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Amino-Terminal Sequence, Synthesis, and Membrane Insertion of Glycoprotein B of Herpes Simplex Virus Type 1

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Glycoprotein B (gB) was purified from cells infected with two strains (KOS and F) of herpes simplex virus type 1. Determination of amino acid sequence at the NH_2 termini revealed, by comparison with amino acid sequence deduced from previously published nucleotide sequence, that gB is made with a cleavable signal sequence of 29 or 30 amino acids, depending on the virus strain. Analysis of gB translated in vitro in the presence and absence of membranes showed that (i) gB is inserted into membranes and glycosylated cotranslationally; (ii) a large portion of the gB polypeptide made in vitro is protected from proteolysis by membranes; (iii) the large protected fragment carries N-linked carbohydrate and is probably the NH_2 terminus based on locations of signals for the addition of N-linked carbohydrate; and (iv) the size of the protected fragment is 93 kilodaltons (kDa) for gB made in vitro and associated with dog pancreas membranes, whereas both 93- and 98-kDa protected fragments can be detected for gB made in vivo. These last results are consistent with a previous proposal that gB may traverse the membrane three times.

Glycoprotein B (gB) of herpes simplex virus type 1 (HSV-1) is one of several glycoproteins present in the virion envelope (for a review, see reference 28). gB is essential for virion infectivity, being required at least in part for penetration of the virus into the host cell (15, 24). Moreover, gB can be one of the major targets of the host immune response (7).

The nucleotide sequences for the gB gene of HSV-1 strains KOS (5) and F (19) have been determined. The deduced amino acid sequences indicate a 903-amino-acid protein with a large NH_2-terminal domain containing six signals for N-linked glycosylation, a long, strongly hydrophobic domain close to the COOH terminus, and a positively charged COOH-terminal domain probably projecting into the virion or cytoplasm.

gB, like other membrane glycoproteins, is thought to be synthesized on membrane-bound ribosomes attached to the rough endoplasmic reticulum (RER). Newly synthesized gB (made during a 10-min pulse with [^{35}S]methionine) has an apparent molecular size of 108 kilodaltons (kDa) contributed in part by N-linked oligosaccharides (13, 21, 27, 32). Very soon after synthesis, gB assembles into dimers and possibly higher-order complexes (6, 25). gB is believed to be transported from the RER to the inner nuclear membrane and at this location inserted into the virion envelope by budding of the nucleocapsid into the perinuclear space (for a review, see reference 28). The assembled virion is thereafter transported to the cell surface via the Golgi apparatus (12), in which N-linked oligosaccharides are processed and O-linked oligosaccharides are added to gB and other herpes simplex virus glycoproteins (13, 17).

In this report we present the NH_2-terminal amino acid sequence for HSV-1 gB (gB-1). This information coupled with the amino acid sequence deduced from the nucleotide sequence (5, 19) demonstrates that processing of the gB-1 translation product includes cleavage of the polypeptide near the NH_2 terminus to remove a peptide (29 to 30 amino acids) that has properties of a signal sequence for translocation across the RER membrane (1, 14). Polypeptides translated from gB-1 mRNA in vitro were characterized. In the absence of membranes, two gB-1-related polypeptides, one of which was large enough to be the entire unmodified translation product and the other of which was probably prematurely terminated, were made. Their apparent molecular sizes were 96 and 94 kDa, respectively. In the presence of membranes, only one glycosylated polypeptide of 108 kDa was made. Deglycosylation of this polypeptide decreased its apparent molecular size to 93.5 kDa, a size consistent with cotranslational cleavage of the NH_2-terminal signal sequence. gB-1 was cotranslationally inserted into membrane vesicles, with the NH_2 terminus in the lumen. Exposure of protease cleavage sites in membrane-associated gB-1 made in vitro and in vivo is consistent with a proposal that gB-1 may have three membrane-spanning domains near its COOH terminus (19).

MATERIALS AND METHODS

Cells and media. Monolayer cultures of HEp-2 cells were grown in the Dulbecco modification of Eagle minimal essential medium containing 10% fetal calf serum, both from KC Biologicals, Lenexa, Kans. The virus strains used were HSV-1(KOS), obtained from P. Schaffer, Harvard Medical School, and HSV-1(F). Both were passaged at low multiplicity in HEp-2 cells.

Preparation of RNA. Infected HEp-2 cells (infection performed as previously described [13] at 5 PFU per cell) were harvested at 8 h after infection by washing twice in cold phosphate-buffered saline (PBS) and scraping into PBS. Cells were pelleted and lysed in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-hydrochloride (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40 (NP-40; Sigma Chemical Co., St. Louis, Mo.), and RNasin at 500 U/ml (Promega Biotec, Inc., Madison, Wis.). The lysate was incubated for 5 min on ice and then centrifuged at 800 × *g* for 7 min. An equal volume of 25 mM HEPES hydrochloride (pH 7.5)–0.3 M NaCl–1% sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Richmond, Calif.) was added to the

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supernatant fraction. The suspension was extracted twice in phenol-chloroform-isoamyl alcohol (49:49:2) and once in chloroform. The RNA was thereafter ethanol precipitated.

In vitro translation. In vitro translation in the reticulocyte lysate system was carried out as specified by the manufacturer (Amersham Corp., Arlington Heights, Ill.). To each 50 μl of translation mix was added 50 μCi of [^{35}S]methionine (800 Ci/mmol; Amersham). The reticulocyte lysate was not free of endogenous membranes, because in vitro translation in the absence of exogenously added membranes resulted in N-linked glycosylation of some translation products (data not shown). Therefore, the lysate was centrifuged before use at 100,000 × *g* for 5 min in a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, Calif.), and the pellet was discarded. In some cases, as indicated below, membranes (canine pancreatic microsomal membranes; Amersham) were added to the translation mix. When membranes were added after initiation of translation (usually after 30 min of incubation), further translation was inhibited by the addition of cycloheximide (Calbiochem-Behring, La Jolla, Calif.) at a final concentration of 10 μg/ml.

Labeling of infected cells and preparation of [^{35}S]methionine-labeled microsomes. Infected HEp-2 cells at 8 h after infection were washed with warm PBS and incubated for 10 min in the presence of [^{35}S]methionine (50 μCi/ml) in medium 199 supplemented with 1% fetal calf serum. To isolate microsomes, the cells were washed once in ice-cold PBS and scraped off the dish. The cells were pelleted, washed once in ice-cold hypotonic buffer (10 mM Tris potassium chloride [pH 7.4], 10 mM KCl, 5 mM $MgCl_2$), suspended in the hypotonic buffer (2×10^6 cells per ml), and then disrupted by gentle homogenization (15 strokes with a Dounce homogenizer). Compensation buffer (40 mM Tris hydrochloride [pH 7.4], 1.1 M sucrose, 40 mM KCl, 5 mM $MgCl_2$) was added to the cell lysate (1 part:4 parts, respectively). Tetracaine (Sigma) was added (at a final concentration of 3 μg/ml) to stabilize the membranes (26). Nuclei and mitochondria were removed by centrifugation at 5,000 × *g* for 10 min. The supernatants were used in the protection experiments.

Trypsin-chymotrypsin treatment of microsomes (protection experiments). Trypsin (TRLTC; Worthington Diagnostics, Freehold, N.H.) and chymotrypsin (Worthington) were dissolved in 70 mM KCl–1 mM $MgCl_2$ (pH 7.1) at a concentration of 500 μg of each per ml. The enzymes were added at 1/10 the volume of the sample (final concentration, 50 μg/ml), and incubation was for 1 h at 0°C. Soybean trypsin inhibitor (three times the concentration of trypsin; Sigma) and phenylmethylsulfonyl fluoride (40 μg/ml) were added to stop the reaction. Membranes were solubilized by 0.5% NP-40 in 20 mM Tris hydrochloride (pH 7.4)–150 mM NaCl, and immunoprecipitations were performed as described below.

Immunoprecipitation and electrophoresis. Rabbit antiserum (R#63) raised against gB-1 dimers purified by sucrose gradient fractionation of virion envelope extracts (25) was used for immunoprecipitation. Immune complexes were brought down by Sepharose-4B-coupled protein A (Pharmacia, Uppsala, Sweden). The pellet was washed three times with 20 mM Tris hydrochloride (pH 7.5)–0.5 M NaCl–0.5% NP-40. Adsorbed antigens were eluted by boiling for 5 min in 200 mM Tris hydrochloride (pH 8.8), 0.5 M sucrose (J. T. Baker Chemical Co., Phillipsburg, N.J.), 5 mM EDTA, 4% SDS (Bio-Rad), 2% β-mercaptoethanol, and 0.01% bromophenol blue (Merck, Darmstadt, Federal Republic of Germany) (sample buffer). Samples were loaded onto 8.5% polyacrylamide gels crosslinked with *N,N'*-diallyltartardiamide (chemicals from Bio-Rad) as described earlier (9). Gels were treated for fluorography (4) with Amplify (Amersham), dried, and placed in contact with Kodak X-Omat AR (XAR-5) film (Eastman Kodak Co., Rochester, N.Y.).

Purification of gB-1. Infected HEp-2 cell monolayers were harvested at 24 h after infection by being washed twice in cold PBS and then scraped into 20 mM Tris hydrochloride (pH 7.4), 0.5% NP-40, 0.5% deoxycholate, 1% Trasylol (FBA Pharmaceuticals, New York, N.Y.) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The supernatant from a spin (100,000 × *g*) of the solubilized material was adsorbed onto a column of monoclonal antibody II-105-2 (18) coupled to Sepharose 4B (Pharmacia). Adsorbed material was washed with 4 column volumes of 10 mM NH_4HCO_3 (pH 7.5)–150 mM NaCl–0.5% deoxycholate, and 2 column volumes of 10 mM NH_4HCO_3–0.5% deoxycholate. Elution was performed with 3 M KSCN (pH 7.1; Sigma), and eluted material was dialyzed against 10 mM NH_4HCO_3–0.01% SDS.

Endo H treatment. Endoglycosidase H (endo H) was from Miles Scientific Div., Miles Laboratories, Inc., Naperville, Ill.). The protein sample was dissolved in 20 μl of 0.1 M Tris hydrochloride (pH 6.8)–0.1% SDS, to which endo H was added at 30 mU/ml. Incubation was for 4 h at 37°C.

NH_2-terminal amino acid sequence determination. Automated sequencing was carried out in an Applied Biosystems gas phase sequencer (APS 470) as described by Hewick et al. (10). The phenylthiohydantoin residues were analyzed in a high-pressure liquid chromatography system, essentially as outlined by Tarr (30).

RESULTS

NH_2-terminal amino acid sequence. From extracts of HEp-2 cells infected with HSV-1(KOS) or HSV-1(F), gB-1 was purified with an affinity column composed of gB-reactive monoclonal antibody (II-105-2 [18]) coupled to Sepharose 4B. Material eluted from the column had the expected migration pattern and oligomer conformation in the absence of heating (Fig. 1). Two forms of gB-1 monomer (110 and 120 kDa), differing in posttranslational processing, accumulate in infected cells (6, 13, 27, 32). Multiple oligomeric forms (220 to 240 kDa) can also be detected when the sample is not heated prior to electrophoresis (6, 25).

About 200 μg of purified gB-1 was applied to a gas phase sequencer, and the first 10 cycles were analyzed by high-pressure liquid chromatography. The resulting sequences for gB-1 of strains F and KOS are shown in Fig. 2, and the yield for each cycle of the F sequence is shown in Table 1.

The sequence obtained for gB-1 of strain F had alternative amino acid residues in two positions (Table 1). Why alternative amino acid residues (of lower average yield) were detected at these two positions is currently unexplained. The analysis performed on gB-1 purified from strain KOS revealed two sequences, one of which is shown in Fig. 2 and is identical with the major sequence obtained for gB-1 of strain F. The second sequence (RPDDPLEPPY) is not homologous to any stretch in the deduced amino acid sequence of gB-1, and we regard it as resulting from a contaminant of unknown source. The NH_2-terminal sequences thus obtained were alignable with an internal stretch of the amino acid sequence deduced from the nucleotide sequence for the gB-1 gene of both strain F and strain KOS (Fig. 2). The alignment starts at amino acid 30 in the gB-1 gene of F and at 31 in the gB-1 gene

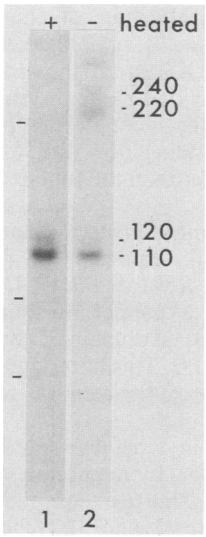

FIG. 1. Material eluted from a gB-affinity column and analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1 shows a sample that was heated prior to electrophoresis. For lane 2, heating was omitted to detect oligomeric forms of gB. Apparent molecular sizes in kilodaltons are shown in this and all subsequent figures as estimated from marker proteins (molecular sizes, 200,000, 92,000, and 69,000), the migration positions of which are indicated at the left of each figure.

of KOS, in both cases an alanine. The amino acid sequence that precedes the alanine displays many features characteristic for a signal sequence, as discussed previously (19) and below.

In vitro translation of gB-1 mRNA. RNA was isolated from HEp-2 cells infected with HSV-1 strains F or KOS and translated in vitro in the reticulocyte lysate system, and gB-related products were characterized after immunoprecipitation. gB-related polypeptides translated from RNA preparations of cells infected with strain F or KOS were indistinguishable (Fig. 3).

In vitro translation in the absence of membranes resulted in two immunoprecipitable products of molecular sizes 94 and 96 kDa (Fig. 3A, lanes 3 and 5), whereas in the presence of membranes, only one larger product of 108 kDa was made (Fig. 3A, lanes 2 and 4). The migration rate of gB-1 synthesized in vitro in the presence of membranes was indistinguishable from that of gB synthesized in vivo, as detected after a 10-min pulse of [^{35}S]methionine (Fig. 3A, lane 1).

TABLE 1. Sequence analysis of HSV-1(F) gB

Cycle	Residue	Yield[a] (pmol)
1	A	150
2	P	175
3	S	5
4	S	—[b]
5	P	130
6	G	96[c]
7	T	15
8	P	75
9	G	93
10	V	100

[a] The yields of serine and threonine residues were, as expected, very low and, in the case of serine in position 4, not quantifiable.
[b] This cycle contained an alternative residue, F, the yield of which was 50 pmol.
[c] This cycle contained an alternative residue, L, the yield of which was 42 pmol.

To ensure that one of the two bands seen upon translation of gB-1 in the absence of membranes was not due to spurious N-linked glycosylation as a result of contaminating reticulocyte membranes, the immunoprecipitated material derived from strain F was digested with endo H prior to analysis. Endo H is known to release N-linked oligosaccharides of the high-mannose type (29). Both the 94- and 96-kDa molecules were completely resistant to endo H (Fig. 3, lanes 5 and 6), whereas material translated in the presence of membranes migrated faster after endo H treatment (lanes 3 and 4). Newly synthesized gB-1 made in vivo and gB-1 made in the presence of membranes in vitro were indistinguishable in migration rate (Fig. 3B, lanes 1 and 3) and were equally sensitive to endo H treatment, which caused an equivalent increase in migration rate for each (Fig. 3B, lanes 2 and 4). The deglycosylated gB-1 (lanes 2 and 4) had an apparent molecular size of 93.5 kDa, which is in good agreement with the size estimated from analysis of the nucleotide sequence of the gB-1 gene (5, 19), assuming cleavage of the signal sequence as described above. Similar results were obtained when the susceptibility to endo H was determined for material derived from strain KOS (not shown).

The size of deglycosylated gB-1 made in vivo or in the presence of membranes in vitro (93.5 kDa) was similar to that of one of the gB-related polypeptides made in the absence of membranes in vitro. This similarity in molecular size may be coincidental, however, based on the following considerations. The gB-1 translation product has a cleavable signal sequence of 3 kDa and cleavage of such sequences

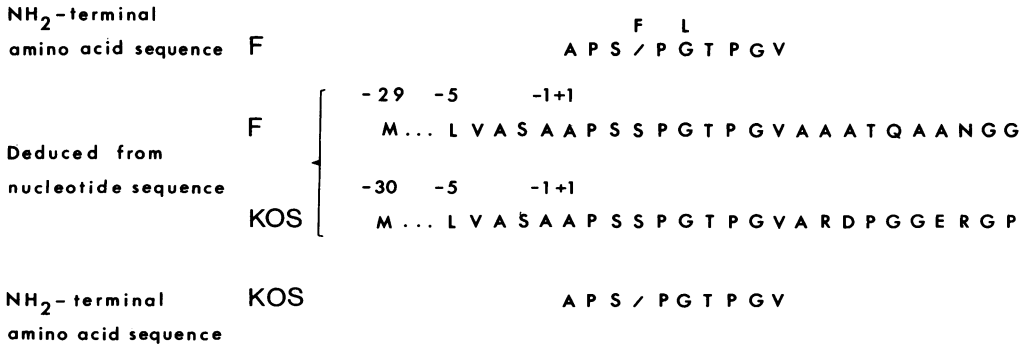

FIG. 2. Amino acid sequences of the NH_2 termini of HSV-1(F) gB and HSV-1(KOS) gB compared with the deduced amino acid sequences of products translated from the respective genes. See also Table 1. The amino acid sequences based on nucleotide sequences are from Pellett et al. (19) for HSV-1(F) and from Bzik et al. (5) for HSV-1(KOS).

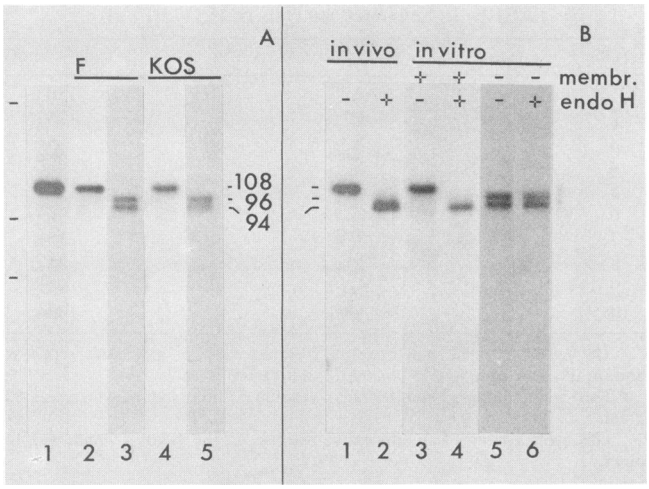

FIG. 3. Immunoprecipitable products resulting from translation of gB-1 mRNA in vivo or in vitro in the presence or absence of membranes. (A) HEp-2 cells were infected with HSV-1 strain F (lanes 1 through 3) or KOS (lanes 4 and 5). At 8 h after infection, a 10-min pulse with [^{35}S]methionine was performed (lane 1), or alternatively, RNA was extracted and used for translation in vitro in the presence of [^{35}S]methionine (lanes 2 through 5). In some cases, membranes were added (lanes 2 and 4). Immunoprecipitates obtained with antiserum 63 were analyzed by SDS-polyacrylamide gel electrophoresis. (B) Samples of in vivo-labeled material (10-min pulse with [^{35}S]methionine; lanes 1 and 2) or material translated in vitro (lanes 3 through 6) were treated with endo H (lanes 2, 4, and 6) or were control incubated (lanes 1, 3, and 5) subsequent to immunoprecipitation with antiserum 63. Samples shown in lanes 3 and 4 were translated in vitro in the presence of membranes.

usually does not occur in vitro unless membranes are present. The 96-kDa product made in vitro in the absence of membranes is probably therefore the complete translation product retaining the signal sequence, whereas the deglycosylated 93.5-kDa product made in the presence of membranes is probably the translation product from which the signal sequence has been removed by cotranslational cleavage. The 94-kDa translation product made in the absence of membranes probably results from premature termination of translation, although proteolytic cleavage cannot be ruled out.

Cotranslational insertion of gB-1 in the membrane. RNA extracted from HSV-1(F)-infected HEp-2 cells was added to an in vitro translation mix to which membranes were added either prior to incubation (time 0) or after 30 min of incubation. In vitro translation in the presence of membranes added at time 0 resulted in production of the 108-kDa band (Fig. 4, lane 1), which was partially protected from proteolysis in the absence of detergent (lane 2), but not after the addition of NP-40 (lane 3).

When membranes were added 30 min after initiation of translation (concomitant with the addition of cycloheximide to block further translation), the glycosylated 108-kDa molecule could not be detected (Fig. 4, lane 4). Instead, only 94- and 96-kDa molecules indistinguishable from those obtained by translation in the absence of membranes (Fig. 4, lane 8) were found upon immunoprecipitation and SDS-polyacrylamide gel electrophoresis analysis. Furthermore, immunoreactive material was detected only in the supernatant (lane 4) and not in the pellet (lane 5) after the membranes were spun out at the end of the translation. Membranes added after 30 min did not protect the translation products made prior to the addition of the membranes against proteolysis (lanes 6 and 7). A similar set of data was obtained for gB-1 from strain KOS (not shown).

Thus gB-1, at least that made in vitro, cannot be inserted into membranes after most of the polypeptide has been synthesized. This finding does not exclude the possibility that fragments smaller than the full-sized translation product can be inserted.

Size of gB-1 fragment protected against proteolysis. The deduced amino acid sequence for gB-1 predicts a long hydrophobic stretch towards the COOH terminus, which is likely to serve as a membrane-spanning region, and a nonhydrophobic charged domain of about 10 kDa right at the COOH terminus (5, 19). This latter domain is predicted to be in contact with the cytoplasm or interior of the virion envelope.

The fragment of in vitro-translated gB-1 that was protected from proteolysis by membranes had a size of 93 kDa, which was 15 kDa shorter than the 108-kDa translation product (Fig. 5, lanes 1 and 3). When N-linked oligosaccharides were removed from the protein core of the undigested translation product or of the protected fragment, a downward shift of 15 kDa was observed in either case (lanes 2 and 4). The 93-kDa protected gB fragment therefore carried most if not all of the N-linked oligosaccharide chains.

Somewhat different results were obtained when the sizes of gB fragments protected by membranes were assessed in other ways. As mentioned in the Materials and Methods section, the reticulocyte lysate system used was contami-

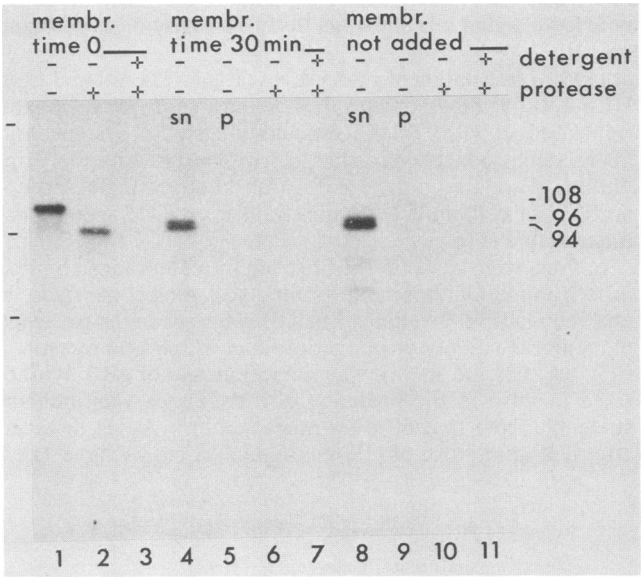

FIG. 4. Effect of membranes added before or after translation on properties of the gB-related products obtained by in vitro translation. The RNA was extracted from HSV-1(F)-infected HEp-2 cells. Membranes were added at the onset of translation for samples shown in lanes 1 through 3. Samples shown in lanes 4 through 7 were incubated in the absence of membranes for 30 min, at which point cycloheximide (10 μg/ml) and membranes were added. Lanes 8 through 11 show samples to which membranes were never added. Some samples were divided into supernatant (sn; lanes 4 and 8) and pellet (p; lanes 5 and 9) fractions by a spin (100,000 × *g*) for 5 min. Trypsin-chymotrypsin treatment was performed on samples shown in lanes 2, 3, 6, 7, 10, and 11. For samples in lanes 3, 7, and 11, membranes were solubilized by the addition of 0.5% NP-40 prior to the addition of the trypsin-chymotrypsin mixture. Immunoprecipitation of gB-1 was performed with antiserum 63.

nated with endogenous membranes, which were routinely removed by centrifugation prior to use of the lysates. When removal of the endogenous membranes was omitted, a different pattern of protected fragments was obtained. gB fragments of either 93 or 98 kDa were protected from proteolysis by the endogenous membranes (Fig. 6, lanes 1 and 2).

The protection experiment was repeated with in vivo-labeled material. Microsomes were isolated from [^{35}S]methionine-labeled infected cells, and the sizes of protected gB-related fragments were determined. In Fig. 6, lanes 3 and 4, is shown the result of a typical experiment. The two protected gB fragments were similar in size to those obtained in vitro in the presence of endogenous membranes. The relative amounts of the two recovered fragments differed, however. Endogenous reticulocyte membranes and HEp-2 cell membranes in vivo partially protect a trypsin-chymotrypsin site which appears to be fully exposed when exogenous dog pancreas membranes are used.

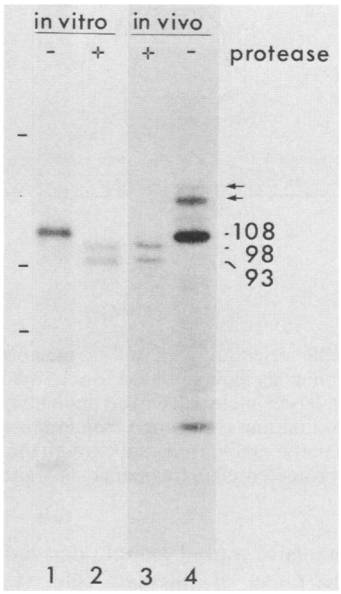

FIG. 6. Fragments of gB-1, made in vitro or in vivo, protected by membranes from proteolysis. RNA was extracted from HSV-1(F)-infected HEp-2 cells and translated in vitro (lanes 1 and 2) in reticulocyte lysate from which endogenous membranes had not been removed. One sample was treated with trypsin-chymotrypsin prior to electrophoresis (lane 2), and the other sample was control incubated (lane 1). Alternatively, microsomes were isolated from infected cells that had been pulse-labeled for 10 min with [^{35}S]methionine at 8 h after infection (lanes 3 and 4). One sample was treated with trypsin-chymotrypsin (lane 3), and the other sample was control incubated (lane 4). The two species indicated by arrows in lane 4 coprecipitate with gB-1 immediately after a pulse-label but not after a chase (6). The lower-molecular-weight band in lane 4 is probably a breakdown product.

DISCUSSION

Comparison of the NH_2-terminal amino acid sequence obtained for purified gB-1 with the amino acid sequence deduced from nucleotide sequence (5, 19) confirms the choice of reading frame for translation of the gene and also demonstrates that the gB-1 translation product is cleaved to yield mature gB-1. The sequence obtained for the NH_2 terminus of purified gB-1 is alignable with the deduced amino acid sequence, starting 31 (KOS) and 30 (F) amino acids downstream of the initiator methionine. The peptide that would constitute the cleaved NH_2 terminus of the translation product has many features characteristic for a signal sequence, as already pointed out by Pellet et al. (19). These include a hydrophobic core sequence of 17 amino acids, preceded by positively charged residues (20), and the sequence Ala-Ser-Ala immediately before the cleavage site (20, 31). Moreover, as we have shown here, the NH_2 terminus of gB-1 synthesized in vitro is translocated cotranslationally across membranes.

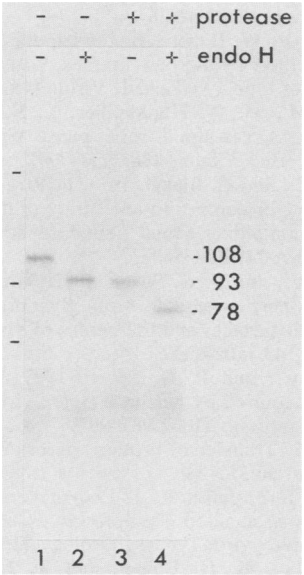

FIG. 5. Endo H sensitivity of gB-1 synthesized in vitro in the presence of membranes and of the gB-1 fragment protected from proteolysis by membranes. RNA extracted from HSV-1(F)-infected HEp-2 cells was translated in vitro in the presence of membranes. A part of the translated material was incubated in the presence of trypsin-chymotrypsin (lanes 3 and 4). Samples of both control and protease-treated samples were incubated in the presence of endo H (lanes 2 and 4) after immunoprecipitation with antiserum 63.

Translation of gB-1 mRNA in vitro, i.e., in reticulocyte lysate and in the absence of membranes, results in two products of 96 and 94 kDa. A similar pattern has been observed by others (22). Because gB-1 appears to have a cleavable signal sequence, it is possible that the molecular size difference between the upper "minus membrane" band (96 kDa) and the single, deglycosylated "plus membrane" band (93.5 kDa) (Fig. 3) results from retention of the signal sequence in the 96-kDa molecule. The signal sequence should not become cleaved unless membranes are provided, as the signal peptidase is located in the lumen of the endoplasmic reticulum (8, 11). It is consequently unlikely that the lower minus membrane band (94 kDa) arises as a result of cleavage of the signal sequence. Such cleavage would indicate the presence of contaminating membranes, in which case at least partial N-linked glycosylation should have occurred. Probably the 94 kDa minus membrane band resulted from premature termination of translation. Any other explanation for the presence of this band (for example, alternative splicing to introduce or delete an extra segment in one of the two minus membrane molecules) has to take into account that the gB-1 polypeptide made in the presence of membranes is homogeneous both in terms of size and charge. Only one spot was observed after two-dimensional

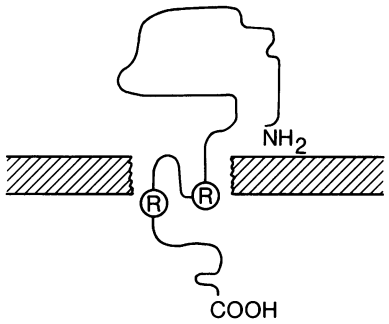

FIG. 7. Possible orientation of gB in membranes, with three membrane-spanning segments, based on a model proposed by Pellett et al. (19). Trypsin cleavage at an arginine (R) exposed in the turn between the first and the second membrane-spanning segment or at an arginine in the cytoplasmic tail close to the membrane could give rise to the protected gB-1 fragments shown in Fig. 6.

gel electrophoresis of a gB-1 sample derived from a 10-min [^{35}S]methionine pulse of infected cells (Claesson-Welsh, unpublished data).

gB-1 synthesized in vitro in the presence of membranes becomes associated with the membranes but apparently cannot be inserted into membranes added after most or all of the polypeptide is synthesized. Posttranslational insertion has been shown to occur for some bacterial, yeast, and plastid proteins (1, 14, 23, 33), whereas most eucaryotic proteins made in the RER become inserted into the RER membrane while they are being synthesized (2, 3). Evidence has been presented that another herpes simplex virus glycoprotein designated gD (16) also falls into the latter category.

A large portion of the gB-1 polypeptide synthesized in vitro in the presence of membranes was protected from proteolysis by the membranes. According to the analysis of endo H-digested and undigested molecules, the large protected gB-1 fragment appeared to contain all the N-linked oligosaccharide of intact gB-1. Because all of the potential signals for N-linked glycosylation were found in the deduced amio acid sequence to be located NH_2 terminal of the proposed membrane-spanning region, our results support the proposed orientation of gB-1 in membranes, which predicts a short COOH-terminal cytoplasmic tail (Fig. 7). It was also proposed that the membrane-spanning region of gB-1 traverses the membrane three times (19). Arg-717 or Lys-721 (located between the first two putative membrane-spanning domains counting from the NH_2 terminus) is apparently the most NH_2-terminal trypsin cleavage site exposed in microsomes, based on the difference of 15 kDa between intact and one form of cleaved gB-1 (Fig. 5–7). Therefore, it seems likely that at least the first of the putative membrane-spanning domains actually does traverse microsomal membranes. Arg-717 may not be 100% exposed for gB-1 synthesized in vivo (or in vitro in the presence of endogenous reticulocyte membranes) because, in these instances, either a 98- or a 93-kDa cleavage product could be detected. The 98-kDa product may result from cleavage at Arg-766 or Arg-770 instead of at Arg-717 or Lys-721. We have shown that gB-1 produced in vivo forms oligomers within minutes of synthesis (6), whereas oligomers have not yet been detected for gB-1 made in vitro (perhaps the concentrations in membranes are too low, particularly when relatively large amounts of microsomes are present). Differences in oligomer and monomer conformations of gB-1 could account, at least in part, for differences in sizes of products protected by membranes from proteolysis. Although our results are consistent with the proposal that gB-1 traverses the membrane three times, additional studies will be required to establish the validity of this model.

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LITERATURE CITED

1. **Blobel, G.** 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA **77**:1496–1500.
2. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. J. Cell Biol. **67**:852–862.
3. **Blobel, G., and D. D. Sabatini.** 1971. Ribosome-membrane interaction in eukaryotic cells, p. 193–195. *In* L. A. Manson (ed.), Biomembranes, vol. 2. Plenum Publishing Corp., New York.
4. **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.
5. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology **133**:301–314.
6. **Claesson-Welsh, L., and P. G. Spear.** 1986. Oligomerization of herpes simplex virus glycoprotein B. J. Virol. **60**:803–806.
7. **Glorioso, J., C. H. Schröder, G. Kumel, M. Szczesiul, and M. Levine.** 1984. Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. J. Virol. **50**:805–812.
8. **Harrison, T. M., G. G. Brownlee, and C. Milstein.** 1974. Studies on polysome-membrane interactions in mouse myeloma cells. Eur. J. Biochem. **47**:613–620.
9. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
10. **Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
11. **Jackson, R. C., and G. Blobel.** 1977. Posttranslational cleavage of presecretory proteins with an extract of dog pancreas rough microsomes containing signal peptidase activity. Proc. Natl. Acad. Sci. USA **74**:5598–5602.
12. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43**:1102–1112.
13. **Johnson, D. C., and P. G. Spear.** 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell **32**:987–997.
14. **Kreil, G.** 1981. Transfer of proteins across membranes. Annu. Rev. Biochem. **50**:317–348.
15. **Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115**:149–158.
16. **Mathews, J. T., G. H. Cohen, and R. J. Eisenberg.** 1983. Synthesis and processing of glycoprotein D of herpes simplex virus types 1 and 2 in an in vitro system. J. Virol. **48**:521–533.
17. **Oloffson, S., J. Blomberg, and E. Lycke.** 1981. O-glycosidic carbohydrate-peptide linkages of herpes simplex virus glycoproteins. Arch. Virol. **70**:321–329.
18. **Para, M. F., M. L. Parish, A. G. Noble, and P. G. Spear.** 1985. Potent neutralizing activity associated with anti-glycoprotein D specificity among monoclonal antibodies selected for binding to

herpes simplex virions. J. Virol. **55**:483–488.
19. **Pellett, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman.** 1985. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J. Virol. **53**:243–253.
20. **Perlman, D., and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eucaryotic and procaryotic signal peptides. J. Mol. Biol. **167**:391–409.
21. **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.
22. **Rafield, L. F., and D. M. Knipe.** 1984. Characterization of the major mRNAs transcribed from the genes for glycoprotein B and DNA-binding protein ICP8 of herpes simplex virus type 1. J. Virol. **49**:960–969.
23. **Rothblatt, J. A., and D. I. Meyer.** 1986. Secretion in yeast: translocation and glycosylation of prepro-a-factor *in vitro* can occur via an ATP-dependent post-translational mechanism. EMBO J. **5**:1031–1036.
24. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B_2) in virion infectivity. J. Virol. **29**:1149–1158.
25. **Sarmiento, M., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. IV. Conformation of the virion glycoprotein designated VP7(B_2). J. Virol. **29**:1159–1167.
26. **Scheele, G., R. Jacoby, and T. Carne.** 1980. Mechanism of compartmentation of secretory proteins: transport of exocrine pancreatic proteins across the microsomal membrane. J. Cell Biol. **87**:611–628.
27. **Serafini-Cessi, F., and G. Campadelli-Fiume.** 1981. Studies on benzhydrazone, a specific inhibitor of herpesvirus glycoprotein synthesis. Size distribution of glycopeptides and endo-β-N-acetylglucosaminidase H treatment. Arch. Virol. **70**:331–343.
28. **Spear, P. G** 1984. Glycoproteins specificied by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Publishing Corp., New York.
29. **Tarentino, A. L., and F. Maley.** 1974. Purification and properties of an endo-β-N-acetylglucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249**:811–817.
30. **Tarr, G. E.** 1981. Rapid separation of amino acid phenylthiohydantoins by isocratic high-performance liquid chromatography. Anal. Biochem. **111**:27–32.
31. **von Heijne, G.** 1984. How signal sequences maintain sequence specificity. J. Mol. Biol. **173**:243–351.
32. **Wenske, E. A., M. W. Bratton, and R. J. Courtney.** 1982. Endo-β-*N*-acetylglucosaminidase H sensitivity of precursors to herpes simplex virus type 1 glycoproteins gB and gC. J. Virol. **44**:241–248.
33. **Wickner, W.** 1980. Assembly of proteins into membranes. Science **210**:861–868.