NOTES

A Human Cytomegalovirus Glycoprotein Complex Designated gC-II Is a Major Heparin-Binding Component of the Envelope

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The purposes of this study were to determine whether heparin would block human cytomegalovirus (HCMV) infection of skin fibroblast (SF) cells and to identify HCMV envelope glycoproteins which might have affinity for heparin. It was determined that soluble heparin in concentrations of 5 to 20 $\mu\text{g/ml}$ was capable of blocking HCMV infection of SF cells. However, after virus had adsorbed to the SF cells, heparin lost its ability to block infection. It was also determined that treatment of SF cells with heparinase to remove cell surface heparinlike moieties prevented HCMV infection of SF cells. These data showed that HCMV, like other herpesviruses, adsorbed to cells by binding cell surface heparin. Heparin affinity chromatography was done to determine which HCMV envelope glycoproteins bound heparin. HCMV envelope glycoproteins were solubilized in a nonionic detergent and applied to a heparin affinity column. An HCMV glycoprotein complex designated gC-II was the major component to bind to immobilized heparin and elute in the presence of soluble heparin.

The envelope of human cytomegalovirus (HCMV) contains several glycoproteins, many of which are associated in disulfide-linked complexes (4, 13). One family of glycoprotein complexes has been designated glycoprotein complex II (gC-II) (4). The designation gC-II does not suggest that these complexes contain individual glycoproteins with homology to any other herpesvirus glycoprotein. gC-II complexes contain two groups of biochemically and immunologically distinct glycoproteins which are disulfide linked to each other (11). These glycoproteins are recognized by human anti-HCMV antibody in sera from convalescent adults. However, sera from congenitally infected infants with persistent infection failed to react with gC-II glycoproteins (10). These data suggested that gC-II may be important to human protective immune responses to HCMV. One of the groups of gC-II glycoproteins may be encoded by a gene or genes in the US6 gene family (US6 through US11, formerly designated the HXLF gene family) (3). The open reading frames US6 through US11 do not show homology to any other known human herpesviruses (19). Cytoplasmic RNAs from all six genes from the US6 gene family were detectable at early and late times after infection (5). The US11 and US10 genes and the US9 and US8 genes are transcribed as bicistronic mRNAs, while US7 and US6 are transcribed as monocistronic RNAs (5). It has also been established that the kinetics of steady-state cytoplasmic RNA was different for each transcription unit from the US6 gene family (8). Furthermore, the US6 transcripts used different initiation sites at early and late times postinfection. There was also evidence for spliced and unspliced messages from this gene family (8). It has recently been established that the US6 gene family is not essential for replication in culture (14). The glycoproteins encoded by the US6 gene family have been designated gp47-52 (3) and have a high content of O-linked

oligosaccharides (9). The gene(s) encoding the second group of glycoproteins (group 2) found in gC-II complexes has not been identified. This group contains glycoproteins with molecular weights of 48,000 to greater than 200,000 (11). How this diverse group of glycoproteins is generated has not been determined.

Nothing has been reported about the functions of the gC-II complexes. Since the gC-II complexes are part of the HCMV envelope, one possibility is that they are involved in adsorption to the host cell and/or penetration of the host cell. Recently, it has been established that the initial interaction of several herpesviruses with the cell surface is mediated by glycosaminoglycans found on the proteoglycans in the cell plasma membrane (6, 16, 17). These glycosaminoglycans are structurally similar to heparin (15). Thus, soluble heparin is capable of blocking the adsorption of these viruses to the host cell. The herpesviruses for which this has been demonstrated are herpes simplex virus (HSV) type 1 (HSV-1) (6), pseudorabies virus (PrV) (16), and bovine herpesvirus 1 (BHV-1) (17). The envelope glycoproteins most important in binding to heparin are glycoprotein C (gC) in HSV-1 and glycoprotein III (gIII) in BHV-1 and PrV. It has been established that HSV gC (7) and PrV gIII (20) glycoproteins are nonessential for replication of these viruses in culture. However, viruses missing these glycoproteins are impaired in their ability to adsorb to the host cell, as judged by their reduced PFU-to-particle ratio (6, 20).

Since it has been established that heparin will block infection of cells by other herpesviruses, we first examined the ability of heparin to block Towne strain HCMV infection of skin fibroblast (SF) cells. Heparin in concentrations of from 5 to 20 $\mu\text{g/ml}$ was effective in blocking HCMV infection when HCMV was preincubated with heparin prior to adsorption to cells (Fig. 1A). These experiments were also done with HCMV strains AD169 and Toledo, and similar results were obtained (data not shown). Because heparin was less effective at blocking HCMV plaque formation than HSV

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FIG. 1. (A) Inhibition of HCMV plaque formation with heparin. HCMV was preincubated with heparin before cultures were infected at an MOI of 0.001. Heparin was present during the entire adsorption period of 1 h. After 1 h, the cultures were washed with PBS and overlaid with agarose for the plaque assay. Plaques were counted 7

days after infection (21), we examined the ability of heparin to prevent plaque formation at multiplicities of infection (MOI) of 0.01 and 0.001. When 10 μ g of heparin per ml at an MOI of 0.01 was used, the number of PFU was reduced by 57%, compared with a reduction of 66% at an MOI of 0.001. These results suggested that the amount of virus present did not influence the ability of heparin to prevent plaque formation. We also pretreated SF cells with heparinase to remove cell surface heparin moieties prior to exposure to HCMV. This was done to determine whether removal of heparinlike moieties from the cell surface would prevent HCMV plaque formation. When this was done, the numbers of plaques produced were also greatly reduced (Fig. 1B). These data suggested that heparin blocked HCMV infection by binding to a heparin-binding component in the envelope of the virus and not by a nonspecific event such as aggregation of virus particles. Heparinase was less effective than heparin at reducing the number of plaques; i.e., the same end point was not obtained. This result was similar to those observed with PrV and HSV (16, 21). It may be that a combination of heparinase and other enzymes, such as heparitinase, would be needed to completely remove all heparinlike moieties on the surface of the cell. Furthermore, neither heparin nor heparinase completely prevented plaque formation. It is most likely that there are other cell surface components which can act as attachment sites for HCMV and that heparin does not prevent attachment by these routes. In contrast to the results described above, when HCMV was allowed to adsorb prior to exposure to heparin, heparin was much less effective at blocking HCMV infection of SF cells (Fig. 1C). The ability of heparin to prevent HCMV infection was greatly reduced when virus was allowed to adsorb to cells for as little as 5 min prior to exposure to heparin. These data suggested that heparin cell surface moieties or heparinlike structures are important for adsorption of HCMV to SF cells.

To determine whether there were HCMV envelope glycoproteins with the ability to bind heparin, heparin affinity chromatography was done. Towne strain HCMV envelope glycoproteins were labeled with [3 H]GlcN. Extracellular virus was extracted with 1.0% Nonidet P-40 (NP-40) to solubilize the envelope glycoproteins as previously described (13). Insoluble material was removed by centrifugation, and the supernatant was applied to a heparin affinity column. The column was washed with several column volumes of phosphate-buffered saline (PBS) containing 0.1% NP-40 prior to the elution of bound material with 2 mg of heparin per ml in PBS-NP-40 buffer. A significant amount of radioactivity bound to the heparin column and was eluted

days after infection. Each point represents the mean of two experiments. (B) Inhibition of HCMV plaque formation by treatment of SF cells with heparinase. SF cells were treated with different amounts of heparinase for 30 min, washed with PBS, and infected with HCMV at an MOI of 0.001. HCMV was allowed to adsorb for 1 h prior to a rinse with PBS. Cultures were overlaid with agarose for plaque assays. Seven days after infection, plaques were counted. Each point represents the mean of two experiments. (C) Effect of heparin added at different times after virus inoculation. SF cells were incubated with 10 μ g of heparin per ml at different times after infection with HCMV at an MOI of 0.001. Virus was allowed to adsorb for a total of 1.5 h. Each point represents the mean of two experiments, except the last two points, which represent single experiments. Towne strain was used in all experiments.

FIG. 2. (A) Elution profile of HCMV envelope glycoproteins applied to a heparin column. HCMV envelope glycoproteins were labeled by growing the virus in the presence of [3 H]GlcN. Virus was purified from infected cell culture media. Purified virus was extracted with 1.0% NP-40. Insoluble material was removed by centrifugation, and the supernatant was applied to a column of immobilized heparin. The column was washed with several column volumes of PBS containing 0.1% NP-40. Bound material was removed by eluting with 2 mg of heparin per ml in PBS containing 0.1% NP-40. FT and RT fractions are indicated. Arrow indicates when soluble heparin was applied. (B) Fluorogram showing glycoproteins in the FT (lane 1) and RT (lane 2) fractions. Both fractions were immunoprecipitated with the gB-specific MAb 41C2. The immunoprecipitated glycoproteins are shown in lanes 3 and 4. (C) Fluorogram done as described for panel B except that lanes 3 and 4 show the glycoproteins immunoprecipitated with the gC-II MAb 27B4. All samples were reduced prior to electrophoresis.

with heparin (Fig. 2A). The specificity of this binding was demonstrated by the fact that no radioactivity was bound to a column of *N*-acetylglucosamine immobilized on agarose (data not shown). These data showed that there were HCMV envelope glycoproteins with an affinity for heparin. To identify the heparin-binding glycoproteins, both the flowthrough (FT) and the retained (RT) fractions were analyzed by immunoprecipitation with monoclonal antibodies (MAbs) specific for gC-II complexes and for complexes containing the HCMV gB glycoproteins. Complexes containing the gB glycoproteins were immunoprecipitated with the gB-specific MAb 41C2 (12), and gC-II complexes were immunoprecipitated with the gC-II group 2-specific MAb 27B4 (11). The glycoproteins immunoprecipitated from the FT and RT fractions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The total amounts of glycoproteins in the FT and RT fractions are shown in Fig. 2B and C, lanes 1 and 2. Of the total amount of radioactivity immunoprecipitated from both peaks with a gB-specific MAb, 81% was immunoprecipitated from the FT fraction. Those glycoproteins immunoprecipitated by the gB-specific MAb from the FT fraction accounted for some of the most abundant glycoproteins in this fraction (Fig. 2B; compare lanes 1 and 3). Only a small amount of the gB gp93-130 glycoprotein was detected in the RT fraction by fluorography (Fig. 2B, lane 4). In contrast to this, the majority of the radioactivity (93%) immunoprecipitated by the gC-II-specific MAb was immunoprecipitated from the RT fraction. The glycoproteins immunoprecipitated by the gC-II MAb appeared to account for most of the glycoproteins detected in the RT fractions (Fig. 2C; compare lanes 2 and 4). These data showed that gC-II glycoprotein complexes were effective at binding to heparin and most likely play a major role in the binding of HCMV to heparin cell surface moieties.

Like several other herpesviruses, HCMV adsorbs to cell surface heparinlike moieties. This was demonstrated in two ways. First, soluble heparin was capable of preventing infection of SF cells when HCMV virions were preincubated with heparin. The inhibitory activity of heparin was lost when HCMV was allowed to bind to SF cells before exposure to heparin. Second, removal of cell surface heparin moieties with heparinase also reduced infection of SF cells by HCMV. This result strongly suggested that heparin was specifically binding to an envelope component of HCMV in order to block HCMV infection. The envelope of HCMV was shown to contain glycoproteins which bind to immobilized heparin. In our experiments, gC-II was a major envelope component which bound heparin. There may have been other envelope components beyond the gC-II complexes and the gB complexes which bound heparin, but they were not detected in our experiments. The gC-II complexes elute at high salt concentrations from an anion-exchange column (13) and their polysaccharides contain sialic acid (9), both of which suggest that gC-II is an anion. Therefore, it seems unlikely that gC-II would nonspecifically bind to a strong anion like heparin. These data also suggest that the blocking of HCMV infection by soluble heparin was specific and not the simple aggregation of virus particles by heparin. For HSV-1, gC was the major envelope glycoprotein responsible for heparin binding (6). The gIII glycoproteins of PrV (16) and BHV-1 (17) were responsible for binding to heparin cell surface moieties. It is interesting that the gIII glycoproteins in both PrV and BHV-1 are structural homologs of HSV-1 gC (2, 18). Furthermore, at least two of these glycoproteins were found to be nonessential for replication in culture, but

strains missing these glycoproteins were much less efficient at replication in culture (5, 6, 18). The US6 through US11 genes which may encode the gC-II gp47-52 glycoproteins are also nonessential for replication in culture (14). While the genes encoding gp47-52 do not share sequence homology with other herpesvirus genes (19), the gp47-52 glycoproteins do have other structural similarities. For example, both gp47-52 and gC from HSV-1 contain a cluster of O-linked oligosaccharides on the peptide backbone (1, 9). These clusters of O-linked oligosaccharides make a large portion of these glycoproteins resistant to proteolysis. These data suggest that HSV-1 gC and gp47-52 undergo similar post-translational processing. The gC-II complexes are also structurally more complicated than these other viral heparin-binding glycoproteins. gC-II complexes contain two groups of immunologically and biochemically distinct glycoproteins which are associated by disulfide bonds (11). It cannot be established from the current data which of these groups of glycoproteins mediates binding to heparin.

A small amount of the HCMV gB glycoproteins was also found in the RT fraction of the heparin column. This result was similar to those reported for HSV-1 and PrV in that the HSV gB and a small amount of the PrV gII (the PrV homolog of HSV gB) were found in the heparin column RT fractions along with gC and gIII, respectively (6, 16). In contrast, only BHV-1 gIII was found in heparin column RT fractions (17). It may be that gB is an alternate heparin-binding glycoprotein or that a complex forms between gB and the other heparin-binding glycoproteins, so that it is secondarily associated with the heparin column. Whether the HCMV gB plays a significant role in heparin binding is uncertain.

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