A Mutant Herpes Simplex Virus Type 1 Unable To Express Glycoprotein L Cannot Enter Cells, and Its Particles Lack Glycoprotein H

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Herpes simplex virus type 1 (HSV-1) glycoprotein H (gH) is essential for virus entry into cells and forms a hetero-oligomer with a newly described viral glycoprotein, gL. Normal folding, posttranslational processing, and intracellular transport of both gH and gL depend upon the coexpression of gH and gL in cells infected with vaccinia virus vectors (L. Hutchinson, H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson, *J. Virol.* 66:2240–2250, 1992). Homologs of gH and gL have been found in herpesviruses of all subgroups, and thus it appears likely that the gH-gL complex serves a highly conserved function during herpesvirus penetration into cells. To examine the role of gL in the infectious cycle of HSV-1, a mutant HSV-1 unable to express gL was constructed by inserting a *lacZ* gene cassette into the coding sequences of the UL1 (gL) gene. Because gL was found to be essential for virus replication, cell lines capable of expressing gL were constructed to complement the virus mutant. In the absence of gL, virus particles were produced, and these particles reached the cell surface; however, gL-negative particles purified from infected cells were also deficient in gH. Mutant virions lacking gH and gL were able to adsorb onto cells but were unable to enter cells and initiate an infection. Further, the role of gL in fusion of infected cells was reexamined. A mutation in HSV-1 (804) which produces the syncytial phenotype had previously been mapped to a region of the HSV-1 genome which includes the UL1 gene and no other open reading frame. However, in contrast to this previous report, we found that the syncytial mutation in 804 affects the UL53 gene, which encodes gK, a gene commonly mutated in syncytial viruses.

Entry of herpesviruses into cells appears to be a complex process involving numerous viral components, principally envelope glycoproteins, and more than a single cell surface receptor. Herpes simplex viruses (HSV) adsorb onto cell surface glycosaminoglycans, predominately heparin sulfate proteoglycans (59, 66, 68), which are numerous components of the extracellular matrix and plasma membrane. Other herpesviruses, including human cytomegalovirus (HCMV) and pseudorabies virus, also adsorb onto cell surface heparin (40, 50, 52). Ensuing interactions between the viral envelope glycoproteins and other cell surface receptors occur, leading to fusion of the virion envelope with cellular membranes, which causes the viral nucleocapsid and tegument proteins to be delivered into the cytoplasm. The virion envelope of HSV contains at least nine membrane glycoproteins (reviewed in references 32 and 63), and five of these proteins (gB, gD, gH, gK, and gL) are necessary for virus entry into cultured cells (7, 16, 31, 43; this report). In addition, gC-negative HSV exhibit reduced levels of binding and infectivity (26). We have presented evidence that HSV gD promotes an interaction between virus and cell surface receptors which is critical for virus entry into cells (34, 35), and a candidate gD receptor protein has been described (4). The hypothesis that gD acts as a receptor-binding protein is further supported by observations that cells expressing gD are resistant to infection with HSV (8, 38).

Homologs of HSV gB and gH exist in herpesviruses of all subgroups (6, 9, 10, 25, 26, 47, 53), and the available information suggests that all members of the herpesvirus

family utilize gB and gH to enter cells. Studies with temperature-sensitive mutants, monoclonal antibody (MAb)-resistant mutants, syncytial mutants, and null mutants have suggested that HSV gB functions directly in membrane fusion events during virus entry (6a, 7, 14, 27, 58), although gB also appears to play a role in virus adsorption (26, 37, 42, 50). MAbs directed to gH neutralize HSV infectivity, inhibit penetration of virus into cells, block membrane fusion, and can inhibit cell-to-cell spread of virus (5, 18, 21). A temperature-sensitive mutant of HSV containing a substitution in gH produced extracellular virus particles which were devoid of gH and noninfectious (15). An HSV mutant in which the gH coding sequences were deleted was able to adsorb onto but could not enter cells and could block superinfection with wild-type HSV (16), in contrast to gD-negative viruses (35), suggesting that gH participates in virus entry but not as a receptor-binding protein.

Recently, a novel HSV glycoprotein, gL, encoded by the UL1 gene was described (29). Most or all of the gH in infected cells was found to be complexed with gL, and coexpression of gL with gH corrected defects in the folding, posttranslational processing, and cell surface transport of gH, which when expressed in the absence of other HSV proteins was aberrantly folded, processed, and transported (17, 22, 54a). Similarly, when gL was expressed without gH, the protein was not processed and did not reach the cell surface (29). These data suggested that the gH-gL hetero-oligomer should be considered a functional unit acting to mediate virus entry into cells. We tested this hypothesis and found that a gL-negative virus failed to incorporate gH into the virion envelope and was unable to enter cells, although the mutant could adsorb onto the cell surface.

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MATERIALS AND METHODS

Cells and viruses. Vero and human R-970 cells were grown in alpha minimal essential medium (alpha-MEM) (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 7% fetal calf serum (FCS). VL303, VL201, and VL206 cells capable of expressing gL were maintained in Dulbecco's modified Eagle medium lacking histidine (DMEM-His) supplemented with 0.3 to 0.5 mM histidinol (Sigma Chemical Co., St. Louis, Mo.) and 7% FCS and were passaged once in alpha-MEM containing 7% FCS before they were infected. HSV-1 wild-type strains F (obtained from P. G. Spear, University of Chicago, Chicago, Ill.) and KOS (obtained from J. Smiley, McMaster University, Hamilton, Ontario, Canada) and a syncytial mutant of HSV-1 KOS, 804 (obtained from P. Schaffer, Dana-Farber Institute, Boston, Mass.), and HSV-2 333 (obtained from P. G. Spear, University of Chicago) were propagated and titered on Vero cells. The recombinant virus KOS-gL β was propagated on VL303 cells.

Plasmids. Plasmid pSG-1 (generously provided by M. Levine, University of Michigan) contains the *EcoRI* JK fragment of HSV-1 KOS inserted into pBR325 (19). Plasmid pUL1 was constructed by cutting pSG-1 with *EcoRV* and ligating a 3.4-kb *EcoRV* fragment (containing the UL1 open reading frame) into the unique *EcoRV* site of plasmid pBR322. Plasmid pD6p (a generous gift of S. Weller, University of Connecticut) contains an ICP6:*lacZ* cassette bounded by *Bam*HI sites which has been used to insertionally inactivate HSV genes (20). Plasmid pUL1 β was constructed by blunt-end ligation of a *Bgl*II oligonucleotide (5'-AGATCT-3', purchased from The Institute for Molecular Biology and Biotechnology, McMaster University) into the unique *Hpa*I site of pUL1 followed by ligation of the 4.3-kb *Bam*HI ICP6:*lacZ* cassette from pD6p into this unique *Bgl*II site. pSV2HIS containing the *Salmonella typhimurium* histidinol dehydrogenase gene under control of the simian virus 40 early promoter and flanked by simian virus 40 polyadenylation sequences was provided by R. A. Weinberg, Whitehead Institute, Cambridge, Mass. (24). Plasmid pSV2HIS-UL1 was constructed by restriction digestion of pUL1 with *EcoRI* and *Bam*HI; the 3.7-kb *EcoRI*-*Bam*HI fragment containing the UL1 gene was then inserted into the *EcoRI*-*Bam*HI region of pSV2HIS. Plasmid pSV2HIS-syn contains a 3.4-kb *EcoRI*-*Bam*HI fragment including the HSV-1 UL53 gene derived from pSG-28 (19) and inserted into pSV2HIS (31). Plasmids pUL1-804 and pUL53-804 were constructed by ligating *Bam*HI fragments derived from HSV-1 KOS (804) (44) into *Bam*HI-digested pUC19. Colony hybridizations (45) were used to identify appropriate bacteria, using oligonucleotide AB1427 (5'-GCC CGA CCC AAC CCA AAA TCC CCA T-3'), which hybridizes at the 5' end of the UL1 open reading frame, or using oligonucleotide AB924 (5'-GCT GCA GGG AAC GGA CGG CGA GCA T-3'), which hybridizes at the 5' end of the UL53 open reading frame (both purchased from The Institute for Molecular Biology and Biotechnology, McMaster University). Plasmid pKBL-BR contains an *Nru*I-*Bam*HI fragment of HSV-1 KOS containing the UL53 gene (map coordinates 0.732 to 0.745) subcloned into pBR322 (3), and plasmid pS20BL-NB contains the equivalent *Nru*I-*Bam*HI fragment derived from syn20 cloned into *Sma*I-*Bam*HI-digested pUC9 (3); both plasmids were generously provided by Tom Holland (Wayne State University, Detroit, Mich.).

Synthetic peptides, antipeptide sera, and other antibodies. Two synthetic peptides, UL1-1 (YVIRSRVAREVGDIK

VPC) and UL1-2 (CATKSRRRRPHSRRL), were synthesized and purified by Bachem Inc. (Torrance, Calif.). Rabbit antisera to these peptides were produced as described previously (29). MAbs LP11 and 52S, directed to HSV gH (5, 60), and LP2, which reacts with HSV gD (51), were kindly provided by A. C. Minson (Cambridge, United Kingdom). An anti-gH polyclonal rabbit serum specific for gH, which had been purified by immunoaffinity chromatography, was a gift of R. Eisenberg and G. Cohen (University of Pennsylvania, Philadelphia). A rabbit antiserum directed to HSV thymidine kinase (TK) produced in *Escherichia coli* (anti-TK) was a generous gift from W. Summers (Yale University, New Haven, Conn.). A rabbit antiserum directed to the large subunit of ribonucleotide reductase (anti-ICP6) (28) was kindly provided by S. Bacchetti (McMaster University).

Construction of VL303, VL201, and VL206 cells capable of expressing gL. Subconfluent monolayers (60-mm-diameter dishes) of Vero cells were transfected with 10 μ g of pSV2HIS-UL1 by using the CaPO_4 technique (23) followed by a glycerol shock as described previously (43). After 2 days, the cell monolayers were confluent, and the cells were trypsinized and plated in 100-mm-diameter dishes with DMEM-His supplemented with 0.2 mM histidinol and 7% FCS. After 15 days, isolated colonies of cells were trypsinized by using cloning cylinders, and the cells were placed in 24-well dishes containing DMEM-His supplemented with 0.15 to 0.3 mM histidinol and 7% FCS. Transformants were screened for production of gL by infecting the cells with HSV-2 333, radiolabeling the cells with [^{35}S]methionine and [^{35}S]cysteine, and immunoprecipitating HSV-1 gL by using anti-UL1-2 serum, which does not react with HSV-2 gL (29). VL303, VL201, and VL206 were chosen from 34 transformants, 6 of which produced gL at levels comparable to those observed in cells infected with HSV-1.

Construction of a gL-negative HSV-1. Two cell lines capable of expressing gL (VL201 and VL303) were cotransfected with infectious HSV-1 KOS DNA obtained by extracting cytoplasmic DNA from virus-infected cells (61) and plasmid pUL1 β DNA, using the CaPO_4 method (23) followed by a glycerol shock (43). Viral cytopathic effect was observed after 2 days, and the monolayers were harvested after 4 days. Recombinant viruses which expressed β -galactosidase were plaque purified by using VL303 or VL201 cells. Cells were infected with viruses and 48 h later overlaid with alpha-MEM containing 3% FCS, 0.5% agarose, and 300 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Boehringer Mannheim Canada Ltd., Dorval, Quebec, Canada) per ml. Blue plaques appeared after 8 to 24 h, and these viruses were selected and propagated in 24-well dishes of VL303 or VL201 cells as previously described (43). Viral DNAs extracted from infected cells (61) were analyzed by Southern blot analysis (45), using end-labeled oligonucleotide AB1427 as a probe.

Radiolabeling of viral proteins in infected cells, immunoprecipitation, and gel electrophoresis. R-970 cells, Vero cells, or Vero cells which had been transfected with pSV2HIS-UL1 were infected with HSV-1 or HSV-2, using 10 or 20 PFU per cell. At 6 to 7 h after infection, the cells were washed twice with medium 199 lacking methionine and cysteine and supplemented with 1% FCS (labeling medium). The cells were then incubated for 5 h with labeling medium containing 100 μ Ci of both [^{35}S]cysteine and [^{35}S]methionine per ml. Cell extracts were made 5 or 6 h later, using NP-40/DOC buffer (1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate [DOC], 50 mM Tris HCl [pH 7.5], 100 mM NaCl) containing 2 mg of bovine serum albumin (BSA) per ml and 1 mM

phenylmethylsulfonyl fluoride and stored overnight at -70°C . The extracts were then thawed, clarified by ultracentrifugation, and in some instances precleared by incubation with rabbit sera (directed to gE and gI) and protein A-Sepharose (Pharmacia Chemicals, Dorval, Quebec, Canada). The precleared extracts were mixed with mouse ascites fluid or rabbit sera and protein A-Sepharose as described previously (35). Immunoprecipitates were washed either three times with NP-40/DOC buffer or with more stringent washes (29). Electrophoresis involved 10 or 12% polyacrylamide gels as described elsewhere (35). The gels were impregnated with En^3Hance (Dupont, Montreal, Quebec, Canada) and exposed at -70°C to XAR film (Eastman Kodak Co., Rochester, N.Y.).

Purification of radiolabeled virions and immunoprecipitation of viral proteins from radiolabeled virions. Virions were purified from Vero or VL303 cytoplasmic extracts by using Dextran T-10 (Pharmacia) gradients as described previously (37, 64) except that the cells were infected with a multiplicity of infection of 5, and some dishes of cells were labeled from 3 to 24 h postinfection in medium 199 lacking cysteine and containing 2% FCS and 50 μCi of [^{35}S]cysteine per ml. Cell extracts were centrifuged at low speed to remove nuclei and then loaded onto dextran gradients (approximately 1×10^8 to 3×10^8 cells per gradient). The gradients were centrifuged at 20,000 rpm with a Beckman SW28 rotor at 4°C for 1 h; then each gradient was fractionated into approximately 35 1-ml aliquots, and fractions were counted in a liquid scintillation counter. Fractions containing labeled virions were pooled and diluted 10-fold with 1 mM phosphate buffer (pH 7.4), and the virus was pelleted at $50,000 \times g$ for 2 h. For immunoprecipitation of viral glycoproteins, pelleted virus was resuspended in NP-40/DOC containing 2 mg of BSA per ml and 1 mM phenylmethylsulfonyl fluoride, sonicated briefly, and clarified by ultracentrifugation. The extracts were immunoprecipitated as described above.

Adsorption of radiolabeled virions onto cells. ^{35}S -labeled virions were resuspended in alpha-MEM supplemented with 2% FCS so that there was approximately 10^6 cpm/ml, and the virus suspension was sonicated briefly at 4°C . Replicate cultures of R-970 cells (or Vero cells) in 24-well dishes were exposed to 150 μl of radiolabeled virus; after various times, the medium containing unadsorbed virus was removed, the cells were washed three times with alpha-MEM supplemented with 2% FCS, and the cells were extracted with 50 mM Tris HCl (pH 7.5)–100 mM NaCl containing 1% NP-40. The unadsorbed virus and virus bound to cells was quantitated by drying material on glass fiber filters and counting the filters in a liquid scintillation counter.

Electron microscopy. Monolayers of Vero or VL303 cells were infected with KOS or KOS-gL β for 20 h, washed with phosphate-buffered saline (containing 1 mM CaCl_2 and 1 mM MgCl_2), and fixed for 30 min at room temperature with 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde. The cells were scraped from the dishes, collected by low-speed centrifugation, and resuspended in cacodylate buffer containing 2% glutaraldehyde. The cells were then pelleted in an Eppendorf tube and processed for electron microscopy (36).

Marker transfer experiments. Vero cells were cotransfected by the CaPO_4 technique (23, 43) with infectious viral DNA derived from wild-type HSV-1 KOS or syncytial mutant 804 and plasmids containing the UL1 derived from either HSV-1 KOS (pUL1) or HSV-1 804 (pUL1-804) or the UL53 gene from HSV-1 KOS (pSV2-HIS-Syn or pKBL-BR), HSV-1 804 (pUL53-804), or HSV-1 syn20 (p20BL-NB).

Preparations of viruses were harvested 3 days after these transfections, sonicated, and plated on 150-cm 2 dishes of Vero cells. Two days after infection, the Vero cell monolayers were fixed with ethanol and glutaraldehyde, stained with crystal violet, and photographed by using an inverted microscope.

RESULTS

Construction of a mutant HSV-1, KOS-gL β , unable to express gL and cells which complement this mutant. To attempt to isolate an HSV-1 mutant in which the UL1 (gL) gene was insertionally inactivated, we constructed plasmid pUL1 β , in which an ICP6:*lacZ* gene cassette, from plasmid pD6p (20), was inserted at a unique *HpaI* site, 279 nucleotides from the start of the UL1 open reading frame (Fig. 1) (46). The insert in UL1 was designed so as not to affect the UL2 open reading frame and its adjacent promoter sequences. Further, this insertion was well downstream of the ICP0 coding sequences and polyadenylation site. Attempts to transfer this *lacZ* marker into infectious HSV-1 were unsuccessful. Viruses staining blue under X-Gal overlays were observed, but efforts to purify such mutant viruses from wild-type viruses were unsuccessful.

From these results, we suspected that the UL1 gene might be essential for virus replication and directed our efforts toward constructing cell lines able to express gL in order to complement gL-negative viruses. We constructed plasmid pSV2HIS-UL1, in which an *EcoRI-BamHI* fragment of HSV-1 KOS, containing the UL1 gene, was inserted into plasmid pSV2HIS, which contains the histidinol dehydrogenase gene coupled to the simian virus 40 promoter (24). Vero cells were transfected with pSV2HIS-UL1, and transformants were selected in medium containing histidinol and lacking histidine. Cells containing the pSV2HIS sequences can metabolize histidinol (toxic to cells) to histidine (an essential amino acid). Transformants were screened for expression of gL by infecting the cells with HSV-2 (to induce expression of the HSV-1 gL gene), and then the HSV-1 gL was immunoprecipitated with peptide serum anti-UL1-2, which reacts with HSV-1 but not HSV-2 gL (29). Cell lines VL102, VL206, and VL303 produced gL at levels comparable to those observed in HSV-1-infected Vero and R-970 cells (Fig. 2). In the lanes involving HSV-1-infected R-970 and Vero cells, anti-UL1-2 serum was reacted with cell extracts in the presence or absence of UL1-2 peptide, and as in previous experiments with this serum (29) and other rabbit sera (32), the HSV-1 immunoglobulin G (IgG) Fc receptor proteins gE and gI (60 to 90 kDa) were major products of the immunoprecipitation reactions. The immature form of HSV-1 gL, pgL, predominated in the HSV-2-infected VL201, VL206, and VL303 cells (Fig. 2b). This observation may reflect preferential association of HSV-2 gH and HSV-2 gL, leaving HSV-1 gL in the immature form because it is less efficiently associated with the HSV-2 gH present in these cells. However, we also observed that anti-UL1-2 serum preferentially immunoprecipitated the immature form of gL from cells infected with certain HSV-1 strains, e.g., HSV-1 strain F (Fig. 2a).

To construct a gL-negative HSV-1 mutant, VL201 and VL303 cell monolayers were transfected with pUL1 β and infectious HSV-1 KOS DNA. Recombinant viruses were selected by overlaying monolayers infected with viruses derived from such transfections with agarose containing X-Gal and selecting blue plaques. An HSV-1 mutant, B1B, derived from VL303 cells was judged to be free of wild-type

FIG. 1. Schematic representations of the HSV-1 genome and of the ICP6:*lacZ* cassette insertion in the UL1 gene. The positions of genes encoding glycoproteins gL, gH, gB, gK, and gD (which are essential for virus replication in cultured cells) as well as the UL1, UL24, UL27 (gB), and UL53 (involved in the syncytial phenotype) genes are indicated. A deletion in the UL20 gene also gives rise to the syncytial phenotype (2). The direction of transcription of the syncytial genes is indicated by arrows. The region encompassing the *EcoRI* J fragment was enlarged so as to indicate position of the ICP6:*lacZ* cassette which was inserted into the UL1 gene at a *HpaI* site (located 279 nucleotides into the UL1 open reading frame). Brackets around the *BamHI* sites indicate that these sites were lost when the ICP6:*lacZ* cassette was cloned into the *HpaI* site in UL1.

viruses because only blue plaques were produced on VL303 cells; this virus was subjected to two further rounds of plaque purification and then designated B1B11. A second recombinant virus was produced after transfection of VL201 cells and, at a stage when this virus produced approximately equal numbers of blue and white plaques, was designated D1. Following subsequent rounds of purification in which only blue plaques were produced, the mutant virus was designated D1B. Southern blot analysis in which viral DNAs were probed with an oligonucleotide, AB1427, capable of hybridizing with the first 25 nucleotides of the UL1 open reading frame was performed. The presence of 13.3-kb *BamHI* and 4.2-kb *EcoRV* fragments and the absence of 8.9-kb *BamHI* and 3.4-kb *EcoRV* fragments indicated that B1B and B1B11 contained only the interrupted form of the UL1 gene (Fig. 3). Minor 7.5-kb *BamHI* and 3.8-kb *EcoRV* fragments observed in B1B and B1B11 DNA preparations were probably due to cross-hybridization or incomplete digestion. As expected, the D1 isolate showed a mixture of wild-type and mutant fragments, and after further plaque purification, the D1B isolate contained only the mutant fragments. Both B1B11 and D1B virus isolates failed to produce any detectable plaques on Vero and human R-970 cells, suggesting that the UL1 gene was essential for replication in these cells. The B1B11 virus isolate was designated KOS-gL β and used in all subsequent studies. KOS-gL β stocks were routinely titered on VL303 or VL201 cells as well as on Vero cells, in which wild-type viruses will produce plaques; these stocks routinely produced titers of 0.5×10^9 to 2×10^9 PFU/ml on the complementing cells, whereas no more than 1×10^2 to 2×10^2 PFU/ml were detected on Vero cells. However, there is the potential for increases in the proportion of wild-type viruses to mutant viruses in KOS-gL β stocks in part as a result of recombination events between viral and cellular copies of the gL gene.

Virus mutant KOS-gL β does not produce glycoprotein gL, and gH is not recognized by a gH-specific MAb in the absence

of gL. To verify the disruption of the UL1 gene in KOS-gL β , Vero or VL303 cells were infected with KOS-gL β or wild-type KOS and radiolabeled with [35 S]methionine and [35 S]cysteine, and gL was immunoprecipitated with two anti-peptide sera, UL1-1, which recognizes residues 26 to 44 of gL, and UL1-2, which recognizes the carboxyl terminus of gL (29). Two forms of gL, the mature 40-kDa form and the immature 30-kDa form, pgL, were both precipitated from extracts of KOS-infected Vero cells or KOS-gL β -infected VL303 cells by using both anti-peptide sera and rabbit anti-gH serum and the gH-specific MAb LP11 (Fig. 4). As noted previously (29), anti-UL1-1 serum inefficiently precipitates the mature form of gL. In contrast, gL was not observed in extracts from KOS-gL β -infected Vero cells by using either of two anti-gL anti-peptide sera or anti-gH polyclonal antibodies (Fig. 4). Of special interest was the observation that gH produced in KOS-gL β -infected Vero cells, i.e., in the absence of gL, was not recognized by MAb LP11, which recognizes a conformationally dependent epitope in gH (5, 22), or could conceivably recognize a hybrid epitope composed of residues contributed from gL as well as from gH. This result further supports the conclusion that gH and gL are dependent upon one another for proper folding, processing, and transport to the cell surface.

A protein of approximately 28 kDa was detected in KOS-gL β extracts by using anti-UL1-1 serum, and this protein was also inefficiently precipitated by anti-gH serum (Fig. 4) and in other experiments (not shown) using a polyclonal serum directed to a TrpE-gH fusion protein (15). This 28-kDa protein was not precipitated by anti-UL1-1 serum preincubated with UL1-1 peptide, suggesting that the protein was antigenically related to gL and the 28-kDa protein was not recognized by anti-UL1-2 serum. Analysis of the DNA sequences at the insertion of the ICP6:*lacZ* gene cassette in plasmid pUL1 β suggested that an in-frame fusion had occurred between the UL1 gene and the UL38 gene, which was present at the 5' end of the ICP6/*lacZ* cassette. This fusion

FIG. 2. Expression of HSV-1 gL in Vero cell transformants after infection with HSV-2. Human R-970 cells or VL201, VL206, and VL303 cells were infected with HSV-1 strain F or KOS or with HSV-2 strain 333, as indicated at the bottom. After 6 h, the cells were labeled with [35 S]cysteine and [35 S]methionine for 6 h. HSV-1 gL was immunoprecipitated from cell extracts by using rabbit anti-UL1-2 serum, which recognizes HSV-1 gL but not HSV-2 gL (29). Anti-UL1-2 antibodies were preincubated with buffer (-) or with UL1-2 peptide (+) before being mixed with R-970 cell extracts. Sizes are indicated in kilodaltons.

protein would be composed of 94 amino-terminal residues of gL fused to 162 residues at the carboxyl terminus of the UL38 open reading frame and would therefore contain the UL1-1 peptide but not the UL1-2 peptide. A rabbit anti-serum directed to a TrpE fusion protein containing the carboxyl terminus of the HSV-2 UL38 gene product (70) also precipitated the 28-kDa protein (results not shown), confirming that UL38 protein sequences were present in this protein. The observation that anti-gH polyclonal serum precipitated a fraction of the 28-kDa fusion protein from KOS-gL β -infected Vero or VL303 cells (Fig. 4) indicates that the first 94 amino acids of gL are sufficient to allow complex formation between the fusion protein and gH, albeit inefficiently. However, MAb LP11, which recognizes a conformationally dependent epitope in gH, did not precipitate gH from KOS-gL β -infected Vero cells, indicating that the 94 residues at the amino terminus of gL were not sufficient to produce the LP11 epitope.

Production of virus particles in the absence of gL. When Vero or R-970 cells were infected with stocks of KOS-gL β that had been derived from VL303 cells, the cells showed cytopathic effects identical to those produced by wild-type KOS, suggesting that HSV replication progresses in the absence of gL. To address the question of whether virions were formed in the absence of gL, two types of experiments were performed. Initially, virus particles were examined in electron micrographs of infected cells. We found that KOS-gL β (derived from complementing cells) could infect Vero cells and produce virus particles which appeared qualitatively and quantitatively identical to particles produced by KOS-gL β in VL303 cells or by wild-type KOS in Vero cells (Fig. 5). At magnifications higher than those shown in Fig. 5,

we could not discern alterations in the envelope of virions lacking gL. In addition, particles lacking gL were distributed throughout the cytoplasm and on the cell surface in a fashion similar to that found for cells infected with KOS. In other experiments, the production of virus particles was quantitated by purifying radiolabeled virus particles from infected cells with use of dextran gradients. Similar quantities of virus particles was produced by KOS-gL β in Vero cells and VL303 cells (Fig. 6) and in other experiments by wild-type KOS in Vero cells (results not shown).

Virus particles lacking gL adsorb onto the cell surface but do not enter cells. To evaluate the ability of gL-negative virions to adsorb onto cells, radiolabeled virions were purified and incubated with R-970 cells growing in 24-well dishes. KOS-gL β particles derived from Vero cells (lacking gL) adsorbed onto cells as efficiently as did KOS-gL β derived from VL303 cells (containing gL) (Fig. 7). Similar results were obtained in binding experiments using Vero cells (results not shown). These experiments demonstrate that gL is not required for adsorption of HSV-1 onto the cell surface.

To examine whether HSV-1 lacking gL could enter cells and initiate an infection, Vero cells were infected with KOS-gL β derived from Vero or VL303 cells or with KOS from Vero cells, and then early viral proteins were quantitated. Infected cells were radiolabeled with [35 S]cysteine and [35 S]methionine, and ICP6 (an immediate-early protein), TK (an early protein), or gD (a delayed-early protein) was immunoprecipitated from cell extracts. Virions lacking gL (KOS-gL β derived from Vero cells) were unable to induce the synthesis of early HSV-1 proteins (Fig. 8), suggesting that they were unable to enter cells.

Glycoprotein gL is part of the virion envelope, and gH is not present in purified virus particles lacking gL. Previous work established that gL forms a complex with gH in membranes of infected cells, although no evidence was presented as to whether gL was present in the virion envelope (29). To determine whether gL was present in virions and whether gH would be incorporated into virions in the absence of gL, we purified [³⁵S]cysteine-labeled KOS or KOS-gL β virions from Vero cells and immunoprecipitated gH or gL. A complex of gL and gH was readily detected in purified KOS virions with use of either anti-gL (UL1-2) or anti-gH (52S) antibodies (Fig. 9). In contrast, KOS-gL β virions derived from Vero cells did not contain detectable levels of gL or gH. The levels of gD and the IgG Fc receptor proteins gE and gI precipitated by rabbit antibodies were comparable, and TK was not present in the purified virion preparations. Previous results have suggested that TK is not a component of the virion (64, 65). In Fig. 9b, a protein which was apparently gD appeared in one of the 52S lanes; this was an isolated observation and appears to have originated from overspill of the neighboring (LP2) lane. In other experiments, we confirmed the absence of gH in KOS-gL β particles derived from Vero cells by using a polyclonal anti-gH serum and found that KOS-gL β particles derived from VL303 cells contained a normal complement of both gH and gL (results not shown). Therefore, these experiments demonstrated that gL is a component of the virion envelope and that gL must be present in infected cells in order for gH to be incorporated into virions or to stabilize gH present in the virion envelope.

HSV-1 mutant 804 contains a mutation in the UL53 gene rather than in the UL1 gene. Previously, a syncytial mutation in mutant 804 was mapped to the *Hpa*I fragment O of the unique long (UL) component of HSV-1, using gel-purified DNA fragments (44). On the basis of this observation and DNA sequence analysis of this region, it was proposed that the syncytial mutation in 804 altered the UL1 gene (46). More recently, we found that 804 expresses gL at normal levels (29), and in this study we observed that KOS-gL β does not produce syncytial plaques. These results, coupled with observations that most HSV-1 syncytial mutants possess mutations in the UL53 gene (3, 27a), prompted a reinvestigation of syncytial mutation in 804. Initially, we performed a marker rescue experiment in which Vero cells were cotransfected with DNA derived from mutant 804 and plasmids containing either the wild-type UL1 gene or the wild-type UL53 gene. Viruses derived from such transfections were plated on Vero cells, and the frequency of wild-type plaques was scored. In these transfections, it was often difficult to detect wild-type (nonsyncytial) plaques against a background of syncytial plaques, which outnumbered the wild-type plaques, and in some cases it was difficult to distinguish plaques produced by slowly growing syncytial viruses from plaques produced by nonsyncytial viruses. However, no wild-type plaques were detected in 3,616 syncytial plaques produced after cotransfection of 804 DNA and a plasmid containing the wild-type UL1 gene (Table 1, experiment 1). In contrast, 26 nonsyncytial plaques were observed among a total of 4,840 plaques after cotransfection of 804 DNA and a plasmid containing the wild-type UL53 gene.

To extend these observations, the UL1 and UL53 genes from mutant 804 were cloned, and these plasmids (pUL1-804 and pUL53-804) were cotransfected with wild-type KOS DNA. In this experiment, syncytial plaques were easily distinguished on a background of nonsyncytial plaques. No

FIG. 3. Southern blot analysis of mutant viral DNA. Viral DNAs were extracted from VL303 or VL201 cells infected with mutant viruses or Vero cells infected with wild-type HSV-1 KOS and then digested with *Bam*HI (upper blots) or *Eco*RV (lower blots), subjected to electrophoresis through a 1% agarose gel, transferred to nitrocellulose, and hybridized with ³²P-labeled UL1 oligonucleotide AB1427, which is antisense to the UL1 message and hybridizes with the first 25 nucleotides of the UL1 open reading frame. Lanes: KOS, wild-type HSV-1; D1, a mutant purified after transfection of VL201 cells and at an early stage of plaque purification so that there is some contamination with KOS; D1B, a mutant virus purified from D1 and no longer contaminated with KOS; B1B, a mutant virus purified on VL303 cells; B1B11, a mutant virus further purified from B1B on VL303 cells; pUL1, a plasmid in which an *Eco*RV fragment containing the UL1 and UL2 genes from KOS was inserted into the unique *Eco*RV site of pBR322; pUL1 β , plasmid in which the ICP6:*lacZ* cassette is inserted into a *Hpa*I site in the UL1 gene of pUL1 (see Fig. 1). The *Bam*HI digest of pUL1 yields a 7.7-kb fragment because *Bam*HI sites are present in pBR322 sequences flanking the KOS *Eco*RV fragment, and the *Bam*HI digest of pUL1 β yields a 12-kb fragment because the *Bam*HI sites flanking the ICP6:*lacZ* cassette were lost during ligation into the *Hpa*I site in UL1. The 8.9-kb KOS *Bam*HI fragment is derived by cuts in the ICP0 gene and 3' of the UL2 gene, and the 13.3-kb KOS-gL β *Bam*HI fragment reflects the insertion of the ICP6:*lacZ* cassette.

syncytial plaques were observed when plasmids containing either the 804 UL1 gene or the wild-type UL53 gene were transfected, whereas either the 804 UL53 gene or the UL53 gene derived from syn20 (a virus containing a mutation in UL53 gene) produced syncytial plaques (Table 1, experiment 2). Figure 10 shows the morphology of syncytial plaques produced after transfection with the 804 UL53 gene (panel 4) or with the UL53 gene of syn20 (panel 2). Wild-type, nonsyncytial plaques obtained when KOS was trans-

FIG. 4. Expression of gL in cells infected with KOS-gL β . Vero and VL303 cells were infected with KOS or KOS-gL β and labeled with [35 S]cysteine and [35 S]methionine for 6 h beginning 6 h after infection. Cell extracts from these cells were precleared with rabbit anti-gE/gI serum and protein A-Sepharose and then mixed with anti-UL1-1 serum (–), anti-UL1-1 serum preincubated with UL1-1 peptide (+), anti-UL1-2 serum (–), and anti-UL1-2 serum preincubated with UL1-2 peptide (+), anti-gH rabbit serum, MAb LP11 specific for gH, or MAb LP2 specific for gD. The antigen-antibody complexes were precipitated with protein A-Sepharose and washed under stringent conditions (see Materials and Methods). The precipitated proteins were subjected to electrophoresis on 12% polyacrylamide gels. The positions of pgL, gL, gD, gH, the IgG Fc receptor proteins gE and gI, and molecular mass markers of 200, 92, 69, 46, and 30 kDa are indicated.

ected with the 804 UL1 gene or the wild-type UL53 gene are shown in Fig. 10, panels 1 and 3.

In other experiments, we have shown that cell lines expressing gK, the UL53 gene product, can suppress fusion induced by HSV-1 mutants with syncytial mutations in the UL53 gene (30). In these experiments, mutant 804 produced wild-type, nonsyncytial plaques on a cell line, gK9, which expresses gK, whereas syncytial plaques were produced on VL303 cells, which express gL. Together, these results demonstrate that mutant 804 possesses a mutation in the UL53 gene rather than in the UL1 gene.

DISCUSSION

Recent observations that the essential HSV-1 glycoprotein, gH, forms a complex with the UL1 gene product, gL, and that the normal folding, processing, and cell surface expression of gH required the coexpression of gL (29) suggested that gL might be essential for replication of HSV-1. In this study, we found that viruses lacking gL were unable to infect monkey Vero cells and human R-970 cells, and, given the critical role that the gH-gL complex plays during virus entry into the cultured cells, we suspect that gH- and gL-negative viruses will be unable to enter most mammalian cells. Viral particles were produced at normal levels in KOS-gL β -infected Vero cells (in which gL was not expressed), and these particles appeared identical to wild-type KOS virions. In addition, gL-negative virions were distributed in membrane vesicles throughout the cytoplasm and on the cell surface as with wild-type HSV-1. However,

gL-negative virions lacked the normal complement of gH, and although gH was produced in cells infected with KOS-gL β , the protein was not properly folded, as judged by the inability of MAb LP11 to recognize this gH. These observations add further support to the hypothesis that gH and gL act as a functional complex or hetero-oligomer and that the two proteins are dependent upon one another for the inter-related processes of folding, processing, intracellular transport, and incorporation in virions. It could be suggested that gL acts as a chaperone, facilitating folding and cell surface transport of gH before dissociating from gH. We have been unable to detect a substantial fraction of gH which is not associated with gL in infected cells (29) and in virions (Fig. 9); therefore, we prefer a model in which gH and gL are intimately complexed in cellular membranes, both shortly after synthesis and after transport to the cell surface, and in the virion envelope. This model could be analogous to that of the major histocompatibility complex class I proteins, in which the heavy chain and β_2 -microglobulin proteins associate, in conjunction with peptide, and are transported to the cell surface. Certain major histocompatibility complex class I heavy-chain molecules are not efficiently transported to the cell surface in the absence of β_2 -microglobulin (67a).

In studies of HSV-1 mutant tsQ26, which expresses a temperature-sensitive gH, Desai et al. (15) observed intracellular virus particles which had incorporated gH and were infectious, whereas secreted virions were devoid of gH and noninfectious. They suggested that mutant gH might be unstable and proteolytically degraded or, alternatively, that mutant gH is unable to reach cytoplasmic membranes,

FIG. 5. Electron micrographs of cells infected with KOS-gL β . Monolayers of Vero or VL303 cells were infected with either wild-type KOS or KOS-gL β (10 PFU per cell), incubated at 37°C for 20 h, fixed with 2% glutaraldehyde, and processed for electron microscopy. (1A and 1B) Vero cells infected with KOS; (2A and 2B) Vero cells infected with KOS-gL β ; (3A and 3B) VL303 cells infected with KOS-gL β . Note that panels 1B, 2B, and 3B show virus particles that have reached the cell surface. Bars: 500 nm in panels 1A, 2A, and 3A; 100 nm in panels 1B, 2B, and 3B.

FIG. 6. Gradient purification of radiolabeled virus particles. Vero cells or VL303 cells were infected with KOS-gL β , labeled with [35 S]cysteine at 3 h postinfection, and harvested at 24 h postinfection. The infected cells were homogenized, nuclei were removed by centrifugation, and cell extracts were loaded onto dextran T-10 gradients. After a 1-h centrifugation, the gradients were fractionated and the radioactivity in each fraction was quantitated in a liquid scintillation counter. Radioactivity at the top of the gradient was not quantitated. Symbols: Δ , Vero cells infected with KOS-gL β ; $\bullet$, VL303 cells infected with KOS-gL β .

where, one model of herpesvirus egress suggests (39, 67), nucleocapsids become reenvoloped. In the absence of gL, we were unable to detect intracellular virions containing gH, perhaps because gH does not fold properly in the absence of gL and may aggregate rather than become incorporated into virions. Alternatively, gH may be rapidly degraded after incorporation into the virion envelope. It is possible that there are low levels of gH in preparations of purified virus; however, the difficulties associated with purifying radiola-

FIG. 7. Adsorption of purified virus particles lacking gL to cells. [35 S]cysteine-labeled KOS-gL β virus particles lacking gL or containing gL were purified from Vero cells or VL303 cells and incubated with human R-970 cells for various times. The cells were washed, and radiolabel associated with the cell monolayer or left in the culture supernatant was quantitated. Symbols: $\square$, Vero cells infected with KOS-gL β ; $\circ$, VL303 cells infected with KOS-gL β . Results are averages from an experiment done in triplicate; error bars represent 1 standard deviation.

FIG. 8. Expression of early HSV proteins in cells infected with mutant KOS-gL β . Vero cells were infected with wild-type KOS produced on Vero cells, KOS-gL β produced on Vero cells (lacking gL), or KOS-gL β produced on VL303 cells (containing gL). The cells were labeled with [35 S]cysteine and [35 S]methionine from 2.5 to 5.5 h after infection, and cell extracts were mixed with anti-ICP6 rabbit serum, anti-TK rabbit serum, or MAb LP2, specific for gD. Antigen-antibody complexes were precipitated with protein A-Sepharose and washed under stringent conditions; then the precipitated proteins were electrophoresed on a 12% polyacrylamide gel. Molecular mass standards (in kilodaltons) are indicated on the right; positions of ICP6, gD, and TK are indicated on the left.

beled virions from disrupted cells have prevented us from more closely examining this point.

Previous work demonstrated that the amino-terminal methionine of gL was removed, supporting the hypothesis that the protein is cleaved by a signal peptidase (29). Because no other hydrophobic domains capable of acting as a transmembrane domain are present in gL and charged residues near the signal sequence might preclude its use as a transmembrane domain (46), we suggested that gL might be tethered to membranes through interactions with gH. We are currently testing this hypothesis. However, it was of interest that the fusion protein produced in KOS-gL β -infected Vero cells associates, albeit poorly, with gH. This finding suggests that gL sequences required for interactions with gH are present in the first 94 (or approximately 69 residues after signal sequence removal) amino acids of gL, although other sequences probably also contribute. Moreover, we observed that anti-UL1-1 sera preferentially immunoprecipitated the immature form of gL and inefficiently coprecipitated gH compared with anti-UL1-2 sera. These observations suggest that anti-UL1-1 antibodies may interfere with interactions between gH and gL or recognize an epitope in gL masked by interactions with gH. The fusion protein-gH complex was not recognized by MAb LP11; therefore, the LP11 epitope in gH or gH-gL must require sequences other than those at the amino terminus of gL.

It was not altogether surprising that virions lacking both gL and gH adsorbed onto the cell surface but could not enter cells. Forrester et al. (16) recently characterized a mutant

FIG. 9. Incorporation of gL and gH into virions. Vero cells were infected with KOS or KOS-gL8 and labeled with [35 S]cysteine from 3 to 24 h postinfection, and virus particles were purified by using dextran gradients. The virus band was diluted in buffer, virus was pelleted in an ultracentrifuge, and the pellet was suspended in NP-40/DOC extraction buffer. Detergent extracts of radiolabeled virus particles were mixed with anti-UL1-2 serum, MAb 52S specific for gH, MAb LP2 specific for gD, or anti-TK serum. Antigen-antibody complexes were precipitated with protein A-Sepharose and washed under stringent conditions, and the immunoprecipitated proteins were eluted and electrophoresed on 11% polyacrylamide gels. Molecular mass markers (in kilodaltons) are indicated on the right; positions of gH, IgG Fc receptor proteins gE and gI, gD, TK, gL, and pgL are indicated on the left.

HSV-1 with gH coding sequences deleted and found a similar or identical phenotype. One would suspect that their virus particles also lack gL. Therefore, it is clear that gH-gL oligomers are not required for interactions between HSV and heparin sulfate or other glycosaminoglycans during virus

adsorption onto the cell surface; however, the complex is required for entry into cells. Furthermore, Forrester et al. (16) found that the gH-negative mutant differed from a gD-negative virus in that the gH mutant was able to block entry of superinfecting virus, although less efficiently than wild-type HSV-1. Thus, it appears that the gH-gL complex acts during HSV entry into cells but at a stage different from that of gD, which apparently acts to engage cell surface protein receptors. One possibility is that the gH-gL oligomer acts in membrane fusion, a process which apparently follows interactions between gD and gD receptors (4, 34, 35). There is also ample evidence that gB performs an essential function during virus entry and is, perhaps, directly involved in membrane fusion.

It is tempting to speculate that the gH-gL oligomer performs a similar function during virus entry for all the members of the herpesvirus family. Homologs of HSV-1 gH have been observed in HSV-2 (48), varicella-zoster virus (11, 47), HCMV (10), and Epstein-Barr virus (1, 12, 47). Similarly, there are homologs or positional homologs of gL in HSV-2 (49), varicella-zoster virus (gene 60) (11), Epstein-Barr virus (BKRF2) (12), and HCMV (UL115) (41, 62). In the absence of other viral glycoproteins, the HCMV and Epstein-Barr virus gH homologs are not folded properly and not expressed on the cell surface (17, 22, 69), and the HCMV UL115 gene product has recently been shown to form a complex with HCMV gH, leading to cell surface expression of both proteins (41, 62).

If the gH-gL oligomer participates directly in the membrane fusion process during HSV penetration into cells, it would not be surprising that mutations affecting gL or gH might produce the syncytial phenotype. In support of this idea, high-level expression of gH or expression early in the infection leads to the syncytial phenotype (16). Previously, marker transfer experiments had suggested that HSV-1 mutant 804 possessed a syncytial mutation mapping to the UL1 (gL) gene (44, 46). However, this conclusion had to be qualified because gel-purified DNA fragments were used in marker transfer experiments (before cloned viral DNA sequences were available), and thus contamination of DNA fragments was possible. Using cloned DNA sequences, we demonstrated that the syncytial phenotype of mutant 804 was determined by a mutation in the UL53 gene. Other studies have suggested that a large fraction and possibly a majority of syncytial mutations in HSV-1 are caused by mutations to the UL53 gene (3, 27a). Since we know of no other cases in which syncytial mutations have been mapped

TABLE 1. Marker transfer experiments involving HSV-1 mutant 804 and cloned UL1 and UL53 genes

Expt	Viral DNA ^a	Plasmid ^b	No. of plaques		
			Wild type	Syncytial	Total
1	804	None	0	824	824
	804	pUL1	0	3,616	3,616
	804	pSV2HIS-Syn	26	4,814	4,840
2	KOS	None	2,650	0	2,650
	KOS	pUL1-804	3,702	0	3,702
	KOS	pUL53-804	5,139	7	5,146
	KOS	pS20BL-NB	5,962	38	6,000
	KOS	pKBL-BR	2,508	0	2,508

^a Viral DNA was extracted from infected cells and cotransfected with plasmids containing HSV-1 genes onto Vero cells monolayers. The viruses derived from these transfections were then plated on Vero cells, and syncytial and wild-type plaques were scored.

^b Plasmid pUL1 contains the wild-type KOS UL1 gene, pSV2HIS-Syn contains the wild-type KOS UL53 gene, pUL1-804 contains the UL1 gene from mutant 804, pUL53-804 contains the UL53 gene from mutant 804, pS20BL-NB contains the UL53 gene from syn20, and pKBL-BR contains the UL53 gene from wild-type KOS.

FIG. 10. Morphology of plaques produced by viruses derived in marker transfer experiments involving the UL1 or UL53 genes of mutant 804. Two plasmids, pUL1-804 and pUL53-804, containing the UL1 and UL53 genes, respectively, from the syncytial mutant 804 were constructed. Vero cell monolayers were cotransfected with HSV-1 KOS viral DNA and one of the following plasmids: pUL1-804 (panel 1); pS20BL-NB, containing the UL53 gene derived from the syncytial virus syn20 (pane 2); pKBL-BR, containing the wild-type UL53 gene (panel 3); or pUL53-804 (panel 4). Virus stocks derived from these transfections were plated on Vero cells, and the cells were fixed and stained 48 h after infection. The syncytial plaques shown here were rare (0.1 to 1% of the total plaques).

to the UL1 gene or adjoining region, the number of genes associated with the syncytial phenotype should be revised to four: UL53 (gK) (13, 54), UL27 (gB) (6, 56), UL24 (33, 57), and UL20 (2). There has also been a report that a fifth syncytial locus is present in the inverted repeats bounding the unique long segment of the HSV-1 genome (55), although the gene product involved has not been identified to date.

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