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Proteins Specified by Herpes Simplex Virus, IV. Site of Glycosylation and Accumulation of Viral Membrane Proteins*

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Abstract. The membrane glycoproteins specified by herpes simplex virus are synthesized concurrently with structural viral proteins and accumulate in the cytoplasm and in the membranes lining it. Analyses of free and membrane-bound polyribosomes, the cytoplasmic pool of soluble proteins, and purified smooth membranes showed that viral membrane proteins bind to membranes soon after synthesis and become glycosylated *in situ*.

Introduction. Pertinent to this paper are the following findings reported previously. (1) Human epidermoid carcinoma no. 2 cells infected with herpes simplex virus cease to manufacture proteins and glycoproteins characteristic of uninfected cells but make instead new proteins including structural components of the virus.[1,2] (2) Some of the structural proteins contained in the envelope of the virus are glycosylated.[2–4] (3) Glycosylated proteins made after infection also bind to cellular membranes.[2,3] (4) The structure of the protein moiety of the glycoproteins is genetically determined by the virus. This conclusion was based on the finding that the glycoproteins bound to membranes and those contained in the virion varied quantitatively and qualitatively depending on the virus strain. Of particular interest were two observations: first, herpes viruses, which differ in their surface properties and in their effects on the social behavior of cells, specify different glycoproteins, and second, the binding of glycoproteins to membranes is not random but ordered since glycoproteins contained in the virion and derived from the nuclear membrane differed from those in smooth membranes.[3] The work described in this paper deals with the site of accumulation and glycosylation of viral glycoproteins.

Materials and Methods. Chemicals: Mixtures of leucine, isoleucine, and valine, containing equal activities of either the ^{3}H or ^{14}C isotope in each amino acid, and ^{14}C-reconstituted protein hydrolysate were obtained from Schwarz BioResearch, Orangeburg, N.Y. The 6-^{3}H-D-glucosamine (1300 mCi/mM) and 1-^{14}C-D-glucosamine (52 mCi/mM) were obtained from New England Nuclear Corp., Boston, Mass. Puromycin-HCl was obtained from Nutritional Biochemical Co., Cleveland, Ohio.

Cells, virus, and infection of cells: Human epidermoid carcinoma no. 2 (HEp-2) cells were used in all the work described here. The pertinent properties of the F strain of herpes simplex virus used in these experiments have been described elsewhere.[3,5] Monolayer cell cultures (approx. 3×10^{7} cells) were infected with a multiplicity of 10–20 plaque-forming units/cell, overlaid with mixture 199 supplemented with 1% calf serum

after a 2-hr adsorption period, and incubated at 37°C. Infection was timed from the beginning of the adsorption period.

Labeling of cells with radioisotopes: Except where otherwise indicated, infected cells were radioactively labeled by incubation in monolayer or after suspension in mixture 199, containing 0.1 or less of the recommended levels of leucine, isoleucine, and valine, 1% dialyzed calf serum, and the appropriate isotopes. In these experiments incorporation of radioactive glucosamine was used to measure the extent of glycosylation. The basis for its use is as follows: (*a*) Glucosamine is incorporated into all herpes simplex virus glycoproteins along with fucose, galactose, and mannose (ref. 3, and Keller, unpublished data). (*b*) Glucosamine is the predominant sugar in the glycoprotein specified by at least one animal virus, Sindbis virus.[6] Glucosamine is incorporated into the glycoproteins of herpes simplex virus as D-glucosamine and D-galactosamine or their acetylated derivatives.[3]

Separation of nuclei from cytoplasm: Dounce homogenization: 12-hr infected cells were suspended in reticulocyte standard buffer[7] at 3×10^7 cells/ml and disrupted with five strokes of a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 1500 rpm for 5 min to sediment the nuclei which were then washed three times with isotonic reticulocyte standard buffer. The washes were discarded. The first supernatant was designated cytoplasmic fraction. Nonidet P-40 (NP-40) fractionation:[8] 12-hr infected cells were suspended in isotonic reticulocyte standard buffer containing 0.5% NP-40 (3×10^7 cells/ml). After 15 min at 0°C, the nuclei were sedimented by centrifugation at 1500 rpm for 5 min and then washed three times with isotonic buffer. The first supernatant was designated cytoplasmic fraction.

Analysis of polyribosomes from infected cells: The procedures were similar to those described previously[9] except that the 15–30% (w/w) sucrose gradient was formed on top of a 2-ml cushion of 50% (w/w) sucrose in order to trap membrane-bound polyribosomes at the 30–50% interface.[10]

Preparation of purified smooth membranes and soluble proteins: The procedure for the purification of smooth membranes has been described.[2,11] Briefly: (*a*) A 4000 × *g* supernatant of infected cells, which had been extensively disrupted by Dounce homogenization, was made 45% (w/w) with respect to sucrose. (*b*) Sucrose solutions of 35, 30, and 25% (w/w) were layered on top in a discontinuous gradient. (*c*) During centrifugation for 20 hr at 25,000 rpm and 5°C in the SW27 rotor, smooth internal membranes from the cells floated to the 25% sucrose layer. This visible band of membranes was removed with a syringe, diluted, and sedimented by centrifugation for 1 hr at 25,000 rpm and 5°C in the 30 rotor. Membranes prepared in this way from infected HEp-2 cells are free from virus, ribosomes, soluble proteins, and other cellular constituents.[2] Soluble proteins remain in the 45% sucrose layer. The supernatant obtained after twofold dilution of the 45% sucrose layer and centrifugation at 25,000 rpm and 5°C in the 30 rotor is designated the soluble protein fraction.

Solubilization of proteins and acrylamide gel electrophoresis: The proteins were solubilized and electrophoresed as previously described[1,12] except that the purified membranes were first dialyzed against a solution containing nine parts dimethylformamide to one part 0.1 N HCl at 0°C for 3 hr, then overnight at room temperature against a solution containing 0.01 M phosphate buffer pH 7.1, 0.5 M urea, 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, and lastly against the same solution but containing 10% sucrose by weight.

Results. Compartmentalization of viral glycoproteins: The following experiment was done to determine whether viral glycoproteins are confined to one compartment of the cell. Cells were incubated with ^{3}H amino acids and ^{14}C-D-glucosamine from 4 to 12 hr after infection. Nuclear and cytoplasmic preparations were made with the aid of a Dounce homogenizer or with NP-40. The electropheretograms shown in Figure 1 indicate that glycoproteins were present in both the nuclear and cytoplasmic fractions prepared by Dounce homogeniza-

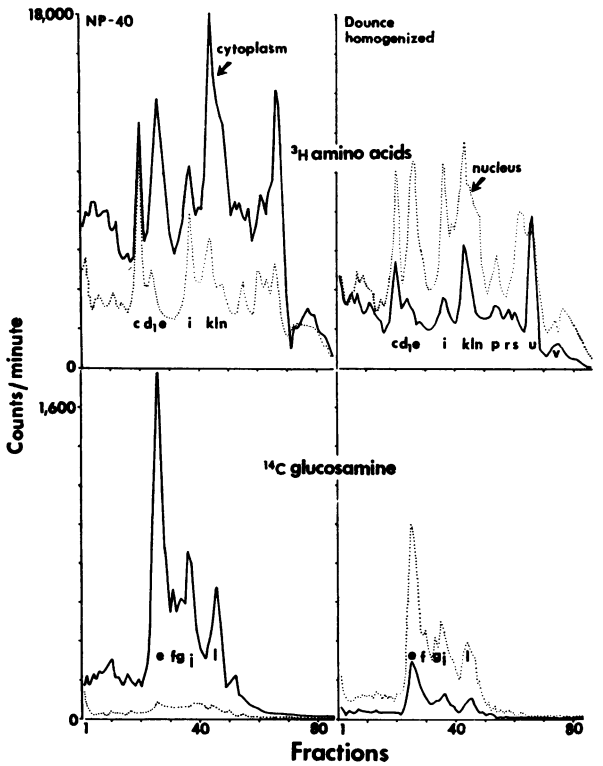

FIG. 1.—Electrophoretograms of proteins in nuclei and cytoplasm prepared from infected cells with NP-40 (*left*) or by Dounce homogenization (*right*). Prior to fractionation replicate cultures (each containing 3×10^7 cells) were incubated 4–12 hr after infection in medium containing ^{3}H-leucine, isoleucine, and valine (2 μCi/ml) and ^{14}C-D-glucosamine (0.4 μCi/ml). To facilitate comparison of the distribution of proteins in the nuclei and cytoplasm obtained by the two techniques, the electrophoretic profiles of ^{3}H amino acid- and ^{14}C-D-glucosamine-labeled proteins were plotted on separate graphs even though the cells were labeled simultaneously with both isotopes.

tion (*lower right gradient*, Fig. 1) but were absent from nuclei prepared with NP-40 (*lower left gradient*, Fig. 1). We conclude from this experiment that viral glycoproteins accumulate only in the cytoplasm and in the membranes lining it and moreover, that NP-40 does not extract proteins from the interior of the nucleus. This conclusion is supported by the following observations: (1) By electron microscopy, cytoplasmic membranes were found to be attached to nuclei prepared by Dounce homogenization and not to those prepared with NP-40. (2) Most of the nonglycosylated proteins, notably c, d, i, and k, were present in both Dounce and NP-40 prepared nuclei (upper half of Fig. 1). (3) Electron microscopic examination of thin sections of the nuclei prepared by the two methods revealed no differences in the contents of the nuclei. (4) We found less leakage of nuclear RNA from nuclei obtained with NP-40 than from those obtained by Dounce homogenization.[13] Although NP-40 may remove glycoproteins from the nuclear membrane, it is unlikely that glycoproteins are found in the interior of the nucleus.

Dissociation of protein synthesis and glycosylation: Two series of experiments were performed to determine whether proteins made after infection are glycosylated during synthesis on polyribosomes. In the first series we examined the nascent peptides attached to polyribosomes from 5-hr infected cells which had been pulse labeled for 5 min with ^{3}H-glucosamine and ^{14}C amino acids. The distribution of the radioactivity in the nascent peptides attached to poly-

ribosomes after centrifugation in sucrose density gradients is shown in Figure 2. The data show: (*a*) By comparison with incorporation of amino acids, the

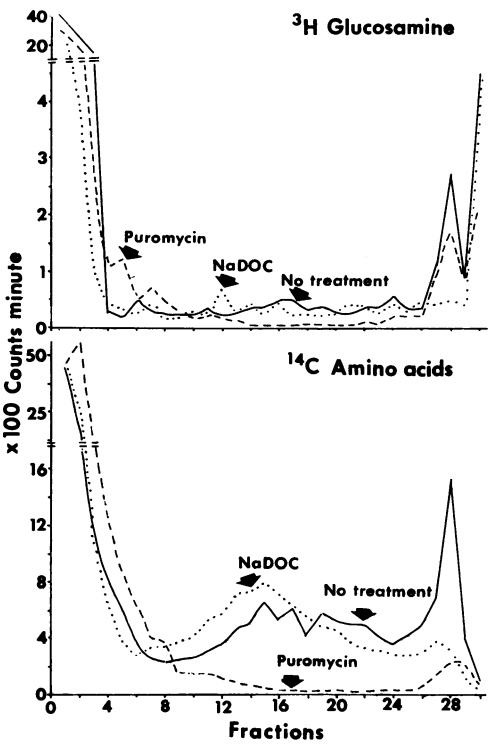

FIG. 2.—Profiles of trichloroacetic acid precipitable radioactivity bound to polyribosomes centrifuged in sucrose density gradients. (Tube top is at left.) Fractions 6–24 contain predominantly free polyribosomes, while fractions 26–30, collected at the 30–50% (w/w) sucrose interface, contain membrane-bound polyribosomes. Three replicate sets (6×10^7 cells/set) of 5-hr infected cultures were suspended and incubated for 5 min in Eagle's medium containing 1/20 the recommended level of amino acids, 10 μCi ^{3}H-D-glucosamine/ml, and 1 μCi ^{14}C reconstituted protein hydrolysate/ml. Incorporation was stopped by pouring on a frozen phosphate-buffered saline slurry. Cytoplasmic extracts were prepared and centrifuged on sucrose density gradients. *Solid line*, no treatment before or after preparation of cytoplasmic extract; *dashed line*, cells incubated with puromycin (100 μg/ml) after labeling period and before preparation of cytoplasmic extract; *dotted line*, Na deoxycholate added to cytoplasmic extract before centrifugation. Distributions of glucosamine and amino acid-labeled nascent peptides bound to polyribosomes in the sucrose density gradients were plotted on separate graphs even though the cells were labeled simultaneously with both isotopes.

incorporation of glucosamine into nascent peptides in the free polyribosome region was negligible. This conclusion may also be deduced from the fact that the amount of ^{3}H-glucosamine precipitable with trichloroacetic acid from the top of the gradient was 100 times that contained in the free polyribosome region whereas the amount of ^{14}C amino acids at the top of the gradient was less than seven times higher. (*b*) There was considerable incorporation of both amino acids and glucosamine into material banding at the 30–50% sucrose interface. However, whereas the bulk of the amino acids at the interface was incorporated into nascent peptides on membrane-bound polyribosomes, the glucosamine is incorporated into macromolecules contained in the membranes and was not incorporated into the nascent peptides. This conclusion is based on the fact that puromycin treatment caused the ^{14}C amino acids, but not the ^{3}H-glucosamine to be released from the interface. The ^{3}H-glucosamine was released only by deoxycholate. The results of these experiments indicated that the proteins made in infected cells are not glycosylated on polyribosomes. One prediction of these results is that glycosylation should not be dependent on protein synthesis. In the second series of experiments designed to test this prediction, we added puromycin to 7-hr infected cells suspended in medium containing

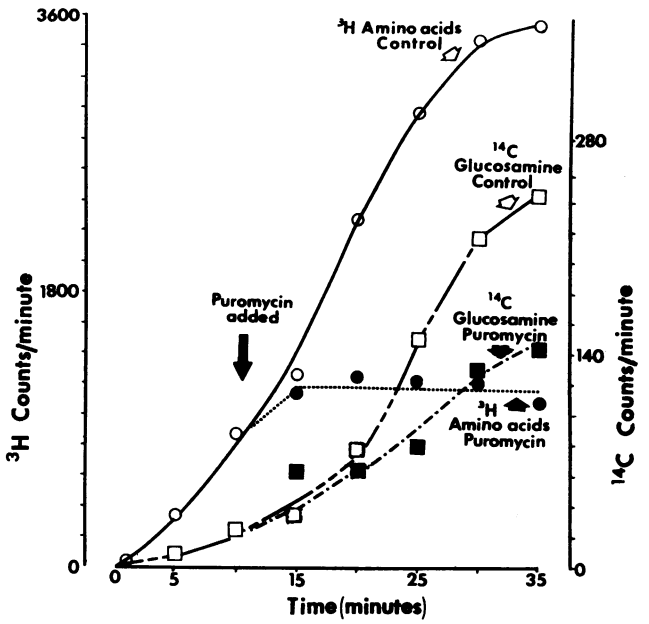

FIG. 3.—Effect of puromycin on the incorporation of amino acids and glucosamine into trichloroacetic acid precipitable material. Cells infected for 7 hr (3×10^7) were suspended in 8 ml of mixture 199 containing ^{3}H-leucine, isoleucine, and valine (5 μCi/ml) and ^{14}C-glucosamine (0.5 μ Ci/ml). After 11 min of incubation the cell suspension was divided in half and puromycin (100 μg/ml) was added to one half. Aliquots (0.4 ml) were removed at the times indicated and precipitated with trichloroacetic acid for radioactivity measurements.

labeled amino acids and glucosamine. As shown in Figure 3, puromycin caused rapid cessation of ^{3}H amino acid incorporation. Puromycin diminished but did not abolish the incorporation of ^{14}C-glucosamine into acid-precipitable material.

Site of glycosylation of viral glycoproteins: The preceding experiment suggested that glycosylation takes place after the proteins are completed, either in transit to or after binding to the membranes. The experimental technique most suitable to test these two possibilities is a pulse with amino acids and glucosamine followed by incubation (chase) in the presence of excess unlabeled precursor. The technique is feasible because, as has been shown repeatedly the intracellular and extracellular amino acid pools rapidly equilibrate.[14,15] On the other hand, in our experiments (unpublished) and as shown here, intracellular glucosamine does not equilibrate rapidly with that in extracellular fluid and glycosylation with labeled glucosamine does continue in medium containing unlabeled precursors. To determine the site of glycosylation, duplicate sets of cultures were incubated for 1 hr with ^{3}H-glucosamine and ^{14}C amino acids at various times after infection. At that time the cells in one set were harvested. The cells in the second set were washed, overlaid with medium containing excess unlabeled precursors, and reincubated until 24 hr after infection. Two experiments were performed; in the second experiment we reversed isotopes. The amounts of

radioactive glucosamine and amino acids incorporated into the purified smooth membranes and into the soluble proteins in both experiments are summarized in Table 1. The data suggest that glycosylation takes place on the membranes and not in the cytoplasmic pool of soluble proteins. This conclusion is based on the following findings: (1) There was considerably more labeled glucosamine in the membrane fraction than in the soluble protein fraction immediately after the pulse. (2) The ratio of glucosamine to amino acids present in the soluble protein fraction did not decrease after the chase. (3) The ratio of glucosamine to amino acids incorporated into the purified membrane fraction increased significantly during the chase. The increase was caused solely by glycosylation; the amount of radioactive amino acids in the membrane fraction remained constant.

To test the hypothesis that glycosylation takes place in the membranes, aliquots of the purified membrane fractions obtained in experiment 2 were solubilized and the proteins subjected to electrophoresis in acrylamide gels. From the results shown in Figure 3 and Table 1 we conclude that: (*a*) Viral membranes proteins are inserted into the smooth membranes before they are extensively glycosylated, (*b*) most or all of the glycosylation takes place in the membranes, (*c*) after a 1-hr pulse only a small fraction of the bound proteins is extensively glycosylated, and (*d*) the protein moieties of the membrane glyco-

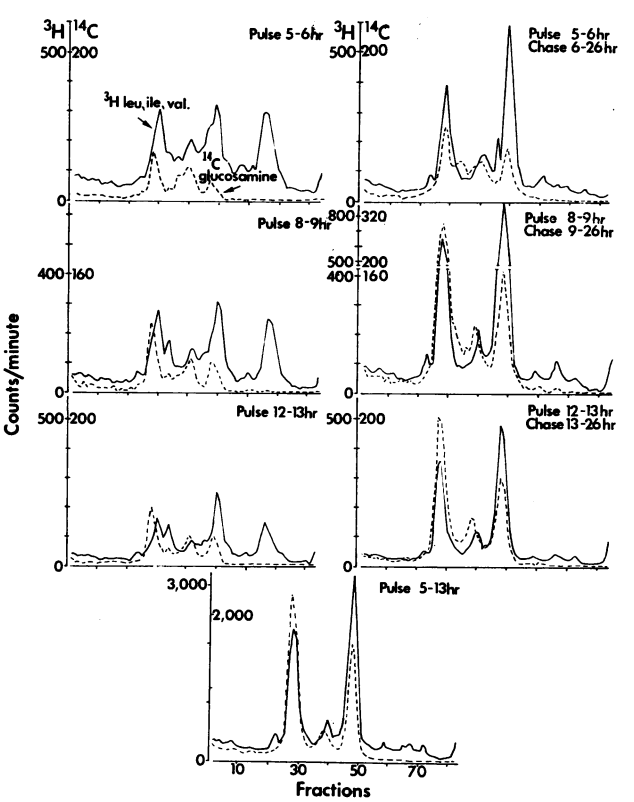

FIG. 4.—Electrophoretograms of radioactive proteins present in smooth membranes purified immediately after the pulse periods or after further incubation in nonradioactive medium, as indicated with each set of profiles. Details of labeling with radioactive percursors, etc., are given in footnotes to Table 1.

proteins are synthesized throughout the time during which structural viral proteins are made.[1,16] The conclusions are based on the following findings: (1) As shown in Table 1, the amount of amino acid label in the membrane frac-

TABLE 1. *Recovery of radioactive precursors in glycoproteins after pulse and chase.*

Preparation	Pulse-labeling period*	Chase period*	CPM Glucosamine After pulse	CPM Glucosamine After chase	CPM Amino Acids After pulse	CPM Amino Acids After chase	Glucosamine Amino Acid Ratio After pulse	Glucosamine Amino Acid Ratio After chase
Expt. 1, †								
Smooth membranes	4–5	5–24	2,180	8,550	6,590	5,850	0.33	1.46
	6–7	7–24	1,980	6,300	5,760	4,980	0.34	1.26
	8–9	9–24	745	4,800	3,100	3,500	0.24	1.37
Soluble proteins	4–5	5–24	180	200	7,080	4,780	0.025	0.042
0.1-ml aliquot	6–7	7–24	260	140	6,680	3,200	0.039	0.044
	8–9	9–24	50	140	3,800	1,680	0.013	0.083
Expt. 2, ‡								
Smooth membranes	5–6	6–26	960	1,730	10,500	8,570	0.091	0.202
	8–9	9–26	1,130	3,540	7,900	11,500	0.143	0.308
	12–13	13–26	970	2,240	5,140	6,150	0.189	0.364
Soluble proteins	5–6	6–26	21	13	9,460	1,800	0.002	0.007
0.1-ml aliquot	8–9	9–26	30	34	5,750	1,610	0.005	0.021
	12–13	13–26	27	16	3,490	750	0.008	0.021

* Hours after infection.

† Replicate sets of cultures (6×10^7 cells/set) were incubated for 1 hr at the times indicated with medium containing ^{3}H-glucosamine (4 μCi/ml) and ^{14}C-leucine, isoleucine, and valine (0.2 μCi/ml). One set was harvested immediately for the purification of smooth membranes and preparation of soluble proteins. The other set was incubated for the times indicated in mixture 199 containing 75 μg nonradioactive glucosamine/ml.

‡ Experiment 2 was performed in the same manner as Expt. 1 except that the radioactive medium contained ^{14}C-glucosamine (0.7 μCi/ml) and ^{3}H-leucine, isoleucine, and valine (3 μCi/ml), and the nonradioactive mixture 199 contained 280 μg of glucosamine/ml.

tion did not become altered appreciably after the chase. (2) Electrophoretograms of membrane proteins immediately after the pulse showed rapidly moving protein bands which contained little or no glucosamine. In addition, proteins more heavily labeled with glucosamine migrated more slowly than the bulk of the proteins which formed broad bands indicating variation in the extent of glycosylation of the proteins. (3) After the chase the rapidly migrating nonglycosylated bands were absent and there was complete coincidence of glucosamine and amino acid label in the major protein bands, without loss of amino acid label from the membrane fractions.

Discussion. In this paper we have shown: (1) Glycoproteins accumulate only in the cytoplasm and in the membranes lining it. This conclusion is based on the finding that purified nuclei free of cytoplasmic debris but containing viral proteins lack glycoproteins.

(2) Proteins are inserted into membranes shortly after synthesis. Specifically, the amount of amino acid-labeled protein in membranes does not increase during the chase. One implication of this conclusion is that there is no cytoplasmic pool of free membrane proteins.

(3) Viral proteins found in the smooth membranes of infected cells are glycosylated most extensively, if not solely, after binding to membranes. Thus, (*a*) we could not detect glycosylation of nascent peptides on either free or bound

polyribosomes, (*b*) glycosylation was not terminated by concentrations of puromycin that inhibited protein synthesis, (*c*) we could not chase glycosylated proteins from the soluble protein pool into membranes, and (*d*) the amino acid label was chased from rapidly migrating bands of nonglycosylated or partially glycosylated proteins in acrylamide gels to more slowly migrating bands of glycoproteins.

(4) There is a significant decrease in electrophoretic mobility of the membrane proteins after glycosylation. Several laboratories have reported an inverse relationship between electrophoretic mobility and the logarithm of molecular weight for proteins in sodium gels dodecyl sulfate.[17,18] In this instance it is clear that the molecular weight of the membrane proteins has increased after glycosylation. However, it is not clear that the decrease in the electrophoretic mobility is caused entirely by an increase in molecular weight. The decrease could also be a result of increased binding of sodium dodecyl sulfate[19] or to a special conformation of glycosylated proteins.

(5) The rate of glycosylation of membrane proteins is slower than the rate of synthesis and insertion of the proteins into membranes. Thus, less than 25% of the amino acid label migrates in acrylamide gels with the fully glycosylated proteins immediately after the pulse.

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