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Characterization of a Recombinant Herpes Simplex Virus Which Expresses a Glycoprotein D Lacking Asparagine-Linked Oligosaccharides

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Glycoprotein D (gD) is an envelope component of herpes simplex virus essential for virus penetration. gD contains three sites for addition of asparagine-linked carbohydrates (N-CHO), all of which are utilized. Previously, we characterized mutant forms of herpes simplex virus type 1 gD (gD-1) lacking one or all three N-CHO addition sites. All of the mutants complemented the infectivity of a gD-minus virus, F-gDβ, to the same extent as wild-type gD. Here, we show that recombinant viruses containing mutations in the gD-1 gene which eliminate the three N-CHO signals are viable. Two such viruses, called F-gD(QAA)-1 and F-gD(QAA)-2, were independently isolated, and the three mutations in the gD gene in one of these viruses were verified by DNA sequencing. We also verified that the gD produced in cells infected by these viruses is devoid of N-CHO. Plaques formed by both mutants developed more slowly than those of the wild-type control virus, F-gD(WT), and were approximately one-half the size of the wild-type. One mutant, F-gD(QAA)-2, was selected for further study. The QAA mutant and wild-type gD proteins extracted from infected cells differed in structure, as determined by the binding of monoclonal antibodies to discontinuous epitopes. However, flow cytometry analysis showed that the amount and structure of gD found on infected cell surfaces was unaffected by the presence or absence of N-CHO. Other properties of F-gD(QAA)-2 were quite similar to those of F-gD(WT). These included (i) the kinetics of virus production as well as the intracellular and extracellular virus titers; (ii) the rate of virus entry into uninfected cells; (iii) the levels of gB, gC, gE, gH, and gI expressed by infected cells; and (iv) the turnover time of gD. Thus, the absence of N-CHO from gD-1 has some effect on its structure but very little effect on its function in virus infection in cell culture.

It is well established that asparagine-linked carbohydrates (N-CHO) present on viral glycoproteins are important for the infectivity of herpes simplex virus (HSV) (35, 51, 64, 65). HSV has at least eight glycoproteins (54), of which glycoprotein B (gB), gD, and gH are essential for replication in cell culture and have been implicated in virus penetration (3, 4, 6, 11, 17, 18, 22, 36, 37, 57) and cell-to-cell fusion (18, 23, 32, 62, 63, 69). Of the remaining glycoproteins, gC (along with gB) has been implicated in virus entry at the attachment step (21).

gD contains three utilized sites (Asn-X-Ser/Thr) for addition of N-CHO. Previously (60, 61), we used oligonucleotide-directed mutagenesis to alter serine (S) or threonine (T) residues to alanine (A) at each N-CHO addition site of HSV type 1 (HSV-1) gD (gD-1) in order to assess the contribution of N-CHO to gD structure and function. Studies with monoclonal antibodies (MAbs) showed that a mutant form of gD lacking all three sites for addition of N-CHO (designated AAA) was structurally altered because of the amino acid change at residue 96 as well as the absence of the N-CHO (60). However, changing asparagine 94 to glutamine (Q) at N-CHO site 1 had less effect on gD structure. We constructed a triple mutant, QAA, which lacked all three N-CHO signals and found that the QAA protein most closely resembles gD produced in tunicamycin-treated cells in its binding to MAbs (61). In addition, wild-type gD and QAA proteins were equally susceptible to digestion by trypsin or *Staphylococcus aureus* V8 protease. In contrast, the AAA protein was more sensitive to trypsin but less sensitive to V8, again suggesting conformational alterations of the AAA protein. Surprisingly, we found that both forms of gD could rescue the infectivity of a gD-minus virus, F-gDβ, in a complementation assay (61). Those studies led to two conclusions: (i) that N-linked CHO on gD are not required for the infectivity of HSV-1 and (ii) that the structural changes observed in the AAA mutant, exhibited as differences in MAb binding and protease sensitivity, do not perturb the functional sites on gD. Furthermore, those studies predicted that viruses containing these mutations would be able to replicate normally in cells in culture.

To test this hypothesis, we carried out recombination experiments and isolated two viruses containing the QAA mutation. An attempt to isolate similar recombinants with the AAA mutation was unsuccessful. The recombinant viruses, F-gD(QAA)-1 and F-gD(QAA)-2, were able to grow on Vero cells, validating our hypothesis that N-CHO on gD are not required for its function in virus infection. However, the mutant plaques formed more slowly than those of the control wild-type virus, F-gD(WT), and at 20 h postinfection (p.i.) were only 50% as large as the wild-type plaques. The mutant and wild-type viruses were remarkably similar in all other properties studied, except that the mutant form of gD found in cytoplasmic extracts exhibited some differences in antigenic structure. However, MAb DL-11 (an antibody to a discontinuous epitope) bound equally well to gD present on the surface of cells infected with F-gD(QAA)-2 and F-gD(WT). We conclude that the N-CHO present on gD are not required for its biological role in HSV infection but

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contribute to some extent to the structure of gD and to efficient plaque formation.

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MATERIALS AND METHODS

Cell culture and viral strains. Five cell lines, CV-1, COS-1, Vero, VD60, and L, were used in this study. Conditions for their growth and maintenance have been described elsewhere (31, 36, 60). F-gDβ virus was propagated and titered in VD60 cells (31, 36). HSV-1 strain F and the two recombinant viruses, F-gD(QAA)-1 and F-gD(QAA)-2 were propagated and titered in Vero cells. Intracellular virus was harvested by scraping the cells into 5% Dulbecco's modified Eagle's medium (DMEM) and then disrupting them by one freeze-thaw cycle followed by sonication in a sonicating water bath (Ultrasonics). Cellular debris was removed by centrifugation at 350 × *g*. The virus stocks were stored at −70°C.

Plaque purification of F-gDβ virus. Stocks of F-gDβ virus contain a small fraction of wild-type virus probably due to recombination between the virus and the resident gD gene (strain F) in the VD60 cells used to maintain the virus (30). To reduce this low level of wild-type virus, which might confound the recombination experiments, F-gDβ was plaque purified by limiting dilution, and a viral stock was grown for one passage in VD60 cells. This low-passage F-gDβ viral stock was then used for recombination.

Isolation of F-gD(QAA)-1 and F-gD(QAA)-2 recombinant viruses. The gD genes containing the AAA and QAA triple mutations were inserted into a plasmid vector (46) which contained HSV-1 DNA sequences which flank the gD gene on either end (Fig. 1). The plasmids were linearized with *Sma*I and transfected into Vero cells. Five hours later, the cells were glycerol shocked, washed with DMEM, and allowed to recover in DMEM containing 5% fetal bovine serum (FBS) for 1 h. The cells were then infected with plaque-purified F-gDβ virus at a multiplicity of infection (MOI) of 0.2. After 18 h, intracellular virus was harvested and replated onto Vero cells. Plaques were observed on two separate plates which had been transfected with the gD gene containing the QAA triple mutation prior to infection by F-gDβ. No plaques were seen on control plates which had been mock transfected prior to infection. Several plaques were seen on plates transfected with the AAA gene prior to infection. Intracellular virus was harvested from each of the QAA- and AAA-transfected plates and plaque purified by limiting dilution on Vero cells. The resulting viruses were named F-gD(QAA)-1 and F-gD(QAA)-2. Plaques obtained from the AAA recombination turned out to be wild type (by Western immunoblot analysis).

Isolation of F-gD(WT). HSV-1 strain F forms nonsyncytial plaques on Vero cells, whereas F-gDβ, F-gD(QAA)-1, and F-gD(QAA)-2 form syncytial plaques. To obtain a control virus with this syncytial phenotype, we plaque purified the background wild-type virus present in the F-gDβ stock (30). This recombinant wild-type virus, called F-gD(WT), which also has a syncytial phenotype, was propagated in Vero cells, and a viral stock was grown.

DNA sequencing of recombinant viruses after DNA amplification. DNA from cells infected with the F-gD(QAA)-2 recombinant virus was amplified by the polymerase chain reaction (55) using primers with the following sequences: 5′ GGTTCTAGAGATCATCAGTTATCCTTAAGG 3′ and 5′ GGTTCTAGATACCGACTTATCGACTGTCCG 3′. Included in the sequences of the two primers used to amplify the gD gene were *Xba*I restriction enzyme sites at the 5′ ends. The resulting 1,400-bp fragment containing the entire gD gene was digested with *Xba*I, cloned into pUC19, and sequenced (56). A similar procedure was used to sequence F(gD)WT. It should be noted that the parent strain of F(gD)WT is strain F and that of F-gD(QAA)-2 is Patton. The gD protein sequences of these two strains were recently shown to be identical (6).

Antibodies. Rabbit polyclonal anti-gD serum R2 (26) was used for Western blotting (2) and for immunoprecipitation. Other gD-specific antibodies used included MAb DL-11 (group Ib [46]) and MAb DL-6 (group II [26, 45]), which were isolated in our laboratory (14). MAb ABD (group III [45]) was kindly provided by C. Desgranges. DL-11 and ABD recognize discontinuous epitopes on the gD protein (14, 44, 45, 58). DL-6 recognizes a continuous epitope within amino acid residues 272 to 279 of gD (26). Other antibodies to discontinuous epitopes that were tested in immunoblot assays (but not shown) included HD1, RIP, 11S, DL-1, DL-2, DL-15, 45S, and 1S (45).

In some cases, Western blots were probed with anti-gB (R69), anti-gC (R46), anti-gE (1BA10), anti-gH (R83), or anti-gD (R2) antiserum and then with iodinated protein A. R69, R46, R83, and R2 are polyclonal antisera to immunosorbent purified glycoproteins (15, 26, 53). MAb 1BA10 was kindly supplied by G. Dubin and H. Friedman.

Quantitation of antigenic activity in cell extracts. Cytoplasmic extracts prepared 14 to 16 h p.i. (9) were dotted onto nitrocellulose and then incubated with a gD-specific MAb and then with iodinated protein A (8). Antibody binding was quantitated as described previously (60). MAb DL-6 was used to normalize for the total amount of gD in each extract.

Enzyme digestions and Western blot analysis. Cytoplasmic extracts containing gD were digested with endo-β-*N*-acetylglucosaminidase F (endo F), which removes high-mannose and complex forms of N-linked sugars, as described elsewhere (60). Other samples were digested for 1 h with neuraminidase, which removed sialic acid, and then for 2 h with endo-*N*-acetyl-galactosamine hydrolase (*O*-glycan-peptide hydrolase or *O*-glycanase), which removes O-linked sugars, as described elsewhere (10, 60, 68). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins under denaturing conditions was performed in 10% acrylamide gels (10). The proteins were transferred to nitrocellulose (2) and probed with R2 (anti-gD) antibody and then with iodinated protein A (ICN Biomedicals, Inc., Irvine, Calif.).

Pulse-chase analysis of gD synthesized in infected cells. Vero cells in a 12-well plate were infected with F-gD(WT) or F-gD(QAA)-2 at an MOI of 5. At 5 h p.i., the medium was replaced with DMEM lacking methionine, and the cells were incubated for 1 h. The medium was again replaced with DMEM lacking methionine and containing 50 μCi of [^{35}S]methionine-cysteine (Tran^{35}S-label; ICN Biomedicals, Inc.) per well. After 15 min, cytoplasmic extracts were prepared (pulse). For the chase, pulse-labeled cells were washed with DMEM and incubated in unlabeled DMEM containing 5% FBS for 1, 3, 5, or 6 h. Cytoplasmic extracts were prepared, immunoprecipitated with R2 anti-gD serum, and electrophoresed on denaturing SDS-polyacrylamide gels (10% acrylamide) (45).

One-step viral growth curve. Vero cells in a 12-well plate were infected with F-gD(WT) or F-gD(QAA)-2 at an MOI of

3 in DMEM containing 5% FBS. After 1 h, the virus inoculum was removed and the cells were washed with DMEM, resuspended in fresh DMEM containing 5% FBS, and incubated at 37°C. At various times thereafter, the medium containing extracellular virus was removed, and intracellular virus was harvested as described above. Virus titers on Vero cells were determined.

Penetration assay. The rate of virus penetration at 37°C was measured by the procedure of Highlander et al. (22) as modified by Long et al. (38).

Analysis of viral glycoprotein expression by flow cytometry. To analyze gI expression, L cells were infected with F-gD(WT) or F-gD(QAA)-2 at an MOI of 5 and harvested 14 h p.i. by treatment with 1 mM EDTA. Flow cytometric analysis was performed with anti-gI MAb 3104 as described previously (13). The antibody was kindly supplied by D. Johnson.

To analyze gD expression, Vero cells were infected with F-gD(WT) or F-gD(QAA)-2 at an MOI of 5 and harvested 6 h p.i. by treatment with 1 mM EDTA. The cells were incubated with MAb DL-6 or DL-11 in DMEM containing 10% FBS for 30 min at 4°C, washed twice with phosphate-buffered saline (PBS) containing 0.1% sodium azide (to prevent capping), and then incubated with goat anti-mouse immunoglobulin G (IgG) fluorescein-labeled conjugate (diluted in PBS containing 10% FBS and 0.1% sodium azide) for 30 min at 4°C. The cells were washed twice with PBS containing 0.1% azide and fixed in 1% paraformaldehyde. Cells were examined for fluorescence staining with a FACSTARPlus flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). Cells which gave fluorescence signals brighter than those seen on 95% of the appropriate control cells were considered to be positive.

RESULTS

Isolation of recombinant viruses F-gD(QAA)-1 and F-gD (QAA)-2. Previously, we constructed and characterized mutants of gD-1 which were altered at N-CHO addition sites (60, 61). We determined that N-CHO are not required for biological activity of gD-1; the N-CHO mutant proteins, when provided in *trans*, could complement a gD-less virus (61). These earlier studies predicted that a virus recombinant containing a form of gD which lacked all N-CHO would be viable and might provide further insights into the role of the carbohydrate in gD function in virus infection. For this recombination, we chose the QAA gene because the QAA protein most closely resembled wild-type gD expressed in cells treated with tunicamycin (61). We first transfected Vero cells with a linearized plasmid containing the QAA gene and then infected the cells with F-gDβ virus (36) (Fig. 1). Only virus containing a functional gD gene replicates on Vero cells. Virus isolates designated F-gD(QAA)-1 and F-gD (QAA)-2 were obtained from two separate plates, and each was plaque purified. Sequence analysis (56) of the gD gene amplified by the polymerase chain reaction (55) verified that the three expected mutations were indeed present in the virus. No other changes were observed in the gD gene. A similar but unsuccessful attempt to derive recombinants with the AAA plasmid was made. As a control wild-type syncytial virus, F-gD(WT) was isolated from stocks of F-gDβ virus.

Analysis of the oligosaccharides present on gD in cells infected with F-gD(QAA) recombinants. Vero cells were infected with HSV-1 strain F, F-gD(WT), F-gD(QAA)-1, or F-gD(QAA)-2. Cytoplasmic extracts were prepared, incubated with various glycosidases, and then subjected to SDS-PAGE and Western blotting (Fig. 2). gD from HSV strain F or F-gD(WT) was susceptible to endo F and therefore contained N-CHO (Fig. 2A). In contrast, the mobility of gD from F-gD(QAA)-1 or F-gD(QAA)-2 was not altered. Thus, gD expressed in cells infected by either of these two strains did not contain N-CHO.

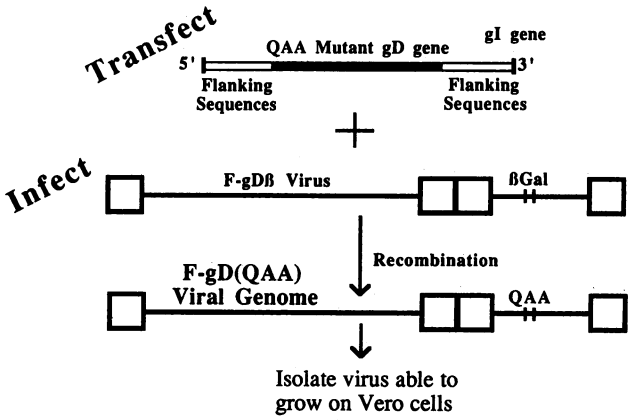

FIG. 1. Recombination of the QAA gene with the F-gDβ genome. The QAA triple mutation was inserted into the flanking sequence vector (46), linearized with the restriction enzyme *SmaI*, and transfected into Vero cells. After 5 h, the cells were glycerol shocked and allowed to recover in medium containing 5% FBS for 1 h. The cells were then infected with plaque-purified F-gDβ virus. After 18 h, intracellular virus was harvested and plated onto a second confluent plate of Vero cells, where recombinant virus would be able to form plaques.

We next examined the types of oligosaccharides present on the wild-type and QAA proteins. Cytoplasmic extracts were digested with neuraminidase and then with *O*-glycanase prior to electrophoresis and Western blotting (Fig. 2B). As previously observed in transfected cells (61), the mobilities of both the wild-type and QAA proteins were altered after enzyme treatment. Since the QAA protein contains no N-CHO, the shift in mobility must be due to the removal of sialic acid attached to *O*-glycans. We conclude that the QAA

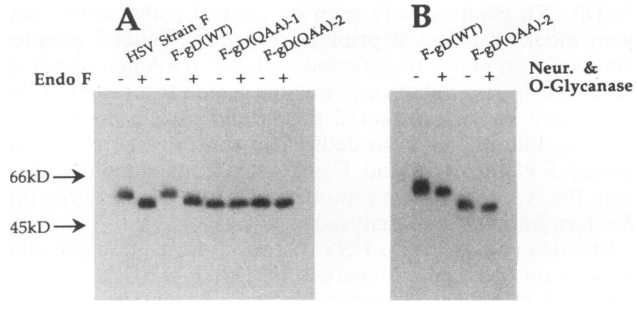

FIG. 2. Western blot analysis of gD-1 from infected cells. Cytoplasmic extracts were prepared from Vero cells 24 h after infection with HSV-1 strain F, F-gD(WT), F-gD(QAA)-1, or F-gD(QAA)-2, electrophoresed on a 10% acrylamide denaturing gel, transferred to nitrocellulose, and then reacted with anti-gD-1 serum and iodinated protein A. (A) Extracts were incubated with endo F (+) for 1 h to remove N-CHO or were untreated (−). (B) Extracts were diluted 1:1 with distilled water and then were treated with neuraminidase (Neur.) for 1 h and *O*-glycanase for 3 h (+) to remove sialic acid and remaining O-linked carbohydrate or were untreated (−).

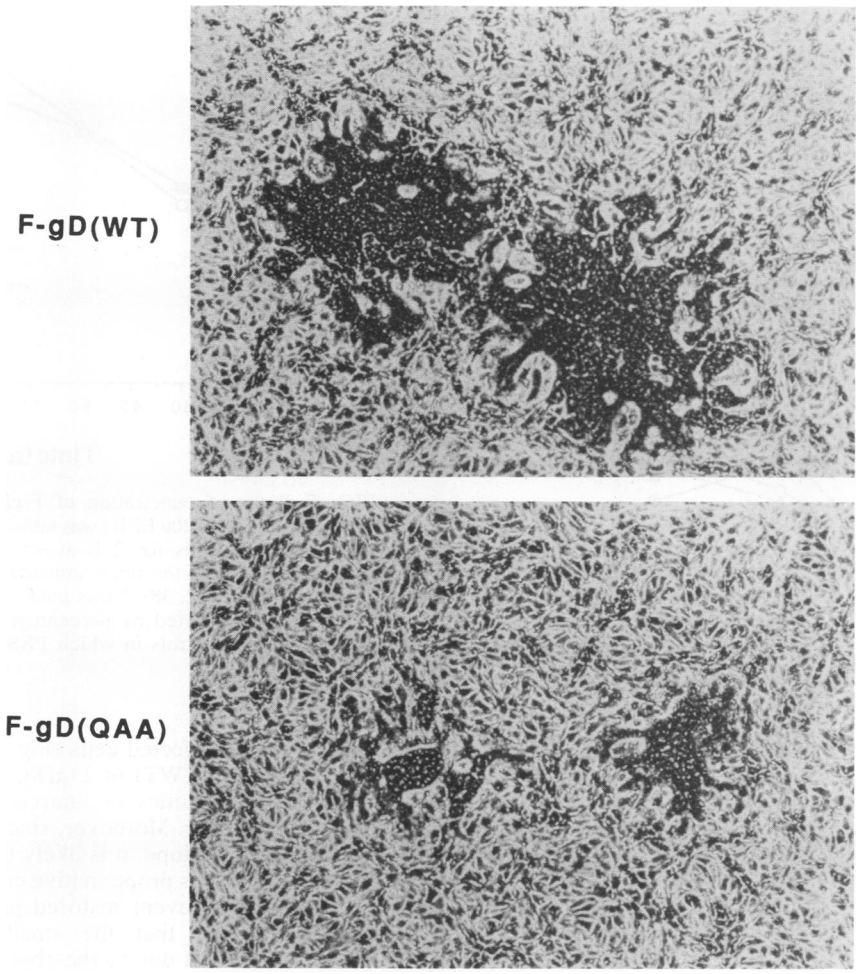

FIG. 3. Syncytial plaque formation by F-gD(WT) and F-gD(QAA)-2 on Vero cells. Confluent monolayers of Vero cells in 35-mm^2 dishes were infected with 50 PFU of virus. Photographs were taken at 20 h p.i. with a Nikon phase-contrast microscope at a magnification of ×100. Essentially the same results were seen with F-gD(QAA)-1 (not shown) as with F-gD(QAA)-2.

protein produced in infected cells is modified through the addition of *O*-glycans.

Plaque formation by F-gD(WT) and F-gD(QAA)-2. Both the F-gD(WT) and F-gD(QAA) viruses form syncytial plaques (as does the F-gDβ parent [30, 31, 36]). We observed that both mutants formed small plaques. The plaques formed by F-gD(QAA)-2 on Vero cells had a circumference which was less than half of that formed by F-gD(WT) (Fig. 3). On CV-1 cells there was a greater range of plaque sizes; however, the average F-gD(QAA)-2 plaque on CV-1 cells was smaller than the average F-gD(WT) plaque (data not shown). The results suggest that the small plaque size may be associated with the absence of N-CHO on gD. Alternatively, the change in plaque size might be a secondary consequence of the mutations in gD.

To determine whether the absence of N-CHO on gD affected other parameters of viral infection, we compared the following properties of F-gD-(WT) and F-gD(QAA)-2: (i) the ability to grow in cultured cells, (ii) the rate of virus penetration, (iii) expression of viral glycoproteins and other viral proteins, (iv) stability of QAA gD in infected cells, and (v) the antigenic structure of QAA gD in the cytoplasm and on the plasma membrane of infected cells.

One-step growth curve of F-gD(QAA)-2. To determine the rate and amount of virus production, we compared the one-step growth curves of F-gD(WT) and F-gD(QAA)-2 (Fig. 4). In two separate experiments (Fig. 4A and B), the time course of virus appearance and the amount of both cell-associated and extracellular virus were similar for F-gD(WT) and F-gD(QAA)-2. Other HSV small-plaque mutants, such as those which contain an alteration in the UL24 gene, exhibit a reduction in viral burst size which explains the small plaque size (28). In one experiment (Fig. 4A), there appeared to be a small difference (less than one log unit) between the burst size of extracellular F-gD(QAA)-2 and that of the wild-type control. However, in a second experiment (Fig. 4B), there was no difference. Thus, the variation seen in Fig. 4A is probably within the range of experimental error. We conclude that the small-plaque phenotype of F-gD(QAA)-2 does not result from a reduction in the rate of virus production or the total amount of virus produced.

Role of N-CHO on gD-1 in viral penetration. gD is involved in HSV-1 penetration (18, 22, 31) and cell-to-cell fusion (43, 48). We speculated that if cell-to-cell fusion is partially inhibited in the F-gD(QAA)-2 virus, then the rate of penetration of the virus into cells might also be inhibited. We

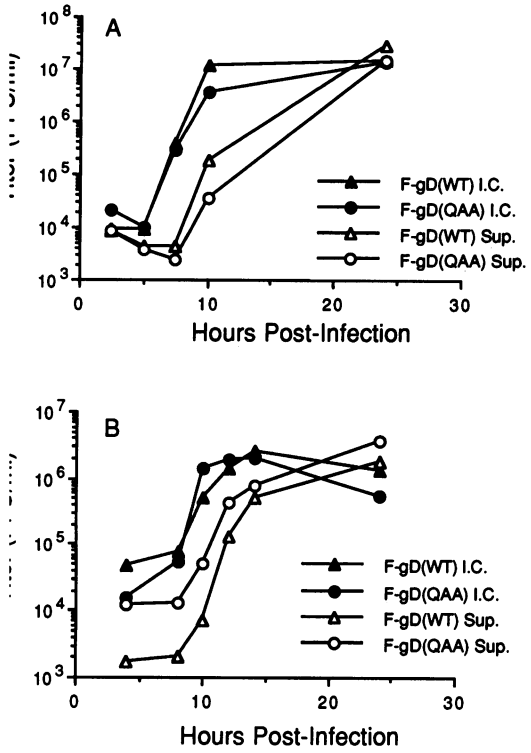

FIG. 4. One-step viral growth curves of F-gD(WT) and F-gD(QAA)-2 in Vero cells. Vero cells were infected at an MOI of 5 PFU per cell. At various times p.i., the supernatant (Sup.) was removed and the intracellular virus (I.C.) was collected by scraping the cells into medium containing 5% FBS. Intracellular virus was released by one freeze-thaw cycle followed by sonication in a sonicating water bath (Ultrasonics). Virus titers on Vero cell monolayers were determined. Panels A and B show the results of two separate experiments.

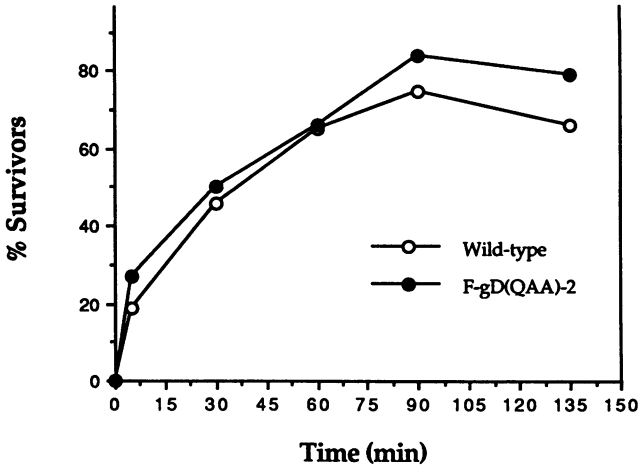

FIG. 5. Rates of penetration of F-gD(WT) and F-gD(QAA)-2 viruses. Virus (100 to 200 PFU) was added to Vero cells and allowed to adsorb to the cells for 2 h at 4°C. The cultures were then incubated at 37°C. At the times indicated, cells were washed with acid-glycine buffer (22, 38). Virus plaques were counted after 24 h. The results are reported as percentages of plaques surviving in comparison with controls in which PBS was substituted for acid-glycine buffer.

compared the ability of F-gD(WT) and F-gD(QAA)-2 to penetrate cells at 37°C. We found no significant difference between the rates of penetration of F-gD(WT) and F-gD(QAA)-2 (Fig. 5); in both cases, 50% of the input virus became resistant to acid inactivation within 30 to 40 min of the beginning of incubation. We conclude that the absence of N-CHO on gD has no apparent effect on the rate of virus penetration.

Expression of viral glycoproteins (and other proteins) by F-gD(QAA)-2. The exact role of each HSV glycoprotein in cell-to-cell fusion has not been established. Because gD has been implicated in this process (43, 48), it seemed reasonable that the altered gD protein was responsible for the small-plaque phenotype of F-gD(QAA)-2 because cell-to-cell fusion was less efficient for this virus than for F-gD(WT). However, it is possible that the expression of other viral glycoproteins was somewhat altered in the F-gD(QAA)-2 recombinant. For example, in addition to lacking the entire gD gene, the parent virus F-gDβ also lacks part of the gI gene and does not express gI protein (13, 31, 36). Thus, the small-plaque phenotype of F-gD(QAA)-2 could be due to the absence of gI. The flanking sequence vector used in the recombination contains the missing portion of the gI gene, and therefore F-gD(QAA)-2 should contain an intact gI gene. MAb 3104 does not react with gI on Western blots (12), so we employed flow cytometry to assay for the presence of gI at the surface of infected cells (Fig. 6). Virtually every cell infected with F-gD(WT) or F-gD(QAA)-2 expressed gI. In addition, the intensities of fluorescence (x axis) for both viruses were similar. Moreover, since MAb 3104 reacts with a discontinuous epitope, it is likely that the gI expressed by F-gD(QAA)-2 has its proper native conformation. Therefore, the recombination event restored proper expression of gI, and we conclude that the small-plaque phenotype of F-gD(QAA)-2 is not due to the absence of gI.

We also examined the expression of other HSV glycoproteins in F-gD(QAA)-2-infected cells. Cytoplasmic extracts from equivalent numbers of F-gD(WT)- and F-gD(QAA)-2-infected cells were subjected to SDS-PAGE and Western blotting. The blots were probed with polyclonal antisera that react specifically with either gB, gC, gE, gH, or gD (Fig. 7). No difference between the two viruses was seen in the amount of any of these glycoproteins. Moreover, the mobil-

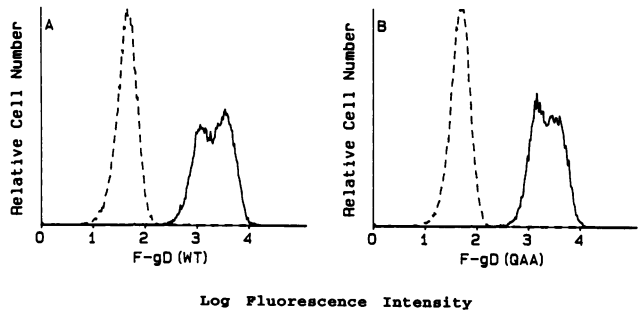

FIG. 6. Expression of gI at the surface of infected cells as demonstrated by flow cytometry. L cells infected with either F-gD(WT) or F-gD(QAA)-2 were harvested at 14 h p.i. and treated with anti-gI MAb 3104 followed by FITC-conjugated goat anti-mouse IgG (——) or treated only with FITC-conjugated goat anti-mouse IgG (– – –) (13). Uninfected cells labeled with antibody and FITC conjugate were negative (not shown).

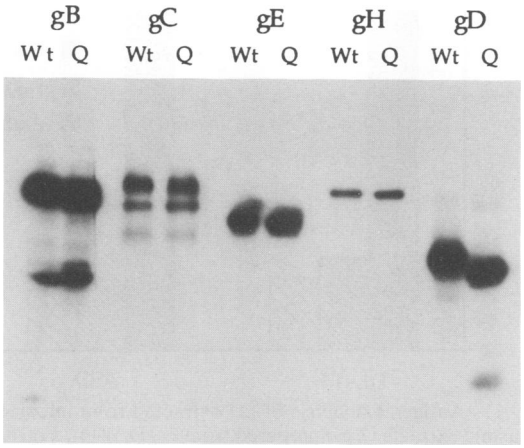

FIG. 7. Western blot analysis of gB, gC, gE, gH, and gD from F-gD(WT)- and F-gD(QAA)-2-infected cells. Cytoplasmic extracts were prepared from Vero cells 14 h p.i. with either F-gD(WT) or F-gD(QAA)-2, electrophoresed on a 10% polyacrylamide denaturing gel, transferred to nitrocellulose, and then reacted first with antiserum to either gB, gC, gD, gE, or gH and then with iodinated protein A.

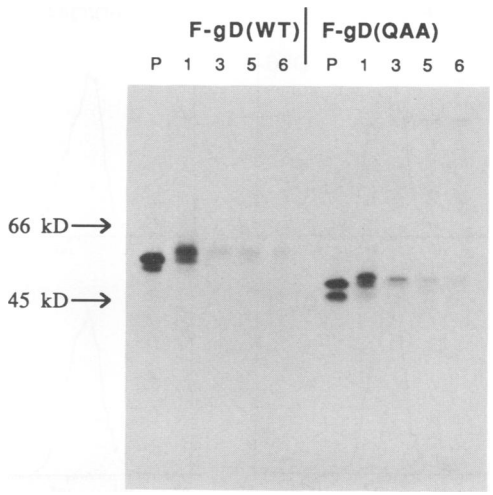

FIG. 9. Pulse-chase analysis of gD proteins from infected cells. Vero cells were infected with either F-gD(WT) or F-gD(QAA)-2, pulse-labeled (P) with Tran^{35}S-label for 15 min, and then chased in unlabeled medium for 1, 3, 5, or 6 h. Extracts were immunoprecipitated with R2 polyclonal anti-gD-1 serum and electrophoresed on a 10% polyacrylamide denaturing gel.

ities of glycoproteins other than gD were similar for F-gD(WT) and F-gD(QAA)-2, suggesting that their patterns of processing are similar.

To determine whether any other viral (or virus-induced) protein was altered after infection by the mutant, we compared the patterns of total proteins synthesized by cells infected with F-gD(WT) and F-gD(QAA)-2. Cells were labeled for 15 min with Tran^{35}S-label at either 2.5 or 6 h p.i., and cytoplasmic extracts were prepared. Samples were examined on SDS–10% polyacrylamide gels (Fig. 8). At 2.5 h p.i., there was no discernible difference between the labeling pattern of the mutant-infected cells and that of the wild-type infected cells (compare lanes 1 and 2). However, at 6 h p.i. (lanes 3 and 4), the patterns differed in the position of a band with the mobility of gD. For the wild type (lane 3), this band was of the expected size in the pulse-labeled sample, and it chased into a polypeptide with the expected size of the mature form of gD (not shown). For F-gD(QAA)-2, a smaller band was seen in the pulse-labeled extract (lane 4), and it chased into a larger band (not shown). The smaller band seen in lane 3 had the expected size of the QAA protein and also exhibited the same intensity of labeling as wild-type gD. We conclude that with the exception of gD, all of the viral proteins that incorporate enough label to be seen on the gel are identical in size and amount in extracts of cells infected by the two viruses.

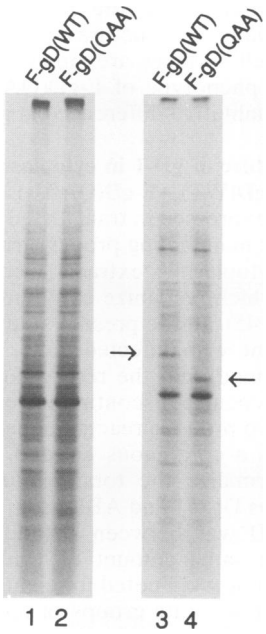

FIG. 8. Analysis of proteins synthesized after infection with F-gD(WT) or F-gD(QAA)-2. Vero cells were infected with 5 PFU of either F-gD(WT) (lanes 1 and 3) or F-gD(QAA)-2 (lanes 2 and 4) per cell. At either 2.5 h p.i. (lanes 1 and 2) or 6 h p.i. (lanes 3 and 4), the medium was removed and the cells were labeled for 15 min with Tran^{35}S-label. Cytoplasmic extracts were prepared and electrophoresed on a 10% polyacrylamide denaturing gel.

Role of N-CHO in gD-1 stability after synthesis. One role ascribed to N-CHO is protection of proteins from proteolytic degradation (7, 49, 52, 66, 70). We noted that the QAA protein was no more susceptible to protease digestion than wild-type gD in transfected cells (61). However, it is possible that the absence of N-CHO leads to an increase in the rate of QAA gD degradation in infected cells, and this in turn might reduce the amount of protein available at the cell surface for participation in cell-to-cell fusion. To test this possibility, we carried out a pulse-chase experiment comparing the kinetics of gD synthesis, processing, and turnover in cells infected with the mutant virus and cells infected with the wild-type virus (Fig. 9). The processing observed for the QAA protein after 1 h of chase was due to the addition of O-linked glycans (Fig. 2B). The amount of label present in each protein declined dramatically, starting at 3 h of chase (9 h p.i.), and was nearly undetectable after 6 h of chase (12 h p.i.). We found that there was slightly more of the QAA gD than the wild-type gD left after 5 h of chase (11 h p.i.) (Fig. 9). This suggests that the turnover rate of the mutant gD is not any

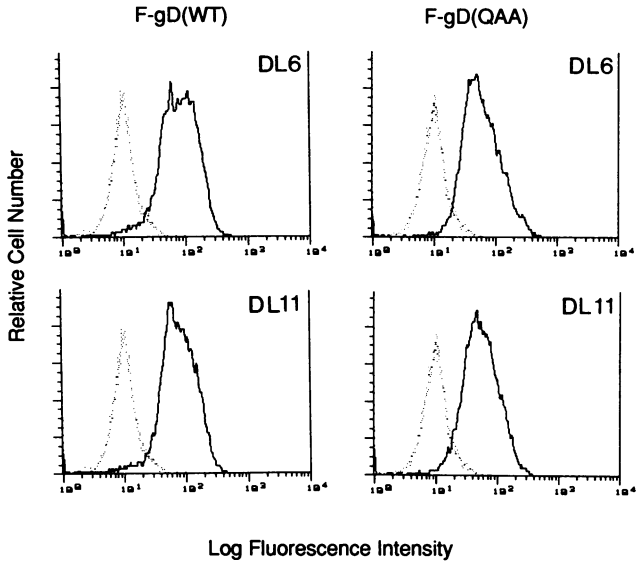

FIG. 10. Expression of gD at the surface of infected cells as demonstrated by flow cytometry. Vero cells infected with either F-gD(WT) or F-gD(QAA)-2 were harvested at 6 h p.i. and treated with MAb DL-6 or DL-11 followed by FITC-conjugated goat anti-mouse IgG (——) or treated only with FITC conjugated goat anti-mouse IgG (· · · · ·). The mean fluorescence values for the infected cells were as follows: DL-6 + F-gD(WT), 504; DL-11 + F-gD(WT), 499; DL-6 + F-gD(QAA)-2, 482; and DL-11 + F-gD(QAA)-2, 466. Infected cells incubated with conjugate alone were negative (not shown). In a second experiment, a third negative control consisted of uninfected cells treated with both antibody and conjugate.

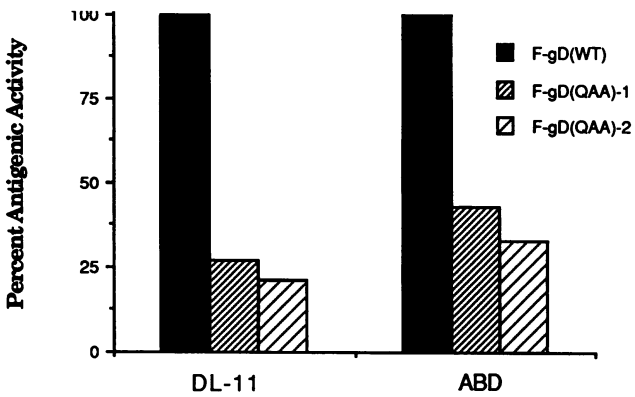

FIG. 11. Antigenic analysis of gD extracted from infected cells. Cytoplasmic extracts (2 μl) prepared from F-gD(WT)-, F-gD(QAA)-1-, or F-gD(QAA)-2-infected cells were dotted onto nitrocellulose. Each nitrocellulose strip was incubated with a MAb (DL-6, DL-11, or ABD) and iodinated protein A. The dots were cut out, the amount of radioactivity was determined in a gamma ray counter, and the antigenic activity was quantitated (60). DL-6 recognizes a continuous epitope within residues 272 to 279 (22); DL-11 (group Ib) and ABD (group III) recognize discontinuous epitopes (14, 44, 45, 58).

higher and may actually be lower than the turnover rate of wild-type gD. There are two ways in which the label would be expected to decline at 9 to 11 h p.i.: (i) from export of the protein as part of the virus, which begins at about 8 to 10 p.i., and (ii) from turnover of the protein by intracellular degradation. Since extracellular virus production occurred at similar rates for the two viruses (Fig. 4), the pulse-chase results suggest that the degradation rates of the two proteins are similar or that the rate for wild-type gD is slightly greater. We conclude that the absence of N-CHO probably does not decrease the amount of gD available for transport to the cell surface.

Role of N-CHO in gD-1 cell surface transport. The pulse-chase study provided only suggestive evidence that gD lacking N-CHO is transported efficiently to the infected cell surface. The absence of N-CHO from the G protein of vesicular stomatitis virus does not alter its stability (40) but does cause the protein to aggregate intracellularly (42) and also inhibits its cell surface transport (41). We observed that gD lacking N-CHO could be detected at the surface of transfected cells (60), but we did not quantitate the amount of protein transported. In the present work, we used flow cytometry to study the levels and the antigenic structure of mutant and wild-type gD present at the surface of infected cells (Fig. 10). MAb DL-6 (Fig. 10) was used to examine the total amount of gD, and two MAbs to discontinuous epitopes, DL-11 (group Ib) (Fig. 10) and DL-1 (group VI) (data not shown) were used to compare the structure of gD. The gD-specific cell surface fluorescence (x axis) of both the F-gD(WT)- and F-gD(QAA)-2-infected cells was at least 10-fold higher than that found on infected cells incubated with only a fluorescein isothiocyanate (FITC) anti-mouse conjugate (Fig. 10). The peak levels of fluorescence were quite similar for the F-gD(WT)- and F-gD(QAA)-2-infected cells when either DL-6 or DL-11 was used as the MAb. Similar levels of fluorescence for mutant- and wild-type-infected cells were also found with MAb DL-1 (data not shown). This experiment was repeated twice with essentially the same results. Thus, the amount of gD found on the cell surface is not affected by the presence or absence of N-CHO. In addition, the structures of mutant and wild-type gD on infected-cell surfaces are similar. We conclude that the small-plaque phenotype of F-gD(QAA)-2 is not due to quantitative or qualitative differences in gD found on infected-cell surfaces.

Antigenic structure of gD-1 in cytoplasmic extracts of cells infected with F-gD(WT), F-gD(QAA)-1, or F-gD(QAA)-2. N-CHO on gD-1 expressed in transfected cells are important for folding and/or maintaining proper structure (60, 61). For those studies, cytoplasmic extracts were analyzed with a panel of MAbs which recognize continuous and discontinuous epitopes (14, 45). In the present study, we carried out a similar experiment with infected cells. Binding of eleven MAbs was examined, and the results for DL-11 and ABD (both of which recognize discontinuous epitopes) are shown in Fig. 11. The two proteins reacted equally well with DL-6, which recognizes a continuous epitope, and this antibody was used to normalize the total amount of gD in each extract. For MAbs DL-11 and ABD, the reactivity of QAA-1 gD or QAA-2 gD was between 18 and 42% of the level observed for the same amount of wild-type gD. Similar reductions in binding were noted for eight other MAbs tested (representing four separate groups of antibodies to discontinuous epitopes) (data not shown) (14, 40). The reduced binding indicates that the overall antigenic structure of the QAA gD protein which accumulates in the infected cells is altered. The results suggest that N-CHO play an important role in the proper folding of gD. However, since the protein which reached the cell surface did not exhibit these alterations (Fig. 10), we cannot relate the small-plaque phenotype

to differences in the antigenic structure of gD. The results suggest that the protein which does get transported to the cell surface is properly folded, whereas the total protein which is present in the cytoplasm is a mixture of properly and improperly folded gD. Alternatively, extraction of the cells to prepare the cytoplasmic extracts may have more deleterious effects on gD molecules which lack N-CHO than on gD which is N glycosylated.

DISCUSSION

The studies presented in this article prove that the N-CHO on gD are not necessary for its function in virus infection. In previous studies (60, 61), we constructed mutant forms of gD which lacked one or all three N-CHO and studied their properties in transiently transfected cells. Using a complementation assay, we found that all of the mutant forms were able to rescue the infectivity of a gD-minus virus (61). These results predicted that one should be able to isolate a recombinant virus in which gD-1 would lack all N-CHO. This indeed was the case, and two mutants, designated F-gD (QAA)-1 and F-gD(QAA)-2, were isolated. The latter virus was studied in detail and exhibits few differences from the wild-type control. Although this result may seem surprising, it is important to note that the gp50 glycoprotein of pseudorabies virus, a gD homolog, has no N-CHO (50). The amino acid sequences of gD and gp50 show only limited homology (50); however, the spacing of cysteine residues in gp50 is highly conserved compared with gD, implying that the proteins are structurally similar. Thus, it is possible that N-CHO are not critical for function of any other gD homolog in other herpesviruses. Indeed, there is variability in the number and position of N-CHO in those gD homologs of other herpesviruses which have been sequenced (16, 33, 67).

One interesting difference was that for F-gD(QAA)-1 and F-gD(QAA)-2, the syncytial plaques formed more slowly on Vero cells than those of F-gD(WT). An HSV small-plaque mutant which contains an alteration in the UL24 gene shows a concomitant reduction in burst size (28). In contrast, F-gD (QAA)-2 produced the same amount of intracellular virus as F-gD(WT). Another possible reason for the smaller syncytial plaque size of F-gD(QAA)-2 is that cells infected by the mutant virus are less efficient in cell-to-cell fusion. The concept that the N-CHO on gD are important for cell-to-cell fusion relates our work with two previous sets of observations: (i) MAbs to gD inhibit fusion of infected cells (43, 48), and (ii) N-CHO are important for cell-to-cell fusion of HSV-infected cells (5, 19, 20). Fusion of infected cells was inhibited by treatment with tunicamycin (20) or 2-deoxy-D-glucose (19) and was found to be altered in a cell line defective in *N*-acetylglucosaminyltransferase I activity (needed for processing of N-CHO) (5).

Other studies have implicated gD in viral penetration (18, 22, 31, 36). The possibility that cell-to-cell fusion and viral penetration involve a similar mechanism prompted us to assess the ability of F-gD(QAA)-2 to penetrate uninfected cells. However, the rate of penetration of F-gD(WT) is similar to that of F-gD(QAA)-2, indicating that there is no correlation between plaque size and penetration efficiency. However, the penetration assay may have failed to detect subtle differences in the ability of the mutated protein to carry out its function in virus entry. gD-1 is believed to bind a high-affinity receptor at the cell surface, an interaction which then promotes viral penetration (31, 36). It is possible that the absence of N-CHO from gD-1 reduces the affinity of gD for its putative receptor. In the penetration assay, complete binding might still be able to take place during the 2-h preincubation at 4°C. Identification of the receptor to which gD-1 binds is needed in order to properly assess the role of N-CHO in this interaction.

For other glycoproteins, N-CHO serve a protective role, and their absence causes an increase in protein degradation (49, 52, 66, 70). However, we found that the QAA protein was as stable as wild-type gD in infected cells at 6 h p.i. In other studies, we found the QAA protein is no more sensitive to proteolysis by trypsin or *S. aureus* V8 protease than wild-type gD (61). We conclude that the N-CHO on gD-1 do not serve a role in preventing protein turnover. Furthermore, the small plaque size of F-gD(QAA)-2 is not accounted for by an increased rate of QAA gD degradation.

We did note differences in the antigenic structure of the QAA protein in cytoplasmic extracts. We found that the binding of MAbs to a variety of discontinuous epitopes of the QAA protein was diminished compared with that of wild-type gD and was comparable to that found for gD produced in tunicamycin-treated transfected cells (60, 61). However, using flow cytometry to examine the amount and the quality of gD on infected-cell surfaces, we found that binding of two MAbs to discontinuous epitopes on gD was unaffected by the absence of N-CHO. This result implies that although some misfolded gD is present in the cytoplasm, only properly folded gD is transported to the cell surface. In other systems, it has been demonstrated that misfolded proteins are retained in the endoplasmic reticulum, often in association with resident proteins such as heavy-chain binding protein (BiP) (24, 25, 39). It would be of interest to determine whether BiP associates permanently with a fraction of the QAA protein present in cell extracts. We also observed that the QAA protein is present at the surface of infected cells at a level comparable to that of wild-type gD. In contrast to our results, it has been reported that N-CHO are necessary for transport and cell surface expression of simian virus 5 hemagglutinin-neuraminidase (47) and vesicular stomatitis virus G (40, 41) protein.

Although it is devoid of N-linked sugars, the QAA gD protein contains O-linked oligosaccharides. gD-1 has been shown to contain two *O*-glycans, and gD-2 contains three (59). These appear to be present in the carboxyl half of each protein; however, their precise locations are unknown. It is of interest to note that gp50, the gD homolog of pseudorabies virus which lacks N-CHO, has also been shown to contain *O*-glycans (50). The possible importance of O-glycans for the structure and function of gD has yet to be assessed. In the case of the HA glycoprotein of influenza virus, it has been proposed that O-glycans are necessary to maintain a stiff extended stalk structure (29). We are now in the process of mapping the O-glycans of the wild-type and QAA forms of gD. Once these are located, mutagenesis techniques will be used to eliminate these sites to determine the effect of their absence on gD structure and function. Further experiments are also planned to determine the transport characteristics of the QAA form of gD by using a cell line which is defective in the addition of O-glycans (34).

The fact that every isolate of HSV-1 and HSV-2 in which gD has been sequenced shows that the protein contains the three N-CHO addition sites is evidence that the N-CHO on gD may be important for HSV pathogenesis. Recent work implicates gD in HSV neuroinvasiveness (27). Another HSV variant with a small-plaque morphology exhibits reduced pathogenicity (1), providing additional impetus for investigating the pathogenesis of F-gD(QAA).

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