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Intracellular Transport of Herpes Simplex Virus gD Occurs More Rapidly in Uninfected Cells than in Infected Cells

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A mouse L cell line which expresses the herpex simplex virus type 1 immediate-early polypeptides ICP4 and ICP47 was cotransfected with a cloned copy of the *Bgl*II L fragment of herpes simplex virus type 2, which includes the gene for gD, and the plasmid pSV2neo, which contains the aminoglycosyl 3'-phosphotransferase (agpt) gene conferring resistance to the antibiotic G418. A G418-resistant transformed cell line was isolated which expressed herpes simplex virus type 2 gD at higher levels than were found in infected cells. The intracellular transport and processing of gD was compared in transformed and infected cells. In the transformed Z4/6 cells gD was rapidly processed and transported to the cell surface; in contrast, the processing and cell surface appearance of gD in infected parental Z4 cells occurred at a much slower rate, and gD accumulated in nuclear membrane to a greater extent. Thus, the movement of HSV-2 gD to the cell surface in infected cells is retarded as viral glycoproteins accumulate in the nuclear envelope, probably because they interact with other viral structural components.

Herpes simplex viruses (HSVs) acquire an envelope composed of viral glycoproteins and cellular lipids as viruses bud into the perinuclear space (8, 26). Viral glycoproteins are thought to be synthesized on membrane-bound ribosomes attached to the rough endoplasmic reticulum and the outer nuclear membranes. These two membrane systems have very similar biochemical compositions (reviewed in references 11 and 14) and are contiguous (2, 11, 14). HSV glycoproteins destined to be part of nascent virions must be transported into the inner nuclear membrane, which lacks ribosomes (14), probably via the pore complexes which join the inner and outer nuclear membranes.

Virions are transported from the perinuclear space to the Golgi apparatus, where the envelope glycoproteins are further processed. Asparagine-linked (N-linked) oligosaccharides are converted from the high-mannose form to the complex form (17, 35, 41) and serine- or threonine-linked (O-linked) oligosaccharides are attached to the glycoproteins (17, 28). In addition, at least one of the HSV glycoproteins, gE, is fatty acid acylated (17). Processing of HSV glycoproteins from immature to mature forms in the Golgi apparatus is accompanied by discrete shifts in the electrophoretic mobility of the polypeptides on sodium dodecyl sulfate (SDS)-polyacrylamide gels (5, 9, 17, 37). From the Golgi apparatus virions move to the cell surface, where they are released into the extracellular space or remain stuck to the cell surface. If the transport of cellular membranes from the Golgi apparatus to the cell surface is blocked by treating cells with the ionophore monensin, virions containing partially processed glycoproteins accumulate in distended, cytoplasmic vacuoles (16). Viral glycoproteins not part of the virion envelope also appear at the cell surface (29) and probably follow a similar pathway, although these glycoproteins may not pass through the inner nuclear membrane.

The intracellular transport and processing of the HSV glycoproteins differs in a number of ways from that described for the vesicular stomatitis virus G protein, which has been extensively studied as a model for cellular membrane glycoprotein biogenesis. G protein is synthesized on membrane-bound ribosomes of the rough endoplasmic reticulum (13, 27), where two N-linked oligosaccharides are added to the polypeptide (10, 23). Evidence has also been presented that G protein accumulates in blebbed regions of the outer nuclear membrane and can diffuse into the inner nuclear membrane (2). G protein is then transported, possibly in clathrin-coated vesicles (33), from the rough endoplasmic reticulum-nuclear envelope to the Golgi apparatus, where N-linked oligosaccharides are processed (4, 32, 39, 40) but no O-linked oligosaccharides are attached. From the Golgi apparatus the polypeptide moves to the plasma membrane, where it participates in the envelopment of viral nucleocapsids which are assembled in the cytoplasm (20, 21). The intracellular transport and processing of G protein occur very rapidly. G protein can be detected in the Golgi apparatus ca. 5 to 10 min after its synthesis and reaches the cell surface 10 to 20 min later (2, 21).

In contrast, HSV glycoproteins are transported to the Golgi apparatus and are processed much more slowly (5, 17, 42), and pulse-labeled glycoproteins do not reach the surfaces of infected HEp-2 cells until ca. 3 to 4 h after polypeptide synthesis (D. C. Johnson and P. G. Spear, unpublished data). The slow intracellular transport of HSV glycoproteins in infected cells may reflect the period of time in which viral glycoproteins accumulate in nuclear membranes. It also seems possible that HSV glycoproteins are targeted or specifically transported to the inner nuclear membrane, where they participate in the envelopment process, and that this process slows the movement of viral glycoproteins to the Golgi apparatus and cell surface.

To test these hypotheses we characterized the intracellular transport and processing of HSV type 2 (HSV-2) gD in transformed cells which constitutively express high levels of this glycoprotein. In these cells gD was rapidly processed and transported to the cell surface, whereas in the untransformed parental cells infected with HSV-2 processing and transport of gD to the cell surface was retarded, and gD accumulated in nuclear membranes. These results argue that HSV-2 gD accumulates or pools in nuclear membranes of infected cells before it is transported to the Golgi apparatus and cell surface. Accumulation of gD in the nuclear envelope

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is probably induced by viral structural polypeptides synthesized in infected cells and does not solely depend upon properties of the gD molecule itself.

MATERIALS AND METHODS

Cells and virus. Vero cells were grown in alpha minimal essential medium (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), penicillin-streptomycin (GIBCO), 0.3% glutamine, 0.075% $NaHCO_3$, and 0.01M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (complete minimal essential medium). Z4 cells were grown in complete minimal essential medium supplemented with 5% fetal bovine serum, hypoxanthine, thymidine, and methotrexate (38). HSV-2, strain 333, and HSV-1, strain F, were propagated at low multiplicity and assayed by plaque formation on Vero cell monolayers.

Antibodies. A rabbit serum which immunoprecipitates HSV-1 and HSV-2 gD was kindly provided by G. Cohen and R. Eisenberg. Ascites fluids containing the monoclonal antibodies 17βA3, an HSV-2 type-specific anti-gD antibody, and 13αC5, which immunoprecipitates p94K, were provided by W. Rawls and S. Bacchetti. The type-common anti-gE monoclonal antibody II-481 was a gift of P. Spear.

Isolation of cell lines expressing HSV-2 gD. Subconfluent 60-mm dishes of Z4 cells (30a) were cotransfected with 0.5 μg of pSV2neo DNA, which contains the aminoglycosyl 3'-phosphotransferase (agpt) gene (36), and 4.5 μg of pBB71 DNA, which contains a cloned copy of the *Bgl*II L fragment (map units 0.88 to 0.94) of HSV-2 (L. Loh and S. Bacchetti, unpublished data), by using the $CaPO_4$ precipitation technique (12). One day after transfection the medium was replaced with medium containing 400 μg of G418 (geneticin; GIBCO) per ml, and after 19 days individual colonies were trypsinized by using a cloning cylinder and expanded into cell lines in medium containing G418 (100 μg/ml). These cell lines were labeled with [^{35}S]methionine (50 μCi/ml, 1,100 to 1,400 to Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in medium containing no methionine for 5 h and extracted with 50 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl–1% Nonidet P-40 (NP40)–0.5% sodium deoxycholate (DOC)–1 mg of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) per ml–0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma)–1 mg of aprotinin (Sigma) per ml (NP40-DOC extraction buffer), and gD was immunoprecipitated.

Pulse-chase labeling experiments, immunoprecipitations, and gel electrophoresis. Z4 cells or Z4/6 cells were infected with 10 PFU per cell of HSV-2 (strain 333) or HSV-1 (strain F), respectively, in complete medium, and the virus was removed after 1.5 h. Infected cells (6 to 8 h after infection for HSV-2 or 11 h for HSV-1) or uninfected cells were labeled with [^{35}S]methionine (200 μCi/ml) in medium containing no methionine for 10 min or were labeled with [^{35}S]methionine for 10 min and then washed once with medium and incubated with medium containing a fivefold excess of unlabeled methionine for 20, 40, or 90 min. The cells were extracted immediately with NP40-DOC extraction buffer (ca. 2×10^6 cells per ml) and sonicated, and the lysates were clarified by centrifugation at 68,000 × *g* for 1 h. For immunoprecipitation, 0.2 to 0.5 ml of the lysates were mixed with 5 μl of anti-gD rabbit serum or 5 μl of ascites fluid for 1 h at 4°C, and then protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.) were added for 1 to 2 h at 4°C on a rotating wheel. The beads were washed twice in NP40-DOC extraction buffer, once in RIPA buffer (50 mM Tris-hydrochloride [pH 7.2], 150 mM NaCl, 0.1% SDS, 1% DOC, 1% Triton X-100), and once with 50 mM Tris-hydrochloride (pH 6.8) containing 0.1% SDS. The precipitated proteins were eluted from the beads by adding twofold-concentrated sample buffer (100 mM Tris-hydrochloride [pH 6.8], 4% SDS, 4% β-mercaptoethanol, 20% glycerol, and bromophenol blue). Samples were electrophoresed in 8.5% *N*,*N'*-diallytartardiamide cross-linked polyacrylamide gels as described by Heine et al. (15) at 50 V for 12 h. Gels were infused with 2,5-diphenyloxazole by the procedure of Bonner and Laskey (3) and then dried and placed in contact with Kodak XAR film.

Immunofluorescence. Monolayers of Z4/6 cells, Z4 cells or Z4 cells infected with HSV-2 (8 h after infection) growing on glass cover slips in 35-mm dishes were washed twice with phosphate-buffered saline (PBS [10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$ 1 mM $CaCl_2$ [pH 7.4]) containing BSA (10 mg/ml) and then incubated with 1 ml of PBS containing 10 μl of 17βA3 ascites fluid and BSA (10 mg/ml) at 4°C for 1 h. The cells were washed three times with PBS containing BSA (10 mg/ml) and then incubated with affinity-purified, fluoresceinated rabbit anti-mouse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) at 4°C for 1 h, washed three times with PBS containing BSA (10 mg/ml), and fixed with 2% glutaraldehyde. The cover slips were mounted in 50% (vol/vol) glycerol–50% (vol/vol) PBS containing *p*-phenylenediamine (10 mg/ml) (19) and viewed in a Zeiss fluorescence microscope.

Cell surface iodination. Monolayers of Z4 cells, Z4/6 cells, and Z4 cells infected for 7 h with HSV-2 grown in 25-cm^2 plastic flasks were labeled with ^{125}I by the procedure of Sefton et al. (34). Cells were subsequently washed once with PBS containing 10 mM NaI and twice with PBS and extracted with NP40-DOC extraction buffer.

Trypsin treatment of cell surfaces. Cells grown in 35-mm dishes which had been labeled for 10 min with [^{35}S]methionine or labeled for 10 min and incubated with medium containing unlabeled methionine for various times were washed twice with PBS and then incubated with 1.5 ml of PBS or 1.5 ml of PBS containing trypsin (0.5 mg/ml; type XIII; Sigma) for 10 min at 37°C. Ten microliters of PMSF (50 mg/ml in EtOH) was added immediately, and then the cells were washed twice with 50 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl containing soybean trypsin inhibitor (2.5 mg/ml; Sigma), BSA (5 mg/ml; Sigma), aprotinin (1 mg/ml; Sigma), and 0.5 mM PMSF at 4°C. The cells were extracted in NP40-DOC extraction buffer, and the extracts were frozen at −70°C.

Subcellular fractionation of cells. Monolayer cultures of Z4/6 cells or Z4 cells infected with HSV-2 (7 h after infection) grown in 150-cm^2 flasks were labeled with [^{35}S]methionine for 10 min or labeled for 10 min and then incubated in medium containing an excess of unlabeled methionine for 20, 40, or 90 min. The cells were washed twice with ice-cold PBS and then scraped into cold PBS, centrifuged at 1,000 × *g* for 5 min, resuspended in 10 mM Tris-hydrochloride (pH 7.5)–1 mM $MgCl_2$ for 15 min at 4°C, and disrupted by Dounce homogenization (usually 20 to 30 strokes with a tight-fitting pestle). Cell breakage was monitored by phase microscopy and was always >95%. Less than 10% of the nuclei were disrupted. Nuclei and cytoplasmic fractions were purified by the procedures of Lyles and McConnell (24). Briefly, the homogenate was centrifuged at 500 × *g* for 10 min. The pellet was suspended in 10 mM Tris-hydrochloride (pH 7.5)–1 mM $MgCl_2$ with a loose-fitting

Dounce homogenizer, layered over 45% sucrose ([wt/wt] in 10 mM Tris-hydrochloride [pH 7.5]–1 mM $MgCl_2$), and centrifuged at 1,600 × *g* for 30 min. The pellet contained the nuclear fraction. Supernatants were pooled and centrifuged at 100,000 × *g* for 1 h and contained the cytoplasmic membrane fraction. The nuclear and cytoplasmic membrane fractions were extracted with NP40-DOC extraction buffer, and gD was immunoprecipitated.

RESULTS

Construction of cell lines expressing HSV-2 gD. A mouse cell line, known as Z4, was derived after cotransfection of Lta cells with the *Xho*I D fragment of HSV-1, which contains the genes for ICP4 and ICP47, and a recombinant fragment of HSV-1, which contains the thymidine kinase (*tk*) gene linked to the late VP5 promoter, and after selection of *tk* positive cells (30a). Z4 cells express high levels of ICP4 and barely detectable amounts of ICP47, and these immediate-early polypeptides activate in *trans* delayed early genes, including gB, gD, gE, tk, ICP6, and ICP8, after the cells are infected with HSV under cycloheximide blockage-reversal conditions (30a). Therefore, we chose this cell line as a recipient for transformation with the HSV-2 gD gene, as it seemed possible that the transfected gene would be expressed at a high level. Z4 cells were cotransfected with plasmid pBB71, which contains a cloned copy of the *Bgl*II L fragment of HSV-2 (Loh and Bacchetti, unpublished data), and plasmid pSV2-neo (36), which contains the aminoglycosyl 3′-phosphotransferase gene (agpt) conferring resistance to the antibiotic G418, by using the $CaPO_4$ procedure of Graham and van der Eb (12). Six cell lines resistant to G418 (400 μg/ml) were selected, and two were found to express high levels of gD-2 (data not shown). One of these two cell lines, Z4/6, was chosen for further study. The level of expression of gD in Z4/6 cells was compared with that in infected Z4 cells by labeling cells with [^{35}S]methionine for 5 h (from 3 h after infection until 8 h after infection in the case of the infected cells) or for 10 min (5 h after infection in the case of the infected cells). The cells were extracted with detergent, gD was immunoprecipitated with a polyclonal anti-gD antibody, and the precipitates were subjected to electrophoresis on SDS-polyacrylamide gels (Fig. 1). When the cells were labeled for 5 h, we detected larger amounts of gD in infected Z4 cells than in Z4/6 cells. However, after the short pulse of [^{35}S]methionine, significantly more of the immature form of gD, pgD, was labeled in Z4/6 cells than in Z4 cells infected with HSV-2. Therefore, it would appear that Z4/6 cells synthesize higher levels of gD than infected Z4 cells and that gD turns over more rapidly in the transformed cells.

The gene for HSV-2 gE has not yet been precisely mapped in the viral genome, although it is assumed that it is colinear with the position of HSV-1 gE which maps in the S component of HSV, very close to gD-1 (22). This assumption is supported by the results of experiments with HSV-1-HSV-2 recombinants (25, 30). Similarly, the gene for an HSV-2 glycoprotein p92K (25) and an HSV-2 glycoprotein, pl24K, newly designated gG (31), have been mapped to the S component of HSV-2. It has been suggested that p92K and gG may be the same polypeptide and may differ in apparent molecular weight due to differences in the compositions of the polyacrylamide gel systems used (31). Since *Bgl*II-L spans a region of the S component of the HSV-2 genome which may encode the structural genes for these glycoproteins, we determined whether the Z4/6 cell line could also express gE or p92K. Detergent extracts from Z4/6 and infected Z4 cells were subjected to immunoprecipitation with the monoclonal antibodies II-481, which precipitates gE-1 and gE-2 (16; D. C. Johnson, unpublished data), and 13αC5, which precipitates p92K (T. Minson, unpublished data). Both gE and a polypeptide of ca. 125 kilodaltons, precipitated with 13αC5, were detected in infected Z4 cells but could not be detected in Z4/6 cells. However, this experiment does not exclude low-level expression of either of these polypeptides in Z4/6 cells. Although Persson et al. (in press) found that ICP4 and ICP47 were able to activate gE expression, it is not known whether these early gene products are sufficient to activate gG.

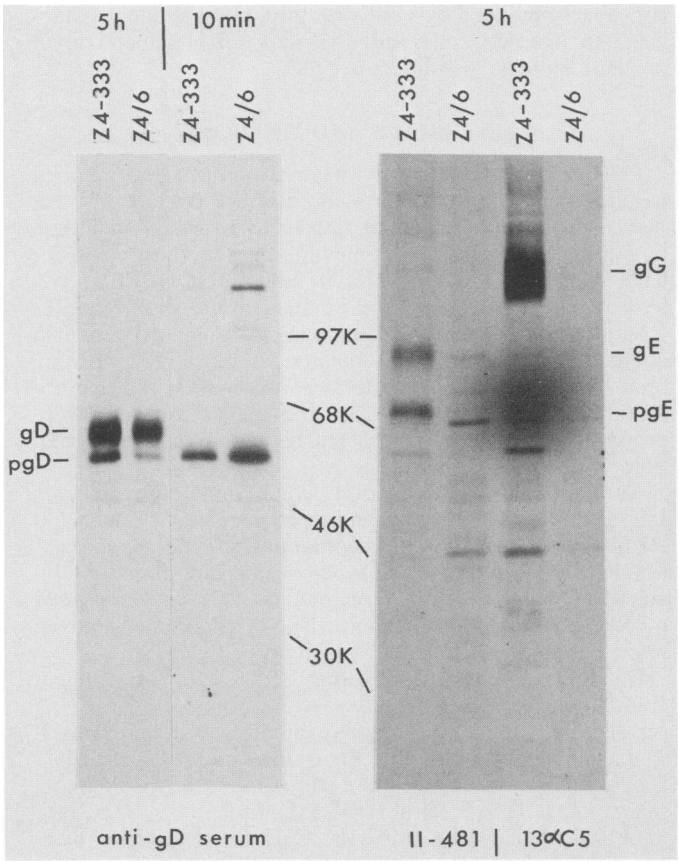

FIG. 1. Synthesis of gD, gE, and p92K in Z4 cells infected HSV-2 (Z4-333) and Z4/6 cells. Cells grown in 25-cm^2 flasks were labeled with [^{35}S]methionine for 5 h (3 to 8 h after infection in the case of Z4-333) or 10 min (5 h after infection in the case of Z4-333). HSV-2 gD was immunoprecipitated with a type-common anti-gD serum, gE was immunoprecipitated with the monoclonal antibody II-481, and p92K was immunoprecipitated with the monoclonal antibody 13αC5. The precipitates were electrophoretically separated on 8.5% polyacrylamide gels cross-linked with *N*, *N*′-diallytartardiamide (15). The immature or precursor forms of gD and gE are indicated by pgD and pgE, respectively, and p92K is indicated by gG.

Expression of gD on the cell surface of Z4/6 cells. The extent to which gD was expressed on the cell surface of Z4/6 cells was determined by immunofluorescence. Unfixed cells were treated with a monoclonal anti-gD antibody, 17βA3 (1), at 4°C, followed by a fluoresceinated rabbit anti-mouse immunoglobulin G, and then fixed with glutaraldehyde. A high degree of variation in the levels of expression of gD on the

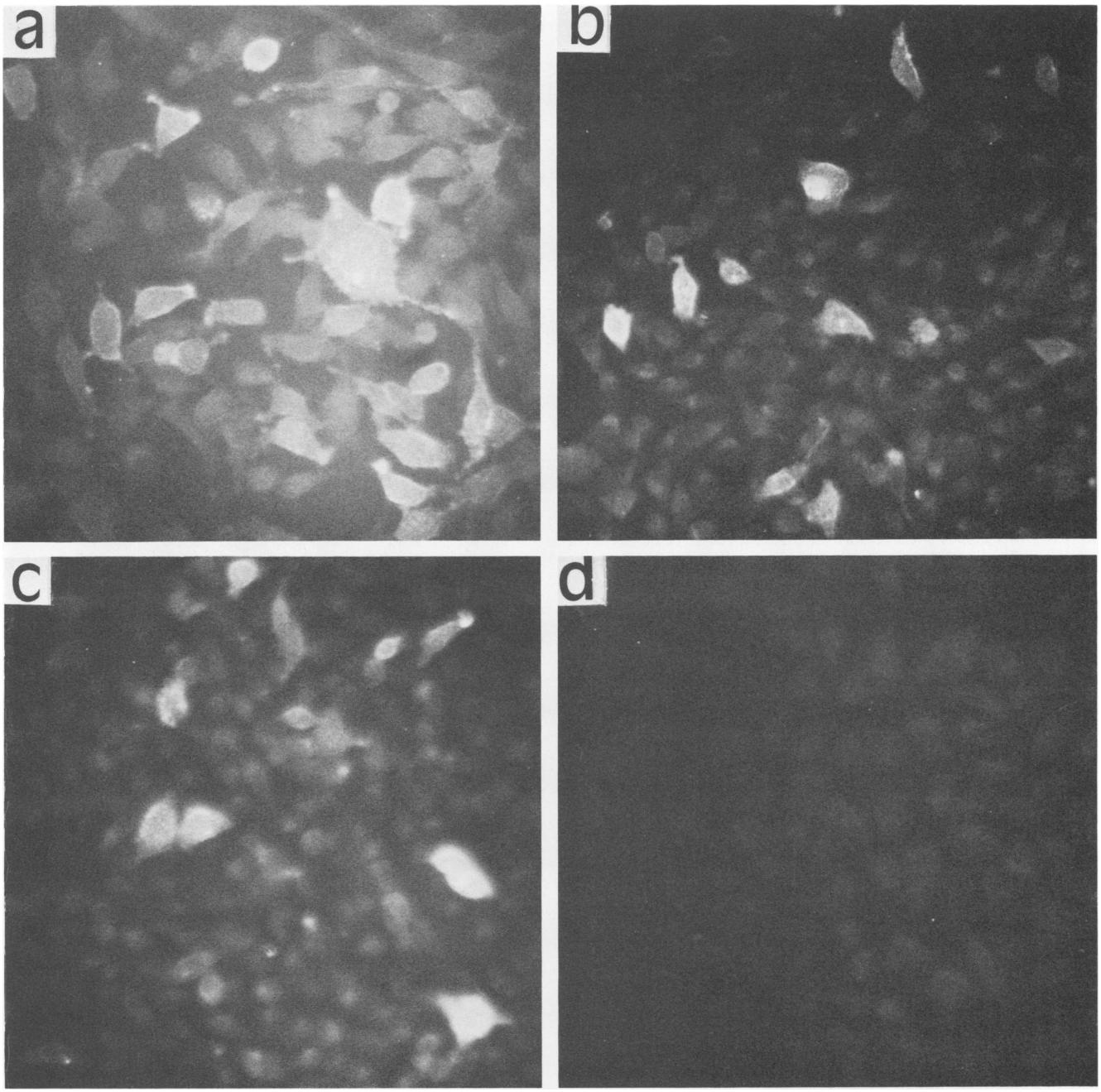

FIG. 2. Cell surface immunofluorescence of Z4 cells, Z4/6 cells, and Z4 cells infected with HSV-2. Monolayers of Z4 cells infected for 8 h with HSV-2 (a), Z4/6 cells (b and c), or Z4 cells (d) growing on glass cover slips were incubated with PBS containing 17βA3 antibodies at 4°C for 1 h and then washed and incubated with fluoresceinated rabbit anti-mouse immunoglobulin G at 4°C for 1 h. The cover slips were washed, fixed in 2% glutaraldehyde, mounted in 50% (vol/vol) glycerol–50% PBS containing *p*-phenylenediamine, and viewed in a fluorescence microscope.

surfaces of individual Z4/6 cells was observed (Fig. 2b and c). Similarly, variations in the level of gD expression with infected Z4 cells were observed (Fig. 2a); however, in no case were the variations observed with infected Z4 cells as great as with Z4/6 monolayers. Immunofluorescence of acetone-fixed monolayers of Z4/6 and infected Z4 cells indicated that, in both cases, all of the cells expressed gD; however, quantitative differences in the levels of gD expression were not noted. When colonies derived from individual Z4/6 cells were examined by immunofluorescence, the same variations in the levels of cell-surface expression of gD were observed among individual cells in each of the colonies (data not shown).

In another series of experiments, Z4/6 cells and Z4 cells infected with HSV-2 were subjected to lactoperoxidase-catalyzed iodination of cell surface proteins. A polyclonal anti-gD antibody was used to immunoprecipitate gD from detergent extracts of ^{125}I-labeled cells, and the precipitated material was separated on SDS-polyacrylamide gels. The level of cell surface expression of gD in Z4/6 cells, as

detected by iodination of the polypeptide, was higher than that found in Z4 cells 8 h after infection with HSV-2 (Fig. 3). In another experiment where infected Z4 cells were iodinated 5 h after infection, the level of iodinated gD was lower than that found at 8 h after infection (data not shown).

Processing of gD in Z4/6 cells. To compare the processing of gD in transformed and infected cells, we performed a pulse-chase experiment in which monolayers of cells were labeled for 10 min with [^{35}S]methionine or labeled for 10 min and then incubated with medium containing excess unlabeled methionine for 20, 40, or 90 min. Detergent extracts of the cells were immunoprecipitated with a polyclonal anti-gD antibody, and the precipitates were electrophoresed on SDS-polyacrylamide gels. In Z4 cells infected with HSV-2 the immature form of gD, pgD, was slowly converted to mature gD (Fig. 4). A large fraction of pgD remained after the 90-min chase period in infected cells. In contrast, pgD was rapidly converted to gD in the transformed Z4/6 cells (Fig. 4). Approximately half of the pgD was processed to gD as early as 20 min after the labeling period.

Intracellular transport of gD in Z4/6 cells. Comparison of the kinetics of processing of gD in transformed and infected cells suggests that transport of gD to the Golgi apparatus, where pgD is converted to gD (17), occurs more rapidly in the transformed Z4/6 cells than in infected cells (Fig. 4). To examine the possibility that gD is also transported to the cell surface more rapidly in Z4/6 cells, two experiments were performed. The first experiment involved a pulse-chase experiment, in which at various times after the pulse cells were treated with trypsin to degrade cell surface polypeptides. Cells were labeled for 10 min with [^{35}S]methionine or labeled for 10 min and then incubated with medium containing excess unlabeled methionine for 20, 40, 90, or 120 min. At the end of the labeling or chase periods, the cells were immediately treated with trypsin for 10 min at 37°C and then washed with buffer containing trypsin inhibitors and extracted with detergent. Detergent extracts of the cells were then immunoprecipitated with polyclonal anti-gD antibody. In this experiment, none of the pgD (Fig. 5) or any of the cytoplasmic viral polypeptides (results not shown) were degraded by trypsin, supporting the notion that trypsin acted solely on cell surface polypeptides. Therefore, acquisition of trypsin sensitivity of gD in these samples signals the appearance of gD at the cell surface. In Z4/6 cells gD rapidly appeared on the cell surface very shortly after pgD was processed to gD (Fig. 5). In these cells, most of the pgD was converted to gD after the 20-min chase period, and a large fraction of gD was already on the cell surface at this time. In contrast, the processing and cell surface appearance of gD in infected Z4 cells were much slower (Fig. 5). In a series of experiments identical to those described above except that HEp-2 cells infected with HSV-1 were treated with trypsin 8 h after infection, it was found that gC, gB, and gD all reached the cell surface only after a 3-h chase period (D. C. Johnson and P. G. Spear, unpublished data).

The second experiment which was performed to compare the intracellular transport of gD in Z4/6 cells with that in infected Z4 cells involved subcellular fractionation of cells into nuclear and cytoplasmic fractions. Again, cells were labeled for 10 min with [^{35}S]methionine or were labeled for 10 min and then incubated for various times in medium containing excess unlabeled methionine. Then the cells were

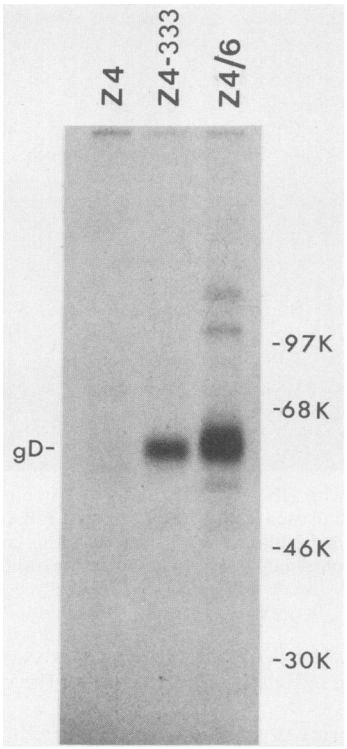

FIG. 3. Iodination of gD on the surfaces of Z4 cells, Z4/6 cells, and Z4 cells infected with HSV-2 (Z4-333). Monolayers of cells growing in 25-cm^2 flasks were subjected to lactoperoxidase-catalyzed iodination of cell surface proteins by the procedure of Sefton et al. (34). The cells were washed, extracts of the cells were made, and gD was immunoprecipitated with rabbit anti-gD serum.

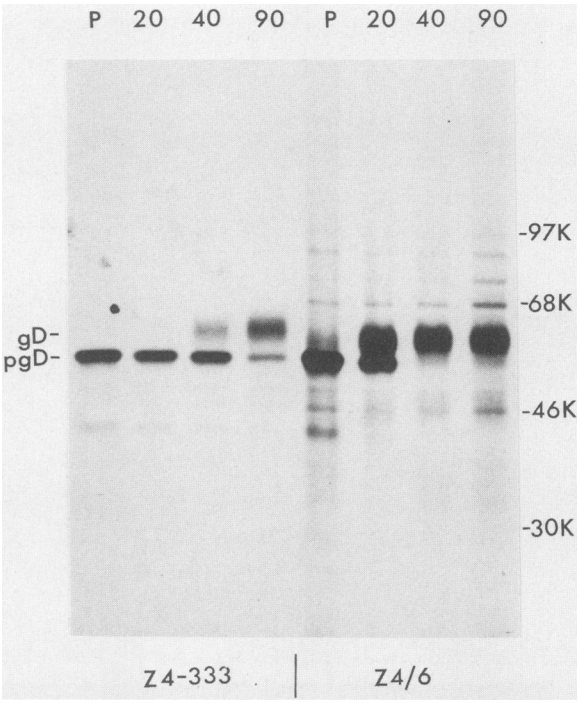

FIG. 4. Processing of gD in Z4/6 cells and Z4 cells infected with HSV-2 (Z4/333). Monolayers of cells growing in 25-cm^2 plastic flasks were labeled with [^{35}S]methionine for 10 min (P) or were labeled for 10 min and then incubated with medium containing excess unlabeled methionine for 20, 40, or 90 min. The cells were extracted with NP40-DOC extraction buffer, and gD was immunoprecipitated with anti-gD serum.

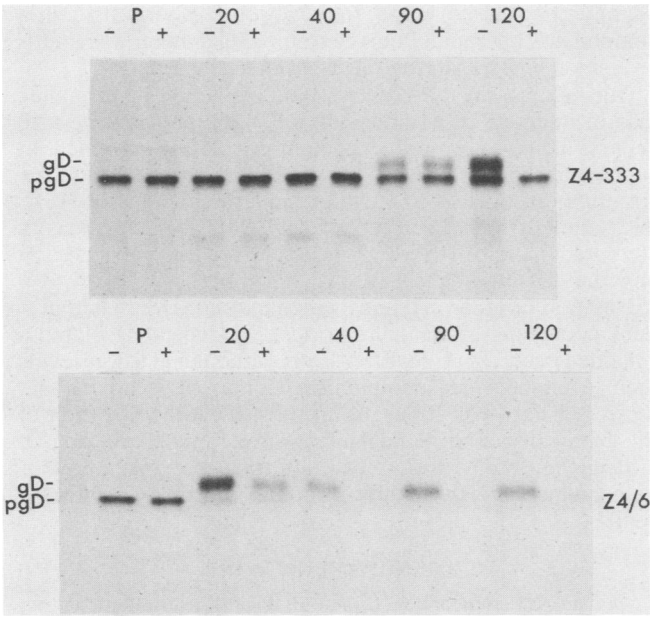

FIG. 5. Treatment of infected and transformed cell surfaces with trypsin. Monolayers of Z4 cells infected for 6 h with HSV-2 (Z4-333), or Z4/6 cells were labeled with [^{35}S]methionine for 10 min or were labeled for 10 min and then incubated in medium containing excess unlabeled methionine for 20, 40, 90, or 120 min. At the end of the labeling or chase period, the cells were immediately treated (+) or not treated (−) with trypsin (0.5 mg/ml) for 10 min at 37°C. The enzyme was inhibited by adding PMSF, and the cells were washed twice in 50 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl containing soybean trypsin inhibitor (2.5 mg/ml), BSA (5 mg/ml), aprotinin (1 mg/ml), and 0.5 mM PMSF. Extracts of the cells were immunoprecipitated with anti-gD serum.

fractionated into two fractions, one enriched in nuclei and the other containing primarily cytoplasmic membranes. These fractions were extracted with detergent, and the extracts were immunoprecipitated with a polyclonal anti-gD antibody. We detected only pgD and no gD in the nuclear fractions (Fig. 6), as has been reported by others (6). Approximately half of the pgD precipitated immediately after cells had been labeled for 10 min was found to be associated with the nuclear fraction in both infected Z4 cells and Z4/6 cells. In infected Z4 cells the amount of pgD in the nuclear fraction decreased slowly, so that a large fraction was still present after the 90-min chase. However, pgD labeled in Z4/6 cells disappeared much more rapidly from the nuclear fraction, coincident with the processing of pgD to gD and appearance of gD in the cytoplasmic fraction (Fig. 6) and on the cell surface (Fig. 5).

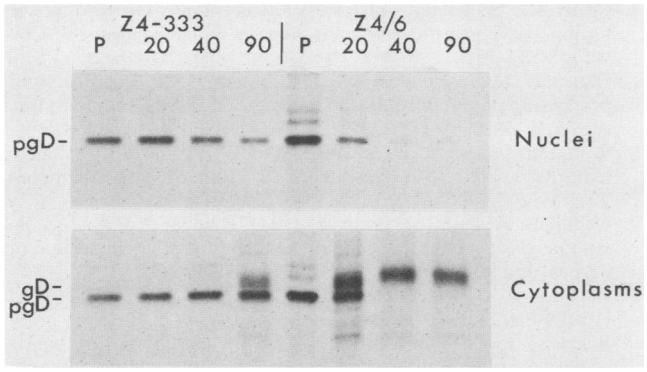

FIG. 6. Subcellular fractionation of infected and transformed cells. Monolayers of Z4 cells infected for 7 h with HSV-2 (Z4-333) or Z4/6 cells growing in 150-cm^2 flasks were labeled with [^{35}S]methionine for 10 min or labeled for 10 min and then incubated in medium containing excess unlabeled methionine for 20, 40, or 90 min. The cells were washed and scraped into cold PBS and then fractionated into a nuclear fraction and a cytoplasmic membrane fraction by the methodology of Lyles and McConnell (24). Extracts of the nuclear and cytoplasmic fractions were immunoprecipitated with rabbit anti-gD serum.

Processing of gD in Z4/6 cells infected with HSV-1. To determine whether the processing of HSV-2 gD expressed in Z4/6 cells could be retarded by viral infection, the cells were infected with HSV-1 (strain F), and the HSV-2 gD (gD-2) was immunoprecipitated with a monoclonal antibody, 17βA3, which does not react with HSV-1 gD (gD-1) (1). Newly synthesized pgD-2 was rapidly processed to gD-2 within 40 min in Z4/6 cells (Fig. 7). However, when these cells were infected with HSV-1, pgD-2 was only partially processed to gD-2 after the 90-min chase. In this experiment we again observed rapid turnover of gD-2 synthesized in Z4/6 cells; however, gD-2 appeared to be somewhat more stable in Z4/6 cells infected with HSV-1 (Fig. 7).

Although it is not completely clear from this exposure of the gel, we observed a reduction in the level of synthesis of gD-2 in Z4/6 cells which were infected with HSV-1 (Fig. 7). To confirm this observation, we excised the radiolabeled bands corresponding to gD-2 in the pulse samples, dissolved the polyacrylamide gel slices in 2% periodic acid, and counted the radioactivity. It was found that the rate of synthesis of gD-2 in Z4/6 cells infected with HSV-1 was reduced by 44% from that in uninfected Z4/6 cells.

DISCUSSION

We have described a cell line which was transformed with HSV-2 DNA sequences and which expresses gD. This cell line was isolated after cotransfecting Z4 cells, which express the HSV-1 immediate-early polypeptides ICP4 and ICP47 (30a), with a plasmid containing the *Bgl*II L fragment of HSV-2 and the plasmid pSV2neo and selecting for resistance to the antibiotic G418. Although the transformed Z4/6 cells

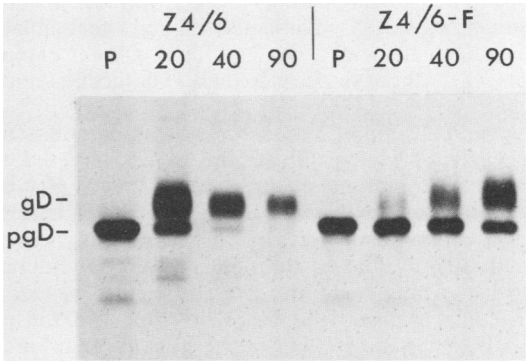

FIG. 7. Processing of gD in Z4/6 and infected Z4/6 cells. Z4/6 cells or Z4/6 cells infected for 11 h with HSV-1 (Z4/6-F) were labeled with [^{35}S]methionine for 10 min or were labeled for 10 min and then incubated in medium containing excess unlabeled methionine for 20, 40, or 90 min. Extracts of the cells were immunoprecipitated with the monoclonal antibody 17βA3, which precipitates HSV-2 gD but not HSV-1 gD.

express high levels of gD, we have no direct evidence that this efficient expression depends upon the HSV-1 immediate-early polypeptides also expressed in these cells. We were surprised to find that the level of synthesis of gD in the transformed cells exceeded that in infected parental cells, since Persson et al. (30a) found that the parental Z4 cell line supported only partial activation of a gD-1 gene introduced by viral infection. If one assumes that transcription of viral structural polypeptide mRNAs take place from a large number of templates after replication of viral DNA, one would expect higher levels of gD to be produced in infected cells. Thus, the relatively high level of gD expression in the transformed Z4/6 cells may reflect a high copy number of the gD gene or very efficient expression of gD from a limited number of copies of the gD gene. We are presently attempting to determine the number of copies of the gD gene integrated into Z4/6 DNA and the role of HSV-1 immediate-early genes in the expression of gD.

Johnson and Spear (18) reported that the translation of HSV-1 gD mRNA is inefficient late in the infection. Thus, the high level of expression of gD may be partially explained if HSV-2 gD mRNA is more efficiently translated in Z4/6 cells than in infected cells. This proposition is supported to some degree by our finding that gD-2 synthesis was inhibited in Z4/6 cells infected with HSV-1 (Fig. 7). However, the inhibition of gD synthesis was not large and may be accounted for by a number of other mechanisms, including inhibition of transcription of the HSV-2 gD mRNA from cellular copies of the gD gene after infection by HSV-1.

The turnover of gD in Z4/6 cells was rapid, especially after the polypeptide had reached the cell surface (Fig. 1 and 7). This result may explain the high degree of variability in the levels of fluorescence among individual Z4/6 cells stained with anti-gD antibody (Fig. 2). Actively growing monolayers of Z4/6 cells exhibited higher variability in the levels of fluorescence than did confluent monolayers (data not shown), perhaps because the turnover of cell surface polypeptides is higher in actively growing cells.

The major finding of this report, however, is that the kinetics of intracellular transport and processing of gD in the transformed Z4/6 cells are markedly different than that observed in infected cells. Processing and transport kinetics of gD in Z4/6 cells parallel those described for the vesicular stomatitis virus G protein, which moves rapidly from its site of synthesis in the rough endoplasmic reticulum-nuclear envelope to the Golgi apparatus and cell surface. In contrast, gD synthesized in HSV-2-infected Z4 cells accumulated or pooled in nuclear membranes to a much greater extent and did not reach the cell surface until 90 to 120 min after synthesis.

These results support a model in which a large fraction of the HSV glycoproteins diffuse into or are targeted to the inner nuclear membrane and accumulate there, most probably in association with viral nucleocapsids or other viral structural components. Regions of the inner nuclear membrane enriched in viral glycoproteins envelope viral nucleocapsids as virions bud into the perinuclear space. Viral glycoproteins, as part of the virion envelope, are transported to the Golgi apparatus (where the glycoproteins are processed and transformed from immature to mature form) and then to the cell surface. In uninfected Z4/6 cells a large fraction of newly synthesized gD is similarly found in the nuclear envelope, and although we have no evidence which excludes the presence of gD in the inner nuclear membrane of these cells, it seems likely that this nuclear gD is transiently deposited by membrane-bound ribosomes attached to the outer nuclear envelope. In the absence of viral structural components gD rapidly moves to the Golgi apparatus and is processed and transported to the cell surface.

Our findings may alternatively be explained by assuming that in infected cells viral structural polypeptides perturb cellular membranes or glycoprotein processing activities. In support of this proposition, morphological alterations in the structures of nuclear and cytoplasmic membranes in HSV-infected cells have been reported (8, 26), and nonglycosylated precursors of HSV-1 gB and gC have been observed in infected cells at 34°C (7).

Isolation of other cell lines which express HSV glycoproteins is ongoing in our laboratories. We recently isolated a cell line which expresses HSV-1 gB, although at lower levels than in infected cells (unpublished data). It is hoped that these cell lines will be useful in further characterization of the pathways of intracellular transport and processing of HSV glycoproteins and, in addition, that they may allow us to examine how these glycoproteins function in infected cells.

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