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Glycosylation of Intracellular Sindbis Virus Glycoproteins†

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ABSTRACT: Oligosaccharides of purified intracellular Sindbis virus glycoproteins have been examined by high-resolution Bio-Gel chromatography. The array of glycopeptides from cellular E1 and E2 appeared similar to the glycopeptides (S1, S2, S3, and S4) found in mature virus glycoproteins described previously [Hakimi, J., & Atkinson, P. H. (1980) *Biochemistry 19*, 5619]. However, compared to its viral counterpart, intracellular E1 glycoprotein also contained larger sized mannosyl oligosaccharides. B and PE2 proteins were found to contain an array of primarily large mannosyl oligosaccharides (8–10 hexose units). No sialyl glycopeptides were found on these proteins regardless of labeling time. By contrast, the products of PE2 cleavage (E2 and E3) contained sialyl glycopeptides similar to those found in mature virus (S1, S2, and S3). E2 also contained smaller mannosyl oligosaccharides (8–5 hexose units) similar to its viral counterpart. Current evidence shows that sialyl and galactosyl transferases are in or near the Golgi region. Thus we conclude that cleavage of PE2 with a Man_8 oligosaccharide structure occurs in the Golgi region and not in the plasma membrane as suggested by others.

Sindbis virus (SbV), a membrane-maturing virus, contains a nonglycosylated nucleocapsid protein (Core) and two structural enveloped glycoproteins (E1 and E2). Chick embryo fibroblasts (CEF) infected with SbV contain six viral structural proteins and their precursors: B, PE2, E1, E2, and Core proteins and a 6000-dalton nonglycosylated polypeptide (6K) which are all translated from a single species of 26S RNA.

The precursor protein PE2 is cleaved prior to virus maturation forming E2 and E3 (or 9.8K) glycoproteins (Schlesinger & Schlesinger, 1972, 1973; Welch & Sefton, 1979). The latter is found in the culture media of SbV infected cells and is analogous to E3 glycoprotein which is associated with Semliki Forest virus (Welch & Sefton, 1979, 1980; Garoff et al., 1974, 1980; Garoff & Soderlund, 1978). The oligosaccharide structures of mature SbV glycoproteins E1 and E2 have been partially characterized (Burge & Strauss, 1970; Sefton & Keegstra, 1974; Burke, 1976; Burke & Keegstra, 1979) and have been shown to contain both sialyl (S1, S2, and S3) and a mixture of oligomannosyl (S4) glycopeptides. The viral S4 glycopeptides have been shown by the use of endoglycosidases to consist of four distinct oligomannosyl chains ranging from Man_8 to Man_5 (Hakimi & Atkinson, 1980; Hakimi et al., 1981). The intracellular glycopeptides of E1, E2, and PE2 have also been partially but not completely characterized (Sefton & Keegstra, 1974). B protein has been previously reported to be a nonstructural and nonglycosylated polypeptide containing within it both E1 and PE2 (Sefton & Burge, 1973; Schlesinger & Schlesinger, 1973). There is also evidence that B protein has a glycosylated form (Lodish, 1980; Welch & Sefton, 1979). To our knowledge no characterization of the

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glycopeptides of SbV B and E3 glycoproteins has been reported.

In this study, we characterize the oligosaccharides of intracellular-derived B, PE2, E1, E2, and E3 (found released in the medium) in order to establish glycosylation patterns during the posttranslational processing of these proteins. The sialyl glycopeptides S1, S2, and S3 and the six major S4 oligosaccharide products of endo-β-*N*-acetylglucosaminidase-H digestion can be separated on Bio-Gel P-4. Only a glycosylated form of B protein from CEF was found which contained large-sized mannosyl oligosaccharides essentially identical with those found in PE2. E3 glycopeptides have been characterized by high-voltage paper electrophoresis and gel filtration. E3 contains only sialylated glycopeptides quite similar to those found associated with mature viral glycoproteins. Since E2 and E3 contain sialyl glycopeptides and are cleavage products of PE2 which contains only mannosyl oligosaccharides, we conclude that PE2 cleavage occurs before association with the plasma membrane in or near the Golgi region where final processing events of glycosylation occur.

Materials and Methods

Cells and Virus. Primary chick embryo fibroblasts and SbV were prepared as previously described (Hakimi & Atkinson, 1980). Cells were grown in plastic tissue culture flasks having 75-cm^2 growth area (Falcon).

Labeling of Infected Cells. Growing CEF were infected with SbV at 300 pfu/cell in 2 mL of serum-free medium containing 3 μg/mL actinomycin D (Calbiochem) at 37 °C. One hour after infection, 16 mL of medium containing 5% fetal calf serum (GIBCO) and 3 μg/mL actinomycin D was added, and the cultures were incubated for an additional 7 h at 37 °C. At this time cells were washed and then incubated with 2.5 mL of medium containing 3 μg/mL actinomycin D, 12.5–25 μCi of ^{14}C-labeled amino acid mix (New England Nuclear), and 125 μCi of D-[2-^{3}H]mannose (Amersham; 2 Ci/mmol). The cultures were incubated up to 5.5 h on a slow moving rocker platform allowing the minimal nutrient volume. Labeling was terminated by the addition of 0.88 mL of concentrated electrophoresis sample buffer (4-fold) to the culture medium as described previously (Welch & Sefton, 1979). The concentrated buffer consisted of 8% sodium dodecyl sulfate ($NaDodSO_4$) (Pierce), 4% β-mercaptoethanol (Sigma), 40% glycerol, 0.4 M dithiothreitol (Sigma), 20 mM sodium phosphate (pH 7) and the proteolytic inhibitors, 8 mM phenylmethanesulfonyl fluoride (Sigma), 4 mM benzamidine (Sigma), 20 mM ε-aminocaproic acid (Calbiochem), 400 μg/mL soybean trypsin inhibitor (Sigma), and 10 units/mL Trasylol (FBA Pharmaceuticals). The solubilized cultures were forced through a 25-gauge needle 3 times, boiled for 5 min and frozen at −70 °C until further analysis.

Purification of Viral Glycoproteins. B, PE2, E1, E2, Core, E3, and 6K proteins were separated by electrophoresis on a 10–15% gradient $NaDodSO_4$–polyacrylamide slab gel as previously described (Hakimi & Atkinson, 1980; Atkinson et al., 1981). The proteins were localized by autoradiography and cut from the gel.

Preparation and Gel Filtration of Glycopeptides. Gel pieces were swollen in 0.15 M Tris-HCl (pH 8) and 0.2 M $CaCl_2$ containing 5 mg/mL Pronase (Calbiochem) and incubated under a toluene atmosphere at 37 °C for 12 h. Additional Pronase was added at 12, 24, and 36 h. Digestion was terminated by boiling samples for 5 min in a water bath. The gel pieces were centrifuged (650*g*, 10 min) and washed 3 times with hot water. The supernatants were combined, lyophilized, and then desalted by Sephadex G-25 chromatography in water (0.9 × 140 cm). Samples were redigested with Pronase as described previously (Muramatsu et al., 1973) and again desalted by Sephadex G-25 chromatography. No amino acid ^{14}C label appeared in the [^{3}H]mannose glycopeptide fractions.

Glycopeptides and oligosaccharides were chromatographed on either Bio-Gel P-6 (Bio-Rad, 200–400 mesh, 0.9 × 175 cm) or Bio-Gel P-4 (−400 mesh, 1.5 × 175 cm) and eluted with 0.1 M NH_4HCO_3. Fraction volumes were approximately 0.55 and 1.22 mL, respectively. On the Bio-Gel P-4 column, sialylated and mannosyl glycopeptides eluted between fractions 95–115 and fractions 125–145, respectively, and endo-β-*N*-acetylglucosaminidase-H-released oligosaccharides appeared between fractions 145 and 185 (Figures 4–7). Bio-Gel P-2 (−400 mesh, 0.9 × 175 cm) chromatography was performed in 1 mM NaN_3, and 0.56-mL fractions were collected.

Enzyme Sources and Incubation Conditions. Endo-β-*N*-acetylglucosaminidase-H (endo-H) from *Streptomyces griseus* was prepared by the method of Tarentino & Maley (1974). Endo-β-*N*-acetylglucosaminidase-D (endo-D) from *Diplococcus pneumonae* was prepared according to the method of Koide & Muramatsu (1974). Endoglycosidase digestions of glycopeptides were performed as previously described (Muramatsu et al., 1976). α-Mannosidase from jack bean meal (Sigma) was prepared and used according to the method of Snaith & Levvy (1968). Neuraminidase (*Vibrio cholerae*) (Grand Island Biological Co.) was used as described previously (Moyer et al., 1976).

High-Voltage Paper Electrophoresis. Hydrolysis of E3 glycopeptides was performed in 0.1 N H_2SO_4 at 80 °C for 30 min prior to electrophoresis. Paper electrophoresis was carried out on a 1 in. wide strip of Whatman No. 1 paper at pH 1.9 (2.5% formic acid, 7.8% acetic acid) or at pH 6.5 (pyridine/acetic acid/H_2O, 100:4:896) at 3500 V for 3 h (Muramatsu et al., 1973).

Results

SbV-Infected CEF Proteins. SbV-infected CEF were labeled with [^{3}H]mannose and ^{14}C-labeled amino acids (mixture). Cellular viral proteins were solubilized by adding an $NaDodSO_4$ sample buffer containing a mixture of proteolytic inhibitors directly to the cell monolayer and incubation medium. Intracellular structural (E1, E2, and Core) and nonstructural (B, PE2, and E3) proteins were well separated by $NaDodSO_4$–polyacrylamide gel electrophoresis and identified by autoradiography (Figure 1A). Separation of purified virus proteins is shown in Figure 1B. E3 is poorly labeled and barely detectable by autoradiography under these conditions. Longer exposure times are required to visualize the small polypeptide. Core and 6K proteins could not be labeled with [^{3}H]mannose. When ^{14}C-labeled amino acids were used, a lower molecular weight form of B protein was not evident, thus suggesting that nonglycosylated B protein must be a minor component in CEF under these experimental conditions. When culture medium was clarified by centrifugation (8000*g*) for 30 min at 4 °C to remove cells and debris and its solubilized proteins were separated by $NaDodSO_4$ gel electrophoresis, only E1, E2, Core, and E3 proteins could be detected by autoradiography (data not shown). This confirmed previous studies (Welch & Sefton, 1979) that show radiolabeled virus and E3 are released into the culture medium following a 2-h pulse period. Pulse-labeling studies confirmed that labeled viral glycoproteins began to appear in the medium at approximately 90 min (data not shown).

Characterization of Cell-Derived SbV Glycopeptides. High-voltage paper electrophoresis of the [^{3}H]mannose-labeled E3 glycopeptides derived from cells and media was performed

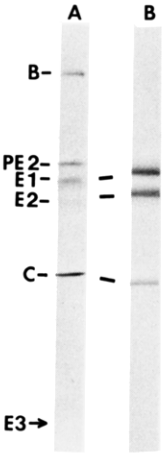

FIGURE 1: Autoradiography of preparative $NaDodSO_4$–polyacrylamide gel electrophoresis of (A) SbV-infected CEF and (B) purified virus. CEF and virus were labeled with ^{14}C-labeled amino acid and [^3H]-mannose for 2 h and their proteins separated on a 10–15% gradient slab gel which was then dried on Whatman 3MM paper. The figure is a composite of two fluorograms of two different preparative slab gels used to isolate viral derived polypeptides. Arrow indicates position where E3 is found after longer exposure times are used.

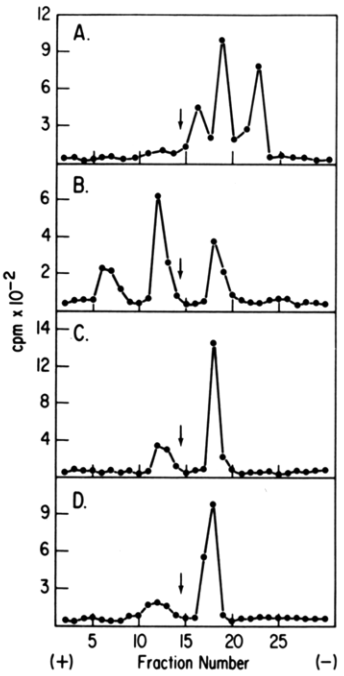

FIGURE 2: High-voltage paper electrophoresis of [^3H]mannose SbV E3 glycopeptides. Glycopeptides were purified on Bio-Gel P-6, pooled, lyophilized, and dissolved in water. Samples were analyzed by paper electrophoresis for 3 h at 3500 V (A) at pH 1.9 or (B) at pH 6.5. Another sample was (C) digested with neuraminidase or (D) hydrolyzed with H_2SO_4 as described and analyzed at pH 6.5. The origin is between fractions (1.5-cm pieces) 14 and 15 as indicated by the arrow.

(Figure 2). The amphoteric behavior of this material at pH 1.9 and pH 6.5 (Figure 2A,B) confirmed that it is glycopeptide in nature. Three glycopeptides were observed upon electrophoresis at each pH. This can be attributed to their heterogeneity with respect to sialic acid content, since neuraminidase and mild acid treatments eliminated most of the acidic behavior at pH 6.5 (Figure 2C,D). The resistant material (at fraction 15) may have been due to incomplete removal of sialic acid. However, at this pH, this electrophoretic behavior could also be consistent with the presence of extra acidic amino acids not removed by Pronase in the peptide moiety. So that it could be determined whether E3 glycopeptides were similar to SbV S1, S2, and S3 glycopeptides, [^3H]mannose-labeled E3 glycopeptides were mixed with [^{14}C]mannose-labeled SbV S1, S2, and S3 glycopeptides, redigested with Pronase, and then cochromatographed on Bio-Gel P-6 (Figure 3). Though S1 glycopeptides coeluted, the E3, S2- and S3-like glycopeptides appeared smaller than SbV S2 and S3 glycopeptides. This may arise from differential Pronase susceptibilities of the two proteins due to different amino acid composition. The glycopeptides were again combined, but digested with endo-D and fractionated on a calibrated Bio-Gel P-2 column (Figure 3B). Endo-D released a major oligosaccharide from both [^3H]- and [^{14}C]mannose-labeled material whose apparent molecular weight corresponded to that of core $Man_3GlcNAc$. The E3 derived material, however, produced an oligosaccharide which did not coelute with any SbV (E1 and E2) derived oligosaccharide, suggesting possible slight differences in oligosaccharide side chains of E3 complex type glycopeptides.

Glycosylation patterns of B, PE2, E1, and E2 were established at 1.5, 2 (data not shown), 3.5 and 5.5 h after [^3H]-mannose was added to infected CEF (Figures 4–7). Glycosylation of all four proteins appeared to reach steady-state labeling between 2 and 3 h. In B and PE2, no sialylated glycopeptides were detected even at 5.5 h while E1 contained sialyl glycopeptides at 1.5 h. Essentially all B and PE2 glycopeptides were endo-H sensitive, yielding an array of three major mannosyl oligosaccharides (Figures 4 and 5) primarily larger than the S4 oligosaccharides derived from SbV glycoproteins (Hakimi & Atkinson, 1980). Relatively short-term (1.5 h) labeled B protein contains $Man_7GlcNAc$, $Man_8GlcNAc$, $Man_9GlcNAc$, $hexose_1Man_9GlcNAc$, and $hexose_2Man_9GlcNAc$. In this array, $Man_9GlcNAc$ and $hexose_1Man_9GlcNAc$ predominate (Figure 4). However, at 5.5 h of labeling, $Man_9GlcNAc$ and $Man_8GlcNAc$ predominate with the appearance of small quantities of $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$.

The molecular weights of the three major PE2 oligosaccharides were determined on a calibrated Bio-Gel column (data not shown) which corresponded to $GlcMan_9GlcNAc$, $Man_9GlcNAc$, and $Man_8GlcNAc$. These species corresponded to the identified early intermediates (20–25 min) of oligosaccharide processing (Sefton, 1977; Kornfeld et al., 1978; Hubbard & Robbins, 1979). Very little $Man_5GlcNAc$, $Man_6GlcNAc$, and $Man_7GlcNAc$ oligosaccharides were found in PE2. The PE2 oligosaccharides were further characterized by identification of their α-mannosidase digestion products on a calibrated Bio-Gel P-4 column. $Man_9GlcNAc$ and $Man_8GlcNAc$ yielded two major products which coeluted with authentic mannose and $Man\beta GlcNAc$ (data not shown). Putative $GlcMan_9GlcNAc$, however, yielded an array of partially digested products (Figure 8), corresponding to the loss of up to five mannose residues and free mannose. No $Man\beta GlcNAc$ was detected. This is consistent with a structure of $GlcMan_9GlcNAc$ as reported by others.

Both E1 and E2 cellular-derived glycopeptides contain sialyl and mannosyl type oligosaccharides (Figures 6 and 7). The appearance of sialyl glycopeptides, S1, on E1 occurs sooner than on E2, and the arrays of mannosyl oligosaccharides on these two glycoproteins are quite different. E2 mannosyl oligosaccharides predominate in a $Man_7GlcNAc$ species with relatively little $Man_8GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ oligosaccharides (Figure 6). A similar situation was found in viral E2 glycopeptides harvested from rapidly growing cells (Hakimi & Atkinson, 1980). The endo-H-resistant material appearing after S3 requires further charac-

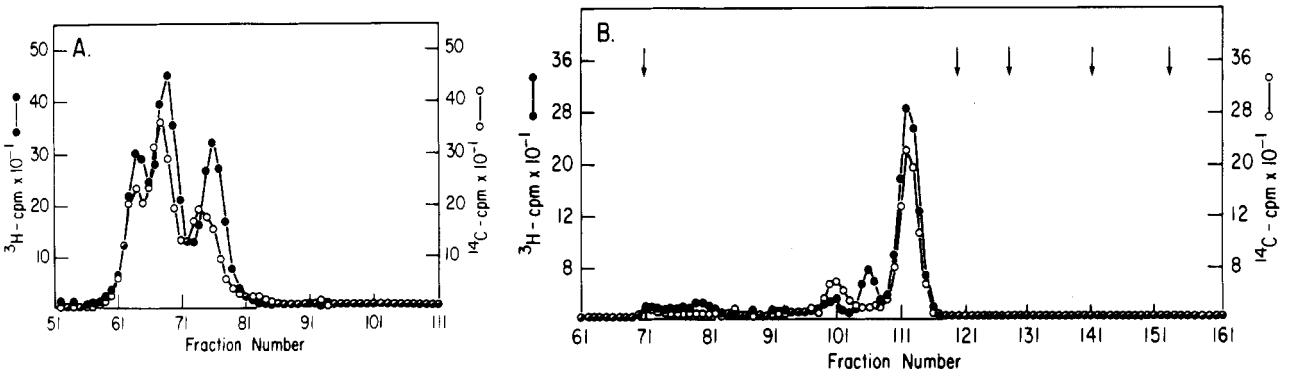

FIGURE 3: Characterization of E3 glycopeptides and its endo-D-released oligosaccharides by Bio-Gel chromatography. [^{14}C]Mannose SbV S1, S2, and S3 glycopeptides were mixed with [^{3}H]mannose E3 glycopeptides (A) redigested with Pronase and fractionated on Bio-Gel P-6 or (B) treated with endo-D and fractionated on Bio-Gel P-2. The arrows from left to right indicate the peak elution fractions of BSA (Vo), stachyose, raffinose, sucrose, and mannose (Vi) which were coeluted with the endo-D products on Bio-Gel P-2 [see Hakimi & Atkinson (1981)].

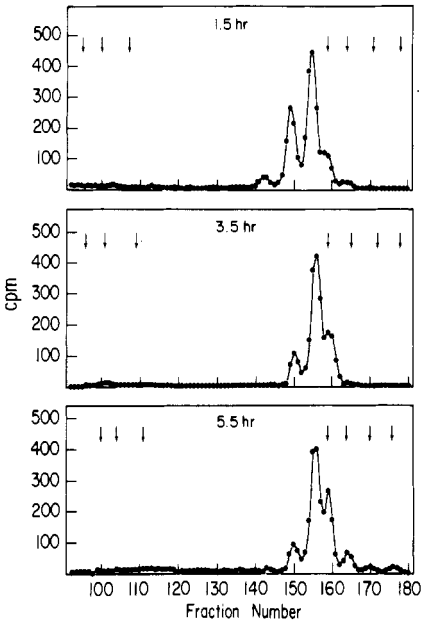

FIGURE 4: Bio-Gel P-4 chromatography of B protein glycopeptides and endo-H-released oligosaccharides. SbV-infected CEF were labeled with [^{3}H]mannose for 1.5, 3.5, and 5.5 h. Viral glycoproteins were purified by NaDodSO$_4$–polyacrylamide gel electrophoresis. Glycopeptides were prepared as described under Materials and Methods, mixed with [^{14}C]mannose SbV glycopeptides, treated with endo-H, and chromatographed on Bio-Gel P-4. The arrows from left to right indicate the peak elution fractions of SbV S1, S2, S3, and S4B (Man$_8$GlcNAc), S4C (Man$_7$GlcNAc), S4D (Man$_6$GlcNAc), and S4E (Man$_5$GlcNAc) [see Hakimi & Atkinson (1980)].

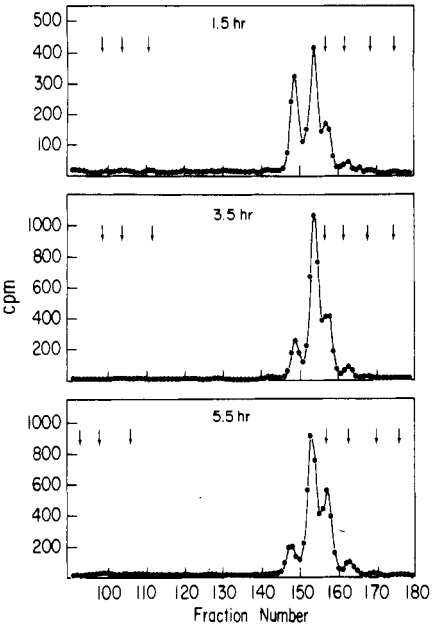

FIGURE 5: Bio-Gel P-4 chromatography of PE2 protein glycopeptides and endo-H-released oligosaccharides. See legend to Figure 4 for details.

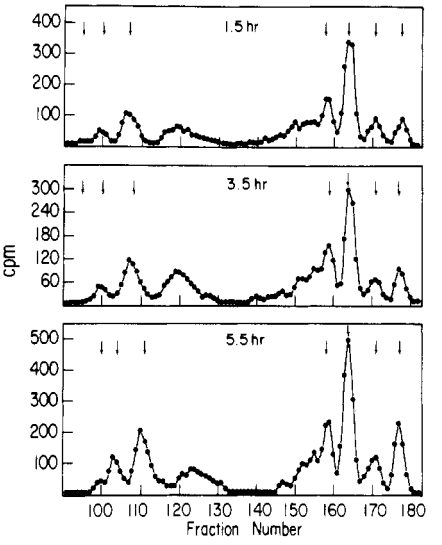

FIGURE 6: Bio-Gel P-4 chromatography of E2 protein glycopeptides and endo-H-released oligosaccharides. See legend to Figure 4 for details.

terization but may be galactose-poor S3. E1 glycopeptides on the other hand contained the larger sized mannosyl oligosaccharide species found in B and PE2 as well as the smaller sized oligosaccharide species found in E2 and viral E1 glycopeptides.

Discussion

We have characterized glycosylation of Sindbis virus glycoproteins at various stages of posttranslational processing. B protein appears to be processed by α-mannosidases, as do other glycoproteins (Robbins et al., 1977; Hunt et al., 1978; Tabas et al., 1978). However, it can exist in two forms, one a glycosylated form and membrane associated and the other nonglycosylated. The latter is probably an abortive form (Kaluza, 1975). If the glycosylated form were a precursor to PE2–6K–E2, cleavage of this polyprotein would be posttranslational. The literature conflicts as to when cleavages occur. While there is good evidence that cleavage is posttranslational in vivo (Clegg, 1975; Soderlund, 1976), an in vitro study concluded the proteins are cleaved cotranslationally (Garoff et al., 1978). In a recent paper, it has been concluded that cleavage of 6K from PE2 and E1 occurs late or post-

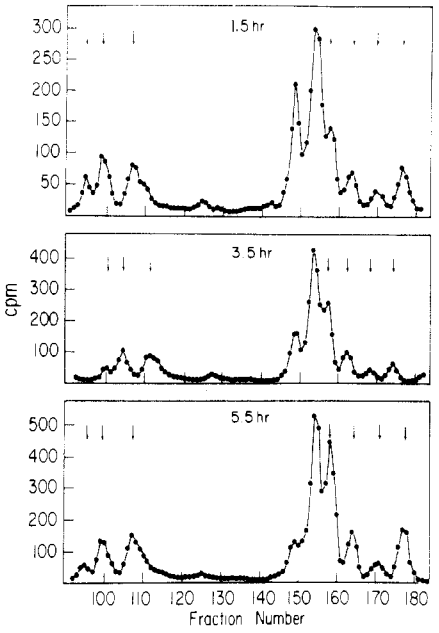

FIGURE 7: Bio-Gel P-4 chromatography of E1 protein glycopeptides and endo-H-released oligosaccharides. See legend to Figure 4 for details.

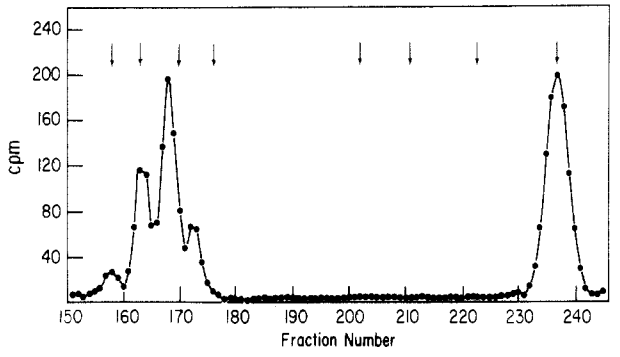

FIGURE 8: Bio-Gel P-4 chromatography of α-mannosidase digestion products of putative $GlcMan_9GlcNAc$. Endo-H-released PE2 oligosaccharides were fractionated on Bio-Gel P-4 as shown in Figure 5. The first peak was pooled, lyophilized, and treated with α-mannosidase. The sample was boiled and mixed with endo-H-treated [^{14}C]Man SbV glycopeptides and chromatographed on Bio-Gel P-4. The arrows from left to right indicate the peak elution fraction of SbV mannosyl oligosaccharides S4B, S4C, S4D, and S4E (see legend to Figure 4 for details), stachyose, raffinose, sucrose, and mannose. BSA and SbV glycopeptides S1, S2, and S3 eluted in fractions 86, 95, 99, and 108, respectively. ManβGlcNAc, if present, would appear between stachyose and raffinose.

translationally based on the distinctly later appearance of PE2 after E1 [Welch et al., 1981; reviewed by Sabatini et al. (1982)].

The major high mannose species on intracellular E1 is Man_9 whereas it is Man_5 in its viral counterpart (Hakimi & Atkinson, 1980). This may be a reflection of a comparatively large pool of precursor E1 resembling PE2 in glycosylation as previously suggested (Sefton & Burge, 1973; Sefton & Keegstra, 1974; Sefton, 1977; Robbins et al., 1977). It would be of interest to know if this form corresponds in glycosylation to PE1 (Cancedda et al., 1981).

In this study all the PE2 glycopeptides are endo-H sensitive, yielding an array of oligosaccharides from $GlcMan_9GlcNAc$ to $Man_8GlcNAc$. Its cleavage products, E3 and E2, contain complex-type oligosaccharides. E3 has a single glycosylation site (Rice & Strauss, 1981). We have shown that the carbohydrate is completely processed to complex oligosaccharides. This has been shown for the N-terminal site on E2 (Rice & Strauss, 1981). The second site on E2 contains high mannose structures ($Man_{8-5}GlcNAc$) thus sharing with PE2 a common oligosaccharide, $Man_8GlcNAc$. Our characterization of E3 and PE2 carbohydrate side chains is in agreement with those described for SbV PE2 (Sefton & Keegstra, 1974) and for Semliki Forest virus E3 (Mattila et al., 1976) and P62 [see Kaariainen & Renkonen (1977)]. In other studies (Gottlieb et al., 1979) the presence of sialyl glycopeptides could have been due to contaminating E1 glycoprotein not fully resolved from PE2 on polyacrylamide gels. We surmise most of PE2 arrives in the Golgi region with a $Man_8GlcNAc$ structure after three glucose residues and one mannose residue are removed in a similar fashion described for thyroglobin (Godelaine et al., 1981). Though α(1,2)-mannosidases active in processing have been found in a Golgi-rich subcellular fraction (Tabas & Kornfeld, 1979), one cannot exclude the possibility that another α(1,2)-mannosidase is present in the rough endoplasmic reticulum. Addition of galactose, fucose (Neutra & Leblond, 1966; Bennett et al., 1974), and sialic acid (Bennett & O'Shaughnessy, 1981; Bretz et al., 1980) occurs in or near the Golgi region. In addition, at the E3–E2 cleavage site, a pair of dibasic amino acid residues occurs (Garoff et al., 1980; Rice & Strauss, 1981) which are also found in many proproteins that are proteolytically processed by Golgi associated proteases (Steiner et al., 1980). We conclude PE2, containing Man_8 high mannose oligosaccharides, must be cleaved in the Golgi region yielding E2 and E3 which are then also processed to sialoglycopeptides. This cleavage can be inhibited by tunicamycin (Leavitt et al., 1977; Wirth et al., 1979). Thus it is apparent that specific changes of oligosaccharide chain lengths and composition are closely linked to translation and proteolytic processing of glycoproteins.

In other studies it has been inferred that PE2 is cleaved on the cell surface. Several lines of evidence supported this view. For example, cleavage is inhibited by antiserum against E1 in intact cells presumably by affecting an E1–PE2 complex (Ziemiecki et al., 1980; Bracha & Schlesinger, 1976; Jones et al., 1977). However, the possibility that the effect was due to ingested antibody was not excluded. Also other laboratories have published evidence which is inconsistent with the formation of PE2/E2 and E1 complexes (Scheefers et al., 1980). Presence of PE2 in plasma membrane was inferred, in cell fractionation studies (Erwin & Brown, 1980; Scheefers et al., 1980). However, plasma membrane fractions containing PE2 were not demonstrated to be free of cross-contaminating rough and smooth endoplasmic reticulum. Through the use of a cell surface lactoperoxidase labeling procedure, only E1 and E2 from intact infected cells could be radioiodinated (Sefton et al., 1973), which is inconsistent with PE2 on the cell surface.

Comparisons of ovalbumin microheterogeneity in individual eggs have suggested that there may be a control mechanism which regulates the degree of processing of carbohydrate chains (Iwase et al., 1981). There may be such a regulation in the display of specific sets of high mannose arrays in SbV E1 and E2 which in turn may be involved in the establishment and preservation of specific protein conformations (Gibson et al., 1981; Kaluza et al., 1980). The occurrence of high mannose side chain glycosylation in mature glycoproteins occurs more distally from the N terminus than complex-type oligosaccharides. The more processed complex oligosaccharides usually do not occur to the C-terminal side of a site substituted with high mannose substances (L. Pollack and P. Atkinson, unpublished results). Thus differences in the orientation and location of oligosaccharide side chains with respect to primary protein structure and secondary structural features (Beely, 1976, 1977; Aubert & Loucheux-Lefebvre,

1976, 1981; Aubert et al., 1976; Rosner et al., 1980) may be important in the degree of their expression.

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