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Cross-Linking of Glycoprotein Oligomers during Herpes Simplex Virus Type 1 Entry

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Herpes simplex virus (HSV) has 10 glycoproteins in its envelope. Glycoprotein B (gB), gC, gD, gH, and gL have been implicated in virus entry. We previously used chemical cross-linking to show that these five glycoproteins were close enough to each other to be cross-linked into homodimeric and hetero-oligomeric forms; hetero-oligomers of gB-gC, gC-gD, gD-gB, gH-gL, gC-gL, and gD-gL were found in purified virions. To better understand the roles of these glycoproteins in viral entry, we have modified a standard HSV penetration assay to include cross-linkers. This allowed us to examine changes in associations of viral glycoproteins during the entry process. HSV-1(KOS) was adsorbed at 4°C to human neuroblastoma cells (SY5Y). The temperature was raised to 37°C and cells were treated with cross-linker at various times after the temperature shift. Cytoplasmic extracts were examined by Western blotting (immunoblotting) for viral glycoproteins. We found that (i) as in virus alone, the length and concentration of the cross-linking agent affected the number of specific complexes isolated; (ii) the same glycoprotein patterns found in purified virions were also present after attachment of virions to cells; and (iii) the ability to cross-link HSV glycoproteins changed as virus penetration proceeded, e.g., gB and gD complexes which were present during attachment disappeared with increasing time, and their disappearance paralleled the kinetics of penetration. However, this phenomenon appeared to be selective since it was not observed with gC oligomers. In addition, we examined the cross-linking patterns of gB and gD in null viruses K082 and KOSgDβ. Neither of these mutants, which attach but cannot penetrate, showed changes in glycoprotein cross-linking over time. We speculate that these changes are due to conformational changes which preclude cross-linking or spatial alterations which dissociate the glycoprotein interactions during the penetration events.

Herpes simplex virus (HSV) contains at least 10 glycoproteins in its envelope which are important for infection and pathogenesis (42). Five of these glycoproteins, gB, gC, gD, gH, and gL, have roles in viral entry; four of these are essential (gB, gD, gH, and gL) (2, 10, 31, 37). The first interaction between virus and cell occurs when gC binds to cell surface heparan sulfate proteoglycans (43, 47). In the absence of gC, gB binds to the cell surface, and in the absence of both glycoproteins, the virus is unable to attach to cells (20, 43). Next, gD presumably interacts with a second cellular receptor (4, 30), possibly the mannose-6-phosphate receptor (1). pH-independent fusion occurs between virus envelope and plasma membrane (46); gB, gD, and gH have all been implicated in this step (35, 42). gL is required for proper expression and incorporation of gH into virions (27, 37) but whether it plays a more specific role in viral entry has not been proven.

Building on the work of others (48, 49), we previously examined glycoprotein-glycoprotein associations in the virion (19). By using chemical cross-linking to stabilize close associations between glycoproteins, both homo- and hetero-oligomers could be identified on the virion surface. Dimer- and trimer-sized species of gD were detected, and the presence of gB and gC dimers, previously seen by Claesson-Welsh and Spear (5) and Zhu and Courtney (48, 49), was confirmed in our study. In addition, hetero-oligomers were found; gB-gC, gC-gD, and gD-gB complexes could all be found in purified preparations of HSV type 1 (HSV-1) (KOS), as could gH-gL, gC-gL, and gD-gL hetero-oligomers. These studies suggested that the HSV glycoproteins which are each important for viral entry have the potential to associate with one another and that these associations may be functionally important in entry.

The presence of so many different membrane glycoproteins in HSV has made it difficult to sort out their functions in entry. Glycoproteins in other viruses, such as influenza virus, are known to require oligomerization into larger complexes to execute fusion and subsequent entry steps (6, 11, 44), and separate glycoproteins are involved in attachment and fusion. At least one study of HSV, however, suggests that individual glycoproteins function at distinct and different points in entry in a sequential, cascade-like mechanism (16). Our data showing close associations among the virion glycoproteins which participate in entry also fit in with the concept of assembly of a fusion machine, proposed by White (45) to occur in other systems involving membrane fusion.

To begin to examine whether the associations of gB, gC, gD, gH, and gL were important for entry, we applied the same cross-linking techniques used to establish their existence in intact virions. We used the human neuroblastoma cell line SY5Y to examine the oligomeric state of the essential glycoproteins during attachment and penetration by wild-type KOS virions, as well as by two mutant viruses, each of which lacked a glycoprotein essential for virus entry (2, 3, 9, 31). Our data suggest that oligomeric associations among the glycoproteins change during entry of the wild-type virus. These changes do not occur when the null viruses lacking either gB or gD are incubated with cells. Thus, our data show a temporal correlation between glycoprotein associations and virus entry.

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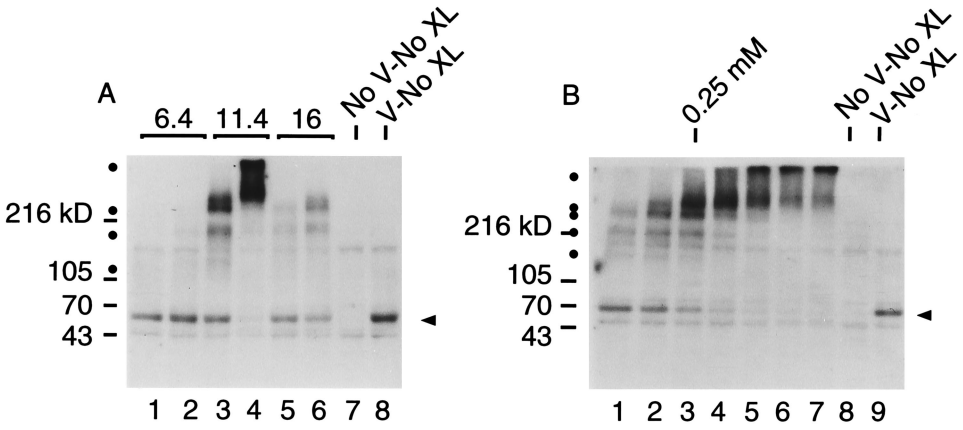

FIG. 1. Effect of cross-linker size and concentration on cross-linking of gD after HSV adsorption. Cell extracts were electrophoresed on denaturing SDS-PAGE (4 to 12% polyacrylamide) gels; this was followed by Western blotting. gD was detected with PAb R1. The results with gD are representative of all three glycoproteins (gB, gC, and gD). (A) Purified HSV-1(KOS) was adsorbed to SY5Y cells for 2 h at 4°C. After being washed, virus and cells were incubated for 50 min at 4°C with three cross-linkers with lengths of 6.4 Å (Sulfo-DST), 11.4 Å (BS^3), and 16 Å (Sulfo-EGS). Lane 1, 0.1 mM Sulfo-DST; lane 2, 1 mM Sulfo-DST; lane 3, 0.1 mM BS^3; lane 4, 1 mM BS^3; lane 5, 0.1 mM Sulfo-EGS; lane 6, 1 mM Sulfo-EGS; lane 7, mock-infected and mock-cross-linked sample (cells only); lane 8, mock cross-linked HSV. (B) Purified HSV-1(KOS) was adsorbed to SY5Y cells for 2 h at 4°C. After being washed, virus and cells were incubated for 50 min at 4°C with increasing amounts of BS^3. Lane 1, 62.5 μM; lane 2, 0.125 mM; lane 3, 0.25 mM; lane 4, 0.5 mM; lane 5, 1 mM; lane 6, 2 mM; lane 7, 4 mM. Controls included a mock-infected, mock-cross-linked sample (lane 8) and an infected mock-cross-linked sample (lane 9). The concentration selected for subsequent experiments was 0.25 mM (lane 3). Arrowheads indicate the position of monomeric gD. The positions of cross-linked oligomers are indicated by bullets. Molecular weight markers are at the left of each panel. No V-No XL, mock-infected and mock-cross-linked; V-No XL, infected mock-cross-linked.

MATERIALS AND METHODS

Cells and virus. Human neuroblastoma-derived SY5Y cells and primate Vero cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum, penicillin-streptomycin solution (100 U/ml; GIBCO/BRL), and L-glutamine (2 mM; GIBCO/BRL) at 37°C. HSV-1 strain KOS was propagated on Vero cells. Complemented mutant viruses KosgDβ and K082 were propagated on VD60 cells and D6 cells, respectively, while uncomplemented stocks were propagated on Vero cells, the parental cell type for both VD60 and D6 cells. KOSgDβ is a gD-null virus in which the β-galactosidase gene replaces the US6 open reading frame (9), and K082 is a gB-null virus in which a linker-insertion mutation results in a truncated gB gene product which is not incorporated into the virion (2, 3). Wild-type KOS was titered on SY5Y cells. Cells and viruses were kindly provided by D. Johnson (VD60 cells), S. Person (K082 virus and D6 cells), D. Pleasure (SY5Y cells), P. Schaffer (KOS virus), and P. Spear (KOSgDβ virus).

Purification of HSV-1(KOS) and null viruses. Roller bottles of Vero cells were infected at a multiplicity of infection (MOI) of 0.1 PFU per cell, and extracellular virus was harvested from the growth medium and purified on sucrose gradients, as described previously (19).

Quantitation of null virus. To accurately compare wild-type and null viruses, we needed to use equivalent amounts of each in our experiments. Purified uncomplemented stocks of KosgDβ and K082 could not be quantitated by plaque forming assay, since they each lack an essential glycoprotein (gD and gB, respectively). We measured equivalent amounts of KOS and null viruses by comparing the quantity of VP5 capsid protein in each, as done by others (21). The amount of VP5 present should be in direct proportion to the number of intact virions in the purified virus sample. Known amounts of wild-type KOS were analyzed along with samples of K082 and KosgDβ on denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels; this was followed by Western blotting (immunoblotting). Blots were probed with polyclonal antibody (PAb) NC1 to VP5 (8); this was followed by incubation with ^{125}I-protein A (ICN Pharmaceuticals, Inc.) (7). Blots were then exposed to a Molecular Dynamics Storage Phosphor Screen, and the amount of capsid protein was quantitated on the Molecular Dynamics PhosphorImager by using ImageQuant software (33).

Cross-linkers. All cross-linkers used were water-soluble, membrane-impermeant, homobifunctional sulfo-*N*-hydroxy-succinimide esters (Pierce Chemical Co.). Disulfo-succinimidyl tartarate (Sulfo-DST) and ethylene glycol*bis*(sulfosuccinimidylsuccinate) (Sulfo-EGS) are cleavable (via periodate or hydroxylamine treatment, respectively); *bis*(sulfosuccinimidyl suberate) (BS^3) is noncleavable.

Penetration assay and cross-linking. (i) Acid inactivation. The rate of virus penetration at 37°C was measured by the HSV penetration assay (26) as modified (25, 32).

(ii) Cross-linking. This assay was carried out by the addition of chemical cross-linking reagents to detect associations between proteins during entry. Virus at an MOI of 5 PFU per cell was incubated with cells at 4°C for 2 h and then the temperature was raised to 37°C for various amounts of time. The medium was removed and replaced with cross-linker in Hank's balanced salt solution at 4°C for 50 min. The cross-linking reaction was quenched with an excess of free amines by overlaying the cells with 1 M Tris-HCl (pH 7.0) for 1 min. Cells were scraped into a small volume of ice-cold phosphate-buffered saline and pelleted by centrifugation at 625 × *g* for 10 min at 4°C. The cell pellets were extracted with ice-cold lysing buffer (0.02 M Tris [pH 7.5], 0.05 M NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) containing 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 0.1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). The nuclei were removed by centrifugation at 625 × *g* for 10 min at 4°C, and the supernatants were stored at −80°C.

SDS-PAGE and Western immunoblotting. Protein extracts were prepared and analyzed by SDS-PAGE and Western blotting as described previously (19). PAbs R69 (anti-gB) (15), R46 (anti-gC) (15), and R1 (anti-gD) (28) were used to detect HSV glycoproteins.

Immunoprecipitation of cross-linked viral proteins. Cross-linked oligomers containing HSV glycoproteins were immunoprecipitated from cytoplasmic extracts with the appropriate immunoglobulin G (IgG) or preimmune IgG as previously described (19). Following SDS-PAGE, Western blots were probed with the precipitating PAb or a second heterologous PAb or monoclonal antibody (MAb) to identify associated glycoproteins; R69, R46, and R1 were used to detect gB, gC, and gD, respectively.

RESULTS

Effect of cross-linker size and concentration on oligomer identification. In our previous work (19), we detected gB, gC, and gD homo-oligomers and hetero-oligomers of gB-gC, gC-gD, gD-gB, gH-gL, gC-gL, and gD-gL. On the basis of these results and the work of others (48, 49), we knew that glycoprotein-glycoprotein associations could be detected in purified HSV-1 virions. In this study, we first asked whether these glycoprotein interactions changed during the initial stages of infection, i.e., when virus attached to cells. In earlier studies we found that a cross-linker of 11.4 Å (1 Å = 0.1 nm) (BS^3) at a concentration of 0.25 mM was optimal for covalently bonding closely associated glycoproteins in purified virions. Therefore, similar titrations were carried out after virions were adsorbed to SY5Y cells at 4°C for 2 h. The cross-linking reaction was carried out with different-sized cross-linkers or different concentrations of BS^3. Cytoplasmic extracts were prepared, and proteins were separated by SDS-PAGE, Western blotted, and probed with PAbs. gB, gC, and gD all had similar results in this assay; the results of gD Western blots are shown in Fig. 1.

Sulfo-DST (6.4 Å), at 0.1 or 1 mM, was ineffective at cross-linking HSV gD (Fig. 1A, compare lanes 1 and 2 to lane 8). BS^3 (11.4 Å) at both concentrations cross-linked gD into high-molecular-weight oligomers (lanes 3 and 4). The Sulfo-EGS (16 Å) pattern (lanes 5 and 6) was similar to that for BS^3, although less material was seen at the highest molecular

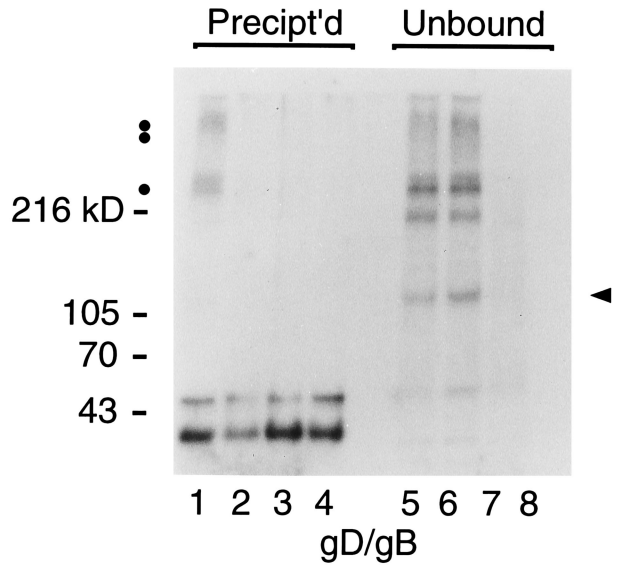

FIG. 2. Detection of gD-gB hetero-oligomers during virus-cell interaction. Virus was adsorbed to SY5Y cells for 2 h at 4°C, after which BS^3 was added. Cytoplasmic extracts were prepared and immunoprecipitated with either R1 IgG against gD-1 or a preimmune IgG. Precipitated proteins (Precipt'd) and unprecipitated fractions (Unbound) were electrophoresed on denaturing SDS-PAGE (4 to 12% polyacrylamide) gels, and Western blots were probed for gB with R69 serum. Lane 1, R1 IgG precipitation of cross-linked virus and cells; lane 2, preimmune IgG precipitation of cross-linked virus and cells; lane 3, R1 precipitation of cross-linked uninfected cells; lane 4, R1 precipitation of buffer alone. Lanes 5 to 8 represent the proteins not precipitated from lanes 1 to 4. The position of monomeric gB is denoted by an arrowhead. Positions of oligomers are indicated by bullets. Molecular weight markers are on the left.

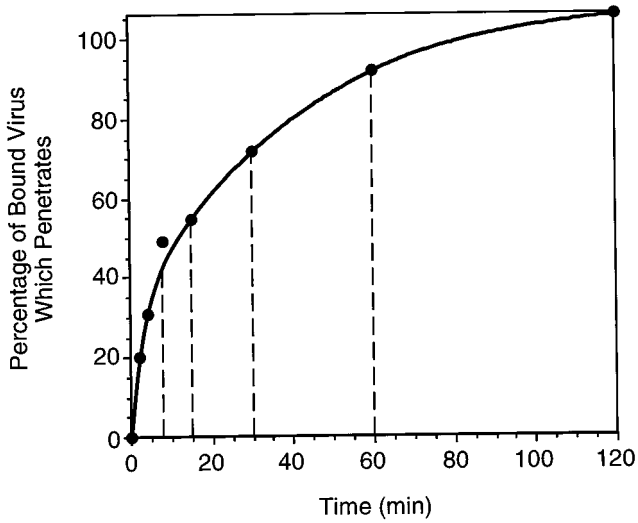

FIG. 3. Kinetics of HSV-1(KOS) entry into SY5Y cells. Sucrose-gradient-purified HSV-1(KOS) was tested for rate of penetration into human neuroblastoma-derived SY5Y cells. A total of 10^2 PFU of virus was adsorbed to SY5Y monolayers for 2 h at 4°C (MOI of 6.3×10^{-5} PFU per cell). Plates were shifted to 37°C, and penetration was allowed to proceed for the times indicated. At each time point, virus which had not penetrated was inactivated by a low-pH wash (pH 3.0). Plates were scored for plaque formation at 40 h postinfection. Values at each time point were determined in triplicate for each experiment and the curve represents the average value for each time point. For reference to subsequent experiments, cross-linking time points of 8, 15, 30, and 60 min are denoted on the curve with dashed lines.

weights (compare lanes 3 and 4 with 5 and 6). Thus, as we found previously with purified virions in solution, the 6.4 Å cross-linker was insufficient to cross-link HSV glycoproteins, while the 11.4 Å cross-linker (BS^3) was optimal. We next tested various concentrations of BS^3 (Fig. 1B). As the concentration of BS^3 was increased (from 62.5 μM to 4 mM), there was a decrease in the amount of monomer (lanes 1 to 7) and an increase in the proportion of higher-molecular-weight oligomers. At 0.25 mM BS^3 (lane 3), a spectrum of cross-linked species for any particular glycoprotein could be identified. Thus, the same conditions used to study associations in purified virions (19) were also useful for studying the interactions of these proteins when virions are attached to cells. There were no discernible differences in the cross-linking patterns for glycoproteins in either purified virions or virions attached to cells.

Detection of hetero-oligomers after attachment. We next asked if the hetero-oligomeric associations detected in the purified virus were altered after virus attachment. Virus was adsorbed to cells for 2 h at 4°C and cross-linked with 0.25 mM BS^3, and lysates were prepared and analyzed for the presence of these complexes. To detect hetero-oligomers between gD and gB, lysates were incubated with R1 IgG to precipitate gD. Monomeric and oligomeric species of gD were separated on SDS-PAGE, transferred to Western blot, and probed with R69 to detect gB (Fig. 2). Lanes 1 to 4 of Fig. 2 contain the proteins immunoprecipitated by R1 or preimmune IgG, while lanes 5 to 8 contain the proteins that failed to be precipitated. High-molecular-weight species were detected by R69 anti-gB serum, indicating the presence of gB-gD complexes (Fig. 2, lane 1). A larger proportion of oligomeric gB remained in the unbound fraction (lane 5). We conclude that during attachment, a subpopulation of gD and gB are within 11.4 Å of one another. These results are essentially the same as those obtained with purified virus (19). When we carried out similar experiments to look for gB-gC and gC-gD hetero-oligomers, the results also matched those obtained with free virus. Thus, the hetero-oligomers detected in virus alone were the same as when virus was adsorbed to SY5Y cells. Although it is possible that some of the cross-linked species contain cell-associated molecules, these associations did not significantly alter the pattern seen on the gels.

Kinetics of HSV-1(KOS) entry into human neuroblastoma cells (SY5Y). We determined the kinetics of HSV-1(KOS) penetration into SY5Y cells by using a standard penetration assay (25, 26, 32). The time required for 50% of the input virus to enter these neuroblastoma cells ($t_{1/2}$) was determined to be 8 to 10 min (Fig. 3), and well over 90% of the virus penetrated within 60 min. This rate falls within the range reported for HSV-1 penetration into a variety of cell lines (22, 23, 26, 34, 38).

Effect of cross-linking during viral entry. We next asked whether there was a change in the pattern of glycoprotein cross-linking during virus penetration. Virus was adsorbed to cells at 4°C for 2 h, then the temperature was shifted to 37°C, and BS^3 was added at various times posttemperature shift (see Fig. 3), up to 60 min. Cytoplasmic extracts were prepared and were analyzed by SDS-PAGE and Western blotting. Maximum cross-linking of gB or gD occurred at time zero (Fig. 4A and 4C, lanes 1). By 15 min after the temperature shift (Fig. 4A and 4C, lanes 3), there was a significant decrease in the amount of gB or gD present in oligomers larger than 116 kDa. The amount of cross-linked gB or gD continued to decline, and by 60 min (lanes 5), few high-molecular-weight oligomers containing gB or gD were detected. In contrast, gC behaved differently; there was no significant change in the degree of cross-linked proteins during virus entry (Fig. 4B, lanes 1 to 5). Moreover, unlike gB or gD, the majority of gC was present as monomer.

Detection of hetero-oligomers during penetration. In the experiment described just above, all gB, gC, and gD complexes were detected by Western blotting, including both homo-oligomers and hetero-oligomers. To answer the question of what happened to individual hetero-oligomers during penetration, the experiment was repeated and a combination of immuno-

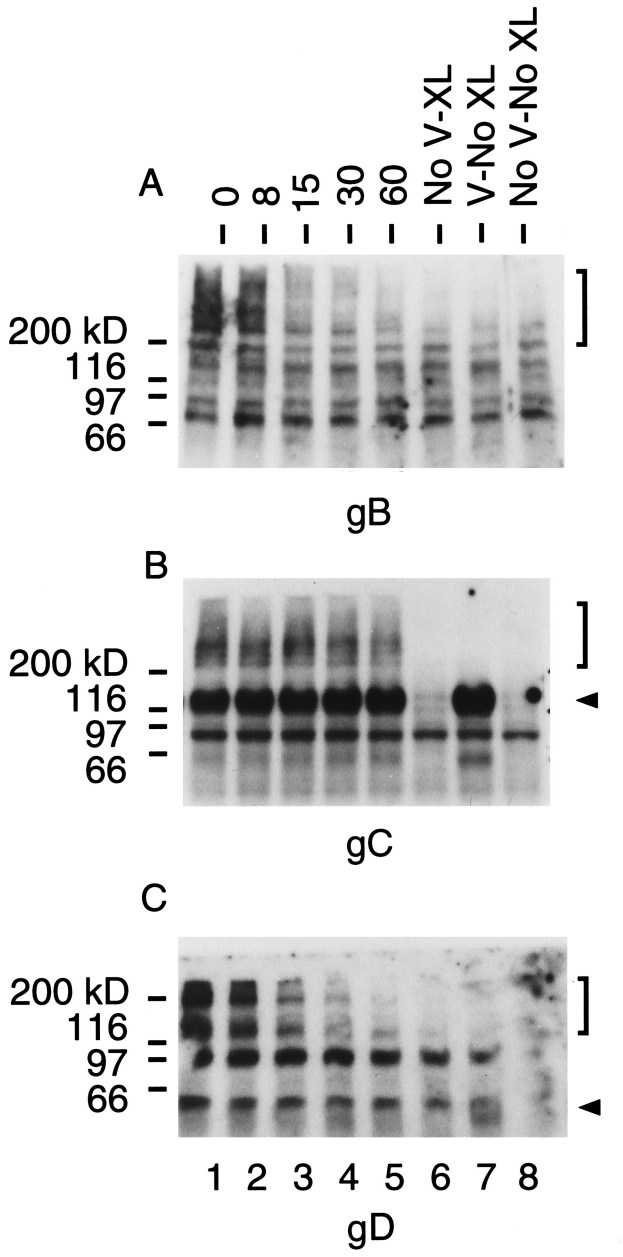

FIG. 4. Detection of glycoprotein oligomers during HSV penetration. HSV-1(KOS) penetration into SY5Y cells was interrupted by cross-linking with BS^3 at times from 0 to 60 min. An MOI of 0.5 PFU per cell was used in this experiment. Western blots of denaturing SDS-PAGE (4 to 20% polyacrylamide) gels were probed with antisera to gB-1 (A), gC-1 (B), and gD-1 (C). Lanes 1 to 5 of each blot represent samples of virus-cell mixtures cross-linked at 0, 8, 15, 30, and 60 min, respectively. Lane 6, mock-infected and cross-linked cells (No V-XL); lane 7, infected and mock-cross-linked cells (V-No XL); lane 8, mock-infected and mock-cross-linked cellular extract alone (No V-No XL). The regions of high-molecular-weight oligomers are indicated by brackets. The positions of the glycoprotein monomers are denoted by arrowheads. Molecular weight markers are on the left.

precipitation and Western blotting was used to follow the hetero-oligomers during virus entry.

gB oligomers were precipitated by R69 anti-gB IgG. We probed the first Western blot with R69 (Fig. 5A). In this experiment, gB monomers and oligomers were detected at time zero. The amount of gB monomer that could be immunoprecipitated decreased between 0 and 15 min of penetration (lane 1) and then remained constant between 15 and 60 min (lanes 2 to 4). In contrast, the ability to cross-link gB oligomers with BS^3 continually decreased as the time of penetration progressed (lanes 1 to 4). When a similar blot was probed with anti-gD serum (Fig. 5B), cross-linking of gB-gD hetero-oligomers also decreased over time. Only a small amount of gB was cross-linked to gC at time zero (Fig. 5C, lane 1), as seen in free virus (19). Less of this complex was seen at later times, and by 60 min, no gB-gC oligomers were detected (lane 4). Thus, a very small subpopulation of gC associated with gB, and this changed during penetration, while the amount of gC oligomers overall did not (Fig. 4B). Similar results were seen for gC-gD hetero-oligomers (data not shown). A larger proportion of gC was complexed to gD than to gB, but this still represented only a small fraction of the total gC or gD. The gC-gD complex also decreased with time, but this change was small (data not shown). Thus gB-gC, gC-gD, and gD-gB hetero-oligomeric associations diminished as entry proceeded. gB-gD hetero-oligomers showed the most dramatic changes. Hetero-oligomers of gC-gB and gC-gD also diminished, but these oligomers represented only a small proportion of the total gC oligomer pool, most of which remained unchanged.

Glycoprotein oligomer formation with HSV null viruses. The possibility exists that we are following a host-directed phenomenon and not a virus-specific series of events linked to entry. To test whether there was a direct relationship between the observed changes in glycoprotein associations and viral penetration, we used two entry-defective null mutants to follow the changes in the ability to cross-link when the virus did not penetrate. These mutants, lacking either the gB gene (K082) or the gD gene (KOSgDβ), attach to cells but fail to enter (2, 31). We infected SY5Y cells with the null mutants at 4°C, shifted the temperature to 37°C, and allowed penetration to proceed for 0, 15, 30, or 60 min. BS^3 was added at each time, and then cytoplasmic extracts were prepared and analyzed by SDS-PAGE and Western blotting (Fig. 6).

In the case of K082, no gB was detected, as expected (Fig. 6A). gD oligomers were detected at time zero (Fig. 6B, lane 1) but the amount did not diminish with increasing time. Likewise in the case of KosgDβ, no gD was detected (Fig. 6D). In contrast, gB oligomers were detected at time zero, and remained at the same level throughout the 60-min incubation at 37°C (Fig. 6C). Thus, the diminishment of the complexes over time, seen with wild-type virus, did not take place with either mutant. We conclude that the apparent disappearance or dissociation of the glycoprotein complexes is correlated with the ability of the virus to penetrate and is not merely due to a cellular effect on the virus occurring independently of virus entry.

Detection of gH and gL oligomers during entry of HSV-1. Previously we showed that we could demonstrate the presence of gH-gL complexes in the virion by increasing the sensitivity of our detection system (19). This included the use of a chemiluminescence assay in combination with more sensitive antibodies to gH (PAb R83) and gL (MAb 8H4) (see reference 14). We used these modifications to reprobe the blots shown in Fig. 4 to determine whether gH-gL complexes were altered in their ability to be cross-linked (19); we detected both the 140-kDa gH-gL complex and a higher-molecular-weight form (greater than 216 kDa) at time zero with MAb 8H4 or R83 (data not shown). However, the signal strength for the gH-gL complexes seen at time zero was quite low, and by 60 min, we no longer detected any gH-gL complexes. Thus, it appears that the ability to cross-link gH-gL into higher-molecular-weight complexes also diminished with time of virus penetration, although the results were not nearly as convincing as with gB and gD.

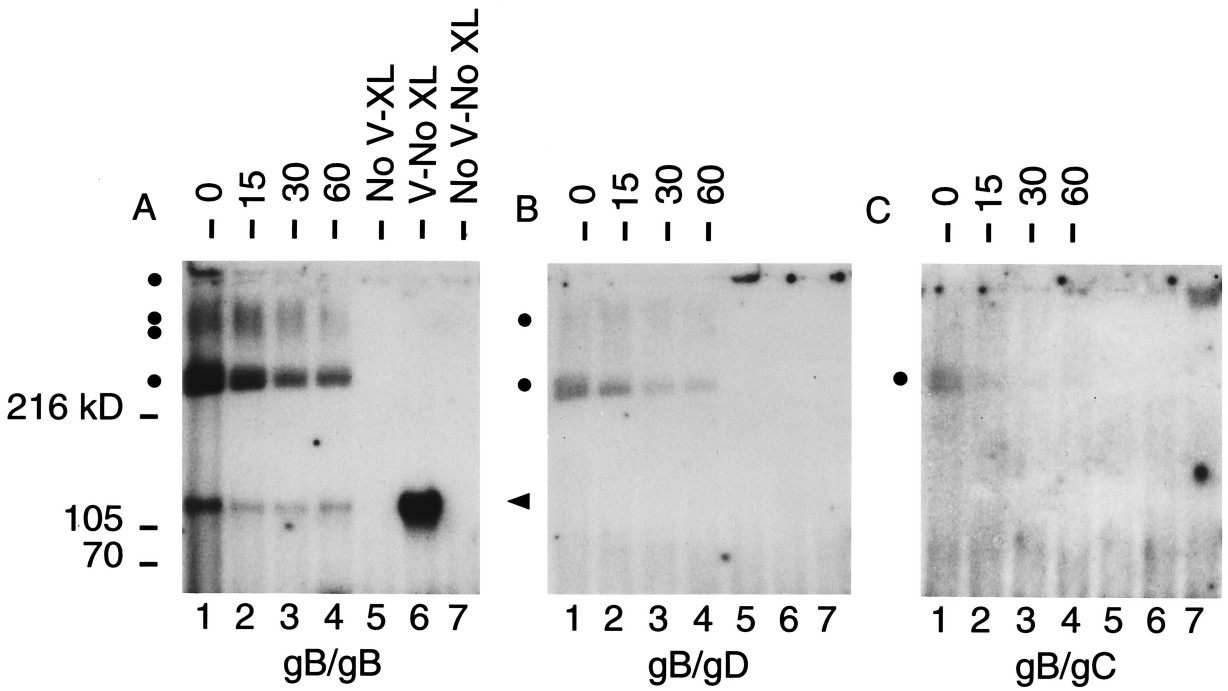

FIG. 5. Cross-linking of gB oligomers and gB-gD and gB-gC hetero-oligomers during penetration. HSV-1(KOS) penetration into SY5Y cells was interrupted by cross-linking with BS^3 at various times from 0 to 60 min. Extracts of cross-linked samples were immunoprecipitated with either R69 IgG against gB-1 or preimmune IgG. Precipitated proteins were separated on denaturing SDS-PAGE (4 to 12% polyacrylamide) gels, and Western blots were probed for gB-1 (A), gD-1 (B), and gC-1 (C). Lanes 1 to 4 of each blot represent penetration times of 0, 15, 30, and 60 min, respectively. Lanes 5 and 6 represent mock-infected and cross-linked (No V-XL) and infected and mock-cross-linked (V-No XL) samples, respectively. In lanes 7, samples of the 15-min time point were precipitated with preimmune IgG. The position of the gB monomer is indicated by an arrowhead. Positions of oligomers are indicated by bullets. Molecular weight markers are on the left.

DISCUSSION

HSV enters mammalian cells by a pH-independent fusion event that occurs between the virion envelope and the plasma membrane (46). Five glycoproteins, gB, gC, gD, gH, and gL, are involved in this complex process and sorting out their function(s) has proven to be formidable. In other systems, virion glycoproteins involved in entry are multimeric, being either homo- or hetero-oligomers (12), and HSV is not an exception. Within the virion, gE and gI (13, 29), as well as gH and gL (14, 27, 37), are heterodimers, and gB (5, 39, 41) is homodimeric. In addition, a proportion of gC can be cross-linked into a complexed form (48). In an accompanying report, we quantitated the amount of gB, gC, gD, and gH in virions. Secondly, we used chemical cross-linking agents to show that gD, as well as gB and gC, exists in a multimeric state in the virion. Lastly, we were able to distinguish hetero-oligomeric complexes between gD and gB and between gD and gC, as well as between gB and gC; gH-gL oligomers were also found (19).

Two methods have been used to study the kinetics of HSV entry. The first uses an acid wash to stop penetration of virus bound to the surface of cells (26). Here, one determines by plaque assay how much virus at any particular time has entered the cell (i.e., that is resistant to the low-pH wash). Certain MAbs to gB, gD, and gH neutralize virus after the attachment step, but the virus is refractory to neutralization once the temperature is raised to 37°C (17, 22, 23, 25). Neutralization by an antibody at this step is accepted as evidence that the glycoprotein is involved in penetration. As a second approach, Fuller and Lee (16) used MAbs to gD and gH in conjunction with electron microscopy (EM) to determine which step in entry was blocked by each antibody. Their data suggest that virus entry occurs as a sequential series of interactions between the virion envelope and cell plasma membrane, such that gD mediates a stable attachment and at a later time gH participates in fusion. We took a third approach to this question. Since we had already shown that the glycoproteins involved in entry are physically close to one another, we wondered if this had functional significance.

Therefore, we asked whether we could detect changes in the cross-linking profile of homo- and hetero-oligomeric complexes of glycoproteins during entry. Essentially, we are probing the surface of the virion with cross-linking reagents and asking what happens to the associations among the proteins during attachment and subsequently as the virus envelope fuses with the cell membrane. Why would we think that there might be changes in the complexes? Highlander et al. (24) speculated that conformational changes in gB occurring at the time of entry might trigger fusion of the viral envelope with the cellular plasma membrane. Shieh et al. (40) proposed that the interaction of cell surface heparan sulfate proteoglycans with an HSV glycoprotein may alter conformation of one or more virion or cell molecules and thereby trigger fusion. During the attachment phase, we found there was no detectable change in the glycoprotein cross-linking patterns of the virion. And EM studies have also failed to discern obvious morphological changes in the virion during this step (16). However, changes in the glycoprotein cross-linking profiles did indeed occur during penetration, such that oligomers of gB, gD, and gH-gL were increasingly less accessible to cross-linking over time. Furthermore, within the entire population of cross-linked glycoprotein complexes, the gB-gD hetero-oligomers were affected to the same extent as the overall oligomer pool. It is likely that some physical change in the envelope occurs during entry which alters the ability of the cross-linker to stabilize the oligomers. Thus, there appears to be a correlation between the extent of penetration and the ability to cross-link the essential glycoproteins. EM

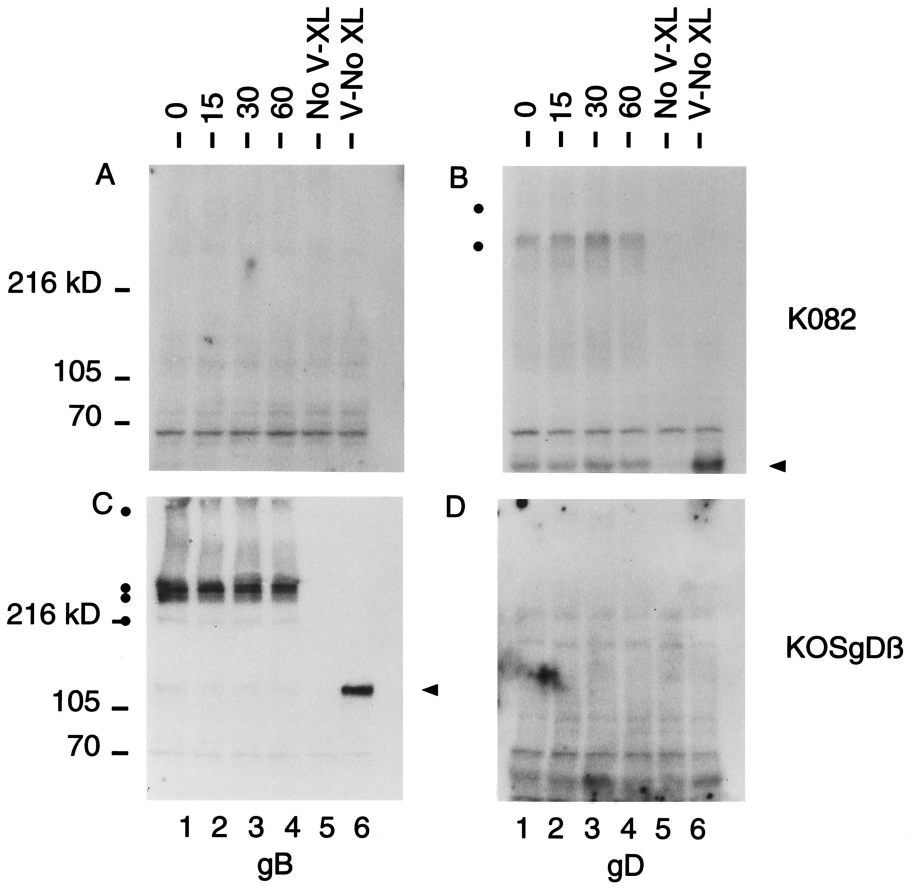

FIG. 6. Accessibility of gB and gD to cross-linking during incubation of HSV-1 null mutants with SY5Y cells. Two mutants, K082 and KOSgDβ, which are unable to penetrate were adsorbed to SY5Y cells for 2 h at 4°C. The temperature was shifted to 37°C, and plates were incubated for times from 0 to 60 min. BS^3 was added at the end of each time point and lysates were prepared, electrophoresed on denaturing SDS-PAGE (4 to 12% polyacrylamide) gels, and Western blotted. (A) Cells infected with K082 and blots probed with R69, (B) cells infected with K082 and blots probed with R1, (C) cells infected with KOSgDβ and blots probed with R69, (D) cells infected with KOSgDβ and blots probed with R1. Lanes 1 to 4 of each blot represent penetration times of 0, 15, 30, and 60 min posttemperature shift, respectively. Lanes 5 and 6 represent mock-infected and cross-linked (No V-XL) and infected and mock-cross-linked (V-No XL) samples, respectively. The positions of the glycoprotein monomers are denoted by arrowheads. Positions of oligomers are indicated by bullets. Molecular weight markers are on the left.

studies have shown marked changes in envelope structure at this time, following the attachment step (16). However, not all of the glycoproteins were affected in this way; oligomeric gC levels did not change. Since the molar quantity of gC and gB are the same in the virion, it does not appear to be a question of amount but more likely the location or accessibility of the protein to the cross-linking reagent. One speculation is that gC may be cross-linked into complexes with heparan sulfate proteoglycans which remain on the cell surface after entry.

Are these changes in virion structure a virus-directed event or a cellular event (e.g., pinocytosis) (4, 18)? First, if it were only a cellular event, then HSV-1 gB- or gD-null virus should show a similar phenomenon as the wild-type virus. However, we found no temporal change in the ability to cross-link gB or gD in the null viruses. The same amount of glycoprotein was cross-linked during attachment as was present at 60 min after raising the temperature to 37°C, strengthening our belief that we are following virus-specific structural changes.

Second, in preliminary experiments, we found that the rate of entry of HSV-1(KOS) on SY5Y cells differed when the infecting virus was grown on different cell lines. When the virus stock was grown on Vero cells the $t_{1/2}$ for entry was 8 to 10 min (Fig. 3). However, if the stock was prepared in L cells, the $t_{1/2}$ for SY5Y cells was 30 min (data not shown). When this slowly penetrating virus was used in cross-linking experiments, the glycoproteins could be cross-linked for much longer times. The kinetics of cross-linking correlated with the slower rate of penetration.

Overall, our data suggest that some definable alteration occurs among the envelope glycoproteins during penetration which makes several of them refractory to cross-linking. We hypothesize that the changes in cross-linking reflect interactions among and between the virion glycoproteins and the plasma membrane of the cell, and that these interactions lead to virus entry. Furthermore, it is entirely possible that the hetero-oligomers of gB, gD, and gH-gL act as a complex in entry, as in the model proposed by White (45). Since there is 10 to 15 times more gD than gB in the envelope (19), gD that is not associated with gB may play an alternate role in entry.

Where were the glycoproteins distributing themselves during penetration to preclude them from being cross-linked? Fuller and Lee showed by EM dramatic morphological changes in the structure of the envelope during the fusion event (16). The virion envelope appears to "melt" into the plasma membrane. Para et al. (36) presented evidence that virion-derived gD is found in the membrane of infected cells following infection. Formation of a fusion pore, as is thought to occur in other viral systems (45), might "bury" the glycoproteins as the nucleocapsid entered the cytoplasm. The "disappearance" of gB and gD (and possibly gH-gL) could thus be due to the formation (and possibly the dissolution) of such a fusion pore. Two other pathways

could be envisioned to explain our observations. First, the proteins might diffuse away from each other at the entry site and remain within the plane of the membrane. Thus, individual proteins would no longer be associated and would be unavailable for cross-linking into oligomers. Second, the glycoproteins might be internalized during entry and thereby become inaccessible to the membrane-impermeant cross-linkers. Future experiments (e.g., by combining the techniques of cross-linking and EM) are needed to evaluate the assumptions underlying these proposed models.

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