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High-Level Expression and Purification of Secreted Forms of Herpes Simplex Virus Type 1 Glycoprotein gD Synthesized by Baculovirus-Infected Insect Cells

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Two forms of herpes simplex virus glycoprotein gD were recombined into *Autographa californica* nuclear polyhedrosis virus (baculovirus) and expressed in infected *Spodoptera frugiperda* (Sf9) cells. Each protein was truncated at residue 306 of mature gD. One form, gD-1(306t), contains the coding sequence of Patton strain herpes simplex virus type 1 gD; the other, gD-1(QAAt), contains three mutations which eliminate all signals for addition of N-linked oligosaccharides. Prior to recombination, each gene was cloned into the baculovirus transfer vector pVT-Bac, which permits insertion of the gene minus its natural signal peptide in frame with the signal peptide of honeybee melittin. As is the case with many other baculovirus transfer vectors, pVT-Bac also contains the promoter for the baculovirus polyhedrin gene and flanking sequences to permit recombination into the polyhedrin site of baculovirus. Each gD gene was engineered to contain codons for five additional histidine residues following histidine at residue 306, to facilitate purification of the secreted protein on nickel-containing resins. Both forms of gD-1 were abundantly expressed and secreted from infected Sf9 cells, reaching a maximum at 96 h postinfection for gD-1(306t) and 72 h postinfection for gD-1(QAAt). Secretion of the latter protein was less efficient than gD-1(306t), possibly because of the absence of N-linked oligosaccharides from gD-1(QAAt). Purification of the two proteins by a combination of immunoaffinity chromatography, nickel-agarose chromatography, and gel filtration yielded products that were >99% pure, with excellent recovery. We are able to obtain 20 mg of purified gD-1(306t) and 1 to 5 mg of purified gD-1(QAAt) per liter of infected insect cells grown in suspension. Both proteins reacted with monoclonal antibodies to discontinuous epitopes, indicating that they retain native structure. Use of this system for gD expression makes crystallization trials feasible.

Glycoprotein gD of herpes simplex virus (HSV) is a component of the virion envelope which plays an essential role in HSV entry into susceptible mammalian cells (28, 48). The evidence to date suggests that gD binds to a cellular molecule, possibly the mannose-6-phosphate receptor (25), following the initial interaction of other HSV glycoproteins, gC and/or gB, with heparan sulfate proteoglycans (23, 26, 27, 48). It was proposed that the interaction between gD and the gD receptor stabilizes the virus-cell complex prior to membrane fusion which is mediated by other essential glycoproteins, such as gB and gH, but possibly also including gD (20, 27, 48). In support of this interaction, it was shown that truncated forms of HSV-1 gD (gD-1t, truncated at residue 275) and HSV-2 gD (gD-2t, truncated at residue 302) both directly bind to cells and also block the binding of virus to cells (26). Using a complementation assay, it was shown that gD-2 can replace gD-1 in the infection process (36), also suggesting that the two glycoproteins interact with the same receptor on Vero cells. Both proteins induce production of neutralizing antibody and stimulate a cross-protective immune response in animal models (reviewed in references 3, 4, 10, 30, 35).

Structural studies of gD of HSV have relied on immunological, biochemical, and genetic approaches to establish working models of this essential protein (10, 15, 18, 32, 37). It is clear that X-ray crystallographic studies are required to precisely define its three-dimensional structure. From the experience of other investigators, it is generally accepted that the likelihood of obtaining X-ray diffraction quality crystals of a glycoprotein such as gD is increased if four conditions are met: (i) the protein is truncated prior to the hydrophobic transmembrane region (TMR); (ii) the protein retains its functionally active or native structure; (iii) sufficient material is available for multiple experiments; and (iv) a single molecular form of the protein can be isolated (i.e., it should not be heterogeneous with respect to carbohydrate or other posttranslational modifications).

To achieve high levels of expression and secretion of gD, we used the insect cell-baculovirus expression system (34, 38, 49, 50), substituting the coding region of the first 306 amino acids of the gD gene (not counting the signal peptide) for the baculovirus polyhedrin gene. Several other laboratories have reported using this system to successfully express full-length gD in large quantity (22, 29, 30). We used the *Autographa californica* nuclear polyhedrosis virus (baculovirus) transfer vector pVT-Bac (50) to insert the gD gene into baculovirus. This vector, which is derived from pAc373 (44), contains the polyhedrin promoter and sequences which normally flank the polyhedrin gene. Of significance, it contains the signal peptide sequence for honeybee melittin and cloning sites adjacent to this sequence which permit in-frame fusion of the coding

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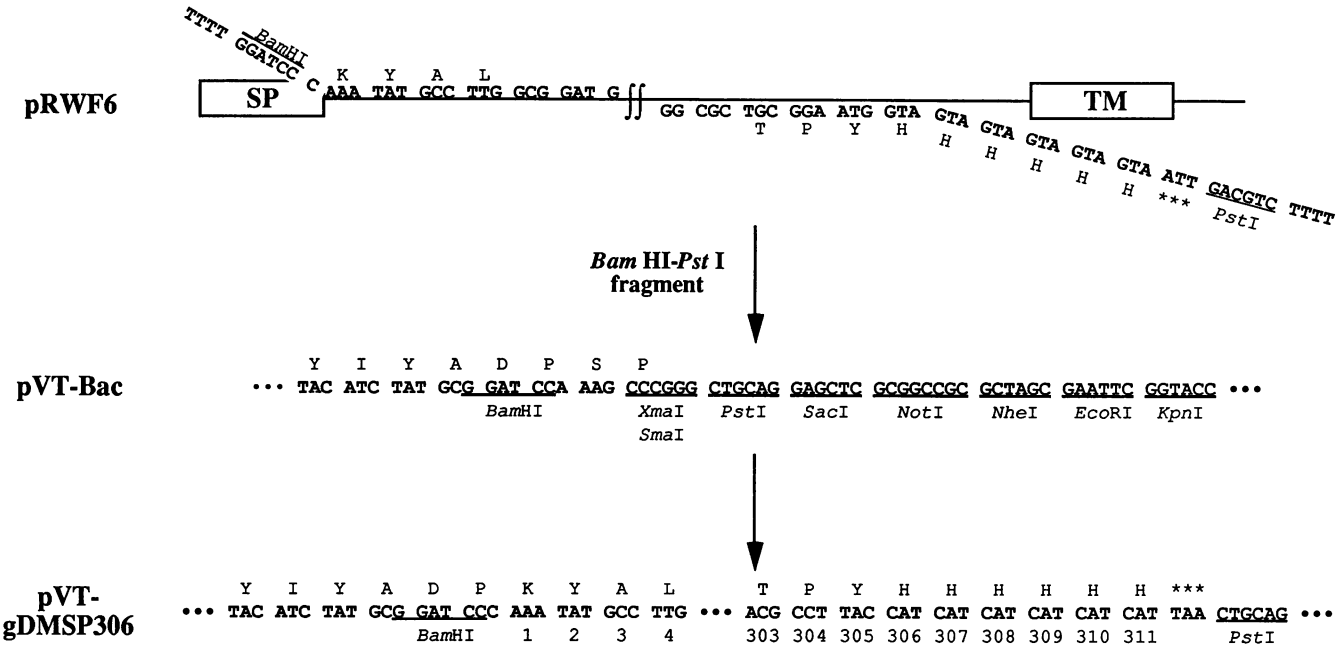

FIG. 1. Gene construction of histidine-tailed gD-1(306t) and gD-1(QAAt). The source of the gD gene was plasmid pRWF6 (54), containing the coding sequence of the Patton strain of HSV-1 gD. SP, gD-1 signal peptide; TM, transmembrane region of the protein. The vector containing the gD DNA sequence coding for the protein gD-1(306t) is called pVT-gDMSP306, and the vector for the protein gD-1(QAAt) is called pVT-gDMSPQAA. Details for the construction of the latter two plasmids are provided in the text.

sequence for a foreign protein with that of the melittin signal peptide. Tessier et al. (50) previously showed that use of this particular signal peptide significantly enhanced expression and secretion of the papain precursor (EC 3.4.22.2) from baculovirus infected *Spodoptera frugiperda* (Sf9) cells (33). We examined the potential of two truncated forms of gD-1 to be expressed in this system. One form, gD-1(306t), has the three signals for addition of N-linked oligosaccharides (N-CHO) present in wild-type gD-1; the other form, gD-1(QAA)t, has the same amino acid sequence except for three mutations which eliminate the three signals for addition of N-CHO (46, 47). Full-length gD-1(QAA), though somewhat different in antigenic properties from fully glycosylated gD, is still functional in virus infection (47). Our reason for overexpressing a truncated form of this protein is to address the fourth concern for crystallography of gD, namely, the possibility that gD might not crystallize because of carbohydrate heterogeneity. For gD-1(306t), we routinely obtain 20 mg of affinity-purified protein per liter of growth medium. In contrast, much of gD-1(QAAt) remains intracellular; therefore, we are able to purify approximately 1 to 5 mg of this protein per liter of growth medium. Both proteins retain the ability to bind most monoclonal antibodies (MAbs) to discontinuous epitopes, though gD-1(QAAt) exhibits reduced ability to bind some group VI MAbs, a property similar to that of full-length gD-1(QAA) produced in mammalian infected cells (46). Lastly, after three steps of purification, both proteins were free of contaminating proteins and appear to be >99% pure. gD-1(306t) exhibits some heterogeneity in gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels, due in large part to N-CHO, whereas gD-1(QAAt) appears to be homogeneous. Thus, both proteins are candidates for crystallization trials according to the four criteria stated above.

MATERIALS AND METHODS

Construction of transfer vectors. Plasmid pRWF6 (53) contains the entire gD gene (Fig. 1). A 918-bp fragment, corresponding to amino acids 1 to 306 of gD (where amino acid 1 is Lys-26 of the amino acid coding region) (12), was amplified by PCR. The amino-terminal primer added a *Bam*HI site just upstream from the codon for amino acid 1; the carboxy-terminal primer added five histidine codons following the naturally occurring histidine at amino acid 306, a stop codon, and a *Pst*I site (Fig. 1). The histidine codons were added to provide a binding site for Ni^{2+}-nitriloacetic acid (NTA)-agarose resin (Qiagen) for possible use in purification protocols. The PCR reaction employed $Vent_R$ DNA polymerase (New England Biolabs) and was carried out for 45 cycles of 1 min at 99°C, 30 s at 52°C, and 2 min at 75°C. The amplification products were analyzed on a 1% agarose gel, and the desired fragment was purified from the gel. The PCR fragment and the vector pVT-Bac (50) were each digested with *Bam*HI and *Pst*I and ligated for 15 h at 15°C, using T4 DNA ligase (New England Biolabs). The ligated plasmid was used to transform *Escherichia coli* XL1-Blue (Stratagene) competent cells. Plasmids from ampicillin-resistant colonies were screened by *Bam*HI and *Pst*I digestions. DNA was then purified by CsCl banding. Plasmid DNA (pVT-gDMSP306) containing the truncated form of gD-1 was recombined into baculovirus, and the recombinant protein was designated gD-1(306t). The recombinant virus was designated bac-gD-1(306t).

Similar constructs were made by using plasmid pDS145 as a source of the gD-1 gene with mutations which eliminated all three N-linked glycosylation signal sites (46). In this mutant, Asn-93 is converted to glutamine (Q), and Ser-122 and Thr-263 are converted to alanine (A). The plasmid was designated pVT-gDMSPQAA, the recombinant virus was designated bac-

gD-1(QAAt), and the recombinant protein was designated gD-1(QAAt).

Cell lines. All experimental work reported in this paper was done in Sf9 cells. In early experiments, we found that gD expression in cell monolayers of infected High Five cells was substantially higher than in infected Sf9 cells. However, High Five cells showed markedly reduced viability and clumping in stirred suspension cultures and were not tested extensively for large-scale (1 liter or more) purification trials. Both cell lines were obtained from Invitrogen. For large-scale production of gD from suspension cultures, we used a second strain of Sf9 cells (kindly provided by G. Godwin, GIBCO), which was specifically adapted to grow in serum-free media.

Media. For small-scale studies, Sf9 cells were cultured in Grace's insect cell culture medium supplemented with 10% fetal bovine serum, 0.1% Pluronic F-68, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (reagents were from GIBCO). The Pluronic F-68 was omitted during transfections and infections. Sf900II medium (GIBCO) (no serum) was used for large-scale growth of Sf9 cells in suspension culture.

Construction and purification of baculovirus recombinants. Cotransfection of Sf9 cells with 1.5 μg of pVT-gDMSP306 or pVT-gDMSPQAA and 200 ng of linearized wild-type baculovirus DNA (*A. californica* nuclear polyhedrosis virus; Invitrogen) was mediated by cationic liposomes (Lipofectin; GIBCO) according to the manufacturer's specifications. The transfection mixture was added to 3×10^6 cells in a 60-mm-diameter dish and incubated with the cells in serum-free medium for 15 h at 27°C. The medium was removed and replaced with serum-containing medium, and then incubation at 27°C was resumed for 48 h. The supernatant virus was reserved for the next step. Separate culture dishes (60-mm diameter) were seeded with 3×10^6 Sf9 cells. The medium was removed, and these cells were incubated for 1 h at room temperature with various dilutions of supernatant virus recovered after cotransfection. The virus was removed, and the cells were overlaid with fresh medium containing 1.5% low-melting-point agarose (Bethesda Research Laboratories) and then incubated at 27°C for 5 days. Occlusion-negative plaques were picked and added to serum-free medium. A portion was analyzed for recombinant virus by PCR as described previously (43). Recombinants were subjected to two additional rounds of plaque purification. Virus stocks were prepared by infecting Sf9 cells in suspension at a multiplicity of infection (MOI) of 0.1 and incubating them for 6 days at 27°C. Supernatant virus was collected, filtered through a 0.45-μm-pore-size filter, titered on monolayers of Sf9 cells, and stored in aliquots at −80°C.

Infections for protein expression. Suspension cultures of Sf9 cells were infected with one of the recombinant viruses at an MOI of 4 and incubated at 27°C for various times up to 108 h. Cells were pelleted by centrifugation, and supernatant proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Gels were analyzed either by staining with Coomassie brilliant blue or by Western blotting (immunoblotting) (6), using polyclonal anti-gD-1 serum (R1) (24).

Radiolabeling and immunoprecipitations. Sf9 cells were infected in monolayers with recombinant baculovirus containing the genes for gD-1(306t) or gD-1(QAAt) at an MOI of 10 and incubated at 27°C. At 24 h postinfection (p.i.), the medium was removed and replaced with methionine-free medium for 1 h. [^{35}S]methionine was added at a final concentration of 50 μCi/ml, and incubation at 27°C was continued until 48 h p.i. Immunoprecipitation studies were done with two MAbs, grouped as described previously (37). Antibody 55306 recognizes a discontinuous epitope and was assigned to group Ia; antibody 55317 recognizes a continuous epitope and was assigned to group VII. An antibody to the fibroblast growth factor receptor (Upstate Biotechnology, Inc.) was used as a control. The supernatants were mixed with an equal volume of radioimmunoprecipitation buffer (0.15 M NaCl, 0.1 M Tris-HCl [pH 7.4], 10% glycerol, 0.5% sodium deoxycholate, 0.5% Nonidet P-40). Antibody was added, and antibody-antigen complexes were collected with protein A-Sepharose. The pellets were washed three times with radioimmunoprecipitation buffer, suspended in SDS sample buffer, and analyzed by SDS-PAGE.

Western immunoblotting. SDS-PAGE under reducing or nonreducing (native) conditions (9) was performed in 10% acrylamide gels. Proteins were transferred to nitrocellulose, probed with either an anti-gD-1 serum (R1) (24) or a MAb, and then probed with [^{125}I]protein A (ICN) as described previously (9). Representative MAbs from several groups (37) were used: HD1 in group Ia (provided by L. Pereira); DL-11 in group Ib, DL6 in group II, and ABD in group III (provided by C. DesGranges); DL-2 in group VI; and 1D3 in group VII.

Analysis of carbohydrates by enzyme digestion. Purified proteins were incubated with glycosidases or with neuraminidase as described previously (57) except that the detergent was omitted from the incubation buffer. The digestion products were analyzed by SDS-PAGE and Western blotting. The following enzymes were used: endo-β-*N*-acetylglucosaminidase H (endo H; Boehringer Mannheim), endo-β-*N*-acetylglucosaminidase F (endo F; Boehringer Mannheim), *O*-glycosidase (Boehringer Mannheim), and neuraminidase (type X, from *Clostridium perfringens*; Sigma Chemical Co.).

Protein purification. (i) Immunoaffinity chromatography. gD-1(306t) and gD-1(QAAt) were purified from supernatants of baculovirus-infected cells grown in suspension. For each protein, the clarified medium was passed over a column of MAb DL6 coupled to Sepharose 4B, washed with 0.1 M Tris–0.15 M saline, pH 7.5 (TS), eluted with 0.1 M ethanolamine, concentrated by using a YM3 membrane (Amicon), and dialyzed (Spectra-Por; molecular weight cutoff of 12,000) against phosphate-buffered saline (PBS) similarly to the method previously described (16, 17).

(ii) Nickel chromatography. Histidine-tailed proteins were purified on NTA-agarose resin (Qiagen) according to the manufacturer's instructions. For small-scale purifications, 0.75 ml of resin was centrifuged and washed in succession with water, 0.2 M acetic acid, and 0.1 M sodium phosphate (pH 8.0). All solutions also contained 0.1 M NaCl and 0.1% Tween 20. One milliliter of supernatant was added to the conditioned resin and mixed for 15 min. The resin was then washed with 0.1 M sodium phosphate (pH 8.0) and twice with 0.1 M sodium phosphate (pH 6.3). Proteins were eluted with 0.1 M sodium phosphate (pH 4.5), centrifuged to remove residual resin, and precipitated with 50% trichloroacetic acid containing 2 mg of sodium deoxycholic acid per ml. Protein was redissolved in SDS-PAGE sample buffer and analyzed on 10% acrylamide gels. Large-scale purification was performed by passing immunosorbent-purified truncated gD over an NTA-agarose column, washing the column with 0.1 M phosphate buffer (pH 7.2) containing 0.3 M NaCl, and eluting with 0.1 M sodium acetate (pH 4.5). The eluate was dialyzed against PBS (pH 7.2) and concentrated by using a PM10 membrane (Amicon).

(iii) Superose 12 chromatography. Proteins purified by immunoaffinity chromatography or by a combination of immunoaffinity chromatography and NTA-agarose chromatography were further purified on a Superose 12 gel filtration column (Pharmacia) in PBS and then concentrated by using a YM3 membrane (Amicon).

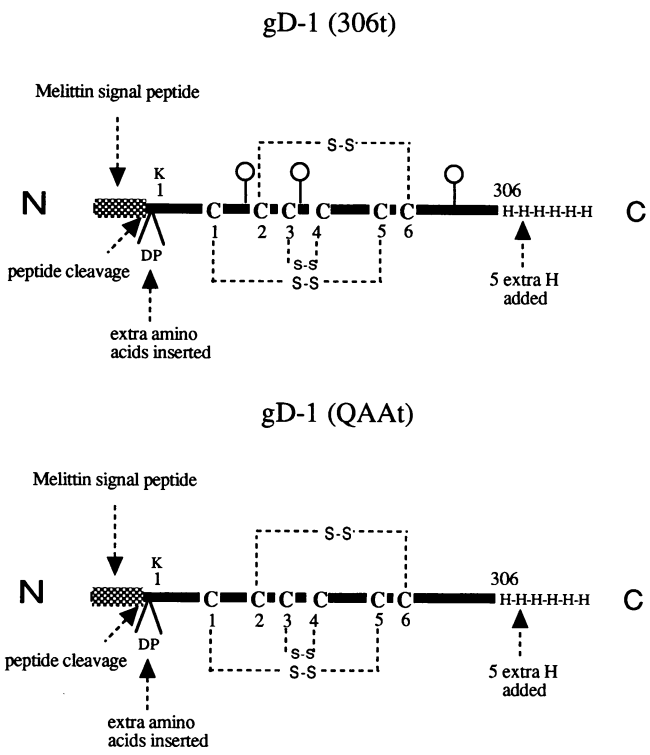

FIG. 2. Schematic representation of two truncation forms of gD-1, gD-1(306t) and gD-1(QAAt). Truncated forms of wild-type gD-1, designated gD-1(306t), and a second form lacking all three signals for addition of N-CHO (46) were cloned into pVT-Bac (50) as indicated in the text. In each case, the normal 25-amino-acid signal peptide of gD (12) has been replaced with that of the honeybee melittin signal. As a result of cloning into pVT-Bac, cleavage of the melittin signal (hatched box) leaves two additional amino acids on the N terminus of gD, aspartic acid and proline, both shown by N-terminal sequencing to precede lysine as shown in the diagram (data not shown). The three disulfide bonds shown to be present in native gD (32) are indicated by dotted lines, and the three sites for addition of N-CHO are shown by balloons.

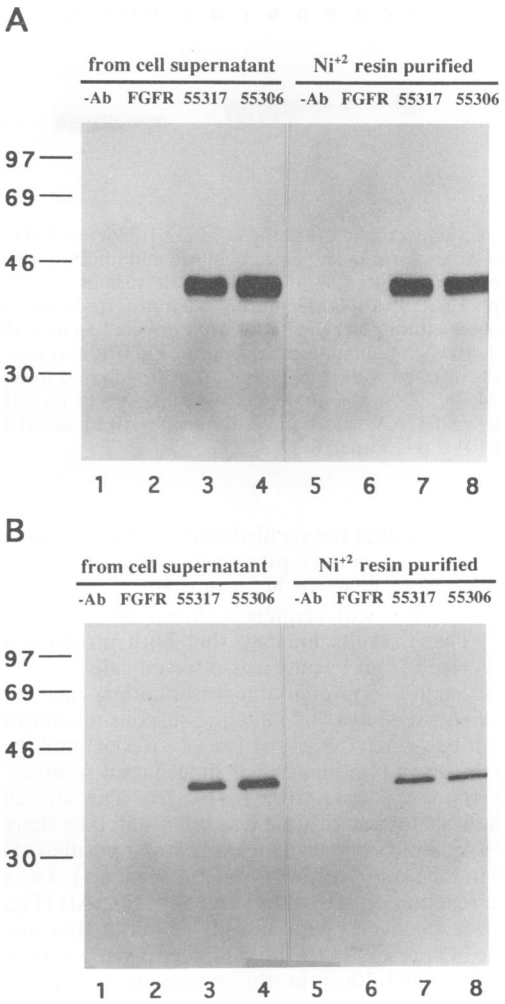

FIG. 3. SDS-PAGE analysis of gD-1(306t) and gD-1(QAAt). (A) Data for gD-1(306t); (B) data for gD-1(QAAt). The radiolabeled truncated forms of gD-1 were immunoprecipitated directly from the supernatant fluids of baculovirus-infected cells (lanes 1 to 4) or were first passed over an NTA-agarose column (lanes 5 to 8). In the latter case, the proteins were eluted with 0.1 M sodium acetate (pH 4.5) and then immunoprecipitated. The first lane of each set was mock immunoprecipitated without antibody (−Ab). The second lane of each set was immunoprecipitated with fibroblast growth factor receptor (FGFR) antibody (as another control), and the last two lanes show the proteins immunoprecipitated with two MAbs that have separate specificities for gD. Sizes are indicated in kilodaltons.

RESULTS

Effect of the melittin signal peptide. Our early attempts to express gD-1(306t) by using the wild-type gD signal peptide and a standard baculovirus vector yielded barely detectable amounts of protein either intra- or extracellularly. We also studied a construct which contained no signal peptide and found that significant amounts of gD were synthesized intracellularly, although as expected, the protein lacked carbohydrate, failed to fold properly, and was not secreted into the medium (data not shown). These experiments suggested that low levels of expression and secretion were associated with the natural gD signal. Significant increases in expression and secretion were observed when the mellitin signal peptide (50) was used instead of the natural gD signal. Figure 2 shows stick models of the two truncated forms of gD that were constructed using pVT-Bac, gD-1(306t), and gD-1(QAAt). Note that in each case, cloning into the *Bam*HI site of pVT-Bac should cause two extra amino acids (aspartic acid and proline) to be present at the N terminus. The 3′ primer was engineered to provide five additional histidine residues (for a total of six in a row) at the C terminus of the protein.

Monolayer cultures of Sf9 cells were infected with either bac-gD-1(306t) or bac-gD-1(QAAt), and at 24 h p.i., the infected cells were labeled for 24 h with [^{35}S]methionine. Supernatants obtained at 48 h p.i. were tested for the presence of secreted gD-1 by immunoprecipitation with either of two MAbs. One, 55317, recognizes a continuous epitope near the N terminus of gD, within antigenic site VII (8), and the other, 55306, reacts with a discontinuous epitope within site Ia (37). Truncated gD-1(306t) and gD-1(QAAt) were detected with both MAbs (Fig. 3), although less gD-1(QAAt) was immunoprecipitated with either MAb (Fig. 3B). These results suggest that both proteins retain their native structure. The glycoproteins were purified from the growth medium of infected cells by NTA chromatography and then immunoprecipitated with either 55317 or 55306. The ability to purify the proteins in this

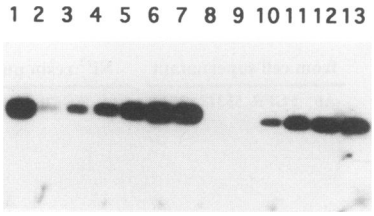

FIG. 4. Time course of synthesis of gD-1(306t) and gD-1(QAAt). Sf9 cells were grown in suspension culture and infected at an MOI of 2. Supernatant fluids (20 μl), sampled at various times p.i., were electrophoresed on a 10% denaturing gel, transferred to nitrocellulose, and probed with a polyclonal antiserum directed against HSV-1 gD. Lanes: 1, 100 ng of immunosorbent-purified gD-1(306t) (see below); 2 to 7, cells infected with bac-gD-1(306t) at the time of infection (0 h) and at 24, 48, 72, 96, and 104 h p.i., respectively; 8 to 13, cells infected with bac-gD-1(QAAt) at the time of infection (0 h) and at 24, 48, 72, 96, and 104 h p.i., respectively.

manner showed that the six histidine residues engineered to be at the C terminus of each protein were present. Furthermore, N-terminal sequencing of NTA-purified gD-1(306t) gave the expected amino acid sequence shown in Fig. 2 (data not shown). These results indicate that both proteins are intact when secreted from baculovirus-infected cells.

To ascertain the optimal time for obtaining each protein, we did time course studies by infecting suspension cultures of Sf9 cells with bac-gD-1(QAAt) or bac-gD-1(306t) and then carrying out Western blot analysis of supernatant samples taken at various times p.i. up to 108 h (Fig. 4). The amount of gD present as a function of time was quantitated by densitometry (Fig. 5A) against a known concentration of purified gD-1(306t) probed in the same Western blot (Fig. 4, lane 1). The small but positive reaction of gD-1(306t) and gD-1(QAAt) (Fig. 4, lanes 2 and 8, respectively) with anti-gD serum at time zero represents the gD present in the initial infecting virus inoculum. The amount of gD-1(306t) in the supernatant fluids increased between 48 and 96 h p.i. and then leveled off by 104 h p.i. (Fig. 4, lanes 4 to 7; Fig. 5A), while the amount of gD-1(QAAt) increased sharply at 48 h p.i. but leveled off by 72 h p.i. (Fig. 4; compare lanes 11 and 12). In both cases, the viability of the cells decreased to 60%, with most of the decrease occurring between 72 and 96 h (Fig. 5B). In this experiment, gD-1(306t) reached a maximum of 35 μg/ml of supernatant and gD-1(QAAt) reached a maximum of 12 μg/ml (Fig. 5A). The amount of gD-1(306t) found in the supernatant is very reproducible between experiments. However, the amount of gD-1(QAAt) present in the supernatant is more variable. In several experiments, we solubilized the cell pellets in SDS sample buffer and examined them by Western blotting. We found that almost no gD-1(306t) was in the infected cell pellet, whereas at least 50% of the gD-1(QAAt) protein was retained intracellularly (data not shown). We infer that the N-CHO are important for efficient secretion of truncated gD from insect cells.

Purification of gD-1(306t) and gD-1(QAAt). Initially, an attempt was made to scale up the NTA chromatography procedure in order to purify gD from the infected cell medium. However, this was impractical because the high concentration of salts in the growth medium caused the nickel to be removed from the column matrix. In contrast, scale-up of the immunoaffinity chromatography step was successful, and the growth medium had no adverse effect on the binding of gD to DL6-Sepharose. Therefore, immunoaffinity chromatography was used as the first step, NTA chromatography was used as

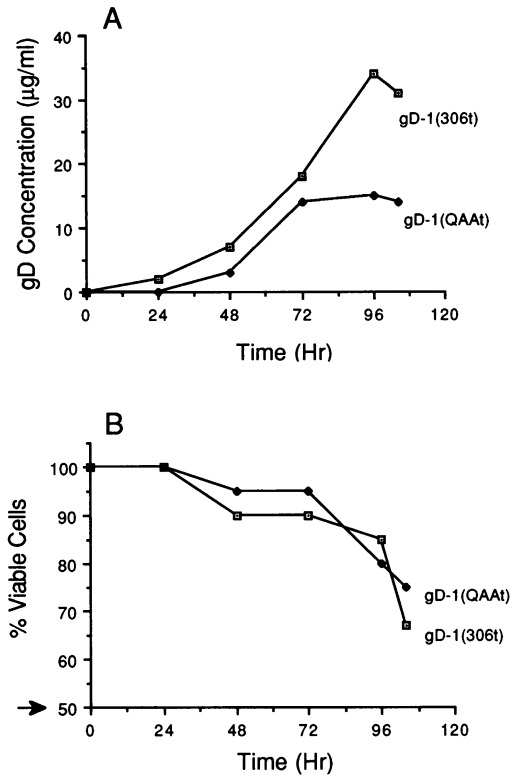

FIG. 5. Expression of gD-1(306t) and gD-1(QAAt) and cell viability after infection by baculovirus recombinants. (A) The data in Fig. 4 were quantitated by densitometry against the gD standard in Fig. 4, lane 1, and plotted as amount of gD present per milliliter of supernatant as a function of time p.i. (B) The viability of Sf9 cells over the time course of infection with gD-1(306t) and gD-1(QAAt) is shown. Cell samples were removed at the time indicated and stained with trypan blue. Cell viability was estimated as the number of cells that excluded the dye versus the total number of cells per field. The arrow indicates the 50% viability mark.

the second step, and gel filtration was used as the third step of purification. Below we will present results based on the final protocol used for purification of gD-1(306t) and gD-1(QAAt).

(i) Immunoaffinity chromatography. Culture supernatants obtained from cells infected either with bac-gD-1(306t) or bac-gD-1(QAAt) (Fig. 6A, lane 1 or 4, respectively) were passed over separate DL6 immunosorbent columns (16, 17), and the flowthrough of each column was collected [Fig. 6A, lane 2 for bac-gD-1(306t) and lane 5 for bac-gD-1(QAAt)]. Each column was washed with at least 5 bed volumes of TS. Bac-gD-1(306t) and bac-gD-1(QAAt) bound to the column were each eluted with 0.1 M ethanolamine (Fig. 6A, lanes 3 and 6, respectively). The eluate was concentrated and dialyzed against PBS, and samples were analyzed by SDS-PAGE. The purified samples were overloaded in the SDS-gel to visualize any contaminating proteins. Small amounts (estimated at less than 5%) of faster- as well as slower-migrating Coomassie-stained proteins were seen along with the affinity-purified products. Western blot analysis of samples from the same purification protocol (Fig. 6B) confirmed the presence of gD in the growth medium and in the DL6 column eluate.

On the basis of electrophoretic mobility, the apparent molecular mass of gD-1(306t) is calculated to be 42 to 43 kDa and that of gD-1(QAAt) is calculated to be 40 kDa. As has

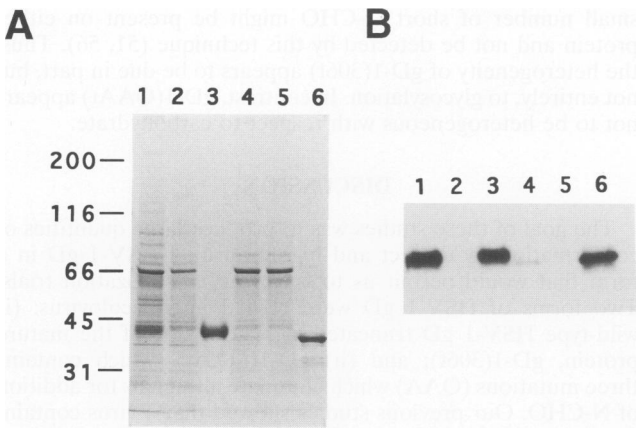

FIG. 6. Immunoaffinity purification of gD-1(306t) and gD-1(QAAt) from the supernatant fluid of baculovirus-infected insect cells. (A) Coomassie blue-stained SDS-polyacrylamide gel; (B) Western blot of smaller aliquots of the same samples that were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal anti-gD-1 serum. In this experiment, 2 liters of Sf9 cells was infected at an MOI of 2 with either gD-1(306t) or gD-1(QAAt) and harvested at 89 h p.i. The cells were removed, and the supernatants were passed over a DL6 immunosorbent. Each column was washed with TS, and gD was eluted with ethanolamine. Lanes 1 to 3 show data for gD-1(306t). Lane 1, 20 μl of supernatant fluid of cells infected by bac-gD-1(306t); lane 2, the DL6 column flowthrough; lane 3, 2 μg of gD-1(306t) eluted from the DL6 column. Lanes 4 to 6 show data for gD-1(QAAt). Lane 4, 20 μl of supernatant fluid of cells infected by bac-gD-1(QAAt); lane 5, the column flowthrough; lane 6, 200 ng of gD-1(QAAt) eluted from the DL6 column. Sizes are indicated in kilodaltons.

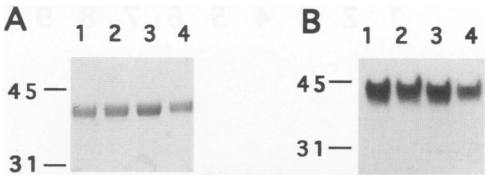

FIG. 7. Further purification of truncated gD-1 by nickel affinity chromatography and gel filtration. Immunoaffinity-purified proteins were subjected to nickel affinity chromatography followed by gel filtration on Superose 12. The purified proteins were analyzed on 10 to 20% gradient polyacrylamide gels. Gels were stained with Coomassie blue, or the proteins were transferred to nitrocellulose and probed with a polyclonal antiserum to gD. Only the data for gD-1(306t) are shown. (A) Coomassie blue-stained gel of 1.5 μg of protein obtained at each step of purification. Lanes: 1, immunoaffinity-purified gD-1(306t); 2, NTA-agarose-purified gD-1(306t); 3, the major peak obtained from the Superose 12 (gel filtration) column for gD-1(306t); 4, the minor shoulder of the gel filtration peak of gD-1(306t). (B) Western blot analysis of 200 ng of the same samples as in panel A. Sizes are indicated in kilodaltons.

been noted before with full-length gD-1 and gD-1(QAA) (45, 54), these sizes of gD are somewhat higher than the predicted molecular masses of fully glycosylated gD-1(306t) (ca. 40 kDa) or of nonglycosylated gD-1(QAAt) (34 kDa) and may be due to the high proline content of the protein (54). We conclude that immunoaffinity purification removed most of the contaminants present in the growth medium, but since small amounts of extraneous protein or other medium components might inhibit crystallization of gD, we explored further purification options by using NTA chromatography and gel filtration.

(ii) Purification of gD-1(306t) and gD-1(QAAt) by NTA chromatography. Immunoaffinity-purified gD-1(306t) and gD-1(QAAt) were bound to the NTA support and eluted with pH 4.5 acetate buffer [data for gD-1(306t) are shown in Fig. 7; data for gD-1(QAAt) are not shown]. Virtually all of the protein was recovered in each case, as shown by Coomassie blue staining (Fig. 7A, lane 2) and also by Western blotting (Fig. 7B, lane 2). However, this step did not substantially increase the purity of the preparation (Fig. 7B). When gels were overloaded with protein, minor contaminants were still present (data not shown).

(iii) Gel filtration. The NTA-purified gD-1(306t) and gD-1(QAAt) were subjected to gel filtration (Fig. 7A and B, lanes 3 and 4) to remove minor contaminants. In the case of gD-1(306t), the major protein peak (Fig. 7A, lane 3) was routinely accompanied by a higher-molecular-weight shoulder (Fig. 7A, lane 4). In some experiments, this shoulder resolved into a minor second peak (data not shown). Western blot analysis (Fig. 7B, lane 4) indicated that the Superose 12 shoulder was gD since it reacted with anti-gD serum. We speculate that it probably represents a more heavily glycosylated form of gD-1(306t) than the majority of the protein. The fractions containing the major peak were pooled and concentrated. A similar protocol was used to purify gD-1(QAAt). In this case, only a single peak was resolved by gel filtration (no shoulder). For both proteins, we recovered >80% of the protein loaded onto the gel filtration column; we estimate that each protein was greater than 99% pure after three steps with excellent overall recovery. In the case of gD-1(306t), an average of 20 mg/liter of supernatant was purified by this protocol and for gD-1(QAAt), 1 to 5 mg/liter of supernatant was recovered in a pure form.

Evaluation of purified gD. (i) Reaction with MAbs. Earlier experiments showed that gD-1, truncated at residue 275, is conformationally correct as determined by antigenic mapping (9). To examine the antigenic structure of gD-1(306t) and gD-1(QAAt), equal amounts of the two purified proteins were electrophoresed on combless denaturing (native) SDS-polyacrylamide gels and transferred to nitrocellulose. Individual strips were probed with one of several MAbs which recognize different discontinuous and continuous epitopes (Fig. 8). MAbs DL6 and 1D3, both of which recognize continuous epitopes, reacted equally well with gD-1(306t) and gD-1(QAAt) (Fig. 8A and B, lanes 1 and 2). Four MAbs that

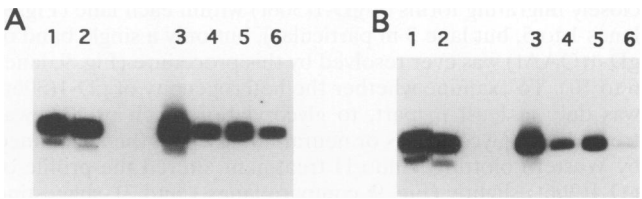

FIG. 8. Western blot analysis of purified truncated forms of gD-1(306t) (A) and gD-1(QAAt) (B). Equal amounts of each of the purified proteins were electrophoresed on combless native or denaturing SDS-polyacrylamide (10%) gels and transferred to nitrocellulose. Nitrocellulose strips were individually probed with separate MAbs and then with iodinated protein A. The first two lanes in each panel were electrophoresed on a denaturing gel and transferred to nitrocellulose, and strips were reacted with MAb 1D3 (group VII; lane 1) or with MAb DL6 (group II; lane 2). Lanes 3 to 6 in each panel were electrophoresed on a native gel and transferred to nitrocellulose, and strips were probed with MAb ABD (group III; lane 3), DL11 (group Ib; lane 4), HD-1 (group Ia; lane 5), or DL-2 (group VI; lane 6).

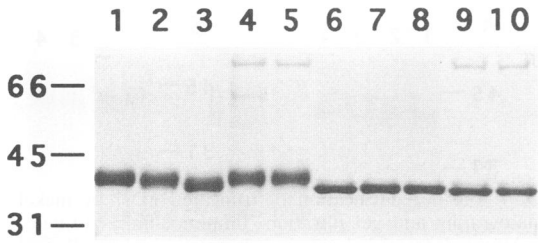

FIG. 9. Effects of glycosidases on the migration of purified gD-1(306t) and gD-1(QAAt). Samples (1.5 μg) of gD-1(306t) or gD-1(QAAt) were treated with glycosidase, neuraminidase, or a combination of neuraminidase and *O*-glycanase, then analyzed on 4 to 20% gradient SDS-polyacrylamide gels, and stained with Coomassie blue. Lanes: 1 to 5, gD-1(306t); lanes 6 to 10, gD-1(QAAt); 1 and 6, untreated samples; 2 and 7, samples treated with endo H; 3 and 8, samples treated with endo F; 4 and 9, samples were treated with neuraminidase; 5 and 10, samples treated with a combination of neuraminidase and *O*-glycanase. Sizes are indicated in kilodaltons.

recognized different discontinuous epitopes representing four different MAb groups (37) reacted well with gD-1(306t) (Fig. 8A, lanes 3 to 6), indicating that protein conformation is intact. In the case of gD-1(QAAt), three of these MAbs bound well (Fig. 8B, lanes 3 to 5) but DL2 (group VI) bound poorly (Fig. 8B, lane 6). Weak binding of group VI MAbs was previously noted for full-length gD-1(QAAt) produced in mammalian cells (46, 47). We also noted that none of the gD-1(306t) or gD-1(QAAt) was present in aggregates on native gels (data not shown). Such aggregates are a sign of faulty protein folding (11, 45, 46). We conclude that the antigenic structure of baculovirus-derived proteins is not noticeably different from the structure of full-length gD-1 or QAA produced in mammalian cells. In addition, neither protein was significantly damaged by the purification procedures.

(ii) Endoglycosidase analysis. gD synthesized in mammalian cells has been shown to contain both N-CHO (7, 45) and O-CHO (42). It is noteworthy that pseudorabies virus gD (also known as gp50 [39]) contains no signals for addition of N-CHO but does contain significant amounts of O-CHO (51). We found that gD-1(QAA) synthesized in mammalian cells does contain O-CHO (46, 47), but whether it is modified on the same amino acids as wild-type gD remains to be determined. Glycoproteins synthesized in insect cells contain N- and O-CHO but generally lack sialic acid (34, 51). Using gradient SDS-polyacrylamide gels, we were able to discern several closely migrating forms of gD-1(306t) within each lane (Fig. 9, lanes 1 to 5, but lane 3 in particular), but only a single band of gD-1(QAAt) was ever resolved by this procedure (Fig. 9, lanes 6 to 10). To examine whether the heterogeneity of gD-1(306t) was due, at least in part, to glycosylation, each protein was treated with glycosidases or neuraminidase and then examined by Western blotting. Endo H treatment altered the profile of gD-1(306t) slightly (Fig. 9; compare lanes 1 and 2), suggesting that most of the N-CHO present on gD were in a complex or an endo H-resistant form. This was confirmed by endo F treatment (Fig. 9, lane 3), which significantly increased the electrophoretic mobility of gD-1(306t), indicating that the protein was modified by complex N-CHO. As expected, the electrophoretic mobility of gD-1(QAAt) was unaffected by treatment with either glycosidase (Fig. 9; compare lanes 7 and 8 with lane 6), confirming that it contains no N-CHO. Neuraminidase and *O*-glycanase treatment had no significant effect on the mobility of either protein (Fig. 9, lanes 4, 5, 9, and 10), suggesting that neither protein contains O-CHO. However, a small number of short O-CHO might be present on either protein and not be detected by this technique (51, 56). Thus, the heterogeneity of gD-1(306t) appears to be due in part, but not entirely, to glycosylation. In contrast, gD-1(QAAt) appears not to be heterogeneous with respect to carbohydrate.

DISCUSSION

The goal of these studies was to produce large quantities of conformationally correct and highly purified HSV-1 gD in a form that would permit us to carry out crystallization trials. Two forms of HSV-1 gD were cloned into baculovirus: (i) wild-type HSV-1 gD truncated at residue 306 of the mature protein, gD-1(306t); and (ii) gD-1(QAAt), which contains three mutations (QAA) which eliminate all signals for addition of N-CHO. Our previous studies showed that a virus containing the full-length form of gD-1(QAA) behaves quite normally in infected cells (47). We were particularly interested in the potential of the QAA mutant to be expressed in this system, because the absence of the N-CHO might provide a less heterogeneous molecule and therefore be more readily crystallizable than gD-1(306t).

The results indicate that the baculovirus expression system developed by Tessier et al. (50) can be used to achieve this goal. We are able to obtain 20 mg of affinity-purified gD-1(306t) per liter of growth medium and somewhat less of gD-1(QAAt). We have developed a three-step purification protocol that permits excellent recovery of both proteins in a highly purified antigenically active form. Purified gD-1(306t) is still somewhat heterogeneous after three steps of purification, since it contains complex N-CHO. On the other hand, gD-1(QAAt) appears to be less heterogeneous because it lacks N-CHO. Neither protein appeared to be extensively O glycosylated. Studies in other laboratories have shown that full-length HSV gD produced in baculovirus-infected cells contain N-CHO (22, 30). Pseudorabies virus gD (gp50) contains no signals for N-CHO (39), and a baculovirus recombinant version of this protein was found to contain short chains of O-CHO (51). gD-1(306t) is able to bind a large number of MAbs to discontinuous epitopes (four shown here), indicating that the protein is properly folded. gD-1(QAAt) is able to bind MAbs of several groups but binds poorly to group VI MAbs, analogous to what was noted before for the full-length form of this protein expressed in mammalian cells (46, 47). In addition, less protein is secreted by bac-gD-1(QAAt)-infected cells, necessitating its purification from a larger quantity of growth medium. On the basis of these considerations, crystallization trials with both proteins will be initiated.

Removal of the TMR of gD. Crystallization of integral membrane proteins with an intact TMR has been successful in a limited number of cases (21, 41). It is generally recognized, however, that removal of the TMR improves the chances for success in crystallization efforts, provided that its absence does not impair the structure or function of the ectodomain. Crystallization of glycosylated proteins is also hampered by heterogeneity of attached oligosaccharides and by the presence of sialic acid. Thus, it is not surprising that the two viral glycoproteins whose structures have been solved, the hemagglutinin and neuraminidase of influenza virus, were each crystallized as truncated forms lacking the TMR. In the former case, the protein lacked sialic acid, and in the latter case, the protein was treated with neuraminidase prior to crystallization (52, 55, 58, 59).

Evidence that gD-1 can be truncated upstream of the TMR and still retain proper antigenic structure was first provided by proteolytic cleavage studies of gD synthesized during HSV

infection (9, 13, 14, 37). Molecular biological approaches were also used to express large quantities of truncated gD-1 in mammalian cells. These truncated forms stimulated an immune response and protected animals from virus challenge (1, 2, 31, 60). We studied one of these truncated forms of gD-1 (residues 1 to 275) and found few differences between its conformational structure and that of the full-length natural product (14). Other studies showed that a truncated form of gD-2 (residues 1 to 302) was still protective in animals (4). As mentioned above, this form of gD-2 also possesses biological activity in in vitro studies (26).

Expression of HSV gD by recombinant means. A number of laboratories have overexpressed truncated forms of gD in transformed mammalian cells (2, 4, 5, 19, 31, 40), and several groups have reported on the expression of full-length gD by baculovirus-infected insect cells (22, 29, 30). In every case in which it was tested, the recombinant gD induced production of neutralizing polyclonal antibodies and protected animals against a lethal challenge of HSV (22, 29, 30). To our knowledge, this is the first report of expression of truncated gD-1 and of truncated nonglycosylated gD-1 using the baculovirus expression system. Landolfi et al. (30) reported that they obtained milligram quantities of HSV-2 gD from an unspecified volume of suspension culture. The other reports of studies using baculovirus recombinants of HSV-1 gD do not specify how much glycoprotein was obtained. Furthermore, in one study, a significant amount of gD was present as smaller-molecular-weight species, possibly due to partial proteolysis of the glycoprotein (22). In the present study, we did not achieve high levels of gD expression and secretion in the standard baculovirus system until we replaced the gD signal peptide with that of honeybee melittin.

Comparison of gD-1(306t) and gD-1(QAAt). gD-1(306t) and gD-1(QAAt) were each secreted into the supernatant of baculovirus-infected cells, but the efficiency of secretion was noticeably different. Virtually all of gD-1(306t) was found in the growth medium, whereas more than half of the gD-1(QAAt) synthesized by baculovirus-infected insect cells was retained inside the cells. This finding suggests that efficient secretion of HSV-1 gD from insect cells requires N-CHO. Does gD-1(QAAt) contain O-CHO? We were unable to document the presence of significant amounts of O-CHO on either recombinant protein and were therefore surprised that gD-1(QAAt) is secreted at all from insect cells. However, a more sensitive method might be needed to clarify this point. It was previously reported that gD synthesized in HSV-1-infected mammalian cells contains two or three O-CHO, detected by chemical methods (42). We used enzymatic methods to demonstrate the presence of O-CHO on gD-1(QAA) synthesized in HSV-1-infected mammalian cells (46, 47). Assuming that baculovirus-expressed gD or QAA contains only two or three O-CHO, their removal would have little effect on the apparent molecular weight of either protein, as these oligosaccharides are known to be far less complex in insect cells than in mammalian cells (51).

Another issue raised by this study is whether inefficient secretion of gD-1(QAAt) is specific for a truncated molecule synthesized by insect cells. In previous studies (46, 47), no difference was found in the kinetics of synthesis and transport of full-length gD-1 versus full-length gD-1(QAA) in mammalian cells. One way to address this issue is to construct baculovirus recombinants containing full-length gD-1(QAA) and full-length gD-1 and to compare the kinetics of their transport to the cell surface with that of the same gD proteins expressed in HSV-infected mammalian cells.

We are encouraged by the fact that both of the truncated glycoproteins appear to retain most of the features of their native structure, especially after several steps of purification. For crystallization, optimally there should be a single molecular species of the protein. Purified gD-1(306t) consists of several closely migrating bands in gradient gels. Some of this heterogeneity is associated with N glycosylation. Other modifications, such as acetylation or phosphorylation, may also contribute to the heterogeneity. We are characterizing the heterogeneity of gD-1(306t) in greater detail and are evaluating additional methods for purification, such as preparative isoelectric focusing, chromatofocusing, and/or ion-exchange chromatography. Of course, none of these measures may be needed if we are able to crystallize gD in a form suitable for X-ray diffraction analysis after the three steps of purification detailed in this report.

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