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Glycoprotein H of Herpes Simplex Virus Type 1 Requires Glycoprotein L for Transport to the Surfaces of Insect Cells

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In mammalian cells, formation of heterooligomers consisting of the glycoproteins H and L (gH and gL) of herpes simplex virus type 1 is essential for the cell-to-cell spread of virions and for the penetration of virions into cells. We examined whether formation of gH1/gL1 heterooligomers and cell surface expression of the complex occurs in insect cells. Three recombinant baculoviruses, expressing gL1, gH1, and truncated gH1 (gH1t), which lacks the transmembrane region, were constructed. It was shown that recombinant gH1/gL1 and gH1t/gL1 heterooligomers were produced in insect cells. As in mammalian cells, gH1 and gH1t were not detected on the surfaces of insect cells in the absence of gL1. When coexpressed with gL1, recombinant gH1 was displayed on the surfaces of insect cells. Coexpression of gH1t and gL1 resulted in secretion of the gH1t/gL1 complex into the cell culture medium, indicating that gH1t is also transported to the surfaces of insect cells. Our results indicate that the process of folding and intracellular transport of gH1 and gL1 is comparable in insect cells and mammalian cells and that the baculovirus expression system can be used to examine the complex formation and the intracellular transport of gH1 and gL1. The availability of secreted gH1t/gL1 complex offers the opportunity to further investigate the immunological properties of this complex.

The glycoproteins B, D, H, and L (gB, gD, gH, and gL) of herpes simplex virus type 1 (HSV-1) are essential for infectivity in vitro (40). Each of these glycoproteins plays a role in the attachment of the virion to the cell and/or penetration of the virion into cells. Homologs of gH1 have been found in members of all three subfamilies of herpesviruses (*Alpha*-, *Beta*-, and *Gammaherpesvirinae*) (3, 18, 21, 23, 28, 29, 36). gH of HSV-1 plays a role in the process of the cell-to-cell spread of virions and is required for the penetration of virions into cells (4, 9, 11, 12, 17, 20). HSV-1 gH produced in mammalian expression systems is retained in the cell, most likely in the endoplasmic reticulum or *cis*-Golgi, and contains oligosaccharide chains that are not fully processed (7, 19). It forms aggregates (32) and is not recognized by the gH1-specific monoclonal antibodies (MAbs) LP11 and 53S (10, 19).

Hutchinson et al. (22) found that gH1 forms a stable complex with gL1, which is encoded by the UL1 gene. Virus particles produced in cells infected with HSV-1 mutants unable to express gL were deficient for both gH and gL (33). These mutant virions lacking gL and gH were able to adsorb to cells but could not penetrate cells and initiate an infection. In addition, coexpression of gL1 and gH1 in mammalian expression systems is required for the transport of gH1 to the cell surface (22) and for the recognition of gH1 by MAb LP11 (1, 22). The formation of a complex by a homolog of gH1 and an additional viral glycoprotein has been found in members of all three subfamilies of herpesviruses (6, 8, 22, 24–26, 33, 39, 43). The biological significance of this complex formation is unclear.

Several groups have investigated the immunological properties of gH1 and/or gL1. MAbs to gH1 are able to neutralize an HSV-1 infection in vitro (2, 17, 37) and can protect mice from a lethal HSV-1 challenge when administered passively (10). However, immunization experiments in mice with recombinant gH1 induced no or minor protective immune responses. Immunization with recombinant vaccinia viruses expressing both gH1 and gL1 induced low levels of neutralizing antibodies. After challenge, HSV-1 virions were cleared from the site of challenge and a reduced amount of latent HSV-1 was observed in the ganglia. Immunization with recombinant vaccinia viruses expressing either gH1 or gL1 did not induce these effects (1, 10). Immunization with gH1 produced in the baculovirus expression system induced HSV-1 neutralizing antibodies and delayed-type hypersensitivity responses, but it did not protect immunized mice against an establishment of latency or a lethal HSV-1 challenge (14, 16). Baculovirus-produced gL1 did not induce neutralizing antibodies, nor did it protect immunized mice against a lethal challenge (15). A mixture of baculovirus-produced gL1 and gH1 did not provide better protection than either glycoprotein alone (15).

In this study, we investigated the production of the gH/gL complex of HSV-1 in insect cells, using the baculovirus expression system. The folding, the complex formation, the cell surface expression, and the secretion of gH1 and of truncated gH1 (gH1t) in insect cells were studied in the absence and the presence of recombinant gL1. Our results indicate that the baculovirus expression system is suited to produce the gH1/gL1 complex and to examine complex formation and intracellular transport of gH1 and gL1.

MATERIALS AND METHODS

Cells and viruses. Virus stocks of HSV-1 strain McIntyre were produced in Vero cells maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum (FCS) and gentamicin (10 mg/ml). The *Spodoptera frugiperda* cell lines Sf-21 and Sf-9 were grown in TC-100 medium supplemented with 10% FCS and gentamicin (10 mg/ml) or in Sf-900II medium (Gibco/BRL) supplemented with gentamicin (10 mg/ml). Recombinant baculovirus AcRDH1 contains the gD1 gene of HSV-1 strain McIntyre under the control of the polyhedrin promoter.

Antibodies. MAb 52S, directed against gH1 (37), was obtained from the American Type Culture Collection (Rockville, Md.). MAb LP11 is also directed against gH1 but recognizes gH1 only in the presence of gL1 and was a gift of

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A. C. Minson (2). Anti-gH1 (rabbit 83), a polyclonal rabbit antiserum specific for gH1, which was purified by immunoaffinity chromatography, was a gift of G. H. Cohen and R. J. Eisenberg (32). Anti-UL1-1 and anti-UL1-2, two rabbit antisera raised against two synthetic peptides of gL, were kindly provided by D. C. Johnson (22). Anti-*trpE*-gH, a polyclonal rabbit antibody directed against a *trpE*-gH fusion protein produced in *Escherichia coli*, was a gift of A. C. Minson (4). MAb A16 is directed against gD of HSV-1 (35).

Construction of recombinant baculoviruses. Recombinant baculoviruses were constructed by using the Bac-to-Bac system (Life Technologies, Inc.) (27). The open reading frame encoding the gL1 glycoprotein was amplified by PCR from DNA isolated from HSV-1 (strain McIntyre)-infected Vero cells. The synthetic oligonucleotide primers gL1 forward (5′ AAGATCTATGGGGATTTTGGGTT 3′) and gL1 reverse (5′ TCCGTCGAGAGATCTTTAGAGGCG 3′) contained UL1 sequences from HSV-1 strain 17 (30) and *Bgl*II endonuclease cleavage sites. The PCR protocol (95°C for 15 s, 45°C for 90 s, 72°C for 120 s; 35 cycles) used a Hybaid thermal reactor HB-TR1 and *Taq* polymerase (HT Biotechnology Ltd.). A full-length form of the gH1 gene and a truncated form encoding amino acids 1 to 791 (gH1t) and lacking the putative transmembrane region and cytoplasmic tail were amplified from a plasmid containing the *Bgl*II M fragment of HSV-1 strain McIntyre. The primers used, gH1 forward (5′ GAGATCTCATG GGGAATGGTTTATGG 3′), gH1 reverse (5′ GAAGCCAAGATCTCTTTAT TCGCGTCTCCA 3′), and gH1t reverse (5′ CTGCGTAGATCTTTATAGCA AATGAATGACGG 3′), contained UL22 sequences from HSV-1 strain HFEM (17) and *Bgl*II endonuclease cleavage sites. The PCR conditions included a preincubation step (99°C for 10 min and 34°C for 5 min) and 40 amplification cycles (95°C for 15 s, 34°C for 90 s, 72°C for 300 s).

The amplified 697-bp fragment encoding gL1, the 2,540-bp fragment encoding gH1, and the 2,396-bp fragment encoding gH1t were ligated into the pGEM-T plasmid (Promega) to generate pGEMgL1, pGEMgH1, and pGEMgH1t, respectively. The *Bgl*II fragments were excised from pGEMgL1, pGEMgH1, and pGEMgH1t and were ligated into the *Bam*HI site of the pFASTBAC1 plasmid of the Bac-to-Bac system. Recombinant baculoviruses were obtained by the procedure described by the manufacturer. Stocks of the recombinant viruses were prepared and the titers were determined by standard protocols (41). Titers ranged from 2×10^7 to 5×10^7 PFU/ml.

Immunoblots. Monolayers of Sf-21 cells were infected and coinfected with the recombinant baculoviruses at a multiplicity of infection (MOI) of 5. After 72 h of infection, the cell culture media were collected and extracts of the cells were prepared. Cell debris was removed from the cell culture media by centrifugation ($20,000 \times g$ for 15 min at 4°C), and aliquots of media were stored at −80°C. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer I (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate [DOC], 0.1% sodium dodecyl sulfate [SDS], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) on ice for 30 min. The extracts were centrifuged ($10,000 \times g$ for 10 min at 4°C). The supernatant contained the soluble fraction of the proteins, and the pellet contained the insoluble fraction of the proteins. The pellets were dissolved in sample buffer with a high detergent concentration (50 mM Tris-HCl [pH 6.8], 8% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue). Sample buffer was added to the supernatants (soluble fractions) to a final concentration of 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue. The insoluble and the soluble fractions were heated at 100°C for 2 min and stored in aliquots at −80°C.

Monolayers of Vero cells were infected with HSV-1 at an MOI of 10. Twenty hours postinfection, the cells were lysed with lysis buffer II (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM PMSF) on ice for 30 min. Cell debris and nuclei were removed by centrifugation at $10,000 \times g$ for 10 min, and the supernatant was stored in aliquots at −80°C.

Extracts and media were analyzed by Western blotting. Proteins were separated on 10% and 12.5% gels by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes with a semidry gel electroblotter (Ancos). Blots were preincubated for 1 h with PBS containing 4% skim milk and 0.1% Tween 20 (PBS-TS). Incubations with anti-UL1-2 (diluted 1:1,000) and anti-gH1 (diluted 1:1,000) were carried out in PBS-TS. After incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (diluted 1:4,000; Dako), antibody binding was visualized by using a luminogenic reaction (ECL-Western light; Amersham) and exposure to Amersham ECL films.

Radioimmunoprecipitation. Monolayers of 2×10^6 Sf-21 cells grown in serum-free medium (Sf900-II) were infected and coinfected with recombinant baculoviruses at an MOI of 5. Medium was removed 41 h postinfection and replaced by Sf900-II medium with 1/10 of the standard cysteine concentration. After 1 h of starvation, cells were labelled for 6 h with 30 mCi of [^{35}S]cysteine (Amersham). Thereafter, medium was removed and clarified by centrifugation at $10,000 \times g$ for 10 min, and aliquots were stored at −80°C. The monolayers were washed twice with ice-cold PBS and subsequently lysed for 30 min on ice with 1 ml of lysis buffer III (1% Nonidet P-40, 0.5% DOC, 100 mM NaCl, 50 mM Tris-HCl [pH 7.5], 2 mg of bovine serum albumin per ml, and 1 mM PMSF). After freezing and thawing, lysates were centrifuged at $10,000 \times g$ for 10 min and aliquots of the supernatants were stored at −80°C. Monolayers of Vero cells were infected with HSV-1 strain McIntyre at an MOI of 10. After an infection period of 6 h, medium was replaced by Dulbecco's minimum essential medium containing 1/10 of the standard cysteine concentration supplemented with 3% dialyzed FCS. After 1 h, 100 mCi of [^{35}S]cysteine (Amersham) was added. After 6 h, a lysate of HSV-1-infected Vero cells was prepared as described for Sf-21 cells.

Lysates and media were pretreated with 1 mg of protein A-Sepharose CL-4B (Pharmacia) for 1 h. Beads were removed by centrifugation at $1,000 \times g$ for 1 min, and monoclonal or polyclonal antibodies were added. After 4 h of incubation, 1.5 mg of protein A-Sepharose CL-4B was added and incubation was continued for 2 h. The immune complexes were washed three times with RIP-A buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 0.5% DOC, and 1% Nonidet P-40) and twice with RIP-B buffer (50 mM Tris-HCl [pH 7.5], 2 M NaCl, 0.5% DOC, and 1% Nonidet P-40). 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) and were separated on 10% gels by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue, incubated with Amplify (Amersham), dried, and exposed to Hyperfilm-βmax (Amersham).

Immunofluorescence assay. Monolayers of Sf-21 cells were infected and coinfected with recombinant baculoviruses. Cells were harvested 46 h after infection and were washed with TC-100 supplemented with 1% FCS. Cells in suspension were incubated for 1 h with polyclonal antibody anti-gH1 or MAb 52S (each diluted 1:150 in TC-100 supplemented with 1% FCS). After being washed twice with TC-100 containing 1% FCS, the cells were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit or rabbit antimouse antibodies (diluted 1:150 in TC-100 supplemented with 1% FCS; Dako). The cells were washed once with TC-100 supplemented with 1% FCS and stained with Evans blue (0.1 mg/ml) for 5 min, followed by an additional wash with TC-100 supplemented with 1% FCS. The cells were viewed by fluorescence microscopy.

FACS analysis. Monolayers of Sf-21 cells were infected and coinfected with recombinant baculoviruses. Cells were harvested 46 h after infection and were washed with TC-100 supplemented with 1% FCS. Cells in suspension were incubated for 1 h with MAb LP11, MAb 52S, MAb A16, or polyclonal antibody anti-gH1 (all diluted 1:150). After being washed twice with TC-100 containing 1% FCS, the cells were incubated for 1 h with FITC-conjugated goat antirabbit or rabbit antimouse antibodies (diluted 1:150 in TC-100 supplemented with 1% FCS; Dako). The cells were washed twice with TC-100 supplemented with 1% FCS, and propidium iodide (PI) (1 mg/ml) was added to determine the cell viability. Infected cells treated as described above, except that incubations with primary antibodies were omitted, were used as controls. A standard fluorescence-activated cell sorter (FACS) analysis was carried out with a FACSTAR (Becton Dickinson). Images of the FACS data were generated with Winmdi 2.0 (The Scripps Research Institute, La Jolla, Calif.). A quadrant was displayed in the images according to the PI fluorescence of the viable and the nonviable control cells and the background FITC fluorescence level of the control cells.

RESULTS

Construction of the recombinant baculoviruses BacgH1, BacgH1t, and BacgL1. In order to examine the production and the complex formation of gH1 and gL1, recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) expressing gL1, gH1, and gH1t (the virus expressing gH1t lacks the putative transmembrane region and cytoplasmic tail) were constructed. The open reading frames encoding gH1, gH1t, and gL1 were cloned into the pFASTBAC1 vector of the Bac-to-Bac system. With the use of this system, recombinant baculoviruses containing the open reading frames encoding gH1, gH1t, and gL1 under control of the polyhedrin promoter were obtained. Recombinant baculoviruses were selected for the production of recombinant HSV proteins by immunoblot analysis and were designated BacgH1, BacgH1t, and BacgL1.

Production of the recombinant proteins. To examine the expression of recombinant gH1, gH1t, and gL1, Sf-21 cells were infected and coinfected with the recombinant baculoviruses. After 72 h of infection, the media were collected and the infected cells were lysed with lysis buffer I. The proteins present in the cell culture media (corresponding to 2×10^4 cells) and in the soluble and insoluble fractions of the lysates (corresponding to 5×10^5 cells) were separated on polyacrylamide gels and stained with Coomassie brilliant blue. Recombinant gH1, gH1t, and gL1 were detected in the insoluble fractions (data not shown). This indicates that high expression levels were achieved for all three recombinant proteins. Ghiasi et al. also reported a high expression level for gH1 in insect cells (13, 15).

Western blotting was performed to identify recombinant gL,

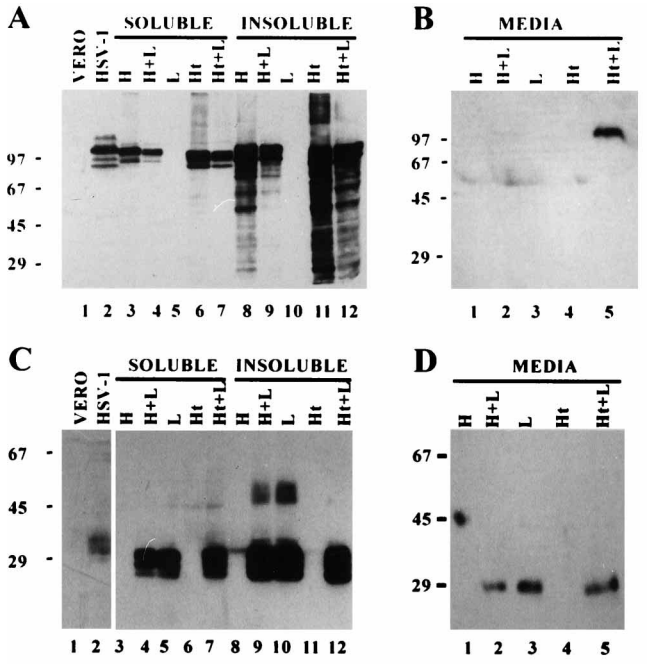

FIG. 1. Western blot analysis of recombinant gH1, gH1t, and gL1 produced in insect cells. Insect cells were infected with the recombinant baculoviruses BacgH1 (H), BacgH1 plus BacgL1 (H+L), BacgL1 (L), BacgH1t (Ht), and BacgHt plus BacgL1 (Ht+L). After 72 h of infection, the media were collected and the insect cells were lysed with lysis buffer I. The media (corresponding to 2×10^4 cells) and the soluble and the insoluble fractions of the lysates (corresponding to 5×10^5 cells) were analyzed (see Materials and Methods). As controls, cytoplasmic extracts of uninfected Vero cells (Vero) and HSV-1 (strain McIntyre)-infected Vero cells (HSV-1) were prepared and analyzed. The proteins present in the cell culture media of insect cells and in the lysates of insect cells and Vero cells were separated on 10% (A and B) and 12.5% (C and D) polyacrylamide gels. The separated proteins were transferred onto polyvinylidene difluoride membranes and probed with the polyclonal antibody anti-gH1 (diluted 1:1,000) (A and B), and with the gL1-specific antipeptide serum anti-UL1-2 (diluted 1:1,000) (C and D). Membranes were subsequently incubated with peroxide-conjugated goat anti-rabbit immunoglobulins (diluted 1:4,000). Antibody binding was visualized by a luminogenic reaction (ECL-Western light; Amersham). The molecular mass markers of 97, 70, 45, and 29 kDa are indicated.

gH, and gHt. The blots were probed with the polyclonal antibody anti-gH1 (Fig. 1A and B), and with anti-UL1-2, an antipeptide serum directed against gL1 (Fig. 1C and D). Recombinants gH1 and gH1t were found in the soluble fractions (Fig. 1A, lanes 3, 4, 6, and 7) and in the insoluble fractions (Fig. 1A, lanes 8, 9, 11, and 12) of the lysates. Soluble recombinant gH1 migrated in two broad, prominent bands with apparent molecular masses of 105 and 90 kDa (Fig. 1A, lanes 3 and 4). The 105-kDa form corresponds to the molecular mass of HSV-1 gH (Fig. 1A, lane 2). The 90-kDa form most likely is the unprocessed form of recombinant gH1. The pattern of bands is similar to the one described in a previous report, in which gH1 was expressed in insect cells (13). Soluble recombinant gH1t migrated in two bands with molecular masses of 100 and 85 kDa (Fig. 1A, lanes 6 and 7). The 5-kDa difference between recombinant gH1 and gH1t is in agreement with the expected molecular mass of the 47 deleted amino acids.

Most of the recombinant gH1 and gH1t was present in an insoluble form (Fig. 1A, lanes 8, 9, 11, and 12). The most prominent bands in the insoluble fractions have apparent molecular masses corresponding to the protein bands in the soluble fractions. Many smaller-sized fragments were present in the insoluble fractions of the lysates, which reacted with the polyclonal serum. These fragments are either not fully translated recombinant gH1 or degradation products of recombinant gH1.

Recombinant gH1t with an apparent molecular mass of 100 kDa is secreted into the cell culture medium of cells coinfected with BacgH1t and BacgL1 (Fig. 1B, lane 5). No recombinant gH1 was found in the culture media of cells infected or coinfected with the other recombinant baculoviruses (Fig. 1B, lanes 1 to 4).

In the soluble and the insoluble fractions of the lysates of cells infected or coinfected with BacgL1, a broad band consisting of at least three bands of recombinant gL1 with apparent molecular masses of approximately 30, 28, and 26 kDa (Fig. 1C, lanes 4, 5, 7, 9, 10, and 12) was present. These three forms of gL1 have been described before by Ghiasi et al. (15), who expressed gL1 in insect cells. A minor part of insoluble recombinant gL1 migrated at 50 to 55 kDa (Fig. 1C, lanes 9 and 10). This broad band could represent dimers of recombinant gL1. HSV-1 gL migrated as a broad band, consisting of two bands with molecular masses of 30 and 35 kDa (Fig. 1C, lane 2). The sizes of the bands correspond to those of immature and mature HSV gL1 found in other studies (5, 15). Recombinant gL1 with an apparent molecular mass of 30 kDa was secreted into the medium independent of coexpression of recombinant gH1 and gH1t (Fig. 1D).

Complex formation between recombinant gH1 and gL1. It has been shown that gH1 and gL1 form a heterooligomeric complex in HSV-infected Vero cells, which is present on the cell surface and in the virion envelope (22). Our aim was to study the complex formation of gH1 and gL1 in insect cells. For this, insect cells were infected and coinfected with the recombinant baculoviruses BacgH1, BacgH1t, and BacgL1 and were metabolically labelled with [^{35}S]cysteine. The cell culture media and the soluble fractions of the cell lysates were immunoprecipitated with a number of antisera with different properties. The polyclonal antibody anti-*trpE*-gH reacts with denatured gH1 and was obtained after immunizations with the *E. coli trpE* fusion protein, which contained residues 270 to 690 of gH1 (4). MAb 52S is directed against a conformationally dependent epitope on gH1 and recognizes both gL-associated and non-gL-associated forms of gH1 (37). MAb LP11 recognizes gH1 only when it is coexpressed with gL1 (19).

Before the complex formation of gH1 and gL1 could be examined, it was necessary to demonstrate that gH1 was correctly folded in insect cells. This was also shown by immunoprecipitation studies. Anti-*trpE*-gH (Fig. 2A, lane 1) and MAb 52S (Fig. 2B, lane 2) precipitated three major polypeptides with apparent molecular masses of 105, 60, and 38 kDa from the cell lysates of insect cells infected with BacgH1. Several minor bands were also precipitated. The 105-kDa species had approximately the same molecular mass as mature HSV-1 gH1, which was precipitated with MAb 52S from a lysate of HSV-1-infected Vero cells (Fig. 2B, lane 1). The 85-kDa polypeptide precipitated from the HSV-1 lysate with MAb 52S probably represents the unprocessed form of gH1 (19). The two other proteins precipitated from the lysate of insect cells infected with BacgH1 represent either fragments of recombinant gH1 or cellular or baculovirus proteins which were complexed to recombinant gH1.

When insect cells were coinfected with BacgH1 and BacgL1, an additional protein with a molecular mass of 30 kDa was precipitated with anti-*trpE*-gH and MAb 52S (Fig. 2A, lane 2; Fig. 2B, lane 6). This protein was not precipitated from lysates of insect cells which had been infected with either BacgL1 or BacgH1 (Fig. 2A, lanes 1 and 3; Fig. 2B, lanes 2 and 4). The additional polypeptide band corresponded in size to fully pro-

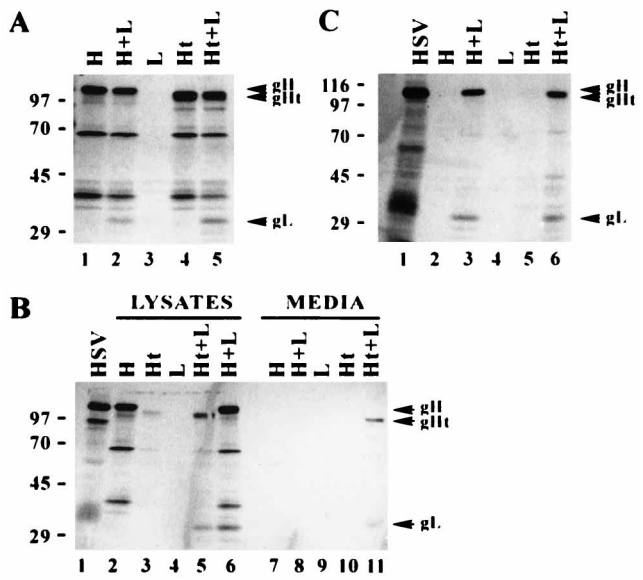

FIG. 2. Immunoprecipitations of gL1 and gH1 from insect cells infected with the recombinant baculoviruses BacgH1 (H), BacgH1t (Ht), and BacgL1 (L) and from insect cells coinfected with the recombinant baculoviruses BacgH1 plus BacgL1 (H+L) and BacgH1t plus BacgL1 (Ht+L). Forty-two hours after infection and coinfection with recombinant baculoviruses, insect cells were labelled with [^{35}S]cysteine for 6 h. As a control, Vero cells were infected with HSV-1 strain McIntyre (HSV). HSV-1-infected Vero cells were labelled 7 h after infection with [^{35}S]cysteine for 6 h. Cell lysates were prepared (see Materials and Methods). The soluble fractions of the lysates were mixed with the polyclonal antibody anti-*trpE*-gH (A), MAb 52S (B [lanes 1 to 6]), and MAb LP11 (C). In addition, metabolically labelled cell culture media were mixed with MAb 52S (B [lanes 7 to 11]). The antigen-antibody complexes were precipitated with protein A-Sepharose and washed. The positions of gH1, gH1t, and gL1 and the molecular mass markers of 97, 70, 45, and 29 kDa are indicated.

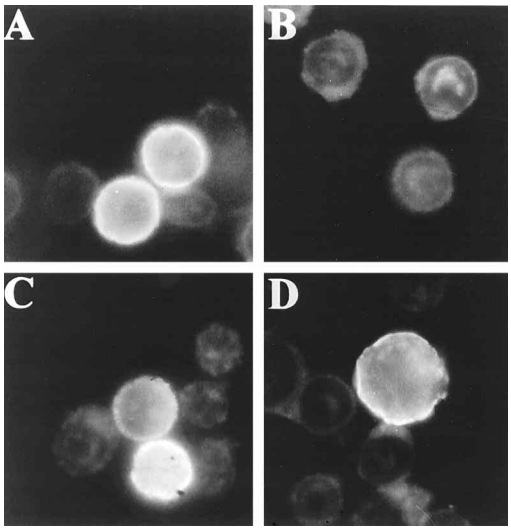

FIG. 3. Immunofluorescence analysis of insect cells infected with BacgH1 (A), BacgL1 (B), and BacgH1 plus BacgL1 (C and D). After 46 h of infection, the insect cells were incubated with the polyclonal antibody anti-gH1 (A to C) or MAb 52S (D) for 1 h and were subsequently incubated with FITC-conjugated goat anti-rabbit antibodies (A to C) or rabbit anti-mouse antibodies (D) for 1 h. The insect cells were counterstained with Evans blue for 5 min and visualized by fluorescence microscopy.

cessed recombinant gL1 found on a Western blot (Fig. 1C). To further analyze whether complex formation of gL1 and gH1 occurs in insect cells, immunoprecipitations were performed with MAb LP11. From lysates of insect cells coinfected with BacgH1 and BacgL1, the 105-kDa recombinant gH1 was precipitated with MAb LP11 (Fig. 2C, lane 3). A protein with a molecular mass of 30 kDa coprecipitated with recombinant gH1 (Fig. 2C, lane 3). No proteins were precipitated to a detectable level when the insect cells were infected with BacgH1 or BacgL1 alone (Fig. 2C, lanes 2 and 4). We conclude that the 30-kDa protein is recombinant gL1, which forms a stable complex with recombinant gH1.

In addition, the effect of the deletion of the transmembrane region and cytoplasmic tail from gH1 on folding and localization was assessed by immunoprecipitations. Truncated recombinant gH1 with an apparent molecular mass of 100 kDa was precipitated with anti-*trpE*-gH from cell lysates of insect cells infected with BacgH1t (Fig. 2A, lane 4). Two additional proteins with molecular masses of 60 and 38 kDa, similar to the two additional proteins precipitated with anti-*trpE*-gH from lysates of insect cells infected with BacgH1 (Fig. 2A, lane 1), were precipitated. A small amount of recombinant gH1t was precipitated with MAb 52S from lysates of insect cells infected with BacgH1t (Fig. 2B, lane 3). This was not the result of less efficient labelling of recombinant gH1t, because approximately the same amounts of labelled recombinant gH1 and gH1t were precipitated with anti-*trpE*-gH (Fig. 2A). When recombinant gH1t was coexpressed with recombinant gL1, a 30-kDa protein was coprecipitated with recombinant gH1t by using anti-*trpE*-gH, MAb 52S, and MAb LP11 (Fig. 2A, lane 5; Fig. 2B, lane 5; Fig. 2C, lane 6). This 30-kDa protein is probably recombinant gL1, and this allows the conclusion that recombinant gH1t also forms a stable complex with recombinant gL1.

A 100- and a 30-kDa protein were precipitated with MAb 52S from the cell culture medium obtained from insect cells coinfected with BacgL1 and BacgH1t (Fig. 2B, lane 11). No proteins were precipitated to a detectable level from the cell culture media of insect cells infected and coinfected with the other recombinant baculoviruses. This suggests that secreted recombinant gH1t is complexed with recombinant gL1.

Cell surface expression of recombinant gH1 and gH1t. In mammalian cells, gH1 is transported to the cell surface only in the presence of gL1 (22, 33). Previously, Ghiasi et al. (13) reported that gH1 is transported to the surfaces of insect cells in the absence of gL1. To investigate whether in this study recombinant gH1 and gH1t are expressed on the surfaces of insect cells, plasma membrane immunofluorescence assays were performed. Insect cells infected with BacgH1, BacgL1, and BacgH1 plus BacgL1 were examined for plasma membrane fluorescence 46 h after infection (Fig. 3). When the polyclonal antibody anti-gH1 was used, a low percentage of insect cells infected with BacgH1 showed fluorescence (Fig. 3A). Insect cells infected with BacgL1 did not show fluorescence (Fig. 3B). The fluorescence staining of insect cells coinfected with BacgL1 plus BacgH1 was not notably different from that of BacgH1-infected cells (Fig. 3C). When MAb 52S was used, however, there was a clear difference between infected and coinfected insect cells. Insect cells infected with BacgH1 or BacgL1 showed no fluorescence with MAb 52S (data not shown). Only insect cells which were coinfected with BacgH1 plus BacgL1 showed plasma membrane fluorescence with MAb 52S (Fig. 3D). Since both antibodies, polyclonal anti-gH1 and MAb 52S, should be able to detect gH1 presented on the surface, we decided to examine the plasma membrane fluorescence further by flow cytometric analyses.

We performed flow cytometric analyses with PI, which allows for discrimination between viable and nonviable cells (34, 43). The infected cells were incubated with the polyclonal

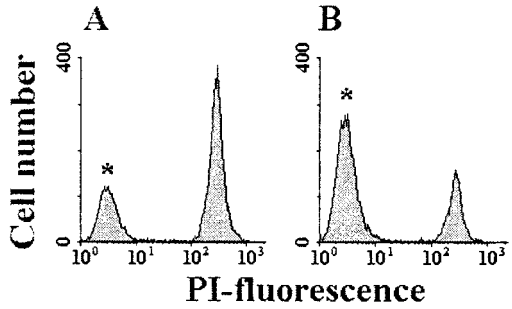

FIG. 4. Analysis of the PI permeability of insect cells infected with BacgH1 by flow cytometry. Insect cells were incubated with the polyclonal antibody anti-gH1 for 1 h, with FITC-conjugated goat anti-rabbit antibodies for 1 h, and subsequently with PI for 10 min (A) or with PI only for 10 min (B). The cells were analyzed on a FACSTAR flow cytometer. The number of cells is plotted along the *y* axis and the PI fluorescence is plotted along the *x* axis of the histogram. Viable cell populations are indicated with an asterisk.

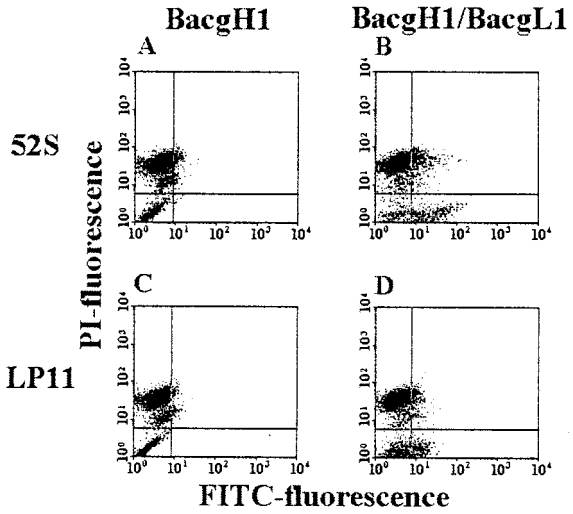

FIG. 6. Analysis of the binding of the MAbs 52S and LP11 on the surfaces of insect cells infected with BacgH1 (A and C), and coinfected with BacgL1 and BacgH1 (B and D). Insect cells were incubated with MAb 52S (A and B) or LP11 (C and D) for 1 h and were subsequently incubated with FITC-conjugated rabbit anti-mouse antibodies for 1 h and with PI for 10 min. The cells were analyzed on a FACSTAR flow cytometer. The PI fluorescence is plotted along the *y* axis, and the FITC fluorescence is plotted along the *x* axis.

antibody anti-gH1, secondary antibody, and PI and were analyzed by flow cytometry. These analyses showed that two cell populations could be distinguished in a PI fluorescence histogram of insect cells infected with BacgH1 (Fig. 4A). Most likely, the cell population with a low PI fluorescence was viable and the population with a high PI fluorescence was nonviable (34, 44). We examined whether the cells were already PI permeable prior to the flow cytometric analysis or had become PI permeable during the procedure. When we treated the cells with PI only and minimized the time between the harvesting of the cells and the flow cytometric analysis, the percentage of PI-permeable cells decreased to about 25 to 30% (Fig. 4B). This indicates that most of the insect cells had become PI permeable as a result of the experimental procedure.

With this in mind, a flow cytometric analysis using the polyclonal antibody anti-gH1 was performed on insect cells infected with either BacgH1 or BacgH1t (Fig. 5A and D). The PI-negative cell population did not show increased FITC fluorescence compared to the control cells. A part of the PI-permeable cell population exhibited increased FITC fluorescence. We compared these results to those of the flow cytometric analysis of insect cells expressing gD of HSV-1 by using the recombinant baculovirus AcRDH1 (Fig. 5F). Recombinant gD1 is expected to be transported to the surfaces of insect cells. The viable cell population had a highly increased FITC fluorescence, indicating that recombinant gD1 was transported to and integrated in the plasma membrane. We therefore conclude that recombinant gH1 and gH1t are not present on the surfaces of infected insect cells.

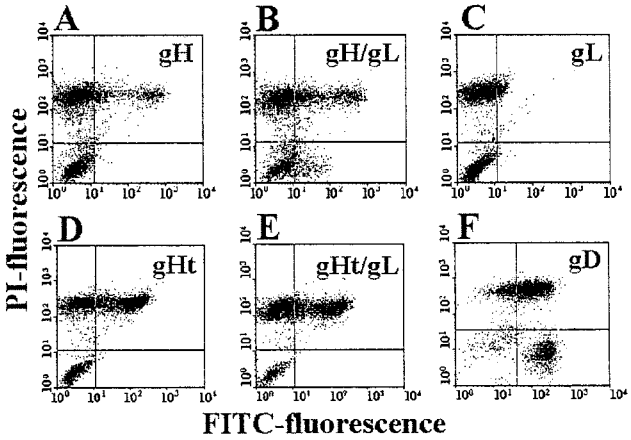

FIG. 5. Flow cytometric analysis of the cell surface expression of recombinant gH1. Insect cells infected with the recombinant baculoviruses BacgH1 (A), BacgH1 plus BacgL1 (B), BacgL1 (C), BacgH1t (D), BacgH1t plus BacgL1 (E), and AcRDH1 (F) were incubated with the polyclonal antibody anti-gH1 (A to E) or MAb A16 (F) for 1 h and were subsequently incubated with FITC-conjugated goat anti-rabbit antibodies (A to E) or FITC-conjugated goat anti-mouse antibodies (F) for 1 h. After incubation with PI for 10 min, the insect cells were analyzed with a FACSTAR flow cytometer. The PI fluorescence is plotted along the *y* axis, and the FITC fluorescence is plotted along the *x* axis.

Flow cytometric analyses performed on insect cells coinfected with BacgH1 and BacgL1 by using the polyclonal antiserum anti-gH1 showed that a part of the PI-negative cells exhibited increased FITC fluorescence (Fig. 5B). This indicated that gH1 was displayed on the plasma membranes of coinfected insect cells. Flow cytometric analyses using MAbs 52S and LP11 showed that the epitopes of these antibodies are present on the surfaces of insect cells after coinfection with BacgL1 and BacgH1 (Fig. 6). However, gH1 was detected on the surfaces of only a percentage of the viable insect cells by anti-gH1 (±30%) or the MAbs 52S (±64%) and LP11 (±34%), despite a high MOI.

When recombinant gH1t was coexpressed with gL1, gH1t was not detected on the plasma membranes of insect cells by the polyclonal antibody anti-gH1 (Fig. 5E). As shown before, gH1t is able to form a complex with gL1 and is secreted into the medium.

DISCUSSION

The formation of heterooligomers between gH1 and gL1 is essential for the infectivity of HSV-1 virions. The biological function of this complex and the molecular mechanism of its formation are yet not understood. This complex formation was studied in insect cells by using the baculovirus expression system. We were able to demonstrate that gH1/gL1 and gH1t/gL1 complexes are produced in insect cells. The gH1/gL1 and gH1t/gL1 complexes were precipitated with polyclonal antibody an-

ti-*trpE*-gH, MAb 52S, and MAb LP11. Two additional proteins with apparent molecular masses of 38 and 60 kDa were precipitated with anti-*trpE*-gH and MAb 52S but not with MAb LP11. A nonspecific precipitation is unlikely, since anti-*trpE*-gH and MAb 52S did not precipitate any proteins to a detectable level from lysates of insect cells infected with BacgL1 (Fig. 2A, lane 3; Fig. 2B, lane 4). These two coprecipitated proteins might be fragments of recombinant gH1 and gH1t. Another possibility is that these two proteins bind gH1 and gH1t to retain gH1 within the insect cell. When gH1 and gH1t are complexed to gL1, the binding with these proteins may be abolished. This might explain why LP11, a MAb that recognizes gH1 only in the presence of gL1, precipitated much less of these two proteins. Complex formation of the human cytomegalovirus homolog of gH1 with the cellular proteins GRP78 and GRP94 has been demonstrated in human fibroblasts (38).

Our study showed that insect cells, like mammalian cells, retained gH1 and gH1t in the cell in the absence of gL1. Others have demonstrated that gH of HSV-1 (13) and gH of human cytomegalovirus (42) produced in insect cells were transported to the cell surfaces. We examined whether the discrepancy between our results and the results obtained by Ghiasi et al. (13) was caused by the differences in cell type and in infection period. In our study, Sf-21 cells were used instead of Sf-9 cells and the cells were assayed 46 h after infection instead of 72 h after infection. We performed flow cytometric analyses of Sf-9 cells infected with BacgH1, and these generated the same results as those found in the present study (data not shown). A longer infection period only increased the percentage of the PI-permeable, FITC-fluorescent cell population. The discrepancy between our study and the earlier study is therefore probably not due to differences in cell type or infection period. It is more likely that this discrepancy is caused by the presence of antibody-permeable cells. A part of the PI-permeable cell population infected with BacgH1 exhibited FITC fluorescence (Fig. 5A). These PI-permeable cells were probably also antibody permeable and may have disturbed the plasma membrane immunofluorescence assays (Fig. 3). The possibility that the differences between the recombinant baculoviruses caused the discrepancy also cannot be excluded.

The coexpression of gH1 and gL1 in insect cells resulted in expression of gH1 at the cell surfaces. The gH1/gL1 complex displayed on the cell surface was recognized by the polyclonal antibody anti-gH and by the conformationally dependent MAbs 52S and LP11. The recognition of the gH1/gL1 complex was different for the different antibodies used. Whether this was due to reduced accessibility of the epitopes on recombinant gH1 or to the absence of the epitopes is not known. The percentage of insect cells which displayed gH1 at the cell surfaces was less than that for gD1. Although a high MOI was used, the possibility that a percentage of the cells did not coexpress gH1 and gL1 cannot be excluded. However, production, complex formation, and transport of gH1/gL1 are probably more complicated than those of gD1, and this may contribute to the lesser cell surface expression of gH1. Perhaps other glycoproteins are required to facilitate the cell surface expression of gH1, as has been reported for varicella-zoster virus (6).

The recombinant gH1t/gL1 complex was also transported to the cell surface, but it was not integrated into the plasma membrane. Instead, gH1t/gL1 was secreted into the medium, most likely because of the lack of a transmembrane anchor. In mammalian cells, gH1t (amino acids 1 to 791) was also secreted into the cell culture medium in the presence of gL1 (5). The observation that insect cells transport gH1 to the cell surfaces in the presence of gL1 indicates that the protein production system and transport system in insect cells, like those in mammalian cells, are capable of producing and transporting gH1/gL1 heterooligomers. Therefore, the baculovirus expression system is suited for further investigation of the complex formation and intracellular transport of gH1 and gL1.

Most of the recombinant gH1 and gH1t produced in insect cells was present in an insoluble form. Coexpression of gL1 did not result in larger amounts of soluble recombinant gH1. The gH homolog of the Epstein-Barr virus (gp85) produced in insect cells also formed insoluble, aggregated protein (31). The failure of recombinant gH1/gL1 to elicit a protective immune response may be attributed to the aggregation of gH1 (1, 10, 14, 16). The availability of the gHt/gL complex offers the opportunity to investigate the immunological properties of gH/gL.

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