Differential Rates of Processing and Transport of Herpes Simplex Virus Type 1 Glycoproteins gB and gC

MARVIN SOMMER† AND RICHARD J. COURTNEY*

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana 71130-3932

Received 7 May 1990/Accepted 9 October 1990

The kinetics of processing and transport of herpes simplex virus type 1 (HSV-1) glycoproteins gB and gC was investigated. The conversion of precursor to mature forms and the appearance of the glycoproteins at the infected-cell surface at different times postinfection (p.i.) were studied. gB, synthesized at 4 h p.i., was converted to the mature form with a half-time ($t_{1/2}$) of 120 min and appeared at the plasma membrane with a $t_{1/2}$ of 270 min. The gB synthesized at later times p.i. (6, 8, and 10.5 h) was transported less efficiently. Less than 50% of gB synthesized at later times p.i. was processed and transported to the cell surface. gB synthesized in transfected cells was transported to the plasma membrane with kinetics similar to that for gB synthesized at early times p.i. gC was processed efficiently when synthesized at both 8 and 10.5 h p.i., with $t_{1/2}$ of conversion of pgC to gC of 40 and 60 min, respectively. Approximately 90 to 95% of the gC synthesized was converted to the mature form. The gC synthesized at 8 h p.i. was also transported rapidly to the cell surface, compared with the transport of gB synthesized at the same time, with a $t_{1/2}$ of 240 min. Greater than 70% of the gC synthesized at 8 h p.i. appeared at the cell surface. The gC synthesized at 10.5 h was transported less efficiently to the cell surface during a 6-h chase.

The genome of herpes simplex virus type 1 (HSV-1) encodes information for at least seven antigenically distinct glycoproteins (1, 2, 13, 20, 30). These glycoproteins, designated gB, gC, gD, gE, gG, gH, and gI, are the major virus-specific components found on the virus envelope and in virus-infected cell membranes (15, 31). While these glycoproteins play important roles in virus entry and egress from the cell, as well as in cell-to-cell spread of the virus (10, 19, 28, 29), a definitive function has yet to be determined for a number of the HSV-1 glycoproteins.

Although much work has been carried out to identify the individual HSV-1 glycoproteins and their processing intermediates, little information is available concerning the actual kinetics of processing and transport of HSV-1 glycoproteins. Transport of HSV-1 glycoproteins can be divided into two steps: (i) transport from the endoplasmic reticulum to the Golgi apparatus and (ii) transport from the Golgi apparatus to the plasma membrane. The work presented in this report seeks to address the kinetics of processing and transport of two representative HSV-1 glycoproteins, gB and gC.

Trypsin treatment of cell surface glycoproteins. Trypsin digestion of cell surface proteins was used to monitor the appearance of gB and gC at the plasma membrane. To show that the HSV-1 glycoproteins present at the cell surface were sensitive to trypsin, HSV-1-infected HEp-2 cells were treated with various amounts of trypsin (0, 0.1, 0.2, 0.5, and 1.0 mg/ml) for different times and at different temperatures. Treatment with 0.2 mg of trypsin per ml at 37°C for 5 min was found to be optimum for detecting gB and gC at the cell surface (data not shown).

HSV-1-infected HEp-2 cells labeled with [³⁵S]methionine

were treated with trypsin as described above. Samples were analyzed by radioimmunoprecipitation, using hyperimmune monospecific rabbit antiserum to gB or gC (11), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and autoradiography (Fig. 1). In the presence of trypsin, the putative plasma membrane-localized gB and gC were degraded (Fig. 1; compare + and – lanes). Small amounts of mature gB and gC remained following trypsin treatment (Fig. 1, + lanes), and these species are believed to represent the intracellular, mature gB and gC that were not associated with the plasma membrane. The digestion of gC reproducibly yielded two cleavage products (Fig. 1, arrowheads). These results demonstrate that plasma membrane-localized forms of gB and gC are accessible to trypsin digestion, while high-mannose, precursor forms of gB and gC (designated pgB and pgC) as well as intracellular mature forms of gB and gC are resistant to trypsin degradation.

To prove that trypsin was not penetrating the intact cell, a number of different criteria were used. First, there was no degradation of the precursor forms, pgB and pgC (Fig. 1, + lanes). Addition of a nonionic detergent, Triton X-100, prior to the addition of trypsin led to the degradation of pgB and pgC (data not shown), demonstrating that pgB and pgC were indeed sensitive to trypsin. Second, the trypsin sensitivity of a non-membrane-associated HSV-1 protein, the 65-kDa DNA-binding protein (21), was monitored by Western immunoblot analysis. The levels of this protein in trypsin-treated and mock-treated HSV-1-infected HEp-2 cells were similar. If cells were treated with detergent plus trypsin, the 65-kDa protein was not detectable (data not shown). These data demonstrated that the trypsin remained extracellular and degraded only those glycoprotein molecules exposed at the cell surface.

Processing and transport of gB. gB is classified as a beta-gamma gene product in that gB can be detected in the

* Corresponding author.

† Present address: Department of Biology and Center for Molecular Genetics, University of California—San Diego, La Jolla, CA 92093.

FIG. 1. Trypsin digestion of plasma membrane-localized gB and gC. Monolayers of HEP-2 cells were infected with HSV-1 (KOS strain) at a multiplicity of infection of 10 to 20 PFU per cell. At 10 h after infection, the cells were pulse-labeled for 10 min with 50 μ Ci of [35 S]methionine per ml. Following a 2-h chase, monolayers were treated (+) or mock-treated (–) with 0.2 mg of trypsin per ml at 37°C for 5 min. Immediately upon removal of the cells from 37°C, 0.5 mM phenylmethylsulfonyl fluoride was added to stop the trypsin reaction. Cells were washed twice in 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl containing soybean trypsin inhibitor (1 mg/ml), bovine serum albumin (2 mg/ml), and 0.5 mM phenylmethylsulfonyl fluoride. The solubilized cell lysate was reacted with anti-gB or anti-gC antiserum, and the immunoprecipitated proteins were analyzed by SDS-PAGE. Arrowheads denote the cleavage products of gC generated by trypsin digestion.

absence of de novo DNA synthesis (7, 25), although DNA replication has been shown to enhance the expression of the gB gene (32). To determine the kinetics of processing and transport of gB, pulse-chase experiments were carried out at different times postinfection (p.i.). The times selected for pulse-chase labeling included early (4 and 6 h) and late (8 and 10.5 h) times in the viral replication cycle.

At different time points during the chase, intact monolayers were treated or mock treated with trypsin. Mock-treated samples were used to determine the rate of processing of the high-mannose, precursor form (pgB) to the mature, complex form (gB). Trypsin-treated samples were used to monitor the time of appearance of gB at the cell surface. gB is synthesized initially as a 110-kDa high-mannose species (30) designated pgB, which is then converted to a 120-kDa mature form. In cells pulse-labeled at early times p.i. (4 h), pgB was

converted rapidly to the 120-kDa mature form, designated gB (Fig. 2A and Fig. 3A). The half-time of conversion of pgB to gB was 120 min for gB synthesized at 4 h p.i. (Fig. 3A and Table 1). Small amounts of mature gB began to appear at the cell surface after 30 min of chase (compare + and – lanes in Fig. 2). The half-time of appearance of gB at the plasma membrane was 270 min (Fig. 3B and Table 1).

The gB synthesized at 6 h p.i. (Fig. 2) was transported somewhat more slowly than gB made at 4 h p.i. Even after 6 h of chase, only 47% of the gB synthesized during the pulse-label was converted to the mature, complex form (Fig. 3A and Table 1), and approximately 40% of the gB molecules reached the cell surface (Fig. 3B). It is of interest that the amount of precursor (pgB) processed to the mature, complex form (gB) appeared to plateau during the chase period (Fig. 3A), while gC was almost completely processed during the chase period (see below).

At later times p.i. (8 and 10.5 h), the gB synthesized was processed at an even slower rate than the gB made at 4 or 6 h p.i. (Fig. 2A and Fig. 3A). Again, the amount of precursor processed to the mature form appeared to peak during the chase period. Even if the chase period was prolonged to 8 h, the amount of pgB synthesized at 8 h p.i. that was converted to the mature form reached only 42% of the total gB synthesized (data not shown). The gB synthesized at 10.5 h p.i. was initially processed to the mature form more slowly than the gB synthesized at 8 h p.i. (Fig. 3A). The total amount of precursor molecules processed to the mature form at 8 and 10.5 h p.i. was comparable at the end of the chase period (Table 1). Although similar amounts of gB were processed, the amount of gB that appeared at the cell surface differed at the two late times. Of the gB synthesized at 8 h p.i., 37% was present at the cell surface, while only 26% of the gB synthesized at 10.5 h p.i. appeared at the plasma membrane (Fig. 3B and Table 1).

Transport and processing of gC. gC is classified as a true late or gamma gene product and therefore requires the onset of viral DNA replication for its expression (7, 25). For this reason, the kinetics of processing and transport of gC was studied at times after the onset of DNA replication (8 and 10.5 h), when detectable amounts of gC are being synthesized.

HSV-1-infected HEP-2 cells were pulse-labeled with [35 S]methionine, chased for various lengths of time, and treated or mock treated with trypsin. Initially, gC is synthesized as a high-mannose precursor molecule designated pgC (5, 33) and is processed to a mature, complex form with a molecular weight of 130,000 (5). The pgC synthesized at 8 h p.i. was rapidly processed to the mature form (Fig. 2B), with a half-time of 40 min (Fig. 3C and Table 1). The conversion of pgC to gC was extremely efficient compared with the conversion of pgB to gB (Fig. 2 and 3), with greater than 90% of the gC being processed during the 6-h chase period (Table 1). The gC made at 8 h p.i. began to appear at the cell surface after 60 min of chase, as evidenced by the appearance of the trypsin digestion products (Fig. 2B, arrowheads). The half-time of appearance of gC at the plasma membrane was 240 min for gC made at 8 h p.i. (Fig. 3D).

The gC synthesized at 10.5 h p.i. was transported and processed at a slightly slower rate than gC synthesized at 8 h p.i. The half-time of conversion of the precursor form to the mature form was 60 min (Fig. 3C and Table 1). Again, the conversion of pgC to gC was efficient, with nearly 90% of the precursor being processed during the chase period (Table 1). It should be noted that although the rate of conversion of pgC to gC decreased at 10.5 h p.i., this conversion was still

FIG. 2. Processing and transport to the plasma membrane of gB and gC synthesized at different times after infection. HEP-2 cell monolayers were infected with HSV-1 at a multiplicity of infection of 10 to 20 PFU per cell and at the indicated times p.i. were pulse-labeled for 10 min with 50 μ Ci of [35 S]methionine per ml. Cultures were treated immediately with trypsin or chased in the presence of excess unlabeled methionine for the times indicated and then treated with trypsin as described in the legend to Fig. 1. Solubilized extracts were reacted with anti-gB antibody (A) or anti-gC antibody (B), and the immunoprecipitated proteins were resolved by SDS-PAGE. Lane P, Pulse. Arrowheads indicate trypsin digestion products.

more efficient than that observed for gB molecules synthesized at the same time. The appearance at the plasma membrane of gC synthesized at 10.5 h was slower than the appearance of gC synthesized at 8 h p.i., with a half-time of more than 360 min (Fig. 3D and Table 1). There was significantly less gC synthesized at 10.5 h p.i. that was transported to the cell surface compared with gC synthesized at 8 h p.i. (50 versus 71%) (Fig. 3D and Table 1). Again, although less gC was transported to the cell surface when the two time points are compared, the relative amount appearing at the cell surface was still significantly greater than the amount of mature gB at the cell surface (Table 1).

Glycoprotein transport in transfected cells. To study the transport of gB in the absence of an HSV-1 infection, COS-1 cells were transfected with a gB expression vector. The vector consisted of the entire gB gene (a kind gift of Neal DeLuca, Harvard Medical School) inserted into the simian virus 40-based expression vector pSV010 (24). At 32 to 42 h after transfection, cells were pulse-labeled, chased for vari-

ous lengths of time, and treated or mock treated with trypsin as described for infected cells. A representative gel of one such experiment is shown in Fig. 4. As in infected cells, gB was initially synthesized as a 110-kDa precursor (Fig. 4, lanes P) that was processed to the mature complex form during the chase period. The relatively poor resolution of the precursor and mature forms seen on this gel may be due to the fact that a relatively small number of transfected cells were producing large amounts of gB or due to differential glycosylation of gB in COS-1 cells. Similar results were observed regardless of when the transfected cells were pulse-labeled at different times after transfection (data not shown). The mature gB appeared at the cell surface, as determined by trypsin sensitivity, after 30 min of chase (Fig. 4, 30 min, + lane). A monoclonal antibody to gB obtained from Melvin Trousdale, University of Southern California, was used in the immunoprecipitation reactions. The use of this monoclonal antibody allowed us to detect the cleavage product of the trypsin digestion of gB (Fig. 4, arrowhead).

FIG. 3. Kinetics of processing and transport of gB and gC. Autoradiograms of polyacrylamide gels such as those shown in Fig. 2 were scanned on a laser scanning densitometer. (A and C) Values obtained for the conversion of pgB (A) or pgC (C) to gB or gC. (B and D) Values obtained for the appearance of gB (B) or gC (D) at the plasma membrane. Each time point represents data obtained from at least two different experiments. Cultures were pulse-labeled at 4 (▲), 6 (●), 8 (◆), or 10.5 (■) h p.i.

The appearance of gB at the cell surface can be more clearly seen at the later times of chase (Fig. 4, + lanes).

The overall goal of this study was to correlate the kinetics of processing of two HSV-1 glycoproteins with the time of

appearance of these glycoproteins at the plasma membrane of HSV-1-infected cells. Four time periods were studied for gB synthesis, and two were studied for gC synthesis. Campadelli-Fiume et al. (3) have observed differential rates of transport for gB, gC, and gD. We have extended these initial studies by measuring the rate of processing at different times after infection and have also determined the rate of appearance of gB and gC at the plasma membrane of infected cells. The time periods were chosen to bracket the most productive times of the HSV replication cycle for the synthesis of these two proteins, as well as a time prior to the onset of a significant degree of cellular destruction.

We have shown that gB is transported and processed most efficiently when synthesized at earlier times p.i. The results obtained suggest that one of the rate-limiting steps in the processing of gB involves the conversion of the high-mannose form to the complex form. If we assess the rate of processing of that portion of gB that is processed to the mature form, a significant difference is still observed for gB synthesized at the times when gC is also synthesized. At these times (8 and 10.5 h p.i.), the half-times of processing of the total mature gB are 80 and 110 min, respectively. This is approximately twofold slower than the rate observed for gC (Table 1).

In contrast, gC is processed efficiently when synthesized at 8 and 10.5 h p.i. Approximately 90 to 95% of the precursor molecules made at these time points are converted to the mature form during the 6-h chase periods. Of the gC synthesized at 8 and 10.5 h p.i., 71 and 50%, respectively, appeared at the cell surface during the chase period. This is considerably greater than the amounts of gB made at 8 and 10.5 h p.i. that appear at the cell surface (37 and 26%). The results obtained with the transport of gC at later times p.i. would

TABLE 1. Processing and transport of gB and gC in HSV-1-infected cells^a

Glycoprotein	Time after infection (h) ^b	Processing ^c		Transport ^d	
		<i>t</i> _{1/2} (min) ^e	% ^f	<i>t</i> _{1/2} (min)	%
gB	4	120	55.5 (2.3)	270	54.4 (5.7)
	6	>360	47.2 (6.0)	ND ^g	42.5 (8.5)
	8	ND	42.6 (9.2)	ND	37.8 (5.0)
	10.5	ND	39.9 (8.3)	ND	25.8 (4.0)
gC	8	40	94.5 (5.8)	240	71.4 (7.8)
	10.5	60	89.4 (7.0)	>360	47.5 (6.1)

^a The autoradiograms shown in Fig. 2 and others from similar experiments were scanned on an LKB Ultrascan LX laser densitometer, and the percentage of glycoprotein processed and transported to the cell surface was calculated.

^b At the indicated times after infection, HSV-1-infected HEP-2 cells were pulse-labeled for 10 min with [³⁵S]methionine and then chased for various time periods.

^c Conversion of the high-mannose precursor form to the mature form of either gB or gC, determined from the molecular weight of the components detected by SDS-PAGE.

^d Appearance of the mature gB or gC at the cell surface as determined by trypsin sensitivity.

^e Length of time required for half of the gB or gC molecules synthesized during the pulse-label to be converted from the high-mannose to the complex form (processing) or to be transported to the cell surface (transport).

^f Percentage of gB or gC processed to the mature form (processing) or transported to the cell surface (transport) after 360 min of chase. Values in parentheses represent the standard deviations.

^g ND, Not determined.

FIG. 4. Processing and transport of HSV-1 gB synthesized in transfected cells. COS-1 cells were transfected with a plasmid containing the gB gene. At 32 to 42 h after transfection, cells were pulse-labeled for 10 min with 50 μ Ci of [35 S]methionine per ml. Cultures were treated immediately with trypsin or chased in the presence of excess unlabeled methionine for the times indicated and then treated with trypsin as described in the legend to Fig. 1. Solubilized extracts were reacted with anti-gB antibody, and the immunoprecipitated proteins were resolved by SDS-PAGE. The arrowhead indicates the gB-derived product generated by trypsin digestion. Lane P, Pulse.

rule out the argument that the decrease in gB processing and transport efficiency is due to the breakdown of the host cell glycosylation machinery as a result of virus infection.

The kinetics of processing, transport, and cell surface expression of HSV-1 glycoproteins, in general, is slow compared with the kinetics of other viral glycoproteins (22, 23). Work carried out by other investigators has demonstrated that HSV-1 glycoproteins are processed and transported much more slowly than are other viral glycoproteins (5, 16). The difference in the rates of transport of HSV-1 glycoproteins compared with other viral glycoproteins may be due to the pool size of the glycoproteins. HSV-1 encodes seven virus-specific glycoproteins, while other viruses such as vesicular stomatitis virus, Newcastle disease virus, and influenza virus encode one or two glycoprotein species. It is therefore possible that during an HSV-1 infection, the glycoproteins are synthesized in such large amounts that the transport machinery becomes saturated. Studies carried out by Johnson and Smiley (16) demonstrated that gD, expressed in the absence of a viral infection, is processed to the mature form and appears at the plasma membrane with faster kinetics than gD in the virus-infected cell. Similar results were obtained with cell lines constitutively expressing gB and gD (3). These results might be interpreted to suggest that the pool size of glycoproteins may influence the rate of transport. It has previously been reported (8) that during an HSV-1 infection there is an accelerated turnover of oligosaccharide lipid as a result of the extensive glycoprotein synthesis and that this leads to the accumulation of nonglycosylated precursor forms of gB and gC (6). Therefore, one may speculate that if the large amounts of glycoproteins being synthesized can saturate the glycosylation machinery in the rough endoplasmic reticulum (RER), then they may also saturate the transport machinery that is responsible for exporting these proteins from the RER to the Golgi apparatus. This saturation may lead to a slower rate of glycoprotein processing and transport for HSV-1 than for other viral glycoproteins.

The observation that gB is transported more slowly than gC is also of interest. gB has been shown to exist as a homodimer (12, 28), and dimerization occurs within minutes

of polypeptide synthesis (4). gC does not appear to oligomerize. It is possible that the oligomerization of gB occurs inefficiently, especially at later times p.i., thereby retarding its movement from the RER. Studies with the HA protein of influenza virus (14) and the G protein of vesicular stomatitis virus (18) have shown that oligomerization is a prerequisite for transport of these glycoproteins from the RER. This feature of the gB molecule may somehow play a role in regulating the rate at which gB is transported through the cell.

Finally, it has been reported by others that the rate-limiting step in the transport and processing of glycoproteins is the rate of movement from the RER to the Golgi apparatus (26). It appears that with gC this is not true. gC synthesized at 8 and 10.5 h p.i. exhibits half-times of transport to the Golgi apparatus of 40 and 60 min, respectively. One would then expect that the half-time of appearance at the cell surface would differ by approximately 20 min for the gC molecules made at the two different time points. However, we have observed that the gC made at 8 h appears at the cell surface with a half-time of 240 min, while gC made at 10.5 h has a half-time of transport to the plasma membrane of more than 360 min. The previous studies involved investigating the rate-limiting steps of glycoprotein transport for cellular proteins or viral proteins that acquire their envelope at the plasma membrane. The unique nature of HSV envelopment (9) and reports that HSV-1 virions are transported through the Golgi apparatus (17), where the virion-associated glycoproteins are processed, could lead us to speculate that HSV-1 glycoproteins may have other rate-limiting steps for their transport in comparison with cellular or other viral glycoproteins. It is possible to envision two separate processing pathways for the HSV-1 glycoproteins. The first would involve the processing of the plasma membrane-localized glycoproteins. The second pathway would process the virion-associated glycoprotein species. Unfortunately, at present it is impossible to distinguish between these two pathways, if they do exist.

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