Synthesis and Processing of Glycoprotein D of Herpes Simplex Virus Types 1 and 2 in an In Vitro System

JAMES T. MATTHEWS,^{1,2,3†} GARY H. COHEN,^{1,2} AND ROSELYN J. EISENBERG^{1,3*}

Center for Oral Health Research¹ and Department of Microbiology,² School of Dental Medicine, and Department of Pathobiology, School of Veterinary Medicine,³ University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received 27 May 1983/Accepted 9 August 1983

We carried out studies of in vitro translation and processing of glycoprotein D (gD) of herpes simplex virus types 1 and 2 by using mRNA from cells infected for 6 h and a reticulocyte lysate translation system. Polypeptides of 49,000 daltons were immunoprecipitated with anti-gD-1 sera. Each in vitro-synthesized molecule had the same methionine tryptic peptide profile as the respective in vivo precursors, pgD-1 and pgD-2. In addition, the polypeptides synthesized in vitro were larger than the corresponding molecules synthesized in the presence of tunicamycin. This suggested that each of the gD polypeptides synthesized in vitro contained a transient N-terminal signal sequence. When the translation mixture was supplemented with pancreatic microsomes, each of the gD polypeptides was converted cotranslationally to a larger-molecular-weight form. Processing involved addition of three *N*-asparagine-linked oligosaccharides and removal of the signal peptide. When trypsin was added after in vitro processing, a polypeptide which was 3,000 daltons smaller than the in vitro-modified form of gD was immunoprecipitated. Experiments with endo- β -*N*-acetylglucosaminidase H showed that this polypeptide still contained the three *N*-asparagine-linked oligosaccharides. Two monoclonal antibodies, 57S (group V) and 17O (group VII), were used to further orient gD in microsomes. The group V determinant was located in the trypsin-sensitive 3,000-dalton fragment, and the group VII determinant was located in the portion of gD which was protected from trypsin. We concluded that gD is oriented with the three glycosylation sites inside the vesicles and that 3,000 daltons containing the group V determinant are located outside. Immunofluorescence studies indicated that the group V determinant of gD is inside the plasma membrane of herpes simplex virus-infected cells and that the group VII determinant is outside. This cellular orientation is consistent with predictions based on the in vitro experiments.

Glycoprotein D (gD) of herpes simplex virus (HSV) is a type-common component of the virion envelope which stimulates production of high titers of virus-neutralizing antibody and is believed to be important in the initial stages of viral infection (5, 6, 8, 12, 34). In previous studies (5, 6, 10, 11, 20, 33, 35, 36), attention was focused on the details of synthesis and processing of gD in HSV type 1 (HSV-1)- and HSV type 2 (HSV-2)-infected cells. These studies indicated that gD is processed from a lower-molecular-weight precursor (pgD-1 for HSV-1 or pgD-2 for HSV-2) to a higher-molecular-weight product (gD-1 or gD-2) in virus-infected cells. This increase is due to the addition of three *N*-asparagine-linked oligosaccharides to both gD-1 and

gD-2 and to the subsequent modification of these oligosaccharides, without significant alteration of the polypeptides (6, 7, 10, 12).

Extensive analysis of gD-1 and gD-2 structures was carried out, including: tryptic peptide analysis of the precursor and product forms (10, 12), analysis of the number and characteristics of *N*-asparagine-linked oligosaccharides (7), and determination of the amino acid composition of the purified glycoproteins (13) and partial N-terminal sequences (R. J. Eisenberg, D. Long, R. Hogue-Angeletti, and G. H. Cohen, submitted for publication). These investigations led us to conclude that gD-1 and gD-2 are very similar, but not identical, in structure. Studies employing both polyclonal and monoclonal antibodies have shown that gD-1 and gD-2 also display antigenic similarities (11, 12). However, there are also a number of type-specific antigenic

† Present address: Department of Pathology, Harvard University School of Medicine, Boston, MA 02115.

determinants in addition to the type-common determinants present in each protein.

Recently, the gene for gD-1 was mapped (19), and its nucleotide sequence was determined (41). The deduced amino acid sequence shows that gD-1 contains two hydrophobic regions, one at the amino terminus, presumed to be a signal peptide (1) or membrane insertion sequence (18, 29), and the other near the carboxy terminus, postulated to be a membrane-anchoring sequence (29). In addition, three carbohydrate acceptance sequences (Asn-X-Thr or Ser) have been identified. Studies in our laboratory have shown that pgD and gD isolated from infected cells lack the first 25 residues of the deduced amino acid sequence. Thus, an early processing event appears to involve removal of a putative signal peptide from the N-terminus. In the present study, we used a coupled *in vitro* translation-processing system to examine specific events in the processing of gD. These events normally occurred too rapidly to be examined in infected cells. In addition, we used the *in vitro* translation-processing system to examine the orientation of the gD molecule in membrane vesicles. Our results indicate that the structure and orientation of gD-1 and gD-2 are very similar and agree with the predictions made from the primary sequence.

(A portion of this work has been submitted by J.T.M. in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Pennsylvania.)

MATERIALS AND METHODS

Cell cultures. Conditions for the growth and maintenance of KB and BHK cells have been described previously (4, 8).

Virus preparation and titration. The procedures used for the preparation of virus stocks of HSV-1 (HF) and HSV-2 (SAVAGE), as well as for the plaque assay, have been previously described (4, 8). For all experiments, a multiplicity of infection of 20 per cell was used for HSV-1, and 10 per cell was used for HSV-2.

Pulse-labeling procedures. The protocols for pulse-labeling with [2, 3-³H]arginine, [³⁵S]methionine, and [³H]methionine, as well as for the preparation of cytoplasmic extracts, have been described elsewhere (5, 6, 10, 12). Briefly, after absorption of virus (2 h at 37°C), monolayers (KB or BHK cells for HSV-1 and BHK cells for HSV-2) were overlaid with Eagle minimal essential medium containing 10% the normal concentration of methionine or arginine. At 6 h postinfection, the cells (35-mm plates) were incubated in 0.5 ml of Hanks salts containing 100 μ Ci of [³⁵S]methionine (1,200 Ci/mmol), 125 μ Ci of [2, 3-³H]arginine (15 Ci/mmol), or 1 mCi of [³H]methionine (80 Ci/mmol) for 15 min. The radiolabeled amino acids were purchased from New England Nuclear Corp. For experiments in which tunicamycin (TM) (2 μ g/ml) (Calbiochem) was

used, BHK cells were used for both HSV-1 and HSV-2 infections (24).

Extraction of total cytoplasmic RNA from HSV-infected cells. At 6 h postinfection, monolayers were rinsed with cold saline, suspended in saline, and then washed three times by centrifugation. Cytoplasmic extracts were prepared, and RNA was extracted by the procedure of Preston (25). RNA recovered by ethanol precipitation was dried, suspended in water (5 to 10 mg/ml), divided into equal portions, and stored at -100°C.

***In vitro* translation.** Rabbit reticulocyte lysates were prepared from New Zealand white rabbits and were digested with micrococcal nuclease (22, 40). Alternatively, we used a lysate preparation from New England Nuclear. Translation of total cytoplasmic RNA was optimized by adjusting the final concentrations of magnesium acetate (0.5 mM), potassium acetate (100 mM), and spermidine (0.05 mM) and by adjusting the temperature of incubation (28°C). Translation was assessed qualitatively with either late adenovirus mRNA (New England Nuclear) or mRNA from Rous sarcoma virus-infected cells (kindly supplied by Susan Weiss, University of Pennsylvania School of Medicine, Philadelphia). No differences were discerned between the two lysate preparations. For both HSV-1 and HSV-2, we used 0.5 to 1.0 μ g of heat-denatured RNA and 50 μ Ci of [³⁵S]methionine (1,200 Ci/mmol) per 25 μ l of assay. For translation in the presence of pancreatic vesicles, we used a protein processing kit (New England Nuclear). The microsome suspension was diluted 1 to 4 in 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.5), and 1 μ l was added per 25 μ l of assay. Conditions for incubation were exactly as described above.

Isolation of microsomes after *in vitro* translation. After translation, the cell-free system was adjusted to 500 mM KCl-10 mM EDTA-10 mM Tris (pH 7.5), and the microsomes were sedimented through a 15% (wt/vol) sucrose cushion at 34,000 rpm at 20°C for 90 min in an SW 50.1 rotor (Beckman Instruments, Inc.) (9, 21). The supernatant was aspirated, and the pellet was solubilized in the 1 $\times$ detergent solution used for immunoprecipitation (see below).

Post-translational proteolysis. Proteolysis studies of the *in vitro* translation products were carried out essentially as described by Scheele et al. (30). Briefly, after translation, the lysates were incubated in the presence of 100 μ g of RNase per ml for 15 min at 28°C. Tetracaine-hydrochloride (Sigma Chemical Co.) was added at a final concentration of 2 mM, and the mixture was incubated for 10 min at 28°C and then chilled at 0°C. Trypsin (50 μ g/ml), chymotrypsin (100 μ g/ml), or both were added to samples either before or after the addition of 0.5% Triton X-100. The samples were incubated for 2 h at 0°C, trasyolol was added (1,500 KIU), and the samples were held at 0°C for an additional 10 min before further processing.

Immunoprecipitation. The preparation and characterization of gD-specific polyclonal (mouse) and monoclonal antibodies used in this study have been described previously (5, 8, 11, 13, 23, 32). Anti-gD-1 serum and group VII (170) antibody react with both gD-1 and gD-2 (11, 12, 23). Group V (57S) antibody reacts only with gD-1 (11, 32). Protein A-Sepharose (Pharmacia Fine Chemicals) was used to collect the antigen-antibody complexes. Briefly, the gel was sus-

pended in NET buffer (140 mM NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride [pH 7.5]) containing 10% sucrose and 0.5% Nonidet P-40, washed three times, and resuspended in NET buffer at a concentration of 10% (wt/vol). After *in vitro* translation, an equal volume of 2× detergent solution (100 mM NaCl–1% sodium deoxycholate–1% nonidet P-40–0.2% sodium dodecyl sulfate (SDS)–20 mM L-methionine in 40 mM Tris-hydrochloride, pH 7.5) was added to the translation mixture. Polyclonal anti-gD-1 serum or monoclonal antibody (170 or 57S) was added, and the mixtures were incubated for 1 h at 25°C. Protein A-Sepharose was added, and the mixtures were incubated for 30 min at 25°C and then sedimented through a 1-ml cushion of 1 M sucrose (in 1× detergent solution) for 5 min at 15,000 × g. The pellets were washed 10 times with 10 mM Tris hydrochloride (pH 7.4) containing 140 mM NaCl, 10 mM L-methionine, 0.1% SDS, and 0.2% Nonidet P-40 and suspended in disrupting buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (5, 6, 12).

Preparation of samples for tryptic peptide analysis. Proteins were eluted from Protein A-Sepharose by boiling for 5 min in 3% SDS and were precipitated with 25% (vol/vol) cold trichloroacetic acid in the presence of carrier bovine serum albumin (6, 11). Trypsinization and ion-exchange chromatography on Chromabeads P (Technicon Corp., Inc.) were carried out as previously described (6, 11, 12).

Digestion with Endo H. Polypeptides were eluted from Protein A-Sepharose by boiling for 5 min in 1% SDS–1% β-mercaptoethanol and were adjusted to pH 6.0 with 0.25 M citrate containing 1 mM phenylmethylsulfonyl fluoride (3, 7). One-half of this sample was treated with 0.5 mU of endo-β-N-acetylglucosaminidase H (Endo H) (per μl of eluate), and the other half served as a control (3, 7). Both samples were incubated at 37°C for 20 h. Bovine serum albumin was added, and the reaction was terminated with 25% trichloroacetic acid. The precipitate was collected by centrifugation, washed successively with 95% ethanol and ether, and dissolved in SDS-PAGE disrupting buffer.

Immunofluorescence analysis. A modification of the indirect procedure described by Reed et al. (26) was employed for fixed cells. Briefly, monolayers of KB cells grown on Lab-Tek slides (Miles Laboratories, Inc.) were infected with HSV-1 (HF) (multiplicity of infection, 10). After 2 h of absorption, the cells were overlaid with complete medium and incubated for an additional 8 h. The slides were fixed in 3.7% (vol/vol) formaldehyde, washed with phosphate-buffered saline (PBS), dehydrated with acetone, and washed again with PBS. The monolayers were overlaid with 50 μl of the appropriately diluted antiserum (prepared in mice) or monoclonal antibody, incubated for 1 h at 37°C, washed with PBS, and then incubated for 1 h with a mixture of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Cappel Laboratories) and rhodamine-conjugated albumin (Microbiological Associates). For unfixed cells, a suspension culture of KB cells was infected with HSV-1 (HF) (multiplicity of infection, = 20). At 12 h postinfection, the cells were washed with cold PBS and then incubated with 100 μl of the appropriately diluted antibody for 1 h at 37°C. The cells were washed with cold PBS and incubated with 100 μl of the fluorescein isothiocyanate-rhodamine staining mixture described above for 1

FIG. 1. SDS-PAGE analysis of gD-1 and gD-2 polypeptides synthesized *in vivo* and *in vitro*. Autoradiogram of a 9 to 12% gradient SDS-polyacrylamide gel. Cytoplasmic RNA from cells infected with HSV-1 (lane 1) or HSV-2 (lane 2) was extracted at 6 h postinfection and translated *in vitro* in a reticulocyte lysate system in the presence of [³⁵S]methionine. The polypeptides were immunoprecipitated with anti-gD-1 serum. BHK cells were infected with HSV-1 (lane 3) or HSV-2 (lane 4) in the presence of TM. At 6 h postinfection, the cells were pulse-labeled for 15 min with [³⁵S]methionine. Cytoplasmic extracts were prepared and immunoprecipitated with anti-gD-1 serum. BHK cells were infected with HSV-1 (lane 5) or HSV-2 (lane 6) and were pulse-labeled with [³⁵S]methionine for 15 min at 6 h postinfection. Cytoplasmic extracts of these cells were immunoprecipitated with anti-gD-1 serum. Lanes 1 and 2 of this gel were exposed to X-ray film for 14 days. Lanes 3 through 6 were exposed for 7 days.

h at 37°C. The cells were washed with cold PBS, resuspended in cold PBS, and placed on fluorescent-antibody test slides.

Electrophoresis of polypeptide products. Proteins were subjected to electrophoresis on slabs of either 10% or 9 to 12% linear gradient SDS-polyacrylamide, cross-linked with 0.4% *N,N'*-diallyltartardiamide (6, 11, 13, 34). After electrophoresis, the gels were stained, dried, and exposed to Kodak XAR-5 film. For fluorography, the procedure of Bonner and Laskey was followed (2). Protein molecular weight markers ranging from 15,000 to 130,000 daltons (15 to 130K) were included on each gel.

RESULTS

Comparison of HSV-1 and HSV-2 polypeptides translated *in vitro* and in infected cells (*in vivo*). Earlier studies showed that synthesis and processing of gD-1 and gD-2 *in vivo* occurred maximally from 5 to 8 h postinfection (6, 12). Consequently, we used total cytoplasmic RNA extracted from cells infected at 6 h postinfection as the source of gD-specific mRNA (hereafter called HSV-1 mRNA or HSV-2 mRNA) for translation *in vitro*. A 49K polypeptide was immunoprecipitated from *in vitro* translations programmed with HSV-1 mRNA (Fig. 1, lane 1) or HSV-2 mRNA (Fig. 1, lane 2). These 49K polypeptides were not immunoprecipitated by preimmune serum or from translations in which RNA from mock-infected cells was used (data not shown). These 49K molecules were also ca. 1,500 to 2,000 daltons larger than the corre-

sponding polypeptides synthesized *in vivo* in the presence of TM (24), as shown in Fig. 1, lanes 3 (TM-gD-1) and 4 (TM-gD-2). In addition, TM-gD-1 (48K) was slightly larger than TM-gD-2 (47.5K). This molecular weight difference was consistent with that seen between the *in vivo* precursors pgD-1 (Fig. 1, lane 5; 53K) and pgD-2 (Fig. 1, lane 6; 52K). Due to the increased resolution of polypeptides with gradient gels, the apparent molecular weights of the gD polypeptides differed somewhat from those previously reported (7, 8, 12, 24). It should also be noted that the apparent molecular weight of the 49K polypeptide synthesized *in vitro* was somewhat greater than the molecular weight of 43,291 predicted from the deduced amino acid sequence (41). A similar molecular weight for gD synthesized *in vitro* was reported by Lee et al. (19) and by Inglis and Newton (15).

Tryptic peptide analysis of *in vitro*-synthesized products. To establish the authenticity of the gD polypeptides synthesized *in vitro*, we analyzed the tryptic peptides by cation-exchange chromatography. The profile of [³⁵S]methionine-labeled tryptic peptides of the gD-1 polypeptide synthesized *in vitro* (Fig. 2A) was identical to that obtained previously for pgD-1 and gD-1 (6, 10, 11). The profile consisted of a flow-through (FT) fraction, a peak which eluted at pH 3.33 (fraction 95, presumably peptide f in reference 11), and a minor peak termed the basic wash (fractions 171 to 180). The proportions of label recovered in the FT fraction (75%) and in fraction 95 (25%) were comparable to those found in previous results (6, 10–13). Apparently, the gD-1 polypeptide synthesized *in vitro* contains no additional methionine-labeled tryptic peptides. The FT fraction was examined further by high-pressure liquid chromatography and by Bio-Gel P6 filtration (data not shown) and was found to contain the two methionine peptides previously found (7) in the Chromabeads P FT fraction of TM-gD-1. Peptide f was previously shown to be an arginine-labeled tryptic peptide (11). To confirm that the methionine peak eluting at pH 3.33 was peptide f, we cochromatographed the [³⁵S]methionine-labeled tryptic peptides derived from the *in vitro* gD-1 polypeptide with [³H]arginine-labeled tryptic peptides derived from pgD-1 (10–12) on Chromabeads P (Fig. 2B). The [³⁵S]methionine-labeled tryptic peptide coeluted with the [³H]arginine-labeled tryptic peptide of pgD-1 previously identified as peptide f (11).

Each of the [³⁵S]methionine-labeled tryptic peptides of TM-gD-2 coeluted from the Chromabeads P column with the [³H]methionine-labeled tryptic peptides of pgD-2 (Fig. 3A and references 12 and 13). The elution profile of [³⁵S]methionine-labeled tryptic peptides derived from gD-2 synthesized *in vitro* (Fig. 3B) was

very similar to profiles of pgD-2 and TM-gD-2 methionine-labeled tryptic peptides (Fig. 3A). No additional methionine-containing tryptic peptides of the *in vitro* product were resolved by this technique; however, a larger fraction of the recovered radioactive label was found in the FT fraction (see Fig. 3A and references 12 and 13). Subsequent analysis of the FT fraction by high-pressure liquid chromatography and gel filtration showed that it contained one tryptic peptide with the same molecular size and hydrophobicity as a methionine-containing tryptic peptide found in the FT fraction of TM-gD-2 (7). In addition, a significant amount of free methionine was found (data not shown). Thus, tryptic peptide analysis confirmed that the 49K polypeptides are the authentic *in vitro*-synthesized precursor molecules of pgD-1 and pgD-2.

***In vitro* processing of translated products.** When microsomes (isolated from dog pancreas) are added to *in vitro* translation mixtures, glycoprotein precursors can be processed by removal of signal peptide sequences and addition of *N*-asparagine-linked oligosaccharides (1, 16, 17, 21, 31, 39). The processed molecules are usually similar in molecular size to the precursor forms of the proteins which are synthesized *in vivo*. When mRNA from HSV-infected cells was translated in the presence of microsomes (Fig. 4), polypeptides (Fig. 4A, lanes 3 and 7) which comigrated with the corresponding *in vivo*-synthesized precursors pgD-1 and pgD-2 (Fig. 4A, lanes 4 and 8) were immunoprecipitated. A comparison of the *in vitro*-translated and *in vitro*-processed forms is shown in Fig. 4B, lanes 1 through 4. It should be noted that modified gD-2 was slightly smaller than modified gD-1.

These size comparisons suggested that *in vitro* processing might be comparable to *in vivo* processing. Recently (7), it was shown that treatment of pgD-1 and pgD-2 with Endo H generated four polypeptides from each pgD molecule, three of which were glycosylated. To determine whether the *in vitro*-modified gD forms contained *N*-asparagine-linked oligosaccharides, we treated the molecules with Endo H under conditions of partial digestion. In preliminary experiments, we found that modified gD-1 and gD-2 were Endo H sensitive. However, these cleavage products had to be detected against a background of unmodified gD polypeptides present in the translation mixture. To overcome this problem, the microsomes (containing processed gD molecules) were collected by centrifugation through a sucrose cushion after *in vitro* translation. The pellets were solubilized, immunoprecipitated, and then treated with Endo H (Fig. 5). The results in Fig. 5 were obtained with modified gD-2. Identical results were obtained with gD-1 (data not shown). In the control (no Endo

FIG. 2. Tryptic peptide analysis of gD-1 polypeptides synthesized in vitro and in vivo. (A) [^{35}S]methionine-labeled polypeptides translated in vitro with HSV-1 mRNA were immunoprecipitated with anti-gD-1 serum (see Fig. 1, lane 1). The immunoprecipitate was disrupted with SDS, oxidized, trypsinized, and chromatographed on a Chromabeads P cation-exchange column. (B) KB cells were infected with HSV-1 and pulse-labeled for 15 min at 6 h postinfection with [^3H]arginine. Cytoplasmic extracts were immunoprecipitated with anti-gD-1 serum, and the precipitate was disrupted, oxidized, trypsinized, and cochromatographed on Chromabeads P with [^{35}S]methionine-labeled tryptic peptides of gD-1 immunoprecipitated from translations using HSV-1 mRNA and prepared as described in (A). Solid lines, [^3H]arginine-labeled pgD-1; dotted lines, [^{35}S]methionine-labeled gD-1 synthesized in vitro.

H), sedimentation of the translation mixture through sucrose resulted in an enrichment of modified gD (Fig. 5, lane 2). This molecule comigrated with pgD immunoprecipitated from infected cell extracts (Fig. 5, lane 1). Endo H digestion of modified gD generated a pattern of four polypeptides (Fig. 5, lane 3) which differed in size from each other by ca. 1,000 to 1,200 daltons. The largest polypeptide comigrated with modified gD-2 (Fig. 5, lane 2), and the

smallest one was slightly larger than TM-gD-2 (Fig. 5, lane 4). This difference in molecular weight was probably due to the presence of the uncleaved *N*-acetylglucosamine remaining on modified gD and was consistent with what was found previously for pgD (7). The results suggest that in vitro processing of gD involves addition of three *N*-asparagine-linked oligosaccharides and removal of signal peptide sequences.

Translocation and processing of in vitro-synthe-

FIG. 3. Tryptic peptide analysis of gD-2 polypeptides synthesized *in vitro* and *in vivo*. (A) BHK cells were infected with HSV-2 in the presence or absence of TM. At 6 h postinfection, the cells were pulse-labeled for 15 min with [^3H]methionine alone or [^{35}S]methionine plus TM. Cytoplasmic extracts prepared from these cells were immunoprecipitated with anti-gD-1 serum, oxidized, trypsinized, and cochromatographed on Chromabeads P. Solid line, [^3H]methionine-labeled pgD-2; dotted line, [^{35}S]methionine-labeled TM-gD-2. (B) [^{35}S]methionine-labeled polypeptides translated *in vitro* with HSV-2 mRNA were immunoprecipitated with anti-gD-1 serum (see Fig. 1, lane 2). The precipitate was disrupted, oxidized, trypsinized, and chromatographed on Chromabeads P.

sized gD. For other membrane glycoproteins, microsomes must be present as soon as the signal peptide emerges from the ribosome for insertion and processing to occur (28–30). Products of *in vitro* translation (Fig. 6A and B, lane 1) were processed normally when microsomes were added to the translation mixture at the same time as was mRNA (Fig. 6A and B, lanes 2 and 3) but were not processed when microsomes were added 1 h after addition of mRNA (Fig. 6A and B, lane 4). Moreover, these unprocessed

molecules were completely degraded by trypsin (Fig. 6A and B, lane 5).

In contrast, *in vitro*-modified gD-1 and gD-2 were partially protected from degradation by trypsin (Fig. 7). Trypsin treatment of modified gD-1 (Fig. 7, lane 1) and gD-2 (Fig. 7, lane 2) reduced the size of each of these molecules by ca. 3,000 daltons (Fig. 7, lanes 4 and 5). When Triton X-100 was added to the mixtures at the end of the coupled translation-processing step and before trypsin treatment, the polypeptides

were completely degraded (Fig. 7, lanes 3 and 6). The results suggest that modified gD-1 and gD-2 had been inserted into microsomal vesicles in such a way that the bulk of the polypeptide was inside the vesicle and was therefore inaccessible to trypsin degradation. For each of these proteins, a 3,000-dalton portion was located outside the vesicle, and, in each case, this portion of each protein contained trypsin-sensitive sites.

Figure 8A shows a similar experiment with chymotrypsin. In this case, modified gD-2 was partially degraded (Fig. 8A, lane 4), and again a portion of ca. 3,000 daltons was removed. However, modified gD-1 appeared to be unaffected by chymotrypsin, even at concentrations as high as 500 μ g/ml and digestion times as long as 4 h (data not shown). Both gD-1 and gD-2 were completely degraded by chymotrypsin when the vesicles were solubilized with detergent (Fig. 8A, lanes 5 and 6). These results indicate that gD-1 and gD-2 differ structurally at the unprotected end of the polypeptide chain. Moreover, the chymotrypsin site(s) present in the unprotected end of gD-2 appears to be physically close to the trypsin site(s), since a similarly sized fragment was removed with each enzyme. Figure

FIG. 4. SDS-PAGE analysis of polypeptides synthesized in vitro in the presence and absence of microsomal vesicles. Autoradiogram of a 9 to 12% gradient gel. All of the polypeptides were labeled with [35 S]methionine, and all immunoprecipitations were carried out with anti-gD-1 serum. (A) Lane 1, gD-1 synthesized in vitro (see Fig. 1 for details of preparation); lane 2, TM-gD-1; lane 3, cytoplasmic mRNA from HSV-1-infected cells translated in the presence of microsomal vesicles; lane 4, pgD-1. Lanes 5 through 8 represent the corresponding gD polypeptides from HSV-2-infected cells. (B) Lanes 1 and 2, comparison of gD-1 and gD-2 synthesized in vitro; lanes 3 and 4, comparison of gD-1 and gD-2 synthesized and processed in vitro.

FIG. 5. Endo H digestion of gD-2 translated and processed in vitro. Fluorogram of a 9 to 12% gradient SDS-polyacrylamide gel. Lane 1, pgD-2 prepared as in Fig. 1, lane 6. Lanes 2 and 3, Cytoplasmic mRNA from HSV-2-infected cells translated in the presence of microsomal vesicles. After translation, the lysate was sedimented through a sucrose cushion, solubilized in 1 $\times$ buffered detergent, and immunoprecipitated with anti-gD-1 serum. The immunoprecipitate was disrupted by boiling for 5 min in 1% SDS-mercaptoethanol and either mock digested (lane 2) or digested (lane 3) with Endo H. Lane 4, TM-gD-2. All polypeptides were labeled with [35 S]methionine. Lanes 1 and 4 of this gel were exposed to X-ray film for 7 days, and lanes 2 and 3 were exposed for 21 days.

8B shows a comparison of the fragments derived from modified gD-2 with trypsin (Fig. 8B, lane 1), chymotrypsin (Fig. 8B, lane 2), or a combination of trypsin and chymotrypsin (Fig. 8B, lane 3). In a similar experiment with proteinase K, only modified gD-2 was found to be sensitive (data not shown). These proteolysis experiments indicated that although a similarly sized portion of the polypeptide chain of in vitro-modified gD-1 and gD-2 was located outside the microsomal vesicles, it was structurally distinct in the two glycoproteins.

Endo H sensitivity of in vitro-modified gD. The *N*-asparagine-linked oligosaccharide chains added to glycoproteins during in vitro processing are located on that portion of the molecule which is resistant to proteolysis, suggesting that they are located within the lumina of the microsomal vesicles (28, 29, 39). To test whether this was the case for gD, the molecule was treated with trypsin and immunoprecipitated (Fig. 9, lane 2). The trypsinized molecule was then treated with Endo H (Fig. 9, lane 3). A series of four bands were generated, the largest of which comigrated with the trypsin-resistant portion of modified gD-2 (Fig. 9, lane 2) and the smallest of which had a molecular size that was ca. 3,000 daltons less than that of TM-gD-2 (Fig. 9, lane 4). This pattern was similar to that observed when modified gD-2 was treated with Endo H (see Fig. 5, lane 3), except that in Fig. 9 each of the Endo H digestion products was ca. 3,000 daltons smaller. The results are consistent with the idea that all three *N*-asparagine-linked oligosaccharides of in vitro-modified gD are located within the lumina of the microsomal vesicles.

FIG. 6. Cotranslational insertion of gD into microsomal vesicles. Fluorogram of a 9 to 12% gradient gel. HSV-1 mRNA (A) and HSV-2 mRNA (B) were translated in vitro, and microsomes were added at different times. After 2 h, the samples were immunoprecipitated with anti-gD-1 serum. Lanes 1, Translation was carried out in the absence of microsomes; lanes 2, microsomes were added at the same time as mRNA (zero time) and incubated for 2 h; lanes 3, microsomes were added at zero time, and RNase was added 1 h later; lanes 4, microsomes and RNase were added 1 h after mRNA was added, and samples were incubated for an additional 1 h; lanes 5, microsomes and RNase were added 1 h after mRNA was added, and the mixture was incubated for 1 h. Trypsin was then added, and the mixture was incubated for 2 h at 0°C.

Orientation of modified gD within the microsomal vesicles. The previous experiments employed polyclonal anti-gD-1 serum to immunoprecipitate gD molecules. We screened a panel of monoclonal antibodies (11) and found that group V and group VII antibodies recognized in vitro-synthesized gD-1 polypeptides. Group VII also recognized in vitro-synthesized gD-2 polypeptides.

Experiments were performed to determine

FIG. 7. SDS-PAGE analysis of gD-1 and gD-2 translated and processed in vitro and treated with trypsin. Fluorogram of a 9 to 12% gradient gel. HSV-1 and HSV-2 mRNA were translated in the presence of microsomes (modified gD). Trypsin (50 μ g/ml) was added to the samples either before or after Triton X-100 was added. The samples were incubated for 2 h at 0°C and then immunoprecipitated with anti-gD-1 serum. Lane 1, In vitro-modified gD-1 (no trypsin); lane 2, modified gD-2 (no trypsin); lane 3, modified gD-2 treated first with 0.5% Triton X-100 and then with trypsin; lane 4, modified gD-1 treated with trypsin and then with Triton X-100; lane 5, modified gD-2 treated with trypsin and then with Triton X-100; lane 6, modified gD-1 treated with Triton X-100 and then with trypsin. Lanes 1 through 5 were exposed to X-ray film for 7 days. Lane 6 was exposed for 12 days.

FIG. 8. SDS-PAGE analysis of in vitro-modified gD polypeptides treated with trypsin and chymotrypsin. Fluorogram of a 9 to 12% gradient gel. HSV-1 and HSV-2 mRNA were translated in the presence of microsomes (modified gD). Chymotrypsin (100 μ g/ml) or trypsin (50 μ g/ml) was added to the samples either before or after Triton X-100 was added. The samples were immunoprecipitated with anti-gD-1 serum. (A) Lane 1, modified gD-2 (no chymotrypsin); lane 2, modified gD-1 (no chymotrypsin); lane 3, modified gD-1 treated first with chymotrypsin and then with Triton X-100; lane 4, modified gD-2 treated first with chymotrypsin and then with Triton X-100; lane 5, modified gD-2 treated first with Triton X-100 and then with chymotrypsin; lane 6, modified gD-1 treated first with Triton X-100 and then with chymotrypsin. Lanes 1 through 4 were exposed to X-ray film for 7 days. Lanes 5 and 6 were exposed for 14 days. (B) Lane 1, modified gD-2 treated with trypsin and then with Triton X-100; lane 2, modified gD-2 treated with chymotrypsin and then with Triton X-100; lane 3, modified gD-2 treated with chymotrypsin, trypsin, and then Triton X-100.

whether these antibodies recognized the protected and unprotected portions of the modified in vitro translation products (Fig. 10). After translation and processing, microsomes were solubilized, and gD-1 was immunoprecipitated with anti-gD-1 serum (Fig. 10A, lane 1), group VII monoclonal antibody 170 (11, 23) (Fig. 10A, lane 2), or group V monoclonal antibody 57S (11, 32) (Fig. 10A, lane 3). When modified gD-1 was treated with trypsin before immunoprecipitation, the protected portion was recognized by anti-gD-1 serum (Fig. 10A, lane 4) and by 170 antibody (Fig. 10A, lane 5) but not by 57S antibody (Fig. 10A, lane 6). These results suggest that the 57S (group V) determinant of gD-1 is located within the unprotected 3,000-dalton fragment and that the 170 (group VII) determinant is located within the fully protected fragment. In the case of modified gD-2, the untreated

ed molecule was recognized by anti-gD-1 serum (Fig. 10B, lane 1) and by 17O antibody (Fig. 10B, lane 2), but not by 57S antibody (Fig. 10B, lane 3). Since group V antibodies are gD-1 specific, this last result was expected. The trypsin-protected fragment of modified gD-2 was immunoprecipitated by anti-gD-1 serum (Fig. 10B, lane 5) and by 17O antibody (Fig. 10B, lane 6). These experiments indicate that gD-1 and gD-2 have a similar orientation within microsomal vesicles.

Orientation of gD in infected cell plasma membranes. Immunofluorescence was used to determine the orientation of group V and group VII monoclonal antibody determinants on gD-1 when the protein was located in the plasma membrane of infected KB cells. The pattern of immunofluorescence observed when fixed HSV-1-infected KB cells were reacted with different antibodies is shown in Fig. 11. Normal mouse serum (Fig. 11a) was unreactive, and the infected cells were visualized only with the aid of the rhodamine counter stain. Polyclonal mouse anti-gD-1 serum (Fig. 11b), as well as group VII (Fig. 11c) and group V (Fig. 11d) monoclonal antibodies, reacted with the fixed infected KB cells, yielding a pattern of moderately intense cytoplasmic fluorescence. None of these antibodies reacted with fixed uninfected KB cells (data not shown).

Unfixed infected KB cells were reacted with the same panel of antibodies (Fig. 12). Again, normal mouse serum (Fig. 12a) was negative. A pattern of intense focal membrane fluorescence was observed with either polyclonal anti-gD-1 serum (Fig. 12b) or group VII (17O) monoclonal

FIG. 9. Endo H sensitivity of the trypsin-resistant fragment of modified gD-2. Fluorogram of a 9 to 12% gradient gel. HSV-2 mRNA was translated in the presence of microsomes (modified gD). Trypsin (50 μ g/ml) was added to one sample after 1 h, and another sample was mock digested. Both samples were incubated for 2 h at 0°C, centrifuged through a sucrose cushion, diluted with buffered detergent, and immunoprecipitated with anti-gD-1 serum. The samples were suspended in buffer containing 1% SDS, and a portion was treated with Endo H. Lane 1, Modified gD-2 mock digested with trypsin (no Endo H); lane 2, modified gD-2 treated with trypsin (no Endo H); lane 3, modified gD-2 treated with trypsin and Endo H; lane 4, TM-gD-2 prepared as described in the legend to Fig. 1, lane 4, and run as a control.

FIG. 10. Orientation of gD-1 and gD-2 in microsomal vesicles. Fluorogram (A) or autoradiogram (B) of a 9 to 12% gradient gel. HSV-1 mRNA (A) or HSV-2 mRNA (B) was translated in the presence of microsomes (modified gD). After translation, one sample was mock digested, and the other was digested with 50 μ g of trypsin per ml for 2 h at 0°C. The samples were solubilized with detergent and immunoprecipitated. pre-gD, In vitro-synthesized form. (A) Lane 1, modified gD-1 mock digested with trypsin and immunoprecipitated with anti-gD-1 serum; lane 2, modified gD-1 mock digested with trypsin and immunoprecipitated with group VII (17O) monoclonal antibody; lane 3, modified gD-1 mock digested with trypsin and immunoprecipitated with group V (57S) monoclonal antibody; lane 4, modified gD-1 digested with trypsin and immunoprecipitated with anti-gD-1 serum; lane 5, modified gD-1 digested with trypsin and immunoprecipitated with 17O antibody; lane 6, modified gD-1 digested with trypsin and immunoprecipitated with 57S antibody. Lanes 1 through 3 were exposed to X-ray film for 7 days. Lanes 4 through 6 were exposed for 14 days. (B) Lane 1, modified gD-2 mock digested with trypsin and immunoprecipitated with anti-gD-1 serum; lane 2, modified gD-2 mock digested with trypsin and immunoprecipitated with 17O monoclonal antibody; lane 3, modified gD-2 mock digested with trypsin and immunoprecipitated with 57S monoclonal antibody; lane 4, modified gD-2 digested with trypsin and immunoprecipitated with anti-gD-1 serum; lane 5, modified gD-2 digested with trypsin and immunoprecipitated with 17O monoclonal antibody.

antibody (Fig. 12c). No fluorescence was observed with group V (57S) monoclonal antibody (Fig. 12d). None of the antibodies reacted with unfixed uninfected KB cells (data not shown). The results indicate that the group VII determinant is located on the outside of infected cells (corresponding to the lumina of microsomal ves-

FIG. 11. Immunofluorescence studies of fixed HSV-1-infected KB cells. Monolayers were infected with HSV-1, fixed with 3.7% formaldehyde, and dehydrated with acetone at 10 h postinfection. The cells were incubated with normal mouse serum (diluted 1:20) (a); mouse anti-gD-1 serum (diluted 1:80) (b); 17O monoclonal antibody (diluted 1:640) (c); or 57S monoclonal antibody (diluted 1:40) (d). Each monolayer was then reacted with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G. A representative field observed with each of these antibodies is shown.

icles), whereas the group V determinant is located on the inside of the cells (corresponding to the outside of the microsomal vesicles). Thus, gD has the orientation in infected cell plasma membranes that would be predicted from *in vitro* processing experiments.

DISCUSSION

In previous studies of the synthesis and processing of HSV glycoprotein D, we documented some of the structural changes in the protein as it was found in HSV-infected cells (5, 6, 10, 12). In those studies, the first precursor that was detected was already glycosylated, even when we used radioactive pulse-labeling times as short as 2 min (6). To detect the unglycosylated precursor to gD-1 in infected cells, TM was added to inhibit the first step of glycosylation (24). The experiments in the present study have provided

details concerning the earliest events in synthesis, as well as the processing and membrane orientation of gD. All of our findings agree with predictions made about the orientation and structure of gD-1 as a transmembrane glycoprotein, based on its primary amino acid sequence (41). Moreover, we have shown that gD-2 has a structure and an orientation similar to those of gD-1.

With polyclonal anti-gD-1 serum, we identified the primary unglycosylated translation products of gD by using an *in vitro* translation system and HSV-1 mRNA or HSV-2 mRNA. Each of the primary products had an apparent molecular size, measured by SDS-PAGE, of 49K. Our results have confirmed and extended the work of Inglis and Newton (15), as well as that of Lee et al. (19), who showed that the primary translation product of gD-1 had a molecular weight of ca. 50K. We found that the [³⁵S]methionine-labeled tryptic peptide profiles of the *in vitro* precursors are indistinguishable from the profiles of the corresponding pgD molecules isolated from HSV-1- and HSV-2-infected cells. Each of the *in vitro*-synthesized molecules was also found to be slightly larger than the corresponding polypeptides produced in the presence of TM. This difference in size, as estimated by SDS-PAGE, is consistent with the presence of transient signal peptide sequences in gD-1 and gD-2 which are missing in the TM-treated gD molecules. The fact that the *in vitro*-synthesized forms of gD-1 and gD-2 did not contain any additional methionine tryptic peptides indicates that these signal peptides probably do not contain methionine.

A difference in signal peptide size would account for the similarity in the molecular weights of *in vitro*-synthesized gD-1 and gD-2 as opposed to the difference in molecular weights of each of the processed forms of the two glycoproteins. Thus, it is possible that the signal peptide of gD-2 has a molecular weight higher than that of gD-1. However, all of the estimations of molecular weight based on migration in SDS-polyacrylamide gels appear to be somewhat inaccurate, since the molecular weight of gD-1 predicted from the deduced amino acid sequence is 43,291 and the size estimated from SDS-PAGE was found to be 49K. A similar discrepancy between predicted and actual molecular weights was noted for gD (41), as well as for gC (14), and in both cases it was suggested that the difference could be due to the high proline content of the protein (14, 41).

In the present studies, we found that each of the *in vitro*-synthesized forms of gD was processed to a higher-molecular-weight form. These *in vitro*-modified polypeptides comigrated on SDS-PAGE with pgD-1 and pgD-2. Experiments

with Endo H showed that each of the in vitro-processed molecules contained three *N*-asparagine-linked oligosaccharides, suggesting that in vitro processing resembles the process which occurs in vivo. Thus, we would predict that all of the potential *N*-asparagine glycosylation sites are used for in vitro and in vivo processing of gD-1. The possibility that gD-2 contains additional potential glycosylation sites will best be assessed from its sequence. For certain glycoproteins, some glycosylation sites predicted from primary amino acid sequence data are not used (37).

Because non-glycosylated forms of gD are not normally isolated from infected cells, it is reasonable to suppose that gD-1 and gD-2, like many other cell and viral glycoproteins (17, 28, 39), are cotranslationally processed. For other glycoproteins, it is thought that this early processing step probably involves simultaneous translocation into the lumen of the rough endoplasmic reticulum (28, 30). Our experiments are consistent with the idea that insertion and processing of gD occur cotranslationally. Thus, we found that in vitro processing did not occur when microsomal vesicles were added 1 h after mRNA was added to the in vitro translation system. Moreover, under these conditions, newly synthesized gD was not inserted into the microsomal vesicles, since it remained totally sensitive to degradation by trypsin.

When membranes were present during translation, the modified forms of gD-1 and gD-2 were only partially trypsin sensitive, and a fragment of ca. 3,000 daltons was removed from each protein. No other fragments were generated by this treatment. We interpret these results to mean that modified gD-1 and gD-2 are each asymmetrically oriented in the vesicles as trans-membrane proteins, with ca. 3,000 daltons exposed to trypsin. The amount of gD on the outside of the vesicles could actually be greater depending on the number of trypsin sites exposed. However, for gD-1, this size estimate agrees quite well with predictions based on the deduced sequence (41). The last 30 residues of gD-1 include 6 arginines and 2 lysines. One would predict that trypsin would probably remove 29 of the last 30 amino acids of gD-1 or ca. 3,600 daltons of the carboxy terminus.

The exposed portions of modified gD-1 and gD-2 appeared to differ in protease susceptibility, implying that they also differed in structure. Thus, only modified gD-2 appeared to be sensitive to chymotrypsin or proteinase K, whereas both gD-1 and gD-2 were sensitive to trypsin. According to the deduced amino acid sequence of gD-1 (41), only the last two residues are potentially chymotrypsin sensitive beyond the membrane insertion sequence. Removal of these

FIG. 12. Immunofluorescence studies of unfixed HSV-1-infected KB cells. Suspension cultures were infected with HSV-1 for 10 h and washed with PBS. The cells were then incubated with 100 μ l of normal mouse serum (diluted 1:10 in PBS) (a); mouse anti-gD-1 serum (diluted 1:20) (b); 170 monoclonal antibody (diluted 1:80) (c); or 57S monoclonal antibody (diluted 1:10) (d). The cells were centrifuged, washed with PBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G.

residues would probably not be detectable in our system. It is somewhat puzzling that modified gD-1 was insensitive to proteinase K, since this enzyme has a broad specificity. It might be that the secondary structure of the carboxy terminus of gD-1 accounts for this result.

Our data suggest that the three *N*-asparagine-linked oligosaccharides added during the in vitro translation and processing of gD are located on a portion of the glycoprotein which is within the lumina of the microsomes. According to the deduced amino acid sequence of gD-1 (41), the membrane insertion sequence is located close to the carboxy terminus. The predicted orientation of gD is thus similar to that reported for a number of viral glycoproteins (27, 28, 38). This orientation also implies that the group V (57S) determinant is located within the last 30 residues of gD-1. The fact that this determinant is also gD-1 specific emphasizes the difference in structure of gD-1 and gD-2 in this region that was implied from the protease experiments. The data

in Fig. 11 and 12 show that gD-1 is oriented in the plasma membrane of HSV-1-infected cells in the manner predicted from *in vitro* processing. Thus, the 57S (group V) determinant (presumably at the carboxy end) faces the cytoplasm, and the 170 (group VII) determinant (presumably at the amino terminus) faces outside. These determinants would be expected to have a similar orientation in the virion envelope. It is noteworthy that group VII antibodies are capable of virus neutralization and that group V antibodies are not. Experiments are now in progress to further delineate the precise locations of these two determinants.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants DE-02623 from the National Institute of Dental Research and AI-18289 from the National Institute of Allergy and Infectious Diseases. J.T.M. was a predoctoral trainee supported by Public Health Service grants NS-07180 from the National Institute of Neurological and Communicative Disorders and Stroke.

We thank Manuel Ponce de Leon for help in preparation of HSV-2 mRNA and Madeline Cohen and Deborah Long for excellent technical assistance. We are indebted to Lenore Pereira, Berge Hampar, and Martin Zweig for supplying monoclonal antibodies used in this study. We also acknowledge the help of William Wunner in preparation of this manuscript.

LITERATURE CITED

1. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67:835-851.
2. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
3. Braell, W., and H. Lodish. 1981. Biosynthesis of the erythrocyte anion transport protein. *J. Biol. Chem.* 256:11337-11344.
4. Cohen, G. H., M. N. Factor, and M. Ponce de Leon. 1974. Inhibition of herpes simplex virus type 2 replication by thymidine. *J. Virol.* 14:20-25.
5. Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg. 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. *J. Virol.* 27:172-181.
6. Cohen, G. H., D. Long, and R. J. Eisenberg. 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. *J. Virol.* 36:429-439.
7. Cohen, G. H., D. Long, J. T. Matthews, M. May, and R. Eisenberg. 1983. Glycopeptides of the type-common glycoprotein gD of herpes simplex virus types 1 and 2. *J. Virol.* 46:679-689.
8. Cohen, G. H., M. Ponce de Leon, and C. Nichols. 1972. Isolation of a herpes simplex virus-specific antigenic fraction which stimulates the production of neutralizing antibody. *J. Virol.* 10:1021-1030.
9. Dobberstein, B., H. Garoff, G. Warren, and P. J. Robinson. 1979. Cell free synthesis and membrane insertion of mouse H-2D histocompatibility antigen and β 2-microglobulin. *Cell* 17:759-769.
10. Eisenberg, R. J., C. Hydrean-Stern, and G. H. Cohen. 1979. Structural analysis of precursor and product forms of type-common envelope glycoprotein D (CP-1 antigen) of herpes simplex virus type 1. *J. Virol.* 31:608-620.
11. Eisenberg, R. J., D. Long, L. Pereira, B. Hampar, M. Zweig, and G. H. Cohen. 1982. Effect of monoclonal antibodies on limited proteolysis of native glycoprotein gD of herpes simplex virus type 1. *J. Virol.* 41:478-488.
12. Eisenberg, R. J., M. Ponce de Leon, and G. H. Cohen. 1980. Comparative structural analysis of glycoprotein gD of herpes simplex virus types 1 and 2. *J. Virol.* 35:428-435.
13. Eisenberg, R. J., M. Ponce de Leon, L. Pereira, D. Long, and G. H. Cohen. 1982. Purification of glycoprotein gD of herpes simplex virus types 1 and 2 by use of monoclonal antibody. *J. Virol.* 41:1099-1104.
14. Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner. 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. *J. Virol.* 45:634-647.
15. Inglis, M. M., and A. A. Newton. 1982. Identification of the polypeptide precursors to HSV-1 glycoproteins by cell-free translation. *J. Gen. Virol.* 58:217-222.
16. Irving, R., F. Toneguzzo, S. Rhee, T. Hofmann, and H. Ghosh. 1979. Synthesis and assembly of membrane glycoproteins: presence of leader peptide in nonglycosylated precursors of membrane glycoprotein of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 76:570-574.
17. Katz, F., J. Rothman, D. Knipe, and H. Lodish. 1977. Membrane assembly: synthesis and intracellular processing of the vesicular stomatitis viral glycoprotein. *J. Supramol. Struct.* 7:353-370.
18. Kreil, G. 1981. Transfer of proteins across membranes. *Annu. Rev. Biochem.* 50:317-348.
19. Lee, G. T.-Y., M. F. Para, and P. G. Spear. 1982. Location of the structural genes for glycoproteins gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. *J. Virol.* 43:41-49.
20. Palfreyman, J. W., L. Haarr, A. Cross, R. G. Hope, and H. S. Marsden. 1983. Processing of herpes simplex virus type 1 glycoproteins: two-dimensional gel analysis using monoclonal antibodies. *J. Gen. Virol.* 64:873-886.
21. Palmiter, R. D., S. N. Thibodeau, G. Rogers, and I. Boime. 1980. Co-translational sequestration of egg white proteins and placental lactogen inside membrane vesicles. *Ann. N.Y. Acad. Sci.* 343:192-209.
22. Pelham, H., and R. Jackson. 1976. An efficient mRNA dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
23. Pereira, L., D. V. Dondero, D. Gallo, V. Devlin, and J. D. Woodie. 1982. Serological analysis of herpes simplex virus types 1 and 2 with monoclonal antibodies. *Infect. Immun.* 35:363-367.
24. Pizer, L. I., G. H. Cohen, and R. J. Eisenberg. 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. *J. Virol.* 34:142-153.
25. Preston, C. M. 1977. The cell-free synthesis of herpes simplex virus-induced polypeptides. *Virology* 78:349-353.
26. Reed, C. L., G. H. Cohen, and F. Rapp. 1975. Detection of a virus-specific antigen on the surface of herpes simplex virus-transformed cells. *J. Virol.* 15:668-670.
27. Rose, J., W. Welch, B. Sefton, F. Esch, and N. Ling. 1980. Vesicular stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the COOH terminus. *Proc. Natl. Acad. Sci. U.S.A.* 77:3884-3888.
28. Rothman, J., and H. Lodish. 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (London)* 269:775-780.
29. Sabbatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins into membranes and organelles. *J. Cell Biol.* 92:1-22.
30. Scheele, G., R. Jacoby, and T. Carne. 1980. Mechanisms of compartmentation of secretory proteins: transport of exocrine proteins across the microsomal membrane. *J. Cell Biol.* 87:611-628.
31. Shields, D., and G. Blobel. 1978. Efficient cleavage and segregation of nascent presecretory proteins in a reticulocyte lysate supplemented with microsomal membranes. *J. Biol. Chem.* 253:3753-3756.
32. Showalter, S. D., M. Zweig, and B. Hampar. 1981. Mono-

- clonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. *Infect. Immun.* **34**:684-692.
33. **Spear, P. G.** 1975. Glycoproteins specified by herpes simplex virus type 1: their synthesis, processing and antigenic relatedness to HSV-2 glycoproteins, p. 49-61. *In* G. De Thé, M. A. Epstein, and H. zur Hausen (ed.), *Oncogenesis and herpesviruses. II. Part 2*. International Agency for Research on Cancer, Lyons, France.
34. **Spear, P. G.** 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. *J. Virol.* **17**:991-1008.
35. **Spear, P. G.** 1980. Herpesviruses, p. 709-750. *In* H. A. Blough and J. M. Tiffany (ed.), *Cell membranes and viral envelopes*, vol. 2. Academic Press, Inc., New York.
36. **Spear, P. G., M. Sarmiento, and R. Manservigi.** 1978. The structural proteins and glycoproteins of herpesviruses: a review, p. 157-167. *In* G. de Thé, W. Henle, and F. Rapp (ed.), *Oncogenesis and herpesviruses. III. Part 1*. International Agency for Research on Cancer, Lyons, France.
37. **Struck, D., and W. Lennarz.** 1980. The function of saccharide-lipids in synthesis of glycoproteins. *In* W. Lennarz (ed.), *The biochemistry of glycoproteins and proteoglycans*. Plenum Publishing Corp., New York.
38. **Sveda, M., L. Markoff, and G. Lai.** 1982. Cell-surface expression of the influenza virus hemagglutinin requires the hydrophobic carboxy-terminal sequences. *Cell* **30**:649-656.
39. **Toneguzzo, F., and H. Ghosh.** 1977. Synthesis and glycosylation *in vitro* of glycoprotein of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* **74**:1516-1520.
40. **Villa-Komaroff, L., M. McDowell, D. Baltimore, and H. Lodish.** 1974. Translation of reovirus mRNA, poliovirus RNA and bacteriophage QP RNA in cell free extracts of mammalian cells. *Methods Enzymol.* **30**:709-723.
41. **Watson, R. J., J. H. Weis, J. S. Salstrom, and L. W. Enquist.** 1982. Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in *Escherichia coli*. *Science* **218**:381-384.