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Oligomeric Structure of Glycoproteins in Herpes Simplex Virus Type 1

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A number of herpes simplex virus (HSV) glycoproteins are found in oligomeric states: glycoprotein E (gE)-gI and gH-gL form heterodimers, and both gB and gC have been detected as homodimers. We have further explored the organization of glycoproteins in the virion envelope by using both purified virions to quantitate glycoprotein amounts and proportions and chemical cross-linkers to detect oligomers. We purified gB, gC, gD, and gH from cells infected with HSV type 1 and used these as immunological standards. Glycoproteins present in sucrose gradient-purified preparations of two strains of HSV type 1, KOS and NS, were detected with antibodies to each of the purified proteins. From these data, glycoprotein molar ratios of 1:2:11:16 and 1:1:14:9 were calculated for gB/gC/gD/gH in KOS and NS, respectively. gL was also detected in virions, although we lacked a purified gL standard for quantitation. We then asked whether complexes of these glycoproteins could be identified, and if they existed as homo- or hetero-oligomers. Purified KOS was incubated at 4°C with *bis*(sulfosuccinimidyl) suberate (BS^3), an 11.4 Å (1Å = 0.1 mm) noncleavable, water-soluble cross-linker. Virus extracts were examined by Western blotting (immunoblotting), or immunoprecipitation followed by Western blotting, to assay for homo- and hetero-oligomers. Homodimers of gB, gC, and gD were detected, and hetero-oligomers containing gB cross-linked to gC, gC to gD, and gD to gB were also identified. gH and gL were detected as a hetero-oligomeric pair and could be cross-linked to gD or gC but not to gB. We conclude that these glycoproteins are capable of forming associations with one another. These studies suggest that glycoproteins are closely associated in virions and have the potential to function as oligomeric complexes.

Herpes simplex virus (HSV) is an enveloped, double-stranded DNA virus which is an important human pathogen (26). It contains in its envelope at least 11 glycoproteins, 5 of which function in viral entry. At least seven glycoproteins function in virus spread, and several participate in immune evasion (10, 32). To better understand the functions of HSV glycoproteins, it is necessary to understand their structural organization in the virus. Most enveloped viruses exhibit, by electron microscopy, a clearly defined morphological spike structure consisting of one or two distinct integral membrane proteins. In HSV, however, a distinctly organized arrangement of glycoproteins has not been reported. In one immunoelectron microscopy study of HSV virions, glycoprotein B (gB) appeared as clustered "T-shaped" spikes, gC appeared to consist of "randomly distributed," long, thin components, and gD was identified in "irregularly clustered" patches (33). gC components were widely distributed over the viral envelope, while gB and gD were in patches which were distinct from one another. The conclusion was that gB, gC, and gD are present in virions in three distinct structures. These investigators and others have pointed out that their findings were consistent with the lack of evidence for stable associations between or among these protein species (16, 20, 24). Subsequent examination of the structure of the envelope with chemical cross-linkers did identify gB multimers and dimers of gC (38). Other studies have identified a gH-gL oligomer which may be present in the virion (18). However, the concept of separate glycoprotein structures, without interaction among most of the heterologous HSV glycoproteins involved in entry, has remained: "Viral gB, gD, and gH function independently. . .and gB and gD (and also gC) are located on morphologically distinct spikes on the viral envelope." (8). Chemical cross-linking has been widely used to examine the oligomeric state of membrane glycoproteins in many other viral systems (4, 15, 36), and we decided to apply similar techniques to further investigate oligomerization of HSV glycoproteins.

Therefore, the long-term objectives of this project were twofold. First, we wanted to use cross-linking to identify potential associations between HSV glycoproteins in purified virions, particularly those which are important in virus entry. Second, we wanted to know what happened to those associations during entry. The first goal is addressed in this paper. The second will be addressed in an accompanying report. In the present study, we determined the molar amount and relative proportions of gB, gC, gD, and gH in two strains of HSV type 1 (HSV-1). We then used cross-linking reagents to covalently bond the nearest neighbor glycoproteins in purified virions. We used immunoprecipitation combined with Western blotting (immunoblotting) to assay for the presence of hetero-oligomers in HSV.

From our results, we calculate that gB, gC, gD, and gH are present in the molar ratio of 1:2:11:16 in HSV-1(KOS) and 1:1:14:9 in HSV-1(NS). We found that glycoproteins gB, gC, and gD could be cross-linked to themselves and to each other using an 11.4 Å (1Å = 0.1 nm) cross-linking reagent. Our results suggest that structural interactions can occur between gB, gC, and gD in the virion. We also detected gL in hetero-oligomeric complexes with gH and other glycoproteins; gH oligomers devoid of gL were not found.

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MATERIALS AND METHODS

Cells and virus. Human neuroblastoma-derived SY5Y cells (obtained from D. Pleasure) and primate Vero cells were propagated 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 (obtained from P. Schaffer) was propagated on Vero cells and titered by plaque assay on SY5Y cells. Purified HSV-1 strain NS was also propagated on Vero cells and was kindly provided by H. Friedman and J. Lubinski.

Purification of HSV-1(KOS). Roller bottles of Vero cells were infected at a multiplicity of infection of 0.1 PFU per cell, and extracellular virus was harvested from the growth medium and purified on sucrose-gradients by using a modification of several methods (1, 2, 9, 30). Briefly, cellular debris was removed by centrifugation at 8,000 × *g* for 10 min, and virus was then concentrated by pelleting at 100,000 × *g* for 1 h from the clarified medium through a 5% sucrose–phosphate-buffered saline (PBS) cushion. Virus pellets were resuspended overnight at 4°C in PBS and then sedimented at 30,000 × *g* for 5 h through a 10%–30%–60% sucrose-PBS step gradient. Purified virions were collected from the 30%–60% interface via side puncture, aliquoted, and stored at −80°C.

Virus particle counts. The particle-to-PFU ratio of purified HSV-1(KOS) was determined with the help of G. Maul, J. Weibel, and Q. Liu by using electron microscopy and the direct grid sedimentation method of particle counting (23). Briefly, copper mesh grids supported by 0.5% collodion films and 25-mm^2 filters (Millipore) were coated with polylysine. Virus suspensions of known titer were sedimented onto the grids by ultracentrifugation in a Beckman EM90 Airfuge rotor for 30 min at 120,000 × *g*. Filters were negatively stained by incubation in 2% phosphotungstic acid and directly examined by electron microscopy. Photographs of 20 representative fields were taken; virus particles were photographed at a magnification of ×21,640. The particle numbers were calculated according to the equation $N = V_f(D/0.1)(25/A)$, where V_f is the average number of particles per field, D is the dilution factor of the sample, and A is the surface area of the field. Most of the particles in the preparation appeared to be enveloped.

Glycoprotein quantitation. An immunoblot assay (7) was used to determine the amount and stoichiometry of gB, gC, gD, and gH in detergent extracts of purified HSV-1(KOS) and HSV-1(NS). The amount of each glycoprotein in the virion was determined by comparing the amount of viral protein with known amounts of glycoproteins which had been immunoaffinity purified from infected cell extracts (12, 13, 25). Virions were suspended in TSN buffer (0.01 M Tris [pH 7.3], 0.15 M NaCl, 0.1% Nonidet P-40 [NP-40]), and dilutions were spotted onto nitrocellulose membranes; four identical strips were made. Known quantities of purified gB-1, gC-1, gD-1, or gH-1, as determined by BCA protein assay (Pierce Chemical Co.) were spotted onto the strips as standards; protein standards were also diluted in TSN. The strips were then probed with the appropriate polyclonal antiserum (PAb): R69 (anti-gB), R46 (anti-gC), R1 (anti-gD), or R82 (anti-gH) (12, 19, 25). These antisera were checked against purified protein standards to ensure that no cross-reactivity existed. This was then followed by detection with ^{125}I-protein A (ICN Pharmaceuticals, Inc.) (7). The immunoblots were exposed to a Molecular Dynamics Storage Phosphor Screen, which was then analyzed, and the results were quantitated on the Molecular Dynamics PhosphorImager (Model no. 445-486) by using the ImageQuant software (version 2.00) (21). A standard curve was constructed relating the known amounts of protein (in nanograms) to the exposure of the phosphor plate. To calculate the amount of each viral protein present per PFU, the signal obtained for PAb binding to purified virus was compared with the standard curve. Nanograms of protein were converted to molar quantities on the basis of the molecular weight of each protein as calculated from the amino acid sequence; values of 97,019 (gB-1), 52,490 (gC-1), 40,854 (gD-1), and 88,394 kDa (gH-1) were used. This gave a mole/PFU ratio for each glycoprotein. The number of molecules per PFU was calculated from the mole/PFU ratio by using the constant 6.022×10^{23} (Avogadro's number, the number of molecules per mole of any substance). The particle-to-PFU ratio was used to determine the protein values per particle. Finally, these numbers were used to determine the stoichiometry of gB, gC, gD, and gH for each strain (the molar ratio).

Cross-linkers and the cross-linking reaction. 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 glyco*bis*(sulfosuccinimidylsuccinate) (Sulfo-EGS) are cleavable (via periodate or hydroxylamine treatment, respectively); *bis*(sulfosuccinimidyl)suberate (BS^3) is noncleavable. Purified virions were incubated with cross-linker at 4°C for 50 min; the cross-linking reaction was quenched with an equal volume of 1 M Tris-HCl (pH 7.0) for 1 min. Virus was sedimented at 100,000 × *g* for 1 h. Virus pellets were resuspended overnight at 4°C in ice-cold lysing buffer (0.02 M Tris [pH 7.5], 0.05 M NaCl, 0.5% NP-40, 0.5% sodium deoxycholate [DOC]) containing 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and then stored at −80°C until analyzed.

SDS-PAGE and Western immunoblotting. Virus extracts were mixed with electrophoresis sample buffer (final concentration = 100 mM Tris [pH 7.0], 20% glycerol, 3% sodium dodecyl sulfate [SDS], 0.002% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 3 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed in 4 to 12% polyacrylamide gradient Tris-glycine gels (Novel Experimental Technology) under reducing conditions. Proteins were electrophoretically transferred to nitrocellulose and probed with the appropriate antiserum; this was followed by ^{125}I-protein A.

Chemiluminescence. To improve the sensitivity of our detection of gH and gL, blots were stripped and reprobed either with PAbs R82 or R83 to gH or with monoclonal antibody (MAb) 8H4 to gL (kindly provided by G. Dubin [11]); this was followed by chemiluminescence (Amersham) to detect the glycoproteins.

Immunoprecipitation of cross-linked viral proteins. Cross-linked oligomers containing HSV glycoproteins were separated from solubilized virus preparations by immunoprecipitation first with PAb and then with protein A-Sepharose (25, 29). Briefly, 50 μl of extract containing cross-linked proteins was incubated in an equal volume of binding buffer (10 mM Tris [pH 8.0], 50 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.25% gelatin) with purified polyclonal immunoglobulin G (IgG) raised against HSV glycoproteins for 1 h at 4°C. Antigen-antibody complexes were then collected by incubation with 50 μl of protein A-Sepharose (50 mg/ml) for 1 h at 4°C; this was followed by centrifugation at 13,000 × *g* for 1 min. Antigen-antibody complexes were washed four times with high-salt buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.25% gelatin) to remove nonspecifically bound proteins, and pelleted by centrifugation at 13,000 × *g* for 1 min. The antigen-antibody complexes were disrupted by boiling the pellet in sample buffer for 3 min. Supernatants containing precipitated cross-linked proteins were recovered after centrifugation for 1 min, boiled for 3 min, and analyzed on SDS-PAGE. Western blots were probed with the PAb used for immunoprecipitation or a second heterologous PAb to identify associated glycoproteins. R69, R46, R1, and R82 or R83 were used to detect gB, gC, gD, and gH, respectively, on the Western blots, and MAb 8H4 was used to detect gL.

RESULTS

Quantity of glycoproteins in HSV. To examine the quantities of glycoproteins present in HSV virions, we compared the immunoreactivity of four individual glycoproteins in detergent-extracted, purified HSV with known quantities of glycoproteins purified from infected cells. We examined two virus strains, HSV-1(KOS) and HSV-1(NS). Dot blots were prepared in which twofold dilutions of purified virus in TSN buffer were spotted on nitrocellulose; the concentrations of KOS samples ranged from 2.7×10^3 to 2.8×10^6 PFU, while those of NS ranged from 1.2×10^3 to 1.2×10^6 PFU. Immunoaffinity-purified glycoproteins were diluted in TSN buffer and spotted onto nitrocellulose as standards. After incubation with the appropriate PAb and iodinated protein A, the blots were exposed to film (Fig. 1), and quantitated by phosphorimaging. Values from the virus dilutions were then compared with the protein standard curve to obtain the amount of protein (in nanograms) per PFU. This experiment was repeated three times for each virus, and the average of the three experiments is presented in Table 1. From these data and calculations, we have determined a gB/gC/gD/gH molar ratio for KOS of 1:2:11:16; the ratio for NS is 1:1:14:9. Both the molar ratios and the absolute amounts of each glycoprotein per PFU were similar for both virus strains. The number of particles for HSV-1(KOS) was determined by direct sedimentation and negative staining; this was followed by electron microscopy. The particle-to-PFU ratio for this virus was calculated to be 48:1; this agrees with previously reported ratios for purified KOS propagated in Vero cells (5). Thus, we calculate that there are approximately 3,200 molecules of gB per particle of KOS and a similar amount of gC (4,900 molecules) but considerably more gD and gH present in the viral envelope.

Examination of glycoprotein associations in the virion. Immunoelectron microscopy studies (33) have suggested that the envelope of HSV is relatively unorganized in structure. Spike structures characteristic of other viral systems were not detected, and instead, gB, gC, and gD were present in morphologically distinct "patches" over the virion surface. However, Zhu and Courtney (38), using chemical cross-linking, found dimers of gC, and oligomers of gB, in purified virions. These studies confirmed and extended those of Claesson-Welsh and Spear (6), who first reported gB oligomers in infected cells. We

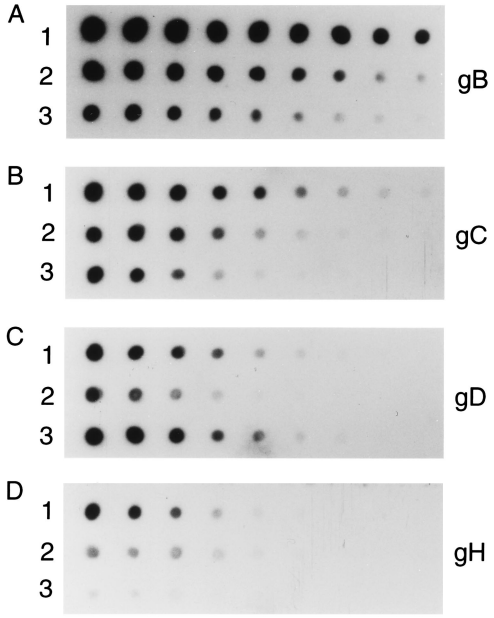

FIG. 1. Quantitation of HSV glycoprotein content. HSV-1 strains KOS and NS were purified on sucrose gradients and suspended in detergent buffer (NP-40). Twofold dilutions of purified glycoproteins gB, gC, gD, and gH were spotted on nitrocellulose and compared with twofold dilutions of virus. The amount of glycoprotein ranged between 0.07 and 18.4 ng for gB-1, 0.21 and 54 ng for gC-1, 0.16 and 41 ng for gD-1, and between 2.1 and 532 ng for gH-1. In each blot, row 1 contains the purified glycoprotein, while rows 2 and 3 contain dilutions of wild-type HSV-1(KOS) and (NS), respectively. Each blot was first reacted with the appropriate PAb and then with ^{125}I-protein A as follows: panel A, R69 (gB), panel B, R46 (gC), panel C, R1 (gD), and panel D, R82 (gH). Blots were exposed to film to obtain autoradiographs and then exposed to a Storage Phosphor Screen (Molecular Dynamics) to allow quantitation of the results via phosphorimaging (see Table 1).

examined the glycoprotein arrangement further, also testing the potential for gD and gH to exist as oligomers.

We found that cross-linker size and concentration were critical in the interpretation of our results. Here we document some studies which led to the conditions used.

(i) Effect of cross-linker size. We tested cross-linkers of several different cross-linking distances. The spanning arm of a cross-linker is the length between its two reactive end groups; the length affects the distance over which the reagent can establish a bond between two primary amines of closely associated proteins. Sulfo-DST (6.4 Å), BS^3 (11.4 Å), and Sulfo-EGS (16 Å) were first tested at two concentrations, 0.1 and 1 mM. HSV-1(KOS) (2×10^7 PFU) was incubated with cross-linker, the reactions were terminated, the virus was isolated by ultracentrifugation, and the proteins were solubilized in lysing buffer. Proteins were then electrophoresed on linear polyacrylamide gradient gels (4 to 12% polyacrylamide) under reducing conditions, which do not disrupt these cross-linkers, and transferred to nitrocellulose for Western blot analysis. Reactions with PAbs to gD-1, gB-1, and gC-1 are shown in Fig. 2. For gD, the 6.4 Å Sulfo-DST cross-linked a small proportion of monomer into a species of dimeric size at both concentrations (Fig. 2A, lanes 1 and 2). A series of higher-molecular-weight oligomers was obtained with BS^3 (11.4 Å) at 1 mM (Fig. 2A, lane 4). Sulfo-EGS (16 Å) cross-linked gD in a manner comparable to BS^3 (compare lanes 4 and 6). Similar results were obtained with gB (Fig. 2B) and gC (Fig. 2C); the greatest range of oligomers were cross-linked by BS^3 (11.4 Å) and Sulfo-EGS (16 Å) (Fig. 2A to C, lanes 4 and 6). We chose to use BS^3 (11.4 Å) for subsequent studies.

In these initial studies, we had difficulty detecting gH on Western blots. We subsequently found that a 50% increase in the amount of virus was required to detect this protein on

TABLE 1. Glycoprotein content of HSV-1 strains KOS and NS[a]

Strain	Amt purified glycoprotein[b] (ng/count)	Amt viral protein					Molar ratio[h]
		count/PFU[c]	ng/PFU[d]	mol/PFU[e]	Molecules/PFU[f]	Molecules/particle[g]	
KOS							
gB	3.2×10^{-5}	7.0×10^{-1}	2.5×10^{-5}	2.6×10^{-19}	1.5×10^{5}	3.2×10^{3}	1
gC	1.6×10^{-4}	1.3×10^{-1}	2.1×10^{-5}	3.9×10^{-19}	2.4×10^{5}	4.9×10^{3}	2
gD	3.1×10^{-4}	3.6×10^{-1}	1.1×10^{-4}	2.7×10^{-18}	1.6×10^{6}	3.4×10^{4}	11
gH	8.6×10^{-3}	4.3×10^{-2}	3.6×10^{-4}	4.1×10^{-18}	2.5×10^{6}	5.2×10^{4}	16
NS							
gB	3.2×10^{-5}	5.3×10^{-1}	1.7×10^{-5}	1.7×10^{-19}	1.0×10^{5}	ND[i]	1
gC	1.6×10^{-4}	6.2×10^{-2}	9.7×10^{-6}	1.8×10^{-19}	1.1×10^{5}	ND	1
gD	3.1×10^{-4}	3.2×10^{-1}	9.9×10^{-5}	2.4×10^{-18}	1.5×10^{6}	ND	14
gH	8.6×10^{-3}	1.7×10^{-2}	1.5×10^{-4}	1.6×10^{-18}	9.9×10^{5}	ND	9

[a] Purified HSV glycoproteins and detergent extracts of purified HSV-1(KOS) and HSV-1(NS) were analyzed by dot blot with the appropriate polyclonal antisera (see Fig. 1) and quantitated by phosphorimaging. The data represent averages of three separate experiments. The variation among experiments ranged from 10 to 30%.

[b] Standard curves relating the amount of glycoprotein to exposure of the phosphor plate (in counts) were used to calculate the nanograms of protein per count. See Materials and Methods for details.

[c] Data from the binding of PAbs to virion glycoproteins were plotted as number of counts per number of PFU. These values were used to calculate the results presented in the subsequent columns.

[d] To calculate nanograms per PFU, only values within the linear ranges of the standard curves were considered but at least five (and as many as nine) values were included in each calculation.

[e] To calculate moles of protein, molecular weights predicted from the amino acid sequence of each glycoprotein were used. For example, moles per PFU of gB in HSV-1(KOS) were computed by dividing the nanograms per PFU of gB by its molecular weight: $(2.5 \times 10^{-5})/(9.7 \times 10^{13}) = 2.6 \times 10^{-19}$ mol/PFU.

[f] Avogadro's number (6.022×10^{23}) was used to convert moles per PFU to molecules per PFU. For example, molecules per PFU of gB were calculated by multiplying the moles per PFU by Avogadro's number: $(2.6 \times 10^{-19}) \times (6.022 \times 10^{23}) = 1.5 \times 10^{5}$ molecules/PFU.

[g] To determine molecules per particle, we determined the particle number for purified HSV-1(KOS) by electron microscopy. This yielded a particle/PFU ratio of 48:1.

[h] Molar ratios were determined by setting the number of molecules of gB per PFU in each preparation equal to 1. The other three glycoprotein values were then determined in proportion to gB.

[i] ND, not done.

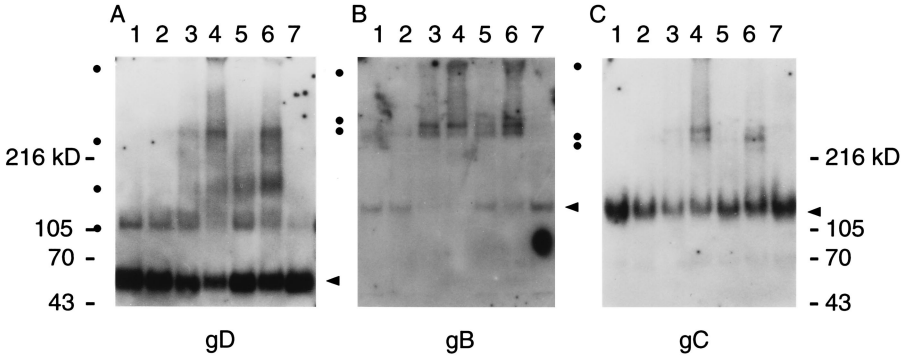

FIG. 2. Effect of cross-linker reagent size on cross-linking of glycoproteins in HSV. Purified HSV-1(KOS) was incubated for 50 min at 4°C with three cross-linkers with lengths of 6.4 Å (Sulfo-DST), 11.4 Å (BS^3), and 16 Å (Sulfo-EGS). Each cross-linker was tested at two concentrations, 0.1 and 1 mM. Samples of the virion glycoproteins were separated on denaturing SDS–4 to 12% PAGE gels; this was followed by Western blotting. Blots were probed with PAbs to gD-1 (A), gB-1 (B), and gC-1 (C); this was followed by incubation with ^{125}I-protein A. Lanes 1, 0.1 mM Sulfo-DST; lanes 2, 1 mM Sulfo-DST; lanes 3, 0.1 mM BS^3; lanes 4, 1 mM BS^3; lanes 5, 0.1 mM Sulfo-EGS; lanes 6, 1 mM Sulfo-EGS; lanes 7, mock-cross-linked HSV (no cross-linker). The positions of monomer migrations are indicated by arrows. Higher-molecular-weight forms are indicated by bullets. Molecular weight markers are at left of panel A and at right of panel C.

Western blots with R82. Further, a switch to chemiluminescence detection was ultimately necessary to reliably find gH oligomers. Since gH seems to be present in the same molar ratio as gD, these results highlight the limitations of the immunologic reagents available to detect gH. Experiments using ^{125}I-protein A to detect the antigen-antibody complexes will be described first, and the higher sensitivity chemiluminescence assay for gH-gL will be described separately.

(ii) Effect of cross-linker concentration on oligomer formation. Ideally, the concentration of cross-linking reagents is chosen according to the amount of each protein to be linked, but because of the complexity of this system, we had to empirically determine the optimal reagent concentration. Cross-linking was performed with concentrations of BS^3 (11.4 Å) from 31.25 μM to 2 mM. Fig. 3A to C show that, first, only monomers were detected in the absence of cross-linker (lanes 8), and second, increasing the concentration of BS^3 resulted in a decrease in the amount of monomeric gD, gB, or gC, with a concomitant increase in oligomers (lanes 1 to 7). By increasing the amount of virus used to 6.3×10^6 PFU, we were able to detect gH (Fig. 3D). The amount of monomeric gH decreased with increasing amounts of BS^3. However, no oligomers were detected by using this technique. We chose 0.25 mM BS^3 (Fig. 3, lanes 4) for subsequent experiments, since a wide range of cross-linked species could be identified at this concentration.

Examination of glycoprotein oligomers. The multimeric species we detected (Fig. 2 and 3) could represent (i) homo-oligomers, such as dimers, trimers, or higher order oligomers of a single glycoprotein and/or (ii) hetero-oligomers composed of different glycoproteins. To determine if hetero-oligomers are present, we performed immunoprecipitations on detergent extracts of cross-linked virus; this was followed by SDS-PAGE and Western blotting. By immunoprecipitating with one antibody (e.g., against gD) and then probing the Western blot with a second (e.g., against gB), we would be able to detect hetero-oligomers (in this example, gB-gD).

(i) Immunoprecipitation of gD-gB and gD-gC hetero-oligomers. Anti-gD-1 IgG was used to immunoprecipitate gD-1 from detergent extracts of virus cross-linked with 0.25 mM BS^3. The oligomers were separated on denaturing SDS-PAGE, Western blotted, and probed separately for gD, gB, and gC. The same set of eight samples was run in lanes 1 to 8 of each gel (Fig. 4A to C). Lanes 1 to 4 contain the immunoprecipitated proteins. Lane 1 contains protein precipitated by anti-gD IgG; it should contain gD monomer and oligomers. Lane 2 represents the same extract, incubated instead with preimmune IgG; it should contain no gD. Lane 3 is mock-cross-linked HSV; only monomeric gD should be present. Lane 4 is the preimmune IgG immunoprecipitation of the lane 3 extract. Lanes 5 to 8 contain those glycoproteins remaining in the extracts of lanes 1 to 4 after immunoprecipitation. Lane 5 should not contain gD if all of the gD was precipitated but other HSV glycoproteins should remain. All HSV glycopro-

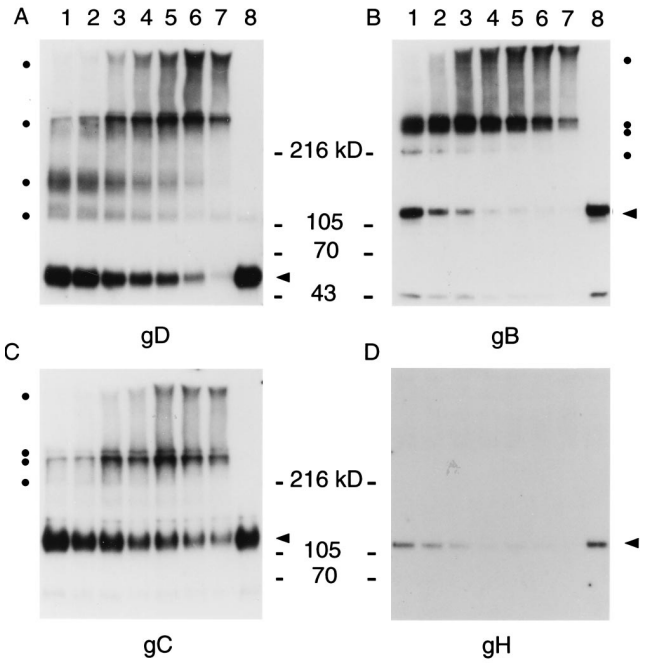

FIG. 3. Effect of cross-linker concentration on cross-linking of glycoproteins in HSV virions. A total of 6.3×10^7 PFU of purified HSV-1(KOS) was incubated with various amounts of BS^3; concentrations used were 31.25 μM (lanes 1), 62.5 μM (lanes 2), 0.125 mM (lanes 3), 0.25 mM (lanes 4), 0.5 mM (lanes 5), 1 mM (lanes 6), and 2 mM (lanes 7). Mock-cross-linked samples incubated without cross-linker are shown in lanes 8. Samples of the virion glycoproteins were then analyzed on denaturing SDS–4 to 12% PAGE gels and Western blots. Detection with antisera to gD-1 (A), gB-1 (B), gC-1 (C), and gH-1 (D) is shown. The positions of monomer migrations are indicated by arrows. Higher-molecular-weight forms are indicated by bullets. Molecular weight markers are between panels A and B and panels C and D, respectively.

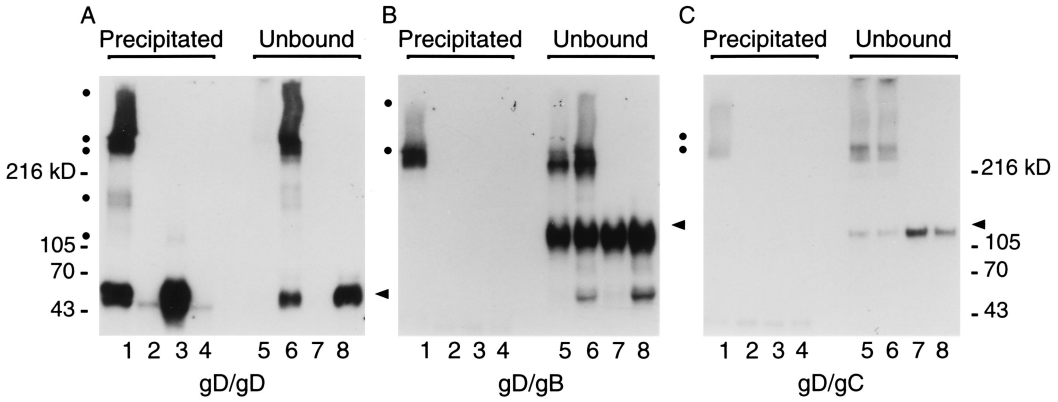

FIG. 4. Detection of gD-gB and gD-gC hetero-oligomers in purified HSV virions. HSV-1(KOS) was incubated with BS^3, or with Hanks balanced salt solution alone, for 50 min at 4°C. Extracts of virus samples were immunoprecipitated with antiserum to gD-1 (R1) or with a preimmune antiserum. Precipitated proteins (Precipitated) or unprecipitated fractions (Unbound) were separated on denaturing SDS–4 to 12% PAGE gels, and Western blots were probed for gD-1 (A), gB-1 (B), or gC-1 (C); this was followed by incubation with ^{125}I-protein A. Lanes 1, R1 precipitation of cross-linked virus; lanes 2, preimmune precipitation of cross-linked virus; lanes 3, R1 precipitation of mock-cross-linked virus; lanes 4, preimmune precipitation of mock-cross-linked virus. Lanes 5 to 8 represent the proteins remaining in the supernatants of lanes 1 to 4. The positions of the glycoprotein monomers are denoted by arrows. Positions of oligomers are indicated by bullets. Molecular weight markers are at left of panel A and at right of panel C.

teins should be left in lane 6, since the preimmune IgG (lane 2) should not have immunoprecipitated any HSV glycoproteins. Lanes 7 and 8 contain the proteins remaining in mock-cross-linked virus extracts after R1 and preimmune immunoprecipitation, respectively. Lane 7 should contain gB and gC monomers, but no gD. Lane 8 should contain monomers of all glycoproteins.

Several high-molecular-weight species containing gD were immunoprecipitated by R1 and recognized by R1 Western blotting (Fig. 4A, lane 1). The band pattern matches that seen by Western blotting alone (Fig. 3A, lane 4). Preimmune IgG did not precipitate any gD oligomers (Fig. 4A, lane 2). Immunoprecipitation of mock-cross-linked virus (Fig. 4A, lanes 3) demonstrates that only gD monomer was present. All of the gD originally present in the extract was immunoprecipitated by R1 (compare lanes 5 and 7 with lanes 1 and 3). gD monomers and/or oligomers were still present after treatment with preimmune IgG (lanes 6 and 8). We conclude that gD was cross-linked into several oligomers of various sizes, which could be detected with this procedure. We then tested whether any of the gD oligomers contained other glycoproteins.

As shown in Fig. 4B, we probed for gB with R69 anti-gB serum. Lane 1 shows the presence of gB oligomers in the gD-precipitated fraction. Thus, in the virion, a proportion of gD and gB are within 11.4 Å of one another. Lane 5 shows that not all of the cross-linked gB oligomers were precipitated by anti-gD IgG. We believe these gB oligomers did not contain gD since all of the gD present in the viral lysate was immunoprecipitated by R1 (Fig. 4A, compare lanes 1 and 5). Thus, not all gB could be covalently cross-linked to gD, even though 15-fold more gD was present in virions (Table 1). Lanes 5 and 7 also contained gB monomer not precipitated by R1. These controls exclude the possibility of cross-reactivity of the anti-gD IgG (R1) with gB.

A proportion of gC was also present in hetero-oligomers immunoprecipitated by anti-gD R1 IgG (Fig. 4C, lane 1). As before, virus was cross-linked and immunoprecipitated with anti-gD IgG or with preimmune IgG, and blots were probed with R46 anti-gC serum. Two high-molecular-weight bands were recognized by R46 in the R1 immunoprecipitation lane (lane 1). These bands contain hetero-oligomers of gD and gC. The remaining controls in lanes 2 to 4 were all negative. A large proportion of gC, both monomers and oligomers, did not contain gD (lane 5). Moreover, monomeric gC remained in the unbound fraction (Fig. 4C, lanes 5 and 7), so recognition of gC by R1 serum was not occurring.

In an effort to detect gH, we increased the amount of virus to 7×10^6 PFU. However, no gD-gH oligomers were detected in R1 immunoprecipitates by using ^{125}I-protein A detection (data not shown).

(ii) Immunoprecipitation of gB-gD and gB-gC hetero-oligomers. To confirm the gD-gB interaction, and to further investigate gB oligomer formation, anti-gB IgG was used. gB oligomers were immunoprecipitated by R69 IgG and the Western blot was probed by R69 (Fig. 5A, lane 1). This precipitation was specific (compare lanes 1 and 2), and oligomers were only found after virus was treated with BS^3 (compare lanes 1 and 3).

When probed for the presence of gD, R1 identified hetero-oligomers of gB and gD (Fig. 5B, lane 1). These hetero-oligomers represented only a portion of the gD oligomers in the extract (compare lanes 4 and 1). Thus, the results of this experiment (Fig. 5B, lane 1) were similar to those shown in the converse experiment where R1 IgG was used for immunoprecipitation and R69 serum for Western blotting (Fig. 4B, lane 1). Thus a proportion of gD and gB exists in a hetero-oligomeric arrangement in the virion.

When we probed the Western blot with R46 PAb, gB-gC hetero-oligomers were seen (Fig. 5C, lane 1). Most of the oligomeric gC remained unbound after incubation with R69 (lane 4). Preimmune IgG (lanes 2 and 5) did not precipitate gC. Since monomeric gC was not precipitated by R69 (lane 3), no cross-reactivity exists between gC and the anti-gB IgG.

To detect gB-gH hetero-oligomers (Fig. 5D), we probed a Western blot of identical samples with R82 anti-gH serum. gH monomer was detected, but no oligomers were seen (data not shown).

Detection of gH and gL oligomers in HSV-1. In initial experiments, we expected to find a cross-linked complex of gH and gL in purified virus, since these two proteins are associated in the virus and in virus-infected cells as a heterodimer (11, 18, 27). However, we found that our reagents for detecting the gH in a gH-gL complex were less sensitive than antibodies for the other glycoproteins. Moreover, the gH antiserum R82 was not as effective for detecting gH on a Western blot of a denaturing

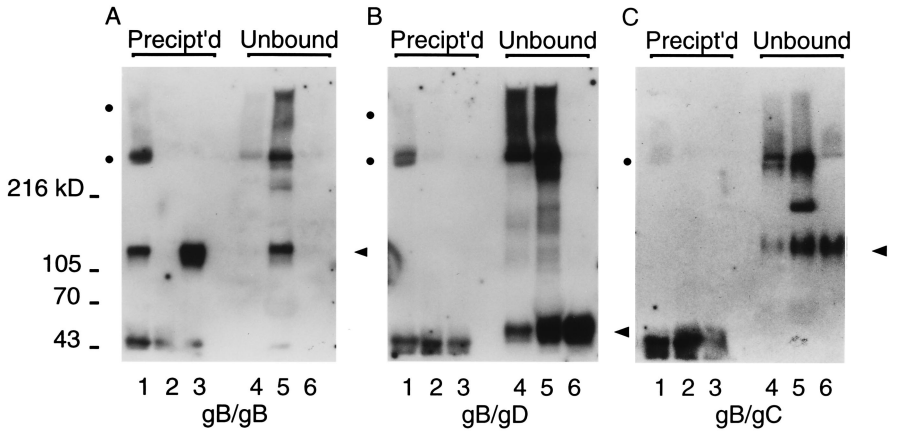

FIG. 5. Detection of gB-gD and gB-gC hetero-oligomers in purified HSV virions. Sucrose-gradient purified HSV-1(KOS) was incubated with BS^3, or with Hanks balanced salt solution alone, for 50 min at 4°C. Extracts of virus samples were immunoprecipitated with antiserum R69 against gB-1 or with a preimmune antiserum. Precipitated proteins (Precipt'd) or unprecipitated fractions (Unbound) were separated on denaturing SDS–4 to 12% PAGE gels, and Western blots were probed for gB-1 (A), gD-1 (B), or gC-1 (C); this was followed by incubation with ^{125}I-protein A. Lanes 1, R69 precipitation of cross-linked virus; lanes 2, preimmune precipitation of cross-linked virus; lanes 3, R69 precipitation of mock-cross-linked virus. Lanes 4 to 6 represent the proteins not precipitated from the extract supernatants of lanes 1 to 3. The positions of the glycoprotein monomers are denoted by arrows. Positions of oligomers are indicated by bullets. Molecular weight markers are at left of panel A.

gel when visualized by ^{125}I-protein A. After the original study was completed, we approached this problem in a different fashion. First, we switched to chemiluminescence for detection, and second, we used a different gH anti-serum (R83) because it recognized gH more effectively by Western blot. We stripped the antibody from the Western blots that we had already probed with other antibodies, and then reprobed them with R83 antiserum. Lastly, we used a gL MAb (8H4) which recognizes a linear epitope, to reexamine the Western blots for gL oligomers. A composite of the results is shown in Fig. 6. These experiments were designed to optimize cross-linker concentration and virus multiplicity.

At concentrations of BS^3 ranging from 0.25 mM to 2 mM, four gH-specific bands were detected in virions with R83: a 110-kDa gH monomer band, a 140-kDa band, and a doublet migrating above the 216-kDa marker (Fig. 6A, lanes 1 to 4). Thus, gH was present in three separate-sized species. Uncross-linked virus contained only the gH monomer (Fig. 6A, lane 5). When the blot was reprobed with MAb 8H4 (Fig. 6B, lanes 1 to 4), gL was present in the 140-kDa band and in the two complexes above 216 kDa. The antibody did not react with the 110-kDa band seen in Fig. 6A (lane 5), confirming that this is gH monomer. Monomeric gL was not detected on this blot because it ran off the gel. In a second experiment comparing different concentrations of virus (Fig. 6C), gH was found as a 110-kDa band and in the >216-kDa doublet. When this blot

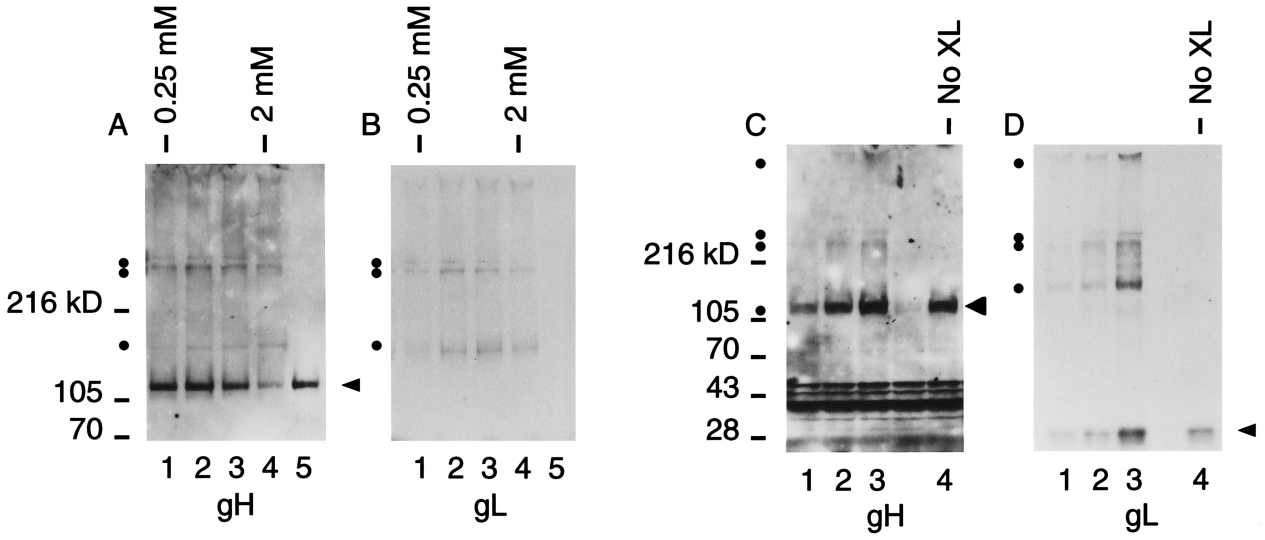

FIG. 6. Detection of gH and gL oligomers by chemiluminescence. In the first experiment (A and B), purified HSV-1(KOS) was incubated with various amounts of BS^3; concentrations used were 0.25 mM (lanes 1), 0.5 mM (lanes 2), 1 mM (lanes 3), and 2 mM (lanes 4). Mock-cross-linked samples incubated with no cross-linker are shown in lanes 5. In the second experiment (C and D), various amounts of virus were cross-linked with 0.25 mM BS^3; amounts used were 6.3×10^7 PFU (lanes 1), 1.3×10^8 PFU (lanes 2), and 3.2×10^8 PFU (lanes 3). Mock-cross-linked samples incubated with no cross-linker are shown in lanes 4. Samples of the virion glycoproteins were analyzed on denaturing SDS–4 to 12% PAGE gels and Western blots. Blots were stripped and reprobed with antisera to gH-1 (panels A and C) and gL-1 (panels B and D). The positions of the glycoprotein monomers are denoted by arrows. Positions of oligomers are indicated by bullets. Molecular weight markers are at left of panels A and C.

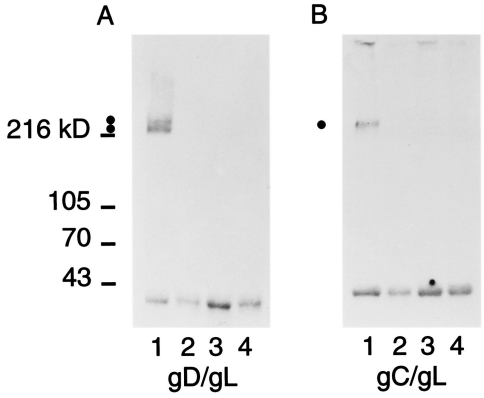

FIG. 7. Detection of gD-gL and gC-gL hetero-oligomers in purified HSV virions by chemiluminescence. HSV-1(KOS) was incubated with BS^3, or with Hanks balanced salt solution alone, for 50 min at 4°C. Extracts of virus samples were immunoprecipitated with antiserum to gD-1 (R1) (A), or gC-1 (R46) (B) or with a preimmune antiserum. Precipitated proteins were separated on denaturing SDS–4 to 12% PAGE gels, and Western blots were stripped and reprobed for gL. Lanes 1, antisera precipitation of cross-linked virus; lanes 2, preimmune precipitation of cross-linked virus; lanes 3, antisera precipitation of mock-cross-linked virus; lanes 4, preimmune precipitation of mock-cross-linked virus. Positions of oligomers are indicated by bullets. Molecular weight markers are at left of panel A.

was reprobed with MAb 8H4 (Fig. 6D), gL was detected in a 140-kDa band, in the >216-kDa doublet, and in a monomeric gL band of 30 kDa. We speculate that the 140-kDa species is a hetero-oligomer consisting of one molecule each of gH and gL. At the highest virus multiplicity (Fig. 6D, lane 3), there was also reactivity with a 110-kDa band that comigrated with gH monomer. For both gH and gL, only a proportion of the total monomers were cross-linked in oligomeric form, regardless of cross-linker concentration or virus multiplicity, as evidenced by the residual amounts of the corresponding monomers. However, this study tells us that we were working below the optimal range of detection for gH and gL.

Next we reexamined the immunoprecipitation-Western blotting experiments done to detect hetero-oligomers. Again, the original blots were stripped and reprobed first with R83 and then with 8H4 (Fig. 7). Both blots showed reactions with MAb 8H4, but no reactions were detected with R83. When gD oligomers were immunoprecipitated with R1 and then probed with 8H4 for gL (Fig. 7A, lane 1), gD-gL hetero-oligomers were detected in a doublet of bands migrating above 216 kDa. A gC-gL hetero-oligomer band larger than 216 kDa was also detected (Fig. 7B, lane 1). Interestingly, no hetero-oligomers of gB-gL were detected (data not shown). Although R83 failed to detect hetero-oligomers of gH in these Western blots, it is likely that hetero-oligomers which contained gL also contained gH, since these two proteins were shown to complex with each other in previous experiments (Fig. 6).

DISCUSSION

Few studies have addressed the organization of glycoproteins in the HSV envelope. Immunoelectron microscopy studies showed little if any organization or association among the virion glycoproteins (33). Subsequent studies using purified virus, however, clearly demonstrated that gB and gC could each be detected directly on nondenaturing gels as oligomers or cross-linked into homodimeric and higher order oligomeric forms (6, 38, 39). Our objective was to advance the understanding of HSV virion envelope structure and determine if any associations occurred between the viral glycoproteins which might be important for entry into cells. To do this, we examined the content of gB, gC, gD, gH, and gL in purified virus and then performed cross-linking studies to determine if there were associations among these five glycoproteins.

How much glycoprotein is present in the virus? Knowing the amount of glycoprotein present is important for both a structural understanding and to gain insight into possible functional roles for the membrane proteins in infection. We used purified virus of two HSV-1 strains to estimate the quantities of gB-1, gC-1, gD-1, and gH-1. The molar ratios of these four were 1:2:11:16 for the KOS strain and 1:2:14:9 for NS. Since purified gL was unavailable to use as a standard to quantitate gL in the virion, we suggest that there is a 1:1 correspondence between gH and gL because of (i) the 140-kDa band (Fig. 6), which likely contains equimolar amounts of each protein, and (ii) the work of Hutchinson et al. (18), which originally suggested a 1:1 relationship in infected cells and probably in virions. Therefore, we propose that the molar ratio of gB/gC/gD/gH:gL in the KOS strain is 1:2:11:16:16. The observation that two different virus strains, which were grown in different cell lines and purified in two separate labs, should yield similar glycoprotein ratios implies that those proportions are reasonable estimates. Secondly, it implies that this molar ratio has some intrinsic importance. At least, it is improbable that the amount of each glycoprotein incorporated into virions during maturation is random. Assembly and acquisition of the envelope glycoproteins are more likely to be a well-orchestrated event, as has been suggested previously (35). On the basis of our calculations, the total number of glycoprotein molecules in the envelope probably exceeds 100,000, since the sum of gB, gC, gD, and gH content already equals 9.4×10^4 molecules. There is only a limited amount of information available regarding the stoichiometry or amounts of glycoproteins in enveloped viruses. However, in the case of Sindbis virus, 240 copies of each of the two glycoproteins, E1 and E2, which comprise the virion spikes, are present in the envelope, as determined by cryoelectron microscopy studies (14). Sindbis virus is a member of the alphavirus family, which are isometric enveloped RNA viruses which are considerably smaller in size than herpesviruses. The icosohedral shape of the nucleocapsid of Sindbis virus makes it somewhat analogous to herpesviruses but smaller in size and without the tegument. The surface area of the HSV envelope is approximately 40 times greater than that of Sindbis virus. If E1 and E2 were present in the same ordered way on a virus particle the size of HSV, one would expect there to be approximately 10,000 copies of each of these proteins in the envelope. Thus, it is possible that the envelope of HSV contains a somewhat greater total concentration of glycoprotein molecules than are present on alphavirus envelopes, although the amounts of individual glycoproteins such as gD are in similar proportions to E1 and E2.

One surprising result is the large amount of gH relative to the other three glycoproteins. Previous impressions were that this protein was less abundant in the virion than the other glycoproteins (3, 27). In the present study, R82 was used to detect gH. We determined that it was significantly less reactive when used to detect denatured gH. Thus, this rabbit PAb (prepared to immunopurified gH) detects mostly conformational epitopes. The quantitation studies were done with a detergent (NP-40) which does not markedly alter native conformation, and by immunodot blot, we were able to detect gH by using as little as 8.8×10^4 PFU of virus. In the subsequent Western blot analysis, the amount of virus analyzed exceeded this minimum (ranging from 2×10^6 to 6.3×10^6 PFU). However, these samples were run under denaturing conditions,

with SDS, reducing agents, and boiling. Thus, at least 6.3×10^6 PFU of HSV was necessary to detect gH monomer on a Western blot of a denaturing gel (Fig. 3D); gH was much more difficult to detect when less virus was used (Fig. 2). However, even when gH was present in sufficient amounts to detect the monomer, we were unable to detect higher-molecular-weight oligomers. It was possible that R82 does not detect gH in such complexes or that the fraction of gH in complexes was still below the threshold necessary to be detected by R82. This result was quite unsatisfying, so we intensified our efforts to find gH and also enlisted a recently obtained MAb to gL (8H4) to aid our efforts.

Examination of glycoprotein associations in the virion. We chose the BS^3 cross-linker (11.4 Å) at a particular concentration (0.25 mM) to study the possible interactions between the glycoproteins. We found that these conditions resulted in a wide range of interactions and identified different-sized oligomers. While we cannot exclude the possibility of intervirion cross-linking, we believe that the use of purified virus and low concentrations of cross-linker favor oligomer formation within single virus particles. It is important to bear in mind that increasing the concentration of BS^3 above 1 mM drove virtually all of the monomers and oligomers into very large oligomers. We also found that the distance between neighboring molecules was greater than 6.4 Å, since Sulfo-DST failed to significantly cross-link gB, gC, or gD. And while Sulfo-EGS, at 16 Å, seemed to cross-link just as well as BS^3, use of these longer cross-linkers has been found to result in a greater number of nonspecific reactions (34). We therefore settled on BS^3 at 0.25 mM.

Our examination of protein associations in the presence and absence of cross-linkers confirms prior studies which detected gB (6) and gC in high-molecular-weight species in the virion (38, 39). Furthermore, it extends our understanding of gD and gH-gL. For example, several gD oligomeric species were detected by cross-linking. This is the first demonstration of dimeric- and trimeric-sized oligomers of this protein in purified virions, and it confirms earlier predictions of the ability of gD to form higher-molecular-weight complexes (13). Moreover, this study shows that a proportion of oligomeric cross-linked gD also contains gB and gC. Initially, no oligomers of gH were detected. But the existence of gH associations was suggested by the reduction in the amount of gH monomer that was detected after cross-linking with increasing amounts of BS^3. Different antibodies and the more sensitive detection afforded by chemiluminescence allowed us to pursue this observation.

It is well established that gH associates with gL as a hetero-oligomer in HSV-infected cells and in transfected cells (11, 18, 27). Our data confirm this association. We show that gH can be cross-linked with gL in virions, both as a 140-kDa complex and in higher-molecular-weight species.

Cross-linking also stabilized homo-oligomers of gD and gB. Two gD oligomers, migrating between 105 and 216 kDa, are likely to be, from their size, a homodimer and homotrimer, respectively (Fig. 3A), since we could not detect hetero-oligomers of gD below 216 kDa. Previous work from our laboratory indicated that gD might exist as a dimer (13). It has also been previously demonstrated that gB forms a noncovalently bound, detergent-stable, heat-dissociable dimer and higher-molecular-weight oligomers (6, 28, 31). We detected a dimer-sized cross-linked species of gB which is likely to be this homodimer, as well as larger complexes.

Hetero-oligomers of glycoproteins in HSV virions. One question raised by previous immunoelectron microscopy studies (33) relates to whether organized complexes of the essential viral glycoproteins exist. These complexes might come together to form a functional unit, as proposed by White (37). We found that a proportion of gB, gC, gD, and gH-gL can be found within 11.4 Å of each other. We hypothesize that these close associations may have functional significance, in that they might form a fusion complex. Supporting evidence for glycoprotein associations within the herpesvirus envelope has been reported for pseudorabies virus. Mettenleiter et al. (22) reported that gB-gC associations may occur in pseudorabies virus, as seen by co-immunoprecipitations of the two glycoproteins gII (gB) and gIII (gC). Attempts to directly identify a complex containing more than two proteins, by using sequential immunoprecipitation, gave inconclusive results (data not shown). In this regard, it is interesting to note that gL could be cross-linked to gC or gD but not to gB. This could reflect an organization which precludes associations of gH-gL with gB. Further experiments will be needed to explore these possibilities. In light of cooperative models of viral glycoprotein function (37), it is also of interest to determine the fate of the glycoprotein complexes during HSV infection of cells. Experiments to investigate HSV-1 penetration, and to determine if the structural glycoprotein interactions documented here play a role during viral entry, are described elsewhere (17).

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