh *et al.*, 1983b). These studies are consistent with the hypothesis that complex acidic-type oligosaccharides are the end product of processing at glycosylated sites where the precursor oligosaccharide is relatively

exposed, whereas high-mannose, neutral-type oligosaccharides are the end products where the precursor oligosaccharide are relatively inaccessible to processing enzymes (Hsieh *et al.*, 1983a). We hypothesize that the glycosylation sites on the Sindbis-virus E1 and Hazelnut VS-virus_{NJ} G proteins with a mixture of acidic-, hybrid- or neutral-type oligosaccharides on different mature polypeptides may represent sites with intermediate accessibility, so that the extent of processing at these sites might be especially sensitive to possible host-cell-dependent differences in levels of processing enzymes or in intracellular transit times.

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