Growth-Dependent Alterations in Oligomannosyl Glycopeptides Expressed in Sindbis Virus Glycoproteins†

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ABSTRACT: Sindbis virus was used to examine host cell growth-dependent expression of oligomannosyl glycopeptides within single species of viral glycoproteins. Four mannosyl oligosaccharides ranging from 5 to 8 mannose residues were separated on Bio-Gel P4 column chromatography. Virus derived from rapidly growing chicken embryo fibroblasts displayed greater quantities of larger sized oligomannosyl glycopeptides in intact virus and in purified Sindbis virus glycoproteins E1 and E2 compared with virus from nongrowing cells. The pattern of mannosyl oligosaccharides in the two glycoproteins differed remarkably. E1 and E2 contained predominantly $Man_5GlcNAc$ and $Man_7GlcNAc$, respectively; in addition, E1 relative to E2 contained more complex-type glycopeptides than mannosyl-type glycopeptides. These data clearly demonstrate that host-dependent alterations in glycosylation are expressed in Sindbis virus and that differences in specific glycosylation patterns are obscured in oligosaccharides derived from mixtures of glycoproteins. It is apparent that processing of these cotranslated glycoproteins can yield different patterns of oligosaccharide structures.

Growth-dependent alterations in oligomannosyl or "high mannose" glycopeptides of animal cell surface glycoproteins have been characterized. Such oligosaccharide species (four–five) consist of only two monosaccharides, mannose (Man) and *N*-acetylglucosamine (GlcNAc). In rapidly growing cells compared with nongrowing density-inhibited cells, there is a general tendency to express markedly more of the larger sized oligosaccharides [Muramatsu et al., 1976; see also review, Atkinson & Hakimi (1980)]. Our interest in such expression derives from the as yet unproven notion that various oligosaccharides, including these, are involved in growth regulation mediated by the cell surface. Also, the cell biological problem of how cells go about such expression seems important.

Cell surface glycopeptides can be obtained either by isolation of plasma membranes or by removal of surface glycopeptides by mild proteolytic digestion. Using the latter approach, one cannot distinguish whether growth-related differences in the relative amounts of oligosaccharides are occurring due to alterations in the synthesis and turnover of glycoprotein species within a glycoprotein mixture or whether there are specific size alterations of oligosaccharide within individual glycoproteins. Thus, individual glycoproteins must be purified, a task made simpler by the use of Sindbis virus (SbV). SbV is a membrane-maturing enveloped virus, containing only two glycoproteins, E1 and E2, which can be purified [reviewed by Lenard (1978); Kääriänen & Renkonen, 1977; Strauss & Strauss, 1977)]. Viral infection inhibits host cell RNA and protein synthesis, and thus radioactive precursors incorporate into virus rather than into host molecules. E1 and E2 grown in chick embryo fibroblasts (CEF) contain complex glycopeptides (S1, S2, and S3) and oligomannosyl (S4) glycopeptides (Burke & Keegstra, 1976, 1979). Glycosylation of SbV glycoproteins are primarily regulated by the host cell as shown by the differential expression of viral glycopeptides derived from normal vs. transformed cells (Keegstra et al., 1975; Burge & Strauss, 1970).

To our knowledge there is no study on growth-dependent changes in oligosaccharides derived from a single purified species of membrane glycoprotein. In this paper we have demonstrated that E1 and E2 express different relative quantities of four mannosyl oligosaccharides and that the relative amounts of these species are quite markedly growth dependent.

Materials and Methods

Cells and Viruses. Primary chick embryo fibroblasts were prepared from 9–11-day-old embryos (Spafas). Embryos were removed aseptically, decapitated, eviscerated, and washed several times in phosphate-buffered saline. The tissue was resuspended in Eagle's minimal essential media (Gibco, F-11) and forced twice through a 50-mL syringe. The tissue suspension was incubated at 37 °C in a final concentration of 0.25% trypsin (Difco, MI) and stirred for 25 min. The suspension was passed through cheesecloth and centrifuged (650*g*) for 10 min. Cells were suspended in Eagle's media supplemented with 5% fetal calf serum, penicillin, and streptomycin. The equivalent of two–three embryos was diluted to 75 mL and poured into glass roller bottles with 700-cm^2 growing surface area (Bellco). These cells were grown to confluency, and all experiments described were performed with CEF subdivided with 0.25% trypsin and 0.04% EDTA in Earle's salts supplemented with amino acids and vitamins. CEF growth characteristics were established by following total cell protein in stoppered-glass T-15 flasks at 37 °C for 32 days as previously described (Oyama & Eagle, 1956; Muramatsu et al., 1973) in order to define growing and nongrowing density-inhibited states of these cells. CEF begin to grow exponentially within 24-h postinoculation. Cell growth was monitored as a measure of mg of protein/cm^2 surface area in glass T-15 flasks. Doubling occurred every 45–54 h, as shown in Figure 1 and confirmed in two additional independent studies. Maximum cell protein concentration of approximately 90 $\mu g/cm^2$ was achieved, and cultures at this stage were used as nongrowing or density-inhibited cells. Arrows on the growth curve (Figure 1) show where cells were labeled for growing and nongrowing characteristics.

Stock Sindbis virus (SbV) source was prepared by infecting CEF at a multiplicity of 0.01–0.1 pfu/cell (pfu: plaque forming units, determined on CEF) at 37 °C for 16 h. The

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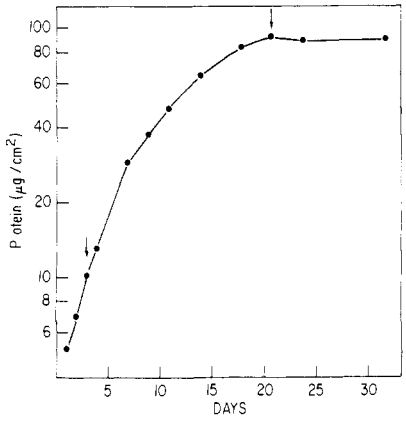

FIGURE 1: Growth characteristics of CEF. Cells were plated at a cell density of 2×10^5 cells/T15 culture flask and grown at 37 °C as described under Materials and Methods. Cells were fed every other day. Each point is the average of two protein determinations from replicate culture flasks. Arrows indicate time when cells were used to harvest SbV.

media were collected and clarified by centrifugation (8000*g*) for 30 min at 4 °C. Virus stocks were stored at −80 °C and contained 10^9–10^{10} pfu/mL. Purified radiolabeled virus was prepared by infecting growing or nongrowing CEF with SbV at 10–100 pfu/cell for 1 h at 37 °C in the absence of serum. Actinomycin D (Calbiochem) was added to a final concentration of 1.0 μg/mL for an additional 30 min. Growth media was added containing D-[2-^{3}H]mannose (2 Ci/mmol) or [^{14}C]mannose (200 mCi/mol) (both isotopes were obtained from either New England Nuclear or Amersham), and infected cells were incubated approximately 15 h at 37 °C. The media was clarified as described above, and virus was concentrated by centrifugation at 186000*g* for 90 min at 4 °C. The viral pellet was resuspended in TNE buffer (0.15 M NaCl, 0.05 M Tris HCl, 1 mM EDTA, pH 7.5) and purified on a 10–30% sucrose–TNE gradient in a Spinco SW27 rotor at 25 000 rpm for 3 h. The visible light-scattering band was aspirated with a needle and syringe, diluted with TNE, and finally centrifuged at 186000*g* for 90 min. The resulting pellet was resuspended in a minimal volume of TNE and stored at −80 °C. Purity was monitored by polyacrylamide gel electrophoresis and autoradiography (Maizel, 1971).

Viral titers (measured as pfu/cell or pfu/mg of cell protein) from growing and nongrowing CEF were compared. In three independent experiments, growing CEF yielded around twice the pfu/cell than nongrowing cells (data not shown). Whether this indicates that growing cells produce more viral particles or more infectious particles than nongrowing cells has not been determined. However, it seemed unlikely that virus production in nongrowing cultures was due to a minor proportion (less than 10%) of growing cells. Such a quantity of growing cells would have readily been detected as an increase of protein in the growth curve (Figure 1).

Purification of Viral Glycoproteins. E1 and E2 were separated on $NaDodSO_4$ 7.5–15% gradient polyacrylamide slab gels by electrophoresis in a modification of the Reid & Bieleski (1968) apparatus as essentially described by Maizel (1971). Gels were fixed with 50% methanol and 7% acetic acid for 2 h and dried on Whatman 3MM paper in a gel slab dryer (Model 224, Bio-Rad). Autoradiography was performed with Kodak XR X-ray film. E1 and E2 glycoprotein bands were located and cut from the gel. Gel pieces were reswollen in electrophoretic Tris–glycine running buffer (Maizel, 1971), and the glycoproteins were electrophoretically eluted. Glycoproteins were precipitated at −20 °C in 70% acetone (v/v).

Table I: Gel Filtration of Molecular Weight Markers on Bio-Gel P2

marker	mol wt
a, mannose	180
b, sucrose	342
c, raffinose	504
d, stachyose	666
e, $Man_5GlcNAc$[a]	1031
f, $Man_6GlcNAc$[a]	1193
g, $Man_5GlcNAc_3$[a]	1437
h, $Man_5GalGlcNAc_4$[a]	1802
S4A	(2170)[b]
S4B	(1530)
S4C	(1350)
S4D	(1220)
S4E	(1050)

[a] Oligosaccharide products from endoglycosidase digestion of ovalbumin glycopeptides: AC-E3(e), AC-D3(f), AC-C3b(g), and AC-A3(h). [b] Numbers in parentheses are deduced molecular weights from the calibration curve.

The precipitate was pelleted at 12000*g* for 1 h at −10 °C, washed once in 100% acetone, and repelleted. The protein was used immediately.

Preparation and Gel Filtrations of Glycopeptides. Intact virus or isolated glycoproteins (E1 or E2) were digested with 5 mg/mL Pronase (Calbiochem) in 0.15 M Tris-HCl and 0.20 M $CaCl_2$, pH 8, at 37 °C for 12 h under a toluene atmosphere (Muramatsu et al., 1973). Additional Pronase was added at 12, 24, and 36 h. Digestion was terminated by boiling the digest for 3 min in a water bath.

Glycopeptides were chromatographed on Bio-Gel P6 (Bio-Rad, −400 mesh, 0.9 × 175 cm) and eluted with 0.1 M NH_4HCO_3. Fraction volumes were between 0.53 and 0.58 mL. Aliquots were removed, diluted to 1 mL, and added to 10 mL of Aquasol-2 (New England Nuclear) to determine radioactivity. Oligosaccharides were chromatographed either on Bio-Gel P2 (−400 mesh, 0.9 × 175 cm) collecting 0.52–0.57-mL fractions or on Bio-Gel P4 (−400 mesh, 1.5 × 175 cm) collecting ~ 0.82–0.88-mL fractions, and both were eluted with 1 mM sodium azide. Desalting was carried out by Sephadex G10 or Bio-Gel P2 chromatography (1.5 × 100 cm). It should be noted that all Bio-Gel P6 profiles of glycopeptides have been drawn so that the S3 peak heights of both mannose labels appear equal (Figures 2 and 6). All Bio-Gel P4 profiles (Figures 3, 4, and 7) have been normalized so that the S4C peak heights of both mannose labels appear equal. This allows easier comparative analysis of growing and nongrowing Bio-Gel P6 and P4 profiles.

Enzyme Sources and Incubation Conditions. Endo-β-*N*-acetylglucosaminidase H (endo-H) from *Streptomyces griseus* was prepared by the method of Tarentino & Maley (1974), and digestions of glycopeptides were performed as previously described (Muramatsu et al., 1976) at 37 °C. Endo-β-*N*-acetylglucosaminidase D from *Diplococcus pneumoniae* was prepared according to the method of Koide & Muramatsu (1974), and digestions were performed as previously described (Muramatsu et al., 1976). A highly purified α-mannosidase containing no other glycosidase activity was prepared from Jack Bean meal (Sigma) according to the method of Snaith & Levvy (1968), generously donated by Dr. C. Ceccarini, Hunter College, New York. This enzyme was used as described previously (Snaith & Levvy, 1968).

Results

Molecular Weight Determination of S4 Oligosaccharides. Bio-Gel P4 and P2 columns were calibrated with eight oligosaccharide markers (Table I). Approximately 200 μg of

Table II: Quantities of S4 Oligosaccharides from Glycoproteins of Sindbis Virus Harvested from Growing and Nongrowing CEF Cultures[a]

oligosaccharide[b]	SbV-S4[c]		SbV-E1-S4		SbV-E2-S4	
	growing	nongrowing	growing	nongrowing	growing	nongrowing
S4B	13	10	11	0	19	12
S4C	43	32	25	17	53	43
S4D	15	16	16	13	13	18
S4E	29	42	48	70	15	27

[a] Numbers listed are percent of total oligosaccharide derived from endo-H digestion of S4 glycopeptides and fractionated on Bio-Gel P4. [b] Oligosaccharides were purified as detailed under Materials and Methods and legend to Figure 1. [c] Mixed oligosaccharides from E1 and E2 in intact virus.

each marker was applied to the columns. Oligosaccharide markers were prepared by endo-H hydrolysis of purified homogeneous ovalbumin glycopeptides assayed by gas–liquid chromatography and ^1H nuclear magnetic spectroscopy at 360 MHz (P. H. Atkinson et al., unpublished results). S4 oligosaccharides (endo-H digestion products of S4 glycopeptides) were cochromatographed with markers in order to estimate their molecular weights. Elutions of the oligosaccharide markers were followed by the phenol–sulfuric method of Dubois et al. (1956). Radioactivity was followed to determine the elution of the S4 oligosaccharides. On Bio-Gel P2 chromatography a linear relationship between log molecular weight and elution volume was obtained with the oligosaccharide standards. The estimated molecular weights (Table I) of the SbV-S4 oligosaccharides corresponded to $Man_8GlcNAc$ (S4B) as the largest oligosaccharide and $Man_7GlcNAc$ (S4C), $Man_6GlcNAc$ (S4D), and $Man_5GlcNAc$ (S4E) which coeluted with authentic $Man_5GlcNAc$. The identity of S4A is unknown. These compositions were supported by analysis of α-mannosidase digestion products of S4 oligosaccharides resolved on Bio-Gel P2. Two products were obtained: free mannose determined by gas–liquid chromatography and Manβ1→4GlcNAc (which eluted between stachyose and raffinose). The identity of the disaccharide was inferred by ^1H NMR analysis of identically derived material from AC-E3 ovalbumin glycopeptide (Table I) which coeluted with SbV-derived material on Bio-Gel P2 (P. H. Atkinson et al., unpublished results). Thus, the S4 oligosaccharides terminate in mannose and contain only mannose and one GlcNAc residue, and Bio-Gel P2 chromatography fractionates different oligomers of mannose linked to one GlcNAc. A linear molecular weight calibration curve for these oligosaccharide markers on Bio-Gel P4 could only be obtained when the molecular weight of the terminal GlcNAc residues of oligosaccharides g and h (Table I) was calculated for two hexose units rather than one GlcNAc residue.

Analysis of SbV Glycopeptides and Oligosaccharides from Growing and Nongrowing CEF. Glycopeptides from virus harvested from rapidly growing ([^3H]mannose) and nongrowing ([^{14}C]mannose) cells were fractionated into five peaks on Bio-Gel P6 (Figure 2). Though molecular weights were the same, relative proportions differed markedly within the species, reflecting a growth-dependent expression of SbV glycopeptides. Among the three complex-type glycopeptides (S1, S2, and S3), S1 appears to be enriched in virus harvested from nongrowing cells compared with its growing counterpart. The oligomannosyl glycopeptides, S4, were resolved into two peaks. Virus harvested from growing cells contained more larger sized oligomannosyl glycopeptides while that from nongrowing cultures expressed more smaller glycopeptides (Figure 2).

The growth-dependent expression in quantity of the various SbV-S4 glycopeptides (Figure 2) is better illustrated when examining their oligosaccharide counterparts, which are well

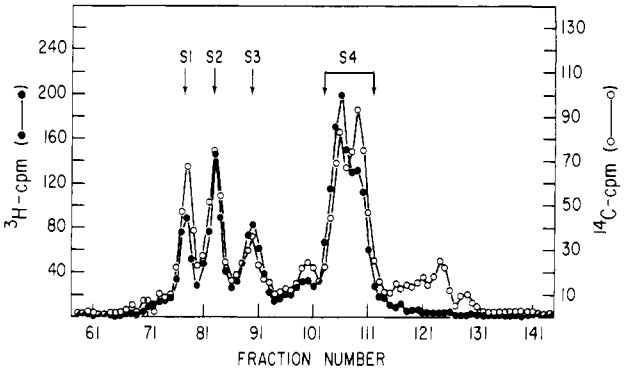

FIGURE 2: Bio-Gel P6 chromatography of SbV glycopeptides. SbV infected growing CEF were labeled with [^3H]mannose (150 μCi/mL) and nongrowing cells were labeled with [^{14}C]mannose (5 μCi/mL). Virus was purified, mixed, Pronase digested, desalted, and applied to Bio-Gel P6 as described under Materials and Methods.

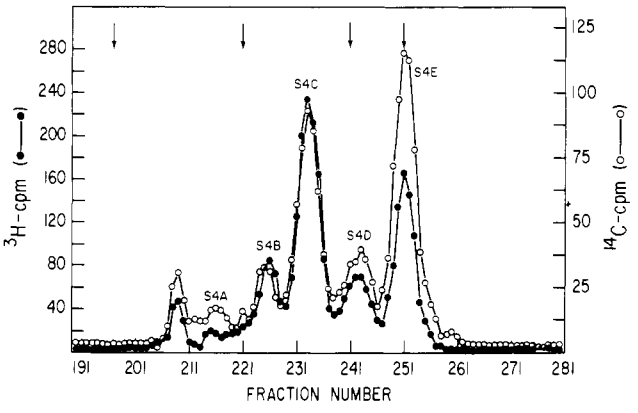

FIGURE 3: Bio-Gel P4 chromatography SbV-S4 oligosaccharides. SbV-S4 glycopeptides from growing and nongrowing cells were copurified on Bio-Gel P6 and digested with endo-H. The S4 oligosaccharide products were compared on Bio-Gel P4. The arrows from left to right indicate the peak elution fraction of ovalbumin oligosaccharides A3, C3b, D3, and E3 (see Table I). BSA (V_0), stachyose, raffinose, sucrose, and mannose (V_i) eluted in fractions 120, 285, 299, 316, and 332, respectively.

resolved in Bio-Gel P4 (Figure 3). When SbV oligosaccharides derived from nongrowing cells are compared to those from growing cells, there is a distinct trend toward relatively greater quantity of oligosaccharide with fewer mannose residues. The areas of each oligosaccharide peak (S4B, S4C, S4D, and S4E) were calculated as the percent of the total oligosaccharides (4) from SbV derived from growing and nongrowing CEF (Table II). S4B, S4C, S4D, and S4E from SbV derived from growing cells consisted of 13, 43, 15, and 29% of total oligosaccharide, respectively. In the same sequence, the nongrowing counterparts consisted of 10, 32, 16, and 42% of total oligosaccharide. It is apparent that SbV from growing cells contains more $Man_7GlcNAc$ than $Man_5GlcNAc$ species while relatively more $Man_5GlcNAc$ is expressed in

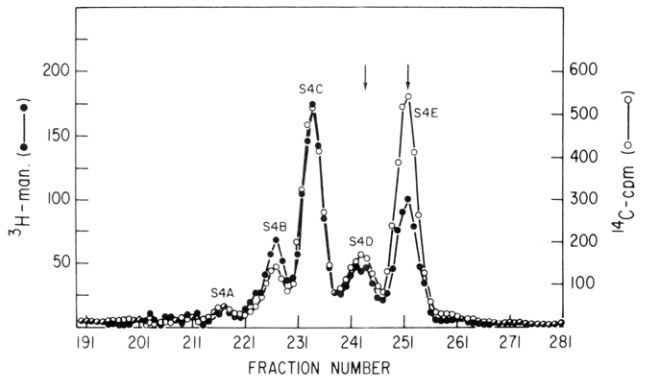

FIGURE 4: Bio-Gel P4 chromatography of SbV-S4 oligosaccharide. Nongrowing CEF were infected with SbV at 10–100 pfu/cell (as described under Materials and Methods) and labeled with [^{14}C]-mannose (0.5 mCi/mL) 90-min postinfection. At 4.5-h postinfection, purified [^{3}H]Man-SbV (10^5 cpm) derived from growing CEF was added to the infected cells. Virus was purified 18.5-h postinfection. S4 glycopeptides were isolated from Bio-Gel P6, digested with endo-H, and chromatographed on Bio-Gel P4. The arrows from left to right indicate the peak elution fraction of ovalbumin oligosaccharides D3 and E3. BSA (V_0), stachyose, raffinose, sucrose, and mannose eluted in fractions 121, 285, 300, 315, and 333, respectively.

nongrowing viral counterparts. Reversal of the mannose label (^{14}C or ^{3}H) produced similar results. Thus, it appears that the expression of SbV oligosaccharides is carefully regulated by the growth state of the host cell.

We questioned whether nongrowing culture media contained glycosidases that may account for the expression of smaller sized oligomannosyl glycopeptides from SbV derived from nongrowing cells. Nongrowing CEF were infected with SbV and labeled with [^{14}C]mannose. At 4.5-h postinfection, purified [^{3}H]Man-SbV harvested from rapidly growing cells (same virus preparation used in Figures 2 and 3) was added back to the cultures. Newly synthesized [^{14}C]SbV and the added [^{3}H]SbV were purified 18.5-h postinfection. Viral glycopeptides and oligosaccharides were prepared, and the S4 oligosaccharides were fractionated on Bio-Gel P4 (Figure 4). The pattern of oligosaccharides characteristic of growing cells (Figure 3) was the same. Growth-related size expression observed in SbV-S4 oligosaccharides from growing and nongrowing cells is, therefore, not due to the concentration of an exogenous α-mannosidase in nongrowing cultures and absent (or diluted) in growing cultures. S1, S2, and S3 glycopeptides were also unaltered, as demonstrated by their Bio-Gel P6 profiles (data not shown).

Purification of SbV Glycoproteins. [^{3}H]Man-SbV and [^{14}C]Man-SbV from growing and nongrowing CEF, respectively, were mixed, and E1 and E2 were separated by Na-DodSO$_4$–polyacrylamide gel electrophoresis (Figure 5). Only three viral proteins were observed by autoradiography or Coomassie Blue staining in all SbV preparations used. Apparently core protein is being labeled by ^{14}C label from mannose which presumably must have been reutilized into protein. The glycoproteins were eluted from acrylamide by electrophoresis and concentrated by acetone precipitation. Recovery was approximately 56% as determined by recovery of total radioactivity.

Comparison of Glycopeptides and Oligosaccharides from E1 and E2. E1 and E2 purified from SbV harvested from growing and nongrowing cultures were mixed, digested with Pronase, and fractionated on Bio-Gel P6. As observed with glycopeptides from intact SbV, growth-dependent differences were expressed in both E1 and E2 except the differences were more accentuated (Figure 6; cf. Figure 2). S1 and S2 appeared enriched in the nongrowing state in both glycoproteins. These

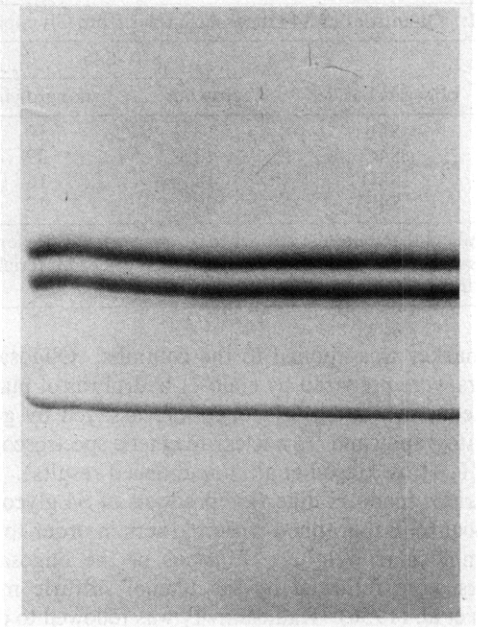

FIGURE 5: Autoradiography of preparative NaDodSO$_4$–polyacrylamide gel electrophoresis. [^{3}H]Man-SbV and [^{14}C]Man-SbV were pooled and separated on 7–15% gradient gels with a 3% stacking gel. The slab gel was fixed in 50% methanol–7% acetic acid, dried on Whatman 3MM paper, and autoradiographed for 3 days as described under Materials and Methods.

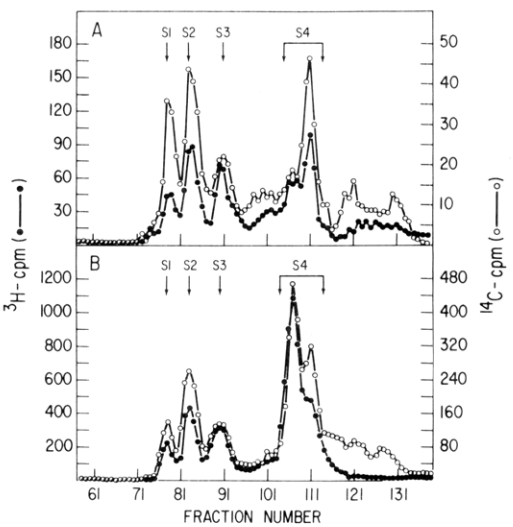

FIGURE 6: Bio-Gel P6 chromatography of SbV glycopeptides from purified glycoproteins (A) E1 and (B) E2. [^{3}H]Man-SbV and [^{14}C]Man SbV from growing and nongrowing cells, respectively, were mixed. E1 and E2 were purified by NaDodSO$_4$–polyacrylamide electrophoresis as described in the text (see Figure 5). The glycoproteins were individually digested with Pronase, desalted, and chromatographed on Bio-Gel P6: (●) SbV glycopeptides from growing CEF; (○) SbV glycopeptides from nongrowing CEF.

differences are reproducible and are not due to interconversion of [^{14}C]mannose radioactivity into other saccharides (data not shown). When the E1- relative to E2-derived glycopeptides were compared, E1 contained more complex glycopeptides than oligomannosyl glycopeptides, as previously reported (Burke & Keegstra, 1976). E2 mannosyl glycopeptides (S4) fractionated into two size classes with relatively more of the larger sized species which is even further emphasized in virus harvested from rapidly growing cells. E2 oligosaccharides derived from these glycopeptides fractionated into four oligosaccharides species, S4B, S4C, and S4E (Figure 7B, Table II). E2 predominated in $Man_7GlcNAc$ (S4C) and, relative to it,

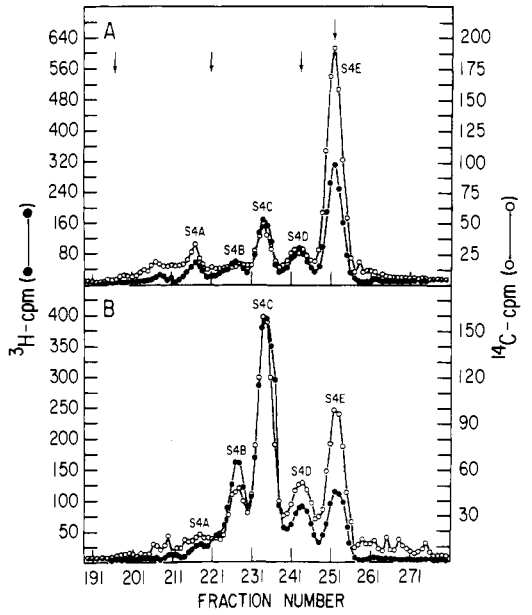

FIGURE 7: Bio-Gel P4 chromatography of SbV-S4 oligosaccharides derived from glycoproteins (A) E1 and (B) E2. SbV-S4 glycopeptides from E1 and E2 (Figure 6) were individually digested with endo-H and chromatographed on Bio-Gel P4; (●) from growing and (○) from nongrowing cells. The arrows from left to right indicate the peak elution position of ovalbumin oligosaccharides A3, C3B, D3, and E3, respectively, for E1. BSA (V_0) stachyose, raffinose, sucrose, and mannose (V_i) eluted in fractions 120, 284, 297, 313, and 330, respectively.

growth-related expression of the other oligosaccharides (S4B, S4D, S4E) is clearly exhibited. Again as with intact SbV, there is an overall expression of the smaller oligomannosyl species in virus obtained from nongrowing density-inhibited cultures. E1 contained three major oligosaccharides (S4C, S4D, S4E) with a small quantity of S4B present only in virus harvested from growing cells. The predominant E1 oligosaccharide is $Man_5GlcNAc$ (S4E), and growth-dependent expression of overall smaller oligomannosyl species in virus from nongrowing cells is again evident (Table II, Figure 7A). It is apparent that the protein-bound oligosaccharides of the two membrane glycoproteins must be processed differently and that the expression of both E1 and E2 oligomannosyl glycopeptides is regulated by the growth state of the host cells.

Discussion

Oligomannosyl glycopeptides are well-known constituents of a variety of secreted glycoproteins and recently have been found as components of membrane glycoproteins. Ceccarini et al. (1975) observed these species associated with the plasma membrane of human diploid fibroblasts. Debray & Montreuil (1977) and Kawai & Spiro (1977) have also reported occurrence of cell surface oligomannosyl glycopeptides in rat hepatocytes and in rabbit fat cells, respectively. The visual pigment rhodopsin, a membrane glycoprotein, has also been shown to be rich in oligomannosyl side chains (Liang et al., 1979; Fukuda et al., 1979). Though the importance of such glycopeptides in cell processes remains obscure, several studies have given insight as to their possible biological involvement as recognition components. In two different systems involving embryonic development, oligomannosyl glycopeptides have been implicated in cell–cell interactions. A cell surface lectin-like component in teratocarcinoma stem cells with specificity for oligomannosyl glycopeptides has been demonstrated to participate in cell–cell adhesion (Grabel et al., 1979). This process is inhibited by a series of mannose-rich glycoproteins. In sea urchin embryos, oligomannosyl glycopeptides have also been implicated in cell–cell interactions occurring during gastrulation (Heifetz & Lennarz, 1979). In rat liver, a recognition system for the rapid clearance of glycoproteins containing oligomannosyl side chains has been reported (Steer & Clarenburg, 1979; Day et al., 1980).

With respect to growth-dependent expression of such substances, larger sized oligomannosyl glycopeptides (up to nine mannoses) were more enriched in growing human diploid cells compared to their density-inhibited counterparts (Muramatsu et al., 1976). It was, however, unknown whether specific mannosyl oligosaccharide chain lengths changed or whether populations of different glycoproteins varied (see below). Uninfected CEF cultures were similar in the growth-dependent expression of oligomannosyl glycopeptides (P. H. Atkinson et al., unpublished results). Using SbV derived from BHK cells, Keegstra et al. (1975) reported a slight increase in the relative amount of S1 glycopeptide from growing cells compared to nongrowing cells. With CEF as host, no growth-dependent expression of SbV glycopeptides was observed. When murine leukemia viruses were used, no growth-dependent alterations of glycosylation was observed either (Rosner et al., 1980). In either of the above studies, no data were included which would allow accurate assessment of host cell growth status. In the current studies, careful examination of CEF growth properties reveals that cultures appearing confluent visually were still increasing in total cell number and protein. These cells were not contact inhibited, and little differences in glycosylation patterns of SbV in them were observed. In subsequent studies, analysis of purified E1 and E2 revealed that both proteins exhibited growth-dependent expression of oligomannosyl glycopeptides.

Purified [^{3}H]Man SbV derived from growing cells added back to nongrowing infected cultures resulted in glycopeptide profiles characteristic of growing cultures. Therefore, it must be concluded that growth-dependent expression is controlled by intracellular rather than extracellular enzymes. This is in agreement with the observation that glycosylation of cellular glycopeptides, i.e., those which remain after mild proteolysis of intact cells, are cell density dependent (Muramatsu et al., 1976). An intracellular cell density dependent α-mannosidase found only in nongrowing cultures has been reported (Robbins, 1979). The existence of such an enzyme emphasizes that growth-dependent expression is not random but carefully regulated. Mannosidases involved in such expression would have to be highly specific, since S4 glycopeptides do not contain structures shorter than $Man_5GlcNAc_2Asn$. High-field ^{1}H NMR analysis of α-mannosidase digestion products of ovalbumin glycopeptides (unpublished results) confirms previous reports (Trimble et al., 1978) demonstrating that $\alpha(1,3)$-linked mannose residues have different susceptibilities to Jack Bean meal α-mannosidases. Apparently these enzymes are not only linkage specific but are also sensitive to the surrounding oligosaccharide structure also. At least three different cellular α-mannosidases, occurring in lysosomes, cytosol, and Golgi membranes, play a role in the biosynthesis of asparagine-linked glycopeptides (Touster, 1978). Thus, enzymes specific for different linkages with variable activities may play a role in final expressed structures.

Characterization of E1 and E2 oligosaccharides as reported here reveals some interesting information on glycosylation. The relative amounts of complex and oligomannosyl glycopeptides in each glycoprotein are distinctly different. The general patterns of S1, S2, and S3 glycopeptides are very similar in E1, E2, and intact SbV. A relative increase in S1

and S2 glycopeptides was observed from both glycoproteins derived from nongrowing CEF. These findings are opposite to those reported previously (Meezan et al., 1969; Buck et al., 1971; Muramatsu et al., 1973) in that nongrowing cells showed smaller sized complex-type glycopeptides. A mixture of oligosaccharides from an unknown number of different glycoproteins was examined in these studies, rather than only two glycoproteins or a single purified glycoprotein as in this current study. This difference may provide an explanation of the conflicting observations. Growth-dependent changes in different oligosaccharide quantity tend to be obscured in mixtures of glycoproteins, but when purified, true variation is readily discernible. Both glycoproteins display a strikingly different array of oligomannosyl glycopeptides regardless of cell density because E1 and E2 contained predominantly $Man_5GlcNAc$ and $Man_7GlcNAc$, respectively.

It is quite curious that the glycosylation products of SbV glycoproteins are so different. PE2 (precursor to E2) and E1 are sequentially translated (after core protein) by a single species of 26S RNA containing a single initiation site. Once PE2 is translated, nascent E1 is cleaved from it and then inserted into the membrane by some unknown mechanism possibly in the same membrane compartment (Bonatti et al., 1979; Wirth et al., 1977, 1979). Glycosylation is also occurring during translation (Sefton, 1977; Katz et al., 1977; Toneguzzo & Ghosh, 1977; Rothman & Lodish, 1977) by an en bloc transfer of a presumably single lipid oligosaccharide intermediate species, $Glc_3Man_9GlcNAc_2$, onto protein. The oligosaccharide is subsequently processed (or trimmed) to yield both complex and oligomannosyl type glycopeptides (Robbins et al., 1977; Hunt et al., 1978; Tabas et al., 1978).

How are two glycoproteins, presumably in the same compartment, processed so differently with respect to their carbohydrate? Are the two proteins actually translated and/or glycosylated in separate compartments? This would at least require the ability of different compartments to package varying amounts of different specific α-mannosidases.

Alternatively, protein configuration could dictate the degree of processing of the oligosaccharide, or an oligosaccharide structure could affect the structure to which it is attached. Such interactions may play some role in oligosaccharide processing and synthesis.

Either explanation (conformation or compartmentation) does not give insight into the heterogeneous expression of these glycopeptides. This complex interconnection between protein translation and glycosylation still remains to be studied.

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