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Proteins Specified by Herpes Simplex Virus

VI. Viral Proteins in the Plasma Membrane

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Previous papers in the series have shown that the surface membranes of herpesvirus-infected cells acquire new immunological specificities and that purified infected cell membrane preparations, characterized by their physical properties rather than topology in the cell, contain new glycoproteins genetically determined by the virus. In this study, we prepared purified plasma membrane identified by its 5'nucleotidase, fucose, and reduced nicotinamide adenine dinucleotide-diaphorase content. Analysis of the membrane proteins and glycoproteins by electrophoresis in acrylamide gels indicated the following. (i) Purified plasma membranes from infected cells contained two sets of proteins, i.e., host proteins were present both before and after infection and viral proteins were present only after infection. (ii) After infection, no appreciable selective or nonselective loss of host proteins from membranes was demonstrable. However, no new host proteins were made. (iii) Electropherograms of plasma membrane proteins from infected cells indicated the presence of at least 12 virus-specific proteins ranging in molecular weight from 25 $\times$ 10^3 to 126 $\times$ 10^3 daltons. Of these, at least nine were glycosylated. Proteins and glycoproteins with similar electrophoretic mobilities but in somewhat different ratios were also present in preparations of highly purified virions.

This paper deals with the membrane proteins specified by herpes simplex virus (HSV). The circumstances which led to this study may be summarized as follows. Early studies on the interaction of infected cells among themselves led to the prediction (16) and subsequent demonstration of new antigens on the surface of infected cells (15, 21). Several lines of evidence based on immunological tests indicated that the antigens on the surface of the cell are similar, if not identical, to those present on the surface of infectious virus particles (15, 21). The presence of new surface antigens was subsequently confirmed by other laboratories (29). The appearance of new surface antigens may be a general property of herpesvirus-infected cells, particularly since immunologically identical antigens appear on the surface of Burkitt's lymphoma cells and on the herpesvirus associated with them (14). In an attempt to relate the new surface antigens to specific membrane components, purified membranes of infected cells were analyzed by electrophoresis in acrylamide gels. These analyses showed that the purified membranes are free from soluble proteins, enveloped and naked nucleocapsids, ribosomes, etc., and contain several glycoproteins made after infection and absent from uninfected cells (23). Moreover, the number and electrophoretic mobilities of the glycoproteins are genetically determined by the virus (11, 18). These studies, however, suffered from two shortcomings. First, the purification procedure deliberately selected a membrane fraction characterized by its low density and not by its topological origin within the cell. The question arises of whether virus-specific glycoproteins are incorporated into a topologically defined membrane such as the plasma membrane and the endoplasmic reticulum of the infected cell. Secondly, as indicated in the preceding paper of this series (24), the procedure for the acrylamide gel electrophoresis used in those earlier studies did not have sufficient resolution to permit us to determine with a high order of precision the number of virus-specific glycoproteins present in the membranes of infected cells. The question arises as to the number and electrophoretic mobility of the glycoproteins in the membranes as compared with those of the virion. This paper deals with these questions.

Pertinent to the understanding of the experimental design employed in our studies are two points concerning the binding of the virus-specific glycoproteins to membranes of infected cells and purification of the plasma membranes, respectively. The first point addresses itself to the stability of the viral glycoprotein-membrane bond particularly in the course of extensive manipula-

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tive procedures. Our experience to date is based on studies of the interaction of viral glycoproteins with smooth membranes defined on the basis of physical characteristics. The data have been discussed in detail elsewhere (19, 20). Briefly, (i) virus-specific membrane proteins bind to membranes during or after synthesis and become glycosylated in the membranes; glycoproteins not bound to cellular membranes cannot be found in the cytoplasm of the infected cell (20, 24). (ii) The binding of proteins to membranes is specific. This is based on the observation that capsid proteins and numerous other proteins present in the cytoplasm of infected cells do not bind to the membranes (20, 24). (iii) The binding of proteins to membranes appears to be selective. Thus, in cells infected with the MP strain of HSV, the glycoproteins present in the envelope of the virion and derived from the nuclear membrane are different from those that bind to the smooth membranes of the infected cells (11, 20). (iv) The binding of the glycoproteins to the plasma membrane is tenacious. We have not been able to effect the release of glycoproteins from the membranes except by procedures which compromise the integrity of the membrane itself. It is noteworthy that the infected cell membrane-viral glycoprotein complex withstands considerable hydrodynamic stress even when augmented by the presence of antibody bound to the glycoprotein (19, 20). The second point stems from the fact that purification of plasma membranes is not standardized. The techniques used for the preparation of plasma membranes fall into two categories. The first is based on the preparation of fixed and unfixed cell ghosts. Identification of the plasma membrane is visual. The purity of such preparation, however, is debatable and subject to definition as to whether anything fixed or bound to the plasma membrane is really a structural component of the membrane. The second technique is based on fractionation of membranes according to physical properties and selected markers. The markers used for differentiation of membranes have been reviewed by Steck et al. (25); those most commonly used are 5′ nucleotidase, reduced nicotinamide adenine dinucleotide (NADH)-diaphorase, and, more recently (1), the presence of fucose in the membrane glycoproteins. Membranes prepared by these techniques yield a product of measurable purity, but the topological origin of the product cannot be confirmed visually. In the studies described in this paper, we define plasma membranes as rich in 5′ nucleotidase and fucose but poor in NADH-diaphorase activity.

MATERIALS AND METHODS

Cells. Human epidermoid carcinoma no. 2 (HEp-2) cells were grown on monolayer cultures in 32-oz (ca. 900 ml) bottles in Eagles minimal essential medium supplemented with 10% calf serum, 0.001% ferric nitrate, and 1% sodium pyruvate.

Virus. The properties, procedures for the production, and assay of the F prototype of HSV subtype 1 were reported elsewhere (5, 12, 24).

Infection. Cells were infected at 37 C with a multiplicity of 5 to 10 plaque-forming units per cell and then overlaid with a medium consisting of mixture 199 supplemented with 1% calf serum and incubated further at 37 C.

Labeling of cells prior to extraction and purification of membranes. Uninfected cell proteins were labeled for 24 hr in mixture 199 containing 25% of the usual concentration of leucine, isoleucine, and valine but supplemented with 10% dialyzed calf serum and ^{14}C-leucine, -isoleucine, and -valine (0.4 μCi/ml). The labeled cells were then incubated for 4 hr in complete nonradioactive growth medium prior to either virus infection or mock infection.

Uninfected cell glycoproteins were labeled for 18 hr with ^{3}H-fucose (3 μCi/ml). The cells were then incubated for 12 hr in a medium containing unlabeled fucose (3 mg/liter) before infection. Previous studies in our laboratory (Keller, *unpublished data*) have shown that labeled fucose incorporated into HEp-2 cells is quantitatively recovered as fucose.

Infected cell proteins were labeled from 4 to 18 hr postinfection in mixture 199 containing 10% of the usual concentration of leucine, isoleucine, and valine but supplemented with 1% dialyzed calf serum and ^{14}C-leucine, -isoleucine, and -valine (0.3 μCi/ml).

Infected cell glycoproteins were labeled between 4 and 18 hr postinfection in mixture 199 supplemented with 1% calf serum and ^{14}C-glucosamine (0.3 μCi/ml). We previously reported that, under these conditions of labeling, 92% of the radioactivity in proteins was recovered as glucosamine and galactosamine (11, 23).

Preparation of the plasma membrane. The procedure for the purification of the plasma membrane involved two steps. In the first step, we followed the procedure of Kamat and Wallach (10) which has several advantages over most other procedures. First, the procedure is very gentle in that it permits rupturing the cells with minimal discharge of lysosomes. The procedure for disruption of HEp-2 cells was standardized in the same fashion as that reported for L cells by Heine and Schnaitman (9). The ruptured cells were monitored for glucose-6-phosphate dehydrogenase and acid phosphatase as markers for ruptured cells and discharged lysosomes, respectively. The N_2 pressure and other conditions selected ruptured 95% of the cells with minimal discharge of lysosomes. Secondly, the most important advantage of this procedure is that it allows the separation of two smooth membrane populations which are similar in many physical properties. In these experiments, microcavitation caused the formation of microvesicles from surface membranes which were separated from the nucleus, endoplasmic reticulum complex, mitochondria, and large cytoplasmic membrane fragments by low-speed centrifugations. The supernatant fluid, i.e., the microsomal fraction, was enriched in plasma membrane and contained as its major contaminant endoplasmic

reticulum. The plasma membrane was separated from the bulk of microsomal components and other smooth cytoplasmic membranes with divalent cations followed by centrifugation through a Dextran 110 barrier. After centrifugation, the plasma membrane remained at the buffer-dextran interphase, whereas most of the smooth cytoplasmic membranes along with the other microsomal components were found in the pellet (10). Operationally, the cells were harvested 18 hr after infection, washed, suspended in 10 volumes of a solution containing 0.25 M sucrose, 0.02 mM $MgSO_4$, 0.5 g of bovine serum albumin per liter, and buffered with 1 mM HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethane sulfonic acid) at *p*H 7.4. The cells were then placed in a pressure bomb (Parr Instrument Co., Moline, Ill.), allowed to equilibrate in nitrogen for 20 min at 750 psi and 4 C, and then ruptured by rapid decompression. The suspension of ruptured cells released from the nitrogen bomb was allowed to stand for 5 min. At that time, 0.1 M ethylenediaminetetraacetic acid (EDTA) at pH 7.0 was added to yield a final concentration of 1 mM. The ruptured cells were then subjected first to a low-speed centrifugation (600 × *g* for 10 min) to remove nuclei and then to a higher-speed centrifugation (15,000 × *g* for 15 min) to remove mitochondria. A third centrifugation of 105,000 × *g* for 45 min pelleted the microsomes. These were washed with a series of tris(hydroxymethyl)-aminomethane (Tris) buffers differing in ionic strength, first with 1 mM Tris at *p*H 7.4, then with 10 mM Tris at *p*H 8.3, and finally with 1 mM Tris at *p*H 8.3 to release as many soluble proteins as possible from the membrane vesicles. The washed microsomal suspension in 1 mM Tris buffer (pH 8.3) was dialyzed overnight against 1 mM Tris buffer (*p*H 8.6) containing 1 mM $MgSO_3$ to bring about the density alteration of the smooth cytoplasmic membranes. Fractions (2 ml) of the sample were then centrifuged at 51,000 × *g* and 2 C for 16 hr onto a 3-ml Dextran 110 (Pharmacia Fine Chemicals, Piscataway, N.J.) barrier (1.0766 g/ml) prepared in the dialysis buffer. The band at the buffer-dextran interphase was harvested, pelleted, and suspended in 1 mM Tris buffer (*p*H 8.3) containing 1 mM EDTA.

The second step in the purification of plasma membranes involved a fractionation of the membranes banded in Dextran 110 according to their physical properties and served to remove residual virus particles still associated with the membranes. In this step, the partially purified plasma membranes suspended in Tris-EDTA buffer were made 45% (w/w) with respect to sucrose and floated through a discontinuous sucrose gradient made up by layering on top of the sample layer sucrose solutions of 35, 30, 25, and 0% (w/w) prepared in 1 mM Tris buffer (*p*H 8.3). The tubes were then centrifuged at 78,000 × *g* for 20 hr as described by Spear et al. (23). The purified plasma membranes banded at the 25 and 30% sucrose interphase.

Acrylamide gel electrophoresis. The preparation of the sample for electrophoresis and the preparation of the acrylamide gels was described by Spear and Roizman (24). Electrophoresis was done in the discontinuous system as described by Dimmock and Watson (4) and by Laemmli (13). The samples (40 to 60 μg of protein in about 50 to 70 μliters) were solubilized by boiling for 4 min in a solution containing 2% sodium dodecyl sulfate and 5% β-mercaptoethanol and buffered with 0.05 M Tris-hydrochloride at *p*H 7.0. The solubilized membrane samples were subjected to electrophoresis alongside each other on a single 8.5% acrylamide flat gel slab (2 mm in thickness by 100 mm in width by 120 mm in length including the main gel and stacker) as described elsewhere (24). After electrophoresis, the proteins were fixed and stained by soaking the gel slab overnight in 0.03% Coomassie Brilliant Blue containing 10% acetic acid and 25% isopropanol and destained in several changes of 10% acetic acid and 10% isopropanol as described by Fairbanks et al. (7). For the analysis of radioactive proteins in the acrylamide gel slabs, autoradiographs were prepared by Fairbanks et al. (6). Absorbance tracings from both of the gels stained with Coomassie Brilliant Blue and the autoradiograms developed on X-ray film were done with a double-beam recording microdensitometer (Joyce Loebel and Col, Burlington, Mass.).

It is pertinent to note that acrylamide gel slabs offer a significant advantage over cylindrical gels in that higher gel concentrations can be used and as many as eight samples can be subjected to electrophoresis at the same time. Moreover, the relative position of the bands formed by proteins in each sample is unaffected by the shrinkage or expansion of the gel during fixation, staining, destaining, and drying. However, the resolution obtained on cylindrical gels is better than that obtained on gel slabs.

Enzyme assays. The 5'nucleotidase assay followed the procedure of Weaver and Boyle (30); the enzyme was assayed in a total volume of 0.5 ml containing 40 to 70 μg of membrane proteins, 2.5 μmoles of adenosine monophosphate, 5 μmoles of $MgCl_2$, and 25 μmoles of glycine at *p*H 9.1. After 30 min of incubation at 37 C, the reaction was stopped by the addition of 2.5 ml of 8% trichloroacetic acid and placed in an ice bath. The solution was filtered through Whatman 42 filter paper, and 1-ml samples were used for the determination of inorganic phosphate by the method of Fiske and Subbarow (8).

NADH-diaphorase was assayed spectrophotometrically at 25 C by the procedure of Wallach and Kamat (28). To identical 1-ml cuvettes the following solutions were added: 0.4 ml of 0.2 M Tris buffer (*p*H 7.4), 0.4 ml of 0.28 mM NADH in Tris buffer, 0.2 ml of 0.33 mM potassium ferric cyanide, and 0.05 ml of 4 to 20 μg of membrane protein. The rate of oxidation was followed at 340 nm.

Electron microscopy. Membrane preparations were monitored for the presence of virus particles by examination of thin sections by electron microscopy. Purified membrane pellets were suspended in a small volume of 1 mM Tris buffer (*p*H 8.3) and transferred to microcentrifuge tubes half filled with 2% glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.4) containing 0.2 mM $CaCl_2$. The tubes were then centrifuged for 5 min in a model 152 Microfuge (Beckman Instruments Inc., Palo Alto, Calif.) operated at 110 v. The pellets were removed and placed in fresh glutaraldehyde for 2 hr. The pellets were then washed repeatedly and postfixed in 1.5% OsO_4 in the same buffer for 2 hr. The fixed pellets were dehydrated in an ethanol series

and embedded in Epon 812. Thin sections were stained with lead citrate and uranyl acetate prior to examination with the aid of an AEI-EM6B microscope.

RESULTS

Purification of plasma membranes. The extent of purification of plasma membranes from the crude homogenate of infected cells was monitored by assaying the specific activities of the 5′ nucleotidase, the diaphorase, or the fucose contained in the starting material, the intermediate, and the end products of the purification procedures. The results obtained in two experiments are summarized in Table 1. In the first experiment, approximately 8×10^8 18-hr infected cells were processed as described above. Fractions of the initial material and the intermediate and end products were assayed for enzyme activity. In the second experiment, approximately 4×10^8 cells were incubated for 16 hr in a medium containing ^{2}H-fucose (3 μCi/ml) and for an additional 16 hr in a medium containing unlabeled fucose (3.3 mg/liter). At that time, the cells were infected and then labeled from 4 to 18 hr postinfection with ^{14}C-glucosamine. The results may be summarized as follows. (i) The degree of purification indicated by the ratios of the specific activities of 5′ nucleotidase to diaphorase was approximately 26-fold. The extent of purification obtained with infected cells was very similar to that of uninfected cells, suggesting that there is no massive redistribution of marker enzymes after infection. (ii) The degree of purification indicated by the fucose content was approximately 14-fold, i.e., less than that observed with the enzyme markers. It should be noted here that the discrepancy could be expected. The experimental design required that cells be labeled with fucose before infection since viral glycoproteins contain fucose (18, 20). Although the cells were incubated in unlabeled fucose for 12 hr before infection and acrylamide gel electrophoresis of the doubly labeled plasma membranes failed to show appreciable amounts of fucose in the viral glycoproteins, we cannot exclude the possibility that some labeled fucose remained in the nucleotide sugar pool and was available to the viral glycoproteins at all membranes or that fucose was removed from host glycoproteins and reutilized. (iii) Thin sections studied by electron microscopy indicated that the purified plasma membranes were free from naked and enveloped nucleocapsids. The absence of virus particles was also evident from the absence of the major capsid protein from the acrylamide gel electropherograms of membrane proteins reported in the next section of this paper.

Protein composition of plasma membranes of infected and uninfected cells. The major objectives of these experiments were to determine the protein composition of plasma membranes extracted from infected and uninfected cells and the fate of host membrane proteins after infection. In the first series of experiments, four sets of cell cultures each containing 4×10^8 cells were treated as follows. The first set of cell cultures was replenished with labeling medium containing ^{14}C-amino acids at 0 time. After 24 hr of incubation, the cells were washed and incubated for an additional 22 hr in a medium containing unlabeled amino acids. The second set of cell cultures was treated from time 0 to 24 hr exactly as the first set. At that time, the labeling medium was removed, and the cells were incubated for 4 hr in unlabeled medium and then infected and reincubated in unlabeled medium for an additional 18 hr. The cells in the third set were labeled with ^{14}C-amino acids between 32 and 46 hr after 0 time. The cells in the fourth set were infected at 28 hr and labeled between 32 and 46 hr after 0 time, i.e., between 4 and 18 hr postinfection. At 46 hr after 0 time, all cell cultures were harvested, and the plasma membranes were purified, solubilized, and subjected to electrophoresis on a flat acrylamide gel slab as described above. Figure 1 shows the absorbance

TABLE 1. *Purification of plasma membrane*

Materials tested	Expt 1		Expt 2	
	NADH-diaphorase (ΔOD per min per mg of protein[a])	5′-Nucleotidase (μmoles of PO_4 per mg of protein released per hr)	Ratio of specific activity of 5′-nucleotidase to that of NADH-diaphorase[b]	^{3}H-fucose[c] (counts per min per mg of protein)
Microsomal fraction	0.403	36.92	1.0	11
Dextran 110 band	0.098	86.20	9.6	90
Plasma membrane	0.038	92.80	29.6	151

[a] OD, optical density.
[b] Normalized with respect to that of the microsomal fraction.
[c] Values are expressed $\times 10^4$.

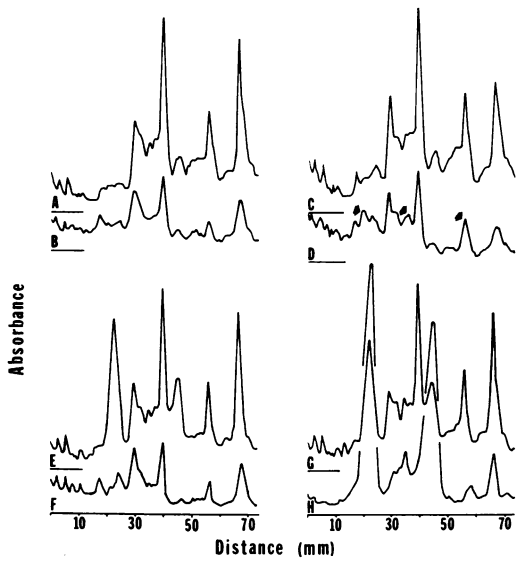

FIG. 1. *Electropherograms of purified plasma membrane proteins of infected and uninfected cells labeled with ^{14}C-amino acids before and after infection. Cells were either infected or mock-infected at 28 hr and harvested at 46 hr after 0 time. The absorbance profiles of a Coomassie Brilliant Blue-stained flat gel (curves A, C, E, G) and of autoradiograms (curves B, D, F, H) developed from the same gel are shown. (A) Absorbance of stained bands of plasma membrane proteins of cells incubated from 0 to 24 hr in a radioactive medium followed by incubation in nonradioactive medium until 46 hr after 0 time; (B) autoradiographic tracing of labeled proteins in A; (C) absorbance of stained bands of plasma membrane proteins of cells incubated from 32 to 46 hr after 0 time in a radioactive medium; (D) an autoradiographic tracing of labeled proteins in C (the arrows point out the proteins reduced in B); (E) absorbance of stained bands of plasma membrane proteins of cells incubated from 0 to 24 hr in a radioactive medium followed by infection at 28 hr and incubation in a nonradioactive medium from 24 to 46 hr after 0 time; (F) an autoradiographic tracing of labeled proteins in E; (G) the absorbance of stained bands of plasma membrane proteins of cells infected at 28 hr after 0 time and incubated in radioactive medium from 32 to 46 hr; (H) an autoradiographic tracing of labeled proteins in G.*

of the bands stained with Coomassie Brilliant Blue and of the autoradiograms developed in the X-ray film. The results may be summarized as follows.

(i) The proteins in the plasma membranes of uninfected cells differed with respect to turnover rate. This conclusion is based on two sets of observations. On one hand, the absorbance of the stained protein bands (Fig. 1A, C) from membranes of uninfected cells labeled between 0 and 24 hr (set 1) and after mock infection between 32 and 46 hr after 0 time (set 3) appeared to be identical. However, minor differences were apparent in the absorbance tracings of their autoradiograms (Fig. 1B, D). Thus, the membranes of the cells labeled after mock infection (set 3), i.e., immediately before harvesting, contained three prominent bands of labeled proteins (Fig. 1D) which were present in reduced amounts relative to others in the autoradiograms of membranes labeled from 0 to 24 hr (set 1).

(ii) The plasma membranes of infected cells contained two sets of protein bands, i.e., proteins characteristically present in uninfected cells and new proteins, absent from uninfected cells. This conclusion is based on the comparison of the absorbance tracings of the stained bands of plasma membrane proteins from infected cells (Fig. 1E, G) with those of uninfected cells (Fig. 1A, C).

(iii) In infected cells, the synthesis of host plasma membrane proteins ceased and only the new membrane proteins were made. This emerged from comparisons of the autoradiographs of the gels of plasma membranes from uninfected cells and infected cells labeled before infection (Fig. 1B, D, F, sets 1, 2, 3) and infected cells labeled after infection (Fig. 1H, set 4). Direct comparison of the autoradiographic absorbance tracings from the gels of infected and uninfected cell membranes (Fig. 2) indicated that infected cell membrane proteins differ in number and electrophoretic mobilities from those of uninfected cell membranes and resemble the electropherograms of the membrane proteins of smooth membranes as published previously (11, 18, 23). In the earlier studies, it was demonstrated that the number and electrophoretic mobility of the membrane proteins made after infection were genetically determined by the virus and hence were virus-specific (11, 23).

(iv) Host proteins were not selectively removed or expelled from the membranes of infected cells. This conclusion is based on comparisons of the autoradiograms from gels of membranes from infected cells labeled before infection, i.e., between 0 and 24 hr (set 2) and uninfected cells labeled during the same interval (set 1). On the contrary, the data suggest that infection may slightly decrease the rate of cellular protein turnover in the plasma membrane. This conclusion is based on two observations. First, the Coomassie Brilliant Blue stain profiles of infected cell membranes (Fig. 1E, G) revealed that the virus-specified proteins were a significant fraction of the total protein in the membranes (approximately 10%). The specific activities of membrane proteins from infected and uninfected cells labeled during the 0 to 24 hr interval prior to infection or mock infection were the same (Table 2). The ob-

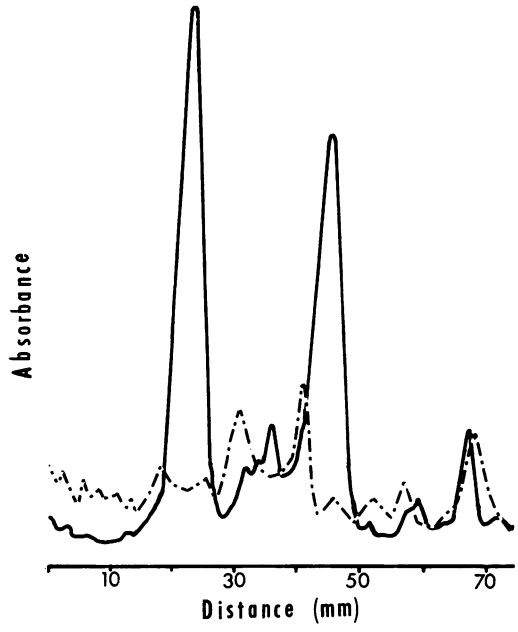

FIG. 2. *Electropherograms of purified plasma membrane proteins of infected cells labeled with ^{14}C-amino acids from 32 to 46 hr (solid line) and uninfected cells labeled as above from 0 to 24 hr (dashed line) past 0 time. The absorbance profiles of the autoradiograms of Fig. 1B and H were superimposed to demonstrate that the electrophoretic mobilities of the virus-specified membrane proteins are different from the membrane proteins of uninfected cells.*

TABLE 2. *Specific activities of ^{14}C-amino acid-labeled protein of purified plasma membrane*

Origin of plasma membrane	Interval of exposure to radioactive medium (hr)	Specific activity (counts per min per mg of protein)
Infected cells	0–24	136.4[a]
Uninfected cells	0–24	132.1
Infected cells	32–46[b]	184.8
Uninfected cells	32–46[b]	161.6

[a] Values are expressed × 10^4.
[b] After 0 time as defined in the text.

servation that this ratio remained constant in the face of measurable entry of new proteins into membranes is consistent with the hypothesis that the rate of host protein removal as a consequence of turnover is reduced after infection.

Nature of the plasma membrane: proteins made after infection. The purpose of these experiments was to determine how many virus-specific membrane proteins were made and bound to the plasma membrane, how many were glycosylated, and how they were related to the structural proteins and glycoproteins of the virion. Approximately 4 × 10^8 cells were labeled either with ^{14}C-amino acids or with ^{14}C-glucosamine, respectively, between 4 and 18 hr postinfection. The cells were then harvested, and the plasma membranes were extracted and purified as described above. The purified plasma membranes were solubilized and subjected to electrophoresis on a flat gel slab concurrently with solubilized proteins from highly purified preparations of enveloped virus labeled with ^{14}C-glucosamine. The batch of purified virus used in this experiment was the same as that used in one of the experiments (Fig. 6C, D) reported in the preceding paper of this series (24). The absorbance tracings of the stained gel and of the autoradiograms of the gels are shown in Fig. 3. The results of this experiment may be summarized as follows.

(i) Comparison of the autoradiographic tracing of the amino acid-labeled proteins in the plasma membrane (Fig. 3C) with the absorbance tracings of the stained bands of the virion proteins (Fig. 3D) suggested that the proteins made after infection and binding to plasma membrane were also present in the virion, albeit in somewhat different proportions. This conclusion was reinforced by comparisons of the autoradiograms of the ^{14}C-glucosamine-labeled proteins in the virion and in the plasma membrane (Fig. 3A, B). (ii) In view of the correspondence of the electrophoretic mobilities of the plasma membrane proteins and glycoproteins with proteins and glycoproteins in the virion, it is convenient to adopt for the plasma membrane structural proteins the numerical designation of the viral structural proteins prefixed with the letters MP. On the basis of examination of the electropherograms presented in this and the preceding paper, we conclude that the plasma membrane of infected cells contains at least 12 proteins, i.e., MP7, MP8, MP11, MP12, MP13, MP14, MP17, MP18, MP19, MP22, MP23, and MP24. The electropherograms (Fig. 3) show an additional band of glycosylated protein which migrated slightly slower than MP22. This band, however, was not found consistently, and we are not certain whether it should be included among the invariant population of virus-specific membrane proteins. The estimated molecular weights of the MP series of viral proteins are summarized in Table 3. Several points should be made in connection with these data. First, the differentiation of proteins MP 7, 8, 11 to 14, and 17 to 19 was based on visual examination of both the autoradiograms and the stained gels containing different concentrations of acrylamide. Second, the molecular

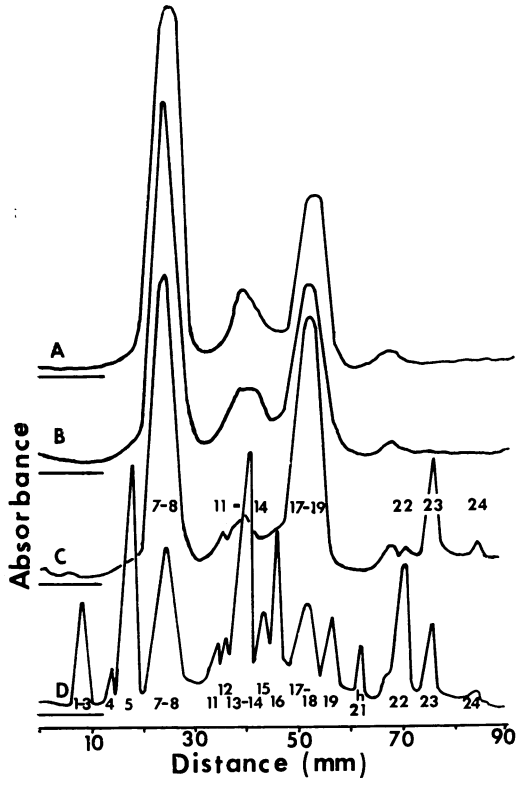

FIG. 3. *Electropherograms of purified plasma membrane proteins of infected cells labeled with ^{14}C-amino acids or ^{14}C-glucosamine and of purified enveloped nucleocapsids of HSV prototype F labeled with ^{14}C-glucosamine. The preparation of purified enveloped nucleocapsids used in this experiment was the same as that subjected to electrophoresis on cylindrical gels and shown in Fig. 6C and D of the preceding paper in this series (24). Note that the resolution obtained in the flat gel slab as shown here is not as good as that obtained in cylindrical gels. In this instance, some of the minor virion proteins are unresolved. The absorbance profiles of Coomassie Brilliant Blue-stained protein bands of the purified virion (D) and of autoradiograms of labeled proteins (A, B, C) are shown. (A) Purified plasma membrane proteins labeled with ^{14}C-glucosamine; (B) purified enveloped nucleocapsid proteins labeled with ^{14}C-glucosamine; (C) a purified plasma membrane protein labeled with ^{14}C-amino acids; (D) stained protein profile of purified enveloped nucleocapsids.*

weight estimates were based on the molecular weight estimates of virion proteins which were co-electrophoresed with six proteins of known molecular weight in gels varying in concentrations of acrylamide from 6 to 14%. Therefore, all of the precision and shortcomings which apply to the molecular weight estimates of the virion proteins as stated (24) also apply to the viral plasma membrane proteins. Third, it seems very likely that MP8, 17, and 18 were extensively glycosylated. We are not certain how many of the proteins in the MP11 to 14 groups were glycosylated, but it seems likely that glucosamine was present in at least three of the proteins in this group. Lastly, it seems pertinent to note that proteins 13, 14, 19, and 22 were present in larger amounts in the virion than in the plasma membrane.

DISCUSSION

This paper deals with the electrophoretic properties of the plasma membrane proteins from herpesvirus-infected cells. In conclusion, the following points should be made.

The procedure for the purification of the plasma membranes used in these studies was selected for two reasons. First, the major constituents of the washed microsomal fraction were cytoplasmic membranes and plasma membranes, which allowed us to monitor the purification procedure with specific enzyme markers. Second, the membrane fractions could be easily separated by alternating the density of the smooth cytoplasmic membrane fragments by divalent cations and followed by centrifugation over a Dextran 110 barrier (10, 28). We chose for monitoring the purification of plasma membranes three markers,

TABLE 3. *Properties of virus-specific membrane proteins and corresponding virion proteins*

Virus-specific membrane protein designation	Corresponding virion proteins	Estimated[a] molecular weight of proteins
MP7	VP7	126
MP8	VP8	
MP11	VP11	93
MP12	VP12	83
MP13	VP13	78
MP14	VP14	
MP17	VP17	59
MP18	VP18	57
MP19	VP19	53
MP22	VP22	37
MP23	VP23	33
MP24	VP24	25

[a] Molecular weights of the viral proteins were estimated as described in the preceding paper of this series (24) by co-electrophoresis of viral proteins with proteins (myosin, β-galactosidase, lactoperoxidase, albumin, ovalbumin, and α-chymotrypsinogen A) of known molecular weight in gels containing 6, 7, 8.5, 9, and 14% acrylamide. The migration of the viral proteins relative to known standards was independent of acrylamide gel concentration, suggesting that mobility was related to size and not to some peculiarity of the conformation. Values are expressed $\times 10^{-3}$.

i.e., 5'nucleotidase, NADH-diaphorase, and fucose. The selection of these markers was based in part on a report of purification of HeLa plasma cell membranes by Atkinson and Summers (1) and in part on the conclusions of several studies summarized by Steck et al. (25) that the best markers for measuring the extent of separation of plasma membranes and of cytoplasmic membranes are the 5' nucleotidase associated with the plasma membrane and the NADH-diaphorase associated with the endoplasmic reticulum which constitutes the bulk of cytoplasmic membranes. The enzyme assays were sensitive, precise, and reproducible. The extent of purification as measured by the extent of separation of the plasma membrane enzyme marker from the endoplasmic reticulum enzyme marker was 26-fold, i.e., as good or better than that reported for other kinds of cells (2, 10, 30). The extent of purification as measured by monitoring fucose was only 14-fold, i.e., somewhat less than that reported by Atkinson and Summers (1). As indicated earlier in the text, this was to be expected since labeled fucose was added before infection, and we cannot be absolutely sure that it labeled or remained bound to host proteins only.

The purification procedure was based largely on the differential physical properties of the endoplasmic reticulum and plasma membranes, whereas identification was based on their enzymatic content. The observation that neither the physical properties of the membrane fraction nor the relative enzymatic content changed after infection suggests that the physical properties of the infected cell membranes do not change and that there is no indiscriminate redistribution of the enzymes or outright exchange of membrane material between the two membrane populations.

The plasma membrane preparations from infected cells contained two sets of proteins, i.e., host proteins and virus-specific proteins. The host proteins were the same as those present in the plasma membranes of uninfected cells. Two points should be made in this connection. First, there was complete cessation of insertion of new host proteins into the membranes; this event was accounted for by the cessation of host protein synthesis described elsewhere (17, 26, 27). Second, we found no evidence for the expulsion, selective or random, of host proteins from the plasma membranes of infected cells.

As indicated earlier, the virus-specific membrane proteins were the only membrane proteins made beginning at 4 hr postinfection. Three points should be made concerning viral membrane proteins. First, the overall electrophoretic profiles of viral proteins in membranes presented in this paper are very similar to those described in the preceding papers of this series except that the higher resolution of the improved acrylamide gel electrophoresis technique revealed more bands. The second point concerning the viral membrane proteins is that despite the increased resolution we are still not entirely certain both with respect to their number or with respect to the proportion that is glycosylated. The electropherograms showed that the plasma membranes contained appreciable amounts of at least 12 proteins of which 9 are glycosylated. We could not exclude trace amounts of other proteins or that other proteins are also glycosylated. Indeed, the extraction of glycoproteins from whole infected cells yielded appreciable amounts of two glycoproteins with electrophoretic mobilities of virion proteins 15 and 16, respectively, suggesting that these two proteins were also glycosylated but not present in appreciable amounts in plasma membrane (Savage, Heine, and Roizman, *manuscript in preparation*). The last point is that the electrophoretic mobilities of virus-specific proteins and glycoproteins present in the plasma membrane could not be differentiated from those present in the highly purified virion preparations described in the preceding paper of this series (24). Several lines of evidence indicate that in the virion the virus-specific glycoproteins were structural components of the envelope derived usually, but not exclusively, from the inner lamella of the nuclear membrane (3, 22).

In contrast to the plasma membrane, the envelope contained only viral proteins and no appreciable amounts of host proteins (24). This observation raised two related questions. The first is whether the envelope was derived from a newly synthesized adjunct to the nuclear membrane or from a region of the nuclear membrane from which host proteins had been expelled. We should point out that, although we were not able to detect appreciable expulsion of host proteins from the plasma membrane, it is conceivable that such an expulsion did take place in the nuclear membrane, perhaps at the time the nuclear membrane became morphologically altered (3, 22). This usually occurred at the time the nucleocapsid came in apposition to the nuclear membrane (17, 22). The second question arises directly from the observation that in the envelope the virus-specific proteins could by themselves form the structural edifice for a trilaminar membrane. Specifically the question arises as to whether in the plasma membrane the virus-specific membrane proteins are in juxtaposition and occupy a topologically unique membrane space or patch or whether they are dispersed throughout the plasma membrane. We cannot answer this question at present. In principle, however, the structural integrity of membranes requires specific interaction of membrane proteins. It is clear that such inter-

action exists among viral proteins in the envelope, and, if the viral proteins are dispersed throughout the plasma membrane, similar interactions must also exist between viral and host membrane proteins.

As indicated earlier, the sequential findings which ultimately led to these studies began with the observation that the plasma membranes of infected cells contain new surface antigens and these cannot be differentiated from the antigens present on the surface of the virion (15, 21). Subsequently, it was shown that infected cell membranes of defined physical properties but of unknown topology contained virus-specific glycoproteins similar to those present in the virion (23) and that these membranes reacted with antiviral sera (19). The data presented in this paper are the first direct demonstration of virus-specific proteins in the plasma membrane and the first direct link between surface immunological specificity and structure of the membranes and of the virion. It remains to be shown which virus-specific proteins are responsible for the modification of the plasma membrane, resulting in the altered immunological specificity and social behavior of infected cells.

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