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J. Virol. **1995, 69(7):4564.**

JOURNAL OF VIROLOGY, July 1995, p. 4564–4568
0022-538X/95/$04.00+0

Vol. 69, No. 7

Expression of Herpes Simplex Virus Type 1 Glycoprotein L (gL) in Transfected Mammalian Cells: Evidence that gL Is Not Independently Anchored to Cell Membranes

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Received 5 December 1994/Accepted 6 April 1995

We expressed herpes simplex virus type 1 glycoprotein L (gL) in transfected cells to investigate whether it is independently anchored to plasma membranes or is membrane associated as a result of complex formation with gH. gL was detected by immunofluorescence microscopy at the surfaces of cotransfected cells when it was expressed with gH but not when it was expressed in the absence of gH or with a truncated form of gH, $gH_{Trunc}(792)$, which lacks the membrane-spanning region and terminates at amino acid 792. Immunoprecipitation studies of transfected-cell culture media revealed that gL was secreted from cells when expressed in the absence of gH and was secreted from cotransfected cells complexed with $gH_{Trunc}(792)$. These observations demonstrate that gL is not independently anchored to plasma membranes but is membrane associated as a result of complex formation with gH.

Herpes simplex virus type 1 (HSV-1) encodes at least 10 glycoproteins which are expressed on the virion envelope and the infected-cell surface (for a review, see reference 28). Of these, glycoprotein B (gB), gD, gH, and gL are essential for infection in vitro, and each has been implicated in the processes of virus entry into cells and cell-to-cell spread of virus, although their individual roles have not been defined (3, 8, 11–14, 17, 19, 21, 24, 26).

Recent studies demonstrate that gH and gL form a heterooligomeric complex which is expressed on the virion envelope and on the surfaces of infected cells (18, 26). Furthermore, coexpression of gH and gL is required for normal posttranslational processing and intracellular transport of both glycoproteins. In the absence of gL expression, gH is incompletely processed, misfolded, and retained intracellularly (9, 15, 25). Hutchinson et al. (18) showed that gL is similarly dependent on gH for its posttranslational processing, since cells infected with gL-expressing recombinant vaccinia virus or recombinant HSV-1 lacking the gH-coding sequences produce an incompletely processed form of gL.

The mechanism of association between the gH-gL complex and plasma membranes has not been fully characterized. Although gH has a carboxy-terminal hydrophobic transmembrane anchor, the amino acid sequence of gL predicts a single hydrophobic domain at the amino-terminus (22). Hutchinson et al. (18) demonstrated that the amino-terminal methionine residue of gL is cleaved during posttranslational processing, suggesting that the hydrophobic domain of gL functions as a signal peptide. Since there are no additional hydrophobic regions of gL that might serve as membrane anchors, it has been postulated that gL may be membrane associated as a result of complex formation with gH (18, 26). Alternatively, a portion of the amino-terminal hydrophobic domain of gL might persist in the mature protein and function as a membrane anchor. Spaete et al. (27) recently demonstrated that the human cytomegalovirus (CMV) UL115 gene, a positional homolog of the HSV-1 gL gene, encodes a protein which is secreted from transfected cells when the protein is expressed with a carboxy-terminally truncated form of CMV gH, suggesting that the gL homolog of CMV is not independently anchored to plasma membranes but is membrane associated as a result of complex formation with CMV gH. However, no studies investigating if HSV gL is membrane anchored when expressed in the absence of gH have been reported.

To examine the mechanism of association between gL and plasma membranes, we used plasmid vectors to express gH and gL in transfected mammalian cells. pCMV3gH-1 was kindly provided by G. H. Cohen and R. J. Eisenberg and has the 3.1-kbp *Hin*dIII fragment of pSR95 (which contains the entire gH-1-coding region from HSV-1 NS) (25) ligated into pCMV3 (1) at the *Hin*dIII site in the polylinker. For some experiments we used $pCMV3gH_{Trunc}(792)$, a plasmid which encodes $gH_{Trunc}(792)$, a truncated form of the protein which terminates at amino acid 792 and lacks the transmembrane region and cytoplasmic domain. This plasmid was constructed by inserting a 14-mer *Spe*I linker containing termination codons (New England BioLabs, Beverly, Mass.) into a unique *Nhe*I site in the gH-1-coding region of pCMV3gH-1, as previously described (25).

To construct a gL expression plasmid, PCR was used to amplify the UL1 open reading frame encoding gL-1 (22) from viral DNA. Two synthetic oligonucleotide primers containing *Xba*I restriction sites (underlined) were designed to facilitate cloning: 5′-TGCTCTAGAGCGCTATGGGGATTTTG GGT-3′ (upstream primer) and 5′-TGCTCTAGAGGTTTCC GTCGAGGCATCGT-3′ (downstream primer). As a template for amplification, HSV-1 (NS) virions (4) were lysed by heating at 95°C for 10 min (to expose viral DNA) and were added to a 100-μl reaction mixture containing 2.5 U of *Taq*I DNA polymerase (Perkin-Elmer Cetus), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM each deoxynucleoside triphosphate, and 1.0 μM each oligonucleotide primer. The reaction mixture was subjected to 35 cycles of amplification (94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min), and the 718-bp amplified DNA product was

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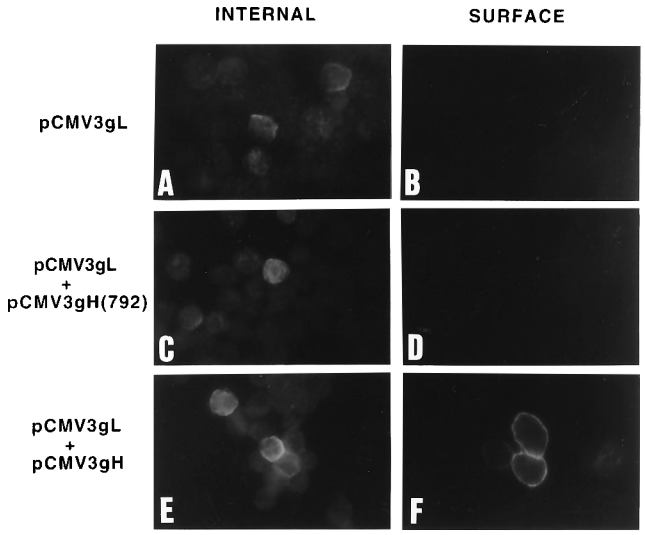

FIG. 1. Cellular localization of gL in transfected cells as determined by immunofluorescence microscopy. L cells were transfected with pCMV3gL-1 (pCMV3gL) alone (A and B), pCMV3gL-1 and pCMV3gH_{Trunc}(792) [pCMV3gH (792)] (C and D), or pCMV3gL-1 and pCMV3gH-1 (pCMV3gH) (E and F). To evaluate cell surface expression of gL, unfixed cells were incubated with a 1:20 dilution of anti-gL serum for 30 min at 4°C, washed twice with phosphate-buffered saline, incubated with a 1:50 dilution of goat anti-rabbit immunoglobulin G $F(ab')_2$ fluorescein-labeled conjugate at 4°C for 30 min, washed twice with phosphate-buffered saline, and viewed with a Leitz epifluorescence microscope. To evaluate internal expression of gL, cells were allowed to adhere to glass microscope slides and were fixed in acetone prior to incubation with anti-gL serum and conjugate.

digested with *Xba*I to generate cohesive termini, was purified from low-melting-point agarose by gel electrophoresis, and was ligated into the *Xba*I site of pCMV3 (1) to create pCMV3gL-1.

We used immunofluorescence microscopy to determine if gL is detected at the surfaces of cells cotransfected to express gL and gH_{Trunc}(792). Since previous studies demonstrated that processing and cell surface association of gL are dependent on gH expression (18), we postulated that gH_{Trunc}(792) would rescue the processing of gL but would be unable to anchor gL to cell membranes, allowing us to determine if gL is independently anchored to cell membranes. Cells were transiently transfected by the calcium phosphate precipitation method (16) with pCMV3gL-1 alone or in combination with either pCMV3gH_{Trunc}(792) or pCMV3gH-1 and were assayed for cell surface expression of gL by immunofluorescence microscopy with rabbit antiserum to the gL-1 UL1-2 peptide (anti-gL serum), kindly provided by D. C. Johnson (18). Experiments were performed with three different cell lines: L, COS, and Vero cells. Figure 1 shows the results of a representative experiment using transfected L cells and demonstrates that when gL was expressed alone (Fig. 1B) or with gH_{Trunc}(792) (Fig. 1D), gL was not detected at the surfaces of unfixed cells. However, gL was easily detected at the cell surface when expressed with the full-length form of gH (Fig. 1F). Under each set of transient-transfection conditions, internal expression of gL was confirmed in approximately 10% of cells by performing antibody and conjugate incubations after acetone permeabilization as shown in Fig. 1A, C, and E; each of these panels shows cells displaying background levels of fluorescence (as defined by viewing mock-transfected cells; not shown) as well as cells that stain more intensely than the background level. Immunofluorescence staining patterns similar to those observed with L cells were obtained with acetone-fixed or unfixed Vero and COS cells (not shown). This experiment demonstrates that association of gL with the plasma membranes of several different types of cells requires coexpression of the transmembrane-anchored form of gH.

We next performed immunoprecipitation studies with transfected-cell culture media to determine if the inability to detect gL at the cell surface when expressed in the absence of gH or with gH_{Trunc}(792) was due to the secretion of gL. For these studies, L, Vero, and COS cells were transfected with pCMV3gL-1 alone or in combination with either pCMV3gH_{Trunc}(792) or pCMV3gH-1, and proteins were radiolabeled with [^{35}S]cysteine. At 18 h posttransfection, the cells were incubated in Dulbecco's modified Eagle medium lacking cysteine (GIBCO BRL, Gaithersburg, Md.) for 30 min, after which the culture media were supplemented with 200 μCi of [^{35}S]cysteine (New England Nuclear, Wilmington, Del.) per ml and the cells were incubated for an additional 24 h. Cell culture media were removed, centrifuged at 5,520 × *g* for 10 min to remove any nonadherent cells or damaged cell membranes, concentrated 10-fold by centrifugation at 5,520 × *g* for 1 h in Centricon-10 concentrator tubes (Amicon, Inc., Beverly, Mass.), and immunoprecipitated with anti-gL serum with heat-killed Cowan 1 strain *Staphylococcus aureus* cells (Calbiochem, San Diego, Calif.) in buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% (wt/vol) Nonidet P-40, and 0.25% (wt/vol) gelatin. A 30-kDa protein was immunoprecipitated from the culture media of L, COS, and Vero cells expressing gL alone, gL with gH_{Trunc}(792), and gL with full-length gH but not from the medium of mock-transfected cells

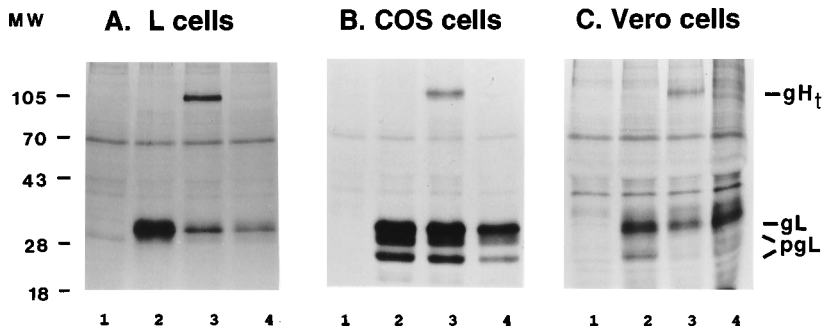

FIG. 2. Detection of gL in transfected-cell culture media by immunoprecipitation. L, COS, and Vero cells were transiently transfected with pCMV3gL-1 alone (lane 2) or in combination with either pCMV3gH_{Trunc}(792) (lane 3) or pCMV3gH-1 (lane 4). The cells were labeled with [^{35}S]cysteine, and concentrated cell culture media were immunoprecipitated with anti-gL serum. As a control, mock-transfected cells were also labeled and immunoprecipitated (lane 1) in this way. Immunoprecipitated proteins were separated by SDS–12% PAGE. MW, molecular weight (numbers are in thousands); gH_t, gH_{Trunc}(792); pgL, precursor forms of gL.

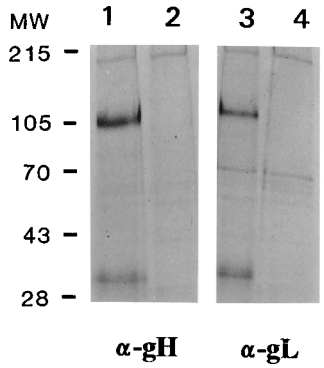

FIG. 3. Analysis of transfected-cell culture media for secretion of gL and $gH_{Trunc}(792)$. L cells were transiently transfected with pCMV3gL-1 and $pCMV3gH_{Trunc}(792)$ (lanes 1 and 3) or with $pCMV3gH_{Trunc}(792)$ alone (lanes 2 and 4) and were labeled with [^{35}S]cysteine. Cell culture media were collected, concentrated 10-fold, and immunoprecipitated with either anti-gH serum (R83) (α-gH) or anti-gL serum (α-gL). Immunoprecipitated proteins were resolved by SDS-PAGE. MW, molecular weight (numbers are in thousands).

(Fig. 2). Anti-gL serum also immunoprecipitated several smaller proteins (ranging in size from 25 to 28 kDa) from the media of COS and Vero cells (Fig. 2B and C). These proteins likely represent precursor forms of gL that undergo incomplete or slower processing in these cell lines. The secretion of gL into culture media of cells expressing the full-length form of gH likely reflects overexpression of gL relative to gH in transfected cells (Fig. 2A to C, lanes 4).

A 105-kDa protein, presumed to be $gH_{Trunc}(792)$, coprecipitated from culture media with the 30-kDa protein (Fig. 2A to C, lanes 3), indicating that the two proteins are secreted from transfected cells in a stable complex. The full-length form of gH did not coprecipitate from culture media with gL, demonstrating that this form of gH is indeed membrane anchored (Fig. 2A to C, lanes 4). Additional immunoprecipitation studies were performed as described above with R83, an anti-gH-1 serum (26), and the culture medium of cells cotransfected with $pCMV3gH_{Trunc}(792)$ and pCMV3gL-1. The antibody immunoprecipitated the same 30-kDa–105-kDa-protein complex that was immunoprecipitated with anti-gL serum, confirming the identities of the 30- and 105-kDa component proteins as gL and $gH_{Trunc}(792)$, respectively (Fig. 3, lanes 1 and 3). The culture medium of cells transfected with $gH_{Trunc}(792)$ alone was also assayed with anti-gH or anti-gL serum, and under these conditions neither the 30- nor the 105-kDa protein was immunoprecipitated (Fig. 3, lanes 2 and 4). This finding further demonstrates the specificity of the immunoprecipitations, since Roberts et al. (25) previously showed that in the absence of gL, carboxy-terminal truncations of gH are not secreted from cells. Taken together, these experiments indicate the following. (i) gL is secreted from transfected cells that do not express gH and, therefore, is not independently anchored to plasma membranes. (ii) Cell surface association of gL results from complex formation with gH. (iii) Posttranslational processing and intracellular transport of gL in transfected cells do not require gH expression; however, the processing of gL in transfected cells appears to be cell type dependent.

The observation that gL is posttranslationally processed and secreted from transfected cells in the absence of gH was unexpected, since previous studies of gL indicated that its processing was dependent on gH expression (18). To further investigate the processing and secretion of gL in transfected cells, we performed a pulse-chase analysis. L cells were transfected with pCMV3gL-1 and after 42 h were labeled with

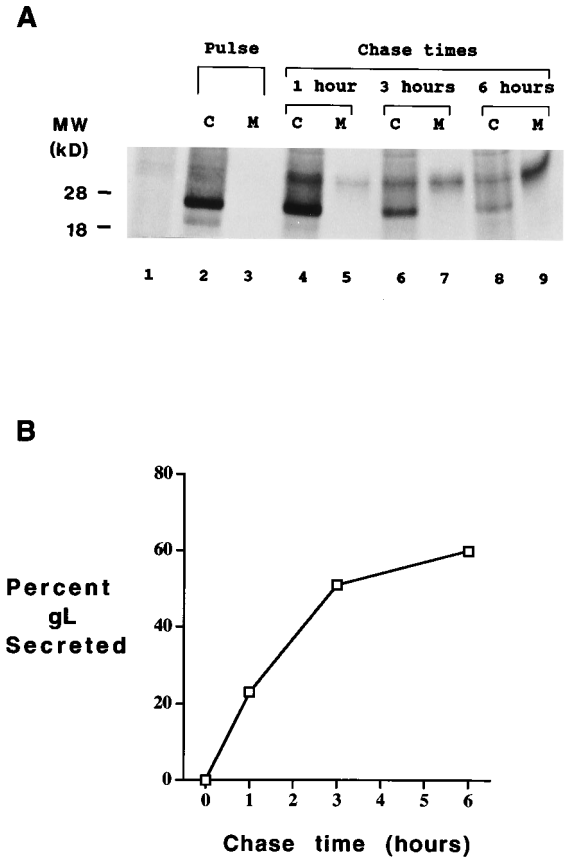

FIG. 4. Pulse-chase analysis of gL produced in transfected L cells that do not express gH. (A) L cells were transiently transfected with pCMV3gL-1, pulse-labeled with [^{35}S]cysteine, and then not chased (lanes 2 and 3) or chased as indicated. Cell culture media (M) were concentrated and cell extracts (C) were prepared such that the final volumes of each extract and medium sample were equivalent. The samples were immunoprecipitated with anti-gL serum, and the immunoprecipitated proteins were resolved by SDS-PAGE. Extracts of mock-transfected cells were immunoprecipitated as a control (lane 1). (B) The intensities of the autoradiograph bands in panel A were measured with a laser densitometer, and the percentage of labeled gL secreted from transfected cells was determined at each time point by comparing the intensity of the gL signal in culture media with the combined intensities of the 25- and 30-kDa gL signals in cell extracts and culture media.

[^{35}S]cysteine for 30 min. Either the cells and cell culture media were immediately collected or the cells were washed with Dulbecco's modified Eagle medium containing cysteine and incubated for a chase period of 1, 3, or 6 h prior to the collection of the cells and cell culture media. The culture media were concentrated as described above and cell extracts were prepared (5) such that the final volumes of each culture medium and cell extract sample were equivalent. The samples were immunoprecipitated with anti-gL serum by using protein A-Sepharose (Pharmacia Chemicals, Dorval, Quebec, Canada) in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 0.1% Nonidet P-40, 1 mM EDTA, and 0.25% sodium azide. As shown in Fig. 4A, gL is initially produced as a 25-kDa precursor (lane 2) which undergoes posttranslational processing to a 30-kDa form which is detected in both the cell extracts and culture media at 1, 3, and 6 h of chase (lanes 4 to 9). Figure 4B shows a densitometric analysis of the autoradiograph bands in Fig. 4A and demonstrates the kinetics of gL secretion. After 6 h of chase, approximately 60% of labeled gL is detected in cell culture media as the 30-kDa form of the protein, while the

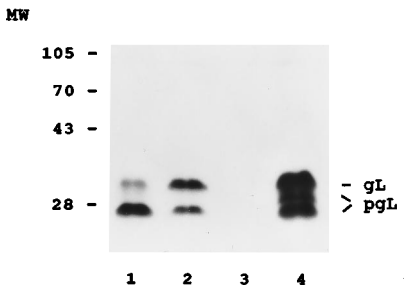

FIG. 5. Expression of gL in transfected and HSV-1 (NS)-infected L cells. Cytoplasmic extracts were prepared as previously described (5) from L cells transiently transfected with pCMV3gL-1 alone (lane 1) or with pCMV3gL-1 and pCMV3gH-1 in combination (lane 2) or from L cells infected for 24 h with HSV-1 (NS) at a multiplicity of infection of 10 (lane 4). Proteins were resolved by SDS-PAGE under denaturing conditions, transferred to a nylon membrane, and probed with gL monoclonal antibody 8H4 (unpublished data). Bound antibody was detected with goat anti-mouse antibody-horseradish peroxidase conjugate and a chemiluminescent substrate solution (New England Nuclear). Lane 3 contains gel loading buffer alone. MW, molecular weight (numbers are in thousands); pgL, precursor form of gL.

remainder of labeled gL is detected in cell extracts. The 25- and 30-kDa proteins correspond to precursor and fully processed forms of gL, respectively, produced in cells infected with HSV-1 (NS), a wild-type strain of virus from which the gL open reading frame of pCMV3gL-1 was derived (Fig. 5). As demonstrated in Fig. 5, infected cells also express a protein with an intermediate molecular weight, which may represent a partially processed form of gL.

Our studies demonstrate that in transfected cells, gL derived from strain NS of HSV-1 is initially produced as a 25-kDa precursor protein which, in the absence of gH, undergoes post-translational processing to a 30-kDa form that is secreted into the cell culture medium. In conjunction with the observation that gL cannot be detected at the surfaces of transfected cells when expressed in the absence of gH, our findings indicate that gL is not independently anchored to plasma membranes. Furthermore, in cotransfection experiments with a plasmid that encodes a truncated form of gH which lacks the transmembrane and cytoplasmic domains, we showed that gL is secreted into cell culture media complexed with the gH truncation mutant. This observation provides additional evidence that gL is membrane associated as a result of complex formation with gH and that the gH-gL complex is anchored to plasma membranes by the gH membrane-spanning domain.

There are several unresolved issues concerning the processing of gL which are raised by our studies. Previous studies by Hutchinson et al. (18) demonstrated that in the absence of gH, gL produced in infected cells is expressed as an incompletely processed precursor which is not converted to the mature form of the protein. Why then are transfected cells able to post-translationally process gL in the absence of gH, while gH is required for efficient processing of gL in infected cells? Our studies also indicate that the apparent molecular masses of the precursor and mature forms of gL produced in both transfected and infected cells are 25 and 30 kDa, respectively (Fig. 4 and 5); these molecular masses differ from those reported by Hutchinson et al., i.e., 30 and 40 kDa, respectively (18). Both in our studies and in those of Hutchinson et al., gL was expressed in a variety of cell lines (including Vero cells), suggesting that these differences were not related to cell-type-dependent processing. Perhaps the apparent discrepancy results from the fact that the studies used different strains of viruses as sources of gL. In our studies gL was derived from HSV-1 (NS), while in those of Hutchinson et al. gL was derived from HSV-1 (KOS), HSV-1 (17), and HSV-1 (SC16) (18); the processing of these gL proteins may therefore differ from gL processing in our study. Alternatively, the lack of agreement among the reported molecular masses of gL may simply reflect differences in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) conditions in each study.

Homologs of genes that encode HSV-1 gH and gL have been identified in many herpesviruses, including human CMV (6), varicella-zoster virus (7, 10, 23), and Epstein-Barr virus (2, 23). The BKRF2 gene of Epstein-Barr virus is a positional homolog of HSV-1 gL and encodes a protein that associates with gp85, the Epstein-Barr virus gH homolog (29). The CMV UL115 gene, a positional homolog of HSV-1 gL, encodes a protein that complexes with CMV gH and which is secreted from cells when coexpressed with a carboxy-terminal truncation mutant of CMV gH (20, 27). Additional studies will be necessary to determine if other gH and gL homologs interact in a similar fashion. Although the mechanism by which the HSV-1 gH-gL complex participates in viral entry and cell-to-cell spread of virus has not been established, the conservation of gH and gL throughout members of the herpesvirus family suggests a central role for these glycoproteins in viral pathogenesis.

We thank G. H. Cohen and R. J. Eisenberg for rabbit antisera to gH and pCMV3gH-1; D. C. Johnson for anti-UL1-2 serum; and G. H. Cohen, R. J. Eisenberg, and H. M. Friedman for critical review of the manuscript.

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