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Limited Proteolysis of Herpes Simplex Virus Glycoproteins That Occurs During Their Extraction from Vero Cells

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Herpes simplex virus glycoproteins extracted from infected Vero cells can be smaller in apparent size than the same viral products extracted from infected HEp-2 cells. Here we show that the differences in size result primarily from limited proteolysis, during or after their extraction, of the viral glycoproteins made in Vero cells. In the absence of appropriate protease inhibitors, both mature and immature forms of four different glycoproteins specified by herpes simplex virus type 2 were significantly smaller (based on electrophoretic mobilities in acrylamide gels) when extracted from Vero cells than when extracted from HEp-2 cells. Inclusion of certain protease inhibitors in the extraction buffer, however, permitted isolation of immature forms from Vero cells that were indistinguishable in size from the immature forms extracted from HEp-2 cells. Under these conditions, the mature forms of glycoproteins B and E were also indistinguishable by electrophoretic sizing from those made in HEp-2 cells, whereas the mature forms of glycoproteins D and F were smaller, indicating the possibility of differences between Vero and HEp-2 cells in the post-translational processing of glycoproteins D and F.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) specify several glycoproteins which are expressed on both virion and infected cell surfaces (16). Keller et al. (6) first observed differences between HSV glycoproteins produced in infected Vero cells and those made in infected HEp-2 cells. More recently, several investigators (2, 11, 12, 15, 19) have used monoclonal antibodies in immunoprecipitation experiments to show that the electrophoretic mobilities of several HSV glycoproteins depend on the cell type which produced them, and these investigators have also discussed evidence of limited proteolytic cleavages of some HSV glycoproteins made in certain cell types, particularly Vero cells or rodent cells. Pereira et al. (11, 12) compared the synthesis and processing of the HSV glycoprotein designated gB in HEp-2 cells and Vero cells, found differences in products consistent with the proteolytic cleavage of gB made in Vero cells, and detected a proteolytic activity in lysates of uninfected Vero cells that could cleave gB from HEp-2 cells to yield products similar to those detected in Vero cells. They suggested (12) that this protease might act in vivo during the post-translational processing of the HSV glycoproteins in Vero cells.

We undertook comparisons of extraction conditions for immunoprecipitation of glycoproteins made in HEp-2 and Vero cells in the course of our ongoing studies (10, 17, 18) of HSV-2 gF (1, 2) and then extended these comparisons to other HSV-2 glycoproteins. Use of particular protease inhibitors in the extraction buffer permitted isolation from Vero cells of HSV-2 glycoproteins that had electrophoretic mobilities similar or identical to the equivalent species isolated from HEp-2 cells. Therefore, it seems likely that many, but not all, of the observed differences between HSV glycoproteins produced in Vero and HEp-2 cells can be attributed to proteolytic cleavages occurring at the time of extraction.

For all results reported here, monoclonal antibodies identified in the figure legends and previously described elsewhere (9, 10) were used for immunoprecipitation of HSV-2 gB, gD, gE, and gF. When the extraction buffer (10 mM sodium phosphate buffer [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mg of ovalbumin per ml, and 100 U of trasylol [Mobay Chemical Corp., New York] per ml) previously used for solubilization of gF from HSV-2-infected HEp-2 cells was used for continuously labeled HSV-2-infected Vero cells, we were unable to detect any of the forms of gF (stable immature form of 65,000 [65K] molecular weight and heterogeneous mature forms ca. 75K molecular weight) characteristic of HEp-2 cells (17). Instead, a polypeptide of ca. 57K molecular weight was precipitated by the anti-gF antibody from extracts of the infected Vero cells (Fig. 1).

In preliminary experiments with pulse-labeled infected Vero cells, we found that substitution of the phosphate buffer and sodium salt in the extraction buffer with a Tris-hydrochloride buffer and potassium salt resulted in precipitation by the anti-gF antibody of 67K and 69K polypeptides characteristic of the forms seen in HEp-2 cells (17), in addition to a faster-migrating polypeptide (59K) previously detected (data not shown). Addition of 10 mM EDTA to the extraction buffer increased the amounts of the 67K and 69K polypeptides relative to the 59K protein, but addition of phenylmethylsulfonyl fluoride (0.1 mM), pepstatin A (0.1 mM), or *p*-nitrophenyl-*p'*-guanidinobenzoate (1 mM) to the extraction buffer did not have any apparent effects on the relative amounts of the gF-related polypeptides (data not shown).

Effects of several other protease inhibitors added at the time of extraction were then examined. Vero or HEp-2 cells infected with HSV-2(333) at 10 PFU per cell were maintained at 37°C in medium 199 supplemented with 1% inactivated calf serum (199V) and were labeled by the addition at 4 h after infection of [^{35}S]methionine to 30 μCi/ml in medium containing one-tenth the usual concentration of methionine. At 24 h after infection, cell monolayers (in 25-cm^2 flasks) were washed three times with cold phosphate-buffered saline (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, pH 7.4) for sample 1, with 20 mM Tris-hydrochloride buffer (pH 7.4)–

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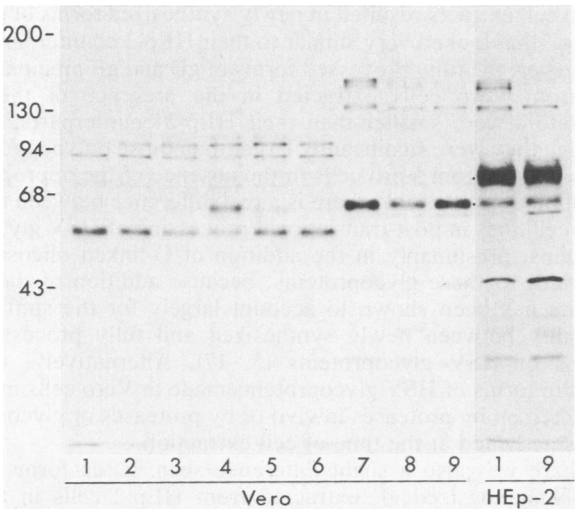

FIG. 1. Polypeptides precipitated by anti-gF(III188) monoclonal antibody from extracts of Vero or HEp-2 cells infected with HSV-2(333) at 10 PFU per cell. The extracts were prepared under a variety of conditions as described in the text. Immune precipitates were solubilized and analyzed on 8.5% sodium dodecyl sulfate-polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide (4). Gels were fixed and stained and then impregnated with 2,5-diphenyloxazole (3). Cronex medical X-ray film was prefogged (8) and exposed to the dried gel at −70°C. The molecular weight markers used here and in all other figures were myosin (200K), β-galactosidase (130K), phosphorylase *b* (94K), bovine serum albumin (68K), and ovalbumin (43K). All samples were run on the same slab gel; the last two lanes were exposed to film for a shorter period of time.

150 mM KCl and including 10 mM EDTA for samples 3, 4, 5, 6, and 9, or without EDTA for samples 2, 7, and 8. Cells were extracted by the addition of 1 ml of extraction buffer. All extraction buffers contained 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mg of ovalbumin per ml, and 100 U of trasylol per ml, but they differed in that buffer 1 contained 10 mM sodium phosphate buffer (pH 7.4)–150 mM NaCl, whereas buffers 2 through 9 contained 20 mM Tris-hydrochloride buffer (pH 7.4)–150 mM KCl. Buffers 2 through 9 differed by the addition of other components as follows: 2, no addition; 3, 10 mM EDTA; 4, 30 mM EDTA; 5, 10 mM EDTA, 10 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA); 6, 10 mM EDTA, 20 mM glycine; 7, 1 mM tosyl-lysyl-chloromethyl ketone (TLCK); 8, 1 mM *p*-hydroxy-mercuribenzoate; and 9, 10 mM EDTA, 10 mM EGTA, 20 mM glycine, 1 mM TLCK, 1 mM *p*-hydroxymercuribenzoate. Extracts were held on ice for 15 min, centrifuged at 25,000 rpm in an SW27.1 rotor for 1 h to remove insoluble material, and held at −70°C until immunoprecipitations were performed. For immunoprecipitations, 100 μl of extract was mixed with 1 μl of anti-gF(III188) antibody and held on ice for 1 h. A 35-μl amount of 20% Formalin-fixed *Staphylococcus aureus* (7) in extraction buffer 1 was added to sample 1, in buffer 2 was added to samples 2, 7, and 8, and in buffer 3 was added to samples 3, 4, 5, 6, and 9. After an additional 15 min on ice, bacterial cells were pelleted and washed three times in 0.1 M Tris-hydrochloride buffer (pH 8.0)–0.5 M LiCl–1% mercaptoethanol, and including 10 mM EDTA for samples 3, 4, 5, 6, and 9 and without EDTA for samples 1, 2, 7, and 8. Pellets were solubilized for electrophoretic analysis.

The first lane in Fig. 1 shows gF immunoprecipitated from Vero cell extracts prepared with 10 mM phosphate buffer (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mg of ovalbumin per ml, and 100 U of trasylol per ml (buffer 1). Substitution of a Tris-hydrochloride buffer (20 mM, pH 7.4) and potassium salt in the extraction buffer (buffer 2) had no effect on the appearance of stable forms of gF that accumulated under continuous labeling conditions (in contrast to the results described above for pulse-labeled forms of gF). Addition of other components to this modified buffer (buffers 3 through 9) had various effects. Addition of 10 mM EDTA (buffer 3) or 10 mM EDTA–20 mM glycine (buffer 6) did not affect the appearance of gF, in contrast to expectations from preliminary experiments with EDTA; however, the addition of 30 mM EDTA (buffer 4) or 10 mM EDTA–10 mM EGTA (buffer 5) to the extraction buffer resulted in the appearance of a gF-related polypeptide in Vero cell extracts that comigrated with the stable immature form of gF (65K) obtained from infected HEp-2 cells (the forms of gF or other glycoproteins detected in HEp-2 cells are indicated by dots in all figures). A greater effect was seen with the addition of 1 mM TLCK or 1 mM *p*-hydroxymercuribenzoate (buffers 7 and 8, respectively) in that the 57K form of gF disappeared completely, presumably converted to the slower-migrating 65K form. Use of a combination of protease inhibitors (buffer 9: 10 mM EDTA, 10 mM EGTA, 20 mM glycine, 1 mM TLCK, and 1 mM *p*-hydroxymercuribenzoate) in the extraction buffer appeared to give optimal results with respect to increasing the intensity of the 65K form of gF at the expense of smaller-molecular-weight bands. The forms of gF immunoprecipitated from HEp-2 cells extracted in the presence of these inhibitors (buffer 9) did not appear to be different from those seen under usual extraction conditions (buffer 1). The 75K mature form of gF obtained from HEp-2 cells was not detected in Vero cell extracts, even in the presence of all protease inhibitors.

HSV-2 glycoproteins gB, gD, and gE immunoprecipitated from Vero cells extracted under various conditions were then examined and compared with their counterparts extracted from HEp-2 cells. The differences seen in electrophoretic mobilities of glycoproteins extracted from Vero cells in buffers 2 through 6, as compared with extraction in buffer 1, varied slightly for each glycoprotein examined (data not shown), but for all three glycoproteins, extraction in either buffer 7 or 8 resulted in complete conversion of immunoprecipitable material from polypeptides of higher to lower electrophoretic mobility (smaller to larger size), and buffer 9 was slightly better in this respect than either buffer 7 or buffer 8. Comparisons of gB, gD, and gE extracted from Vero and HEp-2 cells in buffer 1 or 9 are shown in Fig. 2. For both gB and gE, the polypeptides precipitated from Vero cell extracts prepared in the presence of the inhibitors (buffer 9) were very similar in size to the forms obtained from HEp-2 cells. In contrast, only one form of gD was obtained from Vero cells in the presence of the inhibitors, and this form had the same electrophoretic mobility as the stable immature form of gD detected in HEp-2 cells. For both gD and gF, therefore, mature forms similar in size to those detected in HEp-2 cells could not be extracted from Vero cells even with the combination of protease inhibitors used. Extraction of HEp-2 cells with or without these protease inhibitors (buffer 9 or 1) had no apparent effect on the sizes of the glycoproteins precipitated.

Newly synthesized and fully processed forms of these glycoproteins were then examined. Figure 3 shows gD and gF precipitated from Vero or HEp-2 cells that had been

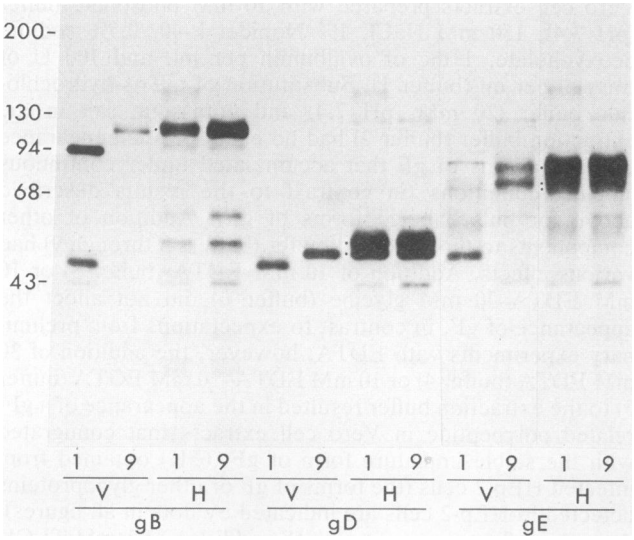

FIG. 2. Polypeptides precipitated by anti-gB(II105), anti-gD(III114), and anti-gE(III347) monoclonal antibodies from extracts of Vero (V) or HEp-2 (H) cells infected with HSV-2(333) and extracted in two different ways (buffers 1 and 9) as described in the text. Cells were infected and labeled, and immunoprecipitations were performed as described in the legend to Fig. 1. All samples were run on the same slab gel; lanes containing gE polypeptides precipitated from infected Vero cells were exposed to film for a longer period of time than the other lanes.

labeled with [^{35}S]methionine for 10 min at 6 h after infection and then harvested immediately (pulse) or incubated in nonradioactive medium for 4 h before harvesting (chase). Extraction was done in the presence (buffer 9) or absence (buffer 1) of the protease inhibitors previously indicated. Addition of these protease inhibitors to the pulse-labeled Vero cell extracts resulted in newly synthesized forms of gD and gF that looked very similar to their HEp-2 counterparts. However, the fully processed forms of gD and gF precipitated from Vero cells extracted in the presence of these inhibitors were smaller than their HEp-2 counterparts, although they were significantly larger than those polypeptides precipitated from Vero cells in the absence of the appropriate inhibitors. Possibly there is a real difference between the two cell lines in post-translational processing of HSV glycoproteins, presumably in the addition of O-linked oligosaccharides to these glycoproteins, because addition of these sugars has been shown to account largely for the shift in mobility between newly synthesized and fully processed forms of HSV glycoproteins (5, 17). Alternatively, the mature forms of HSV glycoproteins made in Vero cells may be acted on by proteases in vivo or by proteases or glycosylases activated at the time of cell extraction.

There was also a slight difference seen in the forms of newly synthesized gF extracted from HEp-2 cells in the presence of inhibitors (buffer 9) compared with those seen in cells extracted without these inhibitors (buffer 1). This was the only instance in which we saw evidence of a protease activity in HEp-2 cells at the time of extraction.

Newly synthesized and fully processed forms of gB and gE made in Vero and HEp-2 cells and extracted in the two ways previously described are shown in Fig. 4. Similar to the results obtained with gD and gF, all forms of gB and gE extracted from Vero cells in the absence of appropriate inhibitors (buffer 1) were smaller than those extracted in the presence of inhibitors (buffer 9). In contrast to the results obtained with gD and gF, however, both immature and mature forms of gB and gE extracted from Vero cells in the presence of inhibitors were similar in electrophoretic mobility to equivalent forms obtained from HEp-2 cells. The newly synthesized form of gB was not efficiently extracted from either Vero or HEp-2 cells.

Finally, the total complement of infected cell proteins

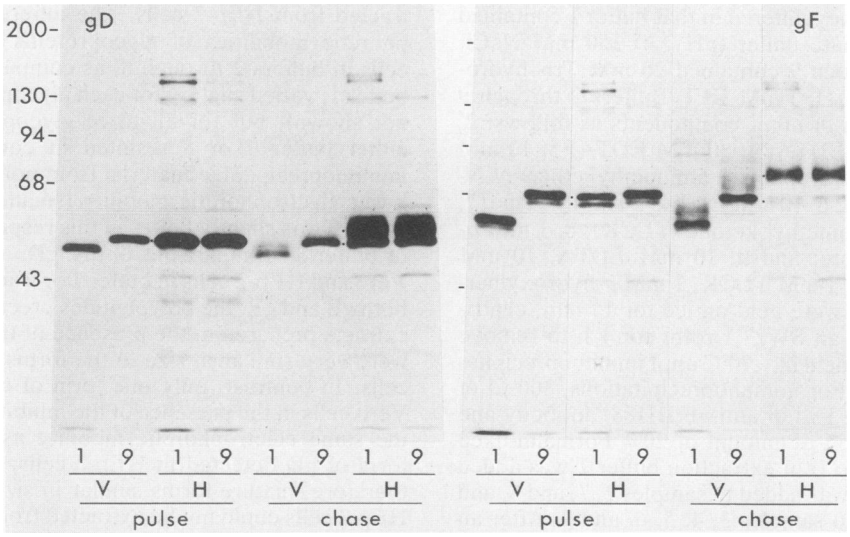

FIG. 3. Newly synthesized and processed forms of gD and gF precipitated by anti-gD(III114) or anti-gF(III188) monoclonal antibodies, respectively, from extracts of Vero (V) or HEp-2 (H) cells infected with HSV-2(333) and extracted in two different ways (buffers 1 and 9) as described in the text. All procedures were as described in the legend to Fig. 1, except for labeling of the cells. Infected cells were washed three times at 6 h after infection with 199V lacking methionine, incubated for 10 min with [^{35}S]methionine at 75 μCi/ml in this medium, and then harvested immediately (pulse) or washed three times with 199V containing a 10-fold excess of unlabeled methionine with incubation continuing in this medium for 4 h before harvesting (chase). The gD and gF polypeptides were each run on a single slab gel; the lanes containing gF polypeptides from infected Vero cells were exposed to film for a longer period of time than the other lanes.

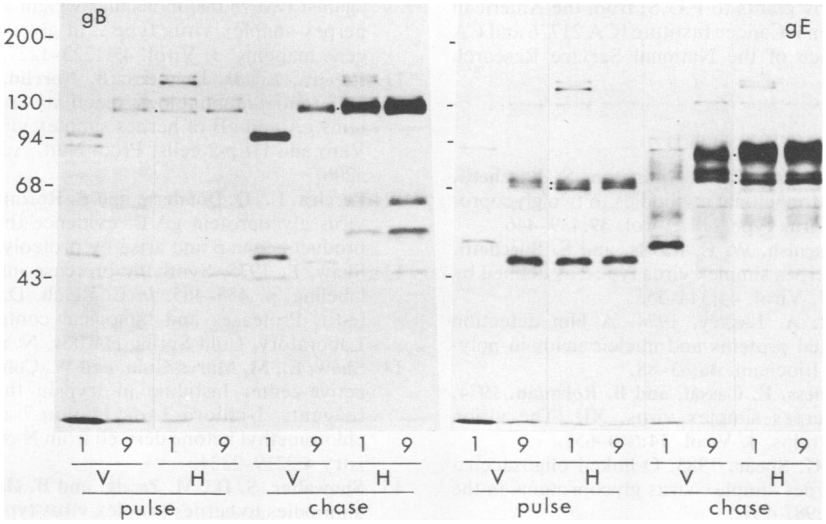

FIG. 4. Newly synthesized and processed forms of gB and gE precipitated by anti-gB(II105) or anti-gE(III347) monoclonal antibodies, respectively, from extracts of Vero (V) or HEp-2 (H) cells infected with HSV-2(333) and extracted in two different ways (buffers 1 and 9) as described in the text. Labeling of cells was as described in the legend to Fig. 3, and all other procedures were as described in the legend to Fig. 1. The gB and gE polypeptides were each run on a single slab gel. The lanes containing newly synthesized gB from infected Vero cells and gE from infected Vero cells were exposed to film for a longer period of time than the other lanes.

extracted from cells continuously labeled, pulse-labeled, or pulse-labeled and then chased for 4 h was examined to determine whether differences seen in glycoproteins from Vero cells extracted under different conditions applied to infected cell proteins in general. The electrophoretic profile of major infected cell polypeptides is similar for extracts prepared from HEp-2 cells with either buffer and for the extract prepared from Vero cells with buffer 9 (combination of protease inhibitors present; Fig. 5). In contrast, many of the major polypeptides present in the extract obtained from Vero cells with buffer 9 are missing or reduced in amount in the extract prepared without the appropriate inhibitors (buffer 1). Moreover, in the latter extract there appears to be an accumulation of smaller-molecular-weight material that could have resulted from limited proteolysis of the major polypeptides.

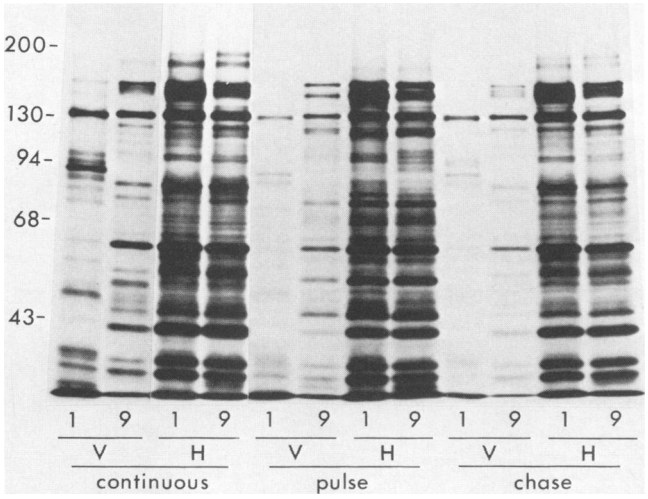

FIG. 5. Infected cell extracts from Vero (V) or HEp-2 (H) cells infected with HSV-2(333) and extracted in two different ways (buffers 1 and 9) as described in the text. Continuously labeled, newly synthesized (pulse) and processed (chase) infected cell proteins are shown. Procedures for infection and labeling of cells and preparation of cell extracts are described in the legends to Fig. 1 and 3. All samples were run on one slab gel; lanes containing proteins from continuously labeled infected HEp-2 cells were printed differently with a shorter exposure time than the other lanes.

The nature of the protease activity or activities present in Vero cells and active against HSV glycoproteins (after cell lysis) can be explored by examining the inhibitors which prevent such activity. EDTA and EGTA, metalloprotease inhibitors, had a slight inhibitory effect. *p*-Hydroxymercuribenzoate, a sulfhydryl protease inhibitor, seemed to prevent most of the protease activity, but so did TLCK, a trypsin-like serine protease inhibitor (14), as was observed previously by others (12). TLCK is known to have side reactions, however, particularly at sulfhydryl groups (13). It should be noted that, in preliminary experiments, the serine protease inhibitors phenylmethylsulfonyl fluoride, *p*-nitrophenyl-*p'*-guanidinobenzoate, and trasylol had no apparent effect on the proteases responsible for cleavage of gF. Therefore, it is possible that a combination of sulfhydryl and metalloproteases is responsible for the phenomena reported here and elsewhere, although no decisive conclusions can be drawn from the data presented here.

Our results demonstrate that the HSV-2 glycoproteins produced in Vero cells are more similar to those produced in HEp-2 cells than previously believed, and that the mixture of protease inhibitors used here should be included in extraction buffers when examining glycoproteins from infected Vero cells (and perhaps other cell types), although TLCK or *p*-hydroxymercuribenzoate alone may be sufficient for inhibition of most proteolytic activity. We assume that similar results would be obtained for HSV-1 glycoproteins.

While this work was in progress, and later while the manuscript was under review, it was shown (10, 17–19) that gF is the HSV-2 counterpart of HSV-1 gC (gC-1). A recommendation was made at the 1983 International Herpesvirus Workshop (Oxford, England) to change the name of this HSV-2 glycoprotein from gF-2 to gC-2.

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