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Influence of Asparagine-Linked Oligosaccharides on Antigenicity, Processing, and Cell Surface Expression of Herpes Simplex Virus Type 1 Glycoprotein D

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Glycoprotein D (gD) is an envelope component of herpes simplex virus types 1 and 2. gD-1 contains three sites for the addition of N-linked carbohydrate (N-CHO), all of which are used. Three mutants were constructed by site-directed mutagenesis, each of which altered one N-CHO addition site from Asn-X-Thr/Ser to Asn-X-Ala. A fourth mutant was altered at all three sites. The mutant genes were inserted into an expression vector, and the expressed protein was analyzed in transiently transfected COS-1 cells. The mutant protein lacking N-CHO at site 1 (Asn-94) had a reduced affinity for monoclonal antibodies (MAbs) to discontinuous epitopes, suggesting that the conformation of the protein had been altered. However, the protein was processed and transported to the cell surface. The absence of N-CHO at site 2 (Asn-121) had no apparent effect on processing or transport of gD-1 but resulted in reduced binding of two MAbs previously shown to be in group VI. Binding of other MAbs to discontinuous epitopes (including other group VI MAbs) was not affected. The absence of N-CHO at site 3 (Asn-262) had no effect on processing, transport, or conformation of the gD-1 protein. The absence of N-CHO from site 1 or from all three sites resulted in the formation of high-molecular-weight aggregates or complexes and a reduction in MAb binding. However, these proteins were modified by the addition of *O*-glycans and transported to the cell surface. We conclude that the absence of the first or all N-linked carbohydrates alters the native conformation of gD-1 but does not prevent its transport to the cell surface.

Glycoprotein D (gD) of herpes simplex virus type 1 (HSV-1) is a structural component of the virion envelope which stimulates high titers of complement-independent neutralizing antibody (4, 6, 17, 19, 23, 34, 42, 54). Studies with anti-gD monoclonal antibodies (MAbs) have implicated gD-1 in virus adsorption (19), penetration (20, 23), and in cell-to-cell fusion (42, 44). A virus lacking the gD gene was used to demonstrate that gD is involved in penetration and not required for the adsorption of virus to cells (33). The gD-negative virus which had adsorbed to the cell surface did not block infectious (gD-containing) virus from entering the cell (27). Johnson and Ligas (27) proposed that gD is capable of binding a limited set of cell surface receptors, an interaction which then promotes virus penetration. Our long-term goal is to determine the regions of gD which are important for its function. Here we began to examine the importance of the asparagine-linked carbohydrates present on gD of HSV-1.

A majority of the proteins which are translocated into the lumen of the endoplasmic reticulum during synthesis contain oligosaccharides which are covalently attached to asparagine residues (3, 48, 50). These asparagine-linked (N-linked) carbohydrate moieties (N-CHO) have been implicated in a variety of functions such as protection of the protein from proteolytic degradation (41, 58) and/or intracellular aggregation (11, 21, 36, 37). There is also evidence that they help establish the native conformation of glycoproteins, including the accurate formation and disulfide bonds (11, 32, 37, 40, 59). Experiments employing tunicamycin (Tm) (49, 57) and 2-deoxy-D-glucose (56, 57) have shown that N-CHO are important for HSV infectivity. Furthermore, endoglycosidase treatment of HSV virions decreases their infectivity (29).

gD from HSV-1 contains three predicted sites for the addition of N-CHO at amino acid residues 94, 121, and 262 (31, 60, 61), all of which are utilized (8, 39); also present are O-linked oligosaccharides (26, 53). We used site-directed mutagenesis to alter each of the N-linked addition sites individually; a fourth mutant was altered at all three sites. The gD N-CHO mutant genes were inserted into an expression vector and analyzed in transient transfection assays in COS-1 cells. The mutants were used to determine the role of each N-CHO in the native conformation and intracellular transport of gD-1.

MATERIALS AND METHODS

Cell culture. COS-1 cells were propagated in Dulbecco minimal medium supplemented with 10% fetal bovine serum at 37°C. The COS-1 cell line gave greater gD expression than other cell lines tested.

Construction of glycosylation mutants of gD by oligonucleotide-directed mutagenesis. A 1,420-base-pair *Hin*dIII fragment containing the entire gD-1 (Patton)-coding sequence (61) was excised from the gD expression vector pRE4 (9) and subcloned into the *Hin*dIII site of M13mp18. Mutagenesis was done by the method of Zoller and Smith (63) as modified by Kunkel et al. (30) to allow for phenotypic selection of bacteriophage containing the desired mutation. Three synthetic oligonucleotide primers were used to change the Asn-X-Ser/Thr N-CHO signals. Each had a single-base mismatch designed to change threonine 96 (N-CHO 1^- mutant, pDS-91), serine 123 (N-CHO 2^-, pDS-92), or threonine 264 (N-CHO 3^-, pDS-93) to alanine (35). The triple mutant lacking all three glycosylation sites (pDS-94) was constructed by using the N-CHO 3^- mutant as a template.

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Mutants were identified, and their complete DNA sequences were determined by the dideoxynucleotide method (51). The mutated gD-1 genes were removed from the M13mp18 replicative-form DNA with *Hin*dIII and inserted into the eucaryotic expression vector pRSV-nt EPA, which has the Rous sarcoma virus long terminal repeat as a promoter and a simian virus 40 polyadenylation signal (9). A unique *Hin*dIII site adjacent to the Rous sarcoma virus long terminal repeat was used for the insertion of the coding region of the gD-1 mutant genes. This expression vector will not replicate in COS-1 cells owing to the absence of a eucaryotic origin of replication. This vector was also the parent vector for pRE4 (wild-type gD-1) (9). All DNA used in transfections was subjected to two consecutive CsCl gradient centrifugation steps (38).

Transfection procedure. The calcium phosphate coprecipitation procedure (22) was used as previously described (9, 62). A 10-μg portion of DNA was used per 60-mm plate of COS-1 cells. Cells grown in the presence of Tm (2 μg/ml) from 15 to 48 h after transfection were devoid of detectable levels of N-glycosylated gD-1. Cells were harvested 48 h posttransfection, and cytoplasmic extracts were prepared as previously described for infected cells (6), except that the high-speed centrifugation step was omitted to avoid pelleting gD-1 present in high-molecular-weight forms.

Polyclonal antibodies and MAbs. Rabbit anti-gD-1 serum (62) was used for Western blotting (immunoblotting), for radioimmunoprecipitation, and for indirect immunofluorescence experiments. Nineteen MAbs from groups Ia, Ib, II, III, IV, and VI were used to analyze the N-CHO mutants. MAbs to gD were grouped by their ability to react with native or denatured gD-1 and gD-2 (4, 5, 24) blocking studies (16), neutralization activity (15, 54), reactions with synthetic peptides or gD fragments (4, 5, 12, 15, 16), and reactivity with MAb-resistant mutants (43). The MAbs tested were HD-1 and LP-2 (group Ia); DL-11, D2, and 4S (group Ib); DL-6 (group II); 11S, ABD, RIP, VID, and I-99 (group III); 1S and 45S (group IV); DL-2, D1, 41S, II-436, II-886, and I-206 (group VI) (5). MAbs were obtained from A. Minson (LP-2), L. Pereira (HD-1), J. Glorioso (D2), P. Spear (II-436, II-886, I-206), and M. Zweig (4S, 11S, 1S, 45S, 41S). MAbs DL-11, DL-2, and DL-6 were developed in our laboratory (5, 12, 16, 24, 43). All the MAbs except DL-6 (group II) recognize discontinuous epitopes on the gD protein (5, 12, 16). DL-6 recognizes a continuous epitope within amino acid residues 272 to 279 of gD (16, 24).

Enzyme digestions and Western blot analysis. The digestion of gD-1 with endoglycosidase F (Endo F) (removes high-mannose and complex forms of N-linked sugars), endoglycosidase H (Endo H) (removes high-mannose N-linked sugars), or neuraminidase (removes sialic acid) was performed as described previously (9, 62). To achieve efficient digestion of O-linked oligosaccharides, cell extracts containing gD were first diluted 1:1 with distilled water and then incubated with 150 mU of neuraminidase for 2 h at 37°C. Then 0.5 mU of endo-α-*N*-acetyl-galactosamine hydrolase (*O*-glycan-peptide hydrolase) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added, and the incubation was continued for 6 h at 37°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins under denaturing and nondenaturing "native" conditions was performed in 10% acrylamide gels as described previously (5). The proteins were transferred to nitrocellulose (2) and then probed with antibody followed by iodinated protein A (ICN Biomedicals, Inc., Irvine, Calif.).

Quantitation of antigenic activity. Samples of cytoplasmic extracts were dotted onto nitrocellulose and then incubated with a gD-specific MAb (4, 5, 16, 24). The nitrocellulose strips were then washed, incubated with iodinated protein A, and exposed to X-ray film to directly observe the MAb reactions. The dots were cut out, and their radioactivities were counted in a gamma counter. Each experiment was performed in duplicate and repeated at least twice. The reaction of gD-1 in each extract (expressed as counts per minute [cpm]) with MAb DL-6 was used to normalize the results obtained with the other MAbs. Background (Bkg) was determined by dotting equal amounts of an extract of nontransfected cells and reacting it with the appropriate test or control MAb. The amount of binding of each test MAb to gD-1 was calculated as follows: (cpm of test MAb − Bkg)/(cpm of DL-6 − Bkg). This equation was used to determine the binding of a test MAb to one of the N-CHO mutant gD-1 proteins (*A*) or to wild-type gD-1 (*B*). The antigenic activity of each N-CHO mutant and Tm-gD was calculated as follows: percent antigenic activity = $(A/B) \times 100$.

Pulse-chase and radioimmunoprecipitation studies of gD. At 42 h after transfection, COS-1 cells were pulsed for 10 min with 200 μCi of [^{35}S]cysteine-methionine (ICN Biomedicals). Cytoplasmic extracts were prepared immediately (pulse) or after the cells were incubated in unlabeled medium for 20, 40, 60, or 80 min (chase). The cell extract (25%) from a 60-mm plate was immunoprecipitated as previously described (10), electrophoresed through 10% polyacrylamide-sodium dodecyl sulfate gels cross-linked with *N,N'*-diallyltartardiamide, and exposed to Kodak XAR-5 film. Densitometric scanning of the autoradiogram was used to determine the percentage of gD which was processed from precursor (pgD) to product (gD) form (7).

Cell surface expression. COS-1 cells were seeded onto cover slips, transfected with the appropriate plasmid, and incubated for 45 h. Cells grown in the presence of Tm were exposed to the drug beginning 15 h posttransfection. The immunofluorescence protocol was modified from that described previously (35). Briefly, cover slips were washed in phosphate-buffered saline, and the cells were fixed in 3% paraformaldehyde-saline solution. For surface staining, cells were incubated with rabbit anti-gD-1 serum (1 h at 37°C) and then with anti-rabbit tetramethylrhodamine isothiocyanate conjugate (Boehringer Mannheim) (1 h at 37°C). The cells were then permeabilized in 0.8% Nonidet P-40–saline solution and incubated first with rabbit anti-gD-1 serum and then with anti-rabbit fluorescein isothiocyanate conjugate (Boehringer Mannheim). The cells were viewed with a Leitz epifluorescence microscope.

RESULTS

A diagram of the N-linked glycosylation mutants of gD-1 is presented in Fig. 1. Each glycosylation site was altered by changing the Asn-X-Ser/Thr to Asn-X-Ala, thus preventing the addition of N-CHO (35). The gD-1 mutants are referred to as N-CHO 1$^-$, N-CHO 2$^-$, N-CHO 3$^-$, and N-CHO 1,2,3$^-$.

Expression of N-CHO mutant gD-1 in transfected cells. We previously showed that the three N-linked addition sites of gD-1 are utilized in infected cells (8, 39). Since gD-1 present in transfected cells has the same electrophoretic mobility as gD-1 in infected cells, we presume that all three addition sites are utilized in the wild-type gD-1-transfected cells. Each N-CHO mutant form of gD-1 was analyzed in transiently transfected COS-1 cells. As controls, cells were transfected with the wild-type gD-1 gene in the presence or

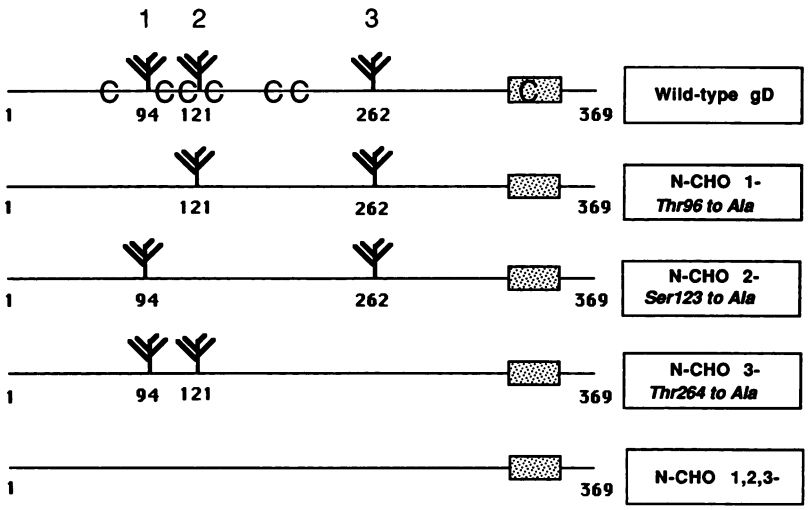

FIG. 1. Schematic representation of gD-1 and the four glycosylation mutants. The essential features of the gD-1 protein are depicted. The protein contains a signal sequence of 25 amino acids (14) which is cleaved in a cotranslational processing event to leave a mature form of 369 amino acids (39). The transmembrane region (dotted box) is located near the carboxy terminus (39). The seven cysteine residues (C) are located at amino acids 66, 106, 118, 127, 189, 202, and 333. gD-1 contains three sites (residues 94, 121, and 262) for the addition of N-CHO, all of which are utilized (8). These are depicted as branched structures.

absence of Tm. Cytoplasmic extracts of the transfected cells were separated on a denaturing gel, Western blotted, and reacted with polyclonal anti-gD-1 serum (Fig. 2A). The wild-type (59 kilodaltons [kDa], lane 6), N-CHO 2$^-$ (57 kDa, lane 2), and N-CHO 3$^-$ (57 kDa, lane 3) proteins each migrated as a single band. The N-CHO 1$^-$ protein migrated as two distinct bands (55 and 57 kDa) (Fig. 2A, lane 1). The lower band might represent a form of gD-1 in which only one of the two remaining N-linked sites is filled. Alternatively, additional *O*-glycans on the processed protein could also result in two bands. The N-CHO 1$^-$, N-CHO 2$^-$, and N-CHO 3$^-$ proteins migrated in the gel to a position consistent with the absence of one N-CHO. After Endo F treatment (Fig. 2B), wild-type gD-1, N-CHO 1$^-$, N-CHO 2$^-$, and N-CHO 3$^-$ migrated to similar positions in the gel (lanes 1 to 4). These results confirm that the altered mobilities observed in Fig. 2A were due to the absence of N-CHO. In a separate experiment, extracts containing the wild-type gD-1, N-CHO 1$^-$, N-CHO 2$^-$, and N-CHO 3$^-$ proteins were digested with Endo H to remove only the precursor (high-mannose) N-CHO. In each case, more then 50% of the protein was Endo H resistant, indicating that all the mutant proteins lacking one N-CHO were processed to the product form (data not shown).

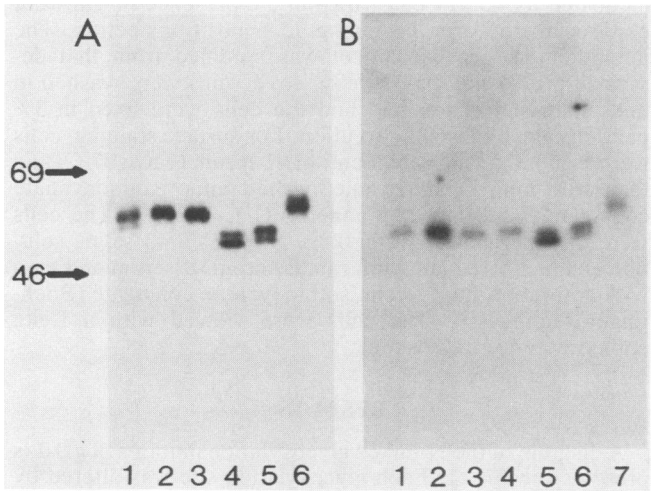

FIG. 2. Western blot analysis of wild-type gD-1 and gD-1 proteins lacking N-CHO electrophoresed under denaturing conditions. Cytoplasmic extracts were prepared from COS-1 cells 48 h after transfection, electrophoresed on a 10% denaturing gel, transferred to nitrocellulose, and then reacted with anti-gD-1 serum and iodinated protein A. (A) Lanes: 1, N-CHO 1$^-$; 2, N-CHO 2$^-$; 3, N-CHO 3$^-$; 4, N-CHO 1,2,3$^-$; 5, gD-1 from Tm-treated transfected cells (Tm-gD); 6, wild-type gD-1. Endo F was used to remove N-CHO present on the gD-1 proteins (18). (B) Lanes: 1, Endo F-treated wild-type gD-1; 2, Endo F-treated N-CHO 1$^-$; 3, Endo F-treated N-CHO 2$^-$; 4, Endo F-treated N-CHO 3$^-$; 5, Endo F-treated N-CHO 1,2,3$^-$; 6, Endo F-treated Tm-gD; and 7, untreated wild-type gD-1. Molecular size markers of 46 and 69 kDa are indicated with arrows.

The N-CHO 1,2,3$^-$ (Fig. 2A, lane 4, 49 and 52 kDa) and Tm-gD (lane 5, 50 and 53 kDa) proteins migrated more rapidly than the single mutants but differently from each other. This latter observation was surprising since both proteins lack all three N-CHO, as evidenced by the inability of Endo F to affect their electrophoretic mobility (Fig. 2B, lanes 5 and 6). The three amino acid changes in N-CHO 1,2,3$^-$ may be responsible for altering its electrophoretic migration in comparison with Tm-gD.

Furthermore, the N-CHO 1,2,3$^-$ protein and Tm-gD were each resolved into two distinct bands in the denaturing gel. We speculated that the slower-migrating band might result from the addition of *O*-glycans which are known to be attached to wild-type gD-1 expressed in infected cells (26, 53). We found that wild-type gD-1 expressed in transfected cells also contained *O*-glycans, since its mobility increased after digestion by neuraminidase and *O*-glycan-peptide hydrolase (Fig. 3, lane 3) compared with that after digestion by neuraminidase alone (lane 2). Similar results were obtained for each of the mutants as well as for Tm-gD. Data for N-CHO 1$^-$ and N-CHO 1,2,3$^-$ are shown in Fig. 3, lanes 4

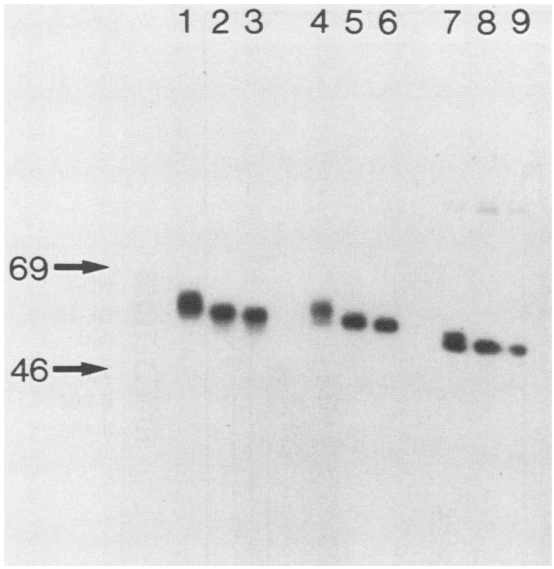

FIG. 3. Effect of neuraminidase and *O*-glycan-peptide hydrolase on gD-1 and gD-1 proteins lacking N-CHO. Cytoplasmic extracts were diluted 1:1 with distilled water, treated with enzymes, electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with anti-gD-1 serum and iodinated protein A. The proteins analyzed were wild-type gD-1 (lanes 1 to 3), N-CHO 1^- (lanes 4 to 6), and N-CHO $1,2,3^-$ (lanes 7 to 9). Proteins were mock digested (lanes 1, 4, and 7), treated with neuraminidase (lanes 2, 5, and 8), or treated with neuraminidase followed by *O*-glycan-peptide hydrolase (lanes 3, 6, and 9). Molecular size markers of 46 and 69 kDa are indicated with arrows.

to 9. Data for N-CHO 2^-, N-CHO 3^-, and Tm-gD are not shown.

Analysis of N-CHO mutant proteins in nondenaturing gel system. Previous results with deletion and cysteine mutants of gD-1 demonstrated that some mutations induce the formation of "aggregates" (9, 62), which can be detected on nonreducing (native) gels (5). This native gel system was used to examine the N-CHO mutants (Fig. 4). Wild-type gD-1 (lanes 1 and 7), N-CHO 2^- (lane 3), and N-CHO 3^- (lane 4) each migrated as a single band. In contrast, much of N-CHO 1^- (lane 2) and N-CHO $1,2,3^-$ (lane 5) did not enter the gel efficiently but remained trapped in the stacking gel or at the stacking/separating gel border (arrow in Fig. 4). A small percentage of the Tm-gD protein was also present in higher-molecular-weight forms (lane 6). These high-molecular-weight forms may be composed entirely of aggregated gD-1 protein or gD-1 complexed with cellular proteins. We conclude that the absence of N-CHO at site 1 is associated with structural alterations of the gD-1 protein.

Antigenic analysis. Previous results with gD-1 mutant proteins demonstrated a correlation between aggregation and reduced binding of MAbs to discontinuous epitopes (9, 62). We used a panel of 18 MAbs to discontinuous epitopes (5, 12, 43) in a dot-blot assay to determine whether the N-CHO mutants showed altered protein folding. Results for MAbs DL-11 and II-886 are shown in Fig. 5B and C, respectively. We also measured binding of the gD-1 proteins to MAb DL-6 (Fig. 5A), which recognizes a continuous epitope from residues 272 to 279 (24). The DL-6 epitope was not altered by the N-CHO mutations and therefore was used to correct for differences in gD-1 concentration in each extract. The N-CHO 1^- (row 2) and N-CHO $1,2,3^-$ (row 5) proteins were much less reactive with DL-11 or II-886 than

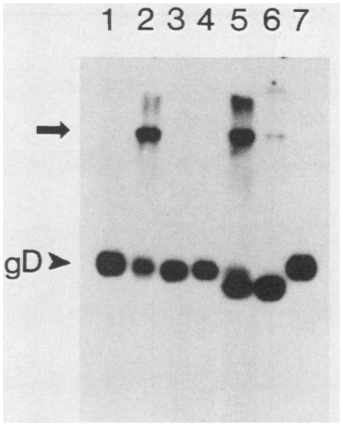

FIG. 4. Western blot analysis of wild-type gD-1 and gD-1 proteins lacking N-CHO electrophoresed under native conditions. Cytoplasmic extracts prepared from transfected cells were electrophoresed on a 10% nondenaturing (native) gel (5). The proteins were transferred to nitrocellulose and reacted with polyclonal anti-gD-1 serum and then with iodinated protein A. Lanes 1 and 7, Wild-type gD-1; lane 2, N-CHO 1^-; lane 3, N-CHO 2^-; lane 4, N-CHO 3^-; lane 5, N-CHO $1,2,3^-$; lane 6, gD-1 from Tm-treated transfected cells. The separation between the stacking and resolving gels is indicated by the arrow.

was wild-type gD-1 (row 1). The N-CHO 2^- protein (row 3) reacted at the wild-type level with DL-11 but did not react with II-886. The N-CHO 3^- protein (row 4) reacted at or near wild-type level with both MAbs. The binding of MAb II-886 was abolished with Tm-gD (row 6). The Tm-gD protein also reacted at a reduced level with DL-11, although this reduction is not easily observed in panel B owing to the deliberate overexposure of this autoradiogram (used to detect reactivity in rows 2 and 5). With each of the N-CHO mutants and Tm-gD, 15 MAbs reacted similarly to DL-11.

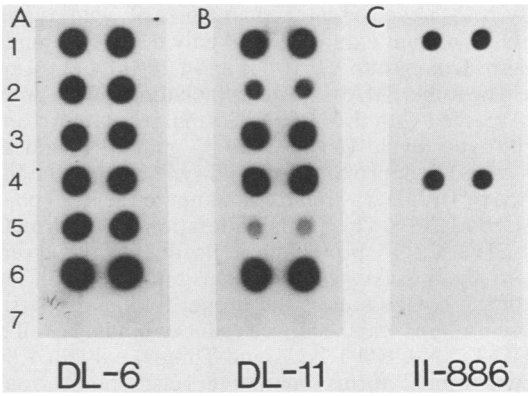

FIG. 5. Dot-blot analysis of wild-type gD-1 and gD-1 proteins lacking N-CHO with MAbs. A 2-μl sample of each cytoplasmic extract prepared from cells transfected with wild-type or mutant gD-1 genes was dotted in duplicate onto nitrocellulose. Each nitrocellulose strip was incubated with a different MAb as follows: (A) MAb DL-6 recognizing a continuous epitope, group II (24); (B) MAb DL-11 recognizing a discontinuous epitope, group Ib (43); (C) MAb II-886 recognizing a discontinuous epitope, group VI. Row 1, wild-type gD-1; row 2, N-CHO 1^-; row 3, N-CHO 2^-; row 4, N-CHO 3^-; row 5, N-CHO $1,2,3^-$; row 6, gD-1 from Tm-treated transfected cells; row 7, cytoplasmic extract from nontransfected cells. The excess MAb was removed, and the strips were incubated with iodinated protein A before being exposed to X-ray film.

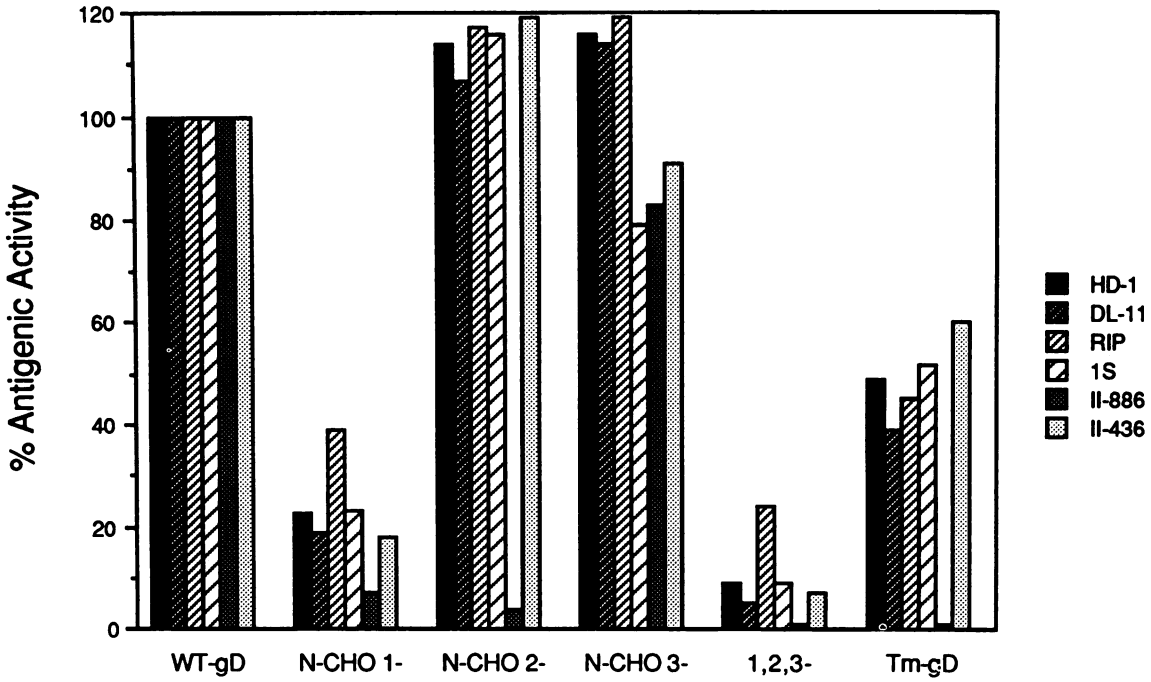

FIG. 6. Quantitative antigenic analysis of wild-type gD-1 and gD-1 proteins lacking N-CHO. Antigenic activity, expressed as percent wild type, was quantitated from dot blots (Fig. 4) as described in Materials and Methods. Each bar represents the average of two experiments. Eighteen MAbs were tested, and results obtained with six of the MAbs are shown here. Except for group VI, each MAb shown represents the results obtained for one MAb group. Two representatives of group VI are shown; II-886 gave the same pattern as I-206, and II-436 gave the same pattern as DL-2, D1, and 41S.

One MAb (I-206) reacted similarly to II-886 (data not shown).

To obtain quantitative data, the dots were cut out and their radioactivities were counted in a gamma counter (Fig. 6). The N-CHO 1^- mutant exhibited decreased binding of all MAbs to discontinuous epitopes tested. Indeed, the binding activity was never greater than 40% of wild type. The N-CHO 2^- mutant exhibited markedly decreased binding of two MAbs from group VI: II-886 and I-206 (3.4 to 9% of wild type). The other MAbs tested (including other group VI MAbs) reacted with the N-CHO 2^- mutant at approximately the wild-type level. The N-CHO 3^- mutant bound all the MAbs tested at approximately wild-type levels. The binding of MAbs to Tm-gD was reduced but not to the level observed for N-CHO $1,2,3^-$. This further alteration of conformation in the N-CHO $1,2,3^-$ protein may be due to the threonine/serine-to-alanine changes. In conclusion, absence of the first or all of the N-CHO alters the proper folding of gD-1.

Those proteins which showed reduced binding of all MAbs (N-CHO 1^-, N-CHO $1,2,3^-$, and Tm-gD) exhibited higher-molecular-weight forms on native gels, and it would be interesting to compare the antigenicity of these forms with that of monomers. Unfortunately, the dot-blot assay does not allow us to determine which forms of the protein are reacting with the MAbs. Experiments designed to assess the reactivity of the different forms were not conclusive; gD-1 lacking N-CHO loses most of its reactivity with MAbs to discontinuous epitopes during native gel electrophoresis and Western blotting (M. I. Muggeridge, T.-T. Wu, D. C. Johnson, J. C. Glorioso, R. J. Eisenberg, and G. H. Cohen, Virology, in press).

Processing and transport. (i) Rate of processing. We next examined the ability of the N-CHO mutants to be processed and transported through the cell. Pulse-chase analysis was used to examine processing of the N-CHO mutants from the precursor to the product form (7, 13, 39, 55; reviewed in references 3, 48, and 50). Transfected cells were pulse-labeled with [^{35}S]methionine-cysteine for 10 min and either harvested immediately or chased for various times (Fig. 7). The wild-type and N-CHO mutant proteins were each processed from a faster-migrating precursor form (pgD) to the more slowly migrating product form (gD); in each case, processing began by 20 to 40 min of chase and was nearly complete after 2 h (data not shown). To quantitate the data, we analyzed the bands by densitometry (Fig. 8). The rate of processing of gD-1 mutants lacking one N-CHO site was indistinguishable from that of wild-type gD-1. The time needed for one-half of the N-CHO to be processed from pgD to gD was approximately 60 min for each of the proteins tested. We conclude that transport to gD-1 to the Golgi apparatus (where further processing occurs) was not significantly affected by the absence of any one N-linked oligosaccharide.

(ii) Immunofluorescence. We next examined the N-CHO mutants using immunofluorescence to observe gD-1 expression internally as well as on the surface of transfected cells. Transfected cells were tagged on the surface with anti-gD serum and tetramethylrhodamine isothiocyanate conjugate (Fig. 9B, D, F, and H; Fig. 10B, D, and F) and then permeabilized with Nonidet P-40 and internally tagged with anti-gD serum and a fluorescein isothiocyanate conjugate (Fig. 9A, C, E, and G; Fig 10A, C, and E). As a control, cells were transfected with a plasmid which expresses a truncated form of gD-1 containing residues 1 to 182. This mutant protein does not contain a transmembrane region. It is neither transported nor secreted but remains inside the cell

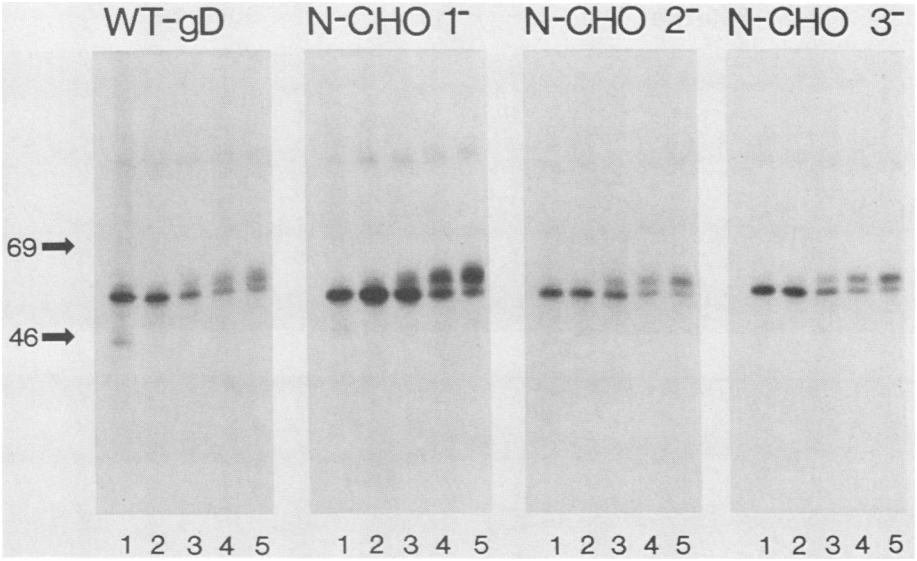

FIG. 7. Pulse-chase analysis of wild-type and mutant gD proteins lacking one N-CHO site. COS-1 cells were transfected with wild-type and mutant gD genes, pulsed-labeled with [^{35}S]cysteine-methionine for 10 min, and then chased in unlabeled medium for various times. Extracts were immunoprecipitated with polyclonal anti-gD-1 serum (10) and electrophoresed on a 10% denaturing polyacrylamide gel. Lane 1, 10-min pulse; lane 2, 10-min pulse followed by 20-min chase; lane 3, 40-min chase; lane 4, 60-min chase; lane 5, 80-min chase. Molecular size markers of 46 and 69 kDa are indicated by arrows. WT, wild type.

(D. Long and D. L. Sodora, unpublished data). The lack of surface labeling (Fig. 10F) and the presence of internal labeling (Fig. 10E) in cells expressing this mutant protein indicated that this technique is reliable.

We found that the pattern of internal labeling was similar in both wild-type (Fig. 9A), N-CHO 1$^-$ (Fig. 9C), N-CHO 2$^-$ (Fig. 9E), and N-CHO 3$^-$ (Fig. 9G) expressing cells. In each case, gD-1 was distributed diffusely throughout the cytoplasm. The internal pattern of N-CHO 1,2,3$^-$ (Fig. 10A) and Tm-gD (Fig. 10C) differed from that of wild-type gD-1 (Fig. 9A); in the former two cases, gD-1 appeared to be concentrated in the Golgi apparatus. Thus, the absence of any one N-CHO did not significantly affect intracellular transport; however, when all three were missing, the internal distribution of gD-1 was altered. Nevertheless, all forms of gD-1, including N-CHO 1,2,3$^-$ and Tm-gD, were found on the cell surface (Fig. 9B, D, F, and H; Fig. 10B and D). We conclude that the N-CHO 1,2,3$^-$ protein is transported to the transfected cell surface in sufficient quantity to obtain an immunofluorescent surface-staining pattern.

DISCUSSION

Many cellular proteins which are translocated into the endoplasmic reticulum are altered through the addition of carbohydrate residues attached through either N- or O-linkages (28, 41, 52). Enveloped viruses make use of this cellular pathway, producing proteins which are glycosylated as they mature in the endoplasmic reticulum and Golgi compartments (3, 32, 35). HSV-1 contains at least seven envelope proteins, each glycosylated by N-linked and possibly by O-linked oligosaccharides (7, 13, 39, 55; reviewed in reference 3). Here, we used site-directed mutagenesis to begin to analyze the role of N-CHO present on the gD protein from HSV-1.

The absence of N-CHO from the first glycosylation site of gD-1 resulted in the appearance of high-molecular-weight forms and affected conformation. Alteration of gD-1 at any

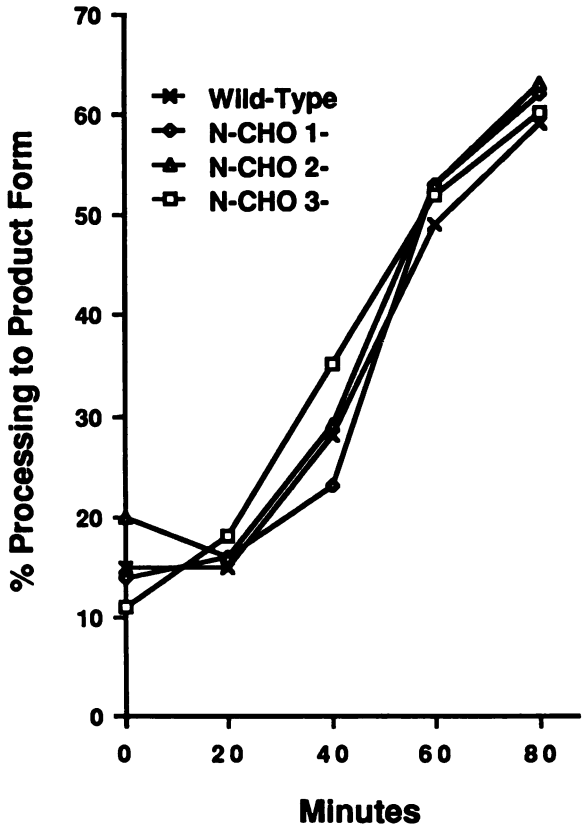

FIG. 8. Rate of processing of wild-type and mutant proteins lacking one N-CHO site. Fluorographs (Fig. 6) were scanned with a densitometer. For each time point, the amount of gD present as product form was calculated as [gD/(pgD + gD)] × 100.

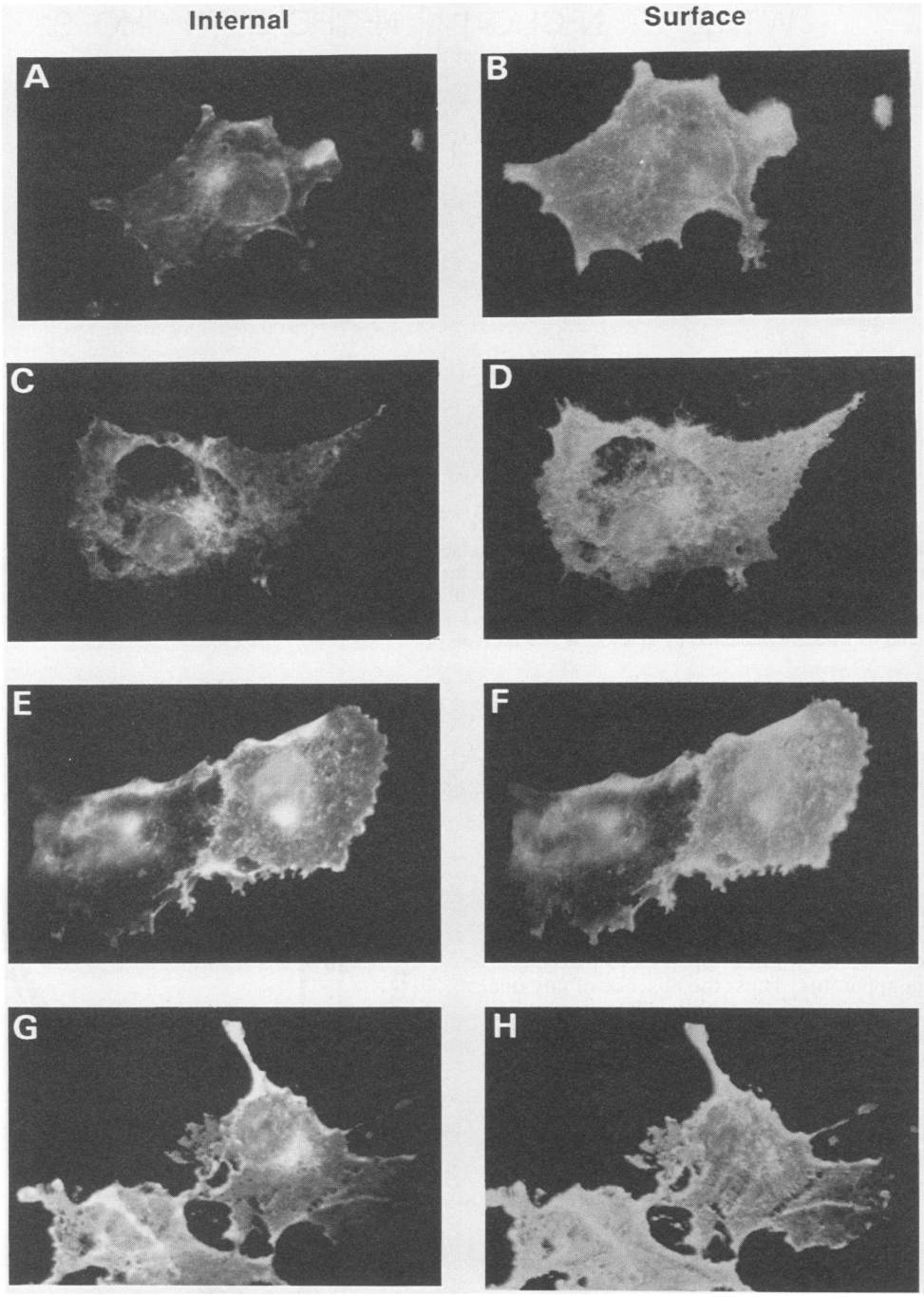

FIG. 9. Immunofluorescence analysis of cells expressing wild-type and mutant forms of gD-1. Double-label immunofluorescence was employed to detect gD-1 on the cell surface and inside cells (34). Cells were fixed with paraformaldehyde and reacted with anti-gD-1 antibody and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G to detect gD-1 on the cell surface (B, D, F, and H). Cells were then permeabilized with Nonidet P-40 and reacted with anti-gD-1 antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G to detect gD-1 within the cell (A, C, E, and G). Proteins analyzed are wild-type gD-1 (A and B), N-CHO 1^- (C and D), N-CHO 2^- (E and F), and N-CHO 3^- (G and H). The cells were viewed with a Leitz epifluorescence microscope.

one of the first six cysteine residues had similar effects (9, 62); we speculated that the mutant gD-1 proteins may be aggregating owing to the presence of an unpaired cysteine which formed intermolecular disulfide bonds (62). The N-CHO at site 1 may be important for proper disulfide bond formation and protein folding since it is located between the first and second cysteine residues. The absence of N-CHO from sites 2 and 3 had little effect on overall gD-1 conformation. However, N-CHO at site 2 (or the serine at position 123) appears to be important for the maintenance of two epitopes (MAbs II-886 and I-206). We favor the idea that the loss of these two epitopes is due to the absence of N-CHO rather than to the alteration of the amino acid because these same epitopes are essentially absent in Tm-gD. Interest-

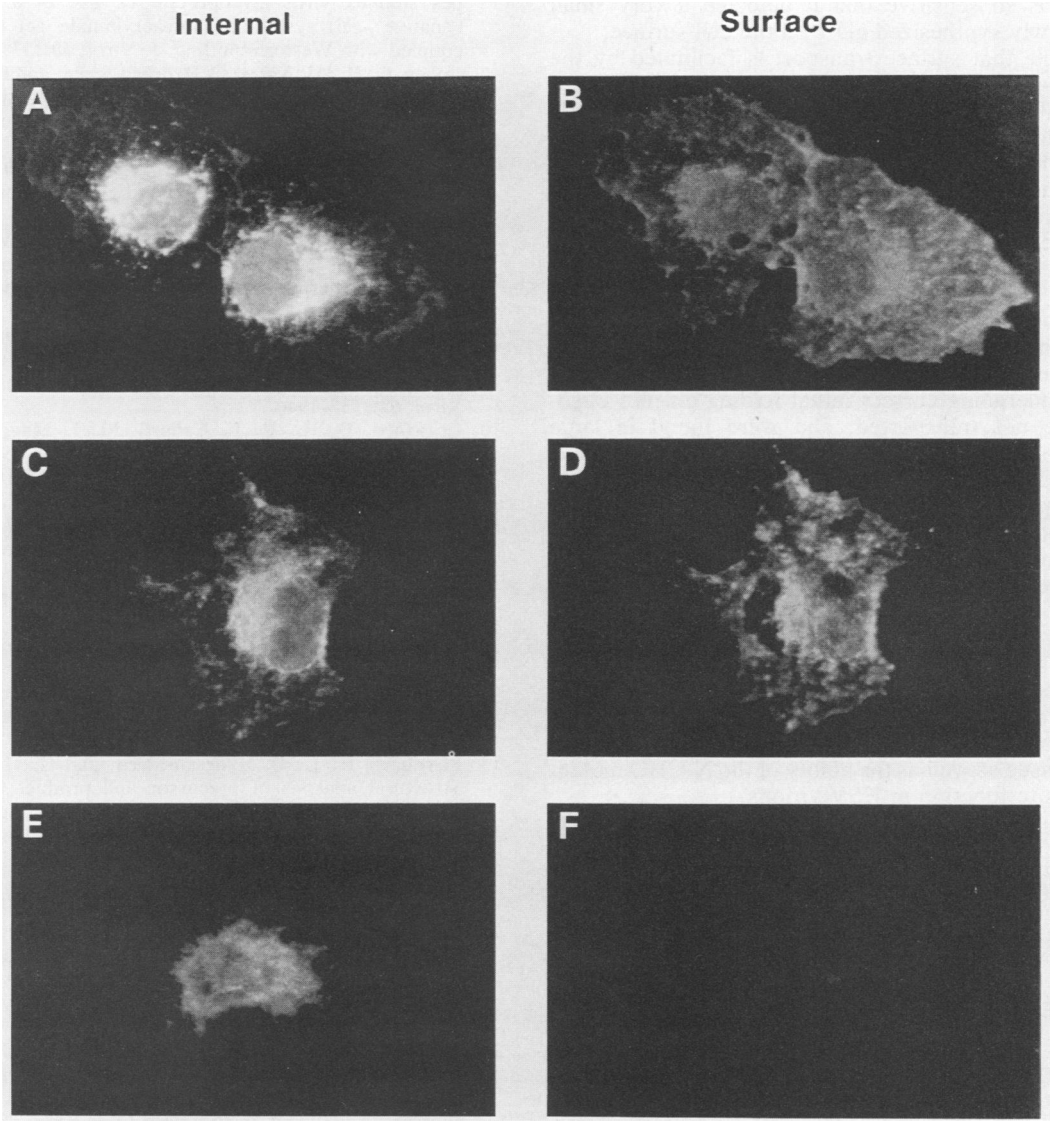

FIG. 10. Immunofluorescence analysis of cells expressing either gD-1 lacking N-CHO or a truncated form of gD-1. Double-label immunofluorescence was employed to detect gD-1 on the cell surface (B, D, and F) and inside cells (A, C, and E) as described in the legend to Fig. 8. Proteins analyzed are N-CHO 1,2,3$^-$ (A and B), gD-1 expressed in Tm-treated transfected cells (C and D), and a gD-1 truncation mutant containing amino acids 1 to 182 (E and F). The gD-1 truncation mutant does not contain a membrane-anchoring region. The cells were viewed with a Leitz epifluorescence microscope.

ingly, MAbs II-886 and I-206 are effective at blocking adsorption of HSV-1 to cells (19).

MAbs in groups Ia, Ib, III, IV, and VI displayed less reactivity with the N-CHO 1,2,3$^-$ protein than with Tm-gD (Fig. 6). Also, the N-CHO 1$^-$ and N-CHO 1,2,3$^-$ proteins tended to have a greater amount of their protein present in high-molecular-weight forms than Tm-gD (Fig. 4). These data suggest that the amino acid changes themselves probably contribute to some extent to the disruption of gD-1 conformation. A similar result was reported for class I HLA (1, 52).

The wild-type gD-1 and each of the N-CHO mutants altered at one site were processed from precursor to product forms at approximately equal rates (Fig. 8). The half-time for gD-1 processing in transiently transfected cells was determined to be 60 min. A similar rate was previously observed for the processing of gD during an HSV-1 infection (7). However, Johnson and Smiley (25) found that gD-2 was more efficiently processed in a transfected cell line than in HSV-2-infected cells. The difference in processing rates observed in transiently transfected cells and in the cell line might be due to an inherent difference in the two expression systems.

N-CHO 1,2,3$^-$ and Tm-gD appeared to accumulate in the Golgi apparatus, but some protein was present on the surface of the transfected cells (Fig. 10). Previously, Norrild and Pederson (45) as well as Peake et al. (46) determined that Tm treatment significantly reduced the amount of gD-1 on the surface of infected cells. Peake et al. (46) attributed this trace amount of surface gD-1 to residual glycoproteins left after fusion of the viral and cellular envelopes. Two possible ways to reconcile our results with previous studies are the following: (i) transport of gD-1 lacking N-CHO is different in infected and transfected cells; or (ii) the technique used in

this study was so sensitive that it detected a very small amount of newly synthesized gD-1 on the cell surface.

It is possible that surface transport is facilitated by the addition of *O*-glycans to the N-CHO 1,2,3$^-$ protein (Fig. 3). A gD homolog (gp50) found in pseudorabies virus has no N-CHO but does contain *O*-glycans (47). A cell line which is defective in the addition of *O*-glycans to glycoproteins (28) will be used to determine the importance of *O*-glycans for cell surface transport of gD-1.

The absence of the first or all three N-CHO alters the conformation of gD-1 but does not prevent processing or transport to the cell surface. Doms and colleagues (11) observed a correlation between protein conformation, oligomerization, and rate of processing of the vesicular stomatitis virus G protein (11). They stated: "Mutant proteins not capable of undergoing correct initial folding did not oligomerize, were not transported, and were found in large aggregates." The oligomeric state of the gD-1 protein is presently unknown. If gD-1 does not have to oligomerize to exit the endoplasmic reticulum, then perhaps proper protein folding is not a requirement for gD-1 transport. Alternatively, the binding of MAbs may be sensitive to very subtle changes in the conformation of the N-CHO 1$^-$ and N-CHO 1,2,3$^-$ proteins, in which case the conformation of these proteins may be altered but not sufficiently to prevent transport. It should also be noted that gD-1 transport was only examined qualitatively. There could be a correlation between the conformation of gD-1 and the amount that is transported to the cell surface. Future studies are planned to address this issue as well as the ability of the N-CHO mutant gD-1 proteins to function in HSV virions.

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