

Cross-linking studies show that herpes simplex virus type 1 glycoprotein C molecules are clustered in the membrane of infected cells

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Chemical cross-linking using ethylene glycol succinimidyl succinate (EGS) and dithiobispropionimidate (DTBP) was performed to determine the association of herpes simplex virus type 1 glycoprotein C (HSV-1 gC) with its nearest neighbours. Human embryonic lung (HEL) cells were infected with HSV-1 strain KOS, treated with EGS, lysed with Nonidet P40, immunoprecipitated with monoclonal antibodies specific for gC, and analysed by SDS-PAGE. These analyses demonstrated the presence of cross-linked complexes that migrated with an apparent M_r in the range 150000

to 260000. Two-dimensional SDS-PAGE (non-reduced and then reduced) analyses of HSV-1-infected HEL cells treated with the cleavable cross-linker DTBP demonstrated that molecules that comigrated with gC were the only components of these high M_r complexes. Immunoelectroblot (Western blot) analyses using polyclonal rabbit antiserum specific for gC verified that the high M_r complexes contained gC. These results indicated that gC molecules may be localized in the infected cell membrane as dimers.

At least seven herpes simplex virus (HSV) glycoproteins have been identified in infected cell membranes and virion envelopes (reviewed by Spear, 1985). The higher order structure of only one of these glycoproteins has been investigated. Thus, glycoprotein B has been shown to form multimers which remain in an oligomeric form in the presence of ionic detergents and reducing agents. These multimers of gB become monomers after reduction and boiling in SDS (reviewed by Spear, 1985), which indicates that these forms of gB are highly stable and tightly associated. Structures with dimensions consistent with dimers of gB have also been observed on virion envelopes using immunogold-labelled antibodies visualized by electron microscopy (Stannard *et al.*, 1987). It is not known whether the oligomeric form of gB is required for the function of this glycoprotein in infection.

In this study the possibility that gC, like gB, exists in a multimeric form was investigated. The method of chemical cross-linking (Wang & Richards, 1974, 1975) was used to analyse the association of gC with its nearest neighbours in infected cells. The results of our study indicate that gC is closely associated with itself in

infected cell membranes and was not cross-linked to other cellular or viral proteins. These data suggest that gC can exist in an oligomeric form although multimers of gC apparently are less tightly bound to one another than are gB multimers.

To analyse the organization of gC in the membranes of infected cells, biosynthetically labelled human embryonic lung (HEL) cells infected with HSV-1 strain KOS or the gC-minus mutant gC⁻ 3, were treated with various concentrations of the homobifunctional chemical cross-linker ethylene glycol succinimidyl succinate (EGS). These cells were lysed and gC was immunoprecipitated with a pool of monoclonal antibodies (MAbs) specific for gC. The gC-containing immunoprecipitates were analysed by SDS-PAGE (Fig. 1). A band corresponding to approximately M_r 130000, which is the apparent M_r of gC on SDS-PAGE, was clearly visible (Fig. 1). In addition, complexes of higher M_r (approx. M_r 150000 to 260000) were visible on SDS-PAGE analyses of HSV-1-infected cells that had been treated with EGS (lanes 2 to 5). These higher M_r complexes were not present in control SDS-PAGE analyses of HSV-1-infected cells that had not been treated with EGS (lane 1). Neither were these complexes present in cells infected with the mutant gC⁻ 3 where gC is not present in the cell membrane but is secreted from the cell (lanes 6 to 10). The strong band of M_r approximately 90000 in lanes 6 to 10 may be the incompletely secreted form of gC from

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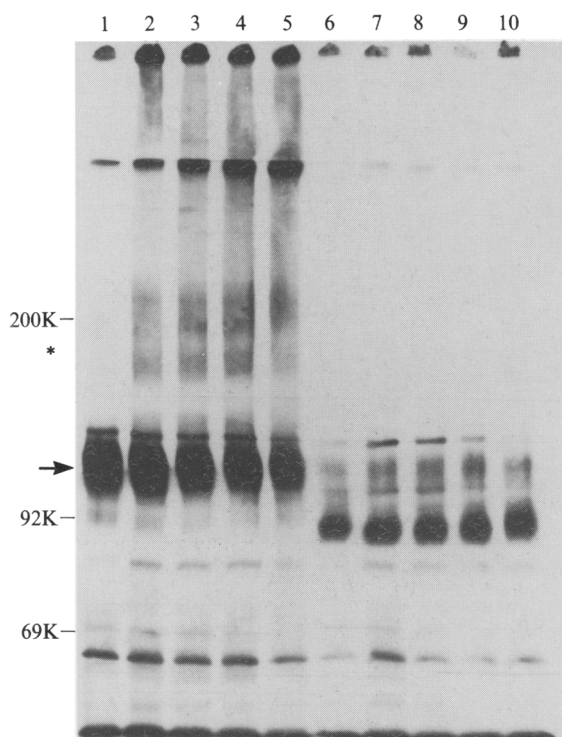


Fig. 1. Cross-linking of gC in cells infected with HSV-1. HEL cells were grown and infected with HSV-1 (KOS 321) (lanes 1 to 5) or the gC-minus HSV-1 mutant (gC⁻ 3) (lanes 6 to 10) (Holland *et al.*, 1983, 1984). The cells were radiolabelled biosynthetically with [³H]arginine and [³H]proline (Kikuchi *et al.*, 1984). After labelling the cells were then treated with the cross-linking reagent EGS at 0.05 mg/ml (lanes 2 and 7), 0.1 mg/ml (lanes 3 and 8), 0.2 mg/ml (lanes 4 and 9) and 0.4 mg/ml (lanes 5 and 10) for 1 h at room temperature. Lanes 1 and 6 represent the controls, i.e. no EGS treatment. The cells were then solubilized by treatment with a buffer containing 150 mM-NaCl, 5 mM-Tris-HCl pH 7.4, 1% NP40, 1 mM-PMSF (NP40 lysis buffer). gC was immunoprecipitated with a pool of MAbs consisting of C3, C10, C13, C16 and C17 (Holland *et al.*, 1984; Marlin *et al.*, 1985). The immunoprecipitates were analysed by SDS-PAGE using a 5.5% acrylamide, 0.47% *NN'*-diallyltartardiamide (DATD) gel (Glorioso *et al.*, 1980). The gel was treated with 1.0 M-sodium salicylate in 0.1 M-Tris-HCl to provide fluorographic enhancement, followed by exposure to type XR5 film (Eastman Kodak) at -70 °C. The migration positions of *M_r* markers are indicated. The position of gC is marked with an arrow and the migration position of the higher *M_r* complexes resulting from cross-linking is shown by an asterisk.

mutant gC⁻ 3. As the high *M_r* complexes seen in lanes 2 to 5 could be immunoprecipitated with gC-specific MAbs, it can be presumed that at least one component of the complex is gC.

We performed two-dimensional SDS-PAGE to analyse further the components of the high *M_r* complexes resulting from treatment of infected cells with the cross-linker. In place of EGS, the imidoester cross-linker dithiobispropionimidate (DTBP), which contains a cleavable internal disulphide bond, was used. This allowed analyses of the immunoprecipitated cross-linked

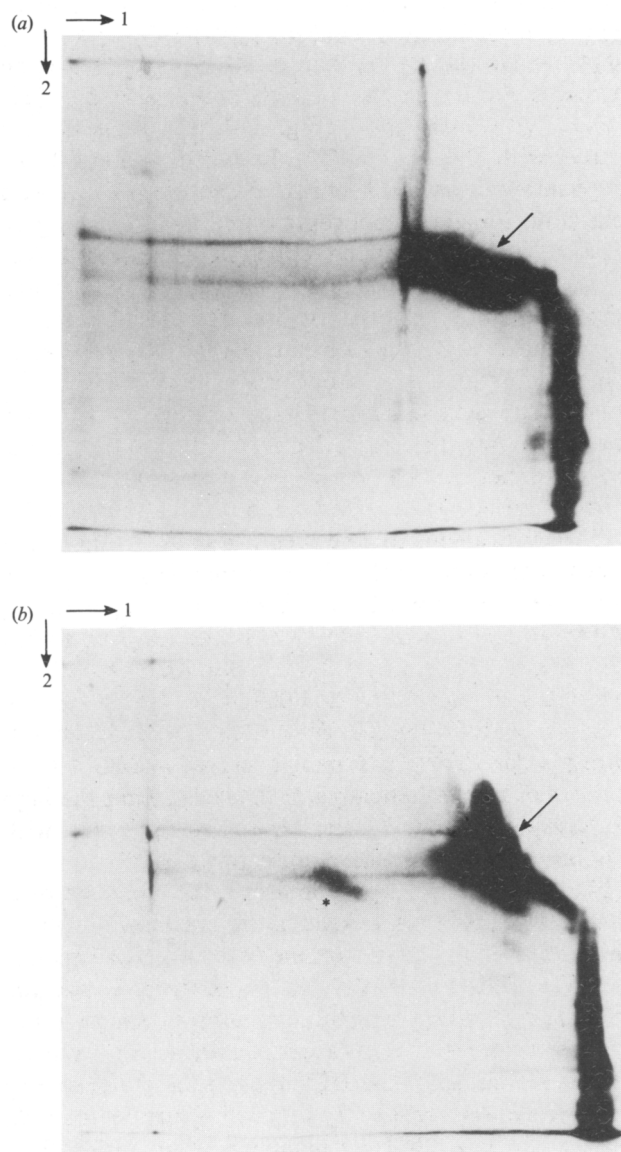


Fig. 2. Two-dimensional SDS-PAGE analysis of cross-linked gC. HEL cells were grown, infected with HSV-1 (KOS) and radiolabelled with [³H]arginine as described in the legend to Fig. 1. At 24 h post-infection, cells were washed three times with phosphate-buffered saline (PBS) and treated either with PBS (a) i.e. the control, or the cross-linker DTBP (b) at 0.1 mg/ml in PBS for 15 min at 4 °C. The cross-linking reaction was then stopped and the cells were solubilized with NP40 lysis buffer; see Fig. 1. The cell lysate was immunoprecipitated with a pool of gC-specific MAbs as for Fig. 1. The immunoprecipitates were first electrophoresed in a slab gel containing 5.5% acrylamide and 0.47% DATD (horizontal direction). The relevant gel lanes were then cut out, soaked in 5% 2-mercaptoethanol and 10 mM-DTT in 0.5 M-Tris-HCl, pH 6.9, for 30 min, placed on top of another slab gel of 8.5% acrylamide and 0.47% DATD using 1% agarose made with 0.5 M-Tris-HCl pH 6.9, and electrophoresed in the second (vertical) direction. The gels were enhanced fluorographically and autoradiographed (see Fig. 1). The migration position of native gC in each gel is indicated with an arrow; the asterisk labels the spot which corresponds to the reduction product of a DTBP-cross-linked gC complex. The streak of material down the right-hand edge of the gel represents material non-specifically immunoprecipitated by the anti-gC antibodies. It migrates differently in the two gels because they are of different acrylamide concentrations.

complex from HSV-1-infected cells by two-dimensional (non-reduced and then reduced) SDS-PAGE (Fig. 2). A large spot corresponding to gC of M_r 130 000 was visible in SDS-PAGE analyses of cells which had not been treated with cross-linker (Fig. 2a) as well as of cells treated with DTBP (Fig. 2b). In addition, a small, off-diagonal spot was visible only in the gel of DTBP-treated cells (Fig. 2b). This spot represented molecules migrating at M_r 130 000 following reduction of the cross-linked complex of M_r 150 000 to 260 000 present in the first dimension. Comigration of this spot in the second dimension with gC suggests that the high M_r cross-linked complex contained gC. As only one off-diagonal spot was seen, we suggest that gC is the only molecule in the cross-linked complex; that is, gC is linked only to itself and not to some other cellular or viral gene product.

Immunoblot (Western blot) analyses provide additional evidence for the presence of gC in the cross-linked complexes. In the Western blot (Fig. 3), a band which reacted with the antibody was visible at M_r 130 000 in analyses of both EGS-treated and untreated cells (Fig. 3, lanes 1 and 2). In addition, the high M_r complexes of 150 000 to 260 000 were visible only in analyses of EGS-treated cells (lane 2). These bands correspond to the high M_r band observed in SDS-PAGE analyses of biosynthetically labelled cells. Thus the high M_r complexes found after cross-linking react with antibodies to gC and therefore contain gC.

The genome of HSV contains nine open reading frames which contain sequences encoding proteins which are likely to be membrane-spanning (McGeoch *et al.*, 1985). Much remains to be learned about the structure and function of these membrane proteins and glycoproteins. Evidence is accumulating for the involvement of a number of the HSV membrane glycoproteins in the process of virus entry into cells (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Highlander *et al.*, 1987, 1989; Ligas & Johnson, 1988) but the mechanism of this at the molecular level is not well understood.

Glycoprotein B has been shown by biochemical and electron microscopic analyses to be associated as homologous multimers in infected cells (Spear, 1985; Stannard *et al.*, 1987) and this may facilitate its role in virus entry. In this report chemical cross-linking methods were used to investigate the possibility that gC, like gB, may exist as a multimer in the membranes of virus-infected cells. Our findings suggest that gC, like gB, is present in infected cell membranes as a cluster of molecules. Cross-linking reagents create stable homologous multimers of gC which can be detected after immunoprecipitation with gC-specific antibodies. However gC is unlike gB, in that the multimeric complexes of gC are not covalent complexes. It appears that both gB and gC are clustered in the infected cell membrane as

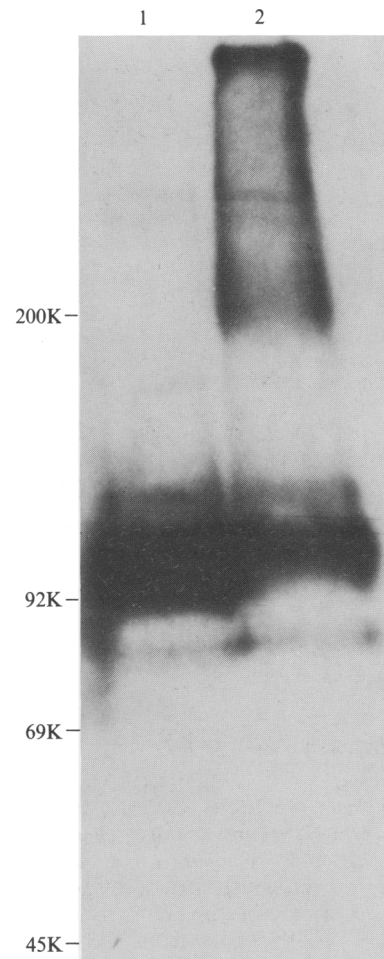


Fig. 3. Immunoblot analysis of cross-linked gC. HEL cells were grown and infected with HSV-1 (KOS). At 24 h post-infection, cells were washed three times with PBS and treated with PBS (lane 1) or 0.2 mg/ml EGS (lane 2) for 1 h at room temperature. The reaction was stopped with NP40 lysis buffer. The lysate was electrophoresed in a 5.5% acrylamide, 0.47% DATD gel as described in the legend to Fig. 1. Proteins were transferred electrophoretically to nitrocellulose (Schleicher & Schuell, BA85) by the method of Towbin *et al.* (1979) in a buffer of 25 mM-Tris-HCl pH 8.3, 192 mM-glycine, and 20% (v/v) methanol using a Bio-Rad Trans-Blot apparatus at 0.25 A for 2 h. The blot was first reacted with polyclonal rabbit immune antiserum specific for gC (Kikuchi *et al.*, 1987) diluted 100-fold in 0.15 M-NaCl, 0.02 M-Tris-HCl pH 7.4, 3% bovine serum albumin and then treated for 1 h with 125 I-labelled Protein A. The blot was autoradiographed at -70°C with an intensifying screen. The migration positions of M_r markers are indicated.

homologous multimers. Perhaps the clustering of these exposed viral glycoproteins into domains of homologous multimers facilitates virus attachment and entry into cells in some way. However, the exact mechanism of how this occurs must await further investigation.

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