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Role of the Extracellular Domain of Human Herpesvirus 7 Glycoprotein B in Virus Binding to Cell Surface Heparan Sulfate Proteoglycans

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In an attempt to identify the human herpesvirus 7 (HHV-7) envelope protein(s) involved in cell surface binding, the extracellular domain of the HHV-7 glycoprotein B (gB) homolog protein was cloned and expressed as a fusion product with the Fc domain of human immunoglobulin G heavy chain γ1 (gB-Fc) in an eukaryotic cell system. Indirect immunofluorescence followed by flow cytometric analysis revealed specific binding of gB-Fc to the membrane of SupT1 cells but not to other CD4$^+$ T-lymphoblastoid cell lines, such as Jurkat or PM1, clearly indicating that gB-Fc did not bind to the CD4 molecule. This was also suggested by the ability of gB-Fc to bind to CD4-negative fibroblastoid Chinese hamster ovary (CHO) cells. The binding was abrogated by enzymatic removal of cell surface heparan sulfate proteoglycans by heparinase and heparitinase but not by treatment with condroitinase ABC. In addition, binding of the gB-Fc fusion protein to CHO cells was severely impaired in the presence of soluble heparin, as well as when heparan sulfate-deficient mutant CHO cells were used. Consistent with these findings, soluble heparin was found to block HHV-7 infection and syncytium formation in the SupT1 cell line. Although the CD4 antigen is a critical component of the receptor for the T-lymphotropic HHV-7, these findings suggest that heparin-like molecules also play an important role in HHV-7–cell surface interactions required for infection and that gB represents one of the HHV-7 envelope proteins involved in the adsorption of virus-to-cell surface proteoglycans.

Human herpesvirus 7 (HHV-7) is a recently identified human T-lymphotropic herpesvirus (3, 16) which utilizes CD4 as a cellular membrane receptor for the infection of primary CD4$^+$ T lymphocytes and of the SupT1 CD4$^+$ T-lymphoblastoid cell line (28). However, the fact that other human CD4$^+$ T-cell lines, including Jurkat and PM1, show poor susceptibility to HHV-7 infection (40, 42a, 51) suggests that multiple interactions must occur between viral envelope HHV-7 glycoproteins and surface antigens of the target cell. Similar events have been previously shown for other members of the *Herpesviridae* family (8, 18, 26, 31, 36, 43, 44, 48). At the present time, the envelope proteins involved in HHV-7 attachment to the cell surface and in the entry process, including the putative CD4 ligand, are still unidentified.

The glycoprotein B (gB) homologs constitute the most highly conserved group among herpesvirus glycoproteins. The high degree of sequence conservation may indicate that these proteins exert common functions in the life cycle of herpesviruses. In fact, it has been shown that gB homolog glycoproteins play an essential role in virus attachment to and penetration of the host membrane for several herpesviruses (4, 5, 21, 26, 33). Therefore, it was of interest to analyze whether, and through which mechanism(s), HHV-7 gB is involved in the early steps of infection by interacting with specific cell surface molecules.

The entire genome of HHV-7 has been recently sequenced (34). The sequence encoding for the HHV-7 gB homolog glycoprotein has been identified (34, 42). HHV-7 gB consists of 822 amino acids (aa) and shows features characteristic of type I integral membrane proteins. Computer analysis predicts that this protein has a signal peptide at the amino terminus, a large external domain which contains 11 potential N-glycosylation sites, and a transmembrane domain followed by a shorter cytoplasmic tail (42). By analogy with the gB of other herpesviruses, it is plausible to hypothesize that the gB of HHV-7 also plays an important role in the early events of virus-cell interaction. To address this issue, we have constructed and characterized an HHV-7 gB fusion protein (gB-Fc) that was used to perform binding studies and to identify the cell surface molecules involved in HHV-7–target cell interactions.

MATERIALS AND METHODS

Cell culture and virus. SupT1 and Jurkat CD4$^+$ T-cell lines and the CHO-K1 fibroblastoid cell line were obtained from the American Type Culture Collection (Rockville, Md.). The PM1 CD4$^+$ T-cell line was previously described by Lusso et al. (29). In addition to parental wild-type CHO-K1 cell line, the following mutant cell lines defective in proteoglycan synthesis were kindly provided by J. D. Esko (University of Alabama at Birmingham): pgsD-677 (12, 27), pgsE-606 (2), and pgsA-745 (13). SupT1, Jurkat, and PM1 cells were maintained in RPMI 1640 containing 10% fetal calf serum (Gibco BRL, Gaithersburg, Md.). Wild-type and mutant CHO cells were routinely grown in Ham's F12 medium supplemented with 10% fetal calf serum (Gibco BRL). The HHV-7 isolate AL, used in this study, has been previously described (28, 40).

Plasmid construction. Cloning procedures were performed as described previously (39) unless otherwise stated. A 1,873-bp DNA fragment containing the nucleotides coding from aa 1 to 608 of the gB protein (predicted to be the extracellular portion of the glycoprotein) was amplified by PCR from a gB cDNA clone that we obtained by screening an HHV-7 cDNA library (42). The following primers, including the restriction sites (underlined nucleotides) *Kpn*I and *Eco*RV, respectively, were used: primer 1, 5′-TACCGGTACCAAATCTGTTA GATCTATGTAGAC-3′, which corresponds to the sequence from −4 to −36 upstream of the presumed initiation codon; and primer 2, 5′-CCAAATGATA

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TCCGTTCGCTTTGCTCAGTTCATC-3′, which corresponds to the sequence from +1803 to +1836 of the HHV-7 gB. The amplified DNA fragment containing a portion of the gB sequence was digested with *Kpn*I and *Eco*RV, separated by electrophoresis on a low-melting-temperature gel, eluted, and ligated in frame to the amino terminus of the Fc fragment of human immunoglobulin G heavy chain γ1 (IgG1) (kindly provided by D. Cosman, Immunex Corporation, Seattle, Wash.) in pBluescript vector. For expression in a eukaryotic system, the gB-Fc cassette was subcloned under the control of a cytomegalovirus (CMV) promoter into pCEP4 vector (Invitrogen, San Diego, Calif.) via sites for *Kpn*I and *Not*I. The correct construction of pCEP4/gB-Fc was checked by restriction enzyme digestion and nucleotide sequencing of the cloning junction.

In vitro translation. One microgram of DNA of plasmid pBluescript containing the gB-Fc fusion gene under the T7 promoter was used for in vitro transcription and translation experiments in a rabbit reticulocyte lysate cell-free system in the presence of [^{35}S]methionine as recommended by the manufacturer (Promega, Madison, Wis.). Posttranscriptional events were investigated by performing the in vitro transcription and translation reaction in the presence of canine microsomal membranes (Promega) according to the manufacturer's recommendations. The product of the reactions was analyzed after being run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, fluorographed, and autographed (−70°C).

Transfections. CHO cells were transfected by using the Lipofectamine procedure (Gibco BRL) according to the manufacturer's instructions and then were either stained to check for protein expression or lysed for protein purification. For the first purpose, CHO cells were seeded onto glass coverslips, transfected 24 h later, and subjected to immunofluorescence staining 2 days posttransfection. At this time point, cells were fixed with cold acetone, incubated for 20 min at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG-Fc antibodies (Kirkegaard & Perry), and extensively washed with phosphate-buffered saline (PBS). After counterstaining with Evans blue, the cells were examined and photographed with a Zeiss fluorescence microscope.

Expression and purification of the fusion protein. For purification of the gB-Fc fusion protein, transfected CHO cells were cultured in serum-free medium and lysed 48 to 72 h posttransfection. For this purpose, monolayers were washed with cold PBS and lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40 and 1% sodium deoxycholate in PBS). Samples were sonicated and centrifuged at 16,000 × *g* for 20 min to remove cell debris. The clarified supernatant obtained from the cellular lysate was applied to a protein A column (Pierce, Rockford, Ill.) and eluted by using the Immunopure buffers provided in the kit. The fractions collected during the elution process were analyzed for the presence of the fusion protein by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting, by staining with goat anti-human IgG1-Fc antibodies and alkaline phosphatase conjugated-rabbit anti-goat antibodies (Pierce). The identified positive fractions were then dialyzed, concentrated, and used for cell membrane binding experiments.

Protein concentration was estimated from the A_{280} (1 A_{280} equals 0.5 μg/ml) or measured with the Bio-Rad protein assay.

Metabolic labeling and endoglycosidase digestion. The recombinant proteins were characterized by labeling CHO cells, 48 h after transfection, with 100 μCi of [^{3}H]glucosamine (Amersham, Arlington Heights, Ill.) or [^{35}S]methionine-cysteine mix (EXPRE^{35}S^{35}S Protein Labeling Mix; Dupont NEN, Boston, Mass.) per ml for 3 to 4 h at 37°C, after a 3-h incubation step in glucose-free or methionine-free medium, respectively. To inhibit protein glycosylation, 5 μg of tunicamycin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml was added to the culture medium throughout both the starving and labeling periods. For immunoprecipitation of cell-associated polypeptides, monolayers were lysed as described above. The fusion protein was recovered from the clarified cellular lysates by incubation with Sepharose-protein A overnight at 4°C. The immunoprecipitate was washed five times with cold radioimmunoprecipitation assay buffer and resuspended in sample buffer containing 2-β-mercaptoethanol. Samples were boiled and analyzed by SDS-PAGE. The protein bands were visualized by standard fluorographic techniques.

For endoglycosidase digestions, immunologically precipitated material from unlabeled cells was resuspended in the digestion buffer, and digestions of denatured proteins (incubated for 5 min at 100°C in 0.01% SDS) with endoglycosidase H and *N*-glycosidase F (Boehringer Mannheim Biochemicals) were performed according to the manufacturer's protocols, in the presence of inhibitors of the proteolytic activity. The products of digestions were analyzed by SDS-PAGE and Western blotting as described above.

Cytofluorimetric analysis. The binding of gB-Fc fusion protein to the cell surface membrane of SupT1, PM1, Jurkat, and CHO cells was investigated by flow cytometry. A total of 10^6 cells were incubated for 30 min at 4°C with approximately 100 to 200 ng of recombinant gB-Fc protein or with 200 ng of human IgG (Pierce). After two washings in ice-cold PBS, cells were labeled with a FITC-conjugated goat anti-human IgG-Fc antibodies (Kirkegaard & Perry). For the experiments of binding inhibition, heparin (Sigma, St. Louis, Mo.) or basic fibroblast growth factor (bFGF; Bachem, Torrance, Calif.) was dissolved in PBS and added to the gB-Fc during the incubation with the cells. Heparinase II, Heparinase III (heparitinase), and condroitinase ABC (Sigma) digestions were carried out by incubating 10^6 SupT1 or CHO cells with 1 U of each enzyme for 1 h at 37°C, followed by at least two washings with PBS prior to the addition of gB-Fc. A minimum of 5,000 cells per sample were analyzed by using a FACScan (Becton Dickinson, San Jose, Calif.).

HHV-7 infection inhibition test. SupT1 cells (10^6) were incubated with 1 ml of cell-free viral stock, represented by the culture supernatant collected from infected phytohemagglutinin-stimulated CD4$^+$ T lymphocytes, with an approximate multiplicity of infection of 0.1. After incubation for 3 h at 37°C to allow virus adsorption, cells were washed and seeded in culture. Various 10-fold dilutions of heparin (10 to 0.1 μg/ml) were added to the cells before, during, or after viral adsorption. The infection level was evaluated by quantitative DNA PCR (41) performed on aliquots of supernatant harvested 6 days postinfection. To test binding inhibition, the viral inoculum was mixed with the indicated concentrations of heparin and incubated for 45 min at room temperature. The virus-heparin mixtures were added to the cells, and incubation continued for 2 h on ice. Alternatively, SupT1 cells were preincubated with 0.1 to 10 μg of gB-Fc protein per ml before infection. After cell-virus incubation (on ice), cells were extensively washed with cold PBS, and cell-associated viral DNA was evaluated by quantitative PCR (41).

The syncytium inhibition test was performed by pretreating 2×10^5 HHV-7-infected SupT1 cells with 10-fold dilutions (100 to 0.1 μg/ml) of heparin for 20 min at room temperature; the infected cells were then cocultured with 10^6 uninfected SupT1 cells in 24-well plates in a total volume of 2 ml. In other cases, uninfected SupT1 cells were treated with 0.5 μg of CD4 monoclonal antibody (MAb) Leu3a (Becton Dickinson) per ml before the coculture with untreated HHV-7-infected cells. Syncytia were scored 20 and 40 h after the beginning of cocultivation.

RESULTS

Cloning and expression of HHV-7 gB-Fc fusion protein. To identify the viral protein(s) involved in the binding of HHV-7 to the cell surface antigens of SupT1, HHV-7 genomic DNA was analyzed in search of sequences showing similarities with envelope glycoproteins of other herpesviruses. A sequence encoding a putative HHV-7 gB homolog protein was identified (42). Due to the lack of specific reagents (i.e., MAb or hyperimmune sera) against HHV-7 glycoproteins, we adopted the strategy to construct a fusion protein by linking the portion of the gene encoding the predicted extracellular domain of HHV-7 gB (from aa 1 to 608) upstream to sequences encoding part of the human IgG1, including the hinge and the CH2 and CH3 constant regions (Fig. 1).

To determine whether the 2.6-kbp chimeric gene (1.9 kbp from the gB gene plus 0.7 kbp from the Fc sequence) was able to encode a protein, in vitro transcription and translation experiments were performed. RNA transcribed from the DNA plasmid gB-Fc cloned in pBluescript under the T7 promoter produced a major polypeptide with an apparent molecular mass of ~90 kDa (data not shown), which closely approximated the predicted molecular mass of the putative fusion protein. When canine microsomal membranes were added to the translation reaction mixture, the polypeptide shifted in mobility to a polypeptide of about 110 kDa, indicating that glycosylation had taken place (data not shown) and confirming that HHV-7 gB is indeed a glycoprotein as suggested by the sequence analysis.

The fusion gene was then subcloned into the mammalian expression vector pCEP4, constituting a versatile system for detection and affinity purification of recombinant proteins expressed in mammalian cells (15, 17, 50).

Characterization of gB-Fc expressed in CHO cells. Expression of gB-Fc in plasmid-transfected CHO cells was first documented by fluorescence microscopy on fixed cells, using FITC-conjugated goat antibodies raised against the human Fc portion of the chimeric protein. Intense cytoplasmic staining was specifically observed in the pCEP4/gB-Fc-transfected cells (Fig. 2).

For additional characterization, the protein encoded by the fusion gene was isolated from cellular lysates of transfected cells, harvested 48 h posttransfection, by affinity chromatography with protein A. Immunoblot analysis of the elution fractions showed that the fusion protein was detected as a doublet

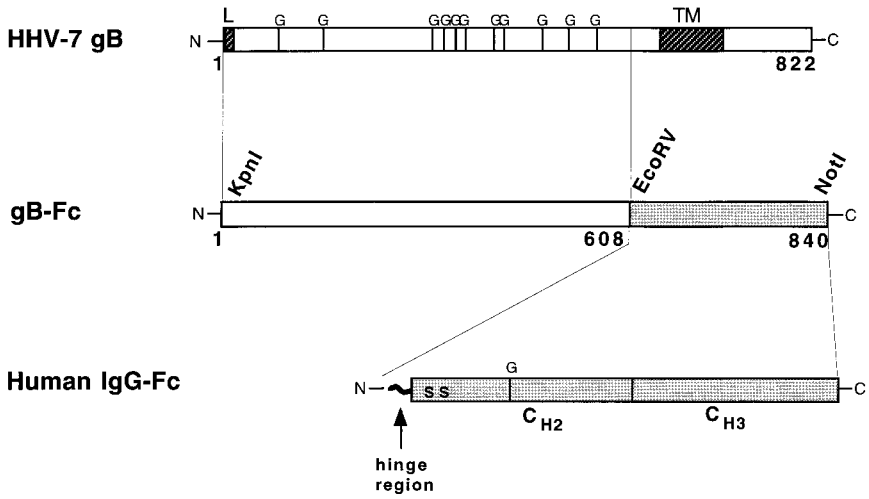

FIG. 1. Schematic representation of the gB-Fc fusion gene obtained by linking the putative extracellular portion of HHV-7 gB (aa 1 to 608) to the Fc of the human IgG1, as described in Materials and Methods. Putative sites for the addition of N-linked carbohydrate (G) and the probable leader (L) and transmembrane (TM) domains in full-length HHV-7 gB (42) are indicated. In the Fc sequence, the hinge region and CH2 and CH3 constant domains are schematically depicted. The glycosylation site (G) and the positions of the cysteine residues involved in disulfide bounds (S) are indicated. For expression in a mammalian system, the chimeric gene has been cloned into the *Kpn*I and *Not*I sites of the pCEP4 vector (Invitrogen).

of bands with apparent molecular masses of approximately 98 and 115 kDa under reducing conditions (Fig. 3). No immunostaining was detected in the lysates obtained from mock-transfected cells (Fig. 3).

When the isolated gB-Fc was analyzed under nonreducing conditions, a predominant band of approximately 250 kDa was observed (Fig. 3). Since the molecular size of the major protein in the nonreduced form was approximately twice the size found under reducing conditions, we hypothesized that gB-Fc is synthesized as a dimer. This hypothesis is in agreement with the existence of non-disulfide-bonded dimer forms of herpes simplex virus (HSV) gB that are disrupted by heat (35, 38). However, we cannot exclude the possibility that the dimerization process arises from the disulfide bonds between the Cys residues near the hinge region of the Fc fragment (Fig. 1).

Glycosylation patterns of gB-Fc. The fusion protein was then analyzed for the presence of posttranslational modifications, which are expected to occur during viral infection of mammalian cells. When the cells were labeled with [^{35}S]methionine or [^{3}H]glucosamine, two bands corresponding to molecular masses of approximately 98 and 115 kDa were identified with both kinds of metabolic labeling (Fig. 4A). Although computer analysis of the amino acid sequence of the fusion protein revealed the presence of one potential glycosylation site also in the Fc portion, it is likely that glycosylation mainly occurred in the gB fragment, which contains 11 putative glycosylation sites (Fig. 1).

The level of glycosylation of gB-Fc was also assessed by metabolic labeling of transfected cells in the presence or absence of tunicamycin (5 μg/ml), which blocks the addition of N-linked oligosaccharides to proteins. Treatment of transfected cells with tunicamycin lowered the apparent molecular masses of the two immunoprecipitation bands from 98 and 115 kDa to 75 and 95 kDa (data not shown).

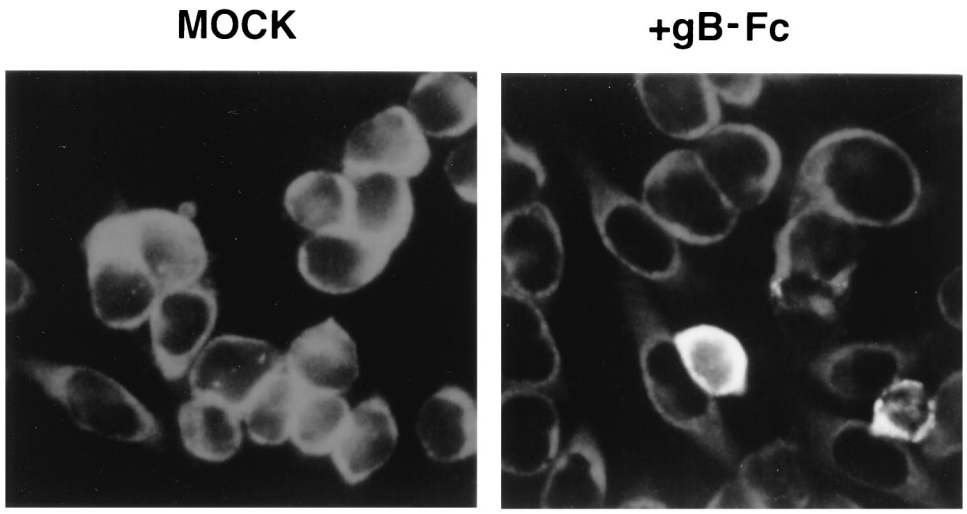

FIG. 2. Expression of the gB-Fc tested in transiently transfected CHO cells. Forty-eight hours after transfection, acetone-fixed cells were assessed by direct immunofluorescence with FITC-conjugated goat anti-human IgG-Fc antibodies. Negative reactions are counterstained with Evans blue.

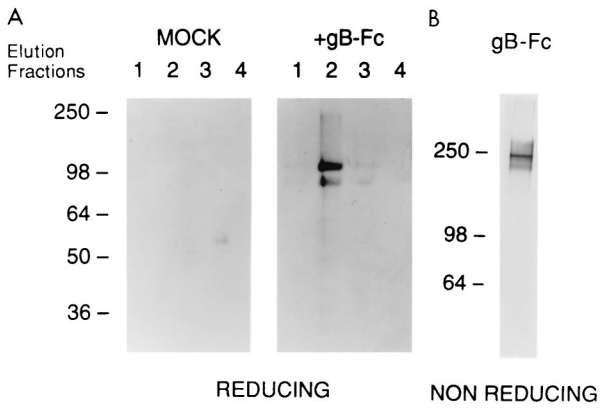

FIG. 3. Purification of gB-Fc from cellular lysates using immobilized protein. (A) Eluates of cellular extracts of CHO cells either mock transfected or transfected with the pCEP4/gB-Fc expression vector were resolved by SDS-PAGE under reducing conditions, electrotransferred onto a nitrocellulose membrane, and incubated with goat anti-human IgG-Fc antibodies followed by alkaline phosphatase-conjugated rabbit anti-goat antibodies. (B) Analysis of the elution fraction containing the fusion protein resolved by SDS-PAGE under nonreducing conditions. Positions of molecular weight markers are shown in kilodaltons at the left side.

To investigate the glycosylation in more detail, the immunoprecipitated proteins were digested with endoglycosidase H or *N*-glycosidase F. The former hydrolyzes N-linked high-mannose oligosaccharides and some hybrid oligosaccharides but does not cleave complex oligosaccharides. Resistance to endoglycosidase H treatment indicates that a glycoprotein has been processed through the Golgi apparatus. *N*-glycosidase F cleaves N-linked high-mannose, hybrid, and complex oligosaccharides from glycoproteins. Following digestion with endoglycosidase H, the immunoprecipitated proteins migrated in SDS-PAGE at approximately 100 and 80 kDa, while *N*-glycosidase F yielded two bands of approximately 95 and 75 kDa (Fig. 4B).

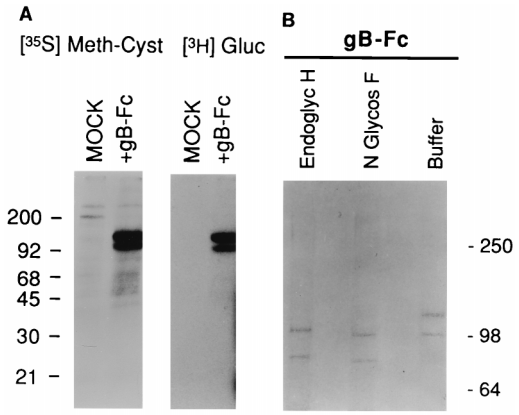

FIG. 4. Glycosylation of gB-Fc. (A) Cells were either mock transfected or transfected with the plasmid expressing the gB-Fc fusion gene and then metabolically labeled with either [^{35}S]methionine-cysteine or [^{3}H]glucosamine. Proteins were immunoprecipitated directly with protein A. All samples were analyzed under reducing conditions by SDS-PAGE and visualized by the standard fluorographic technique. Positions of molecular weight markers are shown in kilodaltons at the left. (B) Endoglycosidase sensitivity of gB-Fc gB-Fc immunoprecipitated from transfected CHO cells was treated with either endoglycosidases (endoglycosidase H and *N*-glycosidase F) or buffer alone. Proteins were size fractionated by SDS-PAGE and detected by Western blotting with goat anti-human IgG-Fc antibodies followed by alkaline phosphatase-conjugated rabbit anti-goat antibodies. Positions of molecular weight markers are shown in kilodaltons at the right.

These data indicate that N-linked glycans, consisting of both complex and high-mannose oligosaccharide side chains, are contained in the gB-Fc synthesized in CHO cells. Apparently, the glycosylation process takes place starting from two different protein precursors of approximately 75 and 95 kDa, respectively.

At the moment, it is unclear whether these two protein precursors are the result of transcriptional-translational events starting at two distinct initiation sites or of a posttranslation cleavage modification, in agreement with the known proteolytic cleavage of human CMV gB and other herpesvirus gB homologs in the center of the extracellular domain (38, 42, 45). An additional possibility to be considered is that members of the gB family usually retain some N-linked glycans as the high-mannose type and acquire O-linked glycans which are not necessarily affected by either tunicamycin or endoglycosidase F treatment (6, 22).

Selective binding of gB-Fc to the cell surface membrane of SupT1 but not Jurkat or PM1 CD4$^+$ T cells. The affinity-purified chimeric protein was used as a probe to identify potential cellular receptor(s) for HHV-7 by FACScan. gB-Fc, but not human IgG protein, specifically bound to the human T-lymphoblastoid cell line SupT1 (Fig. 5). On the other hand, gB-Fc was unable to bind to Jurkat and PM1 human CD4$^+$ T-lymphoblastoid cells, which show surface CD4 expression but a poor susceptibility to HHV-7 infection. Taken together, these findings indicate that (i) binding of the chimeric protein gB-Fc to SupT1 cells did not occur specifically through the Fc portion and (ii) the CD4 antigen is not the surface receptor for gB-Fc.

HS-dependent binding of gB-Fc to the cell surface membrane. To elucidate whether surface proteoglycans were responsible for the observed gB-Fc binding, SupT1 cells were preincubated with heparinase III, which enzymatically removes heparan sulfate (HS) proteoglycans from the cell membrane. Following heparinase III treatment, the binding of gB-Fc to SupT1 cell surface was abolished (Fig. 5).

After this first evidence suggesting that the gB-Fc binding to the cell membrane could be mediated through HS proteoglycans, several approaches were used to confirm and define the nature of the observed interaction(s).

A specific binding of gB-Fc was also observed in assays using the CD4-negative fibroblastoid CHO cell line, further indicating that CD4 or other lymphoid cell-specific surface antigens were not required for gB-Fc binding. When CHO cells were exposed to heparinase II and heparinase III, which cleave heparin and HS glycosidic linkages, respectively, gB-Fc binding was again significantly impaired. In contrast, treatment of CHO cells with chondroitinase ABC, which hydrolyzes glycosidic linkages in the chondroitin sulfate group of molecules, had no effect (Fig. 6A), indicating that gB-Fc does not bind to all cell surface proteoglycans but binds specifically to HS proteoglycans. Consistent with this hypothesis, when CHO cells were incubated with gB-Fc in the presence of increasing concentrations of soluble heparin, a dose-dependent binding inhibition was observed (Fig. 6B). A drastic decline of the gB-Fc binding was also observed by preincubating CHO cells with the cytokine bFGF, which has been shown to interact with HS proteoglycans in the extracellular matrix prior to binding to a high-affinity receptor (52).

These observations were confirmed and expanded by analyzing a panel of CHO cell lines defective in various aspect of glycosaminoglycan biosynthesis: (i) pgsA-745, which produces little or no HS and chondroitin sulfate, being defective in the xylosyltransferase enzyme (9); (ii) pgsD-677, which produces no HS but elevated levels of chondroitin sulfate, being defec-

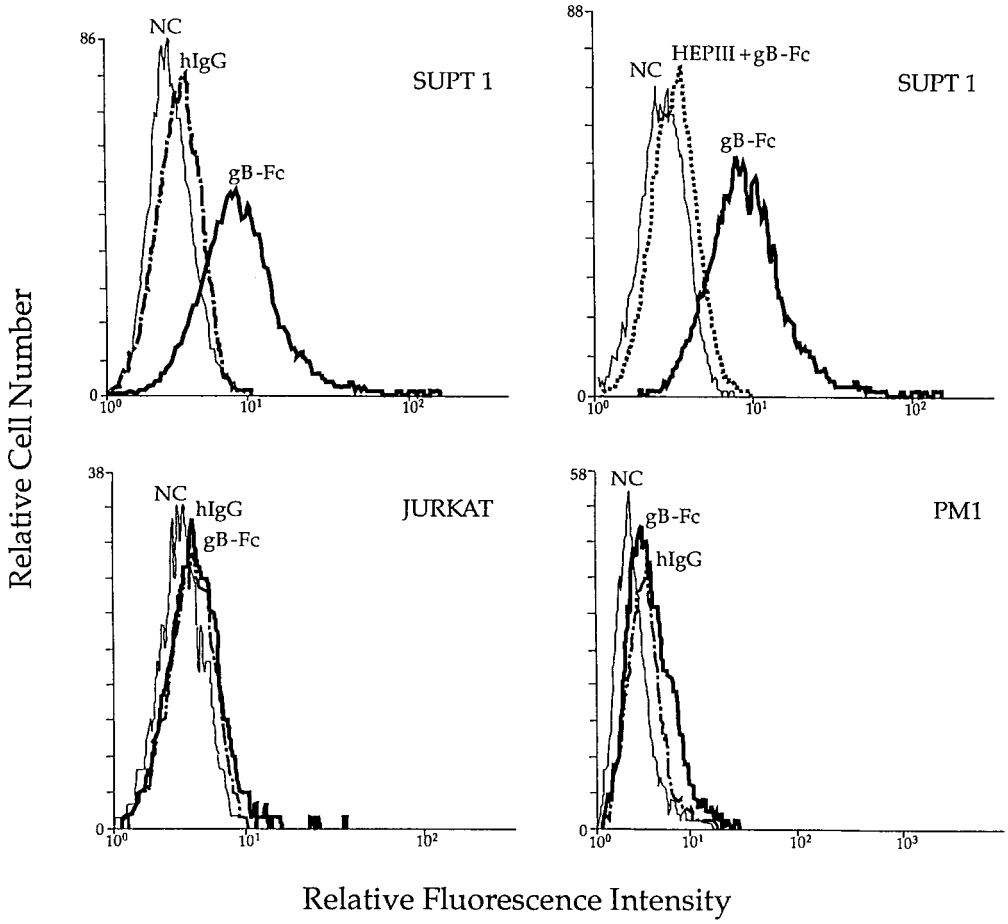

FIG. 5. FACScan analysis of gB-Fc binding to cell membrane. SupT1, Jurkat, and PM1 T-cells (10^6/sample) were incubated with either 200 ng of gB-Fc or 200 ng of human IgG1 (hIgG1), for 30 min at 4°C. The cells were washed, and protein binding to the cellular surface was detected by using FITC-conjugated goat anti-human IgG-Fc antibodies. Cells stained with secondary reagent alone are shown (NC). gB-Fc binding to SupT1 cells, pretreated with 1 U of heparitinase III (HEPIII) per ml for 1 h at 37°C, is also shown.

tive in *N*-acetylglucosaminyltransferase and glucuronosyltransferase enzymes (8, 21); and (iii) pgsE-606, which produces normal levels of both HS and chondroitin sulfate, but the HS is undersulfated (1). This last cell line shows a defect in *N*-sulfotransferase enzyme.

The results of binding experiments obtained with wild-type and mutant CHO cell lines are shown in Fig. 6C. While gB-Fc efficiently bound to wild-type CHO-K1 cells, it failed to bind to pgsA-745 or pgsD-677 cells, which are both deficient in HS biosynthesis. In addition, gB-Fc also bound very poorly to the undersulfated HS pgsE-606 cells, suggesting that a highly sulfated subclass of HS may be required for efficient gB-Fc attachment to the cell membrane.

Effect of heparin on HHV-7 infectivity and syncytium formation in SupT1 CD4$^+$ T-lymphoblastoid cells. Since it has been extensively demonstrated that other members of the *Herpesviridae* family use cell surface HS for binding and entry into their target cells (8, 24, 30, 31, 36, 43, 44, 48, 49, 53), we examined the effect of heparin on HHV-7 infectivity.

Infection experiments were carried out by inoculating SupT1 cells with a cell-free viral stock in the presence or absence of various concentrations (0.1 to 10 μg/ml) of soluble heparin. Pretreatment of SupT1 cells with heparin, which was removed prior to the addition of the viral inoculum, had no inhibitory effect on virus replication monitored as HHV-7 genome copy number released in the culture supernatants (data not shown). On the other hand, a dose-dependent inhibition of viral replication was observed when heparin was added during virus adsorption (Fig. 7A). Moreover, when heparin was added to the cells after virus adsorption and left in the medium for the entire time of culture, a marked inhibition of viral replication was also observed at the lowest dose used (0.1 μg/ml) (Fig. 7A). These findings indicated that heparin does not affect HHV-7 replication by inducing modifications on the SupT1 cell surface but likely interferes with the binding and entry of the virus into the target cells. The ability of heparin to block HHV-7 binding to the cell surface of SupT1 cells was confirmed in experiments in which viral inoculum was pretreated with various concentration of heparin (0.1 to 10 μg/ml) for 45 min and then incubated with the cells for 2 h in ice (Fig. 7B). After extensive washings, we found that the amount of cell-associated viral DNA was greatly reduced in the presence of heparin. Interestingly, a partial (approximately 30 to 35%) inhibition of HHV-7 binding to SupT1 cells was also observed when cells were pretreated with approximately 0.1 to 10 μg of gB-Fc protein per ml before addition of the viral inoculum (data not shown). On the other hand, we were unable to examine in detail entry events, mainly due to the lack of accurate methods that allow to discriminate and quantify the levels of mRNA expression of HHV-7 early regulatory genes.

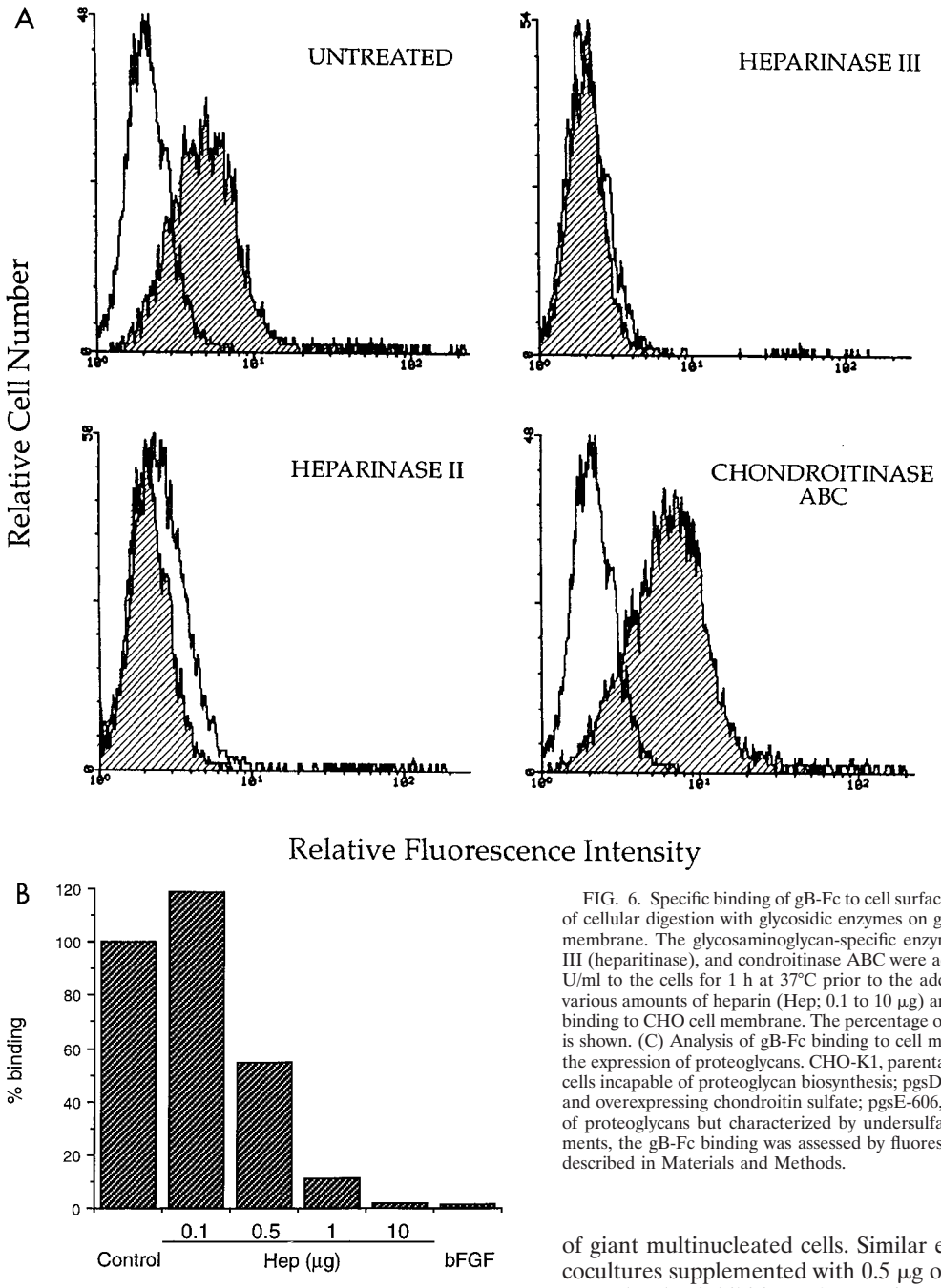

FIG. 6. Specific binding of gB-Fc to cell surface HS proteoglycans. (A) Effect of cellular digestion with glycosidic enzymes on gB-Fc binding to the CHO cell membrane. The glycosaminoglycan-specific enzymes heparinase II, heparinase III (heparitinase), and condroitinase ABC were added at the concentration of 1 U/ml to the cells for 1 h at 37°C prior to the addition of gB-Fc. (B) Effects of various amounts of heparin (Hep; 0.1 to 10 μg) and of bFGF (250 ng) on gB-Fc binding to CHO cell membrane. The percentage of cells showing specific binding is shown. (C) Analysis of gB-Fc binding to cell membrane of CHO mutants for the expression of proteoglycans. CHO-K1, parental wild-type cell line; pgsA-745, cells incapable of proteoglycan biosynthesis; pgsD-677, cells not synthesizing HS and overexpressing chondroitin sulfate; pgsE-606, cells expressing normal levels of proteoglycans but characterized by undersulfation of HS. In all the experiments, the gB-Fc binding was assessed by fluorescence-activated cell sorting as described in Materials and Methods.

We then investigated whether heparin also had some effects on the fusogenic events leading to the formation of the large polykaryocytes which characterize the cytopathic effect induced by in vitro infection with HHV-7 (3, 16, 28, 40). For this purpose, a previously developed syncytium inhibition assay (40) was used. Infections were carried out by coculturing HHV-7 acutely infected (0.2×10^6) with uninfected (10^6) SupT1 cells in the presence or absence of 1 to 100 μg of heparin per ml. After 20 h, the cells were examined for the appearance of syncytia. In the absence of heparin, new syncytia started to be detected at 20 h postcoculture (Fig. 7C), becoming progressively larger with time. In contrast, the addition of 100 μg of heparin per ml completely prevented the formation of giant multinucleated cells. Similar effects were observed in cocultures supplemented with 0.5 μg of anti-CD4 MAb Leu3a per ml. The inhibition of syncytia was absent or weak and disappeared (48 h postcoculture) in the presence of lower heparin concentrations (1 to 10 μg/ml) (Fig. 7C).

DISCUSSION

Our study demonstrates that the gB homolog of HHV-7 is able to bind cell surface HS, which likely functions as an essential coreceptor for a productive infection of $CD4^+$ T-lymphoid cells, the major target of this virus (16, 28). We had previously established that the CD4 antigen, expressed primarily by the helper/inducer subset of T-lymphoid cells, is the cellular receptor for the lymphotropic HHV-7 (28). However, the absent or low susceptibility of several $CD4^+$ T-lymphoid cell lines to a productive HHV-7 infection suggested the pos-

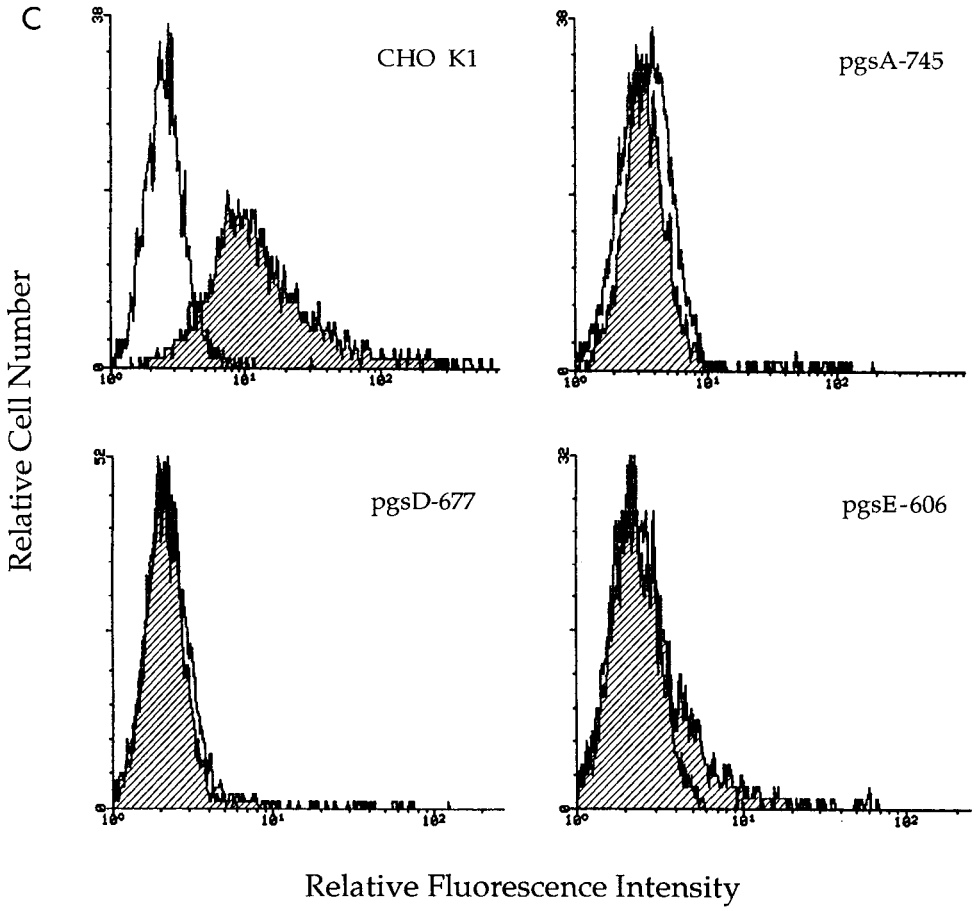

FIG. 6—*Continued.*

sibility that surface molecules other than CD4 are involved as coreceptors for HHV-7 binding and entry. Similar findings have now clearly emerged for human immunodeficiency virus type 1 (7, 9, 10, 14).

In this respect, the participation of surface proteoglycans related to heparin in adsorption of enveloped viruses to the cellular membrane has been extensively documented by different authors (24, 30, 37, 49, 53). For instance, heparin, a related glycosaminoglycan, inhibits the cellular attachment of several herpesviruses and the HS moiety of surface proteoglycans seems required for the initial cell binding of virions (8, 31, 36, 43, 44, 46–49). We likewise found that heparin significantly impaired HHV-7 replication and syncytium formation in SupT1 cells, likely interfering with the early steps of infection (i.e., attachment, entry, and fusion events).

In general, during infection with herpesviruses, more than one envelope protein plays a critical function in (i) virus adsorption to cellular receptors (i.e., attachment), (ii) fusion with the plasma membrane and penetration into the cytoplasm, and (iii) virion assembly and egress of the viral progeny from the infected cells. Viral envelope glycoproteins exhibiting structural homology among various members of the *Herpesviridae* family (i.e., gB and gH) are thought to serve similar functions, although this assumption has not been proven directly. In particular, gB seems to play a central role in early interactions between virion and target cell in different herpesviruses (4, 5, 21, 26, 33, 38).

The essential tool to verify the role of HHV-7 gB in cell surface binding was represented by a chimeric protein, originated by fusion of the hydrophilic amino acidic region of the HHV-7 gB open reading frame with the Fc fragment of human IgG1. gB-Fc was expressed in a mammalian cell system to allow the posttranslational modifications, including glycosylation and intrachain disulfide bond formation, necessary to confer to the recombinant protein the same structural and antigenic properties of wild-type gB.

The ability of gB-Fc to specifically bind HS, and not CD4 or other lymphoid cell-restricted surface molecules, was supported by the following findings: (i) purified gB-Fc protein was able to bind efficiently to the membrane of $CD4^+$ T-lymphoblastoid SupT1 and fibroblastoid CHO cells but not $CD4^+$ T-lymphoblastoid Jurkat and PM1 cells; (ii) heparitinase treatment of both SupT1 and CHO cells completely abolished gB-Fc binding; (iii) gB-Fc cell binding was inhibited in a dose-dependent manner by the addition of soluble heparin or bFGF; and (iv) unlike wild-type CHO cells, CHO mutants, characterized by genetic deficiencies in HS proteoglycan biosynthesis, failed to bind gB-Fc.

Clearly, gB-HS interactions are not sufficient to establish a productive HHV-7 infection, since soluble recombinant CD4, anti-CD4 MAb, and recombinant human immunodeficiency virus envelope protein gp120 (28) are all sufficient to block HHV-7 infection. In any case, among the $CD4^+$ T-cell lines examined in this study, an interesting correlation was observed between gB-Fc binding and susceptibility to a productive HHV-7 infection, suggesting that gB-HS interactions may rep-

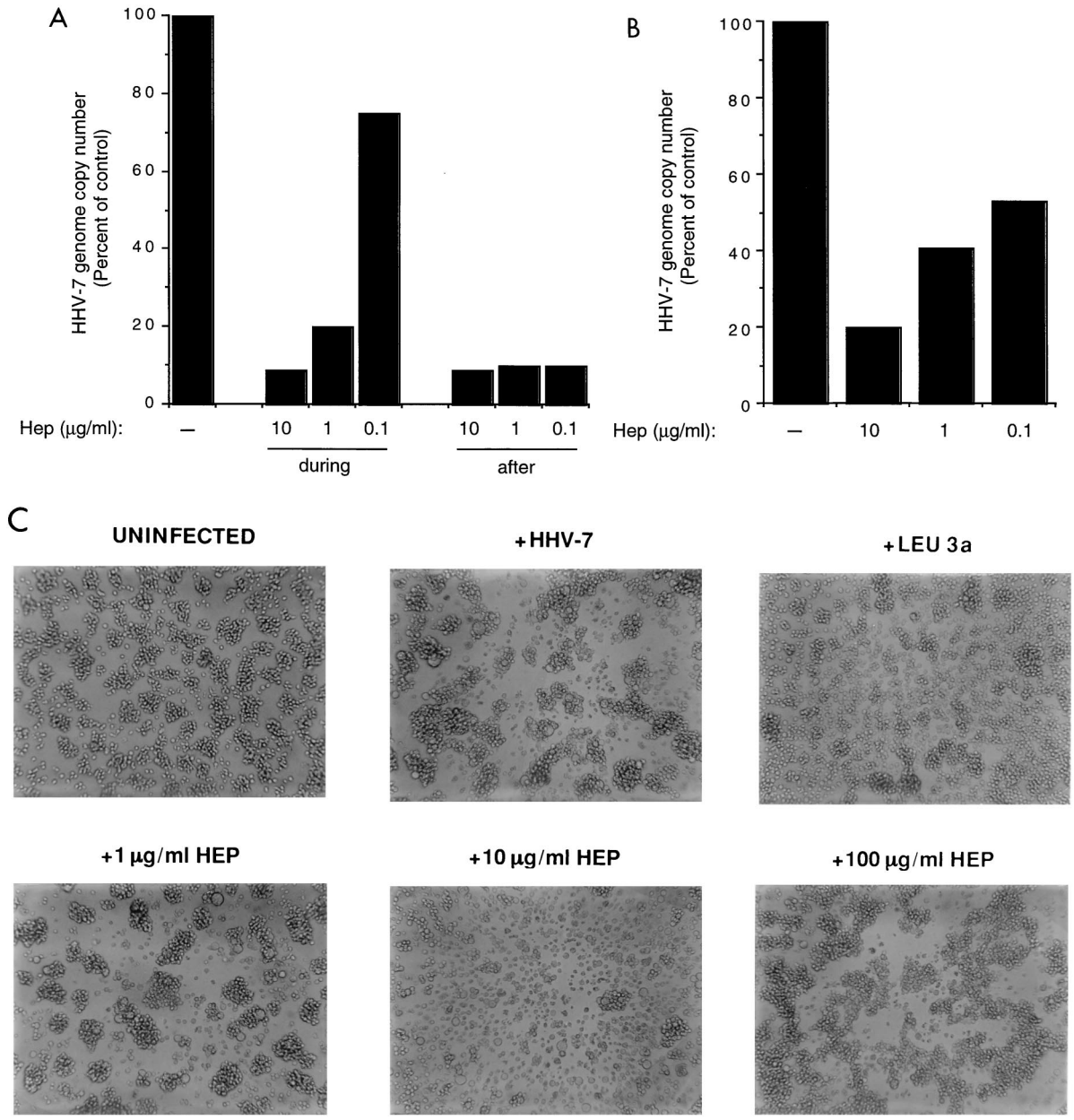

FIG. 7. Effect of soluble heparin on HHV-7 infectivity (A), binding (B), and syncytium formation (C) in SupT1 cells. (A) Cells were incubated with 0.1 to 10 μg of heparin per ml during or after viral adsorption performed for 3 h at 37°C. In all cases, HHV-7 was added to SupT1 cells and washed after incubation. The viral progeny of the different cultures was evaluated by quantitative DNA PCR performed on aliquots of the supernatant collected at 6 days postinfection. Data are reported as means of results of experiments performed in duplicate. (B) Viral inoculum was mixed with the indicated concentrations of heparin and incubated for 45 min at room temperature. The virus-heparin mixtures were added to the cells, and incubation continued for 2 h on ice. After extensive washings with cold PBS, cell-associated viral DNA was evaluated by quantitative PCR. Data are reported as means of results of experiments performed in duplicate. (C) Cells were photographed 20 h after coculture of HHV-7-infected with uninfected SupT1 cells, in the presence or absence of 0.5 μg of anti-CD4 MAb Leu3a or 1 to 100 μg of heparin (HEP) per ml.

resent an important component of the infection process. Consistent with our findings, it was previously shown that the adsorption of HSV also was severely impaired in HS-deficient mutant CHO cells (46), although other cell surface molecules are thought to serve as high-affinity receptors for viral entry (18, 23, 32).

As recently described also for HSV type 1 (32), it seems likely that the entry of many herpesviruses requires a first set of receptor-ligand interactions for binding and a second one for entry. Cell surface HS proteoglycans may function to help to sequester and localize the virions in the vicinity of a specific receptor or other essential components of the cell surface. Our present findings on the binding of gB to HS, together with the identification of the CD4 antigen as an essential receptor for HHV-7 (28), suggest that a similar model of virus-cell interactions may also occur during HHV-7 infection. It is possible that the function of HS is limited to the initial attraction and binding of HHV-7 to the cells by interacting with HHV-7 gB. Alternatively, HS may facilitate the interactions between

HHV-7 virions and some other cell surface molecule(s), i.e., the CD4 receptor.

Envelope proteins other than gB are expected to be required for membrane fusion/binding and entry, and it is also possible that HS interacts with more than one viral protein. Using 0.1 to 10 μg of gB-Fc protein per ml, we have observed only a partial (approximately 30%) inhibition of HHV-7 binding to SupT1 cells. This may be due to the inability of these concentrations of gB-Fc to completely saturate the HS binding sites present on the cell surface of SupT1 cells and/or to the contribution of other viral glycoproteins for the initial binding/attachment step. In fact, for all alpha- and betaherpesviruses studied, it seems clear that at least two glycoproteins have heparin-binding activity, invariably a member of the gB family (reviewed in reference 38) and a member of the gC family for alphaherpesviruses (19, 31, 36) or gC-II for CMV (24).

In agreement with previous findings (20), we also found that the sulfate groups represent critical determinants in the interactions of cell surface HS with HHV-7. HS represents a family of heterogeneous molecules showing significant differences with respect to patterns of expression, chain sulfatation, and chain length (11, 25). Although we have no data on the expression pattern of HS proteoglycans on the surface of SupT1, PM1, and Jurkat $CD4^+$ T cells, a nonuniform distribution of sulfate groups in HS present on the surface of different cell lines (37) may account for the variable efficiency in the virus-cell interactions and may help to explain some of the differences in susceptibility of various $CD4^+$ T-cell lines to gB-Fc binding and productive HHV-7 infection. However, we cannot rule out the possibility that postbinding events or nonpermissiveness for expression of viral genes account for the inability of HHV-7 to infect Jurkat, PM1 $CD4^+$ T cells or HeLa cells transfected with CD4.

On the basis of these observations, we conclude that HHV-7 infection involves binding to cell surface HS proteoglycans, and we suggest that the gB homolog is a potential envelope protein mediating HHV-7–cell membrane interactions. In analogy to other herpesviruses, the binding of HHV-7 to $CD4^+$ T-lymphoid cells is likely a multistep event, and the coexpression of CD4 and HS on a cell membrane may define the cell type specificity of HHV-7 infection.

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