Heterogeneity of Sindbis Virus Glycoprotein E₁ and its Modification by Host Cell Transformation

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SUMMARY

The electrophoretic properties of glycoprotein E₁ of Sindbis virus grown in Rous sarcoma virus-transformed cells were compared with those of Sindbis virus grown in untransformed cells. Isoelectric focusing in a gel containing 9.5 M-urea and 2% Nonidet P40 indicated that the glycoprotein E₁ of Sindbis virus from the untransformed cells was electrochemically heterogeneous and consisted of four components, whose isoelectric points (pis) ranged from 6.2 to 6.7; the E₁ of Sindbis virus from the transformed cells contained an additional component with a pi of 6.1. After treatment with neuraminidase the observed charge heterogeneity disappeared regardless of whether the virus had come from the transformed or the untransformed cells. The five components were digested with Pronase and compared by gel filtration on Bio-Gel P-6; the four components with relatively higher pis released high-mannose-type and complex-type oligosaccharides which differed in their content of sialic acid. In contrast, the pi 6.1 component, which existed only in the transformed cell-derived SV glycoprotein E₁, released only complex-type oligosaccharide. These results indicate that the properties of glycoprotein E₁ derived from both cell types are basically similar but that a small proportion of the E₁ molecules from the transformed cells, with carbohydrate chains elongated by attachment of sialic acid residues, lacks simple-type oligosaccharides either because they are not attached or because they have been processed to the complex type. Glycoprotein E₂ appeared as at least three components in non-equilibrium pH gradient electrophoresis. However, no significant difference was observed between the cell types.

INTRODUCTION

Enveloped RNA viruses mature at the host cell membrane and their envelopes are known to be derived from pre-existing cellular membranes which are modified by the infection. The envelope of a virus that infects a transformed cell retains some properties of the transformed cell membrane (Forchhammer & Turnock, 1978; Lai & Duesberg, 1972; Miki & Kuwata, 1976; Miki, 1980; Moyer & Summers, 1974). In this study Sindbis virus, one of the better characterized enveloped RNA viruses, was used as a model to study modifications of the cell membrane by host cell transformation. The envelope of Sindbis virus contains two glycoproteins designated E₁ and E₂ which have similar molecular weights (Schlesinger et al., 1972). They have been isolated at isoelectric points (pis) 6 and 9, respectively, by isoelectric focusing in sucrose density gradients (Dalrymple et al., 1976). Pronase digestion of Sindbis virus glycoprotein E₁ yielded four size classes of glycopeptides designated S₁, S₂, S₃ and S₄ (Sefton & Keegstra, 1974). S₁, S₂ and S₃ are complex-type glycopeptides; S₃ does not contain sialic acid whereas one or two residues have been demonstrated in S₂ and S₁, respectively (Keegstra et al., 1975). The smallest glycopeptide, S₄, is of the high-mannose-type (Sefton, 1976). Experimental results reported in this paper indicate that Sindbis virus glycoprotein E₁ is electrochemically heterogeneous and strongly suggest that modification of envelope glycoprotein E₁ by host cell transformation does not occur evenly in all glycoprotein molecules; rather, it is restricted to a small proportion of
molecules. The modification involves not only increased attachment of sialic acid residues but also lack of the high-mannose-type oligosaccharides.

**METHODS**

**Viruses and cells.** Wild-type Sindbis virus was provided by Dr B. Shimizu (National Institute of Health, Tokyo, Japan). The non-defective Schmidt-Ruppin strain of Rous sarcoma virus (RSV), subgroup A (nd RSV) and its non-conditional transformation-defective mutant (td RSV) (Kawai et al., 1977) were provided by Dr S. Kawai (The University of Tokyo). White Leghorn resistance-inducing factor-free, gs chick embryos (C/O, C/E) were obtained from the Nippon Institute for Biological Science, Oume, Tokyo. Primary cultures of chick embryo fibroblasts (CEF) were prepared from 10- to 11-day-old embryos according to the method of Rubin (1960). About 4 × 10⁶ cells were plated per 100 mm plastic plate (Falcon) in 10 ml Eagle’s minimal essential medium (MEM) supplemented with 5% calf serum. One day later, the medium was removed and subconfluent monolayers were infected with Sindbis virus at a multiplicity of infection of about 100 in 1 ml MEM containing 2% calf serum. After adsorption for 1 h the cultures were incubated with 10 ml MEM containing 2% calf serum and the media were harvested at 16 h post-infection. Cells were plated before infection at about 2 × 10⁶ per plate. Three h after seeding, the medium was removed and the cells were infected with 1 ml of stock RSV.

**Radioactive labelling of virus.** Radioactive mannose [1-14C]mannose (59 mCi/mmole, Amersham International) at 2-5 μCi/ml; [2-3H]mannose (10 to 20 Ci/mmol, New England Nuclear) at 20 μCi/ml was added 3 h after Sindbis virus infection in MEM containing 1% calf serum and 5 mM-fructose in place of glucose. [3,4,5-3H]-Leucine (50 Ci/mmol, Commissariat a l’Énergie Atomique, Saclay, France) was added 5 h post-infection in 20 μCi/ml in MEM lacking leucine and supplemented with 1% calf serum. The labelling media were harvested at 16 h post-infection (Kaluza & Pauli, 1980). To label the virus with 32P, cells prelabelled for 2 h with 32P (carrier-free orthophosphate, Japan Atomic Energy Research Institute, Ibaraki, Japan) at 20 μCi/ml in phosphate-free MEM containing 10% calf serum were infected with Sindbis virus in 1 ml MEM containing 2% calf serum. After adsorption for 1 h the culture was incubated with 5 ml 32P-containing, phosphate-free MEM at 20 μCi/ml. After 2 h post-infection, 5 ml MEM (not phosphate-free) containing 5% calf serum was added. The labelled media were harvested at 16 h post-infection.

**Virus purification.** Cells and cell debris were removed by centrifugation at 5000 g for 15 min. Polyethylene glycol 6000 and NaCl were added to the supernatant to final concentrations of 10 and 3% (w/v), respectively. The solution was stirred for 60 min (David, 1971) and the virus was collected by centrifugation at 12000 g for 15 min. The pellet was suspended in TEN buffer (10 mM-Tris-HCl pH 7-4, 1 mM-EDTA, 0.2 M-NaCl), homogenized with a Teflon tissue grinder after storage overnight at 4 °C, and centrifuged at 12000 g for 15 min. The supernatant was layered onto a 2 ml cushion of 20% (w/w) glycerol (Moore et al., 1976) in TEN buffer and centrifuged at 25000 rev/min in a Spincò SW27.1 rotor for 90 min at 4 °C. The pellet was covered with 1 ml of TEN buffer, and clarified after storage overnight at 4 °C by centrifugation at 12000 g for 15 min. The supernatant was layered onto a 30 ml linear 0 to 40% (w/w) potassium tetratrate (KT)-30 to 0% (w/w) glycerol (GLY) density gradient (Obijski et al., 1974) in TEN buffer, and centrifuged in a Spincò SW27.1 rotor at 27000 rev/min for 16 h at 4 °C. The virus band at a density of 1.18 to 1.20 g/ml was collected, diluted threefold with TEN buffer, and pelleted at 25000 rev/min in a Spincò SW27.1 rotor and suspended in NP buffer [1% Nonidet P40 (NP40), 0.01 M-Tris-HCl pH 7.5, 0.2 M-NaCl] for glycoprotein preparation.

**Glycoprotein preparation.** Purified virus was treated with NP buffer at 37 °C for 30 min, then centrifuged at 35090 rev/min in a Spincò type 40 rotor for 60 min at 4 °C. After the addition of 250 μg bovine serum albumin, the supernatant was shaken with 2 vol. n-butanol for 1 min using an Omni mixer (Etchison et al., 1977). The aqueous phase was separated from the butanol phase by centrifugation at 2000 g for 2 min. Nine vol. ethanol was added to the aqueous phase, and the precipitated glycoprotein was collected after storage overnight at −20 °C by centrifugation at 3000 g for 20 min. It was resuspended in an appropriate buffer or stored in 95% ethanol at −70 °C.

**SDS-polyacrylamide gel electrophoresis.** Gels were prepared by a modified Laemmli method (Selton & Burge, 1973), the ratio of acrylamide to methylene bisacrylamide being 30:1.6. The resolving gel was 7-5% acrylamide containing 0.188 M-Tris-HCl pH 8.8, and the stacking gel contained 0.062 M-Tris-HCl pH 6.8. Samples were heated to 100 °C for 2 min in the sample buffer (Takács, 1979) with SDS and 2-mercaptoethanol, and were electrophoresed for 3 h at 150 V. After the run, the gel was frozen and fractionated by a parallel array of razor blades to obtain approx. 1 mm slices. The radioactivity of each slice was measured in a liquid scintillation counter after overnight incubation in 3.5 ml of scintillation mixture (143 ml of NCS tissue solubilizer, 3.73 litre PPO) at 37 °C (Takács, 1979).

**Gel isoelectric focusing (IEF).** Gels were prepared in 0.5 × 12 cm glass tubes by O’Farrell’s method (O’Farrell, 1975) with a slight modification. The glycoprotein was dissolved in 50 μl of IEF sample buffer (9.5 M-urea, 2% NP40, 2% Ampholine pH 3-5 to 10, 5% 2-mercaptoethanol), and electrofocused through a polyacrylamide gel containing 9.5 M-urea, 2% NP40 and 2% Ampholine, pH range 3-5 to 10, at 400 V for 16 h. After the run the gel was frozen and fractionated as described above. Each slice was incubated in 0.5 ml 0.1% SDS overnight at 25 °C.
The radioactivity of 25 µl aliquots of the fractions was determined using a liquid scintillation counter in 3.5 ml of Triton X-100/toluene scintillation solution (Patterson & Greene, 1965), and appropriate fractions were combined. After addition of 250 µg of bovine serum albumin as carrier, the glycoprotein was precipitated with 5 vol. ethanol. After overnight storage at -20 °C the glycoprotein was collected by centrifugation at 3000 g for 20 min and resuspended in an appropriate buffer for use or stored in 95% ethanol at -70 °C. Alternatively, the radioactivity was measured in a liquid scintillation counter after incubation of each slice overnight at 37 °C in 3.5 ml of scintillation mixture containing NCS. The pH was measured in 5-mm sections placed in vials containing 2 ml of degassed water and shaken for 5 to 10 min after replacement of air by nitrogen.

Non-equilibrium pH gradient electrophoresis. [3H]Leucine-labelled Sindbis virus glycoproteins were separated in non-equilibrium pH gradient electrophoresis using a slight modification of the procedure of O’Farrell et al. (1977). The glycoprotein was dissolved in 50 µl of sample buffer (9.5 M urea, 2% NP40, 1.6% pH 9 to 11 Ampholine, 0.4% pH 3.5 to 10 Ampholine, 5% 2-mercaptoethanol), and electrophoresed through a polyacrylamide gel containing 9.5 M-urea, 2% NP40, 1.6% pH 9 to 11 Ampholine, and 0.4% pH 3.5 to 10 Ampholine at 400 V for 5 h. After the run, sliced gels were analysed for radioactivity.

Chromatofocusing. Chromatofocusing of Sindbis virus glycoprotein, labelled with [3H]mannose, was performed with Polybuffer 74 and Polybuffer exchanger, PBE 94 (Pharmacia) according to the recommended procedure with a slight modification. PBE 94 was packed into a column (1.0 x 40 cm) and equilibrated with the starting buffer (0.025 M-imidazole-HCl pH 7.4, 8 M-urea, 1% NP40). Urea and NP40 were added to the eluate to final concentrations of 8 M and 1%, respectively, and its pH was adjusted to pH 4 by adding 0.5 M-HCl. The sample was dissolved in 0.3 ml of the starting buffer containing 5% 2-mercaptoethanol and applied to the column. The column was eluted at a flow rate of 0.06 ml/min and 1 ml fractions were collected. The pH and radioactivity of each fraction were determined. Fractions containing the mannose label in the pH range from 6 to 7 were pooled, concentrated using an Immersible CX-10 (Millipore) after adding 250 µg serum albumin as carrier, and diluted fivefold with TEN buffer. After three cycles of concentration–dilution, the glycoprotein was precipitated with ethanol after treatment with n-butanol. The precipitated glycoprotein was suspended in 0.1 M-Tris–HCl pH 8.0 containing 0.01 M-CaCl2 for Pronase digestion.

Glass wool column chromatography. The glycoproteins E1 and E2 were separated by glass wool column chromatography (Bell et al., 1979). The supernatant after high-speed centrifugation of NP buffer-treated Sindbis virus was dialysed against equilibration buffer (0.1% Triton X-100, 0.05 M-sodium succinate pH 5.5, 0.3 M-NaCl, 1 mM-dithiothreitol), with two or three changes, for 2 days. A column (1.5 x 33 cm) was packed with sheared Pyrex glass wool, washed with 1 litre of 1% SDS in water and equilibrated with 1 litre of equilibration buffer. The dialysed sample was applied to the column. The E1 glycoprotein passed through the column and appeared with the front of the equilibration buffer, while the E2 glycoprotein remained bound; it was eluted with 0.1% Triton X-100, 0.05 M-Tris–HCl pH 8.0, 0.8 M-NaCl, 1 mM-dithiothreitol. One ml fractions were collected at a flow rate of approximately 0.1 ml/min. The E2 glycoprotein peak was further concentrated using a Millipore Immersible CX-10 and diluted fivefold with phosphate-buffered saline. After three cycles of concentration–dilution, the glycoprotein E2 was used to immunize mice.

Anti-E1 serum. Antiserum was produced in B10. BR mice by three subcutaneous injections of the glycoprotein E1 preparations in complete Freund’s adjuvant at intervals of 1 week. Immune serum was harvested 7 days after the last injection.

Immunoprecipitation. The supernatant after high-speed centrifugation of NP buffer-treated Sindbis virus, labelled with [3H]mannose, was diluted tenfold with TN buffer (10 mM-Tris–HCl pH 7.4, 0.2 M-NaCl). Thirty µl of anti-E1 serum was added to 0.3 ml aliquots, and the reaction mixture was incubated for 30 min at 37 °C. One-hundred µl of rabbit anti-mouse immunoglobulin (DAKO, Copenhagen, Denmark) was added to precipitate the immune complex, and the reaction mixture was incubated further for 16 h at 0 °C. The resulting precipitate was washed twice with 2 ml cold TN buffer containing 0.1% NP40 (1000 g, 10 min for each wash) and dissolved in 0.2 ml distilled water. The labelled material was precipitated with 5 vol. ethanol, collected by centrifugation at 3000 g for 20 min after overnight storage at −20 °C, and dissolved in IEF buffer for gel isoelectric focusing. The supernatant was precipitated with ethanol and also analysed by gel IEF.

Neuraminidase treatment. One-tenth of a unit of neuraminidase from Arthrobacter ureafaciens (Nakarai Chemicals, Ltd., Kyoto, Japan) was added to the [3H]mannose-labelled Sindbis virus glycoprotein in 0.3 ml 0.1 M-phosphate buffer pH 5.3, and the reaction was allowed to proceed for 1 h at 37 °C. Neuraminidase-treated samples were precipitated with 5 vol. ethanol, collected at −20 °C by centrifugation at 3000 g for 20 min after overnight storage, and analysed by gel isoelectric focusing.

Mild acid hydrolysis. The Pronase digests were adjusted to pH 1 by addition of 2 M-HCl, and incubated for 30 min at 80 °C. The reaction mixture was analysed by gel filtration on Bio-Gel P-6 after neutralization with 2 M-NaOH (Seflon, 1976).

Pronase digestion. Pronase (Kaken Chemicals, Ltd., Tokyo, Japan) was pre-digested in 0.1 M-Tris–HCl pH 8.0 and 0.01 M-CaCl2 at a concentration of 10 mg/ml at 37 °C for 2 h to destroy any glycosidase activity. Pronase
(500 μg in 50 μl) was added to [3H]mannose-labelled glycoproteins in 0.3 ml 0.1 M-Tris-HCl pH 8.0 containing 0.01 M-CaCl₂, and digestion was carried out for 24 to 30 h at 50 °C. Additional Pronase (250 μg in 25 μl) was added after 8 and 16 h of incubation (Keegstra et al., 1975; Sefton, 1976); 10 μl of toluene was also added to inhibit bacterial growth. The released glycopeptides were analysed by gel filtration on Bio-Gel P-6 or further digested with endoglycosidase H.

Endoglycosidase H digestion. Pronase digests were desalted by gel filtration on a Bio-Gel P-2 column (100 to 200 mesh, 1 x 40 cm, Bio-Rad) equilibrated in 0.1 M-NH₄HCO₃ and by several cycles of lyophilization, dissolving the samples in water each time (Liu et al., 1979). The samples were then treated with 0.01 units of endoglycosidase H (Endo-β-N-acetylglucosaminidase H) (approx. 30 units/mg protein, Seikagaku Kogyo Co., Ltd., Tokyo, Japan) in 0.3 ml 0.1 m-sodium citrate buffer pH 5.5, at 37 °C for 16 h (Rosner et al., 1980). The reaction products were analysed by gel filtration on Bio-Gel P-4.

Gel filtration. A Bio-Gel P-6 column (200 to 400 mesh, 1 x 110 cm, Bio-Rad) and a Bio-Gel P-4 column (minus 400 mesh, 1 x 110 cm, Bio-Rad) were eluted with 0.1 M-Tris-HCl pH 8.0 containing 0.5 mM-sodium azide (Liu et al., 1979). Fractions (0.5 ml) were collected at a flow rate of 0.08 ml/min. Bovine serum albumin (1 mg) and 14C-labelled protein hydrolysate (1 x 10⁶ ct/min) were added to samples as internal standards for the void volume (V₀) and inclusion volume (Vᵢ), respectively. The amount of protein was determined by the Coomassie Brilliant Blue G-250-protein complex assay (Bradford, 1976). The radioactivity was determined by liquid scintillation counting in Triton X-100/toluene.

RESULTS

Electrochemical heterogeneity of Sindbis virus glycoprotein E₁

The glycoprotein of Sindbis virus from nd RSV-transformed cells (SV-nd) was labelled with [3H]mannose and that from td RSV-infected cells (SV-td) with [14C]mannose, and their homogeneity was examined by SDS-polyacrylamide gel electrophoresis. No difference was observed between the electrophoretic patterns of the mannose labels derived from SV-nd and SV-td (Fig. 1). The mannose labels appeared as two peaks corresponding to molecular weights of 52000 and 55000 and no radiolabel appeared at other positions; this indicates that contamination with labelled cellular glycoproteins and avian retrovirus glycoprotein was negligible. The [3H]mannose-labelled glycoprotein of Sindbis virus derived from cells not pre-infected with RSV (SV-C) was analysed by gel isoelectric focusing on a polyacrylamide gel in the

![Fig. 1. SDS–polyacrylamide gel electrophoresis of Sindbis virus glycoproteins. The SV-td glycoprotein and the SV-nd glycoprotein labelled with [14C]mannose and [3H]mannose, respectively, were analysed by electrophoresis using a modified Laemmli method. Phosphorylase b (mol. wt. 94000), albumin (67000) and ovalbumin (43000) were run on the same gel as internal markers. After electrophoresis the gel was sliced into 1 mm fractions and counted, in a scintillation fluid containing NCS tissue solubilizer, after overnight incubation. ○, SV-nd glycoprotein; ●, SV-td glycoprotein.](image-url)
Sindbis glycoprotein modified by transformation

presence of 2% NP40 and 9.5 M-urea, thus excluding the possibility of glycoprotein aggregation. The mannose radiolabels were separated into two groups, one with pl's between pH 6-2 and 6-7, and the other with pl's higher than 7-0 (Fig. 2a). These two groups correspond to the glycoproteins E1 and E2 reported by Dalrymple et al. (1976). The glycoprotein E1 appeared heterogeneous and consisted of four components with pl's of 6-2, 6-4, 6-6 and 6-7. When urea was absent, the mannose radiolabels did not penetrate into the gel, indicating glycoprotein aggregation.

Comparison of glycoprotein E1 of Sindbis viruses grown in transformed and untransformed cells by gel isoelectric focusing

To investigate whether the Sindbis virus glycoprotein E1 is electrochemically modified by host cell transformation, the glycoproteins E1 of Sindbis virus grown in nd RSV-transformed cells or in td RSV-infected cells were compared by gel isoelectric focusing. The SV-td glycoprotein E1 was separated into four components (Fig. 2c). Its isoelectric point profile was similar to that seen with the SV-C glycoprotein E1. In contrast, the SV-nd glycoprotein E1 appeared as five components, having an additional component of pl 6-1 (Fig. 2b). To examine whether the pl 6-1 component was a constituent of E1, the SV-nd glycoprotein was treated with anti-E1 serum, and the resulting precipitate and supernatant were analysed by gel IEF. All mannose radiolabels with pl's of 6-1 to 6-7 appeared in the precipitate, while the labels with pl's higher than 7-0 remained in the supernatant (Fig. 3a, b). These results confirm that the two groups of mannose radiolabel are representative of the glycoproteins E1 and E2, respectively, and, combined with the results of SDS-polyacrylamide gel electrophoresis, exclude a possibility that the pl 6-1 component is not a member of the glycoprotein E1 but is a host contaminant. After treatment with neuraminidase, the charge heterogeneity disappeared and the mannose label appeared as a single peak with a pl of 6-7, both for preparations derived from transformed or untransformed cells (Fig. 2d). Phosphorylation of Sindbis virus glycoprotein might be responsible for the observed charge heterogeneity. To examine this possibility, Sindbis virus was grown in 32P-labelled transformed and untransformed cells, and treated with NP40. Eighty to 90% of the radioactivity remained in the supernatant after high-speed centrifugation (39700 ct/min in SV-td; 32870 ct/min in SV-nd). After treatment with butanol, aliquots (8560 ct/min in SV-nd; 8160 ct/min in SV-td) were electrophoresed in SDS-polyacrylamide gels; no labelled peak appeared in the position corresponding to Sindbis virus glycoprotein. Thus, no evidence for the existence of phosphorylated Sindbis virus glycoproteins was obtained. These results indicate that the Sindbis virus glycoprotein E1 is electrochemically heterogeneous and comprised four components; it is modified by host cell transformation, resulting in the appearance of an additional more acidic component.

Non-equilibrium pH gradient electrophoresis was performed to study whether transformation leads to modifications in the Sindbis virus glycoprotein E2. The Sindbis virus glycoprotein, labelled with [3H]leucine, appeared in two groups, one with pl's lower than 7 and the other with pl's higher than 8, corresponding to glycoproteins E1 and E2, respectively (Fig. 4a, b). The glycoprotein E2 appeared as at least three components in both preparations. No significant difference was observed between the electrophoretic patterns of glycoproteins E2 from SV-nd and SV-td.

Gel filtration of glycopeptides

The SV-nd glycoprotein E1 and its five components with pl's of 6-1, 6-2, 6-4, 6-6 and 6-7 were digested exhaustively with Pronase and the resulting glycopeptides were compared by gel filtration on Bio-Gel P-6. The glycopeptides of the SV-nd glycoprotein E1 were resolved into the four species designated S1, S2, S3 and S4 by Sefton & Keegstra (1974) (Fig. 5a). The glycopeptide elution patterns of the five components differed from each other. In the pl 6-7 component the major glycopeptides released were S3 and S4, the amount of S2 was less, and that of S1 negligible (Fig. 5b). The pi 6-6 component released a higher proportion of S2 with less S1 (Fig. 5c). The pi 6-4 and 6-2 components released S1 as the most abundant species with a lower proportion of S2 (Fig. 5d, e). The pi 6-2 component released no S3. In the pi 6-1 component, which existed only in the transformed cell-derived Sindbis virus glycoprotein E1, the mannose label appeared in the
Fig. 2. Gel isoelectric focusing of Sindbis virus glycoproteins. Glycoproteins, labelled with \[^{3}H\]mannose or \[^{14}C\]mannose, were electrofocused at 400 V for 16 h in 9.5 M-urea, 2% NP40 and 2% pH 3-5 Ampholines. After the run the gel was fractionated in 1 mm slices. The pH was measured in 5-mm sections which were placed in vials containing 2 ml of degassed water. (a) Sindbis virus glycoprotein derived from cells not pre-infected with RSV; (b) SV-nd glycoprotein; (c) SV-td glycoprotein; (d) SV-nd and SV-td glycoproteins, labelled with \[^{3}H\]mannose and \[^{14}C\]mannose, respectively, electrofocused after treatment with neuraminidase. ○, SV-nd glycoprotein; ●, SV-td glycoprotein.
Fig. 3. Gel isoelectric focusing profiles of the immunoprecipitate and supernatant of Sindbis virus glycoprotein after treatment with mouse anti-E1 serum. The SV-nd glycoprotein, labelled with [3H]-mannose, was incubated with mouse anti-E1 serum for 30 min at 37 °C. The immune complex was precipitated by incubation with rabbit anti-mouse immunoglobulin for a further 16 h at 0 °C. O, Radioactivity profile of the SV-nd glycoprotein labelled with [3H]mannose. (a) Immunoprecipitate; (b) supernatant after immunoprecipitation.

fractions corresponding to S1; the smallest glycopeptide S4 was completely lacking (Fig. 5f). Prior treatment of glycoprotein E1 with neuraminidase appeared to convert S1 and S2 to S3, while S4 remained unchanged (Fig. 6a). The Pronase digest of the pI 6-4 component was subjected to mild acid hydrolysis (Burge & Huang, 1970) and analysed on Bio-Gel P-6. As observed with the glycoprotein E1, S1 and S2 appeared to be converted to S3 (Fig. 6b). These results are consistent with the suggestion of Keegstra et al. (1975) that S1, S2 and S3 are glycopeptides having related structures but which differ in their content of sialic acid (S1 contains two residues, S2 one and S3 no sialic acid residue). High-mannose-type oligosaccharides are sensitive to endoglycosidase H (endo H) whereas complex-type oligosaccharides are resistant. Accordingly, resistance to endo H was used as a working definition for a complex-type oligosaccharide (Rosner et al., 1980). The Pronase digests of the pI 6-4 component, labelled with [3H]mannose, were analysed on Bio-Gel P-4 before and after endo H digestion. The glycopeptide S4 eluting between fractions 93 and 98 in Fig. 7(a) was converted to a structure eluting between fractions 122 and 128 (Fig. 7b), confirming that S4 is a high-mannose-type oligosaccharide (Sefton, 1976). Partial conversion of S3 to S2 was observed, probably due to partial removal of sialic acid during incubation of the reaction mixture at pH 5-5. Next, the material released as S1 from the pI 6-1 component was examined for sensitivity to endo H. By treatment with endo H, S1 was not extensively degraded (Fig. 7c, d), although partial conversion of S1 to S2 was observed; the extent of the conversion was similar to that observed in S1 of the pI 6-4 component, indicating...
that S₁ of the pI 6.1 component is not a high-mannose-type oligosaccharide. These results indicate that a small proportion of the E₁ molecules from the transformed cells is deficient in high-mannose-type oligosaccharides.

DISCUSSION

Isoelectric focusing in 0.1 % NP40-containing sucrose density gradients revealed the Sindbis virus glycoprotein E₁ as one peak with a pI of 6 (Dalrymple et al., 1976). The finding is inconsistent with the results presented here, where the E₁ glycoprotein appeared as four components with pIs between 6.2 and 6.7. This discrepancy might result from the release of sialic acid residues during glycoprotein preparation. If so, glycopeptides S₁ and S₂ would have been converted to S₃, resulting in a decrease of S₁ and S₂, and a concomitant increase of S₃. However, this was not observed (Fig. 5a). The Sindbis virus glycoprotein could not penetrate into the gel unless urea was present, suggesting that the observed discrepancy is due to glycoprotein aggregation or micelle formation with NP40. The model suggested by Keegstra et al. (1975) in which glycoprotein E₁ contains two carbohydrate attachment sites, one giving rise to the high-mannose-type oligosaccharide S₄ and the second to either the complex-type oligosaccharide S₁, S₂ or S₃ is inconsistent with the present study. The model predicts the presence of three electrochemically different components, but actually more than three were observed, four in the untransformed cell-derived and five in the transformed cell-derived glycoprotein E₁. From the nucleotide sequence of the 26S mRNA of Sindbis virus, two potential glycosylation sites (Asn-139 and Asn-245) have been deduced to be present in glycoprotein E₁ (Rice & Strauss,
Fig. 5. Gel filtration profiles of glycopeptides of Sindbis virus glycoprotein. Glycoprotein E₁, labelled with [³H]mannose, was isolated by chromatofocusing. Five components with pl's of 6.7, 6.6, 6.4, 6.2 and 6.1, labelled with [³H]mannose, were isolated by isoelectric focusing. Glycoproteins were digested with Pronase and applied to a column of Bio-Gel P-6. Fractions (0.5 ml) were collected, and the radioactivity of each fraction was determined by liquid scintillation counting. The void volume (V₀) was at fraction 72, the inclusion volume (V₁) at fraction 162. (a) Sindbis virus glycoprotein E₁; (b) pl 6.7 component; (c) pl 6.6 component; (d) pl 6.4 component; (e) pl 6.2 component; (f) pl 6.1 component.
Fig. 6. Gel filtration profiles of glycopeptides after removal of sialic acid. (a) Glycoprotein E₁, isolated by chromatofocusing, was digested with Pronase after treatment with neuraminidase. The Pronase digest was analysed by gel filtration on Bio-Gel P-6. ○, Radioactivity of [³H]mannose-labelled glycopeptides; (b) pI 6-4 component isolated by gel isoelectric focusing and digested with Pronase. The Pronase digest was analysed by gel filtration on Bio-Gel P-6 after incubation in 0-1 M-HCl at 80 °C for 30 min. The void volume was at fraction 72, the inclusion volume at fraction 162.

1981), but BHK cell-derived Sindbis virus glycoprotein E₁ contains only complex-type chains (Burke & Keegstra, 1976). Possibly, the two glycosylation sites of glycoprotein E₁ are less specific for a particular type of carbohydrate chain. E₂ also focused in one peak at pI 9 (Dalrymple et al., 1976), whereas in my experiments the glycoprotein E₂ was resolved into at least three components, which is consistent with the analysis of the carbohydrate structures carried out by Burke & Keegstra (1979). However, in contrast to the glycoprotein E₁ results, no significant differences were observed between the E₂ glycoproteins derived from the two cell types. In RSV-transformed cells some host proteins have been shown to be phosphorylated by the src gene product pp60src (Collett & Erikson, 1978; Erikson & Erikson, 1980; Sefton et al., 1980). The Sindbis virus glycoprotein might be phosphorylated by this RSV-induced phosphotransferase and the resulting phosphorylated glycoprotein might be partly responsible for the observed charge heterogeneity. However, no evidence that the Sindbis virus glycoprotein was phosphorylated was obtained.

A lipid-linked oligosaccharide chain, a common precursor of the high-mannose- and complex-type asparagine-linked oligosaccharide of glycoprotein, is transferred en bloc to nascent polypeptide chains (Hubbard & Robbins, 1979). The newly synthesized Sindbis virus glycoproteins carry only large oligosaccharides similar in size to the lipid-linked oligosaccharide (Robbins et al., 1977). Subsequent processing to yield the high-mannose- and complex-type oligosaccharides is presumed to occur at sites destined to become mature high-mannose- and complex-type oligosaccharide, respectively, by enzymes of the host cell. The pI 6-1 component which appeared only in the RSV-transformed cell-derived Sindbis virus glycoprotein E₁
contains only sialylated complex-type oligosaccharides, either because large oligosaccharides at sites destined to become high-mannose-type have been processed to complex-type or because they are not attached as a result of modification of the enzyme by host cell transformation. Whether complex-type chains are attached to the presumed attachment site of the high-mannose-type remains to be determined. Sialyl transferase activity has been shown to increase in RSV-transformed cells (Warren et al., 1972, 1973). However, which enzymes are modified by host cell transformation and are responsible for the observed changes is unknown.

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