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Herpesvirus Glycoprotein Synthesis and Insertion into Plasma Membranes

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In the presence of the antibiotic tunicamycin (TM), glycosylation of herpes simplex virus glycoproteins is inhibited and non-glycosylated polypeptides analogous to the glycoproteins are synthesized (Pizer et al., J. Virol. **34**:142–153, 1980). The synthesis of viral proteins and DNA occurs in TM-treated cells. By electron microscopy, nucleocapsids can be observed both in the nucleus and the cytoplasm of TM-treated cells; a small number of enveloped virions were observed on the cell surface. Analyses of the proteins in partially purified virus readily detects viral glycoproteins in the control cells, but neither glycoproteins nor non-glycosylated polypeptide analogs were observed in the virus prepared from TM-treated cells. By labeling the surface of infected cells with ^{125}I, viral glycoproteins were detected as soon as 90 min after infection even when protein synthesis was inhibited with cycloheximide and glycosylation was blocked with TM. Labeling the proteins synthesized in infected cells with [^{35}S]methionine showed that the surface glycoproteins detected in the cycloheximide- and TM-treated cells were not synthesized de novo after infection, but were placed on the cell surface by the infecting virus. Studies with metabolic inhibitors and a temperature-sensitive mutant blocked early in the infectious cycle showed that glycoproteins gA/gB and gD were synthesized soon after infection, but that the synthesis of gC was delayed. Under conditions of infection, in which gC and its precursor pgC are not produced, we have been able to observe the relationships between the glycosylated polypeptides that correspond to pgA/pgB and the nonglycosylated analog made in the presence of TM.

We have reported that the antibiotic tunicamycin (TM) blocks the synthesis of herpes simplex virus (HSV) glycoproteins and the production of infectious progeny (32). Similar results with TM have been observed previously with enveloped RNA viruses (8, 21, 38, 45, 47). Investigations of viral glycoprotein maturation have shown that glycosylation is not an obligatory requirement for the insertion of polypeptide chains into membranes (11–13, 35, 46), and that, depending on the virus strain and temperature of infection, virions may be produced (13, 22, 29). These studies, coupled with the report by Damsky et al. (7) describing the continued appearance of membrane proteins on the surface of TM-treated BHK cells, prompted us to reinvestigate the effect of TM on HSV infection. We were concerned with the possibility that TM was not blocking virus maturation, but that virions lacking the carbohydrate portion of the glycoproteins were being released from the cells as noninfectious progeny. This situation has been reported by Sarmiento et al. for a temperature-sensitive (*ts*) mutant of HSV altered in glycoprotein B (gB), which attaches to cells but fails to penetrate (37). Also having bearing on this question is a recent paper by Katz et al., which shows a single electron microscope picture of a TM-treated cell with virions indistinguishable from those seen in a control cell (18). However, in spite of careful examination of the data it was difficult to determine whether the virus in the TM-treated sample was intracellular or extracellular in nature.

To evaluate the possibility that in TM-treated cells noninfectious virions are being produced which contain the non-glycosylated analogs of the glycoproteins, we investigated viral protein and DNA synthesis and examined infected cells for intracellular virus by electron microscopy. The appearance of viral polypeptides in cell extracts, membranes, and virus preparations was followed by means of a specific antiserum against viral membranes.

From our experiments we have drawn the following conclusions: (i) viral polypeptides, with the exception of the glycoproteins, and viral DNA are synthesized in appreciable amounts in TM-treated cells; (ii) viral nucleocapsids can be readily observed in the nucleus

and cytoplasm of TM-treated cells, whereas unenveloped virions are observed in the cytoplasm and a small number of enveloped virions are observed on the cell surface; (iii) the nonglycosylated analogs of the viral glycoproteins are not inserted into the surface membrane of the cell, nor do they become associated with virion-like structures seen in the cytoplasm in sufficient quantities to be detected by our methods; (iv) viral glycoproteins present on the surface of infected cells early after infection or when de novo protein synthesis is inhibited are placed on the cell by the infecting virus; and (v) the glycoproteins and their non-glycosylated analogs appears to be synthesized early after infection with gA, gB, and gD, preceding gC.

It has been generally accepted by investigators studying the glycoproteins of HSV that the four major species in the virus, gA, gB, gC, and gD, have corresponding precursor forms in the cell: pgA, pgB, pgC, and pgD (10, 17, 40, 43). The labeling conditions we use give mainly the precursors, and pgA/pgB appear after electrophoresis as one band migrating close to pgC. By using conditions of infection under which pgC is not produced, it has been possible to identify a single non-glycosylated analog to pgA/pgB and to confirm that an 85,000-dalton (85K) polypeptide is the non-glycosylated analog to pgC.

MATERIALS AND METHODS

Conditions for infection. HSV type 1 (strain HF) was used in this study with BHK-C13 cells as the host. Because the procedures for obtaining virus, growing cells, and performing the infection have previously been described in detail (32, 44), only a brief description of the experimental conditions will be given below. BHK-13 cells at approximately 75% confluence were infected at a multiplicity of 50 PFU/cell. After 90 min of adsorption the virus was removed and the cultures were overlaid with minimal Eagle medium supplemented with 1% fetal bovine serum and 100 μm arginine. TM at 2 μg/ml was added at this time to the antibiotic-treated cultures. Cycloheximide (CH) was added to the cell cultures at a final concentration of 200 μg/ml 30 min before virus infection. Phosphonoacetic acid, a gift from H. Isom, Pennsylvania State Medical Center, Hershey, Pa., was used at a final concentration of 500 μg/ml and was added to cell cultures 30 min before infection. The temperature-sensitive mutant HSV-1(KOS)B2 and the parent HSV-1(KOS) were obtained from P. Schaffer, Harvard Medical School, Boston, Mass. (5, 43). Virus stocks were grown and titrated in a line of rabbit skin cells obtained from J. Gerdes, University, of Colorado Medical School, Denver.

Conditions for labeling with radioactive compounds. The conditions for labeling cells with radioactive [^{36}S]methionine ([^{35}S]Met) and [^{3}H]thymidine [^{3}H]TdR (New England Nuclear Corp., Boston, Mass.) have been described in our earlier publications (32, 44). The specific information on the quantity of isotope used and the periods of time that the infected cells were exposed to the radioactive compounds has been presented in the text describing each experiment or in the legends to the figures.

Surface labeling of cells with Na ^{125}I was carried out using the procedure described by Markwell and Fox (26). The infected cells in 60-mm petri plates were prepared for labeling by removing the culture medium, washing with phosphate-buffered saline, and overlaying each plate with 3 ml of phosphate-buffered saline containing 5 mM glucose and 500 μCi of Na ^{125}I (New England Nuclear Corp.). The iodinating compound (Iodogen-Pierce Chemical Co., Rockford, Ill.) was coated onto cover slips (100 μg/cm^2) and floated on top of the cells for 10 min at 25°C. The cover slips and the radioactive phosphate-buffered saline were then removed and the cells were thoroughly washed with a phosphate-buffered 7.5% solution of NaI.

Fractionating procedures. Cell extracts were prepared by treating cells with 0.6 ml of lysing buffer containing 50 mM NaCl, 20 mM Tris-hydrochloride (pH 7.5), 0.5% Nonidet P-40, and 0.5% sodium deoxycholate. Nuclei were removed by centrifugation at 10,000 × *g*, and the supernatant fluids were stored at −20°C.

Antiserum against viral envelopes (anti-env serum) was used to precipitate glycoproteins by the *Staphylococcus* (Staph A)-assisted precipitation procedure (2, 10, 32). A two-step procedure was used in which the radioactive cell extracts were pretreated with nonimmune sheep serum and Staph A immune adsorbant (32). The Staph A immune adsorbant was removed by centrifugation, and anti-env serum was added to the supernatant fluids. Subsequently, additional Staph A was added, and the antigen-antibody complexes that adhere to the Staph A were thoroughly washed with 50 mM Tris buffer (pH 7.5), containing 1% Nonidet P-40, 0.5% deoxycholate, and 150 mM NaCl, before being dissociated with electrophoresis buffer containing 3% sodium dodecyl sulfate and 2% mercaptoethanol. Dissociation was at 100°C for 3 min, and the Staph A was removed by centrifugation. Polyacrylamide gel analyses of solubilized immune precipitates, cell extracts, or purified virus were carried out by the method described earlier, using a 12.5% gel crosslinked with *N,N'*-diallyltartardiamide (32). Viral DNA labeled with [^{3}H]TdR was separated from cellular DNA by equilibrium sedimentation in CsCl gradients (44), and cytoplasmic virions were purified by sedimentation on Dextran gradients as described by Spear and Roizman (42). Extracellular virus was obtained from the culture medium by sedimentation at 120,000 × *g* for 2 h. The methods for preparing cells for electron microscopy have been published along with detailed electron microscopic analyses of HSV-infected and TM-treated BHK cells (7, 33).

RESULTS

Synthesis of viral macromolecules. Our previous experiments studying the effect of TM on HSV infection demonstrated that whereas glycoproteins failed to mature, other viral proteins were synthesized in the presence of the antibiotic. In our earlier publication, we made no attempt to enumerate the viral polypeptides synthesized or to quantitate the amounts made in

TM-treated cells. To obtain these data, cells were labeled with [^{35}S]Met for 30 min, 5 h after infection, and cytoplasmic extracts were prepared. Samples of these extracts (20 μl), containing 290,000 cpm for the control cell extract (Fig. 1A) and 260,000 cpm for the TM-treated extract (Fig. 1B), were subjected directly to electrophoresis. Other samples (200 μl) were treated with anti-env serum, and the immune precipitates obtained are shown in Fig. 1, lanes C and D. The tracing of the radioautographs (Fig. 1A and B) shows that with the exception of the glycoproteins (peaks 7, 8 and 17, 18), the viral polypeptides are very similar in number and quantity. Extraction of the nuclear pellet with buffer containing 1% sodium dodecyl sulfate gave similar patterns and failed to detect additional polypeptides in the TM-treated cells. To quantitate the viral polypeptides in lanes C and D, the bands were eluted from gel slices and the radioactivity was counted. The data obtained (not shown) demonstrated the absence of glycoproteins in TM-treated cells, indicating that under our standard conditions the block in glycosylation was complete by 5 h after infection.

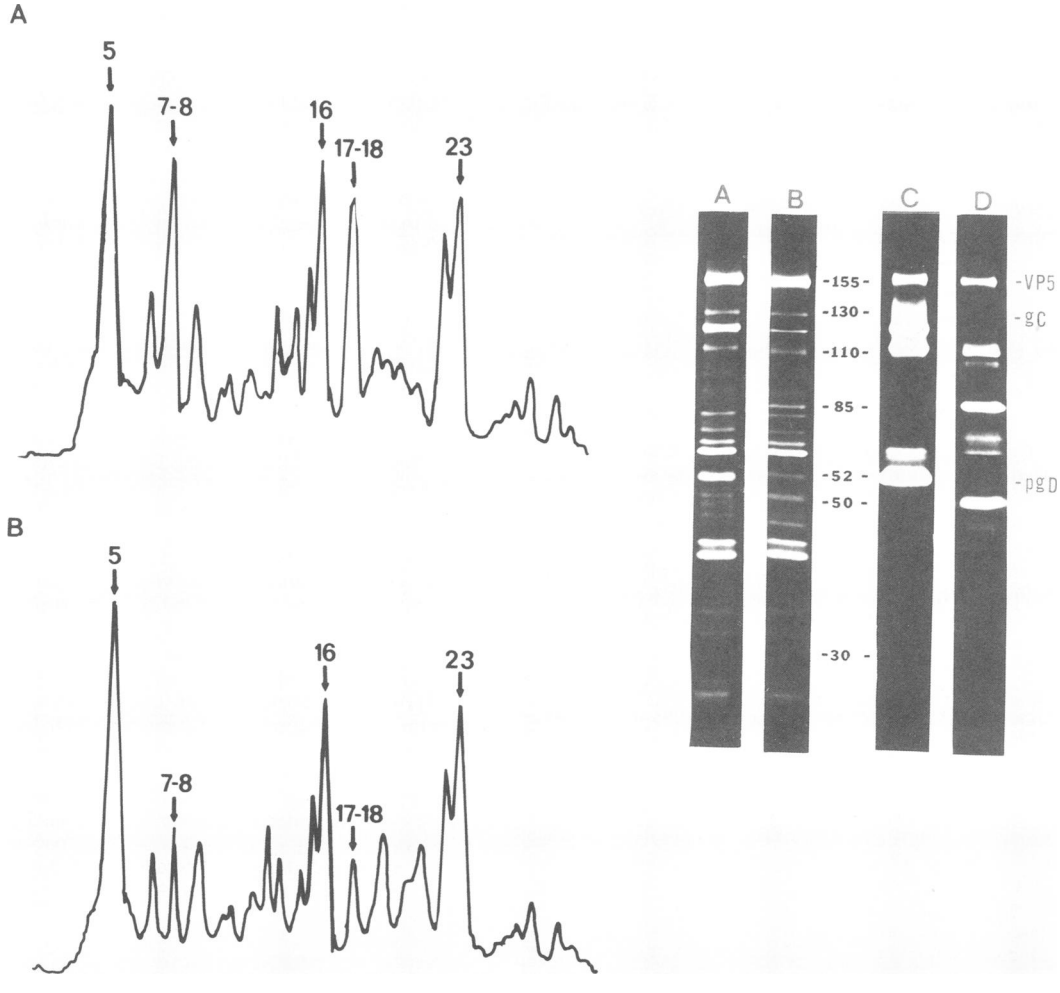


FIG. 1. Densitometer tracing of [^{35}S]Met-labeled polypeptides separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cells were labeled with 200 μCi/ml for 30 min at 5 h postinfection, and cytoplasmic extracts were prepared. After electrophoresis, the gel was fluorographed, and the radioautograph was subsequently scanned with a densitometer. Control cell extract is shown in (A); TM-treated extract is shown in (B). The numbers above the peaks are those used by Spear and Roizman (42). Beside the tracings are shown prints of the radioautograph obtained with cytoplasmic extracts (A, control extract; B, TM-treated cell extract) and the immune precipitates obtained with anti-env serum (C, control; D, TM-treated cell extract).

The incorporation of tritiated TdR into DNA was not affected by TM. When infected cells were labeled with [^{3}H]TdR (100 μCi/ml) from 4 to 6 h after infection, approximately 2×10^5 cpm of thymidine per 10^6 cells was incorporated into the total DNA from both the TM-treated and the control cultures. In both cases, sedimentation in CsCl gradients demonstrated that approximately 50% of the DNA synthesized in the 2-h period was viral (data not shown). To find out whether the DNA was contained in cytoplasmic virus-like structures, we prepared cytoplasmic extracts 18 h after infection from [^{3}H]TdR-labeled cells. The [^{3}H]TdR was added to the cultures 90 min after infection at a concentration of 100 μCi/ml, and extracts were fractionated on 11 to 30% Dextran gradients designed for virus purification. For both the control and the TM-treated culture a peak of radioactive DNA was found at the position for virus, but the amount of radioactivity was reduced by 60% in the extract from TM-treated cells (Fig. 2).

Electron microscopic examination of infected cells. Examination of thin sections of HSV-infected cells in the electron microscope showed differences between the TM-treated culture and controls. Several sections from 13 cells prepared from the TM-treated culture and 8 cells from the control were used for this study. By 12 h after infection, enveloped virus was readily found both inside and on the surface of the control cells (Fig. 3A). In TM-treated cells (Fig. 3B) nucleocapsids were abundant in the nucleus, and also were unexpectedly found in the cytoplasm, but extracellular virus was difficult to find. Higher magnification showed that 75% of the control cells contained enveloped virions in the cytoplasm (Fig. 3C). In contrast, all the TM-treated cells had naked capsids in the cytoplasm (Fig. 3D), and close examination showed that half the cells had a very low number of enveloped virions inside and outside the cell (Fig. 3D). The virions on the surface of the cell may be remnants from the infecting inoculum that did not penetrate the cell membrane. Because of the presence of nucleocapsids and a few enveloped

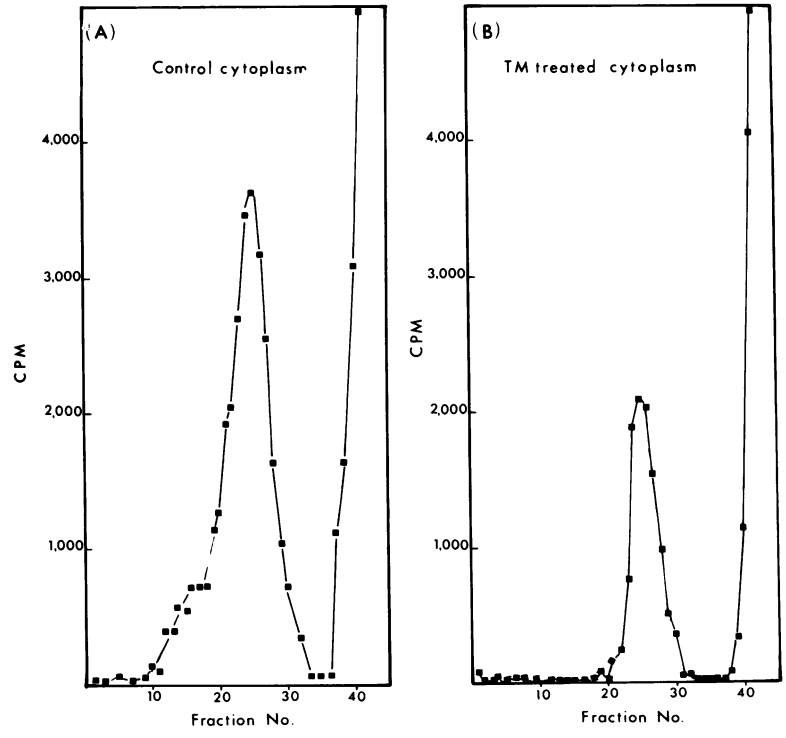


FIG. 2. Sedimentation of [^{3}H]TdR-labeled virion structures from cytoplasmic extracts. About 2×10^7 cells were infected with 50 PFU/cell, and after 90 min of adsorption the virus was removed, medium containing 10 μM Met was placed over the cells, and TM was added to the experimental culture. At 4 h postinfection, 200 μCi of [^{3}H]TdR was added to the 20 ml of medium used to overlay each plate of cells, and the infection was allowed to proceed for 14 h. The cells were then harvested, and cytoplasmic extracts were prepared as for the isolation of virus. Samples of 1.0 ml were placed on 16-ml 11 to 30% Dextran gradients, and the gradients were centrifuged at 20,000 × *g* in an SW27.1 centrifuge rotor for 90 min. From each gradient 50 fractions were collected, and the radioactivity in 20 μl of each fraction was counted in Aquasol (New England Nuclear Corp.).

TABLE 1. Radioactive [^{35}S]Met recovered in cellular and virus fractions

Fraction	Total radioactivity incorporated[a] (cpm × 10^5)		Radioactivity in sample before immune precipitation[b] (cpm × 10^3)		Radioactivity in immune precipitate[c] (%)	
	−TM	+TM	−TM	+TM	−TM	+TM
Cytoplasmic extract	230	223	1,500	1,275	2.3	0.6
Extracellular virus	64.50	10.75	645	430	6.7	0.8
Cytoplasmic virus	7.80	2.25	520	150	10.0	3.4

[a] The acid-precipitable radioactivity present in 20-μl aliquots of each fraction was determined and used to calculate the total radioactivity incorporated in each fraction.

[b] The radioactivity present in samples from control cell and TM-treated cell fractions that were subjected to immune precipitation with anti-env serum.

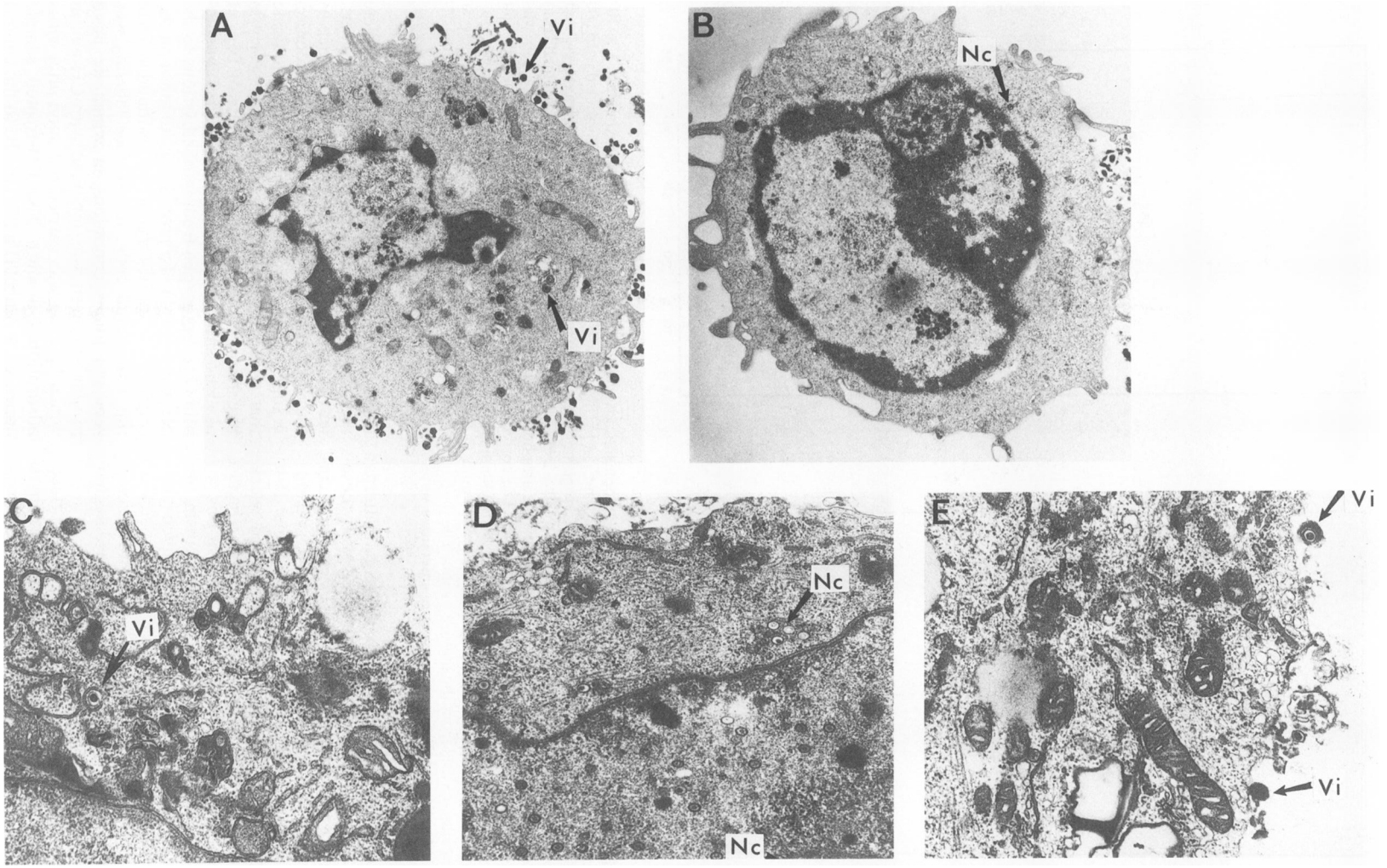
[c] The radioactivity present in 5-μl aliquots of the immune precipitates was measured. The data are expressed in the table as the percentage of the total radioactive extract treated with antiserum. The remaining immune precipitate (50 μl) was analyzed by polyacrylamide gel electrophoresis (Fig. 5).

virus particles in the TM-treated culture, we wished to know whether non-glycosylated analogs to the glycoproteins were being inserted into membranes and whether they were associated with virus particles.

Comparison of virions in TM-treated and control cells. To analyze the proteins in the small amount of virus produced in TM-treated cells, we modified the isolation procedure to include carrier virus from control cultures not treated with TM. Large (150-mm) plates of cells were infected with HSV and subsequently labeled with [^{3}H]TdR. Single dishes of infected cells (100 mm) were either treated with TM or served as a control and, 5 h after infection, were labeled with [^{35}S]Met for 12 h. The [^{35}S]Met-labeled plates were harvested together with the [^{3}H]TdR-labeled plates, and virus was purified from both the cytoplasm and the extracellular medium. The sedimentation patterns for the cytoplasmic virus purified on Dextran gradients are shown in Fig. 4A and B. The amount of [^{3}H]TdR incorporated into cytoplasmic nuclear fraction and fractions containing virus was the same for both cultures, indicating that the recovery of carrier [^{3}H]TdR-labeled virus was the same in both TM and control preparations. Although total [^{35}S]Met incorporation was essentially the same in both cell extracts, the incorporation into the cytoplasmic and extracellular virus fractions was appreciably reduced in the TM-treated cells (Table 1). We used anti-env serum to look for glycoproteins and their non-glycosylated analogs in the virus peak purified on the Dextran gradients and in the extracellular virus fraction. The amount of radioactivity in the immune precipitates (Table 1) indicates that the virus fractions from TM-treated cells contain reduced amounts of glycoprotein. Polyacrylamide gel electrophoresis analyses of these precipitates (Fig. 5) readily detected glycoproteins in the control cell extract and control virus preparations. These bands were not found in the virus preparation from TM-treated cells, but the non-glycosylated analogs of the glycoproteins (i.e., 50K, 85K, and 105K polypeptides) (32) were detected in cell extracts. These polypeptides were not seen initially in the preparations of virions. However, a longer exposure of this gel (30 days) permitted visualization of the 85K and 110K non-glycosylated polypeptides in the cytoplasmic virus preparation, but not in the extracellular virus fraction. These weak bands could result from cytoplasmic contamination of intracellular virus or inefficient incorporation of the non-glycosylated polypeptides into virion structures.

Radioiodination of infected cell surfaces. Even though we could not detect significant quantities of non-glycosylated glycoprotein analogs in virus particles, the important question remained as to whether these viral polypeptides could be inserted into the cell surface membranes. To test for the presence of the non-glycosylated polypeptide analogs on the surface of TM-treated cells, radioiodination of intact cells was carried

FIG. 3. Electron micrographs of thin sections of BHK cells after HSV infection. Cultures were fixed and sections were prepared 12 h after infection. A and B were photographed at a magnification of ×19,000, using the procedures described in reference 22. C, D, and E were taken at a magnification of ×42,000, using the procedure described in reference 2. Control cultures were used for A and C, and TM-treated cultures were used for B, D, and E. Vi, Enveloped virus; Nc, nuclear capsid of virus.


A
Vi
Vi
B
Nc
C
Vi
D
Nc
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E
Vi
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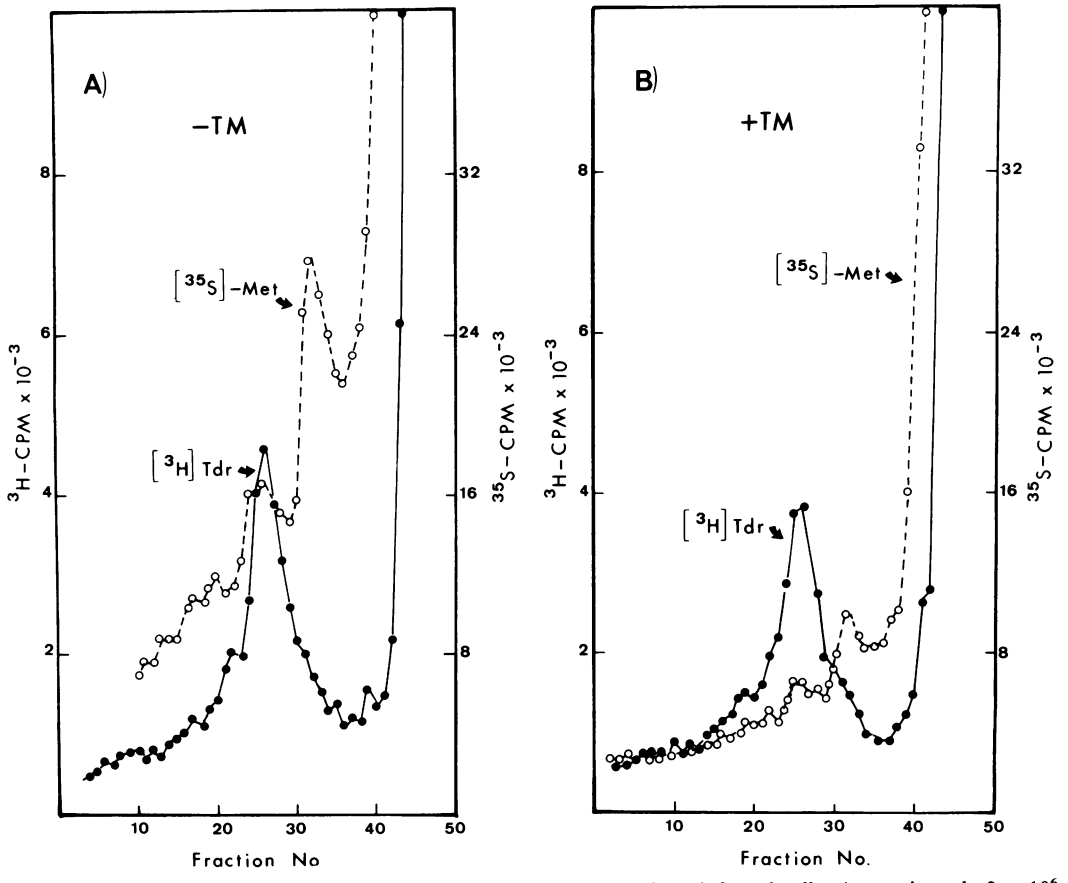

FIG. 4. Dextran gradient sedimentation of cytoplasmic extracts from infected cells. Approximately 3×10^6 cells labeled in 20 μM Met medium for 15 h with 1 mCi of [^{35}S]Met were mixed with 5×10^7 cells labeled with 300 μCi of [^{3}H]TdR for the same period of time. Cytoplasm and nuclei were prepared from the cell mixtures, and cytoplasmic extracts were sedimented on 11 to 30% Dextran gradients. Samples of the fractions obtained from the gradient were counted in a scintillation spectrophotometer, and the radioactivity in each fraction was corrected for [^{35}S]Met in the [^{3}H]TdR channel. The corrected profiles are displayed in (A) for control cells and (B) for TM-treated cells. Fractions 20 to 30, containing the viral components, were pooled, and virus was collected by sedimentation at 120,000 × *g* for 120 min. The same sedimentation conditions were used to harvest extracellular virus from the culture media. The preparations containing the cytoplasm from the control and TM-treated cells had the same number of infectious virus particles as measured by plaque assays.

out at 6 and 24 h after infection. The gel patterns of the immunoprecipitates obtained with these cells (Fig. 6) show radioactive glycoprotein bands in the control infected cells at 6 h postinfection and much stronger bands in the 24-h extract. The TM-treated cells showed weak bands in the 6-h extract and essentially no bands 24 h after infection. Longer exposure of this gel detected small amounts of glycoprotein in the 24-h TM-treated extract, but the non-glycosylated polypeptide analogs easily found in the cytoplasm of cells labeled with [^{35}S]Met and infected under the same conditions were never detected on the cell surface.

The presence of glycoproteins on the surface of the TM-treated cell 6 h after infection is consistent with an incomplete block in glycosylation. It is also consistent with incomplete removal of adsorbed virus or the infecting virus, leaving envelope proteins on the cell surface as they penetrate the cell. The latter interpretation is supported by immunological data that indicate that viral antigens are present on cells early in infection (30, 39), and by the results of Para et al., who showed the transfer of the immunoglobulin G receptor (gE) from purified virions to the cell surface (31). To provide information on the origin of the surface glycoproteins found at 6 h in the TM-treated cells, the surface labeling was repeated under conditions in which de novo protein synthesis was inhibited with CH and in which labeling was carried out soon after virus adsorption (90 min). The CH-treated sample appeared identical (Fig. 7, lane B) to the control

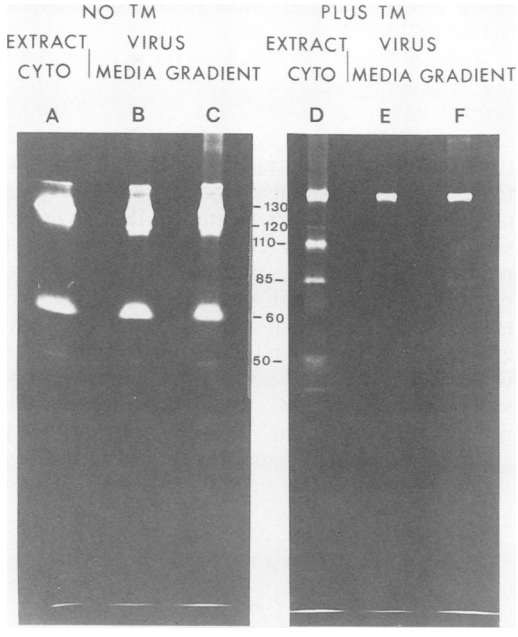

FIG. 5. Immune precipitation of viral glycoproteins from infected cells and virus preparations. Samples of cellular extracts and fractions containing viruses were solubilized with a mixture of buffered detergents (0.5% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris-chloride, pH 7.5) and subjected to the Staph A-assisted immune precipitation procedure. The washed immune precipitates were electrophoresed, and the dried gel was fluorographed. Approximately the same amount of radioactivity was used for immunoprecipitation of the TM-treated and control samples. For the cytoplasmic extracts (lanes A and D) 15×10^5 cpm was treated with anti-env serum, and for the extracellular virus fractions (lanes B and E) 5×10^5 cpm was used. For the virus fractions from the gradients, 5×10^5 and 1.5×10^5 cpm were used from the control cell extract and the TM-treated cell extract, respectively. The radioautogram has been exposed for different periods of time to detect the weaker bands in the samples from the TM-treated cells. The radioautogram was exposed for 3 days with XRS fast film to detect the bands in the control lanes A, B, and C. Thirty days of exposure was used with slow SRP film to detect the bands shown in lanes D, E, and F.

cells labeled immediately after adsorption (Fig. 7, lane A) and the TM-treated extract labeled 6 h after infection (Fig. 7, lane D). Pretreating cells with TM for 4 h before infection gave the same pattern (data not shown). The control cell extract, labeled 6 h after infection (Fig. 7, lane C), showed the expected increase in the amount of glycoproteins on the cell surface. Quantitative measurements of the radioactivity in the glycoproteins were made by cutting the protein bands from the gel and measuring the ^{125}I radioactivity in a γ counter. The data obtained by counting the bands were in complete accord with the visual impression given by the radioautogram (Fig. 7) and showed that the input virus in the absence of protein synthesis leaves a considerable amount of viral glycoprotein on the cell surface. By 6 h after infection, the amount of glycoprotein roughly doubled in the control cultures, but in the TM-treated cells it remained unchanged.

Appearance of glycoproteins synthesized de novo early after infection. To establish when after infection the synthesis of the glycoproteins starts, we have used metabolic inhibitors that block protein and DNA synthesis as well as a *ts* DNA^- mutant (43). The results of the experiment with the inhibitors (Fig. 8) show that barely detectable amounts of the precursors to the glycoproteins gA, gB, and gD (Fig. 8, lane C) were produced 30 min after reversing a CH block. Although the bands are difficult to visualize, cutting the bands from the gel and counting

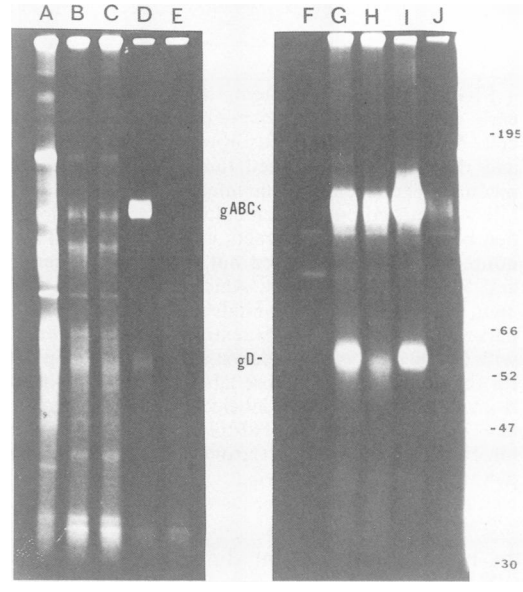

FIG. 6. Labeling of surface proteins in intact HSV-infected cells. The surface components of infected cells were labeled with ^{125}I while they remained attached to the matrix of the petri plates. After thorough washing, the radioactive cells were removed from the dish and solubilized, and a portion of each extract was subjected to immune precipitation. Samples of the extracts (20 μl) are shown in lanes A through E. Immune precipitates from 100 μl of these extracts are shown in lanes F through J. The radioautogram was developed with the aid of intensifying screens for 1 day to detect lanes A through E and for 5 days for lanes F through J. Lanes: A and F, uninfected cell extract; B and G, control cell extract 6 h postinfection; C and H, TM-treated cell extract 6 h postinfection; D and I, control cell extract 24 postinfection; E and J, TM-treated cell extract 24 h postinfection.

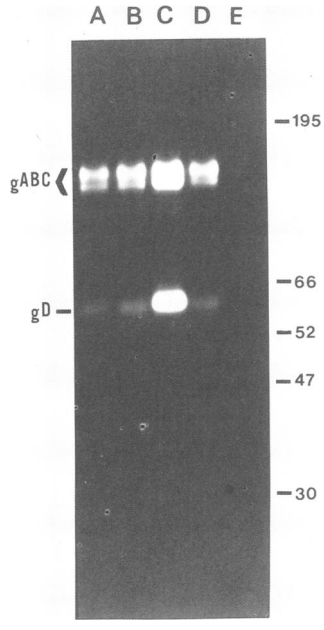

FIG. 7. Viral glycoproteins in the cell membrane early after infection. Cells were infected with 50 PFU/cell. After 90 min the unadsorbed virus was removed and the cells were washed three times with warm medium. The labeling of the infected cell surface with ^{125}I was carried out in each culture at the time specified below. After cell extracts were prepared, immunoprecipitation was carried out with anti-env serum and 50 μl of each extract, which contained 2×10^6 cpm. Lane A, Extract from infected cells labeled and harvested at 90 min; lane B, extract from cells treated with CH 30 min before infection and maintained in CH for the 90-min period before labeling; lane C, extract from the control culture labeled 6 h after infection; lane D, extract from the TM-treated culture 6 h after infection; lane E, extract from the uninfected cell culture.

DNA and that gA, gB, and gD are synthesized before gC.

As an independent check on these results, we investigated glycoprotein synthesis after infection of cells with a DNA$^-$ *ts* mutant blocked early in the infectious cycle. The radioautograph (Fig. 9) shows the immune precipitates obtained from cells infected at 34 and 39°C. The data obtained with the *ts* mutant HSV(KOS)B2 shows that pgA/pgB (120K) and pgD (52K), but not pgC (110K), were synthesized at 39°C. Comparison of the control and TM-treated cell extracts shows that non-glycosylated analogs to gD and gA/gB are found with molecular weights of 50K and 105K, respectively. The non-glycosylated analog to gC (85K) and the normal precursor to gC, pgC (110K) (32), are only synthesized at the premissive temperature.

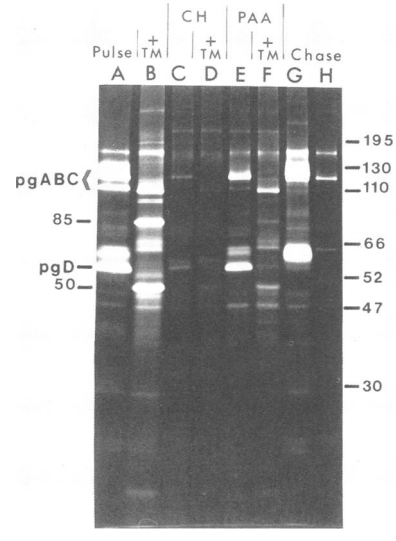

FIG. 8. Effect of metabolic inhibitors on glycoprotein production. Cell cultures to be infected in the presence of CH or phosphonoacetic acid (PAA) were incubated in medium containing 10% Met 2 h before infection and were treated with the compounds 30 min before the addition of virus, during the 90-min adsorption period, and during the 5-h incubation period before labeling. At 5 h postinfection the CH block was reversed by washing with warm medium, and all cultures were labeled for 30 min with 100 μCi of [^{35}S]Met per plate. TM, when present, was added at 90 min after infection, when the unadsorbed virus was removed. The preparation of cell extracts and immunoprecipitation were carried out as described in the text. The immunoprecipitates shown in lanes A through G correspond to the following cell extracts: A, no inhibitor (control); B, plus TM; C, CH; D, CH plus TM; E, PAA; F, PAA and TM; G, no inhibitor, with the 30-min pulse with [^{35}S]Met followed by a 5-h chase.

the radioactivity present showed that pgA/pgB (120K) contained 900 cpm and pgD (52K) contained 1,500 cpm above background. The non-glycosylated analogs to gA, gB, and gD were found in the cultures treated with both TM and CH (Fig. 8, lane D). These bands at 50K and 105K both contained 400 cpm. In neither culture was the precursor to gC, pgC, detected on the radioautograph. In the extracts from cultures treated with phosphonoacetic acid (Fig. 8, lanes E and F), the quantity of glycoprotein and the corresponding non-glycosylated polypeptides was reduced as compared with the extracts from cells making viral DNA (Fig. 8, lanes A and B), but bands were found on the gel corresponding to the precursors for all the glycoproteins, including gC. These results indicate that glycoprotein synthesis starts before the synthesis of viral

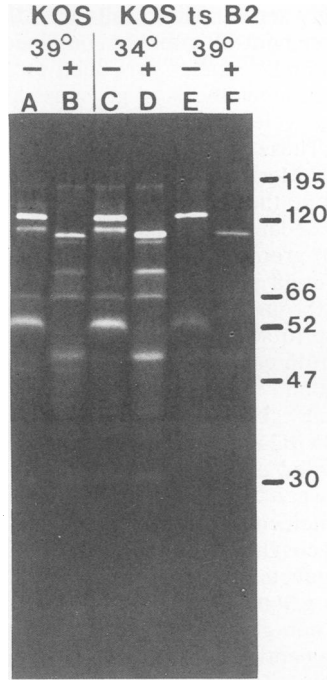

FIG. 9. Immune precipitation of glycoproteins from extracts of cells infected with HSV-1(KOS)B2. Cells were infected at 34 or 39°C with the parent HSV(KOS) or the temperature-sensitive mutant HSV(KOS)B2. Five hours after infection, the cells were labeled with [^{35}S]Met for 30 min, and extracts were prepared. The radioactivity incorporated was approximately the same in all the extracts, and a 100-μl sample containing 2×10^6 cpm was treated with anti-env serum. Lanes A and B contain the immune precipitates from cells infected with wild-type strain KOS. Lanes C, D, E, and F contain the immune precipitates from cells infected with the *ts* mutant (KOS)B2. The presence of TM in the culture is indicated by the + sign above lanes B, D, and F.

DISCUSSION

The experimental results described in this report extend our previous observations on the effect of TM on HSV production (32). In the absence of mature viral glycoprotein synthesis, other viral polypeptides are produced and viral DNA is synthesized. Because the biosynthesis of viral macromolecules continues in TM-treated cells, it was not surprising to see virus structures in the cell, but it was unexpected to find relatively large numbers of nucleocapsids in the cytoplasm at a time where the nuclear membrane appears to be undamaged. The occurrence of the nucleocapsids in the cytoplasm of TM-treated cells indicates that viral glycoproteins are not involved in DNA production or movement of the nucleocapsids from the nucleus to the cytoplasm. Earlier biochemical and morphological experiments using the antimetabolite cytochalasin B demonstrated that under conditions in which viral glycoproteins were not synthesized, nucleocapsids accumulated in the nucleus of infected cells, and the number of enveloped virions in the cytoplasm decreased (9, 25). The straightforward interpretation of these data, that mature glycoproteins are required for nucleocapsid movement into the cytoplasm, is clearly different from the interpretation we draw from our experiment. In discussing the results with cytochalasin B, Dix and Courtney pointed out that the antibiotic alters several aspects of cell structure and transport and that these effects could restrict nucleocapsid movement rather than the absence of glycoproteins (9).

We found very few enveloped virions in TM-treated cells, and it appeared that the particles we saw did not contain detectable amounts of the non-glycosylated polypeptides related to the viral glycoproteins. We do not know whether these virus-like particles utilize cellular glycoproteins in their assembly, but it appears that under our conditions of infection, HSV fails to insert non-glycosylated forms of glycoproteins into membranes. The few nucleocapsids which can be observed may be budding through those patches of membrane which have been modified by the input virus, or they may be using cellular glycoproteins for assembly (23). The virions on the surface could be partially uncoated virions remaining from the infecting virus.

In many respects the synthesis and maturation of HSV glycoproteins appear to follow the same biochemical sequence reported for other glycoproteins. Together with or soon after polypeptide synthesis, carbohydrate residues are added to the polypeptide (10, 17, 40). Subsequently, the glycosylated precursors undergo maturation by altering the structure of the carbohydrate side chains as the glycoproteins migrate through the cell to the surface membranes (10, 17, 19, 40, 41). Our iodination studies of intact cell surfaces indicate that viral glycoproteins or non-glycosylated analogs do not reach the plasma membrane of TM-treated cells. The antigen present on these cells at 6 h after infection must be residual material introduced by the infecting virus. As mentioned above, some of the antigens may be in virions not removed by the washing of the cells. The absence of glycoprotein in the 24-h TM-treated sample, at a time when large amounts are present in the 24-h control, indicates that surface components are shed from HSV-infected cells and that continued replacement of glycoproteins into infected cell membranes is occurring. These results are in keeping with iodination studies of HSV-infected (15) and TM-treated BHK cells (7). Our results are direct

proof for the existence of viral antigens on the cell surface immediately after infection, even when protein synthesis is inhibited, and are consistent with immunological experiments that demonstrate viral surface antigens early after infection (6, 30, 31, 39). They appear to be somewhat inconsistent with the experiments that show that TM blocks the formation of HSV-infected target cells for cytotoxic T cells (1, 20). The apparent inconsistencies probably result from differences in the experimental procedures. To generate the targets for cytotoxic T cells, the multiplicities of infection were low and the time between infection and test of the target cell was long enough (16 h) to permit turnover of the surface membranes with the shedding of the input viral antigens. With TM blocking the insertion of new surface antigens into the membrane of HSV-infected cells, these cells can no longer be recognized by the T-cells involved in cytotoxicity. It should be pointed out that whereas the TM studies indicate that viral glycoproteins are involved in generating the target for cytotoxic anti-HSV T cells, they do not prove that carbohydrate side chains are required for cellular recognition. Because the non-glycosylated polypeptides fail to reach the cell surface, their failure to be involved in cell-cell interaction is most likely due to topological reasons.

Because the non-glycosylated analogs of the viral glycoproteins do not follow the normal maturation sequence which leads to their release from the cell as virus or membrane components, our earlier observation that these polypeptides labeled during a pulse lose radioactivity during a chase can best be interpreted as intracellular degradation. The proteolytic degradation of the non-glycosylated polypeptides appears to maintain a low steady-state intracellular level of these components which might limit their ability to be inserted into membranes or participate in viral maturation. Provided conditions could be found in which degradation of these polypeptides was minimized, a direct test of their role in membrane assembly and intracellular movement of virus could be carried out. Techniques are now available to produce mutants with alterations in specific regions of the genome (2, 36) and to select for mutants defective in glycoprotein synthesis (14, 24). By combining these methods, it should be possible to obtain mutants deficient in specific glycoprotein and test whether individual glycoproteins are required for virus maturation.

Whereas *ts* mutants with blocks in the individual glycoproteins would be useful in studying the stages in viral maturation, the single experiment described in this paper with the early mutant HSV(KOS)B2 has provided useful information on the kinetics of glycoprotein synthesis and relationships between polypeptides precipitated by anti-env serum. The results with the mutant are in agreement with our data obtained with CH and the kinetic studies of Cohen et al., which show that the synthesis of gC starts later in the infectious cycle than that of the other glycoproteins (4). These results, together with the mutant studies of Marsden et al., place the glycoproteins among the "early class" of viral proteins (27, 28). Because mutants in the same complementation group as HSV(KOS)B2 show somewhat different phenotypes (34, 43), it might be possible through the use of other members of this group to separate the synthesis of gA, gB, and gD from each other and study their regulation in detail.

Blocking gC synthesis by infecting with HSV(KOS)B2 at the nonpermissive temperature has provided a clearer picture of the relationships between the polypeptides in the 100,000 to 130,000 molecular weight range. It appears that a non-glycosylated polypeptide of 105K made early in infection corresponds to the 120K precursor to gA/gB. Under permissive conditions, the 85K non-glycosylated polypeptide and the 110K polypeptide (pgC) are synthesized during a 30-min pulse. The relationship between the 85K, the 110K (pC), and the 130K (gC) polypeptides has been previously demonstrated on the basis of tryptic peptide mapping (3, 32). The absence of both the 85K and 110K polypeptides in Fig. 9, lanes E and F, confirms the connection between these molecules. It appears that the non-glycosylated polypeptide analogs and the corresponding glycoproteins are synthesized at the same time after infection. We have not detected additional polypeptides in the immune precipitates that might represent novel glycoprotein precursor made early, and the increase in glycoprotein synthesis observed after DNA synthesis has started is characteristic of many structural proteins destined to become part of the virion (16). In the original paper describing the "cascade regulation" of herpes protein production it was pointed out that some proteins that are placed in the γ class because their rates of synthesis increase after viral DNA synthesis was initiated were synthesized early after infection (16).

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