Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II

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The structure and heparin binding properties of a family of human cytomegalovirus (HCMV) disulphide-linked glycoprotein complexes designated gC-II were analysed. gC-II complexes contain two groups of glycoproteins designated Group 1 and Group 2. These glycoproteins were separated from each other by short exposure of virions to a reducing agent. This showed that the disulphide bonds between these glycoproteins were on the external surface of the virion. Although these glycoproteins were no longer associated they were not released from the virion, suggesting that they were transmembrane glycoproteins. Approximately 75 to 90% of the gC-II complexes and 18% of the complexes containing the HCMV gB glycoprotein obtained from the virion envelope bound immobilized heparin. When virions were incubated with [3H]heparin, gC-II complexes bound more heparin than gB complexes, by approximately threefold. These data showed that gC-II complexes had a greater heparin-binding capacity. After treatment of virions with a reducing agent the affinity of gC-II glycoproteins for heparin was greatly reduced whereas the affinity of gB glycoproteins was only slightly reduced. Thus, higher order structure was important for heparin binding by gC-II complexes but not by those of gB. Relative to gC-II Group 1 glycoproteins, a greater portion of gC-II Group 2 glycoproteins still bound to heparin after reduction, suggesting that Group 2 glycoproteins may be the important heparin binding component of the gC-II complexes. Both gB and gC-II complexes were eluted from immobilized heparin with soluble heparin or 0.65 M-NaCl suggesting that both formed ionic bonds with heparin. Chondroitin sulphate was not effective at eluting HCMV envelope glycoproteins from immobilized heparin. Thus, the structure of the glucosaminoglycan backbone is important to the binding of HCMV glycoproteins to heparin.

Introduction

The initial interaction of herpesviruses with the cell surface is mediated by glycosaminoglycans (GAGs) on the proteoglycans in the plasma membrane of the host cell. These GAGs are structurally similar to heparin, and therefore soluble heparin can prevent herpesviruses from attaching to the host cell. These herpesviruses include human cytomegalovirus (HCMV) (Kari & Gehrz, 1992; Neyts et al., 1992), herpes simplex virus types 1 and 2 (HSV-1 and -2) (Shieh et al., 1992; Herold et al., 1991; WuDunn & Spear, 1989), bovine herpesvirus 1 (BHV-1) (Okazaki et al., 1991), and pseudorabies virus (PRV) (Mettenleiter et al., 1990). The envelope glycoproteins gB and gC in HSV (Herold et al., 1991), glycoproteins gII and gIII in PRV (Mettenleiter et al., 1990) and gI in BHV-1 (Okazaki et al., 1991) all have affinity for immobilized heparin. Because of this, they may all interact with the host cell GAGs. However, gC in HSV and gIII in BHV-1 and PRV appear to play a principal role in binding of the virion to the host cell. The PRV gIII glycoprotein is homologous to gB of HSV, and gIII glycoproteins of BHV-1 and PRV are homologues of the gC glycoprotein of HSV (Fitzpatrick et al., 1989; Robbins et al., 1986). In HSV it has been shown that the interaction of the virion with the cell surface proteoglycans is dependent on the size of the GAG chain and the sulphate content of the GAG (Lycke et al., 1991). Thus, an important component of heparin–virion binding is the formation of ionic bonds. However, the structure of the carbohydrate backbone of the host cell GAG also plays a role as HSV binding was observed using heparan sulphate containing 1.5 sulphate groups/disaccharide unit but not with dermatan sulphate containing 1.3 sulphate groups/disaccharide unit (Lycke et al., 1991). The PRV gIII glycoprotein also contains an arginine-rich region near the amino terminus (Robbins et al., 1986). This domain may be responsible for the binding of gIII to heparin through ionic bonds.

Previously we have shown that HCMV envelope glycoproteins have affinity for immobilized heparin (Kari & Gehrz, 1992). One of these glycoproteins has been variably designated gp55-116 (Britt & Auger, 1986), glycoprotein complex I (gCI) (Gretch et al., 1988a) or gB
This glycoprotein has homology to the HSV gB glycoprotein (Cranage et al., 1986). The other heparin-binding envelope component that we detected has been designated glycoprotein complex II (gC-II) (Gretch et al., 1988b). gC-II complexes contain two groups of distinct glycoproteins designated Group 1 and Group 2. These glycoproteins were placed in groups on the basis of their reactivity with monoclonal antibodies (MAbs) and their biochemical characteristics (Kari et al., 1990b). It is not known whether gC-II complexes contain glycoproteins with homology to other herpesvirus glycoproteins. Analysis of the [3H]glucosamine ([3H]GlcN)-labelled glycoproteins that bound heparin showed that most of the gC-II complexes in a virion extract bound heparin. We also found that gC-II represented a large portion of the total number of [3H]GlcN-labelled proteins in the heparin-binding fraction.

In the present study we have further examined the structure and composition of gC-II and examined the influence of structure on the ability of gC-II to bind immobilized heparin. In some cases parallel studies were done with the HCMV gB glycoprotein. It was determined that both gB and gC-II bind to immobilized heparin by ionic bonds. Anions that released gB and gC-II from immobilized heparin included high NaCl concentrations and soluble heparin. Other polyanions such as chondroitin sulphate were not effective in releasing gB or gC-II from immobilized heparin. The binding of gC-II to immobilized heparin depended on structures maintained by disulphide bonds. In contrast, the binding of gB complexes to heparin was not greatly affected by reduction of disulphide bonds.

**Methods**

**Virus and cells.** Towne strain HCMV was grown in skin fibroblast cells as reported (Kari et al., 1986). Viral proteins were radiolabelled by incubating infected cells with [3H]Ser, [3H]Arg or [3H]GlcN (New England Nuclear) as described (Kari et al., 1986). Virions were purified from culture media by differential centrifugation.

**MAbs.** The two groups of gC-II-specific MAbs used in this study have been described (Kari et al., 1990b). The Group 1 MAbs used were 8B4 and 9E10 and the Group 2 MAbs were 27B4 and 15F9. The gB MAbs used were 41C2 and 9B7 (Lussenhop et al., 1986).

**Extraction of viral glycoproteins and immunoprecipitation with MAbs.** Viral glycoproteins were extracted from virions using a Tris buffer (50 mM-Tris-HCl pH 7.4, 150 mM-NaCl, 2 mM-PMSF) containing 10% NP40. Sometimes the following protease inhibitors (Boehringer Mannheim Biochemical) were added: aprotinin (2 mg/ml), disodium EDTA (0.2 mg/ml), leupeptin (0.5 mg/ml) and pepstatin (0.7 mg/ml). Extraction was carried out for 1 h with constant mixing and insoluble material was removed by centrifugation. Extracts were used directly for immunoprecipitations as described (Kari et al., 1986).

**SDS-PAGE, detection of radiolabelled proteins and Western blotting.** SDS-PAGE was done with 9.0% polyacrylamide gels using a Bio-Rad mini-gel system following the method of Laemmli (1970). Radiolabelled proteins were detected by fluorography using Enhance (New England Nuclear). Western blotting was done as described (Lussenhop et al., 1988).

**Lectin analysis.** A Glycan Differentiation Kit (Boehringer Mannheim) was used to analyse glycoforms of gC-II glycoproteins. Briefly, gC-II glycoproteins were blotted onto nitrocellulose paper. The paper was cut into strips and the strips were incubated with digoxigenin-labelled lectins. After washing, the strips were incubated with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase. The strips were washed and colour was developed according to the manufacturer’s instructions.

**DTT reduction of virions.** A method was used which has previously been used to reduce disulphide bonds in gB complexes in the envelope of HCMV virions (Kari et al., 1990a). Briefly, aliquots of purified [3H]GlcN-labelled virions were suspended in PBS (0.01M-sodium phosphate, 30 mM-KCl, 150 mM-NaCl, pH 7-4) containing 50 mM-DTT and allowed to react at room temperature for 30 and 120 min with constant mixing. Excess iodoacetamide was added, the reaction was left for 30 min and virions were collected by centrifugation. Virions were washed and then extracted with NP40 as described above. Extracts were used for immunoprecipitations using gC-II-specific MAbs.

**Two-dimensional (2D) gel electrophoresis.** A 2D gel electrophoresis system was used to characterize further the disulphide bonds in the gC-II complexes. Virions were labelled with [3H]GlcN and exposed to a reducing agent as described above. Group 1 glycoproteins were immunoprecipitated from reduced virion extracts with MAB 9E10. For the first dimension, SDS-PAGE was done under non-reducing conditions (no 2-mercaptoethanol; ME). For the second dimension, the lane containing these glycoproteins was cut from the first dimension gel and exposed to ME for 30 min before SDS-PAGE a second time. Glycoproteins in the second gel were detected by fluorography.

**Heparin affinity chromatography.** Heparin binding experiments were carried out in two ways. For one method NP40 extracts from DTT-treated and untreated virions, containing HCMV glycoproteins labelled with [3H]GlcN, were applied to a 3 ml column of immobilized heparin (Sigma). The column was washed with several column volumes of PBS containing 0.1% NP40 to remove unbound material. This was designated the flow-through (FT) fraction. Material was eluted by washing the column with 20 mg/ml soluble heparin in PBS containing 0.1% NP40. This was designated the retained (RT) fraction. Fractions were monitored for radioactivity and the percentage of total radioactivity in the FT and RT fractions was determined. In a second method NP40 extracts containing HCMV glycoproteins were incubated with heparin immobilized on agarose beads with constant mixing. The beads were collected by centrifugation and washed with PBS (0.1% NP40). Several reagents were used to elute (or try to elute) bound HCMV glycoproteins. These included 0.65 M-NaCl, 2 mg/ml sialic acid, 2 mg/ml glucosamine (GlcN), 2 mg/ml of chondroitin sulphate A, B or C (Sigma) or 2 mg/ml of five different heparin disaccharides produced by digestion with heparinase I, II and/or III (Sigma). The structures of these heparin disaccharides are given in the following list. Abbreviations used in the list: AUA, 4-deoxy-

**Abbreviations used in the list:**
- AUA, 4-deoxy-
- hex-4-enopyranosyluronic acid; GlcN, D-glucosamine; Ac, Acetyl; NS, 2S-
- 4-GlcNAc; (iv) c-AUA-2S-[l-4]-GlcNS-6S; (v) c-AUA-
- [l-4]-GlcNS-6S (information obtained from Sigma). Bound HCMV glycoproteins were eluted from the beads by mixing them with one of the reagents mentioned above for 30 min. The beads were pelleted by centrifugation and the supernatant was assayed for radioactively labelled HCMV glycoproteins.
New England Nuclear) and the other half with [3H]heparin plus preparation of purified virions was suspended in PBS and divided in specific MAb. Immunoprecipitates were assayed for radioactivity. PBS to remove unbound heparin, and then extracted as described above. The extracts were immunoprecipitated with a gB- or a gC-II-MAbs, showing that all three contained both groups of glycoproteins (Kari et al., 1990b). When gC-II complexes were labelled with [3H]GlcN, [3H]Ser or [3H]Arg, glycoproteins were analysed. No reaction was detected with the glycoproteins recognized by Group 1 MAbs (8B4). Lane 3, probed with GNA recognizing c terminal sialic acid in N- and O-linked glycans. It is clear from these data that Group 2 MAbs reacted with the high Mr glycoproteins and that Group 1 MAbs did not. However, Group 2 MAbs reacted with a glycoprotein with an Mr of 48000 that overlapped in Mr with the glycoproteins recognized by Group 1 MAbs (Fig. 1c, compare lanes 1 and 3).}

**Results**

**Composition of gC-II complexes obtained from virions**

When gC-II complexes were labelled with [3H]GlcN and isolated from virions, three complexes were detected by non-reducing SDS-PAGE (Fig. 1a, lane 1). These complexes formed broad bands and had Mr values of 93000, 130000, and 200000 to 250000. All three complexes were recognized by Group 1 and Group 2 MAbs, showing that all three contained both groups of glycoproteins (Kari et al., 1990b). When gC-II complexes were labelled with [3H]GlcN, [3H]Arg or [3H]Ser the 200K to 250K Mr complexes appeared most abundant (Fig. 1a, lanes 1 to 3). Several glycoproteins were obtained after reduction of the gC-II complexes (Fig. 1b). Most of these glycoproteins tended to form broad overlapping bands in SDS-PAGE gels. The number of glycoproteins detected was not reduced by using several protease inhibitors during the isolation of the gC-II complexes. When [3H]GlcN was used, Group 1 glycoproteins with Mr values of 47000 to 63000 were most heavily labelled (Fig. 1b, lane 1, gp47-52). However, when [3H]Arg or [3H]Ser was used, proteins with Mr of 90000 and greater were more abundant relative to proteins in the Mr range of 47000 to 63000 (Fig. 1b, lane 2). These data showed that gC-II complexes contained two groups of glycoproteins, those which were heavily labelled with [3H]GlcN and those which were more heavily labelled with radioactive amino acids.

Glycoproteins in gC-II complexes were characterized further by Western blotting using MAbs and digoxigenin-labelled lectins. gC-II complexes were immunoaffinity-purified from virions with Group 2 MAb 15F9. Virions were used so that the glycoproteins would be fully processed. Complexes were reduced before SDS-PAGE and Western blotting so that individual glycoproteins were analysed. No reaction was detected with the gC-II glycoproteins when a negative ascites control was used (Fig. 1c, lane 1). The proteins recognized by Group 2 MAb 27B4 and Group 1 MAb 8B4 are shown in Fig. 1(c), lanes 2 and 5, respectively. It is clear from these data that Group 2 MAbs reacted with the high Mr glycoproteins and that Group 1 MAbs did not. However, Group 2 MAbs reacted with a glycoprotein with an Mr of 48000 that overlapped in Mr with the glycoproteins recognized by Group 1 MAbs (Fig. 1c, compare lanes 2 and 5).

Lectins also showed differences in gC-II glycoproteins. For example, *Galanthus nivalis* agglutinin (GNA), which recognizes mannose in high mannose N-linked glycan, and *Datura stramonium* agglutinin (DSA), which recognizes galactose in N- and O-linked glycan, reacted most strongly with the high Mr, Group 2 gC-II glycoproteins (Fig. 1c, lanes 3 and 4). Instead, a band

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**Fig. 1.** Virion glycoproteins were labelled by growing them in the presence of [3H]GlcN, [3H]Ser or [3H]Arg. Virions were extracted with 10% NP40 in PBS. These extracts were immunoprecipitated with Group 2 MAb 27B4. Immunoprecipitated glycoproteins were examined by SDS-PAGE using a 9% polyacrylamide mini-gel under non-reducing and reducing conditions and fluorography. (a) Radiolabelled gC-II complexes examined under non-reducing conditions. Lane 1, [3H]GlcN-labelled gC-II complexes. Lanes 2 and 3 [3H]Ser- and [3H]Arg-labelled gC-II complexes respectively. (b) Same as (a), except that the gC-II complexes were reduced prior to SDS-PAGE. (c) gC-II complexes were also isolated from unlabelled virions and used for glycan analysis. gC-II complexes were reduced and individual glycoproteins separated by SDS-PAGE prior to electroblotting onto nitrocellulose membranes. These membranes were cut into strips and individual strips were probed with MAbs or with lectins as described in Methods. Lane 1, SP2 negative ascites control. Lane 2, probed with a Group 2 MAb (27B4), and lane 5 with a Group 1 MAb (8B4). Lane 3, probed with GNA recognizing N-linked glycans. Lane 4, probed with DSA recognizing galactose in N- and O-linked glycans. Lane 6, probed with PNA, recognizing O-linked glycans. Lanes 7 and 8, probed with *Maackia amurensis* agglutinin and *Sambucus nigra* agglutinin respectively. Both recognize static acid in N- and O-linked glycans.
detected with peanut lectin (PNA), which reacts with the core disaccharide galactose $\beta$-(1-3)-N-acetylgalactosamine in O-linked glycans, was very similar to the band detected with Group 1 MAb 8B4 (Fig. 1c, lane 4). Only a small amount of reactivity was detected with PNA in the higher $M_r$ gC-II glycoproteins (Fig. 1c, lane 6). This confirms our past biochemical analysis, which showed that Group 1 glycoproteins contained a high content of O-linked glycans (Kari & Gehrz, 1988). Lectins that recognized sialic acid also reacted strongly with glycoproteins in the $M_r$ range of the Group 1 glycoproteins (Fig. 1c, lanes 7 and 8). However, these lectins only recognized glycoproteins with $M_r$ values of 52000 to 63000. Most likely this occurred because Group 1 glycoproteins were heterogeneous in their glycan composition, with some containing sialic acid and others little or none. This difference would create heterogeneity in the $M_r$ of the Group 1 glycoproteins which would cause them to form a broad band in SDS-PAGE gels. These differences in glycan composition showed that Group 1 and Group 2 glycoproteins underwent different post-translational processing. These results also confirm the presence of two distinct groups of glycoproteins in the gC-II complexes.

**Structures maintained by disulphide bonds in gC-II complexes**

The structures of gC-II complexes in the virion envelope were examined by exposing $[^3]H$GlcN-labelled virions to a reducing agent to reduce accessible disulphide bonds. Virions were collected by centrifugation and extracted with a non-ionic detergent. These extracts were first immunoprecipitated with gC-II Group 2 MAb 27B4 and then with gC-II Group 1 MAb 9E10. The same results were obtained when these antibodies were used in the reverse order (data not shown). When extracts from non-reduced virions were immunoprecipitated with MAb 27B4, complexes with $M_r$s of 93000 to greater than 200000 were detected by SDS-PAGE under non-reducing conditions (Fig. 2a, lane 1). Significant differences were noted in the material immunoprecipitated with MAb 27B4 after virions had been exposed to reducing agent for 30 min (Fig. 2a, lane 2). The relative amount of complexes with $M_r$ values of 250000 was reduced whereas the amount of the material with $M_r$ values of 93000 to 130000 increased. In addition, a minor glycoprotein with an $M_r$ of 48000 was detected (Fig. 2a, lane 2). This glycoprotein was also detected by
MAb 27B4 in Western blotting (Fig. 1c). Longer exposure of the virions to reducing agent did not significantly alter the number or amounts of glycoproteins immunoprecipitated with MAb 27B4 (Fig. 2a, lane 3). After immunoprecipitation with Group 2 MAb 27B4, extracts were treated with Group 1 MAb 9E10. Little additional material was immunoprecipitated by 9E10 from the extract obtained from non-reduced virions (Fig. 2a, lane 4). This result was expected since Group 1 and Group 2 MAbs recognize the same non-reduced gC-II complexes (Kari et al., 1990b). In contrast to this result, 9E10 immunoprecipitated significant amounts of material from extracts obtained from virions that had been exposed to reducing agent for 30 min. When this material was examined by SDS–PAGE under non-reducing conditions Group 1 glycoproteins with Mr values of 47000 to 63000 (gp47-52) and several high Mr bands were detected (Fig. 2a, lane 5). One of these bands had an Mr of 100000 and was different in Mr from any of the complexes immunoprecipitated with MAb 27B4 from the non-reduced or reduced virions. Again the number and relative amounts of the glycoproteins immunoprecipitated by 9E10 did not change with longer exposure of virions to reducing agent (Fig. 2a, lane 6).

Immunoprecipitated material was also examined by SDS–PAGE under reducing conditions. Glycoproteins gp47-63, gp90, gp130 and gp200 were obtained after reduction of gC-II complexes obtained from unreduced virions (Fig. 2b, lane 1). There was a small increase in the amount of glycoproteins in the 47000 to 63000 Mr range after further reduction of the material immunoprecipitated from reduced virions by Group 2 MAb 27B4 (compare Fig. 2a, lanes 2 and 3 with Fig. 2b, lanes 2 and 3). However, there was not a significant change in the amounts or Mr values of the other glycoproteins. These glycoproteins remained more abundant than the lower Mr glycoproteins. This suggested that either most disulphide bonds in Group 2 glycoproteins were reduced by exposing virions to reducing agent for 30 min, or they would not reduce further even under harsh conditions. In contrast to these results, the material immunoprecipitated from reduced virions by MAb 9E10 showed significant changes after additional reduction before SDS–PAGE (compare Fig. 2a, lanes 5 and 6 with Fig. 2b, lanes 5 and 6). In particular, all the high Mr bands detected under non-reducing conditions were reduced in relative amounts as the amount of gp47-63 increased (compare Fig. 2a, lanes 5 and 6 with Fig. 2b, lanes 5 and 6). These data showed that these glycoproteins contained disulphide bonds that were not reduced by exposure of virions to reducing agent for 2 h. It was also apparent that the sum of the glycoproteins immunoprecipitated from reduced virions by MAb 9E10 plus MAb 27B4 was equal to the number of glycoproteins found in gC-II complexes obtained from non-reduced virions (Fig. 2b, compare lane 1 with lane 2 plus lane 5). This showed that no gC-II glycoproteins were released from the envelope by exposure to reducing agent and therefore are likely to be transmembrane glycoproteins.

2D SDS–PAGE analysis of complexes immunoprecipitated from reduced virions by Group 1 MAb 9E10

A 2D SDS–PAGE system was used to examine further the composition of the glycoproteins and complexes immunoprecipitated from reduced virions by Group 1 MAb 9E10. Glycoproteins were run in the first dimension under non-reducing conditions and under reducing conditions in the second dimension. When this was done bands with apparent Mr's of 47000 to 63000 and 100000 in the first dimension generated only 47000 to 63000 Mr glycoproteins in the second dimension (Fig. 3). Bands representing Mr values of 150000 and greater in the first dimension contained the 47000 to 63000 glycoproteins in the second dimension, but they also contained high Mr glycoproteins (Fig. 3). This suggested that the 100000 Mr band detected in the first dimension was a disulphide-linked complex containing only the 47000 to 63000 Mr glycoproteins (i.e. gp47-52). These data show that disulphide bridges formed between Group 1 and Group
Fig. 4. Extracts were obtained from virions (V) or from virions which had been treated with the reducing agent DTT (DV). These extracts were applied to a column of immobilized heparin. An FT fraction was obtained by washing the column with PBS containing 0.1% NP40. An RT fraction was obtained by eluting bound material with PBS containing 0.1% NP40 and 2 mg/ml soluble heparin. The FT and RT fractions were immunoprecipitated with gC-II MAbs 9E10 or 27B4 and gB MAb 41C2. The amount of radioactivity immunoprecipitated from each fraction is represented as a percentage of the total radioactivity immunoprecipitated from both fractions.

2 as well as between two Group 1 glycoproteins and that disulphide bonds between two Group 1 glycoproteins in the envelope of intact virions were protected from reducing agent, and were only reduced after solubilization of the envelope glycoproteins in a detergent.

Influence of higher order structure on the heparin binding properties of HCMV envelope glycoproteins

Both gB and gC-II complexes have complicated higher order structures which could be necessary for efficient heparin binding. The influence of higher order structure on the heparin binding properties of gC-II and gB complexes was analysed by using complexes isolated from DDT-treated and untreated virions. These extracts were applied to a column of immobilized heparin. FT and RT fractions were obtained as described in Methods. When using extracts from untreated virions 75 to 90% of the total radioactivity immunoprecipitated by gC-II MAbs was found in the retained fraction, suggesting that most of the gC-II complexes bound heparin (Fig. 4). In contrast, when using gB MAb 41C2 only 18% of the total radioactivity was immunoprecipitated from the RT fraction suggesting that most of the gB complexes did not bind heparin (Fig. 4). When DDT-treated virion extracts were used the amount of gC-II complexes to be immunoprecipitated from the RT fraction was greatly reduced when either gC-II-specific MAb was used (Fig. 4). However, Group 2 MAb 27B4 immunoprecipitated more radioactivity from the RT fraction as compared to the Group 1 MAb 9E10 when treated virions were used. When gB complexes were immunoprecipitated from the FT and RT fractions of reduced virions the amount of radioactivity immunoprecipitated from each fraction was very similar to that obtained with non-reduced virions (Fig. 4). These results suggested that disulphide bonds in gC-II complexes were important to the binding of gC-II complexes to heparin, but less important to gB complexes. These data also showed that the binding of gB was not dependent on the presence of intact gC-II complexes.

Affinity of gC-II glycoproteins for heparin after reduction of disulphide bonds

The gC-II glycoproteins and complexes found in the FT and RT fractions obtained from DDT-treated and untreated virions were examined by SDS-PAGE under non-reducing conditions. Only complexes were immunoprecipitated from the FT and RT fractions obtained from untreated virions when Group 1 MAb 9E10 was used (Fig. 5a, lanes 1 and 2). Most of the gC-II complexes were immunoprecipitated from the RT fraction. The FT and RT fractions obtained from untreated virions were then immunoprecipitated with Group 2 MAb 27B4. Little additional material was precipitated since these MAbs recognize the same intact gC-II complexes (Fig. 5a, lanes 5 and 6). In contrast to these results, when reduced virion extracts were used most of the gC-II glycoproteins and complexes were immunoprecipitated from the FT fraction with MAb 9E10 (Fig. 5a, lanes 3 and 4). These fractions were then immunoprecipitated with Group 2 MAb 27B4. When this was done only high Mr glycoproteins were immunoprecipitated from either the FT or RT fractions (Fig. 5a, lanes 7 and 8). The 48 000 Mr glycoprotein originally detected in 27B4 immunoprecipitates of extracts from reduced virions was not detected in either the FT or RT fraction. This glycoprotein was a minor component and there may not have been enough present to detect. The relative amounts of the glycoproteins immunoprecipitated by MAb 27B4 from the FT and RT fractions...
Heparin binding by HCMV gC-II complex

Fig. 5. gC-II complexes and glycoproteins immunoprecipitated from the FT and RT fractions described in Fig. 4 were examined by SDS-PAGE and fluorography. (a) SDS-PAGE under non-reducing conditions. Lanes 1 and 2, gC-II complexes and glycoproteins immunoprecipitated from the FT and RT fractions, respectively obtained using Group 1 MAb 9E10 and untreated virion extracts; lanes 3 and 4, the same as lanes 1 to 2 with DTT-treated virion extracts. Lanes 5 and 6, gC-II complexes and glycoproteins immunoprecipitated using Group 2 MAb 27B4 after MAb 9E10 and untreated virion extracts; lanes 7 and 8, the same as lanes 5 and 6 with DTT-treated virion extracts. (b) SDS-PAGE under reducing conditions. Lane 1, glycoproteins obtained after reduction of complexes shown in lane 2 in (a); lane 2, glycoproteins obtained after reduction of complexes and glycoproteins shown in lane 3 in (a); (c) SDS-PAGE under reducing conditions. Lanes 1 and 2, glycoproteins obtained after reduction of the glycoproteins shown in lanes 7 and 8 respectively in (a). M̄ values are shown on the right and left.

were similar with the exception that the 200000 M̄ glycoproteins appeared to be more abundant in immunoprecipitates from the RT fraction.

Some of the fractions described above were also examined after additional reduction of disulphide bonds prior to SDS-PAGE. The complexes immunoprecipitated by Group 1 MAb 9E10 from the RT fraction of untreated virions contained gp47-52 and the higher M̄ glycoproteins typically found in intact gC-II complexes (Fig. 5b, lane 1). In contrast only gp47-52 was obtained after reduction of the material immunoprecipitated by Group 1 9E10 from the FT fraction of reduced virions (Fig. 5b, lane 2). These data showed that gC-II Group 1 glycoproteins did not have affinity for heparin after virions had been treated with DDT. The M̄ values of the glycoproteins immunoprecipitated from the FT and RT fractions of DDT-treated virions by Group 2 MAb 27B4 were not greatly changed by additional reduction prior to SDS-PAGE (Fig. 5c, lanes 1 and 2). These data showed that a portion of the Group 2 glycoproteins still had affinity for heparin after reduction of virions.

gC-II complexes in the envelope bind more soluble heparin than do gB complexes

It was possible that gC-II or gB complexes were binding heparin through sites that were not exposed until these complexes were removed from the virion envelope. To examine this possibility we exposed intact virions to [3H]heparin in the presence and absence of unlabelled heparin. These virions were extracted with a non-ionic detergent and the extract was immunoprecipitated with a gB- or a gC-II-specific MAb using Protein A-Sepharose. Little radioactivity was precipitated when Protein A-Sepharose was added without an MAb (Fig. 6). Radioactive heparin was immunoprecipitated when a gB- or a gC-II-specific MAb was added, showing that these complexes were capable of binding heparin while they were still in the virion envelope (Fig. 6). However,
Fig. 7. (a) Virion extracts containing [3H]GlcN-labelled glycoproteins were incubated with immobilized heparin. The beads were collected by centrifugation and washed to remove unbound glycoproteins. The beads were incubated with several carbohydrates or anions. All incubations were done in PBS containing 0.1% NP40. The beads were pelleted and the supernatants were assayed for radioactivity to determine which reagents were capable of eluting HCMV glycoproteins from the heparin beads. Con. shows the background c.p.m. when the beads were incubated with PBS--0.1% NP40 only. GlcN, glucosamine (2 mg/ml); NANA, sialic acid (2 mg/ml); 1 to 5 are five heparin disaccharides obtained by digestion of heparin with heparinase I, II and/or III. All five were used at a concentration of 2 mg/ml. Each disaccharide contained one or two sulphate groups; heparin was soluble heparin (2 mg/ml); NaCl was used at a concentration of 0.65 M. (b) Same as (a) except that the ability of chondroitin sulphate to release bound HCMV glycoproteins was compared to that of heparin. All reagents were used at a concentration of 2 mg/ml. Con. represents the background c.p.m. obtained by incubation with PBS--0.1% NP40 only. A, B and C, chondroitin sulphate A, B and C, respectively. Hep., heparin.

the gC-II complexes bound approximately three times as much heparin compared to gB complexes, indicating that gC-II had a greater heparin binding capacity. The gB complexes were immunoprecipitated twice to make sure that all gB complexes were immunoprecipitated. Finally, in both cases the amount of radioactive heparin that bound was reduced when unlabelled heparin was present (Fig. 6). This showed that the binding was specific.

Nature of bonds formed between heparin and HCMV envelope glycoproteins

The interaction of other herpesviruses with cell surface GAGs is in part mediated by ionic bonds (Lycke \textit{et al.}, 1991). Because of this we have analysed the conditions under which HCMV envelope glycoproteins could be eluted from immobilized heparin. Several reagents were tested for their ability to elute them. Among the reagents that did not release these proteins from heparin were GlcN, sialic acid and several disaccharides obtained from heparin by the action of heparinase I, II and/or III (Fig. 7a). All heparin disaccharides contained one or two sulphate groups. GlcN was tested because the GAG backbone of heparin contains GlcN, and sialic acid because it is an anion. Among the reagents that released HCMV envelope glycoproteins were heparin and NaCl at a concentration of 0.65 M (Fig. 7a). The relative amounts of gC-II and gB complexes in the material released by heparin and NaCl were the same (data not shown). These data suggested that the interaction between heparin and the HCMV envelope glycoproteins involved ionic bonds. It has also been established that heparin-like GAGs are the receptors on the host cell to which herpesviruses bind, and not chondroitin sulphate (Lycke \textit{et al.}, 1991; Neyts \textit{et al.}, 1992). Therefore, we examined chondroitin sulphate for its ability to release HCMV envelope glycoproteins from immobilized heparin. Both chondroitin sulphate A (from porcine rib) and chondroitin sulphate C (from shark cartilage) released little or no HCMV glycoprotein from immobilized heparin (Fig. 7b). Chondroitin sulphate B (from porcine skin, dermatan sulphate) was more effective than A or C (Fig. 7b). However, heparin was more than twice as effective at releasing HCMV envelope glycoproteins when compared to chondroitin sulphate C (Fig. 7b). These data show that the carbohydrate backbone of the GAG is also important for binding of the HCMV envelope glycoproteins.

Discussion

The gC-II complexes found in the envelope of HCMV contain two groups of glycoproteins differing in several ways. First, based on glycan analysis, Group 1 glycoproteins contain many more O-linked glycans than do Group 2 glycoproteins. Group 1 glycoproteins also are more heavily labelled with [3H]GlcN than are Group 2. When gC-II glycoproteins were isolated from virions and examined under reducing conditions Group 2 glycoproteins covered a much broader range of Mr values. Some of the Group 2 glycoproteins had Mr values that overlapped those of the Group 1 glycoproteins. However, Group 1 glycoproteins are clearly not derived from the
high \( M \), Group 2 glycoproteins since Group 1 MAbs do not recognize these glycoproteins. It is not clear how the large number of different Group 2 glycoproteins is generated. One possibility is that the high \( M \), Group 2 glycoproteins were not completely reduced, although harsh reducing conditions were used. This might occur if Group 2 glycoproteins were particularly hydrophobic and became difficult to separate once the high \( M \), complexes formed. It is also possible that proteolysis occurs during the isolation of the gC-II complexes. Several protease inhibitors were used during purification of gC-II complexes to prevent proteolysis. This did not affect the number of Group 2 glycoproteins detected. Therefore, proteolysis during the isolation as a mechanism to generate the different \( M \), Group 2 glycoproteins seems less likely. It is also possible that Group 2 glycoproteins could be generated by splicing of an HCMV gene(s).

The present studies show that the higher order structure of the gC-II complexes is extremely complicated. Multiple disulphide bonds appear to maintain these structures. For example, disulphide bonds were formed between Group 1 and Group 2 glycoproteins as well as between Group 1 glycoproteins only. In the virion, disulphide bonds between Group 1 and Group 2 were very susceptible to reduction which suggested that these bonds are on the external surface of the virion. After reduction of disulphide bonds between Group 1 and Group 2 glycoproteins these glycoproteins could be separated from each other by extracting them from the virion envelope with a non-ionic detergent. This was different from the results previously obtained with gB complexes. Although the disulphide bonds between the glycoproteins in the gB complexes were reduced, these glycoproteins could be separated from each other only by boiling them in the presence of SDS (Kari et al., 1990a). Thus, glycoproteins in the gB complexes appear to form strong non-covalent bonds whereas Group 1 and Group 2 glycoproteins in gC-II complexes do not.

A potential function of the gC-II complexes is to bind cell surface heparin-like proteoglycans. Other biologically active molecules such as fibroblast growth factors also use cell surface proteoglycans as receptors. These molecules have affinity for immobilized heparin and can be eluted from immobilized heparin by NaCl gradients (for review see Burgess & Maciag, 1989). As we have shown in this report, HCMV envelope glycoproteins can also be eluted from immobilized heparin with NaCl showing that these glycoproteins form ionic bonds with heparin. Ionic bonds are also important in HSV–heparin binding (Lycke et al., 1991). Thus, in general the ability to form ionic bonds appears to be one important property for heparin binding proteins. It was also clear that heparin disaccharides were ineffective at eluting HCMV glycoproteins from immobilized heparin. This observation was similar to that of Lycke et al. (1991). They found that HSV binding decreased with decreasing molecular size of heparin oligosaccharides and could not be detected for saccharides containing less than 10 monosaccharide units. Binding was also not detected with desulphated heparin. Thus, chain length and charge density are important for the binding of herpesviruses to heparin. Although soluble heparin was capable of eluting HCMV envelope glycoproteins from immobilized heparin, other polyanions such as chondroitin sulphates were much less or not effective. It is important to note that chondroitin sulphate does not block binding of HCMV to the host cell (unpublished observation) and removal of chondroitin GAGs from the cell surface does not affect HCMV binding to the host cell (Neyts et al., 1992). A major difference between heparin and chondroitin GAGs is their hexosamine content; heparin contains sulphated N-acetyl-glucosamine and chondroitin sulphated N-acetyl-galactosamine. Furthermore, chondroitin sulphate B was much less effective than heparin at eluting bound HCMV glycoproteins but was more effective than either chondroitin sulphate A or C. A difference between chondroitin sulphate B and A or C is that chondroitin sulphate B contains \( \beta \)-iduronic acid whereas A and C contain \( \beta \)-glucuronic acid. Therefore, the structure of the GAG backbone is also important in heparin binding of HCMV envelope glycoproteins and other herpesviruses.

Of the gC-II complexes found in virions, 75 to 90% bind to immobilized heparin whereas only 18% of the gB complexes bound heparin. While still part of the virion envelope, gC-II complexes also bound more soluble heparin than did gB complexes. These data would suggest that gC-II has a greater affinity for heparin and is a major heparin binding component of the envelope. However, which set of complexes plays the principal heparin binding role remains to be determined. Reduction of disulphide bonds in gC-II complexes significantly reduced the affinity of gC-II glycoproteins and complexes for heparin. Therefore, higher order structure was important for the heparin binding by gC-II complexes. It is conceivable that the structures maintained by disulphide bonds in gC-II complexes were needed to expose a region rich in basic amino acids in either Group 1 or 2 glycoproteins. Binding to heparin may occur through ionic interaction with Group 2 glycoproteins since more of these proteins still had affinity for heparin after reduction of disulphide bonds. In contrast to these results structures maintained by disulphide bonds were much less important to the heparin binding properties of the gB complexes. It has been proposed that there is some redundancy in viral functions that can mediate the binding of HSV to cells.
and that HSV gB may substitute for gC in the absence of gC (Herold et al., 1991). The same may be true for HCMV, i.e. gC-II and gB complexes may have redundant attachment functions.

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References


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