Virology **261,** 96–105 (1999)
Article ID viro.1999.9860, available online at http://www.idealibrary.com on IDEAL®

Domains of Glycoprotein H of Herpes Simplex Virus Type 1 Involved in Complex Formation with Glycoprotein L

Douwe F. Westra,* H. Bea Kuiperij,* Gjalt W. Welling,* Albert Jan Scheffer,* T. Hauw The,† and Sytske Welling-Wester*,[1]

*Departments of *Medical Microbiology and †Clinical Immunology, University of Groningen, P.O. Box 30.001, 9700 RB, Groningen, The Netherlands*

Received March 9, 1999; returned to author for revision April 29, 1999; accepted June 17, 1999

The complex formation between glycoproteins H (gH) and L (gL) of herpes simplex virus type 1 (HSV-1) was studied by using five recombinant baculoviruses expressing open reading frames that contain deletions in the coding region of the extracellular domain of gH. In addition, the gH-deletion mutants contained a C-terminal tag. Complex formation of gL and the gH-deletion mutants was studied by immunoprecipitations with anti-tag monoclonal antibody (MAb) A16 and with the gH-specific MAbs 37S, 46S, and 52S. All gH-deletion mutants were complexed to gL when analyzed by MAb A16. MAb 37S precipitated complexes between gL and the two gH-deletion mutants that contain the epitope of this MAb. When the gH conformation-dependent MAbs 46S and 52S were used, gL was coprecipitated together with the gH-deletion mutant lacking amino acids 31–299, but gL was not coprecipitated with the gH-deletion mutant lacking amino acids 31–473. The data from the precipitation studies do allow at least two interpretations. There is either one site for gL binding on gH (residue 300–473) or gL contacts multiple regions of gH. We were unable to demonstrate gL-dependent cell surface expression of either of the gH-deletion mutants. This suggests that the coassociation of gH with gL is necessary but not sufficient for transport of gH to the cell surface.

INTRODUCTION

Virions of herpes simplex virus type 1 (HSV-1) are able to enter cells either by fusion of the virion envelope with the plasma membrane or by cell-associated spread that involves fusion of plasma membranes of infected cells with plasma membranes of uninfected cells (Spear, 1993). The glycoproteins gB, gD, and gH/gL of HSV are essential for both pathways of entry (Cai et al., 1988; Ligas and Johnson, 1988; Roop *et al.,* 1993; Forrester *et al.,* 1992; Turner *et al.,* 1998). The gH/gL complex has an essential role in the fusion of the involved membranes and not in the attachment of the virion to the cell (Desai *et al.,* 1988; Fuller *et al.,* 1989; Fuller and Lee, 1992; Hutchinson *et al.,* 1992; Roop *et al.,* 1993). The cell-to-cell spread and the virion infectivity may require different domains on the gH/gL complex. It has been reported that specific amino acids in the cytoplasmic domain are required for efficient gH-mediated membrane fusion (Wilson *et al.,* 1994; Browne *et al.,* 1996). Antibodies to gL inhibit only the virion-induced cell fusion, not the virion infectivity (Novotny *et al.,* 1996). Antibodies to gH neutralize virus infectivity and inhibit the cell-to-cell spread of HSV (Gompels *et al.,* 1991).

In addition, gH and gL are involved in the immune response during HSV infections. Mice immunized with the gH/gL complex produced neutralizing antibodies against HSV, showed reduced primary infections, and exhibited no secondary lesions upon challenge with HSV (Peng *et al.,* 1998b). In addition, gH-specific T cell clones have been isolated from patients with herpes simplex virus-mediated acute retinal necrosis (G.M. Verjans, personal communication).

The formation of a hetero-oligomer between gH and gL of HSV-1 is essential for the processing, folding, and cell surface expression of gH (Roop *et al.,* 1993; Hutchinson *et al.,* 1992). In members of all three subfamilies of herpesviruses, complexes are formed between gH and an additional viral glycoprotein (Kaye *et al.,* 1992; Spaete *et al.,* 1993; Yaswen *et al.,* 1993; Liu *et al.,* 1993). Despite this reoccurring theme, there are differences between gH/gL complex formation among the viruses. The association between gH and gL is mediated by a noncovalent interaction among HSV-1, varicella-zoster virus (VZV), and Epstein–Barr virus (EBV) (Hutchinson *et al.,* 1992; Duus *et al.,* 1995; Li *et al.,* 1995). However, gH of human herpesvirus 6 (HHV-6) and of human cytomegalovirus (HCMV) is covalently linked to gL (Kaye *et al.,* 1992; Anderson *et al.,* 1996). An additional difference is that gL of VZV does not contain a signal sequence that directs its synthesis to the biosynthetic pathway but contains an endoplasmic reticulum-targeting sequence (Duus and Grose, 1996). A third protein was found to be associated to the gH/gL complex of HCMV and of EBV (Li *et al.,* 1995; Huber and

[1] To whom reprint requests should be addressed at Hanzeplein 1, 9713 GZ Groningen. Fax: +31-503633528. E-mail: S.Welling-Wester@med.rug.nl.

0042-6822/99 $30.00

Compton, 1997). The third partner of the EBV gH/gL complex, gp42, binds to HLA class II (Spriggs *et al.,* 1996). This presence of gp42 in the gH/gL complex is necessary for EBV infection of B-lymphocytes but not for infection of epithelial cells (Li *et al.,* 1995, 1997b).

HSV-1 gH has the sequence of a typical type 1 membrane protein. It has a predicted signal sequence of 18 amino acids, a large extracellular region, residues 19–804, a C-terminal transmembrane region, and a 14-amino-acid cytoplasmic tail, residues 824–838. The signal sequence directs the synthesis of gH to the biosynthetic pathway and the transmembrane region of gH anchors the gH/gL complex to the cell membrane (Dubin and Jiang, 1995). The C-terminal part of the extracellular region is conserved between gH of HSV-1 and HSV-2. It probably contains, together with the cytoplasmic tail, essential residues for the fusion function of gH (Browne *et al.,* 1996; Galdiero *et al.,* 1997). The extracellular domain probably contains domains involved in binding of gL because gH, which lacks the transmembrane region and the cytoplasmic tail, is still able to bind to gL and is transported to the cell surface in a gL-dependent manner (Dubin and Jiang, 1995; Westra *et al.,* 1997).

Studies have been undertaken to define the functional domains in the extracellular region. Galdiero *et al.* (1997) generated linker-insertion mutants of gH and tested their ability to bind MAb LP11. This MAb recognizes gH only when it is structurally intact and complexed to gL. Results indicated that insertions in the N-terminal half of the external domain induced changes in gH, which inhibited reactions with LP11 but allowed surface expression of gH. In another approach, Peng *et al.* (1998a) studied gH/gL complex formation by using truncated forms of gH and gL. In this study, it was shown that the first 323 amino acids of gH were sufficient for secretion of the gH/gL complex from CHO-K1 cells cotransfected with plasmids for gL and the truncated gH forms.

In our study, we extended the studies described above by using recombinant baculoviruses expressing mutants of full-length gH that contain deletions in the extracellular domain. Full-length gH and the gH-deletion mutants were tagged at the C-terminus with the epitope of MAb A16. The interaction between gL and these gH-deletion mutants was analyzed by immunoprecipitation studies using MAbs directed against the tag and directed against linear and conformation-dependent epitopes of gH. Despite the complex formation that was observed between gL and gH-deletion mutants in the immunoprecipitation studies, no cell surface expression of the complexes could be demonstrated. In addition, we analyzed whether the domains of gH that are involved in gL binding are conserved among alphaherpesviruses. Therefore, gLs from different alphaherpesviruses were studied for their ability to transport gH of HSV to the surfaces of insect cells.

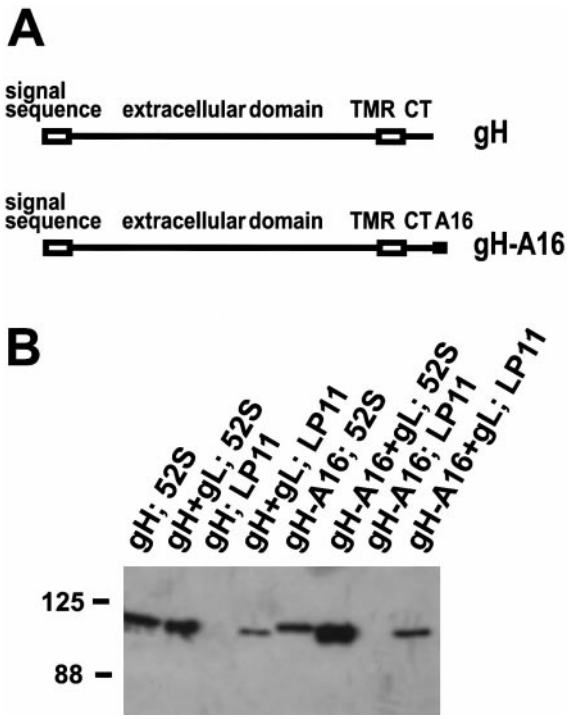

FIG. 1. (A) Schematic representation of gH and gH-A16. The construct gH-A16 contains a linear epitope tagged to the C-terminus, the cytoplasmic tail, of gH. The tag (A16) is recognized by MAb A16, a MAb directed against a group VII epitope of gD. The signal sequence, the extracellular domain, the transmembrane region (TMR), the cytoplasmic tail (CT), and the A16 tag (A16) are indicated. (B) Immunoprecipitations of gH and gH-A16 by MAbs 52S and LP11. Insect cells were infected with recombinant baculoviruses that express gH and gH-A16 or were coinfected with recombinant baculovirus BacgL (gH + gL, gH-A16 + gL). The cells were lysed 48 h after infections. The lysates were immunoprecipitated with conformation-dependent MAbs 52S and LP11, separated by SDS–PAGE, and immunoblotted with polyclonal antibody anti-gH1. The membrane was subsequently incubated with peroxidase-conjugated goat anti-rabbit, and antibody binding was visualized by a luminogenic reaction (ECL-Western light; Amersham). The molecular mass markers are indicated at the left.

RESULTS

Expression of epitope-tagged gH (gH-A16)

To allow detection of gH deletion mutants, gH and mutant gH were tagged with an 11-amino-acid peptide at the C-terminus of the cytoplasmic tail (Fig. 1A). This tag is a linear epitope of gD of HSV-1, which is the original target of MAb A16 (Scheffer *et al.,* 1984). A recombinant baculovirus that contains the open reading frame of gH with the A16 tag under control of the polyhedrin promoter was obtained and designated BacgH-A16. Next we analyzed whether the introduction of the A16-tag to gH (designated as gH-A16) had any effect on the interaction of gH and gL. Insect cells were infected with recombinant baculoviruses expressing either gH-A16-tagged or untagged gH and coinfected with recombinant baculovirus expressing gL (BacgL). To analyze the folding, the infected and coinfected cells were lysed 48 h after infec-

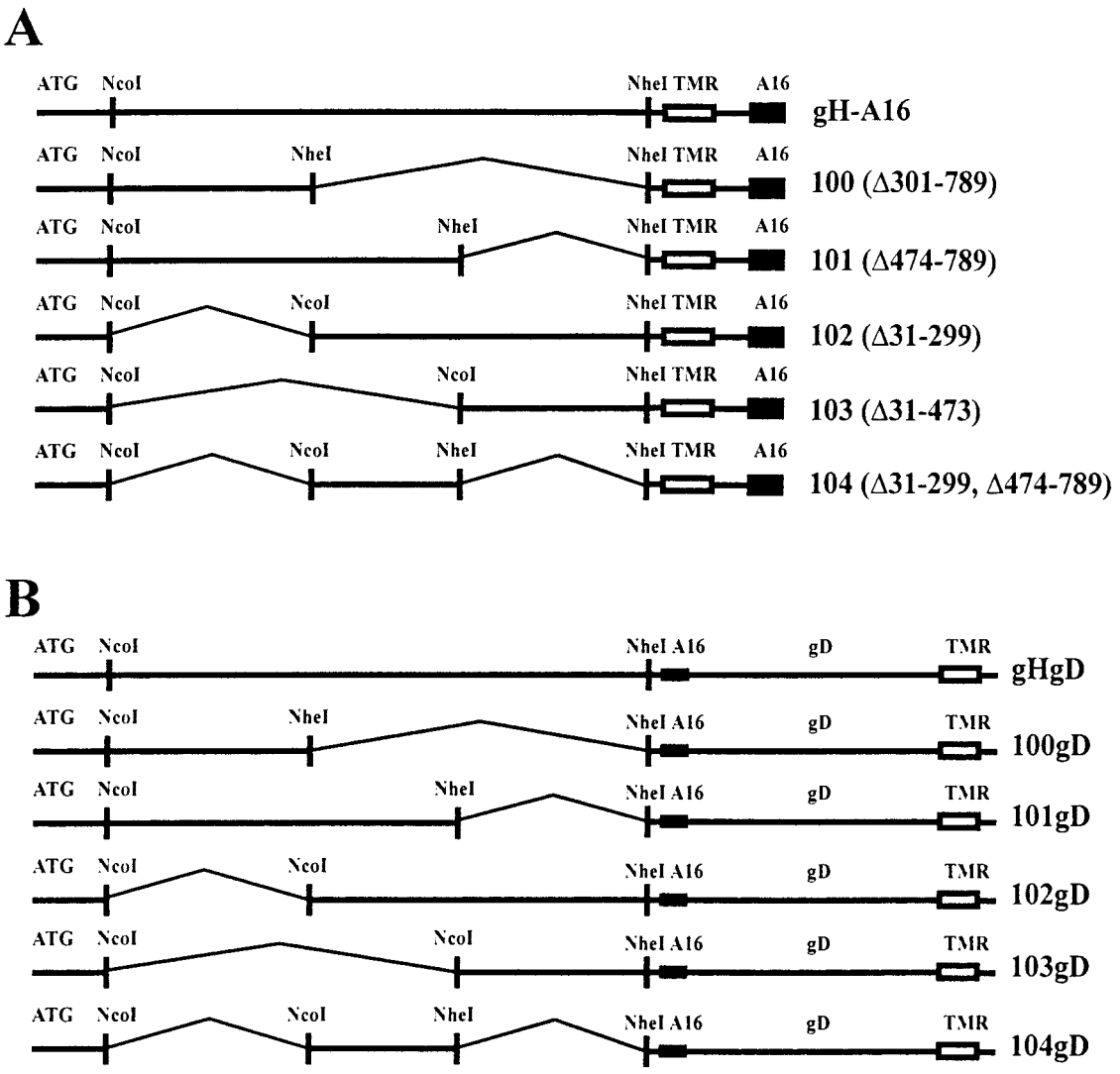

FIG. 2. Schematic representation of the gH-deletion mutants and of the gH/gD fusion proteins. (A) The gH-deletion mutants 100–104 contain deletions in the extracellular domain of gH-A16. The elevated lines indicate the deletions, and the deleted amino acids are indicated in parentheses at the right. (B) The fusion protein gH/gD contains the extracellular region and transmembrane region of gD downstream of the extracellular domain of gH. In addition, gD fusion proteins were made similar to the gH-deletion mutants depicted in A. The deletions present in the extracellular domain of gH in mutants 100–104 were also present in mutants 100gD, 101gD, 102gD, 103gD, and 104gD. The restriction sites *Nco*I and *Nhe*I, the transmembrane regions (TMR), and the A16 tags/epitopes (A16) are indicated.

tion and the lysates were immunoprecipitated with the gH-specific, conformation-dependent MAbs 52S (Roberts *et al.,* 1991) and LP11 (Gompels and Minson, 1989). The immunoprecipitated proteins were separated on SDS–polyacrylamide gels and immunoblotted with anti-gH1, a polyclonal serum raised against the purified gH/gL complex. MAb 52S precipitated recombinant gH and gH-A16 in the presence and absence of gL. MAb LP 11 precipitated recombinant gH and gH-A16 only in the presence of gL (Fig. 1B). This is in agreement with the epitope specificity of these MAbs (Showalter *et al.,* 1981; Gompels and Minson, 1989). The cell surface expression of gH-A16 was analyzed by FACS. Infected and coinfected insect cells were assayed after 46 h of infection for cell surface expression of gH. The epitope-tagged gH was demonstrated by MAbs 52S on the surface of insect cells, which coproduced gH-A16 and gL (see FACS analyses in Fig. 6B). In conclusion, the gL-dependent folding and cell surface expression were not changed by the introduction of the A16 epitope at the C-terminus of gH.

Expression of deletion mutants of gH-A16

To examine the regions of gH involved in interaction with gL, five open reading frames that contain deletions in the extracellular domain of gH-A16 were constructed (Fig. 2A). Recombinant baculoviruses that contain these open reading frames under control of the polyhedrin promoter were generated by using the Bac-to-Bac system and were designated by the numbers 100–104 (Fig.

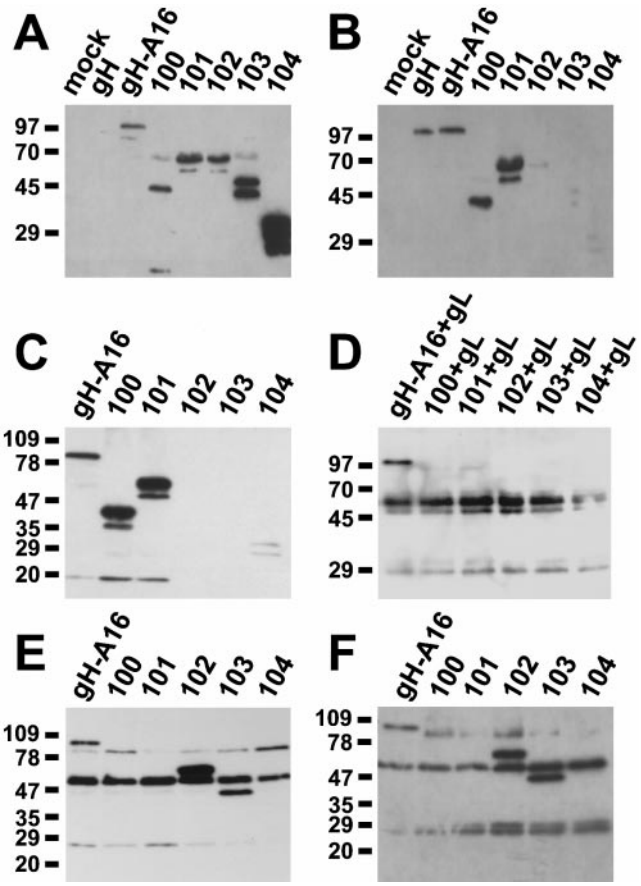

FIG. 3. Expression of the gH-deletion mutants 100–104, analyzed with gH-specific antibodies. Insect cells were infected with recombinant baculoviruses that expressed gH, gH-A16, and the deletion mutants (A–C, E, and F) or were coinfected in combination with recombinant baculovirus BacgL (D). The cells were lysed 48 h after infection. The lysates were either directly separated by SDS–PAGE and immunoblotted with MAb A16 (A), polyclonal anti-gH serum (B), and MAb 37S (C), or lysates were used in immunoprecipitations with the conformation-dependent MAb LP11 (D), MAb 46S (E), and MAb 52S (F). Precipitates were separated by SDS–PAGE and immunoblotted with MAb A16 to detect tagged gH and the deletion mutants of gH. The membranes were subsequently incubated with peroxidase-conjugated goat anti-rabbit (B) or goat anti-mouse (A, C–F). Antibody binding was visualized by a luminogenic reaction (ECL-Western light; Amersham). The molecular mass markers are indicated at the left of the blot.

2A). Insect cells were infected with the recombinant baculovirus BacgH-A16 and with the recombinant baculoviruses 100–104. Cells were lysed after 48 h of infection. Lysates of the infected cells were separated by SDS–PAGE and analyzed by immunoblotting (Figs. 3A–3C). The expression of the gH-deletion mutants was first demonstrated with antibodies that recognized linear epitopes: MAb A16 (Fig. 3A), polyclonal serum anti-gH (Fig. 3B), and MAb 37S (Fig. 3C). All of the gH-deletion mutants could be detected by MAb A16 in the soluble fraction of the cell lysates by immunoblotting (Fig. 3A). The gH-deletion mutants have molecular weights that approximately correspond to the calculated molecular weight. The lysates of cells that were infected with recombinant baculoviruses 103 and 104 contain prominent multiple polypeptide bands. These bands may represent either differentially glycosylated recombinant gH-A16 mutant proteins or degradation products. The polyclonal anti-gH serum (Fig. 3B) predominantly recognized the deletion mutants 100 and 101, which have the N-terminal 300 amino acids in common. Because MAb 37S (Roberts *et al.,* 1991) has a linear epitope in the N-terminal 300 amino acids of gH, the same deletion mutants (numbers 100 and 101) were recognized (Fig. 3C).

In addition, immunoprecipitation studies (Figs. 3D–3F) were performed with the cell lysates of the infected cells. Lysates were treated with conformation-dependent MAbs LP11 (Fig. 3D), MAb 46S (Fig. 3E), and MAb 52S (Fig. 3F). The precipitated proteins were separated by SDS–PAGE and immunoblotted with MAb A16. Only gH-A16 (Fig. 3D) in combination with gL was precipitated by MAb LP11 and none of the gH-deletion mutants, expressed either with or without gL. The conformation-dependent MAbs 46S and 52S precipitated the gH-deletion mutants produced by recombinants 102 and 103 (Figs. 3E and 3F). The additional bands present in all lanes of the immunoblots in which the immunoprecipitates were analyzed (Figs. 3D–3F) are most likely due to the immunoglobulin light and heavy chains.

Interaction between gL and gH-deletion mutants

To study which of the deletion mutants of gH-A16 were able to bind to gL, immunoprecipitation experiments were performed using MAbs against the tag (MAb A16) and against conformation-dependent (MAbs 52S and 46S) and conformation-independent epitopes (MAb 37S) of gH.

Initially, the complex formation between gH-deletion mutants and gL was studied by immunoprecipitation experiments performed with MAb A16, which recognized all of the A16-tagged deletion mutants. Insect cells were infected with the recombinant baculovirus BacgH-A16 and recombinant baculoviruses 100–104 and were coinfected with BacgL. The cells were lysed 48 h after infection. The lysates were immunoprecipitated with MAb A16, and the immunoprecipitated proteins were separated by SDS–PAGE and immunoblotted to detect gL with antiserum W192S5, a polyclonal serum directed against a peptide (residues 213–224) of gL1. These experiments showed that gL coprecipitated together with all of the gH-deletion mutants (Fig. 4). No gL was precipitated from insect cells that expressed gL alone. In addition, the complex formation was studied by immunoprecipitation experiments with gH-specific MAb 37S. This MAb recognizes a linear epitope in the N-terminal 300 amino acids of gH (Roberts *et al.,* 1991b). The results indicated that gL coprecipitated together with the gH-deletion mutants expressed by recombinants 100 and 101 (Fig. 5C). These recombinants lacked residues 301–789 and 474–

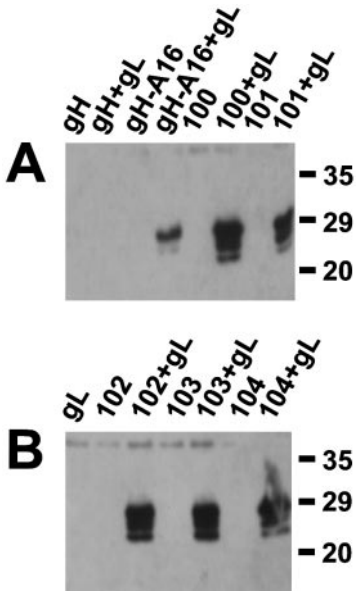

FIG. 4. Analyses of the formation of hetero-oligomerization between gL and the gH-deletion mutants by immunoprecipitation with the anti-tag MAb A16. Insect cells were infected with the recombinant baculoviruses BacgH, BacgH-A16, and 100–104 or coinfected in combination with BacgL. The cells were lysed after 48 h of infection. The lysates were inmmunoprecipitated by MAb A16. The immunoprecipitates were separated by SDS–PAGE and immunoblotted with the anti-gL serum W192S5. The membranes were incubated with peroxide-conjugated goat anti-rabbit. Antibody binding was visualized by a luminogenic reaction (ECL-Western light; Amersham). The molecular mass markers are indicated at the right.

789 of gH, respectively. No reaction of MAb 37S and the mutant gH of recombinants 102–104 was observed because the epitope of MAb 37S is not present in these deletion mutants (see also Fig. 3C). The results of the precipitation studies with MAb A16 suggest that the domains of gH for interaction with gL are, despite the deletions, still present on gH. All five mutants have only two regions in common: residues of amino acids 789–838 plus the A16 tag and the N-terminal 30 amino acids (Fig. 2A). In a previous study, we demonstrated that truncated gH, lacking amino acid residues 789–838, was able to bind gL (Westra *et al.,* 1997).

Because MAbs A16 and 37S are directed against conformation-independent epitopes and will precipitate correctly and incorrectly folded gH, we decided to extend the immunoprecipitation studies with conformation-dependent MAbs 52S and 46S directed against gH. Thus precipitations with MAbs 46S and 52S may limit the detection essentially to conformationally intact gH. The epitopes of both antibodies are present on gH-deletion mutants expressed by recombinants 102 and 103 (Figs. 3E and 3F). The results obtained when MAbs 46S and 52S were used for the precipitation studies proved surprising. The gH-deletion mutant 102, lacking residues 31–299, was able to coprecipitate gL, whereas mutant 103, lacking residues 31–473, was unable to do so (Figs. 5A and 5B). The lack of coprecipitation of gL with mutant 103 was not due to a failure of precipitating the mutant gH. Mutant 103 could be detected in the precipitates by immunoblotting with anti-tag MAb A16 (data not shown). The difference between mutants 102 and 103 suggests that the region of amino acids 300–473 of gH might be involved in the interaction with gL.

Cell surface expression of the gH-deletion mutants

Binding of gL to gH and the gL-dependent cell surface trafficking of gH represent two different protein functions. Both functions may require different domains of gH. However, binding of gL to gH may be one of the prerequisites for the transport of gH to the cell membrane. Therefore, gL-dependent trafficking to the surfaces of insect cells of gH-mutants probably gives information about the gL interaction site on gH.

The display of the gH-deletion mutants on the cell surfaces of insect cells in the presence and absence of gL was assayed by FACS analyses. The cell surface expression of the gH-deletion mutant that lacks the regions of amino acids 31–299 and 474–789 was not investigated due to the lack of an appropriate MAb. The results from these experiments showed that none of the gH-deletion mutants could be detected on the surfaces of insect cells when analyzed with MAb 52S and poly-

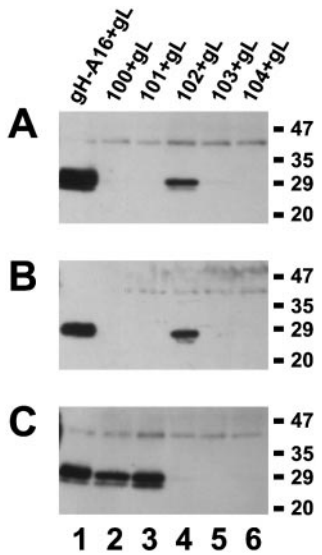

FIG. 5. Analyses of the binding of gL to the gH-deletion mutants by immunoprecipitation with gH-specific MAbs 37S, 46S, and 52S. Insect cells were infected with the recombinant baculoviruses BacgH, BacgH-A16, and 100–104 or coinfected in combination with BacgL. The cells were lysed 48 h after infection. The lysates were immunoprecipitated with MAbs 46S (A), 52S (B), and 37S (C). The immunoprecipitates were separated by SDS–PAGE and blotted onto membranes. The membranes were incubated with an anti-gL serum W192S5, washed, and subsequently incubated with peroxidase-conjugated goat anti-rabbit. Antibody binding was visualized by a luminogenic reaction (ECL-Western light; Amersham). The molecular mass markers are indicated at the right.

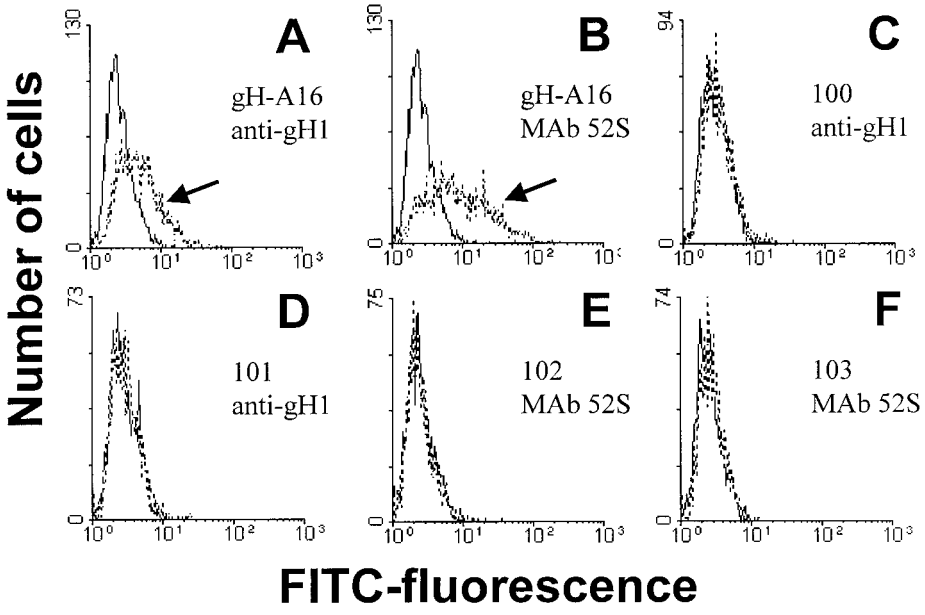

FIG. 6. Flow cytometric analyses of the cell surface expression of the gH-deletion mutants. Insect cells were infected with the recombinant baculoviruses BacgH-A16 (A and B) and recombinants 100 (C), 101 (D), 102 (E), and 103 (F) or coinfected in combination with BacgL (A–F). The cells were incubated with polyclonal antibody anti-gH1 (A, C, and D) or with MAb 52S (B, E, and F) for 1 h and subsequently incubated with FITC-conjugated secondary antibodies for 1 h. After incubation with propidium iodide for 10 min, the insect cells were analyzed with a FACSTAR flow cytometer. The number of viable cells, which have a low propidium iodide fluorescence, is plotted along the *y* axis, and the FITC fluorescence is plotted along the *x* axis of the histogram. Infected cells are indicated by straight lines, and coinfected cells are indicated by dotted lines.

clonal antibody anti-gH1 in either the presence or absence of gL (Figs. 6C–6F). Full-length gH-A16 could be detected on the cell surfaces with these antibodies in a gL-dependent manner (Figs. 6A and 6B).

HSV gD is known to be efficiently transported to the cell surface in insect cells (Westra *et al.,* 1997). It has been shown for PRV that a fusion gH/gD protein, selected by repeating passage of a gL-lacking mutant, was able to mediate the functions of the gH/gL complex (Klupp *et al.,* 1999). We decided to try to facilitate cell surface transport by constructing fusion proteins consisting of the gH-deletion mutants and gD. Open reading frames were constructed in which the coding region of the C-terminal part (residues 789–838 plus A16 tag) of the gH-deletion mutants was replaced by the coding region of amino acids 1–36g of mature gD. Recombinant baculoviruses, designated gH/gD, 100gD, 101gD, 102gD, 103gD, and 104gD (Fig. 2B), were obtained that contained these open reading frames controlled by the polyhedrin promoter. Insect cells infected with these recombinant baculoviruses or coinfected with the recombinant virus BacgL were assayed for cell surface expression of gH by FACS analyses with the use of antibodies A16, 52S, and anti-gH1. The results showed that the gH-deletion mutants fused to gD could not be detected on the cell surfaces of insect cells in either the presence or absence of gL (data not shown). Only the fusion protein gH/gD, which contained the complete extracellular domain of gH fused to gD, was observed on the cell surface in a gL-dependent manner.

Cell surface expression of gH of HSV-1 in the presence of the gL homologs of EHV-1, PRV, and VZV

Although the amino acid sequences of gL of the different alphaherpesviruses are not well conserved, the formation of the gH/gL complex and its trafficking to the cell surface of the complex constitute a requirement for viral infectivity in herpesviruses. We decided to analyze whether functional replacement of gL among different alphaherpesviruses was possible. Recombinant baculoviruses expressing HSV-gL, VZV-gL, PRV-gL, and EHV-gL were used to infect insect cells. The cells were coinfected with gH of HSV-1. The display of gH of HSV on the surfaces of the coinfected cells was analyzed by FACS analyses with the use of MAb 52S. HSV gH could be detected at the cell surface only when cells were coinfected with recombinant baculovirus expressing gL of HSV-1. The gL homologs of VZV, PRV, and EHV were not able to transport gH of HSV-1 to the cell surfaces (data not shown).

DISCUSSION

The molecular mechanism of the gL-dependent cell surface expression of gH is not yet understood. To gain further insight into this process, we studied the location of the gL-binding site on gH. Previous studies have shown that truncated gH that lacks its transmembrane region and cytoplasmic tail was able to bind to gL and was transported to the cell surface in a gL-dependent

manner (Dubin and Jiang, 1995; Westra *et al.,* 1997). From this, we concluded that the gL-binding site is located between residues 1–789 of gH or, more likely, on the extracellular domain of gH. To study this, we constructed a panel of gH open reading frames with deletions in the coding region of the extracellular domain of gH. These open reading frames were expressed in insect cells by using the baculovirus expression system. Previously, we have demonstrated that the protein production and transport systems of insect cells are capable of producing correctly folded and transported gH (Westra *et al.,* 1997).

By using gH-deletion mutants, we were able to roughly locate the epitopes of the gH specific antibodies. The rabbit polyclonal antibody anti-gH1 raised against immunopurified gH/gL complex recognizes linear epitopes that are predominantly located on the N-terminal 300 amino acids of gH. MAb 37S recognizes a linear epitope that is located in the same region of gH. The epitopes of the conformation-dependent MAbs 46S and 52S are present in the gH region consisting of residues 474–790. These findings are in agreement with the observation that MAb 52S-resistant viruses contain mutations in the gH sequence at positions 536 and 537 (Gompels *et al.,* 1991). Another pertinent antibody is MAb LP11, which recognizes only gH in combination with gL. The reaction of LP11 with the gH/gL complex was easily disrupted by changes in the sequence of gH. This LP11 epitope was not present on either of the deletion mutants that were coexpressed with gL. This is in agreement with previous studies (Peng *et al.,* 1998a; Gompels *et al.,* 1991a; Galdiero *et al.,* 1997). For example, Galdiero *et al.* (1997) showed that a gH mutant lacking the region of amino acids 671–838 failed to react with LP11, indicating that this portion of the C-terminus is essential for the LP11 epitope. In addition, mutations in the N-terminal part at amino acid positions 86, 168, 201, 300, 313, 316, 317, 325, 326, and 329 impaired the recognition of MAb LP11 (Gompels *et al.,* 1991; Galdiero *et al.,* 1997). Studies in which truncated forms of gH were used showed that MAb LP11 reacted with the complex of gL and a truncated form of gH consisting of residues 1–648 (Peng *et al.,* 1998a). However, complexes of shorter truncated forms of gH and gL did not react with MAb LP11.

The binding of gH and gL was studied by using deletion mutants of gH. Five gH-deletion mutants were constructed lacking residues 301–789 (mutant 100), 474–789 (mutant 101), 31–299 (mutant 102), 31–473 (mutant 103), and 31–299 plus 474–789 (mutant 104), respectively. The contribution of the disulfide bonds is often important for the structure of proteins. For gH, it has been hypothesized that paired cysteines of residues 90 and 258, 404 and 429, 554 and 589, and 652 and 706 form disulfide bonds C1, C2, C3, and probably C4 (Peng *et al.,* 1998a). According to this assertion, our recombinant deletion mutants 100, 101, 102, and 103 would contain disulfide bonds C1, C1 plus C2, C2 plus C3 plus C4, and C3 plus C4, respectively, with no unpaired cysteines. Most likely, the presence of these disulfide bonds largely maintained the native structure of the gH-deletion mutants, despite the large deletions.

The analysis of the binding of gL to the gH-deletion mutants was performed by immunoprecipitations followed by immunoblotting for the detection of gL. The results were as follows: 1) when precipitations were performed with the anti-tag MAb, all gH-deletion mutants coprecipitated gL. 2) With MAb 37S (directed against the N-terminal regions of gH), gH mutants 100 and 101, lacking residues 301–789 and 474–789, respectively coprecipitate gL. 3) When MAbs 46S and 52S are used, gH mutant 102 (lacking residues 31–299) coprecipitates gL, but mutant 103 (lacking residues 31–473) does not.

At least two explanations can be given for the immunoprecipitation data. One explanation could be that only the experiments in which conformation-dependent antibodies were used give information on the gL-binding regions on gH. All gH-deletion mutants were complexed to gL when analyzed with MAb 37S and anti-tag MAb A16. These MAbs A16 and 37S see linear epitopes on gH and do not discriminate between correctly and incorrectly folded gH. Therefore, the coprecipitation of gL when using these antibodies may be caused by a nonspecific interaction between gL and denatured forms of gH. Correctly folded gH-deletion mutant proteins are recognized only by the conformation-dependent MAbs 46S and 52S. If this assumption is correct, it should be concluded that only the region of amino acids 300–473 of gH is involved in gL binding. However, the possibility that the region delimited by amino acids 300–473 is bound in a nonspecific way to gL despite the use of conformation-dependent MAbs 46S and 52S cannot be excluded.

Another explanation for the immunoprecipitation data is that gL contacts multiple regions or domains of gH. Therefore, deletion of one region or domain of gH may not be sufficient to abrogate the interaction of gH and gL. At least three gL contact regions or domains might be present on gH when multiple gL-binding sites form the explanation for our immunoprecipitation data. One contact region is present within the N-terminal 300 amino acids, the second contact region is present between amino acids 300 and 473, and the third contact region consists of amino acids 475–789. This is consistent with the finding that all gH-deletion mutants, which are recognized by MAbs A16 and 37S, were complexed to gL when analyzed with these antibodies. However, the result that MAbs 46S and 52S did not coprecipitate gL together with gH-deletion mutant 103, lacking the region consisting of residues 31–473, is not consistent with this explanation. This could be explained if binding of MAbs 46S and 52S to gH interferes with that particular binding point of gL to gH.

Recently, Peng *et al.* (1998a) published the results of a study in which truncated forms of gH were used to

determine the minimal regions of both gH and gL required for gL/gH complex formation and secretion. In this study, CHO-K1 cells were cotransfected with plasmids containing series of truncated gH along with a plasmid containing gL. The culture supernatants were analyzed for the presence of the secreted gH-truncated/gL complexes. That study demonstrated that the first 323 amino acids of gH and the first 168 amino acids of gL were still able to form a stable secreted complex.

The findings of Peng *et al.* (1998a) are not in contrast to our data and explanations. The conclusion that the amino acids 300–474 of gH are involved in gL binding is drawn from the immunoprecipitation data obtained with conformation-dependent MAbs 46S and 52S. This region contains an overlap of 23 residues with the region published by Peng *et al.* (1998a). The explanation that gL contacts multiple regions of gH may also be consistent with the study of Peng *et al.* (1998a). The gH-deletion mutant, which lacks amino acids 301–789, was complexed to gL when analyzed with MAb 37S and anti-tag MAb A16. This gH-deletion mutant shares the N-terminal 300 amino acids of gH with the region published by Peng *et al.* (1998a). Therefore, this region may be one of the gL-contact regions of gH.

In another study, Anderson *et al.* (1996) showed that the gL-binding site on gH of another herpesvirus, HHV-6, is located on the first 230 amino acids of the N-terminus of gH. The gH/gL binding of the betaherpesvirus HHV-6 is different from the gH/gL binding of the alphaherpesvirus HSV-1. This disparity is best illustrated by the fact that disulfide bond formation contributes to the HHV-6 gH/gL binding, and not to the HSV-1 gH/gL complex formation. The difference found between the gL-binding locations of HHV-6 and of HSV-1 could well be explained by the differences in gH/gL binding for these herpesviruses.

Additional evidence for the regions involved in interaction of gL and gH could be obtained if one or more of the gH-deletion mutants were to show gL-dependent cell surface expression. In our study, however, we found that none of the gH-deletion mutant proteins were displayed on the cell surface, in either the presence or absence of gL. In addition, we investigated the possibility of facilitating the cell trafficking by fusing the gH-deletion mutants to gD1. Nevertheless, only the complete extracellular domain of gH fused to gD showed gL-dependent cell surface expression. In another study, it was found that amino acids 1–427 of gH that were fused to gC were effectively transported to the cell surface of COS-1 cells in a gL-dependent manner (J. Kuhn, personal communication). At the moment, we do not have a likely explanation for the failure of the gL-dependent cell surface transport of the gH deletion mutants; however, the possibility cannot be excluded that different domains of gH are required for gL binding and for gL-dependent cell trafficking. In addition, gL binding to multiple regions of gH may be a prerequisite for gL-dependent cell trafficking.

In a previous study, we reported that the intracellular transport of gH and gL of HSV-1 is comparable in insect cells and mammalian cells. Furthermore, a heterodimer consisting of VZV gL and a truncated form of VZV gH was secreted into the medium when coproduced in insect cells (L. Maresova, personal communication). In this study, we showed that the gL molecules of the alphaherpesviruses VZV, PRV, and EHV-1 were unable to transport gH of HSV1 to the cell surface of insect cells. Despite the conservation of the mechanism of gH/gL complex formation and gL-dependent gH cell surface expression across herpesviruses, the gH/gL binding of the alphaherpesviruses investigated probably evolved in such a way that gL homologs from other alphaherpesviruses cannot substitute the HSV-gL function. Therefore, the gL binding might be localized on a domain that is not conserved between the alphaherpesviruses. In a previous study, it was shown that the gL molecules of VZV and EBV were functionally interchangeable (Li *et al.,* 1997a). Additional studies are necessary to further elucidate the details of the gL/gH complex formation. Ultimately, the three-dimensional structure of the gH/gL complex will reveal the residues involved in the gH/gL interaction.

MATERIALS AND METHODS

Cells and viruses

The *Spodoptera frugiperda* cell line Sf21 was grown in Xpress medium (BioWhittaker, Walkersville, MD) supplemented with 5% FCS and gentamicin (10 μg/ml). Recombinant baculovirus BacgL1 contains the gL1 gene of HSV-1 strain McIntyre under control of the polyhedrin promoter (Westra *et al.,* 1997). A recombinant baculovirus that expressed gL from EHV-1 was kindly provided by A. Stokes (Stokes *et al.,* 1996). A recombinant baculovirus that expressed gL of PRV was kindly provided by B. Klupp, and a recombinant baculovirus that expressed gL of VZV was kindly provided by L. Maresova.

Antibodies

The gH-specific MAb 52S (Showalter *et al.,* 1981) was obtained from the American Type Culture Collection (Rockville, MD). The gH-specific MAb LP11 was a gift of A. C. Minson (Buckmaster *et al.,* 1984). Anti-gH1 (rabbit 83), a polyclonal rabbit antiserum specific for gH1 that was raised against immunopurified gH/gL complex, was a gift of G. H. Cohen and R. J. Eisenberg (Roberts *et al.,* 1991). The gH-specific MAbs 37S and 46S were also a generous gift of G. H. Cohen and R. J. Eisenberg. A polyclonal rabbit antiserum, W192S5, was raised against the synthetic gL-peptide KSRRRRPHSRRL (residues 213–224 of gL), which was conjugated to ovalbumin. MAb A16 recognizes an 11-amino-acid linear epitope of gD of HSV-1 (Schellekens *et al.,* 1994; Scheffer *et al.,* 1984).

Construction of the open reading frame of gH-A16

The A-16 tag (SLKMADPNRFR) is a linear epitope of gD that is recognized by MAb A16 (Schellekens *et al.,* 1994). The coding sequence was introduced into the open reading frame of gH1 by PCR. The synthetic oligonucleotides gH1 forward (Westra *et al.,* 1997) and gHtag (5′-TAAGCTTAGCGAAAGCGATTGGGGTCGGCCATCT-TGAGGGATCCTTCGCGTCTCCAAAAAAA-3′) were used to amplify a 2567-bp fragment. The oligonucleotide gH1 forward contains UL22 sequences and a *Bgl*II cleavage site, and gHtag contains UL22 sequences, the coding sequence of the A16 tag, and a *Hin*dIII cleavage site. The amplified fragment was digested with *Bgl*II and *Hin*dIII and was cloned into the *Bam*HI and *Hin*dIII sites of pFastbacI, generating pFgH-A16.

Construction of the open reading frames of the deletion mutants of gH

Fragments of gH were obtained by using PCR with the following synthetic oligonucleotides: gH300 reverse (5′-GAACGC-TAGCAAGTCCCCGACCCGATCT-3′), gH473 reverse (5′-TAGAGCTAGCAAGCCCAGCG-CGTGTAAT-3′), gH300 forward (5′-GCATCCATGGGACCCCGCGGAC-GAA-3′), gH474 forward (5′-GCATCCATGGTATCAGCTG-GCCTTCG-3′), gH1 reverse, and gH1 forward. Five fragments were amplified with the following combinations of primers: gH1 forward and gH300 reverse, gH1 forward and gH473 reverse, gH300 forward and gH1 reverse, gH474 forward and gH1 reverse, and gH300 forward and gH473 reverse. The five amplified fragments were digested with *Nco*I–*Nhe*I and cloned into *Nco*I–*Nhe*I-digested pFgH-A16. The resulting plasmids were designated by the numbers 95–99, respectively.

Construction of the gH/gD fusion protein open reading frames

The extracellular part of gD1 was amplified from viral DNA of HSV-1 strain McIntyre by using PCR. The oligonucleotides gD forward (5′-AAGCTAGCCTTGGCGGAT-GCCTCT-3′) and gD1 reverse were used. The amplified fragment was digested with *Nhe*I and *Hin*dIII. This fragment was cloned into pFgH-A16 and the pFastbacI clones 95–99, which were digested with *Nhe*I and *Hin*dIII.

Recombinant baculoviruses

Recombinant baculoviruses were obtained by the procedure described by the manufacturer of the Bac-to-Bac system (Life Technologies) using the pFastbacI constructs described above.

FACS analysis

FACS analyses were performed as described previously (Westra *et al.,* 1997).

Immunoprecipitation and immunoblotting

Monolayers of Sf-21 cells (1×10^6) were infected or coinfected with recombinant baculoviruses at an multiplicity of infection of 5. Medium was removed 46 h after infection, and the cells were washed twice with ice-cold PBS. The cells were lysed by incubation for 30 min on ice with lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 50 mM Tris–HCl, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 10,000 *g* for 10 min, and the supernatant was pretreated with 1 mg of protein A–Sepharose CL-4B beads (Pharmacia) for 1 h. Beads were removed by centrifugation at 1000 *g* for 1 min, and antibodies were added to the supernatant. After 1.5 h of incubation, 1.5 mg of protein A–Sepharose CL-4B was added, and incubation was continued for 1.5 h. The immune complexes were washed twice with RIP-A (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5), twice with RIP-B (1% Nonidet P-40, 0.5% sodium deoxycholate, 2 M NaCl, 50 mM Tris–HCl, pH 7.5), and once with RIP-A. Precipitated proteins were dissolved by boiling for 3 min in sample buffer (150 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue), separated on either 10% or 15% SDS–polyacrylamide gels, and blotted onto PVDF membranes. The membranes were preincubated with PBS containing 4% Protivar (Nutricia) and 0.1% Tween 20 (PBS-PT). Incubations with antiserum W192S5 (diluted 1:4000), MAb A16 (diluted 1:3000), and polyclonal antibody anti-gH (diluted 1:4000) were carried out in PBS-PT. After incubation with peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulins (diluted 1:5000; DAKO), antibody binding was visualized by using a luminogenic reaction (ECL-Western light; Amersham) and exposure to Amersham ECL films.

REFERENCES

Anderson, R. A., Liu, D. X., and Gompels, U. A. (1996). Definition of human herpesvirus-6 betaherpesvirus-specific domain in glycoprotein gH that governs interaction with glycoprotein gL: Substitution of human cytomegalovirus glycoproteins permits group-specific complex formation. *Virology* **217**, 517–526.

Browne, H. M., Bruun, B. C., and Minson, A. C. (1996). Characterization of herpes simplex virus type 1 recombinants with mutations in the cytoplasmic tail of glycoprotein H. *J. Gen. Virol* **77,** 2569–2573.

Buckmaster, E. A., Gompels, U., and Minson, A. (1984). Characterisation and physical mapping of an HSV-1 glycoprotein of approximately 115×10^3 molecular weight. *Virology* **139,** 408–413.

Cai, W., Gu, B., and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* **62,** 2596–2604.

Desai, P. J., Schaffer, P. A., and Minson, A. C. (1988). Excretion of non-infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: Evidence that gH is essential for virion infectivity. *J. Gen. Virol.* **69,** 1147–1156.

Dubin, G., and Jiang, H. (1995). Expression of herpes simplex virus type 1 glycoprotein L (gL) in transfected mammalian cells: Evidence that gL is not independently anchored to cell membranes. *J. Virol.* **69,** 4564–4568.

Duus, K., and Grose, C. (1996). Multiple regulatory effects of varicella-zoster virus (VZV) gL on trafficking patterns and fusogenic properties of VZV gH. *J. Virol.* **70,** 8961–8971.

Duus, K. M., Hatfield, C., and Grose, C. (1995). Cell surface expression and fusion by the varicella-zoster virus gH:gL glycoprotein complex: Analysis by laser scanning confocal microscopy. *Virology* **210,** 429–440.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* **66,** 341–348.

Fuller, A. O., and Lee, W. C. (1992). Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. *J. Virol.* **66,** 5002–5012.

Fuller, A. O., Santos, R. E., and Spear, P. G. (1989). Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J. Virol.* **63,** 3435–3443.

Galdiero, M., Whiteley, A., Bruun, B., Bell, S., Minson, T., and Browne, H. (1997). Site-directed and linker insertion mutagenesis of herpes simplex virus type 1 glycoprotein H. *J. Virol.* **71,** 2163–2170.

Gompels, U. A., Carss, A. L., Saxby, C., Hancock, D. C., Forrester, A., and Minson, A. C. (1991). Characterization and sequence analyses of antibody-selected antigenic variants of herpes simplex virus show a conformationally complex epitope on glycoprotein H. *J. Virol.* **65,** 2393–2401.

Gompels, U. A., and Minson, A. C. (1989). Antigenic properties and cellular localization of herpes simplex virus glycoprotein H synthesized in a mammalian cell expression system. *J. Virol.* **63,** 4744–4755.

Huber, M. T., and Compton, T. (1997). Characterization of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex. *J. Virol.* **71,** 5391–5398.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A. C., and Johnson, D. C. (1992). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. *J. Virol.* **66,** 2240–2250.

Kaye, J. F., Gompels, U. A., and Minson, A. C. (1992). Glycoprotein H of human cytomegalovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. *J. Gen. Virol.* **73,** 2693–2698.

Klupp, B. G., and Mettenleiter T. M. (1999). Glycoprotein L-independent infectivity of pseudorabies virus is mediated by a gD-gH fusion protein. *J. Virol.* **73,** 3014–3022.

Li, Q., Buranathai, C., Grose, C., and Hutt-Fletcher, L. M. (1997a). Chaperone functions common to nonhomologous Epstein-Barr virus gL and varicella-zoster virus gL proteins. *J. Virol.* **71,** 1667–1670.

Li, Q., Spriggs, M. K., Kovats, S., Turk, S. M., Comeau, M. R., Nepom, B., and Hutt-Fletcher, L. M. (1997b). Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. *J. Virol.* **71,** 4657–4662.

Li, Q., Turk, S. M., and Hutt-Fletcher, L. M. (1995). The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells. *J. Virol.* **69,** 3987–3994.

Ligas, M. W., and Johnson, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* **62,** 1486–1494.

Liu, D. X., Gompels, U. A., Nicholas, J., and Lelliott, C. (1993). Identification and expression of the human herpesvirus 6 glycoprotein H and interaction with an accessory 40K glycoprotein. *J. Gen. Virol.* **74,** 1847–1857.

Novotny, M. J., Parish, M. L., and Spear, P. G. (1996). Variability of herpes simplex virus 1 gL and anti-gL antibodies that inhibit cell fusion but not viral infectivity. *Virology* **221,** 1–13.

Peng, T., Ponce-de-Leon, M., Jiang, H., Dubin, G., Lubinski, J. M., Eisenberg, R. J., and Cohen, G. H. (1998b). The gH-gL complex of herpes simplex virus (HSV) stimulates neutralizing antibody and protects mice against HSV type 1 challenge. *J. Virol.* **72,** 65–72.

Peng, T., Ponce de Leon, M., Novotny, M. J., Jiang, H., Lambris, J. D., Dubin, G., Spear, P. G., Cohen, G. H., and Eisenberg, R. J. (1998a). Structural and antigenic analysis of a truncated form of the herpes simplex virus glycoprotein gH-gL complex. *J. Virol.* **72,** 6092–6103.

Roberts, S. R., Ponce de Leon, M., Cohen, G. H., and Eisenberg, R. J. (1991). Analysis of the intracellular maturation of the herpes simplex virus type 1 glycoprotein gH in infected and transfected cells. *Virology* **184,** 609–624.

Roop, C., Hutchinson, L., and Johnson, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *J. Virol.* **67,** 2285–2297.

Scheffer, A. J., Koedijk, D. G. A. M., Abee, T., and Osterhaus, A. D. M. E. (1984). Monoclonal antibodies against herpes simplex viruses. *Dev. Biol. Stand.* **57,** 269–274.

Schellekens, G. A., Lasonder, E., Feijlbrief, M., Koedijk, D. G. A. M., Scheffer, A. J., Welling-Wester, S., and Welling, G. W. (1994). Identification of the core residues of the epitope of a monoclonal antibody raised against glycoprotein D of herpes simplex virus type 1 by screening of a random peptide library. *Eur. J. Immunol.* **24,** 3188–3193.

Showalter, S. D., Zweig, M., and Hampar, B. (1981). Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP4. *Infect. Immunol.* **34,** 684–692.

Spaete, R. R., Perot, K., Scott, P. I., Nelson, J. A., Stinski, M. F., and Pachl, C. (1993). Coexpression of truncated human cytomegalovirus gH with the UL115 gene products or the truncated human fibroblast growth factor receptor results in transport of gH to the cell surface. *Virology* **193,** 853–861.

Spear, P. G. (1993). Entry of alphaherpesviruses into cells. *Semin. Virol.* **4,** 167–180.

Spriggs, M. K., Armitage, R. J., Comeau, M. R., Strockbine, L., Farrah, T., Macduff, B., Ulrich, D., Alderson, M. R., Mullberg, J., and Cohen, J. I. (1996). The extracellular domain of the Epstein-Barr virus BZLF2 protein binds the HLA-DR beta chain and inhibits antigen presentation. *J. Virol.* **70,** 5557–5563.

Stokes, A., Alber, D. G., Greensill, J., Amellal, B., Carvalho, R., Taylor, L. A., Doel, T. R., Killington, R. A., Halliburton, I. W., and Meredith, D. M. (1996). The expression of the proteins of equine herpesvirus 1 which share homology with herpes simplex virus 1 glycoproteins H and L. *Virus Res.* **40,** 91–107.

Turner, A., Bruun, B., Minson, T., and Browne, H. (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.

Yaswen, L. R., Stephens, E. B., Davenport, L. C., and Hutt-Fletcher, L. M. (1993). Epstein-Barr virus glycoprotein gp85 associates with the BKRF2 gene product and is incompletely processed as a recombinant protein. *Virology* **195,** 387–396.

Westra, D. F., Glazenburg, K. L., Harmsen, M. C., Tiran, A., Scheffer, A. J., Welling, G. W., Hauw, T., and Welling-Wester, S. (1997). Glycoprotein H of herpes simplex virus type 1 requires glycoprotein L for transport to the surfaces of insect cells. *J. Virol.* **71,** 2285–2291.

Wilson, D. W., Davis-Poynter, N., and Minson, A. C. (1994). Mutations in the cytoplasmic tail of herpes simplex virus glycoprotein H suppress cell fusion by a syncytial strain. *J. Virol.* **68,** 6985–6993.