Herpes simplex virus (HSV) is a double-stranded DNA virus which encodes information for at least 11 glycoproteins, 10 of which are found in the virion envelope as well as on the surfaces of infected mammalian cells. Because of their surface location, HSV glycoproteins act as major antigenic determinants for the cellular and immune responses of the host (33, 41, 42). Five of the glycoproteins are important for virus entry into mammalian cells. The initial interaction between virus and cell is through the binding of gC with cell surface heparan sulfate proteoglycans (17, 18, 50), which is followed by the specific binding of gD with a cellular receptor, termed HVEM (32). Subsequently, in some undefined manner, gD in complex with gC triggers protease activity on the virion, which is required for uncoating of the viral DNA (31, 34). The proteins gB, gC, gD, gF, and gI are required for viral entry, whereas gA, gK, and gL play a role in the initial phase of herpesvirus replication and nuclear localization, with gA being especially important for nuclear localization (19, 39). The glycoprotein gB is a membrane protein that functions to enable viral DNA to enter the cell nucleus. The proteins gD and gC together induce typical zosteriform lesions. Furthermore, neutralizing antibodies against gB, gD, and gI protect against HSV-1 (20) and HSV-2 (28) infection. In a previous study (35), we described the expression and initial characterization of a recombinant form of the gH-gL complex. We constructed a cell line (HL-7) which expresses and secretes a soluble complex consisting of gH truncated at residue 792 just prior to the transmembrane anchor (gHt) and full-length gL. The purified complex stimulated production of neutralizing antibodies and protected mice challenged with herpes simplex virus type 1 (HSV-1) against development of zosteriform lesions. Furthermore, the purified gHt-gL complex reacted with gH and gL monoclonal antibodies (MAbs), including the anti-gH MAb LP11, indicating that it retains its proper antigenic structure after secretion and purification. These findings suggest that the conformation of gHt-gL in the secreted complex was similar to that of its full-length counterpart produced in HSV-infected cells. This cell system allowed for production of sufficient quantities of conformationally correct purified gHt-gL for biochemical and antigenic analysis.

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HSV-1 gH contains 838 amino acids, the first 18 of which have been postulated to constitute a cleavable signal sequence (12, 27). The protein has seven consensus sites for N-linked oligosaccharides (N-CHO) (22) as well as 11 sites for O-linked glycosylation (O-CHO) (16). Until this study, it was not known how many of the CHO sites were actually utilized by mammalian cells. gH-1 and gH-2 (26) are 77% homologous, especially in the C-terminal one-fourth of the proteins. The spacing of six N-CHO sites is conserved in gH-1 and gH-2. gH-1 has eight...
cysteines (27), seven of which are conserved in gH-2 (26). Whereas the disulfide bond arrangements of gB (32), gC (39), and gD (25) have been solved, nothing is known about the disulfide bond formation of gH. Residues 804 to 824 constitute the transmembrane region (TMR), which is hydrophobic and presumably anchors the protein into membranes. However, a previous study showed that truncated forms of gH lacking the TMR are not secreted from cells (36). Proper transport of full-length gH from the endoplasmic reticulum to the infected cell surface requires cotransport of gL (19).

gL contains 224 amino acids, the first 19 of which have been postulated to constitute the signal sequence (26). The protein has one consensus site for N-CHO, which has been shown to be utilized (19). gL has three potential sites for the addition of O-CHO (16). The location of the N-CHO consensus site and the locations of the four cysteine residues are conserved between gL-1 and gL-2 (26). Lastly, gL does not have a TMR, and it is expressed in the absence of gH. gL is secreted from transfected cells (5). Retention of gL on the surface of the cell therefore requires the coexpression of gH (5, 38).

The goal of the current study was to extend our understanding of native gH-gL structure and to further relate structure to function. We determined the stoichiometry of gH-gL and analyzed its carbohydrate composition. The oligomer consists of 1 mol each of gH and gL, and most sites for N-CHO are utilized. Both proteins are also modified with O-CHO. Using a series of C-terminal truncation mutants of gH and gL as well as a panel of gH and gL MAbs, we localize neutralizing and nonneutralizing epitopes on each protein. With the truncations, we mapped the minimum length of each protein that was needed to form a complex that could be secreted from the cell. We found that the first 323 amino acids of gH and the first 168 amino acids of gL can form a stable, secreted complex which is reactive with MAb LP11. Based on these and other data, a model of gH-gL structure is proposed.

**MATERIALS AND METHODS**

**Cells and virus.** African green monkey kidney (Vero) and mouse L cells were grown in MEM with 10% fetal bovine serum (FBS). Chinese hamster ovary (CHO-K1) cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS). Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were maintained in Eagle medium (DME) supplemented with 5% fetal bovine serum (FBS). Chinese hamster ovary (CHO-K1) cells were grown in Ham’s F-12 medium with 5% FBS. D14 cells (Vero derived), which express HSV-1 ICP-6 (51) were grown in DMEM with 5% FBS and 418 (25 μg/ml) at 37°C. HL-7 cells, which constitutively express HSV-1 ICP-6 (51) were grown in DMEM supplemented with 5% FBS and hygromycin B (50 μg/ml) (35). For protein production, hygromycin B was eliminated from medium. HSV-1 (hrR3) was described previously (51). Rabbit antibodies against peptide sequences of gL, were kindly provided by D. Johnson (19).

**Antibodies used.** The anti-gL MAbs VIII 62, 82, 87, 200, 820, and rabbit polyclonal antibodies (Pabs) RS88 and RS89 were described previously (34). Hybridoma cell lines secreting anti-gH-1 MAbs S25 and S35 (40) were obtained from the American Type Culture Collection. Hybridoma cell lines secreting anti-gH-1 MAbs H1 to H13 and anti-gL MAbs L1 to L3 were kindly provided by D. Johnson (19). Antibodies used in the current study were the following: mouse anti-gH monoclonal antibody (MAb) 8H4, which recognizes a linear epitope on gH-1, cell supernatant as described above. For phosphorimaging analysis, purified [35S]cysteine-labeled gHt-gL was applied to SDS-PAGE gel and the dried gel was scanned with a Storm 840 PhosphorImager (Molecular Dynamics).

**RESULTS**

The stoichiometry of gH and gL in the complex. Previously, we showed that gHt-gL can be purified as a complex of the two proteins (35). To determine whether gH and gL are present in the complex, we took advantage of the 2:1 molar ratio of cysteine residues in gH and gL, respectively. If the two proteins were present in a 1:1 ratio in an oligomeric complex, then stoichiometric analysis should yield twice as many disintegrations per minute in gHt as in gL. HL-7 cells were grown in the...
presence of $[^{35}S]$cysteine. The radiolabeled gHt-gL complex was purified, and the two proteins were separated on denaturing SDS-PAGE gel. The gel was dried, and the disintegrations per minute (dpm) of label in each band were quantitated by phosphorimaging. In three separate experiments, one of which is shown (Fig. 1A), the gHt band contained twice as much radiolabel as the gL band. Since gHt contains eight cysteines per molecule and gL contains four, the disintegrations per minute per cysteine residue were equivalent. Thus, we conclude that the ratio of gHt to gL in the complex is 1:1. This result agrees with an estimate of the ratio, which was based on immunoprecipitation of $[^{35}S]$cysteine-labeled full-length gH-gL from extracts of HSV-1-infected cells (19).

As a second approach, and to determine the overall size of the complex, we subjected purified gHt-gL to Superose 12 gel filtration (Fig. 1B). gHt and gL were each detected in column fractions by ELISA. Ninety percent of gHt and gL eluted from the column in a single peak (Fig. 1B, peak 1), with a mass of approximately 180 kDa. A higher-mass shoulder (Fig. 1B, peak 2) also contained both proteins, suggesting that a small proportion of gHt and gL can also exist as higher-molecular-mass forms, i.e., greater than 180 kDa. A small amount of gL was found in the absence of gHt (Fig. 1B, peak 3), suggesting that the complex can dissociate under relatively mild elution conditions. As a third approach, gHt-gL was electrophoresed on a nondenaturing native gel, followed by Western blotting (Fig. 1C). Both anti-gH and anti-gL antisera reacted with a band migrating at 180 kDa as well as with a band migrating at >200 kDa. These results are in agreement with the gel filtration data. In addition, anti-gH serum reacted with a 110-kDa band, and anti-gL serum reacted with a 35-kDa band, corresponding to the monomeric sizes of the individual proteins. Since this represented the majority of the protein, we believe that much of the complex dissociated during electrophoresis, possibly due to the presence of 0.1% SDS in the native sample buffer. Hutchinson et al. (19) suggested that gH-gL is not disulfide bonded.

As a fourth approach, we attempted to determine the molar ratio of gL by performing N-terminal sequencing of the complex. However, the N terminus of gH was blocked. In contrast, we were able to determine the sequence of the first 20 amino acid residues of mature gL (which begin with GLPSTEYVIR) and found that glycine at amino acid 20 of the predicted sequence was the first amino acid of mature gL. Thus, we formally demonstrated that the predicted signal peptide of gL is cleaved at the predicted site (19).

We demonstrated that the mass of a gHt-gL complex is 180 kDa and that the ratio of gH and gL in the complex is 1:1. Since the molecular size of one gH is 110 kDa and that of one gL is 35 kDa, there is only one gH in the gHt-gL complex. Thus, we conclude that the complex contains one gH and one gL.

Glycosylation of gHt and gL. As the next step in structural analysis of gHt-gL, we determined the extent of glycosylation of the purified complex. The coding sequences for gH and gL contain predicted sites for N-CHO and O-CHO (Fig. 3). It was previously reported that both proteins within complexes obtained from infected-cell extracts or from baculovirus recombinants contain N-CHO (19, 46). Here we determined the type of glycosylation in each protein produced by HL-7 cells and estimated how many of the predicted sites were utilized. Purified gHt-gL was treated with glycosidases and with neuraminidase, either alone or in combination, and resolved by SDS-PAGE followed by Western blotting (Fig. 2A and B). Treatment with EndoH reduced the mass of gHt only slightly and had no effect on gL (Fig. 2A and B, lanes 2). In contrast, EndoF treatment had a more dramatic effect on both proteins, reducing the size of gHt from 108 to 91 kDa (Fig. 2A, compare lanes 1 and 4) and that of gL from 35 to 33 kDa (Fig. 2B, compare lanes 1 and 4). These data indicate that the majority of the N-CHO on gHt and the one N-CHO on gL were in the complex form. Neuraminidase treatment had a greater effect on the mobility of gL than on that of gHt (Fig. 2A and B, compare lanes 1 and 3), indicating that sialic acid was present,
and combined EndoF and neuraminidase treatment increased the mobility of each protein even more, particularly that of gL (Fig. 2A and B, lanes 5). The latter results indicated that sialic acid was probably present on O-CHO, especially on gL. Treatment of gHt-gL with neuraminidase and O-glycanase also resulted in increased gHt mobility (Fig. 2A and B, lanes 6), another indication that both proteins contain O-CHO. When all three enzymes were used, gHt migrated to a molecular size of 86 kDa (Fig. 2A, lane 7), and gL migrated to a molecular size of 27.5 kDa (Fig. 2B, lane 7), values close to the predicted sizes of the unglycosylated molecules.

We estimate that N-CHO contributes approximately 17 kDa to the mass of gHt; O-CHO contributes at least 7 kDa, and sialic acid contributes at least 2 kDa (Fig. 2C). In the case of gL, N-CHO contributes at least 2 kDa, O-CHO contributes 6 kDa, and sialic acid contributes 2 kDa in mass (Fig. 2D). Since there is a single N-CHO site on gL, which is used (mass, 2 kDa), we estimate that the mass of 17 kDa is sufficient to account for seven predicted N-CHO sites on gL. We conclude that most of the predicted N-linked sites on gH are used and that they are present in the mature complex form. Our data show that both proteins also contain both O-CHO and sialic acid.

Antigenic analysis of gH-gL. Thus far, only a limited number of anti-gH or gL MABs have been available for the study of antigenic structure (2, 5, 34, 36, 40). In order to begin mapping antigenic domains on gHt-gL, we decided to generate more gH and gL MABs. Mice were immunized with purified gHt-gL or full-length gH (36), and hybridoma supernatants were screened for production of gH- or gL-specific MABs by an ELISA to detect antibodies which reacted against purified gHt-gL. To confirm their reactivity, we further screened positive clones by immunofluorescence, using cells transfected with gH and/or gL plasmids. Sixteen gH-specific and 4 gL-specific MABs were obtained (Table 1). These 20 MABs recognized gH and gL antigens in immunofluorescence studies.

**TABLE 1.** gH and gL antibodies which react with linear epitopes

<table>
<thead>
<tr>
<th>Antibody</th>
<th>MAb or PAb</th>
<th>Glycogen specificity</th>
<th>Source of antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 to H13</td>
<td>MAb</td>
<td>gH</td>
<td>Purified gHt-gL from HL-7 cells</td>
</tr>
<tr>
<td>MP6, MP7, MP8</td>
<td>MAb</td>
<td>gH</td>
<td>Purified gH from HSV-1-infected cells</td>
</tr>
<tr>
<td>375&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MAb</td>
<td>gH</td>
<td>HSV-1 virion</td>
</tr>
<tr>
<td>L1, L2, L3</td>
<td>MAb</td>
<td>gL</td>
<td>Purified gHt-gL from HL-7 cells</td>
</tr>
<tr>
<td>8H4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAb</td>
<td>gL</td>
<td>Purified gHt-gL from HL-7 cells</td>
</tr>
<tr>
<td>VII 62, 82, 87, 200, 820&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MAb</td>
<td>gL</td>
<td>Purified gHt-gL from Escherichia coli</td>
</tr>
<tr>
<td>RS 88&lt;sup&gt;d&lt;/sup&gt;</td>
<td>PAb</td>
<td>gL</td>
<td>Purified gHt-gL from Escherichia coli</td>
</tr>
<tr>
<td>RS 89&lt;sup&gt;d&lt;/sup&gt;</td>
<td>PAb</td>
<td>gL</td>
<td>Purified gHt-gL from Escherichia coli</td>
</tr>
<tr>
<td>R137&lt;sup&gt;e&lt;/sup&gt;</td>
<td>PAb</td>
<td>gH-gL</td>
<td>Purified gHt-gL from HL-7 cells</td>
</tr>
</tbody>
</table>

<sup>a</sup> Showalter et al. (40).
<sup>b</sup> Dubin and Jiang (5).
<sup>c</sup> Peng et al. (35).
<sup>d</sup> Novotny et al. (34).

FIG. 2. Analysis of carbohydrates on gH and gL. Purified gHt-gL was incubated with no enzyme (untreated control) or with glycosidases and neuraminidase in the indicated combinations. The digests were resolved on a 10% denaturing polyacrylamide gel. Following transfer to a nitrocellulose membrane, one blot was probed with R83 anti-gH antibody (A). The bound antibody was detected with goat anti-rabbit IgG-peroxidase and chemiluminescent substrate. A second blot was probed with anti-gH MAb 8H4 (B) and then with goat anti-mouse IgG-peroxidase and chemiluminescent substrate. The molecular weight after each treatment of gH and gL was calculated according to molecular size markers on the gel (data not shown). The contributions of N-CHO, O-CHO, and sialic acid to the molecular weights of gH (C) and gL (D) were estimated from the difference between the untreated controls (A and B, lanes 1) and the Endo-F-treated (A and B, lanes 4), O-glycanase-treated (A and B, lanes 6), and neuraminidase-treated (A and B, lanes 7) samples.
or gL on Western blots of denaturing gels, indicating that all of them recognize linear epitopes.

A series of gH and gL plasmids containing the gH and gL genes encoding C-terminal truncations of decreasing length (Fig. 3) was used to map these MAbs. In the first set of experiments, CHO-K1 cells were transiently transfected with plasmids expressing truncation or deletion mutants of gH, cell extracts were prepared and separated by SDS-PAGE, and Western blots were probed with gH MAbs. Surprisingly, 16 of the gH MAbs (represented in Fig. 4A by H6) reacted with the longer forms of gH (data not shown) as well as with gH475 (Fig. 4A, lane 3) and gH323 (lane 4). As a control, cells were mock transfected (Fig. 4A, lane 1). The band which reacts with the antibody in all four lanes, including lane 1, is antibody heavy chain (50 kDa). These data suggested that the epitopes for all 16 MAbs are located within residues 19 (at the end of the signal peptide) to 323. These MAbs also reacted with a gH mutant with residues 276 to 323 deleted (Fig. 4A, lane 2).

FIG. 3. Schematic stick figures of full-length HSV-1 gH and gL and the C-terminal truncation mutants. Plasmids were constructed to express truncated forms of gH, a deletion mutant of gH pCMV3del(276–323), full-length gL (pCMV3gL), and truncated forms of gL. The signal peptides (signal), TMR, positions of the cysteine residues (C) and predicted N-CHO sites (open balloons) and predicted O-CHO sites (open hexagons), and the names of the plasmids are indicated.

FIG. 4. Epitope mapping of anti-gH antibodies. CHO cells were transfected with gH truncation or deletion mutants. (A) Cell extracts were immunoprecipitated with R137, electrophoresed on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with MAb H6. Cell extracts from cells mock transfected (lane 1) or transfected with pCMV3gHdel(276–323) (lane 2), pSR123 (lane 3), or pCMV3gHtrunc(323) (lane 4) are shown. (B) Cell extracts were electrophoresed on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with MAb H12. (C) Cell extracts were electrophoresed on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with R137. Secondary antibodies were then added, and the blots were visualized by ECL. Lanes for panels B and C are cell extracts from pSR124- (lane 1), pSR123- (lane 2), and mock- (lane 3) transfected cells.
Together, these results suggest that the epitope for H6 and those for 15 other gH-specific MAbs are located between residues 19 and 276. In contrast, MAb H12 reacted with gH648 but not with gH475 (Fig. 4B, compare lanes 1 and 2), indicating that its epitope is located between amino acids 475 and 648. As a control, a duplicate blot of Fig. 4B was probed with R137, a PAb prepared to purified gHt-gL (35). As expected, R137 reacted with both gH648 and gH475 (Fig. 4C, lanes 1 and 2).

Similar studies were also done for the four newly prepared gL MAbs (L1, L2, L3, and 8H4). Cytoplasmic extracts prepared from pMN115 (encoding gL168)- or pCMV3gL (gL) (lanes 2, 4, 6, 8, and 10). Cell extracts were prepared and resolved on a 12% polyacrylamide denaturing gel. After Western blotting, separate strips of the membrane were probed with antibodies L1, L2, L3, 8H4, or aUL1-1, as indicated below the gel. Secondary antibodies were then added, and ECL was used to visualize the bands.

FIG. 5. Reactivity of anti-gL antibodies with gL168 or with full-length gL. CHO-K1 cells were transfected with pMN115 (gL168) (lanes 1, 3, 5, 7, and 9) or pCMV3gL (gL) (lanes 2, 4, 6, 8, and 10). Cell extracts were prepared and resolved on a 12% polyacrylamide denaturing gel. After Western blotting, separate strips of the membrane were probed with antibodies L1, L2, L3, 8H4, or aUL1-1, as indicated below the gel. Secondary antibodies were then added, and ECL was used to visualize the bands.

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FIG. 6. Mapping of anti-gL antibody epitopes with synthetic peptides. (A) Diagram depicting the sequences of the set of overlapping synthetic peptides mimicking the gL sequence. The location of each peptide within the gL sequence is indicated. (B) Dot blot analysis of anti-gL antibodies with the peptides. Two microliters of each peptide (4 μg/dot) was spotted onto nitrocellulose membrane strips. After blocking, antibodies were added to each strip, and the reactivity was detected by ECL with goat anti-mouse peroxidase or goat anti-rabbit peroxidase.
To determine the shortest fragment of each protein that was necessary to form a complex with the other protein, we knew from previous studies that several truncated forms of gH synthesized in transfected cells in the absence of gL failed to be secreted (36); however, cells transfected with a plasmid containing only gL were able to secrete gL protein (5). Thus, we decided to reexamine the properties of truncated gH, using a transient cotransfection system. We hypothesized that only properly folded protein complexes containing gH should be secreted from cells cotransfected with the gH and gL plasmids. To verify conformation, we examined the secreted complexes with MAbs.

We first transfected CHO-K1 cells with the gH C-terminal plasmids (Fig. 3). Culture supernatants and cytoplasmic (cell) extracts obtained from each transfection were immunoprecipitated with anti-gH MAb H6, followed by Western blotting (Fig. 8). Both blots were then probed with polyclonal anti-gH serum R137. None of the truncated gH proteins were detected in the supernatant (Fig. 8A), despite the fact that the four longest truncations were detected in the cell extract (Fig. 8B, lanes 1 to 4). Thus, as was shown before for gH792 (36), none of these gH truncations was secreted by itself. The shortest fragment, gH102, was not found in the cytoplasmic extract (Fig. 8B, lane 5), and therefore it was not used in any more studies. CHO-K1 cells were then cotransfected with pCMV3gL along with each of the gH plasmids. Culture supernatants were immunoprecipitated with LP11 (Fig. 9B, lanes 1 and 2), but complexes containing gH792-gL and gH648-gL were immunoprecipitated with LP11 (Fig. 9B, lanes 1 and 2), but complexes containing the shorter truncations of gH were not (Fig. 9, lanes 3 to 5). This result suggests that (i) the LP11 epitope remains intact even in the absence of the last two cysteines of gH and that (ii) the LP11 epitope is located upstream of gH648. This result is consistent with the studies of HSV strains (MAb-resistant [MAR] mutants), which do not react with LP11 and contain point or insertion mutations within this region (10, 13). Similar results were also obtained for another conformation-dependent anti-gH MAb, 53S (data not shown).

Next we determined the shortest truncation of gL which was sufficient for complex formation with gH. gL C-terminal truncations (Fig. 5) were constructed, with the Flu HA epitope attached to the C terminus of each protein (34) for the convenience of detection by anti-HA MAb 12CA5 (49). Each construct was transiently cotransfected into CHO-K1 cells along with plasmid pSR162, encoding amino acids 1 to 792 of gH. The assumption was that gH792 would be secreted only if the truncated gL protein formed a proper complex with it. Culture supernatants were immunoprecipitated with 12CA5. After Western blotting, R137 serum was used to detect secreted gH792 (Fig. 10A), and a UL1-1 antibody was used to detect each of the gL truncations (Fig. 10B). We found that gH792 was secreted when cells were cotransfected with pMNN15, encoding gL168 (Fig. 10A, lane 1) or with pMNN110, encoding gL161 (Fig. 10A, lane 2). gH792 was not secreted when cells were cotransfected with pSR162 and plasmids encoding shorter C-terminal truncations of gL (Fig. 10A, lanes 3 to 7). Interestingly, of all the gL plasmids tested by cotransfection, only gL168 and gL161 were detected in the culture supernatant (Fig. 10B, lanes 1 and 2). To show that each of the truncated gL proteins was actually synthesized, we carried out SDS-PAGE and Western blot analysis of the cytoplasmic extracts prepared from each transfection mixture (Fig. 10C and D). gH792 was present in each of the cell extracts (Fig. 10C), and the truncated forms of gL was also synthesized (Fig. 5).

FIG. 5. Positions of the epitopes for MAbs to gH and gL mapped in this study. Schematic figures depict the linear amino acid sequences of gH and gL. The hatched bars depict the locations of the epitopes of anti-gH and anti-gL antibodies. The position of MAb 52S is according to the amino acid change (residue 536) of two MAR mutants selected by 52S (13).

FIG. 8. Expression of gH truncations by transfected CHO-K1 cells. (A) Culture supernatants were immunoprecipitated (IP) with gH-specific MAb H6, electrophoresed on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with R137. (B) Cell extracts were immunoprecipitated (IP) with gH-specific MAb H6, electrophoresed on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with R137. The bands corresponding to the various gH truncations are indicated with arrows. In both A and B, cells were transfected with pSR162 (lanes 1); pSR124 (lanes 2); pSR123 (lanes 3); pCMV3gH323 (lanes 4); and pSR125 (lanes 5).
10D). We conclude that gL161 is the shortest gL truncation able to complex with gH792 and be secreted.

Having demonstrated that gH323 can be secreted when co-expressed with full-length gL and that gL161 is able to form a complex with gH792, we wondered whether a stable, secreted complex could form when these two short forms of each protein were coexpressed. Plasmids pMN115 and pMN110, expressing either gL168 or gL161, respectively, were cotransfected with each of the gH plasmids. Culture supernatants were immunoprecipitated (IP) with MAbs H6 (anti-gH) and 8H4 (anti-gL) (A) or with LP11 (anti-gH-gL) (B). Proteins were resolved on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with R137. Lanes contain culture supernatants of cells cotransfected with pSR162 and pCMV3gL (lanes 1), pSR124 and pCMV3gL (lanes 2), pSR123 and pCMV3gL (lanes 3), pCMV3gL323 and pCMV3gL (lanes 4), or pCMV3gL (lanes 5) or of mock-transfected cells.

**FIG. 9.** Determination of the shortest C-terminal truncation of gH that forms a complex with full-length gL and is secreted. CHO-K1 cells were cotransfected with pCMV3gL, encoding full-length gL, and a plasmid encoding one of the five gH truncations. Culture supernatants were immunoprecipitated (IP) with MAbs H6 (anti-gH) and 8H4 (anti-gL) (A) or with LP11 (anti-gH-gL) (B). Proteins were resolved on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with R137. Lanes contain culture supernatants of cells cotransfected with pSR162 and pCMV3gL (lanes 1), pSR124 and pCMV3gL (lanes 2), pSR123 and pCMV3gL (lanes 3), pCMV3gL323 and pCMV3gL (lanes 4), or pCMV3gL (lanes 5) or of mock-transfected cells.

**FIG. 10.** Determination of the minimal size of gL that forms a complex with gH792 and is secreted. CHO-K1 cells were cotransfected with pSR162, encoding gH792, and plasmids encoding one of the seven gL truncations. (A and B) Culture supernatants were immunoprecipitated (IP) with anti-gH MAb H6 and anti-HA MAb 12CA5 (directed at the HA epitope present in each of the gL truncations). The precipitated proteins were resolved on a 16% polyacrylamide denaturing gel and transferred to nitrocellulose. The blot was cut in half, and the top half was probed with R137 to detect gH (A); the bottom half was probed with αUL1-1 to detect gL (B). (C and D) Cell extracts were immunoprecipitated with H6 and 12CA5. The precipitated proteins were resolved on a 16% polyacrylamide denaturing gel and transferred to nitrocellulose. The blot was cut in half, the top half was probed with R137 to detect gH (C), and the bottom half was probed with αUL1-1 to detect gL (D). Lanes contain cells transfected with pSR 162 and pMN115 (lane 1); pMN110 (lane 2); pMN109 (lane 3); pMN108 (lane 4); pMN107 (lane 5); pMN112 (lane 6); and pMN114 (lane 7).
The positions of IgG heavy chain (HC) and light chain (LC) are also indicated. pSR123 plus pMN110 (lane 3); pCMV3gH323 plus pMN110 (lane 4); and pMN110 (lane 5). The positions of bands representing truncated gHs are indicated by arrows.

nitrocellulose, and probed with R137 and aUL1-1. Lanes in panels B and C are cells transfected with pSR162 plus pMN110 (lane 1); pSR124 plus pMN110 (lane 2); pSR123 plus pMN115 (lane 3); pSR124 plus pMN115 (lane 4); pCMV3gH323 plus pMN115; lane 5; pMN115. (B) Culture supernatants were immunoprecipitated (IP) with anti-gH MAb H6 and anti-HA MAb 12CA5. Protein was resolved on 12% polyacrylamide denaturing gel, transferred to nitrocellulose, and probed with R137. Lanes in panels B and C are cells transfected with pSR162 plus pMN110 (lane 1); pSR124 plus pMN110 (lane 2); pSR123 plus pMN110 (lane 3); pCD3gH323 plus pMN10 (lane 4); and pMN110 (lane 5). The positions of bands representing truncated gHs are indicated by arrows. The positions of IgG heavy chain (HC) and light chain (LC) are also indicated.

**FIG. 11.** The smallest complexes formed and secreted by cells cotransfected with plasmids expressing truncated gH and truncated gL. CHO-K1 cells were cotransfected with gH truncation mutants and pMN115 (A) or pMN110 (B and C). (A) Culture supernatants were immunoprecipitated (IP) with anti-gH MAb H6, and the precipitates were resolved on 12% denaturing polyacrylamide gels, transferred to nitrocellulose, and probed with R137. Lane 1, pSR162 plus pMN115; lane 2, pSR124 plus pMN115; lane 3, pSR123 plus pMN115; lane 4, pCMV3gH323 plus pMN115; lane 5, pMN115. (B) Culture supernatants were immunoprecipitated (IP) with anti-gH MAb H6, and the precipitates were resolved on 12% denaturing polyacrylamide gels, transferred to nitrocellulose, and probed with R137. (C) Cell extracts were immunoprecipitated (IP) with anti-gH MAb H6 and anti-HA MAb 12CA5. Protein was resolved on 12% polyacrylamide denaturing gel, transferred to nitrocellulose, and probed with R137 and aUL1-1. Lanes in panels B and C are cells transfected with pSR162 plus pMN110 (lane 1); pSR124 plus pMN110 (lane 2); pSR123 plus pMN110 (lane 3); pCD3gH323 plus pMN10 (lane 4); and pMN110 (lane 5). The positions of bands representing truncated gHs are indicated by arrows. The positions of IgG heavy chain (HC) and light chain (LC) are also indicated.

**DISCUSSION**

Four of the 11 HSV glycoproteins are essential for the entry of HSV into mammalian cells (3, 8, 24, 38, 44). Determination of the precise contribution of each virion glycoprotein to the entry process has been complicated by the sheer number of proteins involved as well as by the unknown relationship of one to another. It is now established that entry requires interaction between virion gD and one of several gD-specific cell surface receptors (11, 29, 30, 45, 47). This interaction coupled with the gC-glycosaminoglycan attraction may be viewed as a set of coordinated steps which lead to virus-cell fusion. The three remaining essential glycoproteins, gB and the gH-gL complex, are likely to be involved in the membrane fusion step (43). Tuner et al. (44) recently showed that gD, gB, and gH-gL are necessary and sufficient for HSV-induced cell fusion.

Which approaches can be used to shed light on this process and the molecules involved? We continued here by studying the properties of a secreted soluble form of the gH-gL complex. We previously showed that gHt-gL produced by HL-7 cells retains its native structure after purification as judged by its interaction with conformation-dependent MAbs and its ability to induce neutralizing antibodies and protect animals against viral challenge (35). Unlike soluble gD, gHt-gL did not block virus entry (35). Thus, although viral entry probably requires interaction of virion gH-gL with another virion glycoprotein or with a cell surface receptor, soluble gHt-gL failed to block this protein interaction. We decided that we lacked a good working model of gH-gL structure, and the goal here was to obtain data that would fill in details of antigenic structure and oligomerization. Based on the studies described in this article as well as on other known information about gH and gL, we propose a model (Fig. 12) to illustrate what we now know about this glycoprotein complex.

Our first objective was to determine the stoichiometry of the complex. An estimate of 1:1 for the two proteins was made by Hutchinson et al. (19) by immunoprecipitation methods. Here we confirmed and extended these results by three different approaches to show that there is one molecule each of gH and gL in the truncated gH and gL complex. According to the size of the complex determined by gel filtration (180 kDa), it appears that the bulk of the complex exists in solution as a heterodimer. However, a small fraction of the protein eluted at a higher molecular weight, and higher-molecular-weight forms were seen by native SDS-PAGE. Analytical ultracentrifugation studies are now under way to examine this issue more precisely. Interestingly, higher oligomeric forms of gH-gL were not found in the virion by chemical cross-linking techniques (15). Thus, our working model (Fig. 12) contains one molecule each of gH and gL. Here, we show a full-length version of gH-gL which is anchored in the virion envelope via the TMR of gH.

Earlier, we detected complex oligosaccharides on gH during infection (36). In the present study, we showed that both proteins contained complex sugars and sialic acid. Our studies also suggest that most of the consensus sites for the addition of N-CHO in gH are utilized. These sites are distributed throughout the length of the protein (see model, Fig. 12). How much
the N-CHO contributes to function is not known, but the most
C-terminal N-CHO (NGT at residues 783, 784, and 785) is
conserved among other gH homologs (12, 20, 21, 27, 28, 31,
37), although it is not required for infection (10). Furthermore,
both gH and gL contain O-CHO and sialic acid. All of the
predicted sites for O-glycosylation of gL are clustered near its
C terminus. The locations of the oligosaccharides were taken
into consideration when the model was being constructed.

We also incorporated published information about gH and
gL MAbs as well as the new information from this study into
the thinking behind this model. For example, LP11 recognizes
a conformation-dependent epitope which requires proper
oligomerization of gH and gL (14). This MAb protects animals
by passive immunization (9). Because LP11 has a high level of
virus-neutralizing activity, it has been used to isolate MAR
mutant viruses (13). In addition, linker insertion mutants of gH
have been examined for LP11 activity (10). These mutant sites
have been mapped to gH residues 86 to 326 (10, 13). Most of
the continuous epitopes of gH mapped in this study are located
on gH323. Thus, the first 323 amino acids of gH contain a
major antigenic site, with both continuous and discontinuous
epitopes. A second antigenic site is located between amino
carboxyl terminal truncations of gH are secreted from cells
(36), but gL is secreted from transfected cells that do not
express gH (5). We found that the shortest fragment of gH that
supported complex formation with gL and secretion is gH323.
This estimate for gH must be tempered by the fact that the
shortest fragment, gH102, was not detected in the transfected
cell in the presence or absence of full-length gL. Interestingly,
the shortest fragment necessary for gH-gL complex formation in
HHV-6 requires the first 320 residues of gH (1). It is also of
interest that this region of HSV-1 gH overlaps a major anti-
genic site.

Our studies showed that gL161 is the minimal portion of gL
necessary for complex formation and secretion with gH792.
However, gL168 was the shortest gL truncation which could
fold and form a secreted complex with gH323. It should be
noted that the major antigenic site on gL is downstream of the
portion which interacts with gH. Thus, this portion of gL is
probably exposed to the outside, as depicted in Fig. 12. Since
gL161 and gL168 are truncated after the fourth cysteine, we
argue that gL forms two disulfide bonds, both of which are
necessary for proper folding and oligomerization with gH. A
similar conclusion was drawn for varicella-zoster virus gL (6,
23).

The contribution of disulfide bonds to glycoprotein structure
and function is critical. The disulfide bond arrangements of gD
(25), gC (39), and gB (32) are known. What is the contribution
of the cysteine residues to the structure of gHt-gL? It is rela-
tively clear that gH is not disulfide bonded to gL, since the
two molecules dissociate under nonreducing conditions (19).
What do our data suggest about intramolecular disulfide bond-
ing? First, gH792 contains eight cysteines in the full-length
protein, while gH(648) contains six; gH(475) contains four, and
H(323) contains two. We hypothesize that since these four
truncated forms of gH are able to complex with gL, there are
no unpaired cysteines which could result in a malformed pro-
tein (25). According to this hypothesis, gH323 contains one
disulfide bond (i.e., C1-C2), gH475 contains two (C1-C2 and
C3-C4), and gH678 contains three (C1-C2, C3-C4, and C5-C6). Cysteines 7 and 8 are depicted as paired by default. We de-
picted the proposed disulfide bond arrangement as broken
lines between the hypothetically paired cysteines in Fig. 12.
Paired cysteines have also been found in the first 230 amino
of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. 65:1090–1098.


24. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligo- carbohydrates of HHV-6 (1). Clearly, this hypothesis should be tested for the next. PENG ET AL. J. VIROL.


32. Robert, M. C., J. H. Johnson for synthetic peptides UL1-1 and UL1-2 and 19525 from Cancer and Diabetes Centers core support grants (J.D.L.).


44. Turner, A., B. Bruun, T. Minson, and H. Browne. 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J. Virol. 72:873–875.