Herpes simplex virus type 1 glycoprotein L mutants that fail to promote trafficking of glycoprotein H and fail to function in fusion can induce binding of glycoprotein L-dependent anti-glycoprotein H antibodies

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The herpes simplex virus type 1 (HSV-1) glycoproteins H (gH) and L (gL) form a heterodimer and efficient expression of gH at the virion or cell surface is dependent upon gL. Five carboxy-terminal deletion mutants of gL were created and their ability to interact with and mediate cell-surface expression of gH, to promote binding of gL-dependent anti-gH antibodies and to contribute to cell fusion was analysed. All of the gL mutants bound gH, but only two mutants, containing the amino-terminal 161 or 168 aa of gL, mediated cell-surface expression of gH, and only gL161 and gL168 functioned in cell fusion. The binding of gL to gH, therefore, was not sufficient to ensure gH cell-surface expression and it was not possible to separate the gH-trafficking role of gL from gL function in fusion. Co-expression of gH with any gL mutant conferred binding of the anti-gH mAbs 53S and LP11. If the acquisition of 53S and LP11 binding to gH reflects a gL-induced conformational change, such a change is not sufficient to mediate trafficking of the gH–gL heterodimer.

INTRODUCTION

Herpes simplex virus (HSV) virions are enveloped by a lipid bilayer containing numerous surface glycoproteins. The surface glycoproteins mediate the recognition of susceptible cells and the subsequent fusion of the viral membrane with the cell plasma membrane. Fusion can be observed by mixing a population of cultured cells expressing the viral glycoproteins B (gB), gD, gH and gL with cells expressing a gD receptor (Jones & Geraghty, 2004; Pertel et al., 2001; Turner et al., 1998). The development of such plasmid-based fusion assays for HSV-1 fusion glycoproteins has facilitated the study of the role of individual glycoproteins in membrane fusion.

HSV-1 gL is a 224 aa glycoprotein that contains an amino-terminal signal sequence, one N-linked glycosylation site and three potential O-linked glycosylation sites (McGeoch et al., 1991; Peng et al., 1998). Membrane fusion during virus entry and during cell fusion requires gL (Pertel et al., 2001; Roop et al., 1993; Turner et al., 1998). HSV-1 virions lacking gL also lack gH and, whilst they bind to the cell surface, they do not initiate fusion (Roop et al., 1993). The formation of a heterodimeric complex consisting of gH–gL is necessary for localization of HSV-1 gH to the virion or cell surface (Hutchinson et al., 1992; Peng et al., 1998). Upon co-expression with gH and corresponding heterodimer formation, gL is associated with the plasma membrane (Dubin & Jiang, 1995). However, gL lacks a transmembrane domain and is secreted from cells when expressed in the absence of gH. Heterodimer formation may be important for proper folding of gH because the anti-gH mAbs 53S and LP11 bind gH only when gL is present (Gompels & Minson, 1986, 1989). The four cysteine residues of gL form intramolecular disulfide bonds and do not form intermolecular disulfide bonds with gH (Hutchinson et al., 1992). All of the cysteines are critical for gL to function in fusion (Cairns et al., 2003, 2005); however, whether they are important for gH binding or transport of gH to the cell surface has not been examined directly.

Recently, heptad-repeat sequences predicted to form α-helical coiled coils were identified within gH of human cytomegalovirus and a peptide containing that region blocked virus entry (Lopper & Compton, 2004). Shortly thereafter, heptad repeats and a putative fusion peptide were identified in HSV-1 gH (Galdiero et al., 2005; Gianni et al., 2005a, b). Peptides from the heptad-repeat region of HSV-1 gH also inhibited virus entry (Gianni et al., 2005b). Heptad repeats and fusion peptides are common features of viral glycoproteins involved in membrane fusion and their presence in gH further emphasizes the importance of gH in the fusion process (Galdiero et al., 2005; Gianni et al., 2005a, b; Lopper
As gH is extremely important for fusion, one role for gL in fusion is to mediate appropriate localization of gH to the surface of the virion or the cell. However, mAbs to HSV-1 gL can block cell fusion (Novotny et al., 1996), suggesting that HSV-1 gL may have another role, possibly to interact with a viral or cellular protein, to promote fusion.

HSV-1 gL deletion mutants have been analysed for their chaperone-like activity (Peng et al., 1998). The ability of gL truncation mutants to bind and promote trafficking of a truncated and secreted form of gH (gH792) was determined. The gL mutant containing 161 amino-terminal amino acids was the smallest mutant that bound and promoted trafficking of gH792. A mutant containing 147 amino-terminal amino acids did not promote trafficking of gH792 (Peng et al., 1998). Whilst this study investigated the domains of gL required for trafficking of a truncated form of gH, it did not determine the ability of gL deletion mutants to interact with wild-type gH, to mediate trafficking of wild-type gH to the cell surface, to confer binding of gL-dependent mAbs to gH or to mediate cell fusion.

The goal of this study was to determine the regions of gL required for appropriate trafficking of wild-type gH and to determine the regions of gL required for fusion. We created five gL mutants with progressive truncations from the carboxy terminus. We analysed the chaperone-like activity of the gL mutants and their ability to confer 53S and LP11 binding to gH and to promote cell fusion. All of the gL deletion mutants co-immunoprecipitated with wild-type gH in co-expressing cells. However, only two of the mutants mediated surface expression of gH, indicating that the ability to bind gH was separable from the ability to mediate cell-surface expression. All of the corresponding gH–gL mutant complexes bound mAbs 53S and LP11, regardless of whether they trafficked to the cell surface, thus indicating that the ability to confer 53S and LP11 binding did not guarantee gH localization to the cell surface. Our deletion analysis indicates that gL function in fusion is linked to the ability to promote gH cell-surface expression. Also, the region between aa 155 and 161 is required for gL function.

**METHODS**

**Cell lines and antibodies.** CHO-K1 cells (ATCC) were grown in F12 medium, 7 % fetal bovine serum (FBS) and penicillin/streptomycin. Anti-gH mAb 52S and 53S hybrids (Showalter et al., 1981) were purchased from the ATCC. Anti-gH mAb LP11 was a gift from A. Minson (University of Cambridge, UK). Rat anti-haemagglutinin (HA) was from Roche. Rabbit anti-calnexin was provided by A. Sinai (University of Kentucky, KY, USA). Rabbit anti-giantin was from Covance and anti-KDEL mouse mAb was from Stresgen. The rabbit anti-rat IgG, goat anti-mouse fluorescein isothiocyanate (FITC)-, goat anti-rat FITC- and goat anti-rabbit FITC-conjugated antibodies were from Jackson ImmunoResearch. The goat anti-mouse and goat anti-rat–Texas red antibodies were from Molecular Probes. The anti-mouse biotin-conjugated antibody was from Sigma. The donkey anti-rabbit 35S-conjugated antibody was from Amersham Biosciences.

**Plasmids.** Plasmids expressing HSV-1 gB (pPEP98), gD (pPEP99) or gH (pPEP100) and the plasmid expressing T7 RNA polymerase (pT7pol) (Pertel et al., 2001) were provided by P. Pertel (Bayer Pharmaceuticals, West Haven, CT, USA). The plasmids expressing nectin-1 (pC4) and CD4 (pBG53) have been described previously (Geraghty et al., 2000, 2001). The plasmid expressing β-galactosidase (β-gal) under the control of the T7 promoter (pG1NT7β-gal) (Nussbaum et al., 1994) was provided by E. Berger (National Institutes of Health, MD, USA). The plasmid expressing an HA epitope-tagged gL (pMN116) (Novotny et al., 1996) was provided by P. Spear (Northwestern University, IL, USA).

The gL mutants were created by amplifying pMN116 with primer HSVgL5 (5'-CTGAGGCTTATGGGATTTGGTGGTGC) and one of the following primers: gl68 (5'-AGAATTTCAATCTCCAAAA- TCTCTGCACC), gl61 (5'-AGAATTCTCATACAGGGCCGCGTGG- CGCG), gl147 (5'-AGAATTGACCTTACAGGGTGTGTTGCG), gl150C (5'-TGAATTTCAACACACCCAGCTCAGAGTTG) or gl155C (5'-TGAATTTCAATCTAGTGAAGTTCAAGTTACACCCCA). The PCR products were gel-purified and digested with enzymes EcoRI and HindIII. The products were ligated into pMN116 that had previously been digested with EcoRI/HindIII. All newly created plasmids were verified by DNA sequencing (Davis Sequencing, Davis, CA, USA).

**Transfections.** In each well of a six-well plate, approximately 80 % confluent CHO-K1 cells were incubated with 1.5 μg plasmid DNA and 5 μl Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The cells were incubated with the transfection reagents for 6–8 h and the transfection medium was replaced with F12/20% FBS.

**Flow cytometry.** CHO-K1 cells were transfected with plasmids expressing gH and gL, and gH and a gL mutant, gH and control plasmid or gL and control plasmid (1:1 molar ratio). Forty-eight hours post-transfection, a single-cell suspension was generated by incubating the cells in PBS/4 mM EDTA at 37 °C for 15 min. The cells were washed in FACS buffer (PBS/2 % heat-inactivated FBS), incubated for 10 min on ice in 100 μl primary antibody diluted in FACS buffer (anti-gH 52S or 53S; 4 μg ml⁻¹), washed in FACS buffer, incubated for 10 min on ice in 100 μl FITC-conjugated secondary antibody diluted in FACS buffer (anti-mouse–FITC at 1:200) and washed in FACS buffer; 20 μl propidium iodide (50 μg ml⁻¹) was added before analysis.

**Co-immunoprecipitation/Western blot.** CHO-K1 cells were transfected with plasmids expressing gH and gL, and a gL mutant, gH and control plasmid or gL and control plasmid (1:1 molar ratio). Co-immunoprecipitations were carried out with mAb 52S as described previously (Jones & Geraghty, 2004). Equivalent volumes of immunoprecipitates (except wild-type gL–gH, where one-third of the volume was loaded) were separated on an 8–16 % SDS/polyacrylamide-gradient gel and transferred to nitrocellulose for Western blot analysis using an anti-HA mAb as described previously (Jones & Geraghty, 2004).

For the quantification of gL mutant expression, one-quarter of the cells transfected for use in flow cytometry were prepared for Western blot as described previously (Jones & Geraghty, 2004). The antibodies used were rat anti-HA antibody (1:2500 dilution), rabbit anti-rat (1:2000) and donkey anti-rabbit 35S-conjugated antibody [0.2 μCi (7.4 kBq) ml⁻¹]. Sixty micrograms of lyase protein was loaded for each sample, except for the gH–gL sample, where 30 μg was loaded. Quantification was performed by using a Molecular Dynamics phosphorimager.

**Immunofluorescence.** CHO-K1 cells at 24 h post-transfection were replated onto 22 × 22 mm square glass coverslips. The next day, immunofluorescence was performed as described previously (Subramanian et al., 2005). Antibody incubations (purified anti-gH
53S, 4 μg ml\(^{-1}\); 52S, 4 μg ml\(^{-1}\); LP11 hybridoma supernatant, neat; anti-HA, 1:200; anti-calnexin, 1:1000; goat anti-mouse–FITC, 1:200; goat anti-rat–Texas red, 1:500; goat anti-mouse–Texas red, 1:500; goat anti-rabbit–FITC, 1:1000, where applicable) were performed at 37°C. For the immunofluorescence co-localization with giantin, cells were processed as described by Oka et al. (2004) and Ungar et al. (2002), except that the antibody incubations and washes were performed in PBT (PBS, 2% BSA, 0-1% Triton X-100). The antibodies used were anti-giantin (1:500), anti-KDEL (1:50), goat anti-rabbit–FITC, goat anti-rat–Texas red and goat anti-mouse–Texas red (all at 1:500). The coverslips were mounted on glass slides with 30 μl p-phenylenediamine mounting solution [0-5% p-phenylenediamine in 20 mM Tris (pH 8.0) with 75% glycerol] and observed via fluorescence microscopy using a Zeiss Axioscope fluorescence microscope.

**Cell-mixing fusion assay and cellular ELISA (CELISA).** The cell-mixing fusion assay was described previously (Jones & Geraghty, 2004) and used with a few modifications. CHO-K1 effector cells were transfected with the plasmids expressing the HSV-1 fusion glycoproteins (gB, gD, gH and gL) and T7 RNA polymerase (at a 1:1:1:1:1 molar ratio). A plasmid expressing a gL mutant or a control plasmid (pcDNA3; Invitrogen) was substituted individually for the wild-type gL plasmid. In some fusion experiments, reduced amounts of wild-type gL plasmid were used (0.005 μg for comparison with gL168 transfections and 0.0025 μg for comparison with gL161 transfections), but total DNA was maintained with control plasmid (pcDNA3). Target CHO-K1 cells were transfected with the plasmid expressing nectin-1 and pG1NT7β-gal (3:1 molar ratio). Twenty-four hours post-transfection, effector and target cells were mixed in a 1:1 ratio and co-cultivated for 18 h on a 96-well dish. β-Gal activity was measured by using the substrate CPRG (0-7 mg ml\(^{-1}\) in PBS with 0.5% Nonidet P40) and spectrometry. Multiple readings of each sample were taken to ensure the linear relationship of β-gal activity over time.

The CELISA assay was performed as described previously (Jones & Geraghty, 2004). CHO-K1 effector cells, transfected for the fusion assay, were replated in a 96-well plate. The anti-gH 52S ascites fluid was used at a 1:500 dilution. Biotin-conjugated anti-mouse secondary antibody was used at a 1:500 dilution. AMDEX streptavidin-conjugated horseradish peroxidase (Amersham Biosciences) was used at a 1:20 000 dilution.

**RESULTS**

**Carboxy-terminal deletions of gL**

To investigate regions of gL important for interaction with gH and for fusion, we created five deletion mutants, gL168, gL161, gL155, gL150 and gL147 (Fig. 1). The deletions were made from the carboxy terminus of gL and the deletion name indicates the number of amino acids contained in the mutant. Mutants gL168 and gL161 contain all four conserved cysteine residues, mutants gL155 and gL150 contain three cysteines and mutant gL147 contains two cysteines. A major antigenic site residing in the carboxy terminus of gL (Novotny et al., 1996; Peng et al., 1998) was removed in the creation of the deletion mutants, so, for ease of detection, wild-type gL and mutants have an HA-epitope tag fused to the carboxy terminus.

![Diagram of gL and deletion mutants. Numbers indicate amino acids contained in the mutant. Wild-type gL contains 224 aa. Amino acid numbers, starting with the initiator methionine, were based on GenBank accession no. AAM78607. 'C' indicates cysteine. Wild-type gL and all gL mutants have an HA-epitope tag (black box) fused to their carboxy terminus. Drawing is not to scale.](http://vir.sgmjournals.org)

**Intracellular localization of deletion mutants**

When gL is expressed in the absence of gH, gL enters the secretory pathway and is secreted from the cell (Dubin & Jiang, 1995). We wanted to determine whether the gL mutants also entered the secretory pathway; however, we could not monitor acquisition and processing of carbohydrates because the predicted glycosylation sites for gL were removed in all of our deletion mutants. Therefore, to determine whether the intracellular localization of the gL deletion mutants was similar to that of wild-type gL and consistent with entrance into the secretory pathway, Chinese hamster ovary (CHO-K1) cells were transfected with a plasmid expressing wild-type gL or a plasmid expressing one of the gL deletion mutants and indirect immunofluorescence was performed. The localization of gL and all gL mutants was indicative of endoplasmic reticulum (ER) staining, as demonstrated by co-localization with the ER-resident protein calnexin (Fig. 2a). Golgi staining was demonstrated by co-localization with the Golgi resident giantin (Fig. 2b). The ER and Golgi co-localization of two representative mutants, gL161 and gL155, is shown in Fig. 2(a, b). As expected, a protein not normally secreted from the cell, the ER resident KDEL, did not co-localize with giantin (Fig. 2b). Finally, secretion of all gL mutants and wild-type gL was detected by immunoprecipitation from supernatant of transfected cells by using an antibody against the HA tag (data not shown). These results demonstrate that, similarly to wild-type gL, the gL mutants entered the secretory pathway and were capable of being secreted from the cell. During this analysis, fewer cells appeared to be expressing the mutant proteins when compared with wild-type gL and the mean intensity of staining for cells expressing the mutant forms of gL was lower than that for cells expressing wild-type gL (data not shown). The mutants may have been expressed at lower levels or were less stable than wild-type gL.
Expression of gL mutants and ability to mediate cell-surface expression of gH

To determine the level of gL mutant expression compared with wild-type gL, cell lysates were generated from cells expressing gH and gL or a gL mutant, and the lysates were subjected to SDS-PAGE and transferred to nitrocellulose. The membrane was incubated with an anti-HA mAb, rabbit anti-rat IgG, plus \[^{35}S\]anti-rabbit IgG and analysed by phosphorimaging to determine steady-state levels of protein expression. The mutants were expressed at levels of approximately 1–8% of those of wild-type gL (Fig. 3), indicating that the deletion of the carboxy-terminal region reduced overall protein expression. The band representing gL161 consistently migrated faster than predicted under denaturing conditions in SDS-PAGE (Figs 3 and 5). The reason for the anomalous migration of gL161 is unclear at this time.

The ability of gL deletion mutants to promote gH expression on the cell surface was analysed by flow cytometry. A portion of the cells transfected for Western blot analysis were examined by flow cytometry for cell-surface gH expression. The cells were incubated with anti-gH mAb 52S (Showalter et al., 1981), which recognizes gH in the presence or absence of gL binding (Gompels & Minson, 1989). Prior to analysis, the cells were incubated with FITC-conjugated anti-HA (gL) and anti-calnexin rabbit antibody followed by anti-rat Texas red- and anti-rabbit FITC-conjugated antibodies. The co-localization of the envelope glycoproteins with calnexin is represented as yellow in the ‘merge’ column.

Fig. 2. Co-localization of wild-type gL and gL mutants with calnexin and giantin. (a) CHO-K1 cells transiently expressing gL, gL161 or gL155 were fixed, incubated with rat anti-HA (to detect gL) and anti-calnexin rabbit antibody followed by anti-rat Texas red- and anti-rabbit FITC-conjugated antibodies. The co-localization of the envelope glycoproteins with calnexin is represented as yellow in the ‘merge’ column. (b) CHO-K1 cells transiently expressing the proteins indicated in (a) were fixed, incubated with rat anti-HA or mouse anti-KDEL and anti-giantin rabbit antibody, followed by anti-rat or anti-mouse Texas red- and anti-rabbit FITC-conjugated antibodies. The images shown with the anti-KDEL antibody were from control-transfected cells. The co-localization of the envelope glycoproteins or KDEL with giantin is represented as yellow in the ‘merge’ column.

Fig. 3. Total cellular expression of gL and gL mutants. CHO-K1 cells transfected with plasmids expressing gH and wild-type gL, gH and a gL mutant, gH and control plasmid or gL and control plasmid were processed for SDS-PAGE and Western blotting. Proteins were detected by using rat anti-HA, rabbit anti-rat IgG and \[^{35}S\]donkey anti-rabbit IgG. Approximately 60 μg protein was loaded from each cell lysate except for the gH–gL lysate, where 30 μg protein was loaded. Numbers below the graph indicate the relative expression of gL and mutants and are the means of two independent experiments. Values are expressed as a percentage of that obtained for gH–gL × 2 (gH–gL × 2 set to 100). Quantification was performed via phosphorimaging.
secondary antibody. Cell-surface expression of gH was readily detectable upon co-expression with wild-type gL, gL168 or gL161 (Fig. 4). Mutants gL155, gL150 and gL147 failed to promote gH cell-surface expression above that detected when gH was expressed alone (Fig. 4). Reduced expression levels of the gL mutants cannot account for our inability to detect gH localization to the cell surface for certain mutants, because gL161 and gL168 promoted cell-surface expression of gH and were expressed at levels comparable to the non-functional gL147, gL150 and gL155 (Fig. 4). We were surprised that gL161 and gL168 were expressed at approximately 1 and 8% of the levels of wild-type gL, respectively, but mediated cell-surface gH expression at levels greater than 1–8% of that of wild-type gL. The mutants may be more efficient than gL in mediating the trafficking of gH. A more likely alternative is that the high levels of wild-type gL expression may have resulted in excess gL that was not involved in trafficking of gH. We repeated the flow-cytometry experiments with anti-gH mAbs 53S and LP11, with results identical to those depicted for 52S (data not shown). Our results identify the region between residues 155 and 161 of gL as critical for trafficking of wild-type gH.

**gL mutants bind gH**

To investigate whether the gL mutants formed a complex with gH, we conducted co-immunoprecipitation and Western blot analysis on cells co-expressing gH and a gL mutant or gH and wild-type gL. Co-expressing cells were lysed and immunoprecipitated by using the 52S mAb. The immunoprecipitated proteins were subjected to Western blot analysis using an anti-HA mAb and an antibody-detection system. All gL mutants were immunoprecipitated with the anti-gH antibody, indicating that all mutants interacted with gH (Fig. 5). The mutant gL147 was the smallest form of gL that bound gH, thereby mapping a gH-interacting domain to the amino-terminal 147 aa of gL.

**Antibody binding to gH in the presence of gL and mutants**

The binding of anti-gH mAb 53S to gH is thought to reflect a conformational change in gH induced by gL association and that change in conformation promotes the trafficking of the gH–gL heterodimer out of the ER and to the cell surface.
(Gompels & Minson, 1989; Gompels et al., 1991; Peng et al., 1998). Therefore, it was of interest to determine whether gH–gL mutant complexes bound 53S, especially the gH–gL mutant complexes that were not trafficked efficiently to the cell surface. The results shown in Fig. 6 were for cells expressing gH alone, gH and wild-type gL or a gL mutant that efficiently mediates gH cell-surface expression, gL161, or a mutant that does not, gL155. The results for the other gL mutants were identical to those obtained for gL161 and gL155 (data not shown). Virtually all cells that expressed gH and a gL mutant also bound 53S antibody. We obtained identical results with the anti-gH mAb LP11 (data not shown), which, like 53S, binds gH only in the presence of gL (Gompels & Minson, 1986, 1989). No fluorescence was detected by using 53S or LP11 in cells expressing gH alone (data not shown). The gH–gL155 heterodimers that bound 53S co-localized with calnexin and, therefore, remained in the ER when examined by indirect immunofluorescence (Fig. 6). A similar 53S fluorescence pattern and calnexin co-localization were detected in cells expressing gH–gL150 and gH–gL147 (data not shown) and when cells expressed gH alone by using the 52S antibody (Fig. 6). Cells expressing gH–gL or gH–gL161 displayed significant fluorescence that did not co-localize with calnexin and occurred at the cell edges, indicative of heterodimers trafficking to the cell surface (Fig. 6). Similar results were obtained with cells expressing gH–gL168 (data not shown). These results demonstrate that 53S and LP11 recognized all gH–gL heterodimers, even those that are not efficiently trafficked to the cell surface, such as gH–gL155, gH–gL150 and gH–gL147.

Two gL mutants mediate cell fusion

To test the ability of gL mutants to mediate cell fusion, gL or a gL mutant was expressed with the other HSV-1 fusion glycoproteins in a cell-mixing fusion assay. CHO-K1 effector cells were transfected with the plasmids expressing the HSV-1 fusion glycoproteins gB, gD, gH, gL or one of the gL mutants and T7 RNA polymerase. Target CHO-K1 cells were transfected with a plasmid expressing the gD receptor nectin-1 and a plasmid with the Escherichia coli lacZ gene under control of the T7 promoter. CHO-K1 cells are resistant to HSV-induced fusion unless they express a gD receptor (Jones & Geraghty, 2004; Pertel et al., 2001). Effector and target cells were mixed in a 1:1 ratio and β-gal activity was measured as an indication of the extent of fusion. Cell-surface expression of gH was determined in parallel by CELISA analysis. Mutants gL161 and gL168, the only mutants that mediated detectable cell-surface expression of gH (Fig. 7a), were also the only mutants successful in mediating cell fusion (Fig. 7a). Consequently, the ability of gL mutants to mediate the cell-surface expression of gH determined their ability to promote cell fusion.

The relative fusion activity mediated by gL168 and gL161 was higher than the relative gH cell-surface expression for the respective mutants (Fig. 7a). If cells expressing wild-type gL

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Fig. 6. Intracellular localization of gH when co-expressed with gL or a gL mutant. CHO-K1 cells transiently expressing the proteins indicated were fixed, incubated with anti-gH 53S mAb plus Texas red-conjugated secondary antibody and anti-calnexin plus FITC-conjugated secondary antibody, and observed via conventional fluorescence microscopy. Regions positive for both gH and calnexin were depicted as yellow in the merge. Cells expressing gH alone were undetectable by using 53S, so the mAb 52S was used.
gH–gL produce excess surface gH–gL that does not participate in cell fusion, then gH–gL168 and gH–gL161 complexes could mistakenly appear to possess higher levels of fusion activity. To better compare the ability of wild-type gL, gL168, and gL161 to promote fusion without differences in gH cell-surface expression, we determined fusion activity when the levels of cell-surface gH were similar in mutant and wild-type transfections. To accomplish this, we lowered the levels of gL expression plasmid in the fusion assay by approximately 100- to 200-fold. Fusion mediated by gL168 (Fig. 7b) and gL161 (Fig. 7c) was approximately equivalent to that of wild-type gL when the levels of cell-surface gH were equivalent.

**DISCUSSION**

Based upon the results presented here, we report the following findings: (i) removal of a region containing aa 169–224 resulted in a marked reduction in expression of gL; (ii) all gL mutants bound gH, but only mutants containing the amino-terminal 161 aa promoted cell-surface expression, indicating that binding of gL mutants to gH was separable from gL-dependent gH trafficking to the cell surface; (iii) all gL mutants conferred binding of 53S and LP11 mAbs to gH, regardless of whether the mutant promoted gH cell-surface expression; and (iv) the smallest mutant of gL that mediated...
cell-surface expression of gH and functioned in fusion consisted of the amino-terminal 161 aa. The region between aa 155 and 161 was critical for the chaperone-like activity and function in fusion. Therefore, the function of gL in cell fusion was linked to its ability to mediate gH expression at the cell surface.

All gL mutants examined in this study bound gH, including gL147, thereby mapping a gH-binding site to a region within the amino-terminal 147 aa of gL. Only two of the five mutants promoted cell-surface gH expression, possibly because multiple or more complex interactions between gH and gL were required. Alternatively, gL147, gL150 and gL155 may be unable to interact with a cellular protein(s) required for proper localization of the gH–gL heterodimer.

A gL-dependent conformational change in gH has been postulated to precede trafficking of gH–gL out of the ER and to the cell surface. This putative conformational change can be detected by the binding of anti-gH mAbs 53S and LP11, which efficiently bind gH only when gL is present (Gompels & Minson, 1986, 1989). Presumably, the 53S and LP11 epitopes are formed or become accessible after a change in gH conformation. Surprisingly, our immunofluorescence results demonstrate that all gL mutants conferred 53S and LP11 binding to gH, even the three mutants that did not promote efficient gH trafficking to the cell surface. Therefore, if a gL-dependent conformational change in gH facilitates binding of 53S and LP11, that change is not sufficient to advance the gH–gL heterodimer to the cell surface. Additional conformational changes in gH may be required for the gH–gL complex to exit the ER and those changes may not occur upon gH binding to gL147, gL150 or gL155. Another possibility is that the 53S and LP11 epitopes consist of amino acids from both gH and gL, as virus, and therefore probably a gH–gL heterodimer, was used to generate the antibodies (Buckmaster et al., 1984; Showalter et al., 1981). Under that scenario, the 53S and LP11 epitopes would be present upon gH binding and may not be due to a conformational change in gH. This explanation suggests that gH may be retained in the ER for a reason other than improper folding and that gL may play a more active role in gH trafficking than thought previously. All gL mutants examined in this study entered the secretory pathway and were eventually secreted from the cell, indicating that an obvious defect in trafficking of gL mutants was not responsible for the lack of gH cell-surface expression. There is at this time, however, no evidence that 53S or LP11 binds gL. HSV-1 isolates selected to be resistant to neutralization by LP11 have mutations in gH (Gompels et al., 1991) and LP11-resistant isolates without mutations in gH have not been described.

The two gL deletion mutants that promoted efficient trafficking of gH to the cell surface also functioned in cell fusion. Previously published studies identified the region between aa 147 and 161 as important for ensuring trafficking of a secreted form of gH (Peng et al., 1998). Our results further define a region in gL between aa 155 and 161 that is critical for trafficking of wild-type gH and also define that same region as critical for gL to promote fusion. Importantly, the region contains the fourth and final cysteine residue for HSV-1(KOS) gL. The absence of the cysteine residue and the corresponding absence of a disulfide bond are likely to be an important defect for the gL155 mutant. Consistent with our results, the gL cysteine residues have recently been shown to be essential for gL to function in fusion (Cairns et al., 2005). We were unable to separate gL function in trafficking gH from gL function in fusion, suggesting that the main function of gL in fusion is to ensure appropriate expression of gH. However, more extensive gL mutagenesis and further study of those mutants in gH trafficking and in fusion are warranted.

There was a reduction in the steady-state protein level for all gL deletion mutants. The reduction occurred even in the gL161 and gL168 mutants, both of which contained all four cysteine residues, indicating that disulfide-bond formation was not a determinant of stable expression. Deletion of the carboxy-terminal regions of gL probably resulted in proteins that folded inefficiently and were subject to degradation. The reduction in gL mutant-protein levels was not responsible for the lack of function for gL155, gL150 and gL147, because these three mutants were expressed at levels equivalent to or higher than a functional mutant, gL161. The carboxy terminus of gL, extending from the final conserved cysteine to the end of the protein, is rich in proline and arginine residues and does not appear to be present in gL homologues from Bovine herpesvirus 1, varicella-zoster virus and Pseudorabies virus. It has been suggested that this extended region of HSV-1 gL may be involved in a function not retained by other alphaherpesvirus homologues (Duus & Grose, 1996). The region does not appear to have a direct role in HSV-1 gH trafficking or fusion, because it is deleted in the two gL mutants that retained function. However, the region may be important for an unknown gL function whose detection will require studying the gL mutants in a viral context.

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REFERENCES


