JOURNAL OF VIROLOGY, Apr. 1992, p. 2240–2250
0022-538X/92/042240-11$02.00/0

Vol. 66, No. 4

A Novel Herpes Simplex Virus Glycoprotein, gL, Forms a Complex with Glycoprotein H (gH) and Affects Normal Folding and Surface Expression of gH

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Received 13 November 1991/Accepted 10 January 1992

A glycoprotein encoded by the UL1 gene of herpes simplex virus type 1 (HSV-1) was detected in infected cells with antipeptide sera. The UL1 gene has previously been implicated in virus-induced cell fusion (S. Little and P. A. Schaffer, Virology 112:686–697, 1981). Two protein species, a 30-kDa precursor form and a 40-kDa mature form of the glycoprotein, both of which were modified with N-linked oligosaccharides, were observed. This novel glycoprotein is the 10th HSV-1 glycoprotein to be described and was named glycoprotein L (gL). A complex was formed between gL and gH, a glycoprotein known to be essential for entry of HSV-1 into cells and for virus-induced cell fusion. Previously, it had been reported that gH expressed in the absence of other viral proteins was antigenically abnormal, not processed, and not expressed at the cell surface (U. A. Gompels and A. C. Minson, J. Gen. Virol. 63:4744–4755, 1989; A. J. Forrester, V. Sullivan, A. Simmons, B. A. Blacklaws, G. L. Smith, A. A. Nash, and A. C. Minson, J. Gen. Virol. 72:369–375, 1991). However, gH coexpressed with gL by using vaccinia virus recombinants was antigenically normal, processed normally, and transported to the cell surface. Similarly, gL was dependent on gH for proper posttranslational processing and cell surface expression. These results suggest that it is a hetero-oligomer of gH and gL which is incorporated into virions and transported to the cell surface and which acts during entry of virus into cells.

Herpesviruses frequently induce fusion of cells. Fusion of virus-infected cells with uninfected cells may allow for spread of herpesviruses and reduce contact with components of the immune system. The mechanisms by which these viruses influence infected cells to fuse are thought to be analogous to the processes by which the virion envelope fuses with cellular membranes during virus penetration into host cells.

Cell fusion induced by herpes simplex viruses (HSV) appears to involve a number of membrane glycoproteins which are also essential for entry of viruses into cells. Studies utilizing monoclonal antibodies (MAbs), liposomes containing viral proteins, and temperature-sensitive mutants have implicated glycoproteins B, D, and H (gB, gD, and gH) in virus-induced cell fusion and in virus entry (6, 14, 15, 19, 22, 25, 26, 34, 38, 42, 50). In addition, virus mutants unable to express gB, gD, or gH are unable to enter cells (7, 17, 35), and gB^- and gD^- viruses are unable to cause cell fusion (7, 35). Mutations in at least three other regions of the HSV type 1 (HSV-1) genome, including the UL1 (36), UL24 (29), and UL53 (4, 13, 45, 46) genes, can give rise to the syncytial phenotype in which massive cell fusion is observed, in contrast to the low level of cell fusion observed with wild-type viruses. The protein products of these viral genes may act to regulate membrane fusion events during virus entry or egress, perhaps by interacting with viral proteins, such as gB, gD, and gH, which play a direct role in the fusion process.

Mutations in the UL53 gene, including those which affect residue 40 of the predicted protein, frequently give rise to the syncytial phenotype (13, 46). We have recently detected an HSV-1 glycoprotein encoded by the UL53 gene and have named this protein gK (27). The HSV-1 UL1 gene was also implicated in the syncytial phenotype by Little and Schaffer (36), who performed marker transfer experiments involving the syncytial mutant 804. The syncytial phenotype was transferred to a wild-type virus with the *Hpa*I O fragment from mutant 804. Unfortunately, these marker transfer experiments were performed before cloned fragments of HSV-1 DNA were available and, therefore, it is formally possible that contaminating DNA fragments were present in their preparations of DNA. The *Hpa*I O fragment contains the first 94 codons of the UL1 open reading frame and no other open reading frames (40), and it thus appears likely that the syncytial mutation in 804 maps to the NH_2-terminal half of the UL1 gene.

Here, we have utilized antipeptide sera to identify and characterize the protein product of the UL1 gene, which is a glycoprotein that we have named gL. A large fraction of the gL found in infected cells was discovered to be tightly associated with gH. Previously, it had been found that gH expressed in the absence of other HSV proteins was antigenically distinct from gH in infected cells, was not processed properly, and was not transported to the cell surface (18, 23). Similarly, gL was not properly processed when expressed in the absence of other HSV polypeptides and in cells infected with a mutant virus unable to express gH. However, when gL and gH were coexpressed, the proteins were antigenically similar to those found in infected cells, were processed, and were transported to the cell surface. These observations are especially noteworthy because gH,

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which plays a central role in membrane fusion and virus entry into cells, appears to require gL for its activity.

MATERIALS AND METHODS

Cells and viruses. Vero cells, 143 cells, CV-1 cells, and human R-970 cells (47) were grown in alpha minimal essential medium (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 7% fetal bovine serum (FBS). HSV-1 strains F (obtained from P. G. Spear, University of Chicago), KOS (obtained from J. Smiley, McMaster University, Hamilton, Ontario, Canada), and 17 (obtained from H. Marsden, University of Glasgow) and the syncytial mutant 804 (obtained from P. Schaffer, Dana-Farber Institute, Boston, Mass.) were passaged, and titers were determined on Vero or BHK cells. The HSV-1 mutant SCgHZ, lacking structural sequences encoding gH which were replaced with the LacZ gene, was propagated on F6 cells that express HSV-1 gH in an inducible fashion (17). The HSV-1 mutant K082, which is unable to express gB, was passaged on D6 cells (8). An adenovirus vector expressing gL was constructed by first excising an *Eco*RV fragment containing the UL1 gene from plasmid pSG-1, which contains the *Eco*RI JK fragment of HSV-1 (KOS) (20), and inserting the fragment into the *Eco*V site of pBR322 to generate plasmid pUL1. A 1.95-kb *Avr*II-*Bam*HI fragment derived from pUL1 was inserted between the *Xba*I and *Bam*HI sites of plasmid pAB26, which contains the right end of adenovirus type 5 (Ad5) DNA (3), yielding pABgL. Human 293 cells were cotransfected with pABgL and pFG173, which contains the left end of Ad5 DNA (24), and 14 days later, adenovirus plaques arose. Recombinant viruses were plaque purified, viral DNA was analyzed, and a virus isolate able to express gL (AdgL) was used in further studies. Vaccinia virus strain WR and thymidine kinase (TK)-negative recombinants derived from it were propagated in CV-1 or Vero cells and were assayed in Vero cell monolayers. A recombinant vaccinia virus expressing gL was constructed by the general strategy described by Mackett et al. (37) as follows. The *Kpn*I B fragment of HSV-1 strain 17 cloned in pAT153 was digested with *Sty*I, and an 825-bp fragment corresponding to unique long nucleotides 9329 to 10154 (40) was purified. The initiating ATG of UL1 lies at unique long nucleotide 9339. This fragment was end repaired and subcloned into pRK19 such that the gL coding sequence, under the control of the vaccinia 4b late promoter, was inserted within the vaccinia virus TK gene. TK^- viruses were selected from the progeny by growth in 143 TK^- cells in the presence of bromodeoxyuridine, individual plaques were picked, and recombinants were identified by hybridization with the *Sty*I fragment. A hybridization-positive virus was subjected to three rounds of plaque purification, and its genotype was confirmed by restriction analysis. This virus was named Vac4b-gL. Recombinant vaccinia viruses expressing gH from the 4b promoter, named Vac4b-gH, and the 7.5 promoter, named Vac7.5-gH, have been described previously (18).

Synthetic peptides, antipeptide sera, and other antibodies. Two synthetic peptides, UL1-1 (YVIRSRVAREVGDILK VPC) and UL1-2 (CATKSRRRRPHSRRL), were synthesized and purified by Bachem Inc. (Torrance, Calif.). The peptides were conjugated to keyhole limpet hemocyanin or bovine serum albumin (BSA) via the cysteine residues with maleimidobenzoic acid-*N*-hydroxysuccinimide ester as described previously (16). New Zealand White rabbits were injected initially with 1.0 mg each of peptide conjugated to keyhole limpet hemocyanin in Freund's complete adjuvant and then subsequently (after 4 to 6 weeks) with peptide conjugated to BSA in RIBI adjuvant (RIBI Immunochemicals, Hamilton, Montana). The animals were bled 9 to 10 days after each injection, and sera from animals which reacted with gL were pooled. MAbs LP11 and 52S against HSV-1 gH (5, 51) and 15βB3 against HSV-1 gB (32) have been described elsewhere. Rabbit polyclonal serum specific for gH (anti-gH) purified by immunoaffinity chromatography was a kind gift of Roselyn Eisenberg and Gary Cohen. Rabbit antiserum directed to a trpE-gH fusion protein (anti-trpE-gH) has been described elsewhere (15).

Radiolabelling of cells, immunoprecipitation, and gel electrophoresis. R-970 or Vero cells were infected with HSV-1 with 10 or 20 PFU per cell. At 7 h after infection, the cells were washed three times with medium lacking methionine and cysteine and containing 1% FBS (labelling medium) and incubated for 4 to 5 h with labelling medium containing 100 μCi of [^{35}S]methionine and 100 μCi of [^{35}S]cysteine per ml. Pulse-chase experiments were performed by first washing cells three times with labelling medium and labelling cells with 200 μCi of [^{35}S]methionine and 200 μCi of [^{35}S]cysteine per ml in labelling medium for 20 min, and then cell extracts were made (pulses) or the cells were washed once with alpha minimal essential medium containing 1% FBS and the cells were incubated for 100 or 240 min in alpha minimal essential medium containing 1% FBS (chases). Cell extracts were made with NP40-DOC buffer (1% Nonidet P-40 [NP40], 0.5% sodium deoxycholate [DOC], 50 mM Tris-HCl [pH 7.5], 100 mM NaCl) containing 2 mg of BSA per ml and 1.0 mM phenymethylsulfonyl fluoride and stored overnight at −70°C. The extracts were thawed, centrifuged at 82,000 × *g* for 60 min, and in some instances precleared by incubation with rabbit sera directed to gE and gI and protein A-Sepharose. Extracts derived from 5×10^5 to 10×10^5 cells were mixed with rabbit sera or mouse ascites fluids (5 to 15 μl) for 90 to 120 min on ice. In some experiments, antipeptide sera (5 to 15 μl of pooled sera) were preincubated with 100 to 150 μg of synthetic peptide for 30 to 60 min at 4°C. Protein A-Sepharose (Pharmacia Chemicals, Dorval, Quebec, Canada) was added, and the samples were rotated end over end for a further 60 to 90 min. Immunoprecipitates were washed either three times with NP40-DOC buffer or with the following more stringent washes: twice with RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% DOC, 1% NP40), once with 2 M NaCl–50 mM Tris-HCl (pH 7.5)–0.5% DOC–1% NP40, once with phosphate-buffered saline (PBS) containing 1% NP40–0.5% DOC, once with 1 M $MgCl_2$, and once with 1 mM Tris-HCl (pH 7.5). The precipitated proteins were eluted by adding 50 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, bromophenol blue, and 2% β-mercaptoethanol and boiling the sample for 5 min. Electrophoresis involved 10 or 12% polyacrylamide gels as described elsewhere (30). The gels were impregnated with Enhance (Dupont, Montreal, Quebec, Canada) and exposed to Kodak XAR film.

Endoglycosidase treatment of immunoprecipitated proteins. Immunoprecipitated proteins were eluted from protein A-Sepharose by boiling in 0.1 M sodium phosphate buffer, (pH 7.5) containing 0.5% β-mercaptoethanol and 1% SDS and then diluted with 2 volumes of 0.1 M sodium phosphate buffer containing 1% octyl glucoside, 150 μM phenanthroline, and 10 mM EDTA. Endoglycosidase F (2 or 10 U/ml; Boehringer Mannheim) was added, and the material was incubated for 2 h at 37°C; then 2% SDS–2% β-mercaptoethanol–10% glycerol was added to the samples, which were then subjected to electrophoresis.

Cleveland partial proteolysis of glycoproteins. Proteins were immunoprecipitated, eluted, and electrophoresed on preparative polyacrylamide gels. Bands corresponding to gB, gH, gL, or proteins associated with either gL or gH were located by using X-ray film, excised, and rehydrated as described previously (30). Samples were digested with 5, 20, or 100 μg of V8 protease per ml during early stages of electrophoresis through 18% polyacrylamide gels as described previously (30).

Western immunoblot analysis. Extracts from Vero cells infected with HSV-1 (KOS) or SCgHZ were made 16 h after infection and then in some cases immunoprecipitated with the following MAbs: LP11 or 52S, anti-gH rabbit serum, and anti-UL1-2 or anti-UL1-2 in the presence of UL1-2 peptide. Cell extracts or immunoprecipitated proteins were electrophoresed with 4 to 15% gradient polyacrylamide minigels (Bio-Rad, Mississauga, Ontario, Canada) or 10% linear polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, and the membranes were blocked for 10 h with PBS containing 1% skim milk and 1% BSA and then for 1 h with PBS containing 1% BSA and 0.5% gelatin. The blots were incubated for 2 h with anti-trpE-gH serum diluted 1:200 in PBS containing 1% BSA or with anti-UL1-2 serum diluted 1:50 in PBS containing 1% BSA followed by five washes with PBS containing 0.2% NP40. The membranes were incubated with 0.15 μCi of ^{125}I-labelled protein A (New England Nuclear, Montreal, Quebec, Canada) per ml for 1.0 h and then washed five times with PBS containing 0.2% NP40, dried, and exposed to film.

Immunofluorescence. 143 cells were seeded and infected on glass coverslips in 24-well trays. The cells were fixed in 2% formaldehyde in PBS for 10 min and washed three times with PBS containing 1% FBS. The fixed cells were permeabilized by incubation in PBS containing 1% Triton X-100, 10% sucrose, and 1% FBS for 5 min and then washed three times with PBS containing 1% FBS. All subsequent incubations and washes were in PBS containing 1% FBS. Coverslips were incubated for 1 h in antibody 52S (culture supernatant diluted 1:2), washed three times, incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (IgG) (DAKO) at a dilution of 1:50 for 45 min, and then washed three times. All treatments were at room temperature.

RESULTS

Identification and characterization of the protein product of the UL1 gene, gL. In order to identify and further characterize the protein predicted to be encoded by the UL1 gene (40), we had synthesized two peptides: UL1-1, which includes residues 26 to 44, and UL1-2, which contains residues 200 to 224 (the carboxy terminus) of the UL1 open reading frame. Sera from rabbits injected with either UL1-1 or UL1-2 conjugated to carrier proteins reacted with two protein species, of 30 and 40 kDa, which were labelled with [^{35}S]methionine and [^{35}S]cysteine in cells infected with HSV-1 (KOS) (Fig. 1). The 30- and 40-kDa protein species were not detected in uninfected cells, with preimmune serum, or when immune serum was mixed with the relevant peptide. The 30- and 40-kDa proteins were also detected in cells infected with the syncytial mutant, 804. The anti-UL1-2 serum reproducibly precipitated a larger fraction of the 40-kDa protein than did the anti-UL1-1 serum, and therefore, the anti-UL1-2 serum was used in most of the subsequent experiments. Prominent protein bands in the range of

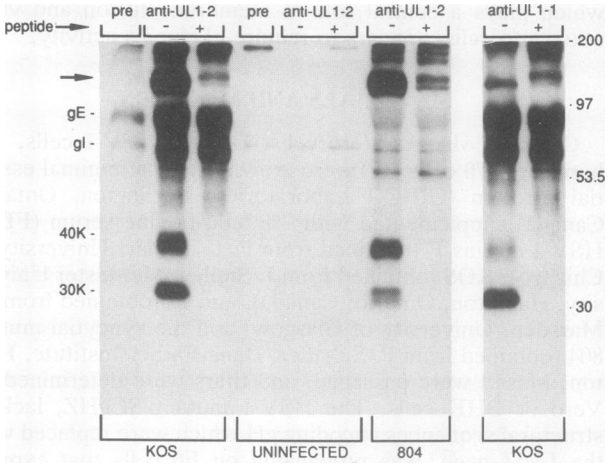

FIG. 1. Immunoprecipitation of the UL1 gene product from HSV-infected cells with antipeptide sera. Human R-970 cells were left uninfected or infected with wild-type HSV-1 (KOS) or the HSV-1 syncytial mutant 804. After 4.5 h, the cells were radiolabelled with [^{35}S]methionine and [^{35}S]cysteine until 10.5 h postinfection. Cell extracts were partially precleared with a rabbit anti-gE/gI serum and protein A-Sepharose and then mixed with anti-UL1-2 serum (−), anti-UL1-2 serum preincubated with UL1-2 peptide (+), pooled preimmune serum (pre), or anti-UL1-1 serum with (+) or without (−) UL1-1 peptide. Antigen-antibody complexes were precipitated with protein A-Sepharose and washed under stringent conditions (see Materials and Methods), and the precipitated proteins were electrophoresed by using 12% polyacrylamide gels. The positions of the 30- and 40-kDa proteins, HSV IgG Fc receptor proteins gE and gI, and marker proteins of 97, 68, 46, and 30 kDa are indicated. The arrow indicates the presence of a 100- to 110-kDa protein associated with gL.

60 to 90 kDa were derived from the HSV IgG Fc receptor which is precipitated in association with rabbit Ig (30, 31).

We investigated the relationship between the 30- and 40-kDa species by performing a pulse-chase experiment in which infected cells were radiolabelled for 20 min, the label was then chased for 100 or 240 min, and cell extracts were immunoprecipitated with anti-UL1-2 serum. After the pulse, the 30-kDa species was detected, and during subsequent chase periods, a large fraction of the 30-kDa protein was converted to the 40-kDa species (Fig. 2, right panel).

To further demonstrate that the 30- and 40-kDa proteins were derived from the UL1 gene and to confirm the specificities of our antipeptide sera, we expressed the UL1 gene product using an adenovirus vector, AdgL, in which the UL1 gene was inserted into the E3 region of Ad5. The anti-UL1-2 serum recognized a 30-kDa protein expressed in AdgL-infected cells which was not observed when antisera were preincubated with the appropriate peptide; however, the 40-kDa protein was not detected (see Fig. 6). It appeared that the 30-kDa protein was not processed to the 40-kDa form in AdgL-infected cells (see below). Three proteins ranging in molecular masses from 75 to 115 kDa were also precipitated from AdgL-infected cells; however, these proteins did not disappear when the sera were preincubated with peptides. The largest and most prominent of these proteins (marked by an arrow in Fig. 6) was apparently the adenovirus hexon protein (115 to 120 kDa), which has been a common contaminant in immunoprecipitation experiments involving rabbit serum (54).

The UL1 gene product contains only a single region near

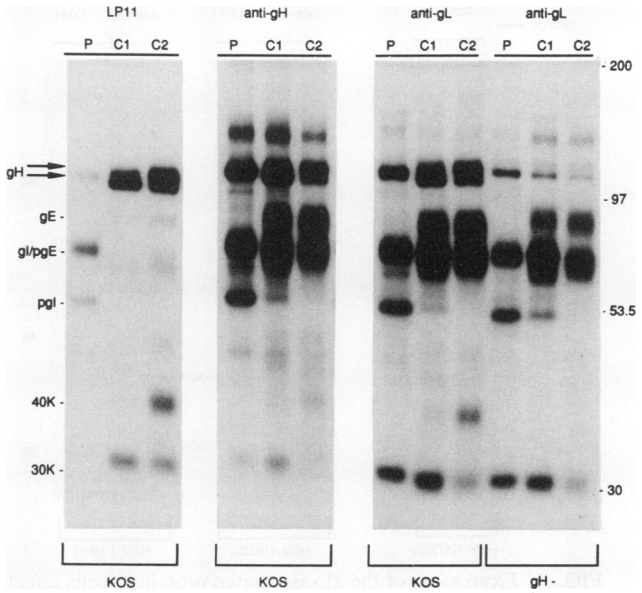

FIG. 2. Pulse-chase analysis of the 30- and 40-kDa UL1 proteins in extracts from cells infected with wild-type HSV-1 or gH$^-$ HSV-1. Human R-970 cells were infected with HSV-1 (KOS) or the gH− mutant virus SCgHZ, and after 6 h were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min. The cells were immediately lysed with detergents (P) or were washed with medium containing methionine and cysteine and incubated for 100 min (C1) or 240 min (C2) in this medium before cell extracts were made. Cell extracts were mixed with MAb LP11 specific for gH, anti-gH rabbit serum, or anti-UL1-2 (anti-gL), and immunoprecipitated proteins were subjected to electrophoresis on 10% polyacrylamide gels. The positions of the 40- and 30-kDa gL proteins, gH, HSV Fc receptor proteins, gE, gI/pgE, pgI, and molecular mass markers of 200, 97, 53.5, and 30 kDa are indicated.

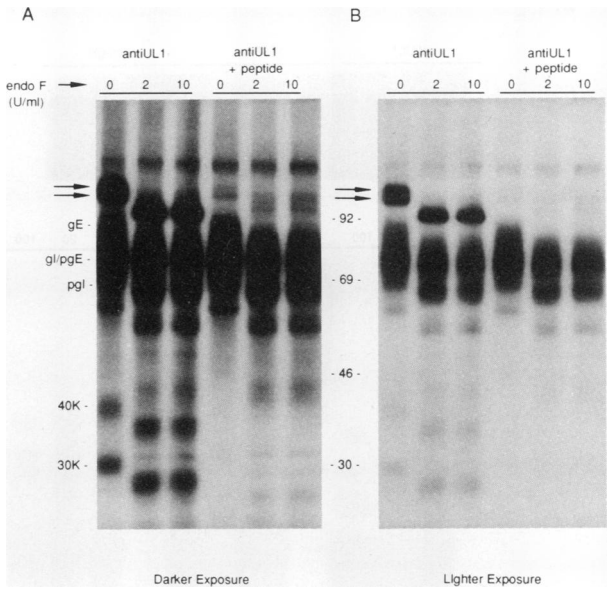

FIG. 3. Endoglycosidase F treatment of proteins immunoprecipitated with anti-UL1-2 serum. Human R-970 cells were infected with HSV-1 (F) and then radiolabelled with [^{35}S]methionine and [^{35}S]cysteine between 5 and 10 h postinfection. Cell extracts were immunoprecipitated with anti-UL1-2 serum or anti-UL1-2 serum mixed with UL1-2 peptide. Immunoprecipitated proteins were eluted and incubated in buffer (0) or were treated with endoglycosidase F (endo F) by using 2 or 10 U/ml for 2 h at 37°C and then subjected to electrophoresis in 12% polyacrylamide gels. A lighter exposure (B) of panel A is included so that heavier bands near the top of the gel can be more readily visualized. The double arrows indicate a pair of protein bands which disappeared when serum was incubated with peptide, in addition to the 30- and 40-kDa proteins. Components of the HSV-1 IgG Fc receptor, gE, gI/pgE, pgI, and molecular mass markers of 92, 69, 46, and 30 kDa are indicated.

the NH2 terminus which would be predicted to act as a signal or transmembrane spanning domain. If this region were cleaved, then the protein might be secreted. However, in a number of experiments (results not shown) we were unable to immunoprecipitate proteins using either anti-UL1-1 or anti-UL1-2 serum from cell culture supernatants (from which virus particles had been removed), suggesting that the UL1 gene product was not secreted from cells in detectable amounts. In these same experiments, we detected cleaved forms of other HSV proteins (gE and gI) which were secreted from the cells in small quantities.

The UL1 gene product, gL, is glycosylated. It has been noted that the UL1 gene product has one potential site for attachment of N-linked oligosaccharides (40). We treated samples containing the 30- and 40-kDa species with endoglycosidase F and observed a shift in the electrophoretic mobilities of both protein species (Fig. 3), suggesting that both are modified with N-linked oligosaccharides. In other experiments (data not shown), a single protein species of 25 kDa was immunoprecipitated by anti-UL1-2 antibodies from HSV-infected cells which had been treated with tunicamycin. This observation was consistent with the predicted size of the UL1 protein before glycosylation (40). Furthermore, both the 30- and 40-kDa species were labelled with ^{3}H-glucosamine (28). Together, these results suggest that a 25-kDa UL1 gene product is initially modified by addition of an N-linked oligosaccharide so that a protein species with an apparent molecular mass of 30-kDa is observed immediately after synthesis. Subsequent modifications to N-linked oligosaccharides and addition of O-linked oligosaccharides during transport to the cell surface apparently contribute to the reduced electrophoretic mobility of the 40-kDa species, as has been noted for other HSV glycoproteins (33). Evidence suggesting that gL is modified with O-linked oligosaccharides comes from the observation that treatment of the 40-kDa form with endoglycosidase F produced a 37-kDa species (Fig. 3) rather than a 25-kDa species, which might be expected if all the oligosaccharides were removed.

We propose to name this newly described HSV-1 glycoprotein gL, following the customs agreed upon at the 1983 Herpesvirus Workshop in Oxford, England, and at subsequent workshops. An HSV glycoprotein, gJ, encoded by the US5 gene, has been previously referred to in a review (48). Further, we recently identified a novel HSV glycoprotein, denoted gK, which is encoded by the UL53 gene (28). On the basis of past experience, it would seem highly likely that a protein analogous to the HSV-1 gL protein is encoded by HSV-2. Unfortunately, the anti-UL1-1 and UL1-2 sera do not react with an analogous protein in extracts from HSV-2-infected cells, most probably because of extensive differences in the amino acid sequences in the regions chosen to produce antipeptide sera (39).

gL forms a complex with gH in infected cells. Two protein species, with apparent molecular masses of approximately 100 and 110 kDa, were consistently observed in immunopre-

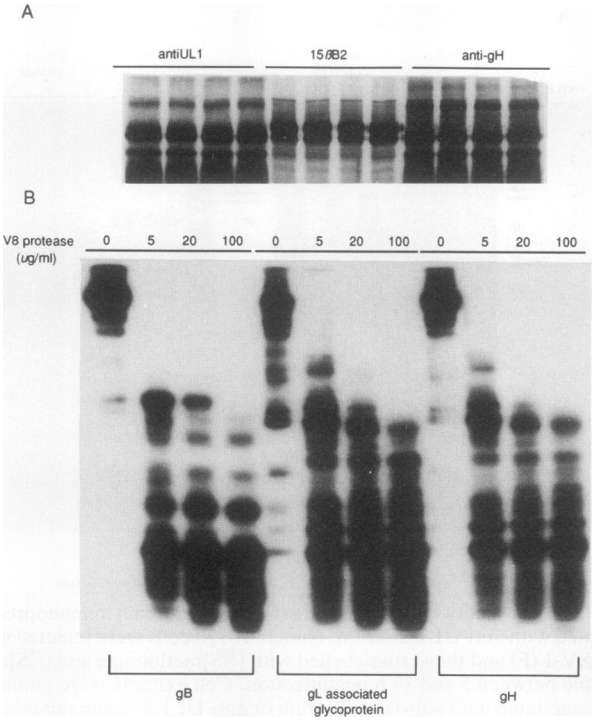

FIG. 4. Cleveland partial proteolysis of gB, gH, and a glycoprotein associated with gL. (A) Radiolabelled extracts from HSV-1-infected cells were immunoprecipitated with MAb 15βB2, which is specific for gB, anti-gH rabbit serum, or anti-UL1-2 serum, and were electrophoresed in polyacrylamide gels. (B) Gel fragments containing gB, gH, or the 100- to 110-kDa protein which was precipitated by anti-UL1-2 were excised and subjected to partial proteolysis with 0, 5, 20, or 100 μg of V8 protease per ml during electrophoresis through 18% polyacrylamide gels.

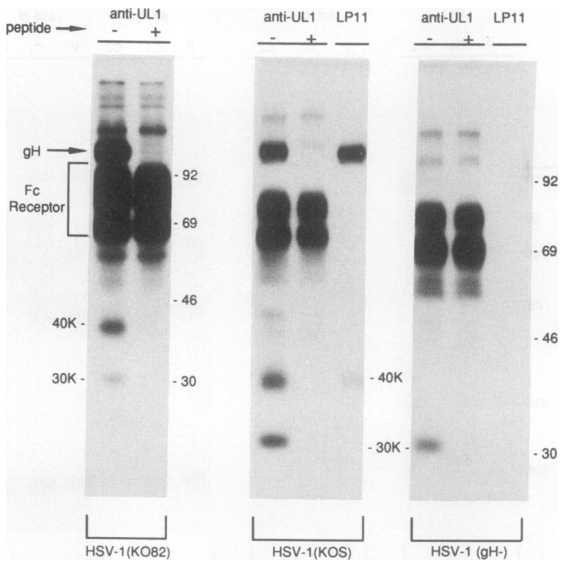

FIG. 5. Expression of the gL-associated protein in cells infected with HSV-1 mutants unable to express gH or gB. Vero cells were infected with wild-type HSV-1 (KOS), the HSV-1 gB$^-$ mutant K082, or the HSV-1 gH$^-$ mutant SCgHZ and labelled with [^{35}S]methionine and [^{35}S]cysteine for 6 h, beginning 5 h after infection. Cell extracts were prepared and mixed with anti-UL1-2 serum (−), anti-UL1-2 serum which had been preincubated with the UL1-2 peptide (+), or MAb LP11 specific for gH. Immunoprecipitated proteins were separated on 12% polyacrylamide gels. The positions of the IgG Fc receptor proteins, gH, the 30- and 40-kDa gL proteins, and molecular mass markers of 92, 69, 46, and 30 kDa are indicated.

cipitations involving the UL1-2 serum (Fig. 1, 2, and 3, arrows). This protein was not observed when anti-gL antibodies were preincubated with the UL1-2 peptide. Anti-UL1-1 antibodies also precipitated this higher-molecular-mass protein in some experiments, although in most cases there was less protein than that observed with anti-UL1-2 serum (Fig. 1). The 100- and 110-kDa protein species were converted to a single faster-migrating protein species by endoglycosidase F (Fig. 3) and, thus, were apparently derived from a single HSV glycoprotein. Two obvious candidates for the gL-associated protein were gB and gH, which have similar or identical electrophoretic mobilities in this gel system. To determine which of these glycoproteins might be associated with gL, we immunoprecipitated gB (using MAb 15βB2), gH (using polyclonal anti-gH serum), and the gL-associated protein (using anti-UL1-2), subjected the proteins to electrophoresis, excised the protein bands, and subjected the proteins to V8 partial protease analysis (9). This analysis clearly demonstrated that the gL-associated protein is structurally similar to gH and not to gB (Fig. 4).

To further examine the origin of the gL-associated protein, we precipitated gL from extracts of cells infected with HSV-1 mutants unable to express either gB or gH. The gL-associated protein was readily detected in extracts from cells infected with HSV-1 (K082), which is unable to express gB (8) (Fig. 5). In contrast, the gL-associated protein was not detected in extracts from cells infected with the gH$^-$ virus, SCgHZ (17). These observations further supported the conclusion that gH was the gL-associated protein.

It was formally possible that anti-gL antibodies cross-reacted with gH, especially because these antibodies were antipeptide antibodies. To examine this possibility, we infected cells with Vac7.5-gH, a vaccinia virus recombinant which expresses gH (18). The anti-UL1-2 serum did not precipitate gH from Vac7.5-gH-infected cells, yet this gH could be precipitated with anti-gH serum (Fig. 6). These results indicate that gH is precipitated by anti-gL antibodies because gL and gH are associated in infected cells. Further support for this hypothesis came from the observation that the gH-specific MAb LP11 precipitated two proteins with electrophoretic mobilities equivalent to the 30- and 40-kDa gL species from extracts of HSV-infected cells (Fig. 3 and 6). The 40-kDa protein immunoprecipitated by LP11 was subjected to V8 protease analysis and found to be identical to the 40-kDa gL species (Fig. 7). LP11 did not directly react with gL because gL was not precipitated from cells infected with AdgL by this antibody (Fig. 6). Therefore, it appears that both the anti-gL polyclonal serum and the gH-specific MAb LP11 precipitate a complex of gH and gL.

To further examine the hypothesis that gH and gL form a complex, we performed Western blot analysis using anti-gH serum to detect gH in material which was first immunoprecipitated with anti-gL antibodies and, conversely, using anti-gL serum to detect gL in the material which was first immunoprecipitated with anti-gH antibodies. Anti-trpE-gH fusion protein serum (15) reacted with gH precipitated by using the anti-gH MAb LP11 and rabbit polyclonal anti-gH and also detected gH in material immunoprecipitated with anti-UL1-2 serum (Fig. 8). Further, the gH band was not

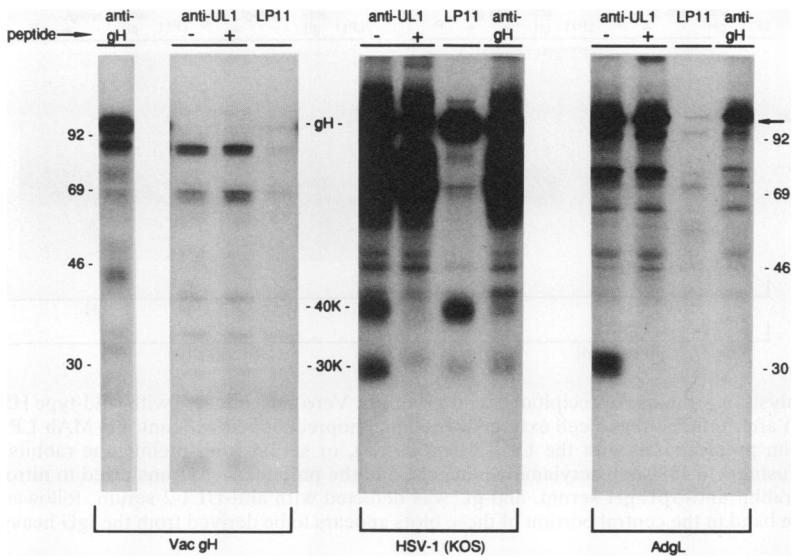

FIG. 6. Expression of gH and gL in cells infected with a vaccinia virus carrying the gH gene or with AdgL. Human R-970 cells were infected with Vac7.5-gH, HSV-1 (KOS), or AdgL. At 6 h after infection with HSV-1 and Vac7.5-gH or at 24 h after infection with AdgL, the cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 5 h and then cell extracts were made. Cell extracts were mixed with MAb LP11, anti-gH serum, anti-UL1-2 serum (−), or anti-UL1-2 serum preincubated with the UL1-2 peptide (+) and subsequently with protein A-Sepharose. Immunoprecipitated proteins were eluted and analyzed with 12% polyacrylamide gels. The positions of gH, the 30- and 40-kDa gL protein species, and molecular mass markers of 92, 69, 46, and 30 kDa are indicated. Also indicated in immunoprecipitates from AdgL-infected cells (arrow) was a contaminating protein of approximately 120 kDa which is routinely observed in extracts from wild-type adenovirus-infected cells immunoprecipitated with rabbit sera.

observed when extracts from cells infected with the gH$^-$ mutant SCgHZ were used. The dark bands centrally located on these blots with an apparent molecular mass of approximately 55-kDa were most probably IgG heavy chains which can bind protein A. Anti-UL1-2 serum reacted with the 30- and 40-kDa gL species in material immunoprecipitated with either anti-gL serum or anti-gH antibodies from extracts of wild-type HSV-infected cells. With extracts from cells infected with the gH$^-$ mutant SCgHZ, gL was not detected in material immunoprecipitated with anti-gH antibodies, and only the 30-kDa form of gL was observed when the immunoprecipitation reactions involved anti-gL antibodies. To-

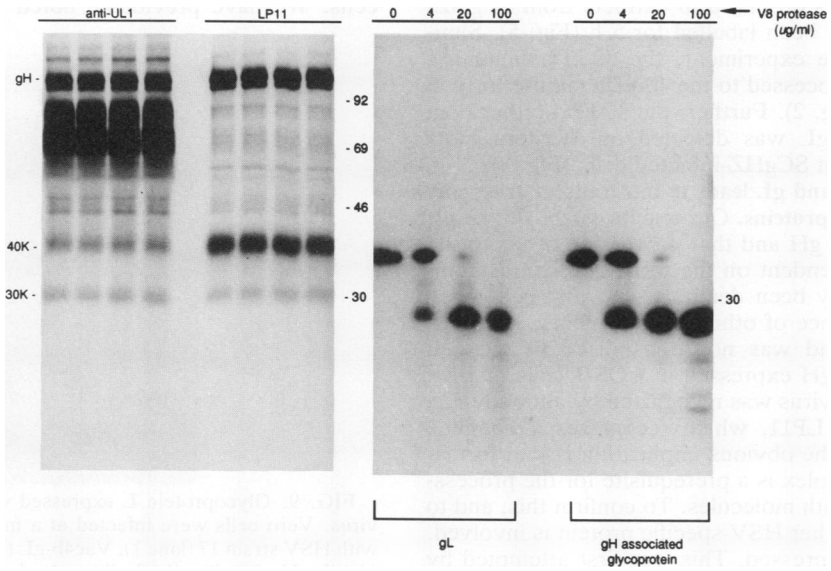

FIG. 7. Cleveland partial proteolysis of gL and a protein associated with gH. Radiolabelled extracts from cells infected with HSV-1 (KOS) were immunoprecipitated with anti-UL1-2 or MAb LP11, specific for gH, and the immunoprecipitated proteins were then electrophoresed by using 12% preparative polyacrylamide gels. Gel fragments containing the 40-kDa gL species precipitated with anti-UL1-2 and the 40-kDa species precipitated with LP11 in conjunction with gH were excised, rehydrated, and treated with 0, 4, 20, or 100 μg of V8 protease per ml during electrophoresis through 18% polyacrylamide gels.

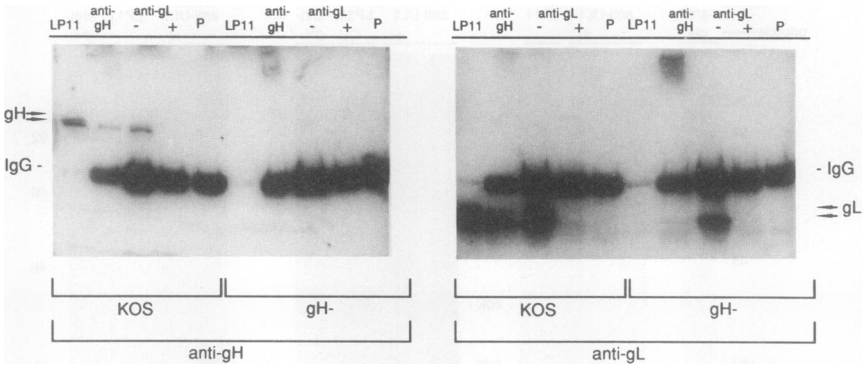

FIG. 8. Western blot analysis of proteins coprecipitated with gH or gL. Vero cells infected with wild-type HSV-1 (KOS) or the gH$^-$ mutant SCgHZ were harvested 16 h after infection, and cell extracts were immunoprecipitated with anti-gH MAb LP11, anti-gH serum, anti-UL1-2 serum (−), anti-UL1-2 serum preincubated with the UL1-2 peptide (+), or serum from preimmune rabbits (P). The immunoprecipitated proteins were separated by using 4 to 15% polyacrylamide minigels, and the proteins were transferred to nitrocellulose membranes. gH was detected on the blots with rabbit anti-trpE-gH serum, and gL was detected with anti-UL1-2 serum, followed in each case by ^{125}I-labelled protein A. The major protein band in the central portion of these blots appears to be derived from the IgG heavy chain which can bind protein A.

gether, these data provide strong evidence that gH and gL form a complex in infected cells. It appears likely that the complex formed between gH and gL does not depend on interchain disulfide bonds, because the electrophoretic mobilities of gH and gL observed in the absence of reducing agents (β-mercaptoethanol) were similar although not identical to those observed in the presence of reducing agents.

Expression and processing of gL in cells infected with an HSV mutant unable to express gH. When gL was expressed in the absence of other HSV proteins by using AdgL, we observed that gL was not processed from the 30-kDa immature form to the 40-kDa mature form. To determine whether this lack of processing was related to the absence of gH, gL was examined in extracts of cells infected with the gH$^-$ mutant SCgHZ. The 30-kDa form of gL but not the 40-kDa mature form of gL was observed in extracts from SCgHZ-infected cells that had been labelled for 5 h (Fig. 5). Similarly, in a pulse-chase experiment, the 30-kDa immature form of gL was not processed to the 40-kDa mature form in the absence of gH (Fig. 2). Further, the 30-kDa rather than the 40-kDa form of gL was detected on Western blots involving extracts from SCgHZ-infected cells (Fig. 8).

Coexpression of gH and gL leads to intracellular transport and processing of both proteins. Our results establish that gL forms a complex with gH and that processing of gL to the 40-kDa species is dependent on the formation of this complex. It has previously been demonstrated that gH, when expressed in the absence of other HSV proteins, was antigenically abnormal and was not transported to the cell surface. Specifically, gH expressed in COS-1 cells or by a recombinant vaccinia virus was recognized by antibody 52S but not by antibody LP11, which recognizes a complex epitope (18, 21, 23). The obvious implication is that formation of the gL-gH complex is a prerequisite for the processing and transport of both molecules. To confirm this, and to demonstrate that no other HSV-specific protein is involved, gL and gH were coexpressed. This was first attempted by coinfecting cells with Vac4b-gH or Vac7.5-gH and AdgL recombinant viruses, but it was apparent that these viruses were incompatible. Therefore, a recombinant vaccinia virus, Vac4b-gL, which expresses gL under the control of the vaccinia virus 4b promoter, was constructed, and cells were infected with Vac4b-gH alone, Vac4b-gL alone, or with the two recombinants together. Lysates were subjected to Western blot analysis with anti-UL1-2 serum (Fig. 9). gL synthesized by Vac4b-gL is primarily in the 30-kDa form, consistent with the results of experiments with AdgL (Fig. 6), although a small fraction of the gL was observed to be in the 40-kDa form when darker exposures of these blots were made. Coexpression of gL and gH resulted in the synthesis of approximately equal amounts of the 30- and 40-kDa forms of gL, a result similar to that observed for HSV-infected cells. We note that the electrophoretic mobilities of gL species from recombinant vaccinia virus-infected cells are slightly different from those of gL in HSV-infected cells. At present, we assume that this is due to small differences in glycosylation in vaccinia virus-infected and HSV-infected cells. We have previously noted minor differences in the

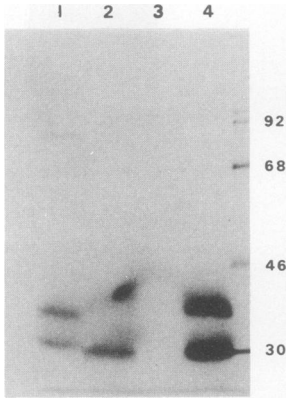

FIG. 9. Glycoprotein L expressed with a recombinant vaccinia virus. Vero cells were infected at a multiplicity of infection of 10 with HSV strain 17 (lane 1), Vac4b-gL (lane 2), Vac4b-gH (lane 3) or Vac4b-gH plus Vac4b-gL (lane 4). Lysates corresponding to 10^5 cells were prepared 16 h after infection and subjected to electrophoresis on 10% polyacrylamide gels, and the proteins were transferred to nitrocellulose. gL was detected with anti-UL1-2 serum. Numbers on the right indicate molecular mass markers of 92.5, 68, 46, and 30 kDa.

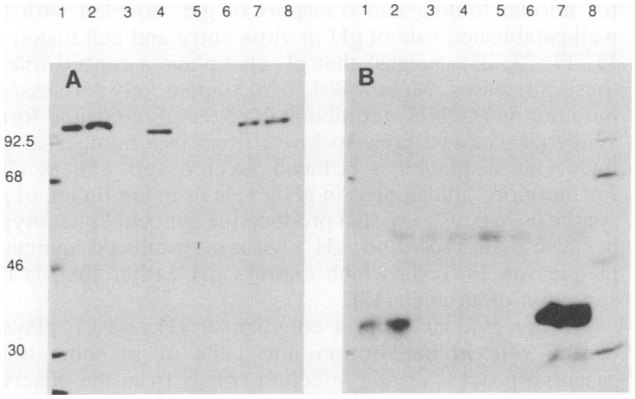

FIG. 10. Antigenic properties of gH expressed in the presence and absence of gL. Vero cells were infected as described in the legend to Fig. 9 with HSV-1 (lanes 1 and 2), Vac4b-gH (lanes 3 and 4), Vac4b-gL (lanes 5 and 6), and Vac4b-gH plus Vac4b-gL (lanes 7 and 8). Lysates were immunoprecipitated with MAb LP11 (lanes 1, 3, 5, and 7) or 52S (lanes 2, 4, 6, and 8), precipitated proteins were separated on 10% polyacrylamide gels, and the proteins were transferred to nitrocellulose. gH was detected on the membranes with rabbit anti-trpE-gH serum (A), and gL was detected with anti-UL1-2 serum (B), followed in both cases with ^{125}I-labelled protein A.

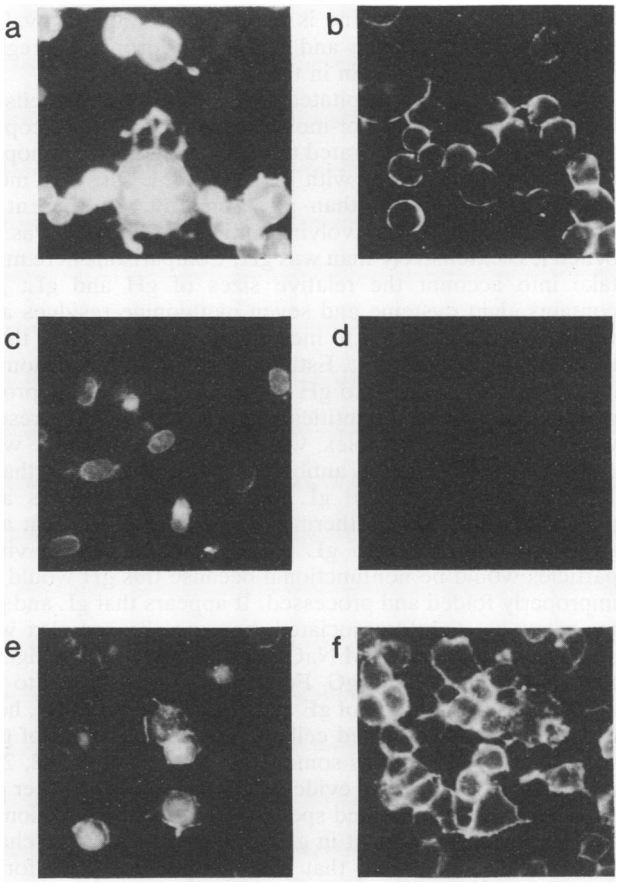

FIG. 11. Cell surface expression of gH requires gL. 143 cells were infected at a multiplicity of infection of 10 with HSV-1 (a and b), Vac4b-gH (c and d), or Vac4b-gH plus Vac4b-gL (e and f). After 16 h, the cells were fixed and the cells shown in panels a, c, and e were permeabilized. gH was detected by incubation with MAb 52S followed by fluorescein-conjugated rabbit anti-mouse IgG.

electrophoretic mobilities of gB synthesized in cells infected with HSV versus gB expressed by an adenovirus vector (53).

To examine the influence of gL on the structure and transport of gH, lysates of infected cells were immunoprecipitated with antibodies 52S and LP11, and the precipitation products were subjected to electrophoresis and transferred to nitrocellulose membranes. gH was detected on the membranes with anti-trpA-gH fusion protein serum, and gL was detected with anti-UL1-2 serum. Vac4b-gH synthesized a form of gH recognized by MAb 52S but not by MAb LP11, as previously reported (18), but coexpression of gH and gL results in a product that was recognized by both antibodies and which is electrophoretically indistinguishable from gH synthesized in HSV-infected cells (Fig. 10A). In addition, gL was immunoprecipitated in conjunction with gH by both anti-gH MAbs (Fig. 10B), demonstrating that the gH-gL complex can form in the absence of other HSV polypeptides. The cellular localization of gH was examined by immunofluorescence. In cells expressing gH alone, the distribution was exclusively intracellular and largely perinuclear, as reported previously (18), but coexpression of gL resulted in efficient cell surface expression (Fig. 11). These results confirm that gH and gL form a complex, that the processing of gL into the 40-kDa form is gH dependent, and that formation of the authentic antigenic structure of gH and its transport to the cell surface is gL dependent. In other immunofluorescence experiments, the cell surface expression of gL was found to depend on coexpression with gH, although a small fraction of gL did reach the cell surface in the absence of gH (results not shown).

DISCUSSION

A novel HSV-1 glycoprotein, encoded by the UL1 gene and designated gL, was detected in extracts from virus-infected cells by using antipeptide sera. Nucleotide sequence analysis of the UL1 gene had previously predicted a protein of 224 amino acids (25 kDa) with a putative signal sequence and a single site for attachment of N-linked oligosaccharides (40). Two different antipeptide sera immunoprecipitated a 30-kDa precursor form of the protein as well as a 40-kDa mature form of the protein. Both protein species were sensitive to endoglycosidase F, suggesting that both contain N-linked oligosaccharides. It would appear that gL behaves in a fashion analogous to the behavior of a number of other HSV glycoproteins, in which the faster migrating, immature protein species is converted to more slowly migrating, mature species during transport of the glycoprotein through the Golgi apparatus, frequently with the addition of O-linked oligosaccharides and the processing of N-linked oligosaccharides (33, 43).

In several experiments, we were unable to label gL with [^{35}S]methionine under conditions in which gL was heavily labelled with [^{35}S]cysteine (28). Since sequence analysis predicts that gL has only a single methionine at the amino terminus of the predicted signal sequence (40), it appears that the putative signal peptide may be removed. There are no other hydrophobic domains present in gL which could act as putative transmembrane domains, and this observation coupled with our inability to detect secreted forms of gL suggests that gL may be membrane associated by virtue of its interactions with gH. However, it is also possible that the

amino-terminal methionine is removed without removal of the entire signal peptide and that this hydrophobic region acts to anchor the protein in the membrane.

gL was immunoprecipitated from HSV-infected cells in conjunction with a higher-molecular-weight viral glycoprotein which was demonstrated to be gH. The gH immunoprecipitated in conjunction with gL by anti-gL sera was more intensely radiolabelled than gL. Similarly, gL present in immunoprecipitations involving anti-gH antibodies was labelled less extensively than was gH. Comparisons here must take into account the relative sizes of gH and gL; gH contains eight cysteine and seven methionine residues and would thus be expected to incorporate approximately three times more label than gL. Estimates of the relative amounts of label incorporated into gH and gL suggest that approximately equal molar quantities of gH and gL were present when the gH-gL complex was immunoprecipitated with either anti-gH or anti-gL antibodies. Thus, we expect that a 1:1 complex of gH and gL exists in infected cells and probably in virions. Furthermore, it would appear that any gH not associated with gL in infected cells or in virus particles would be nonfunctional because this gH would be improperly folded and processed. It appears that gL and gH are relatively tightly associated, because the complex was stable in washes with 2 M NaCl, 0.1% SDS, and 1 M $MgCl_2$. Previously, the HSV IgG Fc receptor was found to be composed of a complex of gE and gI (30, 31); however, here it was found that infected cells contained an excess of gE, which apparently retains some Fc receptor activity (2, 24). At this time, we have no evidence for an excess of either gH or gL in infected cells and speculate that a large fraction of both proteins are present in gH-gL complexes. Pulse-chase experiments suggested that the gH-gL complex forms shortly after synthesis of the proteins.

Expression of both gH and gL was required for the proper processing of both proteins, and the correct folding and cell surface expression of gH was entirely dependent on coexpression with gL. Furthermore, gL expression on the cell surface, evaluated by immunofluorescence experiments, was dependent on the expression of gH (results not shown), although a small fraction of gL reached the cell surface in the absence of gH, consistent with the observation that a minor fraction of gL was processed in the absence of gH.

The observation that gH and gL form a complex solves a long-standing problem involving gH, which when expressed in the absence of other HSV proteins is not properly folded, processed, and transported to the cell surface. A mammalian cell line expressing gH produced a form of gH which was not recognized by conformationally sensitive MAbs, and this protein was not processed and expressed on the cell surface (23). Infection of the cells with HSV led to normal folding, processing, and transport. Similar results were obtained when gH was expressed with a recombinant vaccinia virus vector (18). Together, these results suggested that other HSV proteins intervened during or after synthesis of gH to alter folding and transport of the polypeptide. Previous attempts to detect gH-associated proteins met with little success, partially because gL cannot be labelled with [^{35}S]methionine. The results reported here suggest a methodology for studying the functional and immunogenic properties of gH: gH and gL can be coexpressed by using transfection or virus vectors so that both proteins are properly folded, processed, and localized in the cells.

The role of gL in membrane fusion and virus entry is still by no means clear. However, the observations that gL forms a complex with gH and that complex formation is essential for normal folding and transport of gH, coupled with the well-established role of gH in virus entry and cell fusion (5, 15, 17, 22, 23), suggest that gL also plays a central role in these processes. Mutant 804, which apparently possesses a mutation in the NH_2-terminal half of the gL gene and forms syncytial plaques, grew to lower titers in a number of cell lines and displayed a reduced level of gB dimers (36). Furthermore, imbalances in gH levels or in the timing of gH synthesis in cells may also produce the syncytial phenotype, because wild-type and gH^- viruses produced syncytial plaques on F6 cells which express gH earlier than is observed in other cells (17).

Further evidence that a complex of gH and gL plays a central role in penetration into cells or in some other essential process during infection comes from the observation that genes homologous to HSV gH and gL are observed in most other well-studied herpesvirus families. Homologs of the HSV-1 gH and gL genes have been observed for varicella-zoster virus (11, 41), human cytomegalovirus (10, 52), and Epstein-Barr virus (1, 12, 41). The human cytomegalovirus gH homolog was not expressed on the cell surface in the absence of other human cytomegalovirus proteins (10, 52). Further, the Epstein-Barr virus homolog of gH, gp85, is improperly folded and transported when the protein is expressed in the absence of other Epstein-Barr virus proteins (54), and an Epstein-Barr virus gene (BKRF2) has been suggested as a positional homolog to the HSV-1 UL1 (1, 12, 41).

Recently, we replaced the HSV-1 UL1 gene with a *lacZ* gene construct using a cell line expressing gL to complement the mutant virus (49). The mutant virus is unable to replicate in the absence of gL, suggesting that, like gH, gL is essential for HSV replication. This result might have been expected, given that gL is required for proper folding and cell surface expression of gH, a protein which is essential for virus entry into cells and cell-to-cell spread of virus. Given the results reported here, it will be more appropriate in the future to consider gH and gL as a complex or hetero-oligomer.

ACKNOWLEDGMENTS

We thank Frank Graham and John Rudie, who facilitated the construction of AdgL, Andrew Davison for the *Kpn*I B fragment of HSV-1, and Roselyn Eisenberg and Gary Cohen for rabbit sera specific for gH. We are also indebted to Priscilla Schaffer for mutant 804 and Stan Person for mutant K082 and to Duncan McGeoch for communication of unpublished work.

The work in the United Kingdom was supported by the Medical Research Council and the Wellcome Trust and in Canada by the National Cancer Institute of Canada (NCIC) and the Medical Research Council (MRC). D.C.J. is a research scholar of the NCIC, and L.H. holds a studentship from the MRC.

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