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The Cytoplasmic Domain of Herpes Simplex Virus Type 1 Glycoprotein C Is Required for Membrane Anchoring

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The herpes simplex virus type 1 (HSV-1) glycoprotein C (gC) gene was altered so that it encoded a truncated glycoprotein lacking a cytoplasmic domain but retaining 20 of 23 amino acids of the transmembrane domain. No additional amino acid residues were introduced into the glycoprotein encoded by the altered gene. The gene was recombined into the HSV-1 genome by marker transfer. Two recombinant viruses, *dl*1 and *dl*2, that expressed the mutant gene were isolated. Characterization of these viruses showed that a substantial fraction of the mutant glycoprotein was secreted from infected cells. Pulse-chase experiments showed that the kinetics of posttranslational modification of the mutant glycoprotein were similar to those of the wild type. However, comparison of the kinetics of secretion of gC by *dl*2 and *gC*$^{-}$3, a gC mutant lacking both the transmembrane and cytoplasmic domains, showed that *dl*2 gC was secreted much more slowly than *gC*$^{-}$3 gC. Iodination of plasma membrane glycoproteins showed that *dl*2 gC was initially expressed on the cell surface as a membrane protein and subsequently was slowly released from the membrane into the medium. These data indicate that a major function of the cytoplasmic domain of gC is to ensure the stable anchoring of the glycoprotein in plasma membranes. In contrast to these major changes in the membrane-anchoring properties of gC, characterization of the virions produced by *dl*1 and *dl*2 showed that they contain significant amounts of gC. Thus the cytoplasmic domain does not appear to be essential for incorporation of this glycoprotein into virions.

The specific functions and properties of biological membranes are largely determined by membrane proteins. These may be divided into two groups: peripheral membrane proteins and integral membrane proteins (IMPs). Peripheral membrane proteins are relatively loosely bound either to the surface of the lipid bilayer or to other membrane proteins and may be removed by relatively mild procedures that do not disrupt the bilayer itself. IMPs are associated with the hydrophobic portion of the lipid bilayer, often by a transmembrane domain, and usually can be removed only by procedures that disrupt the membrane (13, 32). Many of the studies on the properties of IMPs have focused on type I IMPs (also called group A IMPs [11]). A type I IMP contains an amino-terminal signal sequence that is cleaved during processing, a large external domain, a single transmembrane domain, and a hydrophilic cytoplasmic domain at its carboxyl terminus. According to current concepts of membrane protein topogenesis (reviewed in references 11 and 34), two functions may be associated with transmembrane domains: a stop transfer function and a membrane-anchoring function. Studies on a number of transmembrane glycoproteins have made it clear that truncation of transmembrane glycoproteins upstream of their transmembrane domains converts them to secreted forms (12, 17, 27). Recent studies have indicated that hydrophobic transmembrane sequences alone may be sufficient to function as stop transfer signals (3, 5). However, the relative contributions of the transmembrane and cytoplasmic domains to membrane anchoring of glycoproteins remains uncertain. Since the transmembrane domain interacts directly with the hydrophobic interior of the membrane, hydrophobic interactions between this domain and membrane lipids may be largely responsible for anchoring. Alternatively, the cytoplasmic domain may contribute to anchoring owing to the difficulty of moving the charged residues of this domain through the bilayer or to an interaction of the domain with structures within the cell.

Glycoprotein C (gC) of herpes simplex virus type 1 (HSV-1) is a type I IMP. It is not essential for virus replication in cell culture, as shown by the isolation of virus mutants that fail to produce gC (16–18). Although gC is nonessential, it has been possible to isolate mutant viruses with a wide spectrum of mutations in the gC gene through the use of antibody selection and screening techniques. Such mutants have been useful in mapping of antigenic sites (17, 18, 24) and sites involved in binding of the complement component C3b (9) and in studies on the regulation of HSV genes (19). Secretion of truncated gC by mutants with nonsense or frameshift mutations upstream of the stop transfer-transmembrane domain has also been demonstrated (17, 20). gC is acquired by virions during budding of the nucleocapsid through the inner nuclear membrane. In this process, the cytoplasmic domain of gC might interact with capsid or tegument proteins to promote specific incorporation of gC into nascent virions. To investigate the role of the cytoplasmic domain of gC in the stop transfer and membrane-anchoring processes and in incorporation of gC into virion envelopes, we constructed mutant viruses encoding gC lacking this domain. We report here direct evidence that the cytoplasmic domain of HSV-1 gC is essential for the stable anchoring of that glycoprotein in the cellular plasma membrane. Although important for membrane anchoring, the domain appears to be dispensable for incorporation of the glycoprotein into virions.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human embryonic lung (HEL) cells were grown in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with nonessential amino acids, 100 μg of streptomycin per ml, 100 U of penicillin per ml (MEM), and 10% fetal calf serum (FCS; GIBCO) as described previously (14). KOS321

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is a plaque-purified isolate of the KOS strain of HSV-1 (18). The isolation and characterization of the gC deletion mutant gC^-39 and the truncated gC mutant gC^-3 have been described elsewhere (17, 20). Virus stocks were prepared by infection of Vero cells at a low multiplicity of infection (MOI), and titers were determined by plaquing on Vero cell monolayers (18).

Plasmid construction. The plasmid pFH46, containing two copies of *Hin*dIII fragment L of HSV-1 (KOS321) DNA (coordinates 0.588 to 0.647) cloned in pBR322, was obtained from F. Homa and M. Levine of the University of Michigan. The plasmid pKE101 was constructed by ligating a single copy of the *Hin*dIII L fragment into the *Hin*dIII site of pUC9. The construction of the mutant gC gene is outlined in Fig. 1A, beginning with pKE101. The *Xba*I-*Hin*dIII fragment containing the 3′ end of the gC gene (coordinates 0.639 to 0.647) was subcloned from pKE101 into the *Xba*I-*Hin*dIII site of pUC13 to create pKE102. A 133-nucleotide *Xba*I-*Rsa*I fragment encoding most of the gC transmembrane domain was excised from pKE102 by digestion with *Xba*I and *Rsa*I, plus *Eco*RI and *Hin*dIII to destroy other fragments with similar termini. A clone containing this small fragment ligated into the *Xba*I-*Hin*cII site of pUC13 was designated pTH105. Ligation of the blunt ends produced by *Rsa*I and *Hin*cII altered codon 498 of the gC reading frame from TAC (tyrosine) to TGA (termination). To rejoin the 5′ end of the gC gene and its upstream sequences with the transmembrane-encoding sequence, we digested pKE101 with *Bam*HI and *Xba*I, and the 5.4-kilobase (kb) fragment containing the 5′ end of the gC gene was ligated into *Bam*HI- and *Xba*I-digested pTH105. The resultant clone contained an altered gC gene encoding a truncated 497-amino-acid glycoprotein and was designated pRL108. To add back sequences from the 3′ end of the gC gene for marker transfer, we constructed pRL110 by ligating a *Hin*dIII linker into the *Xba*I site of pKE101, using a linker tailing procedure (22). *Hin*dIII digestion of pRL110 released a 1.2-kb fragment containing the 3′ end of the gC gene, which was cloned into the *Hin*dIII site of pRL108. A clone in which the fragment was inserted in the correct orientation was designated pRL111. Plasmids pKE101, pKE102, pTH105, and pRL108 were produced by transformation of *Escherichia coli* JM83. Plasmids pRL110 and pRL111 were produced by transformation of *E. coli* HB101. Restriction digests, DNA ligations, agarose gel electrophoresis, and gel purification of DNA fragments were done as described by Maniatis et al. (23). Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and New England BioLabs, Inc. (Beverly, Mass.).

Isolation of recombinant viruses. Viral DNA was purified from Vero cells infected with the gC deletion mutant gC^-39 as described by Sandri-Goldin et al. (29). A calcium phosphate coprecipitate of gC^-39 DNA and *Bam*HI-digested pRL111 DNA was prepared as described by Graham and van der Eb (15). Vero cell monolayers were overlaid with the precipitate and incubated at 37°C for 40 min. Medium was added to the cells, and they were incubated at 37°C for 4 h and shocked with 15% dimethyl sulfoxide (33). When viral cytopathic effect was generalized, the infected cells were scraped into the medium and progeny virus was released by three cycles of freezing and thawing. Released virus was quantified by titration on Vero cell monolayers. The virus was replated on Vero cell monolayers in 100-mm petri dishes with 100 to 1,000 PFU per dish. After adsorption, the monolayer was overlaid with medium containing 1% agarose and plaques were allowed to develop. Plaques formed by recombinant virus were identified by the plaque lift technique described by Homa et al. (19), except that Colony/Plaque Screen filters (New England Nuclear Corp., Boston, Mass.) were used and hybridized according to the instructions of the manufacturer. A 0.9-kb *Eco*RI-*Xba*I gC gene fragment excised from pKE101 was labeled with ^{32}P by the random primer labeling technique (8) and used as a probe.

For genomic blotting of viral DNA, 1 μg of CsCl-purified viral DNA (29) was digested with *Sal*I or with *Hin*dIII and *Eco*RI and electrophoresed on a 0.7% agarose gel. The DNA was electrophoretically transferred to a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.) and hybridized according to the protocol of the manufacturer (Bio-Rad Bulletin 1110). The probe was the same 0.9-kb *Eco*RI-*Xba*I gC gene fragment that was used to probe the plaque lifts.

DNA sequencing was done by the dideoxynucleotide method (30). The *Sal*I fragments containing the gC genes of *dl*1 and *dl*2 were cloned into the *Sal*I site of the vector Bluescribe M13+ (Stratagene, San Diego, Calif.). To bring the region of interest closer to the binding sites for commercially available sequencing primers, we subcloned the *Xba*I-*Sal*I fragment containing the 3′ ends of the mutant gC genes into Bluescribe M13+. Single-stranded template DNA was prepared by infection of the transformed cells with the M13-RV1 helper virus (Stratagene). Dideoxyribonucleotide DNA sequencing reactions were done with a DNA-sequencing kit (Bethesda Research Laboratories) and [^{35}S]dCTP (New England Nuclear Corp.). The reaction products were electrophoresed on 8% polyacrylamide denaturing gels (23).

Protein labeling. HEL cells were infected for protein labeling as described previously (17). A 16-h labeling period was used to label the mature forms of the viral glycoproteins. HEL cell monolayers (10^6 cells) in T25 flasks (Costar, Cambridge, Mass.) were infected with wild-type or mutant viruses at an MOI of 10. Virus was diluted in phosphate-buffered saline (PBS) and adsorbed to cells for 1 h at 37°C. After adsorption, the monolayer was overlaid with 5.0 ml of MEM–10% FCS and incubated at 37°C. After an additional 4 h, the medium was decanted and replaced with 5.0 ml of methionine-free Dulbecco modified Eagle medium (GIBCO) supplemented with 2% FCS and 50 μCi of [^{35}S]methionine (New England Nuclear Corp.) per ml. Approximately 24 h after infection, the labeling medium was removed and reserved. The infected cells were solubilized by the addition of 0.5 ml of lysis buffer (150 mM NaCl, 20 mM Tris [pH 8.0], 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) to the T25 flasks. Both the medium and cell extracts were clarified by centrifugation at 15,000 × *g* for 15 min. Immunoprecipitation of gC was done with gC-specific monoclonal antibody C2 or C8 (18, 24), as noted in Results. Antibody stocks were titrated in immunoprecipitation reactions to determine the amount of antibody required for quantitative immunoprecipitation. Antigen-antibody complexes were allowed to form overnight at 4°C. A 40% suspension of protein A-Sepharose beads (50 μl; Pharmacia, Inc., Piscataway, N.J.) was added to the immunoprecipitation mixtures and mixed by rocking for 1 h at room temperature, after which the beads were washed three times with lysis buffer. An equal volume of 2× electrophoresis sample solution was added to the pelleted beads. Samples were heated to 100°C for 5 min before electrophoresis on 10% polyacrylamide-sodium dodecyl sulfate (SDS) gels (17). Gels were fluorographed as described previously (17).

For pulse-chase labeling of gC, HEL cells were infected as described above. Seven hours after the end of the adsorption

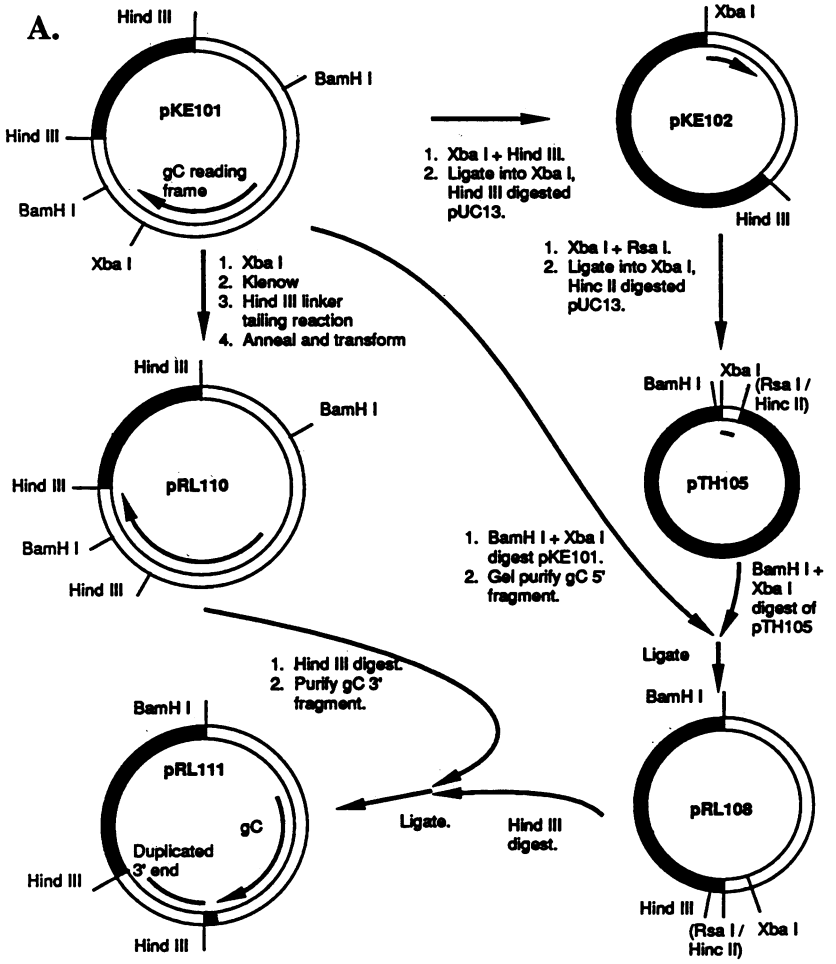

FIG. 1. Construction of a mutant HSV-1 gC gene lacking the cytoplasmic domain. (A) Plasmid sequences derived from pUC9 or pUC13 are represented by solid black lines. Plasmid sequences originally derived from HSV-1 are shown in white. To construct an in-frame termination codon near the end of the HSV-1 gC gene, the 3′ end of the gene was subcloned from pKE101 to form pKE102. Ligation of an *Xba*I-*Rsa*I fragment from pKE102 into the *Xba*I-*Hin*cII site of pUC13 created an in-frame termination codon. The 5′ end of the gene was reconstructed by cloning a *Bam*HI-*Xba*I fragment from pKE101 into pRL105 to form pRL108. Flanking sequences from the 3′ end of the gene were added to the construct by converting the *Xba*I site of pKE101 to a *Hin*dIII site (pRL110) and then inserting the *Hin*dIII fragment containing those sequences into pRL108, forming pRL111. Digestion of pRL111 with *Bam*HI released a fragment containing the altered gC gene together with flanking sequences, allowing recombination with the gC deletion mutant *gC*$^{-}$39. (B) Sequence of the HSV-1 (KOS321) gC transmembrane and cytoplasmic domains and sequence of the *dl*2 gC transmembrane domain are shown. The transmembrane domain sequences are underlined.

period, the medium was decanted and 0.5 ml of prewarmed methionine-free Dulbecco modified Eagle medium containing 100 μCi of [^{35}S]methionine per ml was added to the flasks. After pulse-labeling at 37°C, the labeling medium was decanted and replaced with MEM–10% FCS. Pulse durations of 15 or 30 min were used as indicated in Results. After the indicated chase intervals, the medium was decanted and the cells were solubilized. Immunoprecipitation of gC was done as described above.

Cell surface proteins were labeled by the method of Kessler (21). Briefly, HEL cell monolayers in T25 flasks were infected at an MOI of 10. Seven hours after infection, the medium was removed and the cells were washed twice with PBS supplemented with 1 μM KI (PBS-I). Cultures

were labeled by adding 0.5 ml of PBS-I containing 500 μCi of $Na^{125}I$ per ml, 0.025 ml of PBS containing 1 mg of lactoperoxidase per ml, and 0.025 ml of PBS containing 0.03% hydrogen peroxide to each flask, mixing, and incubating at room temperature for 5 min. A second 0.025-ml portion of 0.03% hydrogen peroxide was added to the flasks, and they were incubated for another 5 min. The labeling mixture was decanted, and the monolayers were washed three times with PBS containing 10 mM KI. A 1-ml portion of MEM supplemented with 10% FCS was added to the flasks, which were incubated at 37°C for the indicated chase period. The medium was decanted and reserved, and the cells were solubilized with 1.0 ml of lysis buffer.

For preparation of labeled virions, roller bottles (850 cm^2; Costar) were inoculated with 10^7 HEL cells and incubated at 37°C until the monolayer was nearly confluent. The cells were infected by decanting the medium and adding 10 ml of PBS containing 5×10^8 PFU (MOI, approximately 10) for 1 h. The virus suspension was decanted, and 50 ml of MEM supplemented with 2% FCS was added. After 4 h, this medium was decanted and 15 ml of Dulbecco modified Eagle medium containing 1/10th the normal concentration of methionine and 15 μCi of [^{35}S]methionine per ml was added to the flasks. After a 20-h labeling period, the infected cells were scraped into the medium, pelleted by low-speed centrifugation, and suspended in 1.0 ml of PBS. Virions were purified by the method of Cai et al. (1), except that PBS was used rather than tricine-buffered saline. Briefly, the cell suspension was freeze-thawed three times to release virus, and cell debris was pelleted by centrifugation at 15,000 × *g* for 5 min. Virions were pelleted by centrifugation at 15,000 × *g* for 30 min and resuspended in 0.1 ml of PBS. The suspension was layered on top of a 10 to 30% dextran (average molecular weight, 9,000; Sigma Chemical Co., St. Louis, Mo.) gradient prepared in PBS and centrifuged in an SW41 rotor at 20,000 rpm for 90 min. Fractions were collected and assayed for radioactivity. The virion peak was approximately two-thirds of the way down the tube. Peak fractions were pooled, diluted with an equal volume of PBS, and pelleted by centrifugation at 15,000 × *g* for 30 min. The virions were disrupted in lysis buffer, and insoluble material was pelleted by centrifugation at 15,000 × *g* for 30 min. Immunoprecipitation and electrophoresis of solubilized gC were done as described above.

Virus neutralizations were done under conditions described previously (18), except that a series of 10-fold dilutions of antibody was used. Normal rabbit serum was used as the source of complement. After neutralization, the fraction of surviving virus was determined by plaque assay on Vero cell monolayers.

RESULTS

Construction of a mutant gC gene. To investigate the properties of the HSV-1 gC cytoplasmic domain, we constructed a mutant gC gene encoding a truncated form of gC lacking this domain. Figure 1A outlines the scheme by which the recombinant plasmid containing the mutated gene was constructed. Examination of the gC gene sequence (6, 10, 20) revealed the presence of a site for the restriction endonuclease *Rsa*I near the 3′ end of the transmembrane domain-coding region. By cleaving the gene at this site and ligating the blunt-ended product with the blunt end produced by *Hinc*II digestion of pUC13, an in-frame termination codon (TGA) was created. The transmembrane domain of wild-type gC consists of a sequence of 23 hydrophobic amino acids, followed by a highly charged cytoplasmic domain consisting of 11 amino acids (Fig. 1B). The mutant glycoprotein encoded by the nucleotide sequences of the mutant gC gene in pRL108 was identical to wild-type gC through amino acid 20 of the transmembrane domain, after which it terminated without the addition of any additional amino acids not found in wild-type gC. Although pRL108 contained a complete copy of the mutant gene and several kilobases of HSV-1 DNA from the region immediately upstream of the gC gene, it did not contain any sequences from the region of the HSV-1 genome downstream of the gC gene. Since homologous downstream flanking sequences were necessary to insert the gene into the HSV-1 genome by marker transfer, downstream sequences were inserted into pRL108 to form pRL111.

Isolation of recombinant viruses. To express and characterize the glycoprotein produced by the mutant gene, we produced recombinant viruses containing the mutant gene. The mutant *gC*$^-$39 has a deletion of 1,702 base pairs, including the entire coding sequence of the gC gene (20). The plasmid pRL111 was constructed to permit homologous recombination between the region flanking the deletion in *gC*$^-$39 and the regions 5′ and 3′ to the mutant gC gene. Purified pRL111 DNA was cotransfected into Vero cells together with purified *gC*$^-$39 DNA, and progeny virus was harvested after cytopathic effect became generalized. Although viruses expressing wild-type gC can be readily detected immunologically (17), the phenotype of viruses expressing the mutant gC was uncertain; hence, the progeny viruses were screened for recombinants by plaque hybridization. Using a cloned fragment of the gC gene as a probe, this method tests directly whether a plaque was formed by a virus carrying the gC gene, regardless of its phenotype (19). A 0.9-kb *Eco*RI-*Hin*dIII fragment of the gC gene (coordinates 0.633 to 0.639) was used as a probe. Since this region has been entirely deleted from *gC*$^-$39, any hybridizing plaques must be recombinants. Several positive isolates were identified among the transfection progeny. After three cycles of purification, 100% of the plaques produced by two isolates hybridized with the probe. Since the mutation introduced into the gC gene effectively deleted the 3′ end of the gC-coding sequence, these isolates were designated *dl*1 and *dl*2.

Confirmation of mutant genotype. To confirm that the recombinant viruses *dl*1 and *dl*2 had the expected genotype, we purified viral DNA from *dl*1- and *dl*2-infected cells on CsCl gradients. Southern blot analysis was used to examine the genomes of *dl*1 and *dl*2 and to compare them with KOS321 and *gC*$^-$39. Samples of the viral DNAs were digested either with *Sal*I or with *Eco*RI and *Hin*dIII, electrophoresed, blotted, and probed with the 0.9-kb *Eco*RI-*Xba*I fragment from the gC gene. As expected from previous characterization of KOS321 and *gC*$^-$39 (17, 20), this probe hybridized with a 3.6-kb *Sal*I fragment from KOS321 and did not hybridize with any fragment from *gC*$^-$39. In the *Sal*I digests of *dl*1 and *dl*2, the probe hybridized with a fragment slightly larger than the 3.6-kb wild-type fragment (data not shown). In double digests of the viral DNAs with *Eco*RI and *Hin*dIII, the probe hybridized with a 2.1-kb fragment of KOS321 DNA and again did not hybridize with any *gC*$^-$39 fragment. In the double digests of *dl*1 and *dl*2, the probe hybridized with a 1.0-kb fragment, owing to the insertion of a *Hin*dIII site as shown in Fig. 1A (data not shown).

To positively confirm that the recombinant viruses carried the expected mutations, we recloned the *Sal*I fragments containing the gC genes of *dl*1 and *dl*2 from purified viral

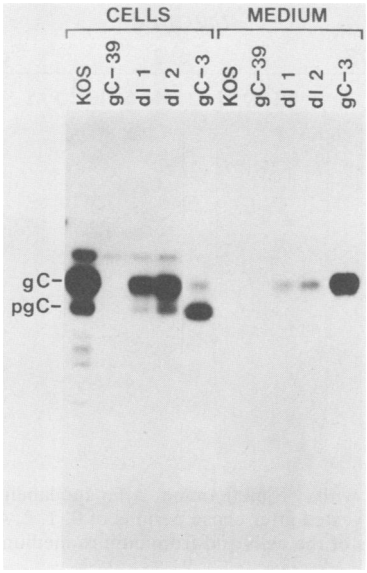

FIG. 2. Synthesis and secretion of gC. HEL cells were infected with KOS321 (wild type), $gC^{-}39$ (a gC deletion mutant), *dl*1, *dl*2, and $gC^{-}3$ (a gC-secreting mutant) at an MOI of 10 and labeled from 5 to 21 h postinfection with [^{35}S]methionine. A gC-specific monoclonal antibody (C2) was used to immunoprecipitate gC from detergent extracts of cells and from the medium. The immunoprecipitated material was analyzed by SDS-polyacrylamide gel electrophoresis. The dried gels were fluorographed.

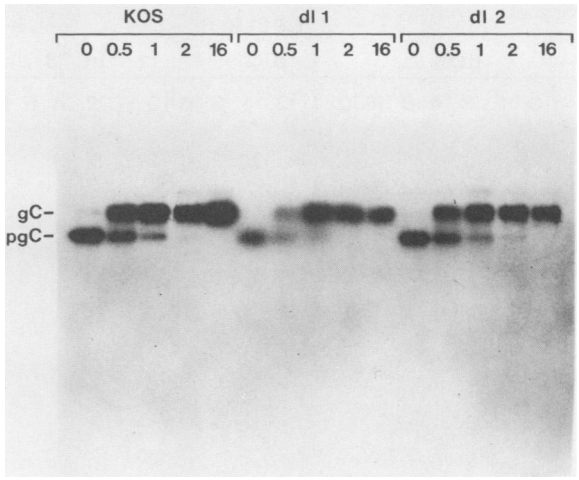

FIG. 3. Pulse-chase labeling of gC. At 7 h after infection, cells were labeled for 15 min with [^{35}S]methionine. The medium was replaced with medium containing unlabeled methionine. Cultures were harvested after chase periods of 0, 0.5, 1, 2, and 16 h. The C8 monoclonal antibody was used to immunoprecipitate gC from detergent extracts of the cells.

DNA. The *Xba*I-*Sal*I fragments containing the 3′ end of the gC gene were subcloned into the sequencing vector Bluescribe M13+. The sequences of the *dl*1 and *dl*2 gC genes from the *Xba*I site to the termination codon were determined and were identical to that expected from the construction scheme shown in Fig. 1 (data not shown).

Synthesis and secretion of mutant glycoprotein. To investigate the effect of the deletion introduced into the gC gene on the properties of the glycoprotein, we examined the synthesis of gC by *dl*1-infected and *dl*2-infected cells. HEL cells were infected with mutant or wild-type virus at an MOI of 10. The infected cells were labeled with [^{35}S]methionine from 5 to 24 h postinfection, at which time the medium was decanted and reserved and the infected cells were solubilized with lysis buffer. The gC-specific monoclonal antibody C2 was used to immunoprecipitate gC from both the labeling medium and the cell extract. The immunoprecipitates were solubilized in SDS sample buffer and electrophoresed on 10% polyacrylamide gels. A fluorograph of the dried gel is shown in Fig. 2. In immunoprecipitates made from extracts of KOS321-infected cells, gC appeared as a major band with an apparent molecular weight of approximately 130,000. A partially glycosylated precursor form of gC (pgC) with an apparent molecular weight of 110,000 was also observed (17, 31). No gC was observed in immunoprecipitates made from cells infected with the gC deletion mutant $gC^{-}39$. In immunoprecipitates made from *dl*1-infected and *dl*2-infected cells, bands of mobility similar to that of wild-type gC and pgC were observed. Although *dl*1 and *dl*2 gC are expected to be shorter than wild-type gC, the difference of less than 2,000 in predicted molecular weights was too small to be detected.

Significantly, a quantity of gC also was immunoprecipitated from the medium of cell cultures infected with *dl*1 and *dl*2, although most of the gC produced by these mutants remained cell associated in this experiment. The apparent molecular weights of the cell-associated form of gC and the gC released into the medium were the same. No detectable gC was released into the medium by KOS321-infected cells, while nearly all the gC produced by $gC^{-}3$ was secreted, as was reported previously (17). Both pgC and a small quantity of mature gC were detected in extracts of $gC^{-}3$-infected cells. The gC produced by $gC^{-}3$ lacks both the transmembrane and cytoplasmic domains of wild-type gC, owing to a frameshift mutation in codon 480 of the gC reading frame (20). It is apparent from Fig. 2 that the quantity of gC produced by *dl*1 and *dl*2 was less than that produced by KOS321 and that *dl*1 produced less gC than *dl*2. This quantitative difference between the two mutants was reproducible and did not depend on the antibody used to immunoprecipitate gC (data not shown). The reason for the difference between the two mutants is not known.

Processing of gC. The reduced accumulation of gC in cells infected with *dl*1 and *dl*2 might have been caused by reduced stability of the mutant glycoprotein. Also, the deletion of the cytoplasmic domain might have had a significant effect on the kinetics of processing of the glycoprotein, as has been noted in other systems (4, 28, 35). A pulse-labeling experiment was used to examine the kinetics of gC processing and the stability of the mutant glycoprotein. Infected cells were pulsed for 15 min with [^{35}S]methionine at 7 h postinfection and then chased with nonradioactive medium for various lengths of time before cell extracts were prepared for immunoprecipitation. The immunoprecipitates were electrophoresed on a 10% polyacrylamide gel. As is evident from the fluorograph of the gel shown in Fig. 3, the kinetics of gC processing by *dl*1 and *dl*2 were similar to that of KOS321. Immediately after the pulse-labeling period, nearly all the immunoprecipitated labeled material was in the form of pgC. After a 0.5-h chase period, a significant fraction of pgC was converted to mature gC, and after a 2-h chase, essentially all the pgC was converted to gC. Neither the kinetics of posttranslational processing nor the stability of gC was significantly affected by deletion of the gC cytoplasmic domain.

Kinetics of secretion. Since the kinetics of gC processing by *dl*1 and *dl*2 appeared similar to those of wild-type and $gC^{-}3$, it was of interest to compare the kinetics of release of

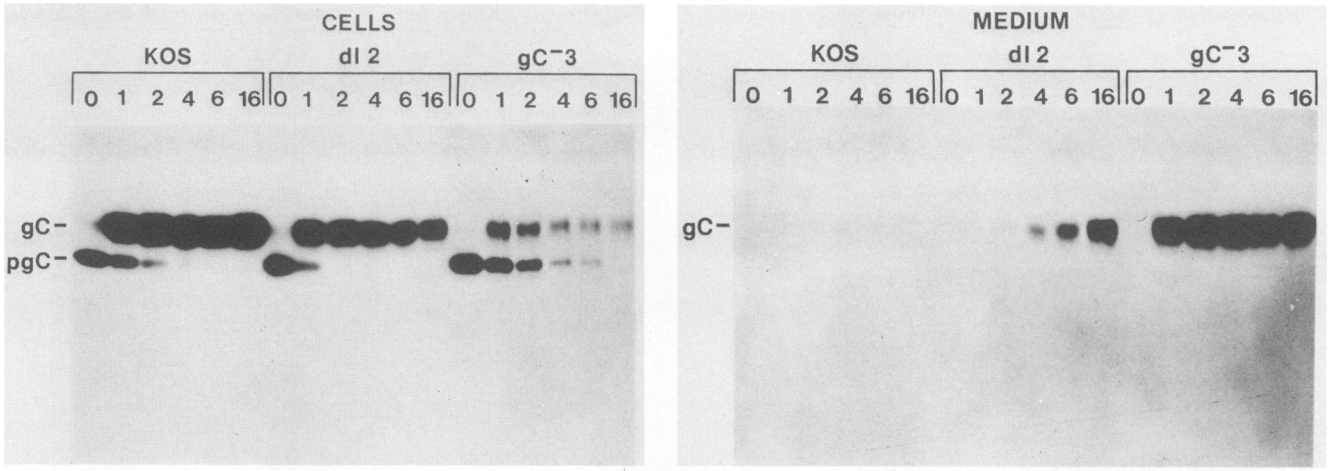

FIG. 4. Kinetics of gC secretion. At 7 h after infection, cells were labeled for 30 min with [^{35}S]methionine. After the labeling period, the medium was replaced with medium containing unlabeled methionine. Cultures were harvested after chase periods of 0, 1, 2, 4, 6, and 16 h. The C8 monoclonal antibody was used to immunoprecipitate gC from detergent extracts of the cells and from culture medium harvested at the same time.

gC from cells infected with the mutant viruses. Since *dl*1 consistently produced less gC than *dl*2, and since only a fraction of this was released into the medium (Fig. 2), only *dl*2 was examined in this experiment. HEL cells were infected with KOS321, *dl*2, or *gC*$^{-}$3 at an MOI of 10. Seven hours postinfection, the cultures were pulsed with [^{35}S]methionine for 30 min. The cultures were chased with nonradioactive medium for 0, 1, 2, 4, 6, and 16 h. After the chase intervals, the medium from one culture from each set was removed and reserved and cell extracts were made with lysis buffer. Immunoprecipitates were made with the gC-specific monoclonal antibody C8 and electrophoresed on 10% polyacrylamide gels. Fluorograms of the dried gels are shown in Fig. 4. Panel A of Fig. 4 shows cell-associated gC and confirmed the KOS321 and *dl*2 gC-processing kinetics shown in Fig. 3. As in the previous experiment, mature *gC*$^{-}$3 gC did not accumulate to any appreciable extent within the cell. Virtually as soon as the Golgi-associated processing occurred (pgC to gC shift), the mature gC was secreted from the cell. This is confirmed in Fig. 4B, which shows that mature gC rapidly accumulated in the medium of *gC*$^{-}$3 cultures. Pulse-labeled *gC*$^{-}$3 gC was readily detected in the medium after a 1-h chase, and there was little additional secretion of labeled gC after a 4-h chase. The kinetics of release into the medium of *dl*2 gC were much different than those of *gC*$^{-}$3 gC. Little *dl*2 gC was detected in the medium after 1- or 2-h chases. Chases of 4, 6, and 16 h resulted in a steady increase in the amount of *dl*2 gC released into the medium, finally reaching approximately 50% of the total labeled gC. This was confirmed by a reduction in the amount of gC precipitated from the *dl*2-infected cell extract after a 16-h chase. The fraction of gC released into the medium in this pulse-chase experiment was higher than the fraction released in the continuous labeling experiment. This may be due to the slow rate of release of *dl*2 gC from cells. Since a significant fraction of *dl*2 gC is not released from the cells until many hours after it is synthesized, gC synthesized late after infection during a continuous labeling experiment may not have time to be released before harvesting of the cells at the end of the labeling period.

Release of *dl*2 gC from the cell surface. The slower rate of appearance of *dl*2 gC in the medium compared with *gC*$^{-}$3 gC might be caused by a slower rate of transport of a soluble form of *dl*2 gC from the Golgi apparatus to the plasma membrane or by slow release of membrane-bound *dl*2 gC from the plasma membrane into the medium. The latter possibility was tested by infecting HEL cell cultures with KOS321, *gC*$^{-}$3, or *dl*2, radioiodinating the cell surface proteins 7 h after infection, and immunoprecipitating gC from the cell surface and culture medium after chase periods of 0, 1, 2, 4, 6, and 16 h. Figure 5A shows a gel of labeled gC immunoprecipitated from cell extracts. No gC was detected in immunoprecipitates from cells infected with *gC*$^{-}$3. This was expected from the soluble nature of this glycoprotein and showed that the large external domain of gC did not mediate any nonspecific binding of gC to the surface of infected HEL cells. Cell surface gC was readily detectable on the plasma membranes of cells infected with KOS321, and the quantity detected remained approximately constant during the chase period. Cell surface gC was also readily detected on cells infected with *dl*2. Note that the 0-h-chase *dl*2 sample was lost in processing. Figure 5B shows a gel of gC immunoprecipitated from the medium. No immunoprecipitates were made from the medium after the 0-h chase. No gC was detected in medium from *gC*$^{-}$3-infected cultures since only gC which was present on the cell surface at 7 h postinfection became labeled. Virtually no gC was found to be released from the surfaces of KOS321-infected cells. However, small quantities of labeled gC were readily detected in the medium of *dl*2 cultures after a chase of 1 to 2 h, and substantial quantities were detected after a chase of 4 to 6 h. Thus, *dl*2 gC was initially expressed on the cell surface in a membrane-bound form, unlike *gC*$^{-}$3 gC, but a significant fraction of the cell surface *dl*2 gC was released into the medium over a period of hours.

Incorporation of gC into virions. To determine whether *dl*1 and *dl*2 incorporated gC into virions, cells were infected at an MOI of 10 and labeled with [^{35}S]methionine from 4 to 24 h postinfection. The infected cells were lysed by freeze-thawing, and virions were purified by centrifugation on dextran gradients. Approximately equal quantities of purified virions were extracted with Nonidet P-40, and the gC-specific monoclonal antibody C8 was used to immunoprecipitate gC from the extracts. The immunoprecipitates were electrophoresed on SDS-10% polyacrylamide gels. A fluorograph of the gel is shown in Fig. 6. Virions produced

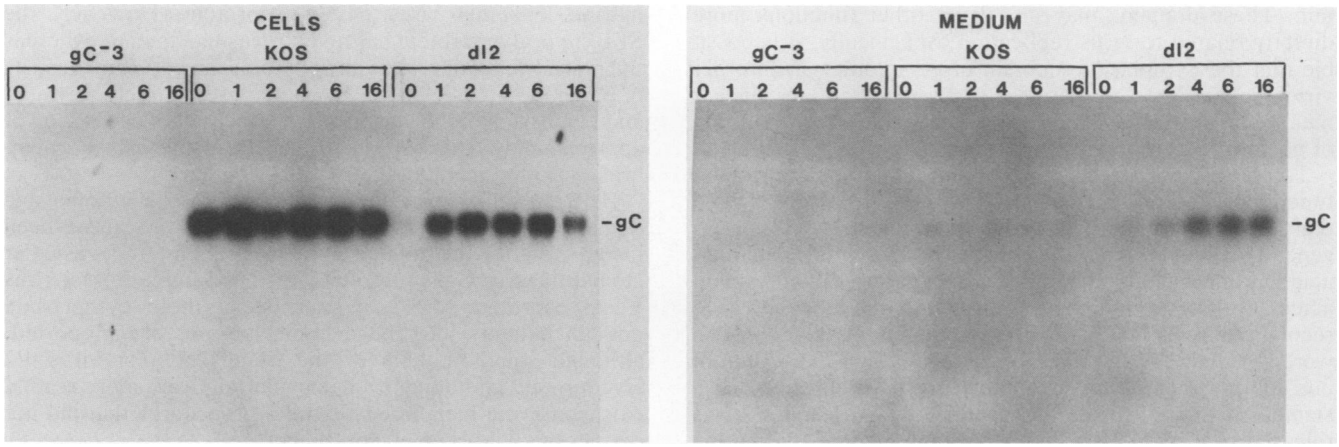

FIG. 5. Release of gC from the plasma membrane. Seven hours after infection, cells were washed and surface labeled with ^{125}I by the lactoperoxidase method. After labeling, the cultures were washed again, fresh medium was added, and the cultures were incubated at 37°C for the indicated chase period (hours). After the chase, the medium was decanted and reserved, and the cells were solubilized with lysis buffer. Monoclonal antibody C8 was used to immunoprecipitate gC from the cell extracts and culture medium. No immunoprecipitates were made from the medium after the 0-h chase. The sample containing the gC immunoprecipitate from *dl*2-infected cells after the 0-h chase was lost in processing. The immunoprecipitates were electrophoresed on 10% polyacrylamide gels and fluorographed.

by *dl*1 and *dl*2 contained gC, although the quantity was somewhat reduced compared with that produced by the wild-type virus, KOS321. The reduction is consistent with the reduced synthesis of gC observed in the experiments described above. The virion preparation appeared not to have been contaminated by cellular membranes, since pgC was not detected, although it was present in infected cells (Fig. 2).

Although gC is not essential for virion infectivity, and gC-specific antibodies alone do not readily neutralize virus, many gC-specific antibodies neutralize virus in the presence of complement (7, 18, 24). A virus neutralization assay was used to test virions for the presence of gC. In the presence of complement, monoclonal antibody C2 neutralized both *dl*1 and *dl*2 nearly as well as KOS321 (Table 1). Virions which lack gC have been shown to be highly resistant in similar assays (17, 18). The susceptibility of *dl*1 and *dl*2 to neutralization provided independent, functional evidence that the mutant gC glycoproteins encoded by these viruses were incorporated into virions. Virus neutralization assays were also conducted on KOS321 and *dl*2 virions that had been incubated for 16 h at 37°C in MEM containing 10% FCS to determine whether *dl*2 virions became resistant to neutralization through the loss of gC from the virion envelope. No decrease in susceptibility to neutralization was observed (data not shown), indicating no detectable loss of gC from the virions. However, this type of assay may not be sensitive to the loss of small or moderate quantities of gC from virion envelopes, since even small amounts of membrane-bound gC may leave the virus susceptible to neutralization.

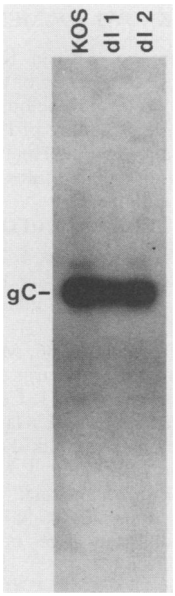

FIG. 6. Analysis of purified virus. Infected cells were labeled with [^{35}S]methionine. Labeled virus was harvested by freeze-thawing the cells and purified on a dextran gradient. The virion envelopes were detergent solubilized, and the C8 monoclonal antibody was used in immunoprecipitations. The immunoprecipitated material was analyzed by SDS-polyacrylamide gel electrophoresis and fluorographed.

DISCUSSION

The purpose of the studies described here was to investigate the structure-function relationships of two domains of HSV-1 gC: the transmembrane domain and the cytoplasmic domain. It was shown previously that truncation of gC upstream of these domains converts it from a membrane-bound glycoprotein into a secreted glycoprotein (17, 20). Current models of membrane protein biosynthesis attribute this to a loss of stop transfer and membrane-anchoring functions (11, 34). However, the relative contributions of these two domains of gC to these functions remained uncer-

TABLE 1. Neutralization of virus by gC-specific monoclonal antibody C2

Virus	% Surviving virus at an antibody dilution of:			
	1,000	10,000	100,000	No C2
KOS321	0	0	34	100
*dl*1	0	0.5	89	100
*dl*2	0	1.2	55	100

tain. These domains may also have other functions more directly related to virus replication. Specifically, it is possible that the cytoplasmic domain of gC interacts with other virion components on the inner surface of the inner nuclear membrane during budding to facilitate specific incorporation of gC into the virion envelope.

To assess the role of the gC cytoplasmic domain in these functions, we constructed a mutant gC gene which lacked the carboxyl-terminal 14 codons found in the wild-type gC gene. The deleted region encompassed the entire 11-amino-acid cytoplasmic domain and the carboxyl-terminal 3 amino acids of the 23-amino-acid transmembrane domain. Two recombinant viruses that expressed the mutant glycoprotein were isolated. These were designated *dl*1 and *dl*2. Continuous and pulse-chase labeling experiments established that a significant fraction of the gC produced by *dl*1 and *dl*2 was released from the infected cells into the medium. When the kinetics of release of *dl*2 gC were compared with those of the $gC^{-}3$ gC, which is truncated upstream of the transmembrane domain (17, 20), *dl*1 and *dl*2 gC were observed to be released into the medium much more slowly than $gC^{-}3$ gC. Surface labeling of infected cells showed that *dl*2 gC was expressed on the plasma membrane and that a significant fraction of the plasma membrane gC was released into the medium with kinetics similar to those of [^{35}S]methionine-pulse-labeled gC.

Our data, particularly the release of *dl*2 gC from the plasma membrane, indicate that deletion of the cytoplasmic domain significantly reduced the stability of anchoring of gC in the plasma membrane. It is possible that a second mechanism of release might also be operating. Since some, if not all, of the mutant gC is initially membrane bound, deletion of the cytoplasmic domain did not eliminate the stop transfer function of the glycoprotein. However, if the efficiency of the stop transfer function was reduced, some nascent glycoproteins would be released into the lumen of the rough endoplasmic reticulum and might subsequently be released into the medium. The difference in the kinetics of release of *dl*2 and $gC^{-}3$ gC rules out this mechanism for the appearance of *dl*2 gC in the medium, unless it is also assumed that *dl*2 gC is also delayed in transport from the Golgi apparatus to the plasma membrane. It also could be argued that the mutant gC was released from the membrane by proteolytic cleavage on the amino-terminal side of the transmembrane domain. However, the lack of any such cleavage of wild-type gC, the location of the altered domain on the opposite side of the membrane from the putative cleavage site, and the similarity of molecular weights of released and membrane-bound gC weigh against proteolytic cleavage. Thus, it appears that deletion of the cytoplasmic domain of gC resulted in a glycoprotein that was synthesized, processed, and transported to the cell surface normally. Without the cytoplasmic domain, the transmembrane domain alone did not provide permanent anchoring of the glycoprotein in the plasma membrane, and the mutant gC was slowly released into the medium.

A mutant glycoprotein with structure and properties similar to *dl*2 gC is the Friend spleen focus-forming virus (SFFV) glycoprotein (25). SFFV is a replication-defective retrovirus with two major lesions within its *env* gene. The first of these is a 585-base-pair in-frame deletion which removes the normal gp70-p15(E) cleavage site. The second, which is of importance here, is a 1-base-pair insertion resulting in premature termination of the *env* gene product immediately after the hydrophobic transmembrane domain. The glycoprotein encoded by the SFFV *env* gene retains the 30-amino-acid hydrophobic transmembrane domain of the murine leukemia virus *env* glycoprotein. However, the SFFV *env* glycoprotein has no cytoplasmic domain, whereas the murine leukemia virus gp70 cytoplasmic domain consists of 32 amino acids. The SFFV gene product is glycosylated and expressed on the plasma membrane, but over a period of approximately a day it is released from the plasma membrane into the medium (25).

Deletion and substitution mutations in the cytoplasmic domains of several other viral glycoproteins have been made, notably the influenza hemagglutinin, the vesicular stomatitis virus G glycoprotein, and the Semliki Forest virus E2 glycoprotein (2–5, 28). Release of these cytoplasmic domain mutants into the medium has not been reported, although some mutants of the Semliki Forest virus E2 glycoprotein and influenza hemagglutinin were more readily extracted from membranes by mild procedures that did not extract the wild-type glycoproteins.

Characterization of *dl*1 and *dl*2 also showed that the cytoplasmic domain was not required for incorporation of gC into virions. The presence of gC in *dl*1 and *dl*2 virions was shown by immunoprecipitation of the glycoprotein from purified virions, and by neutralization of mutants by gC-specific monoclonal antibodies plus complement. Virions acquire their membranes by budding through the inner nuclear membrane (26). This budding process has not been extensively studied, although it has been shown that virions do not contain significant quantities of host proteins (16). Host and viral proteins may segregate into different parts of the inner nuclear membrane. Possible mechanisms of segregation include direct interactions among the viral glycoproteins and interaction of the cytoplasmic domains of the glycoproteins with tegument or capsid proteins. The incorporation of *dl*1 and *dl*2 gC into virions rules out any essential interaction of the cytoplasmic domain of gC with other virion components during envelopment, although such interactions might occur for other HSV glycoproteins.

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LITERATURE CITED

1. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61**:714–721.
2. **Cutler, D. F., and H. Garoff.** 1986. Mutants of the membrane-binding region of Semliki Forest virus E2 protein. I. Cell surface transport and fusogenic activity. J. Cell Biol. **102**:889–901.
3. **Cutler, D. F., P. Melancon, and H. Garoff.** 1986. Mutants of the membrane-binding region of Semliki Forest virus E2 protein. II. Topology and membrane binding. J. Cell Biol. **102**:902–910.
4. **Doyle, C., M. G. Roth, J. Sambrook, and M. J. Gething.** 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. J. Cell Biol. **100**:704–714.
5. **Doyle, C., J. Sambrook, and M. J. Gething.** 1986. Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. J. Cell Biol. **103**:1193–1204.
6. **Draper, K. G., R. H. Costa, G. T.-Y. Lee, P. G. Spear, and E. K. Wagner.** 1984. Molecular basis of the glycoprotein C-negative phenotype of herpes simplex virus type 1 macroplaque strain. J. Virol. **51**:578–585.
7. **Eberle, R., and R. J. Courtney.** 1980. Preparation and charac-

terization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. **35**:902–917.
8. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for labeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
9. **Friedman, H., J. C. Glorioso, G. H. Cohen, J. C. Hastings, S. L. Harris, and R. J. Eisenberg.** 1986. Binding of complement component C3b to glycoprotein gC of herpes simplex virus type 1: mapping of gC-binding site and demonstration of conserved C3b binding in low-passage clinical isolates. J. Virol. **60**:470–475.
10. **Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner.** 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. **45**:634–647.
11. **Garoff, H.** 1985. Using recombinant DNA techniques to study protein targeting in the eukaryotic cell. Annu. Rev. Cell Biol. **1**:403–445.
12. **Gething, M. J., and J. Sambrook.** 1982. Construction of influenza hemagglutinin genes that code for intracellular and secreted forms of the glycoprotein. Nature (London) **300**:598–603.
13. **Gilmore, R., and G. Blobel.** 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. Cell **42**:497–505.
14. **Glorioso, J. C., M. Levine, T. C. Holland, and M. S. Szczesiul.** 1980. Mutant analysis of herpes simplex virus-induced cell surface antigens: resistance to complement-mediated immune cytolysis. J. Virol. **35**:672–681.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of human adenovirus 5 DNA. Virology **52**:456–467.
16. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus XII. The virion proteins of type 1 strains. J. Virol. **14**:640–651.
17. **Holland, T. C., F. L. Homa, S. D. Marlin, M. Levine, and J. Glorioso.** 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. **52**:566–574.
18. **Holland, T. C., S. D. Marlin, M. Levine, and J. C. Glorioso.** 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45**:672–682.
19. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (gamma2) gene lie within bases −38 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6**:3652–3666.
20. **Homa, F. L., D. J. M. Purifoy, J. C. Glorioso, and M. Levine.** 1986. Molecular basis of the glycoprotein C-negative phenotypes of herpes simplex virus type 1 mutants selected with a virus-neutralizing monoclonal antibody. J. Virol. **58**:281–289.
21. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
22. **Lathe, R., M. P. Kieny, S. Skory, and J. P. Lecocq.** 1984. Linker tailing: unphosphorylated linker oligonucleotides for joining DNA termini. DNA **3**:173–182.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Marlin, S. D., T. C. Holland, M. Levine, and J. C. Glorioso.** 1985. Epitopes of herpes simplex virus type 1 glycoprotein gC are clustered in two distinct antigenic sites. J. Virol. **53**:128–136.
25. **Pinter, A., and W. J. Honnen.** 1985. The mature form of the Friend spleen focus-forming virus envelope protein, gp65, is efficiently secreted from cells. Virology **143**:646–650.
26. **Roizman, B., and D. Furlong.** 1974. The replication of herpes viruses. Compr. Virol. **3**:229–403.
27. **Rose, J. K., and J. E. Bergman.** 1982. Expression from cloned cDNA of cell-surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eukaryotic cells. Cell **30**:753–762.
28. **Rose, J. K., and J. E. Bergman.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
29. **Sandri-Goldin, R. M., M. Levine, and J. C. Glorioso.** 1981. Method for induction of mutations in physically defined regions of the herpes simplex virus genome. J. Virol. **38**:41–49.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
31. **Spear, P. G.** 1976. Membrane proteins specified by herpes simplex virus. I. Identification of four glycoprotein precursors and their products in type-1 infected cells. J. Virol. **17**:991–1008.
32. **Steck, T. L., and J. Yu.** 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. J. Supramol. Struct. **1**:220–248.
33. **Stow, N. D., and N. M. Wilkie.** 1976. An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J. Gen. Virol. **33**:447–458.
34. **Walter, P., and V. R. Lingappa.** 1986. Mechanism of protein translocation across the endoplasmic reticulum membrane. Annu. Rev. Cell Biol. **2**:499–516.
35. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus *env* gene that affect the transport and subcellular location of the glycoprotein products. J. Cell Biol. **99**:2011–2023.