Herpes Simplex Virus Type 1-Induced Hemagglutination: Glycoprotein C Mediates Virus Binding to Erythrocyte Surface Heparan Sulfate

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We recently reported that herpes simplex virus type 1 (HSV-1) can cause agglutination of murine erythrocytes (E. Trybala, Z. Larski, and J. Wisniewski, *Arch. Virol.* 113:89-94, 1990). We now demonstrate that the mechanism of this hemagglutination is glycoprotein C-mediated binding of virus to heparan sulfate moieties at the surface of erythrocytes. Hemagglutination was found to be a common property of all gC-expressing laboratory strains and clinical isolates of HSV-1 tested. Mutants of HSV-1 deficient in glycoprotein C caused no specific hemagglutination, whereas their derivatives transfected with a functional gC-1 gene, thus reconstituting gC expression, regained full hemagglutinating activity. Hemagglutination activity was inhibited by antibodies against gC-1 but not by antibodies with specificity for glycoproteins gB, gD, or gE or by murine antiserum raised against the MP strain of HSV-1, which is gC deficient. Finally, purified gC-1 protein, like whole HSV-1 virions, showed high hemagglutinating activity which was inhibited by heparan sulfate and/or heparin and was completely prevented by pretreatment of erythrocytes with heparitinase, providing evidence that gC-1 mediates hemagglutination by binding to heparan sulfate at the cell surface. Thus, HSV-1-induced hemagglutination is gC-1 dependent and resembles the recently proposed mechanism by which HSV-1 attaches to surface heparans on susceptible cells, providing a simple model for initial events in the virus-cell interaction.

The ability of viruses to agglutinate erythrocytes (RBCs) is a potentially simple model for the study of virus attachment to cellular receptors. Inhibition of this activity, hemagglutination (HA) inhibition (HI), is a rapid method for the detection and titer determination of the antibody response against viral hemagglutinins and has been widely used in clinical and laboratory studies. We recently reported that HA can be caused by herpes simplex virus type 1 (HSV-1) (51). HSV-1 agglutinated RBCs from some strains of mice, most notably C57BL, but not the RBCs of many other species, including humans.

Recent studies on early HSV-cell interactions have revealed that heparan sulfate moieties of the cell plasma membrane serve as a receptor for initial binding of HSV. This assertion is based on findings that heparin, a related glycosaminoglycan, inhibits virus attachment (54), HSV adsorption to heparan sulfate-deficient mutant CHO cells is severely impaired (43), and enzymatic digestion of cell surface heparan sulfate reduces HSV binding (54). Binding of HSV to heparan sulfate depends on the degree of its sulfation, and the smallest polymer of heparin able to inhibit virus attachment was reported to be composed of 10 monosaccharide units (35). The contribution of particular envelope glycoproteins of HSV-1 to this process is not fully understood and is complicated by the fact that at least three of them, glycoproteins B (gB), C (gC), and D (gD), bind to cell membrane components in a noncooperative way (29). The importance of gC in the adsorption of HSV-1 to cells is suggested by several observations. First, gC-1 projections

are long and slender (44) and are probably the most externally exposed structures of the virion. In addition, although both gC and gB can bind to heparin (18), adsorption of gC-negative virions only is greatly reduced compared with that of gB-negative (18) and wild-type (18, 30, 45) virions. Purified, truncated gC-1 shows an adsorption profile similar to that of wild-type gC-positive viruses, and pretreatment of this gC-1 with heparin eliminates its binding to cells (45). Moreover, recent data show that monoclonal antibodies (MAbs) specific for gB, gD, and gH inhibit HSV-1 penetration but have little (some anti-gD MAbs) or no effect on attachment (12-14, 20, 21) and that HSV-1 virions devoid of these glycoproteins are defective in penetration but bind to cells normally (4, 10, 32). On the other hand, some studies suggest a facilitating rather than required role of gC in attachment (30). In addition, the ability of gC-negative viruses to replicate in vitro and in vivo (19) indicates the existence of an alternative, gC-independent, mode of adsorption of HSV-1 to cells, a mechanism also proposed for HSV-2 binding to BHK cells (5). Apart from its function in adsorption, gC acts as a receptor for the C3b component of complement (11).

In the work described in this paper, we sought to study the molecular basis of the HA reaction and to determine whether the binding of HSV-1 to RBCs resembles the above proposed model for the initial interaction of HSV-1 with cells. Given the importance of gC in early virus-cell interactions, we focused on the potential role of this glycoprotein in HSV-1-induced HA. Our results demonstrate that gC is critical to the agglutination of murine RBCs by HSV-1 and suggest that this process depends on gC binding to RBC surface heparan sulfate moieties.

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MATERIALS AND METHODS

Cells and viruses. Rabbit skin (RS) and African green monkey kidney (GMK-AH1 and Vero) cells were grown in Eagle's minimum essential medium supplemented with 5% calf serum (for RS and Vero cells) or 0.05% Primatone RL substance (Sheffield Product, Kraft Inc., Norwich, Conn.) and 2% calf serum (for GMK cells), 100 U of penicillin per ml, and 100 mg of streptomycin per ml. The HSV-1 strains used were 17 syn⁺ (3), F (8), McIntyre (42), KJ 502 (53), a plaque-purified isolate of wild-type KOS strain designated KOS 321 (23), ANG (28, 38), the 17syn⁺ × ANG recombinant F1 (16), the F1 × 17syn⁺ recombinants C1 and C2 (made by cotransfection of unit-length F1 DNA with the cloned 17syn⁺ gC region) (17), the gC-negative MP strain of HSV-1 (22), and (Mp)6-9A, a derivative of MP containing a functional gC-1 gene (31). The last two viruses were kindly supplied by P. Spear, Northwestern University Medical and Dental Schools, Chicago, Ill. Unless otherwise stated, 17 syn⁺, ANG, F1, C1, and C2 viruses were grown and subjected to titer determination in RS cells and F, McIntyre, KJ 502, KOS 321, MP, and (Mp)6-9A were grown and subjected to titer determination in GMK cells. The expression of gC by HSV-1 strains was examined either by immunoblotting performed as described previously (2) or by immunoreactive black-plaque assay (24) with rabbit polyclonal anti-gC antibodies.

Preparation of polyclonal antibodies. Anti-HSV-1 serum (K310) was produced by immunization of rabbits with sodium deoxycholate-solubilized HSV-1 membrane antigen, prepared from the F strain grown in rabbit kidney (RK-13) cells (26). Rabbit anti-gB (K861), anti-gC (K642), and anti-gD (K169) antisera were prepared and characterized previously (40, 41). Antigens for the production of these sera as well as for the production of anti-gE (K860) rabbit antiserum and another anti-gC (K922) rabbit antiserum were prepared by immunoaffinity chromatography as described previously (41). MAbs used for purification, that is B3G11 (anti-gB), B1C1 (anti-gC), E5G3 (anti-gD), and B1E6 (anti-gE), have been characterized in detail elsewhere (1). An additional preparation of rabbit polyclonal anti-HSV-1 gC antiserum (here denoted K1) was kindly provided by Gary Cohen, University of Pennsylvania. HSV-specific immunoglobulins in polyclonal rabbit sera were determined by enzyme-linked immunosorbent assay (ELISA) with the use of solubilized HSV-1 membrane antigen (27). Mouse serum, specific for the MP strain, was obtained by infection of animals in the scarified footpad with 3×10^4 PFU, subcutaneous reinoculation with 10^6 PFU 6 weeks later, and sacrifice 9 days after reinoculation.

Preparation of hemagglutination antigen from HSV. Unless otherwise stated, confluent dense monolayers of GMK cells (48 h old) in six-well plates were washed with serum-free Eagle's minimal essential medium and infected with the specified HSV strain at a multiplicity of infection of 0.1 PFU per cell. Following 2 h of adsorption at 37°C, the inoculum was removed and the cells were washed with serum-free Eagle's minimal essential medium and then maintained in 1.5 ml of the same medium for 65 h at 37°C. The cells were then scraped into the culture fluid and centrifuged at $200 \times g$ for 10 min. The cell pellet was resuspended in 150 μ l of an isotonic solution of NaCl buffered with 0.02 M phosphates (Na_2HPO_4 , KH_2PO_4 [pH 7.0]) (PBS) and subjected to three cycles of freezing and thawing at -70°C in ethanol and 37°C in a water bath, respectively. After the suspension was centrifuged at $1,000 \times g$ for 20 min, the HA titer of

cell-associated (CA) virus was determined by using the supernatant from this cell lysate. The HA titer of extracellular (EX) virus was then determined by using the original supernatant after its further centrifugation at $1,000 \times g$ for 20 min. Uninfected cultures were processed identically to serve as controls. If not examined immediately, both EX and CA HA antigens were stored at -70°C .

Titer determination of HA activity. When not otherwise stated, the HA assay was performed as described previously (51) with some modifications. Briefly, the test was carried out by using U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark). HA diluent medium consisted of PBS with 0.2% bovine serum albumin (BSA; Sigma, St. Louis, Mo.) and 0.5 ml of 1% gelatin (Kistner Laboratories, Stockholm, Sweden) per 100 ml. C57BL/6 mice were obtained either from breeding colonies at the Department of Virology, University of Göteborg, or purchased from National Cancer Institute contract facilities. Blood from at least two mice was collected into Alsever solution (24a) and washed three times with PBS. The RBCs were sedimented once more by centrifugation at $400 \times g$ for 12 min prior to the preparation of the 0.4% suspension. The HA test was performed by adding 50- μ l portions of a 0.4% suspension of the RBCs in HA diluent to equal volumes of serial twofold dilutions of the prepared HA reagent (also in HA diluent). After careful shaking, the mixtures were left undisturbed for 1 to 2 h at room temperature before the HA titer was read. Titers are given as the reciprocal of the highest dilution of virus causing HA.

Determination of HI activity. All serum samples were heated at 56°C for 30 min. Unless otherwise stated, they were diluted 1:5 in the HA diluent and mixed with an equal volume of a 25% suspension of acid-washed kaolin to remove nonspecific inhibitors of HA. Following 30 min of agitation at room temperature, the kaolin was sedimented by centrifugation at $1,000 \times g$ for 10 min, and 100 μ l of the supernatant was mixed with 25 μ l of packed mouse RBCs (prepared as described above). After incubation for 1 h at 37°C with occasional manual shaking and then for 30 min at room temperature without shaking, the cells were removed by centrifugation at $1,000 \times g$ for 10 min and the supernatant (now diluted 1:10) was used in the HI test. HA diluent medium was added to every well of the microplate (25 μ l per well) except for the first and the last one in a row, and then 25 μ l of serum was added to the first, second, and last wells (serum control). Serial twofold dilutions of serum were made starting from the second well, and then equal volumes of HA diluent containing 4 HA units of the HA antigen were added. After shaking, the mixtures were incubated for 2 h at 37°C . Subsequently, 50- μ l volumes of 0.4% suspension of RBCs were added, and after shaking, the plates were kept at room temperature for 1 to 2 h before the titer was read. The HI activity is expressed as the highest dilution of serum completely inhibiting HA.

Purification of virions. Roller cultures of GMK cells were infected at a multiplicity of infection of 1 PFU per cell. Viral material was harvested at 72 h after infection and then concentrated and purified by centrifugation through Ficoll 400 (Pharmacia Biotech AB) gradients as described previously (47). An alternate method of virus purification included clarification of [^3H]thymidine-labeled viral material by centrifugation at $15,000 \times g$ for 5 min and separation of the virus peak on a Sepharose CL-2B (Pharmacia Biotech AB) column as previously described (46). The virus was finally concentrated by centrifugation at $80,000 \times g$ for 1 h. Viral particles were counted as described previously (47).

Purification of gC-1 by affinity chromatography. BHK cells in roller cultures were infected with strain F at a multiplicity of infection of 0.1 PFU per cell and harvested when the cytopathic effect was advanced, usually after 3 days. HSV membrane antigens were prepared from infected cells by the method of Jeansson et al. (27) and solubilized with 0.5% Triton X-100 in 0.1 M glycine-NaOH buffer (pH 8.8). Insoluble material was pelleted by centrifugation at $160,000 \times g$ for 1 h, and the supernatant was applied to a column containing *Helix pomatia* lectin coupled to Sepharose 6MB (Pharmacia Biotech AB). After 15 min of adsorption at room temperature, the gel was washed with 10 bed volumes of TBS (0.15 M NaCl, 0.02 M Tris-HCl [pH 7.5]) containing 0.5% Triton X-100 and the adsorbed material was eluted with 0.02 M *N*-acetyl-D-galactosamine in the same buffer. Each fraction was examined by ELISA for the presence of gC-1, and the selected material was subjected to further purification by immunoaffinity chromatography with the use of B1C1 anti-gC MAb as described previously (40, 41). The concentration of protein in this preparation was determined by the procedure of Lowry et al. (33) modified for measurement in detergent-containing solutions (7). A portion of sample containing 35 μ g of protein was precipitated with 2.5 volumes of cold acetone and centrifuged, and the pellet was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (37). The separated proteins were electroblotted onto a polyvinylidene difluoride membrane and stained with 0.1% Coomassie blue R-250.

Effects of heparin and heparan sulfate on HA activity. For both assays, viral HA antigen was adjusted with HA diluent to contain 32 to 64 HA units per 25 μ l. Heparin (159 U/mg; Sigma) was either added to the HA diluent at final concentrations of 0.0075 to 2.0 μ g/ml or mixed with the specified HSV-1 strain at a final concentration of 4 μ g/ml. After incubation for 30 min at 37°C, the mixture was tested for HA activity. In other studies, heparin (40 μ g/ml) was incubated at 37°C for 30 min with a 0.4% suspension of RBCs in HA diluent and the RBCs were then washed three times with PBS before being used as targets in the assay for HA activity. Heparan sulfate, preparation C (1.5 sulfate groups per disaccharide unit), was kindly provided by Ulf Lindahl, Swedish University of Agricultural Sciences, Uppsala. For HI, 25- μ l portions of serial twofold dilutions of heparan sulfate in HA diluent medium were added to equal volumes of serial twofold dilutions of the HA reagent in microtiter U-bottom plates. After shaking, the mixtures were incubated at 37°C for 15 min and then 50 μ l of a 0.4% suspension of RBCs in HA diluent was added.

Enzymatic treatment of RBCs. RBCs were treated with heparitinase (EC 4.2.2.8; Sigma) by a method similar to that described for cells in culture (54). A 1.3-ml portion of a 0.4% suspension of C57BL mouse RBCs in HA diluent was sedimented by centrifugation ($750 \times g$ for 5 min) and then resuspended in PBS containing 1 mM CaCl_2 , 0.5 mM MgCl_2 , 0.05% BSA, and the indicated number of heparitinase units. After incubation for 1 h at 37°C, the cells were sedimented by centrifugation, washed three times with 0.4 ml of HA diluent, and finally resuspended in the original volume of HA diluent for use in the HA assay. Neuraminidase (Sigma) dissolved in PBS was mixed with a 0.4% suspension of mouse RBCs to provide a final concentration of 1 U/ml. Following incubation with neuraminidase for 1 h at 37°C, the RBCs were washed three times with PBS before being tested for HA activity.

Hemolytic and hemofusing activity of HSV-1. To determine

whether hemolytic activity was present, 0.2-ml portions of the CA preparation of HA reagent derived from strain 17 syn⁺, strain ANG, or uninfected cell material were mixed with 1-ml portions of a 0.4% suspension of C57BL/6 mouse RBCs. Following 2 h of adsorption at 4°C, the cells were pelleted by centrifugation at $500 \times g$ for 5 min and resuspended in 0.5 ml of PBS (pH 7.0 or 8.0). After incubation for 30 min at 37°C, the RBCs were pelleted again and the optical density of the supernatant was determined at 540 nm. For hemofusing activity, 0.2-ml portions of the same virus material as used in the hemolysis assay were mixed with 100 μ l of a 0.1% suspension of RBCs. Following 2 h of adsorption at 4°C, the samples were centrifuged at $500 \times g$ for 5 min, resuspended in 100 μ l of PBS (pH 7.0 or 8.0), and, after 15 min of incubation at 37°C, examined for hemofusion by light microscopy.

RESULTS

HA activity, infectivity, particle/HA ratio, and gC-1 expression by HSV-1 strains. Two preparations of each strain were assayed: EX, i.e., infectious culture fluid, and CA, i.e., material released from infected cells into a small volume of PBS by freezing and thawing. Because of the possibility of nonspecific reactions, especially with CA preparations, the viral specificity of HA was always verified by its inhibition by anti-HSV-1 rabbit antiserum (K310). We first determined the HA activity of HSV-1 strains which differ in their expression of gC. HSV-1 F, KOS 321, McIntyre, and the syncytial KJ 502 virus are gC positive whereas MP is gC deficient (6). The phenotype of these strains with respect to the presence of gC was confirmed by immunoblots. All strains expressing gC-1 showed high, specific HA activity and little strain-dependent variation (Table 1). CA virus showed lower infectivity/HA ratios than EX virus did, possibly a consequence of the freeze-thaw procedure used for its preparation. The HA titer range and infectivity of McIntyre strain virions purified by centrifugation through Ficoll gradients were 512 to 1,024 and 3.5×10^9 PFU/ml, respectively (data not shown). Similarly, the six clinical isolates of HSV-1 so far tested in our laboratory were all found by immunoblot to be gC positive and to agglutinate mouse RBCs. The HA titer ranges for their EX and CA HA antigens were 32 to 256 and 128 to 512, respectively (data not shown). In contrast, the gC-negative MP strain, propagated in either RS or Vero cells, caused no HA. When grown in GMK cells, the MP strain induced a low HA titer, which was detected only in the CA preparation. Interestingly, this HA was completely inhibited by heparan sulfate at a final concentration of 1 μ g/ml, and it was fully prevented by the treatment of RBCs with 0.2 U of heparitinase (data not shown). Neither rabbit anti-HSV-1 antiserum (Table 1), polyclonal anti-gB and anti-gC-1 antibodies, nor mouse serum raised against the MP strain (data not shown) was able to inhibit this low level of HA observed in GMK cells, indicating that the hemagglutinins detected were not immunogenic constituents of the MP virions. We also calculated the minimum number of virus particles necessary to induce HA. For the KOS 321 strain, purified on a CL-2B Sepharose column, that value was 1.02×10^5 per HA unit, corresponding to 2.04×10^6 virus particles per ml of the sample.

Inhibition of HA activity by polyclonal antibodies. Having determined that HA is a common property of HSV-1, we sought to identify the virus constituent(s) responsible for it. To this end, rabbit polyclonal antibodies reactive with gB,

TABLE 1. HA activity and infectivity of HSV-1^a

Virus strain	HA titer		Specificity of HA ^b	Infectivity (PFU/ml)	
	EX	CA		EX	CA
F	128	256–512	40	1.6×10^8	9.5×10^7
KOS 321	256	512–1,024	40	4.5×10^8	4.8×10^8
KJ 502	256	256–512	40	2.4×10^8	8.5×10^7
McIntyre	128	256–512	NT ^c	4.7×10^8	2.5×10^8
MP (GMK)	<2	32–64	<10	6.8×10^7	1.5×10^7
MP (RS, Vero) ^d	<2	<2		NT	NT
Control (GMK, Vero)	<2	<2			
Control (RS)	<2	<2–2			

^a EX and CA HA antigens were prepared from infected GMK cells as described in Materials and Methods. Two independent experiments were performed, and the results are given as a HA titer range or mean number of PFU per milliliter.

^b Inhibition of HA by rabbit anti-HSV-1 antiserum (K310). Results are presented as the reciprocal of the HI titer.

^c NT, not tested.

^d HA reagents prepared from MP-infected cells harvested at 48 h (RS cells) or 72 h (Vero cells) after infection.

gC, gD, gE, and whole HSV-1 membrane antigens, as well as mouse serum raised against the gC-negative MP strain of HSV-1, were tested either alone or in combinations for their ability to inhibit HA by strain F (Table 2). HA activity was inhibited by polyclonal antibodies against whole HSV-1 envelope antigens (K310) but not by preimmune (K861) or HSV-1-negative (K68) rabbit sera, suggesting that HA is specific and associated with the viral envelope. The HA-inhibitory action of two preparations of anti-gC-1 polyclonal antibodies, expressing high (K922) or low (K642) levels of anti-HSV-1 ELISA antibody, indicated involvement of this glycoprotein in HA. A third anti-gC-1 polyclonal antibody preparation (K1) also was highly inhibitory to HA induced by HSV-1. It inhibited the HA activity of strain 17 syn⁺ up to a titer of 1:640 (data not shown). In contrast, polyclonal anti-gB (K861 immune serum), anti-gD (K169), and anti-gE (K860) antisera did not inhibit HA either alone or in combination. The levels of anti-HSV-1 ELISA antibodies in these

noninhibitory antisera were equal to or higher than that in the HA-inhibitory K642 anti-gC-1 preparation. Neutralizing mouse anti-MP antiserum, presumably containing antibody against major antigenic determinants of HSV-1 other than gC-1, also failed to inhibit HA. Furthermore, an anti-gC-1 MAb, B1C1, completely prevented agglutination of RBCs by HSV-1 even at IgG concentration as low as 0.12 µg/ml (dilution, 1:4,096).

Effect of molecular transfer of gC expression on HSV-1-induced HA. We compared the HA activity of the gC-negative MP strain with that of its derivative, the (Mp)6-9A mutant, in which a functional gC-1 gene (originated from the F strain of HSV-1) was inserted into the thymidine kinase gene (31). In contrast to the parental gC-negative MP strain, extracellular HA antigen from the gC-expressing (Mp)6-9A mutant showed high HA activity (Table 3). HA induced by both preparations of this mutant, but not the HA induced by CA antigen of strain MP (Table 1), was inhibited by anti-gC-1 antiserum, indicating that the expression of gC-1 was indispensable for the restoration of HA activity to (Mp)6-9A.

We also assayed the HA activity of the virulent gC-

TABLE 2. Antibody effects on HA by HSV-1^a

Antibody or serum	Specificity	Titers ^b in:	
		HI test	ELISA
K310	HSV-1 envelope	40	3,200
K922	gC-1	160	6,400
K642	gC-1	20	400
K861	gB	<10	12,800
K169	gD	<10	800
K860	gE	<10	400
K861/K169/K860	gB/gD/gE	<10	NT ^c
Preimmune (K861)		<10	<100
HSV-negative (K68)		<10	<100
Mouse MP	MP (HSV-1)	<10	NT ^d
Mouse negative		<10	NT ^d
B1C1B4 ^e	gC-1	4,096	NT

^a In all assays the F strain was used as the antigen. Rabbit or mouse polyclonal antibodies were diluted serially in a twofold manner, starting from 1:10 and 1:100 for the HI assay and ELISA, respectively.

^b The results are presented as the reciprocal of the highest dilution of serum inhibiting the HA activity of 4 HA units of the HA reagent (HI test) or giving an optical density at 490 nm of ≥ 0.25 (ELISA).

^c NT, not tested.

^d Antibody titers in the complement-independent neutralization assay with an incubation of serum-virus (100 50% tissue culture infective doses) mixture for 1 h at 37°C were 128 and <4 for mouse MP and mouse negative serum, respectively.

^e The MAb preparation was adjusted with HA diluent to contain 1 mg of IgG per ml, and after treatment with kaolin, it was tested for HI activity (the final dilution of sample in the first well was 1:4).

TABLE 3. Effect of restoration of gC expression on HA activity^a

Virus	Origin	HA activity ^b with:		Specificity of HA ^c
		C57BL/6 cells	BALB/c cells	
MP	EX	<2	NT ^d	NT
	CA	32–64	NT	<10
(Mp)6-9A	EX	128–256	NT	160
	CA	64–128	NT	160
17 syn ⁺	EX	4–8	4–128	NT
	CA	512–1,024	512–1,024	640
F1	EX	<2	<2	NT
	CA	<2	<2–64	NT
F1 gC+1	EX	8–16	8–32	NT
	CA	512–1,024	512–2,048	320
F1 gC+2	EX	8–16	8–32	NT
	CA	256–1,024	512–2,048	NT

^a EX and CA HA reagents were made from RS cultures infected with parental (17syn⁺ and F1) or 17 syn⁺ × F1 recombinant (F1 gC+1 and F1 gC+2) strains and harvested 48 h later. HA antigens of MP and (Mp)6-9A strains were made from GMK cells harvested 65 h after infection.

^b Results are expressed as the reciprocal of HA titer range from three repetitions.

^c HI by K1 or K922 rabbit anti-gC-1 serum [for MP and (Mp)6-9A].

^d NT, not tested.

deficient 17 syn⁺ × ANG recombinant, F1, which contains an ANG insert in the *Eco*RI F region of the 17 syn⁺ strain (16). We were interested in whether the restoration of full gC expression also restored HA activity. The gC-encoding fragment from wild-type strain 17syn⁺ was cotransfected with unit-length F1 DNA as described previously (16, 17), and wild-type gC-positive progeny viruses were identified by using an immunoreactive black-plaque assay. Two plaque-purified progeny viruses (F1 gC+1 and F1 gC+2) which expressed high levels of gC were then tested for their HA activity and compared with the parental strains F1 and 17 syn⁺, using both C57BL and BALB/c RBCs (Table 3). F1 showed no HA activity with C57BL/6 mouse RBCs and little or no HA activity with BALB/c RBCs. The restoration of full gC expression to F1 in F1 gC+1 and F1 gC+2 conferred high titers of HA activity similar to that of the wild-type 17 syn⁺ parent. The higher CA/EX titer ratios compared with the values presented in Table 1 occurred because virus stocks for this experiment were prepared in RS cells and were harvested 48 h after infection when most of the HSV-1 hemagglutinins were cell associated.

HA activity of purified gC-1. Additional proof that gC-1 is involved in HA was obtained by testing the HA activity of gC-1 isolated from the virus envelope. We hypothesized that because solubilized, monomeric viral glycoproteins tend to form micelles or other aggregates in the absence of detergent, mainly owing to hydrophobic interactions of their transmembrane regions (36), such polymeric structures may be capable of cross-linking ligands, including RBCs. Affinity chromatography with *Helix pomatia* lectin and the B1C1 MAb was used to purify gC. Its purity was confirmed by SDS-polyacrylamide gel electrophoresis, in which it migrated as a single diffuse band corresponding to an apparent molecular weight range of 105,000 to 120,000 (Fig. 1). The difference between the molecular weight of this preparation and that of native gC-1 (130,000) is attributed to the fact that *H. pomatia* lectin, used here in the first step of purification, selects glycoforms of gC-1 with much shorter O-linked glycans (34, 41). The purity of the preparation was approximately 90% by densitometric analysis. Purified gC-1 agglutinated mouse RBCs with a titer of up to 1:20,480, equivalent to 1,067 HA units/μg of protein, and this HA activity was inhibited by K642 rabbit anti-gC-1 antiserum (titer, 1:80).

Effects of heparin and heparan sulfate on HA activity of HSV-1. We next sought to determine whether hemagglutination is mediated by interactions between gC and RBC surface heparans. We first tested whether HA can be inhibited by heparin, a molecule closely resembling a characteristic disaccharide repeat sequence of the heparan sulfate glycosaminoglycan chain but showing a higher degree of sulfation (15). Heparin, when present in the HA diluent at 2 μg/ml, completely inhibited the HA activity of the 17 syn⁺ strain (64 HA units); this effect was first noted at a heparin concentration of 0.03 μg/ml (not shown). Complete inhibition of the HA activity of HSV-1 17 syn⁺ was also observed when heparin (4 μg/ml) was directly mixed with 64 HA units of the virus and incubated for 30 min at 37°C before being tested. In contrast, 17 syn⁺ still caused HA with the same titer, 1:64, when tested with RBCs pretreated with heparin (40 μg/ml). These experiments suggested that the interaction of heparin with virions, but not with RBCs, is the basis for HI. Because we had already observed that purified gC-1 caused HA, we hypothesized that this glycoprotein binds to RBC surface heparan sulfate. Indeed, as can be seen from Fig. 2, the HA induced by 32 HA units of purified gC-1 was completely inhibited by heparan sulfate at a final concentra-

FIG. 1. Electrophoretic characterization of affinity-purified gC-1. Affinity-purified gC-1 was precipitated with cold acetone, subjected to electrophoresis in a separation gel of 9.25% acrylamide, and stained with Coomassie blue R-250. The positions of the commercial molecular weight markers, myosin (205,000), β -galactosidase (116,000), phosphorylase *b* (97,000), and albumin (66,000) are indicated on the right.

tion of 0.5 μg/ml. The HA induced by KOS 321 and F strains was somewhat more resistant to heparan sulfate than was the HA induced by purified gC-1; this difference probably resulted from the structural differences between the forms of hemagglutinin particles. Heparan sulfate may have improved access to purified gC-1; alternately, fewer purified gC molecules may have been involved in the cross-linking of RBCs.

Enzymatic treatments of RBCs. We exposed mouse RBCs to enzymatic digestion with heparitinase and then tested their ability to be agglutinated by purified gC-1. Heparitinase shows specificity for N-acetylated units of heparan sulfate glycosaminoglycan chain (15). Pretreatment of mouse RBCs with heparitinase at a concentration of 0.2 U/0.4 ml fully prevented agglutination by the KOS 321 and F strains of HSV-1 and by purified gC-1 (Table 4). In contrast to the effects of heparin and heparan sulfate, the range of HI by treatment of RBCs with heparitinase was narrow, suggesting that a limiting concentration of intact heparan sulfate is required for HA to occur. In contrast to heparitinase, neuraminidase treatment of mouse RBCs did not affect agglutination by HSV-1 (data not shown).

Hemolytic and hemofusing activities of HSV-1. The rationale for the experiments was to determine whether HSV-1 attached to the RBC surface is able to penetrate into the cell. Viral hemolytic and hemofusing properties have been used as markers for such abilities. We studied both the standard

FIG. 2. Effect of heparan sulfate on the HA activity of purified gC-1. Portions (25 µl) of serial twofold dilutions of heparan sulfate were added to equal volumes of serial twofold dilutions of purified gC-1 or HA antigens prepared from HSV-1 KOS 321 or F. After incubation for 15 min at 37°C, 50 µl of a 0.4% suspension of RBCs was added. The mixtures were shaken, and the HA titer was read after a further 1 to 2 h. Results are given as the mean geometric HA titer from two determinations.

17 syn⁺ laboratory strain of HSV-1 and the ANG strain, which produces giant polykaryocytes in cell culture. We tested for these properties both at pH 7.0 and at pH 8.0; the latter pH is known to be optimal for ANG-induced "fusion from without" (9). By using the methods described above, hemolytic and hemofusing activities could not be demonstrated with either strain (data not shown).

DISCUSSION

Our data indicate that the molecular basis of agglutination of mouse RBCs by HSV-1 is the binding of viral gC to RBC surface heparan sulfate, a mechanism closely resembling that of the initial attachment of HSV-1 to susceptible cells. This conclusion emerges from several lines of independently derived evidence.

Only antibodies with specificity for gC-1 inhibited HA. In contrast, anti-gB, anti-gD, and anti-gE sera, alone or in

concert, did not prevent HA. Mouse immune serum raised against the gC-negative MP strain also failed to inhibit HA despite its high virus-neutralizing activity. One of the anti-gC-1 sera (K642) and the MAb (B1C1) which we used here have already been found to inhibit adsorption of HSV-1 to susceptible cells in culture when tested by the ELISA-adsorption method (45). In addition, adsorption inhibition experiments performed in cell cultures (13, 30) revealed an ability of some anti-gC MAbs to inhibit HSV-1 attachment. It has also been reported that polyclonal anti-gD antiserum and some anti-gD MAbs reduced HSV-1 adsorption (13, 21). With our HA model of HSV-1 attachment, such inhibition was not observed, and this is consistent with the observation that virions devoid of gD bind to cells normally (32). One cannot, however, rule out the possibility that attachment is a sequential process and that an initial interaction of gC-1 with the cell may trigger a gD-mediated step.

We next demonstrated that HA activity is strictly dependent on gC-1 expression. Thus, all gC-positive laboratory strains and clinical isolates of HSV-1 showed high levels of HA activity whereas little or no HA was detected in the gC-deficient viruses MP and F1. We also documented the restoration of HA activity to derivatives of the gC-deficient and HA-deficient MP and F1 mutants of HSV-1 by molecular rescue of gC-1 expression. These findings are consistent with recent reports that gC-deficient mutants of HSV-1 attach to susceptible cells at a reduced rate compared with that of wild-type viruses (18, 30, 45). We observed a low level of HA in extracts of GMK cells infected with the MP strain, but this HA was not inhibited by various anti-HSV-1 antisera. Interestingly, although not blocked by antibodies to HSV, these hemagglutinins appeared to bind to a cellular receptor, similar to HSV-1-specific hemagglutinins, as deduced by their inhibition by soluble heparan sulfate and their inability to bind to heparinase-treated RBCs. This HA may be induced by some other physiologically important molecule(s) known to interact with heparan sulfate such as protease inhibitors, plasma lipoproteins, cell-specific growth factors, and lipolytic enzymes (reviewed in reference 25). A possible utilization of such a heparan sulfate-binding factor as a substitute for gC in gC-deficient virions cannot be excluded.

Finally, we demonstrated that affinity-purified gC-1 efficiently caused HA which could also be inhibited by heparan sulfate and prevented by enzymatic digestion of heparan sulfate at the surface of RBCs. Thus, gC-1 itself appears to be capable of mediating HSV-1 binding to cell surface heparan sulfate. Earlier it was observed that heparin and heparan sulfate markedly reduced the adsorption of both HSV-1 and HSV-2 to cells (5, 18, 35, 54), and, because solubilized gC can bind to a heparin-Sepharose column, it was suggested that HSV-1 attachment to cells can be mediated by this glycoprotein and that this function can also be performed by another heparin-binding protein of the virus, gB (18). Our results strongly support the importance of gC as a specialized HSV-1 attachment protein. However, it appears that HA by HSV-1 is strictly gC dependent, since no other viral glycoprotein was able to replace the HA function of gC. This implies that HA may be a very useful method for the study of the attachment functions of gC-1. In contrast, classical methods for the measurement of HSV-1 attachment, i.e., binding of radiolabeled virus to cells, may not discriminate between absorption mediated by gC and that by substitute glycoproteins or other factors (e.g., nonspecific binding).

The ability to agglutinate murine RBCs appears to be a

TABLE 4. Effect of treatment of RBCs with heparitinase on the HA activity of HSV-1 and purified gC-1

Heparitinase units/0.4 ml	HA ^a by:		
	HSV-1 F	HSV-1 KOS 321	gC-1
0	32	32	32
0.006	32	32	32
0.012	32	32	32
0.025	32	32	32
0.05	16	16	32
0.1	16 ^b	16 ^b	16 ^b
0.2	<2	<2	2
0.4	<2	<2	<2
0.8	<2	<2	<2

^a Results are presented as a reciprocal of HA titer.

^b Incomplete HA seen.

property shared by at least some other alphaherpesviruses. Apart from HSV-1, such activity has been described for pseudorabies virus (48, 50), bovine herpesvirus 1 (49), and avian infectious laryngotracheitis virus (39), suggesting the involvement of a similar mechanism in the early interaction of these viruses with cells. Although heparan sulfate and related glycosaminoglycans are ubiquitous cell surface components, an intriguing question is why the ability of RBCs to be agglutinated by HSV-1 and other hemagglutinating herpesviruses is restricted to murine RBCs. Heparan sulfate is a complex and heterogeneous macromolecule (15). In addition to the degree of its sulfation (35, 43), specific spacing of anionic sulfate groups may be important in interactions with HSV. We assume that the form or quantity of this compound present on the mouse RBC surface, but not on RBCs of other species, fulfills the needed criteria. In ongoing studies we have identified heparan sulfate as a target for polycationic substances and demonstrated that an arginine-rich domain at the antigenic site II of gC-1 may, like other polycations, bind to heparan sulfate (52).

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REFERENCES

- Bergström, T., E. Sjögren-Jansson, S. Jeansson, and E. Lycke. 1992. Mapping neuroinvasiveness of the herpes simplex virus type 1 encephalitis-inducing strain 2762 by the use of monoclonal antibodies. *Mol. Cell. Probes* 6:41-49.
- Bergström, T., A. Vahlne, K. Alestig, S. Jeansson, M. Forsgren, and E. Lycke. 1990. Primary and recurrent herpes simplex virus type 2-induced meningitis. *J. Infect. Dis.* 162:322-330.
- Brown, S. M., D. A. Ritchie, and J. H. Subak-Sharpe. 1973. Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. *J. Gen. Virol.* 18:329-346.
- Cai, W. H., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* 62:2596-2604.
- Campadelli-Fiume, G., D. Stirpe, A. Boscaro, E. Avitabile, L. Foa-Tomasi, D. Barker, and B. Roizman. 1990. Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. *Virology* 178:213-222.
- Draper, K. G., R. H. Costa, G. T.-Y. Lee, P. G. Spear, and E. K. Wagner. 1984. The molecular basis of the glycoprotein C negative phenotype herpes simplex virus type 1 macroplaque strain. *J. Virol.* 51:578-585.
- Dulley, J. R., and P. A. Grieve. 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal. Biochem.* 64:136-141.
- Ejercito, P. M., E. D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. *J. Gen. Virol.* 3:357-364.
- Falke, D., A. Knoblich, and Y. Becker. 1985. Fusion from without induced by herpes simplex virus type 1. *Intervirology* 24:211-219.
- Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* 66:341-348.
- Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines. 1984. Glycoprotein C of HSV-1 functions as a C3b receptor on infected endothelial cells. *Nature (London)* 309:633-635.
- Fuller, A. O., R. E. Santos, and P. G. Spear. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J. Virol.* 63:3435-3443.
- Fuller, A. O., and P. G. Spear. 1985. Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. *J. Virol.* 55:475-482.
- Fuller, A. O., and P. G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. *Proc. Natl. Acad. Sci. USA* 84:5454-5458.
- Gallagher, J. T., M. Lyon, and W. P. Steward. 1986. Structure and function of heparan sulphate proteoglycans. *Biochem. J.* 236:313-325.
- Goodman, J. L., M. L. Cook, F. Sederati, K. Izumi, and J. G. Stevens. 1989. Identification, transfer, and characterization of cloned herpes simplex virus invasiveness regions. *J. Virol.* 63:1153-1161.
- Haanes, B., C. M. Nelson, and J. L. Goodman. Unpublished data.
- Herold, B. C., D. WuDunn, N. Soltys, and P. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* 65:1090-1098.
- Hidaka, Y., Y. Sakai, Y. Toh, and R. Mori. 1991. Glycoprotein C of herpes simplex virus type 1 is essential for the virus to evade antibody-independent complement-mediated virus inactivation and lysis of virus-infected cells. *J. Gen. Virol.* 72:915-921.
- Highlander, S. L., W. H. Cai, S. Person, M. Levine, and J. C. Glorioso. 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. *J. Virol.* 62:1881-1888.
- Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. *J. Virol.* 61:3356-3364.
- Hoggan, M. D., and B. Roizman. 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. *Am. J. Hyg.* 70:208-219.
- Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso. 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. *J. Virol.* 45:672-682.
- Holland, T. C., R. M. Sandri-Goldin, L. E. Holland, S. D. Marlin, M. Levine, and J. C. Glorioso. 1983. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. *J. Virol.* 46:649-652.
- Hoskins, J. M. 1967. *Virological procedures*, p. 327. Butterworth and Co. Ltd., London.
- Jackson, R. L., S. J. Busch, and A. D. Cardin. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.* 71:481-539.
- Jeansson, S., H. Elwing, H. Nygren, and S. Olofsson. 1982. Evaluation of solubilized herpes simplex virus membrane antigens in diffusion in gel-enzyme-linked immunosorbent assay (DIG-ELISA). *J. Virol. Methods* 4:167-176.
- Jeansson, S., M. Forsgren, and B. Svennerholm. 1983. Evaluation of solubilized herpes simplex virus membrane antigen by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 18:1160-1166.
- Kaerner, H. C., C. H. Schroder, A. Ott-Hartmann, G. Kumel, and H. Kirchner. 1983. Genetic variability of herpes simplex virus: development of a pathogenic variant during passaging of a nonpathogenic herpes simplex virus type 1 virus strain in mouse brain. *J. Virol.* 46:83-93.
- Kuhn, J. E., M. D. Kramer, W. Willenbacher, U. Wieland, E. U.

- Lorentzen, and R. W. Braun. 1990. Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. *J. Virol.* **64**:2491–2497.
30. Langeland, N., A. M. Oyan, H. S. Marsden, A. Cross, J. C. Glorioso, L. J. Moore, and L. Haarr. 1990. Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to cellular receptor. *J. Virol.* **64**:1271–1277.
31. Lee, G. T.-Y., K. L. Pogue-Geile, L. Pereira, and P. G. Spear. 1982. Expression of HSV gC from a DNA fragment inserted into the thymidine kinase gene of this virus. *Proc. Natl. Acad. Sci. USA* **79**:6612–6616.
32. Ligas, M. W., and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β -galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* **62**:1486–1494.
33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**:265–275.
34. Lundström, M., S. Olofsson, S. Jeansson, E. Lycke, R. Datema, and J. E. Månsson. 1987. Host cell induced differences in O-glycosylation of the herpes simplex virus gC-1. I. Structures of non-sialylated HPA- and PNA-binding carbohydrates. *Virol. J.* **161**:385–394.
35. Lycke, E., M. Johansson, B. Svennerholm, and U. Lindahl. 1991. Binding of herpes simplex virus to cellular heparan sulphate, an initial step in the adsorption process. *J. Gen. Virol.* **72**:1131–1137.
36. Morein, B., and K. Simons. 1985. Subunit vaccines against enveloped viruses: virosomes, micelles and other protein complexes. *Vaccine* **3**:83–93.
37. Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer. 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 $\times$ HSV-2 recombinants. *J. Virol.* **26**:389–410.
38. Munk, K., and G. Ludwig. 1972. Properties of plaque variant of herpes virus hominis strains of genital origin. *Arch. Gesamte Virusforsch.* **37**:308–315.
39. Noda, M., K. Miura, K. Yamanaka, and Y. Inaba. 1990. Hemagglutination with avian infectious laryngotracheitis virus. *Arch. Virol.* **114**:137–142.
40. Olofsson, S., I. Sjöblom, J. C. Glorioso, S. Jeansson, and R. Datema. 1991. Selective induction of discrete epitopes of herpes simplex virus type 1-specified glycoprotein C by interference with terminal steps in glycosylation. *J. Gen. Virol.* **72**:1959–1966.
41. Olofsson, S., I. Sjöblom, M. Lundström, S. Jeansson, and E. Lycke. 1983. Glycoprotein C of herpes simplex virus type 1. Characterization of O-linked oligosaccharides. *J. Gen. Virol.* **64**:2735–2747.
42. Pauls, F. P., and W. R. Dowdle. 1967. A serologic study of herpesvirus hominis strains by microneutralization test. *J. Immunol.* **98**:941–947.
43. Shieh, M. T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear. 1982. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.* **116**:1273–1281.
44. Stannard, L. M., A. O. Fuller, and P. G. Spear. 1987. Herpes simplex virus glycoproteins associated with different morphological entities projecting from the virus envelope. *J. Gen. Virol.* **68**:715–725.
45. Svennerholm, B., S. Jeansson, A. Vahlne, and E. Lycke. 1991. Involvement of glycoprotein C (gC) in adsorption of herpes simplex virus type 1 (HSV-1) to the cell. *Arch. Virol.* **120**:273–279.
46. Svennerholm, B., A. Vahlne, S. Jeansson, R. Lunden, S. Olofsson, G. Svantesson, and E. Lycke. 1980. Separation of herpes simplex virus virions and nucleocapsids on Percoll gradients. *J. Virol. Methods* **1**:303–309.
47. Szilagyi, J. F., and C. Cunningham. 1991. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. *J. Gen. Virol.* **72**:661–668.
48. Tetsu, N., Y. Inaba, M. Yukawa, K. Yoshiki, T. Hirahara, Y. Furuya, S. Ito, A. Yonemocki, and H. Ishikawa. 1989. Hemagglutination with pseudorabies virus. *Arch. Virol.* **106**:321–326.
49. Trepanier, P., C. Seguin, Y. Bastien, G. Boulay, G. Lussier, and M. Trudel. 1985. Hemagglutinating activity associated with bovine herpesvirus type 1. *Vet. Microbiol.* **10**:517–523.
50. Trybala, E. 1989. Hemagglutination by Aujeszky's disease virus and herpes simplex virus type 1. *Med. Vet.* **45**:469–470. (In Polish.)
51. Trybala, E., Z. Larski, and J. Wisniewski. 1990. Hemagglutination by herpes simplex virus type 1. *Arch. Virol.* **113**:89–94.
52. Trybala, E., T. Bergström, B. Svennerholm, S. Jeansson, J. C. Glorioso, and S. Olofsson. Unpublished data.
53. Vahlne, A., J. Blomberg, S. Olofsson, and E. Lycke. 1975. Subtyping of herpes simplex virus. *Acta Pathol. Microbiol. Scand. Ser. B* **83**:506–512.
54. WuDunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63**:52–59.