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A Novel Role for 3-*O*-Sulfated Heparan Sulfate in Herpes Simplex Virus 1 Entry

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Summary

Herpes simplex virus type 1 (HSV-1) binds to cells through interactions of viral glycoproteins gB and gC with heparan sulfate chains on cell surface proteoglycans. This binding is not sufficient for viral entry, which requires fusion between the viral envelope and cell membrane. Here, we show that heparan sulfate modified by a subset of the multiple D-glucosaminyl 3-*O*-sulfotransferase isoforms provides sites for the binding of a third viral glycoprotein, gD, and for initiation of HSV-1 entry. We conclude that susceptibility of cells to HSV-1 entry depends on (1) presence of heparan sulfate chains to which virus can bind and (2) 3-*O*-sulfation of specific glucosamine residues in heparan sulfate to generate gD-binding sites or the expression of other previously identified gD-binding receptors.

Introduction

Heparan sulfate (HS) is widely expressed in animal and human tissues and has diverse roles in development, differentiation, and homeostasis. HS and other glycosaminoglycans are unbranched polymers covalently attached to the protein cores of proteoglycans, which are ubiquitously expressed as integral membrane proteins, glycerol phosphatidyl inositol-linked membrane proteins, and proteins of the extracellular matrix. The HS polymer is assembled by sequential addition of D-glucuronic acid (GlcA) alternating with *N*-acetyl glucosamine (GlcNAc). The chains are then modified heterogeneously and in domains by deacetylation and *N*-sulfation of glucosamine, epimerization of GlcA residues to iduronic acid (IdoA), and sulfation of hydroxyl groups. These modifications provide specific binding sites for a variety of proteins, including cell adhesion molecules, growth factors, chemokines, and factors regulating angiogenesis and blood coagulation. Protein binding to HS may serve to sequester the protein at a particular site or to activate the protein. For example, the binding of antithrombin to a specific pentasaccharide sequence in HS results in striking activation of its anticoagulant activity (reviewed by Rosenberg et al., 1997; Lindahl et al., 1998).

A number of viruses use sites on HS as receptors for binding to cells (Shieh et al., 1992; Compton et al., 1993; Jackson et al., 1996; Byrnes and Griffin, 1998; Chung et al., 1998; Summerford and Samulski, 1998). Viral entry may require interactions with other cell surface receptors as well (Montgomery et al., 1996; Geraghty et al., 1998; Warner et al., 1998; Summerford et al., 1999). In the case of herpes simplex virus types 1 and 2 (HSV-1, HSV-2), which are human herpesviruses of the neurotropic alphaherpesvirus subfamily, the virus binds to cells through interactions of envelope glycoproteins gB and/or gC with cell surface HS (reviewed by Spear, 1993). Following this binding, a third viral glycoprotein, gD, interacts with one of multiple specific receptors, resulting in viral entry by fusion of the virion envelope with a cell membrane. This fusion reaction requires the concerted action of three additional viral glycoproteins, gB, gH, and gL (reviewed by Spear, 1993), and appears to be triggered by the binding of gD to its cognate receptors.

The human gD receptors identified to date include a member of the TNF receptor family, designated HVEM (Montgomery et al., 1996; Whitbeck et al., 1997) or herpesvirus entry protein A (HveA), and officially named TNFRSF14, and two members of the immunoglobulin superfamily (Geraghty et al., 1998; Krummenacher et al., 1998; Warner et al., 1998). The latter two proteins are related to the poliovirus receptor (CD155) (Mendelsohn et al., 1989), were originally designated poliovirus receptor–related proteins 1 (Lopez et al., 1995) and 2 (Eberlé et al., 1995) and more recently named HveC (Geraghty et al., 1998) and HveB (Warner et al., 1998) or nectin 1 and nectin 2 (Takahashi et al., 1999), respectively. Both HveA and HveC serve as gD-binding entry receptors for wild-type HSV-1 and HSV-2 strains, whereas HveB serves as an entry receptor for only a subset of HSV strains (Montgomery et al., 1996; Geraghty et al., 1998; Warner et al., 1998).

Here, we report that a different kind of gD receptor can

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be generated by the action of specific D-glucosaminyl 3-*O*-sulfotransferases (3-OSTs). Members of the family of 3-OSTs are responsible for the addition of sulfate groups at the 3-OH position of glucosamine in HS. These enzymes are present as several isoforms expressed from different genes at different levels in various tissues and cells (Shworak et al., 1999). The 3-OSTs act to modify HS late in its biosynthesis (reviewed by Lindahl et al., 1998), and each isoform recognizes as substrate glucosamine residues in regions of the HS chain having specific, but different, prior modifications, including epimerization and sulfation at other positions (Liu et al., 1999b). Thus, different 3-OSTs generate different potential protein-binding sites in HS. Recently the 3-OST-1 isoform, but not other isoforms (3-OST-2, 3-OST-3_A, and 3-OST-3_B), was shown to generate antithrombin-binding sites in HS (Liu et al., 1996, 1999b; Shworak et al., 1997).

In this study, cells that are resistant to HSV-1 entry were used in expression cloning experiments to identify mouse cDNAs capable of conferring susceptibility to HSV-1 entry. The plasmid isolated in this screen encodes a mouse version of 3-OST-3_B. We show here that both mouse and human forms of 3-OST-3_B and a human form of 3-OST-3_A modified the HS of HSV-1-resistant cells as predicted and made the cells susceptible to HSV-1 infection. Evidence is presented that the enzyme-modified HS rather than the enzymes themselves served as gD-binding receptors to permit HSV-1 entry. Thus, gD-binding receptors needed for HSV-1 entry into cells include 3-OST-3-modified HS or the previously identified protein receptors.

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                  ▼
m/3B (  1) MGQRLSGGRSCLDVPGRFLPQPPPPPPPVRRKLALLFAMLCIWLYMFLYSCAGSCTAAPG
h/3B (  1) .................L.......................V.............A....

m/3B ( 61) LLLLGSGSRATHAQPTLVTAPNETSPKMPFRAPPANSLAAGKDKTVGAGSQEEQSPEAPD
h/3B ( 61) ..........A.DP.A.A...DG.P.RL.......TP..S..EMAE..A.P......V..

m/3B (121) SPSPISSFFSGAGSKQLPQAIIIGVKKGGTRALLEFLRVHPDVRAVGAEPHFFDRSYHKG
h/3B (121) ...........S.............................................D..
h/3A (148) ------------.............................................D..

m/3B (181) LAWYRDLMPRTLKGQITMEKTPSYFVTREAPARISAMSKDTKLIVVVRDPVTRAISDYTQ
h/3B (181) ............D...............................................
h/3A (196) ............D...............................................

m/3B (241) TLSKRPDIPSFESLTFRNRSAGLIDTSWSAIQIGLYAKHLEPWLRHFPLGQMLFVSGERL
h/3B (241) .........T......K..T..............I......H......IR..........
h/3A (256) .........T......K..T..............I......H......IR..........

m/3B (301) VSDPAGELRRVQDFLGLKRIITDKHFYFNQTKGFPCLKKAEGSGKPHCLGKTKGRAHPTI
h/3B (301) I.......G....................K.............SR..........T..E.
h/3A (316) I.......G....................K.............SR..........T..E.

m/3B (361) AREVLRQLRDFYRPFNRKFYQMTGRDFGWD-
h/3B (361) D...V.R..E......L.......H.....-
h/3A (376) D...V.R..E......L.......H.....G
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Figure 1. Amino Acid Sequence of m3-OST-3_B in Comparison with Those of h3-OST-3_B and the Sulfotransferase Domain of h3-OST-3_A

Dots in the latter two sequences indicate identity with the m3-OST-3_B (m/3B) sequence. Single-underlined residues of m3-OST-3_B include the predicted membrane-spanning region, and the double-underlined residues indicate the potential sites for addition of N-linked glycans. The filled triangle marks the beginning of the conserved sulfotransferase domain. The position in this conserved domain of the amino acid substitution in h3-OST-3_A (K162A) that results in loss of enzymatic activity is indicated by the shading. Within the sulfotransferase domain, m3-OST-3_B is 91% identical to both human 3-OST-3s; the remaining N-terminal portion of m3-OST-3_B is 81% identical to that of h3-OST-3_B (h/3B) and unrelated to h3-OST-3_A (h/3A).

Results

A Murine Mediator of HSV-1 Entry Has 3-OST-3 Activity

Wild-type Chinese hamster ovary (CHO) cells are resistant to HSV-1 entry (Shieh et al., 1992). Screening of an embryonic mouse cDNA library for expression of proteins capable of rendering CHO cells susceptible to HSV-1 entry resulted in isolation of pDS43. This plasmid has an insert of 1675 base pairs with one major open reading frame encoding the protein whose sequence is given in Figure 1. A search of the databases revealed that this protein is closely related to the previously described human protein encoding 3-OST-3_B (h3-OST-3_B) (Shworak et al., 1999). We have named the mouse protein m3-OST-3_B because of its similarity in sequence to h3-OST-3_B (Figure 1) and because of its enzymatic activity described below.

Both m3-OST-3_B and h3-OST-3_B are predicted to be type II membrane glycoproteins of 390 amino acids with features described previously (Shworak et al., 1999) and indicated in Figure 1. Evidence that m3-OST-3_B is oriented in membranes as a type II membrane protein was obtained by expressing a tagged version of the protein (Myc epitope fused to the C terminus) in CHO cells and showing that anti-Myc antibodies could bind to live unfixed cells (data not shown). This result also indicates that m3-OST-3_B can be transported to the cell surface even though its principal intracellular location is likely to be the terminal Golgi compartment(s) where HS sulfation occurs. Both the Myc-tagged and untagged versions of m3-OST-3_B were able to convert at least two types of cells (CHO and mouse melanoma cells) from resistance to susceptibility to several strains of HSV-1 (representative data shown in experiments described below).

Evidence that m3-OST-3_B has the predicted enzymatic activity is presented in Figure 2. COS-7 cells were transfected with a plasmid expressing m3-OST-3_B or the empty vector, and extracts from the cells were incubated with HS prepared from CHO cells and the sulfate donor [^{35}S]adenosine 3′-phosphate 5′-phosphosulfate ([^{35}S]PAPS). The labeled HS was then degraded for analysis as shown in Figure 2. Two 3-*O*-sulfated disaccharides characteristic of products generated by h3-OST-3_B and h3-OST-3_A (Liu et al., 1999b) were detected in the reaction mixture containing m3-OST-3_B (Figure 2B) but not in the control reaction (Figure 2A).

Enzymatically Active Mouse and Human 3-OST-3, but Not 3-OST-1, Are Mediators of HSV-1 Entry

Wild-type CHO cells were transfected with plasmids expressing various 3-OSTs and then exposed to a recombinant HSV-1 strain that expresses β-galactosidase upon entry into cells [HSV-1(KOS)gL86]. All three enzymes that catalyze the sulfation reactions described in Figure 2 (m3-OST-3_B, h3-OST-3_B, and h3-OST-3_A) rendered CHO cells susceptible to HSV-1 entry, whereas m3-OST-1, shown in parallel experiments to be expressed as active enzyme (data not shown), had little if any viral entry activity (Figure 3). The 3-*O*-sulfated disaccharides characteristic of HS modified by human or mouse 3-OST-1 are -GlcA-GlcNS±6S3S- (Shworak et al., 1997), whereas those characteristic of 3-OST-3 modifications are -IdoA2S-GlcNH$_2$±6S3S- (Liu et al., 1999b).

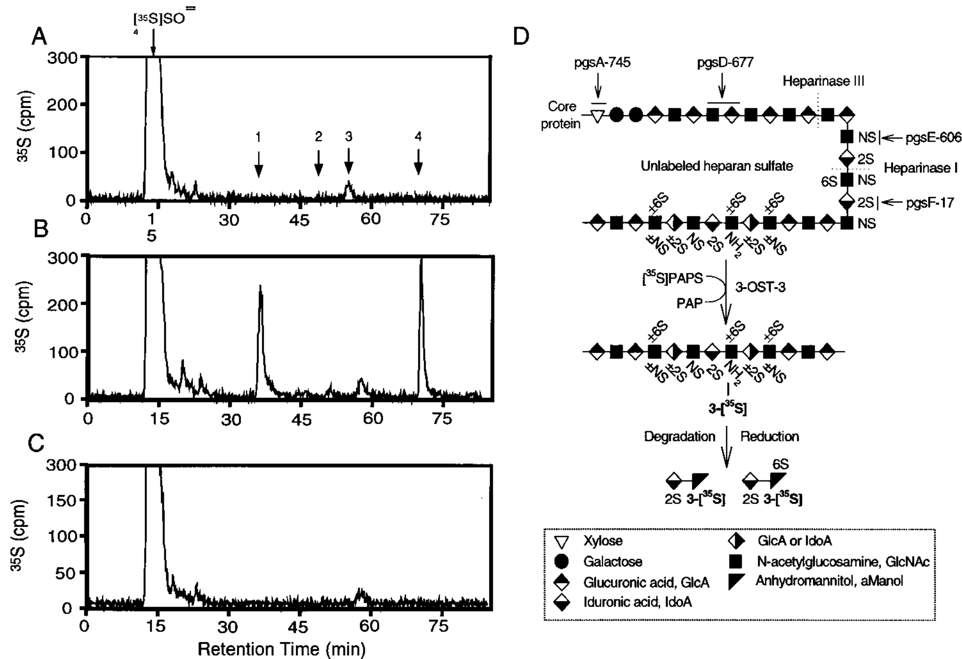

Figure 2. Enzymatic Activity of m3-OST-3_B and Inactivation of h3-OST-3_A Activity by K162A Amino Acid Substitution

Cell extracts from COS-7 cells transfected with the empty plasmid vector pcDNA3 (A) or with plasmids expressing m3-OST-3_B (B) or mutated h3-OST-3_A(K162A) (C) were incubated with HS prepared from CHO cells and [^{35}S]PAPS, and then ^{35}S-labeled degradation products of the HS were generated by nitrous acid treatment and reduction with sodium borohydride, as outlined in (D), and analyzed by HPLC (A–C). This method of degradation results in conversion of glucosamine to anhydromannitol. The arrows in (A) indicate the elution positions of disaccharide standards, where 1 is IdoA2S-aManol3S, 2 is GlcA-aManol3S6S, 3 is IdoA2S-aManol6S, and 4 is IdoA2S-aManol3S6S. IdoA2S-[^{35}S]aManol3S and IdoA2S-[^{35}S]aManol3S6S are the characteristic disaccharides resulting from digestion of h3-OST-3_B- or h3-OST-3_A-modified HS (Liu et al., 1999b). (D) illustrates the composition of HS, presence of sulfated and unsulfated domains in HS, potential cleavage sites for heparinases I and III, and the biosynthetic steps altered in CHO cell mutants pgsA-745 (defective in xylosyltransferase and all GAG assembly), pgsD-677 (defective only in HS formation due to loss of HS copolymerase activity), pgsE-606 (~50% defective in GlcNAc N-deacetylation and N-sulfation due to mutation of one isozyme responsible for this activity), and pgsF-17 (defective in 2-*O*-sulfotransferase).

A mutated form of h3-OST-3_A, in which the Lys at position 162 was changed to Ala, was shown by Western blot analysis to be expressed at a level comparable to that of wild-type enzyme (data not shown) but to have little or no enzymatic activity (Figure 2C) and no viral entry activity (Figure 3C). This particular amino acid substitution in a highly conserved region of the sulfotransferases (Figure 1) was shown previously to dramatically reduce enzymatic activity of a flavonol 3-sulfotransferase with minimal effect on PAPS binding (Marsolais and Varin, 1995). Moreover, mutagenic and structural studies demonstrated that the conserved Lys has a comparable critical role in catalysis by a heparan *N*-sulfotransferase (Sueyoshi et al., 1998; Kakuta et al., 1999).

Function of m3-OST-3_B as an HSV-1 Entry Mediator Depends on HS Production and on 2-*O*-Sulfation of HS

Given that HSV-1 entry activity correlated with 3-OST-3 enzymatic activity, we determined whether entry activity depended on presence of cell surface HS that could be modified by 3-OST-3. Wild-type CHO cells and several mutant cell lines defective for different steps in assembly and modification of HS (Figure 2D) were transfected with plasmids expressing the Myc-tagged version of m3-OST-3_B or HveC, a human protein that can serve as a gD-binding entry receptor for HSV (Geraghty et al., 1998; Krummenacher et al., 1998), or with the empty vector, pcDNA3. The cells were then exposed to HSV-1(KOS)gL86 for quantitation of viral entry. Figure 4B shows that the various cell lines transfected with the m3-OST-3_B-expressing plasmid produced equivalent amounts of the protein. Figure 4A shows that mutations affecting HS biosynthesis had different consequences for HSV-1 entry, depending on whether the cells expressed m3-OST-3_B or HveC, and also that all of the cell lines transfected with control plasmid were resistant to HSV-1 entry. The first point to be made is that expression of m3-OST-3_B permitted HSV-1 entry only in wild-type CHO cells and in pgs606 mutant cells. HS produced by this mutant has the modifications characteristic of wild-type cells, but at a reduced level, because the N sulfation that is prerequisite for other modifications occurs at about 50% of wild-type level (Bame and Esko, 1989; Aikawa and Esko, 1999). The other cell lines either fail to produce HS (pgsA-745 cells produce no glycosaminoglycans [Esko et al., 1985] and pgsD-677 cells produce only chondroitin sulfate [Lidholt et al., 1992]) or fail to modify the hexuronic acid residues of HS by the addition of 2-*O*-sulfate groups (pgsF-17) (Bai and Esko, 1996). Because the substrate for 3-OST-3s is a glucosamine residue adjacent to 2-*O*-sulfated IdoA, incubation of HS from pgsF-17 cells with purified 3-OST-3_A should not yield the characteristic disaccharides resulting from

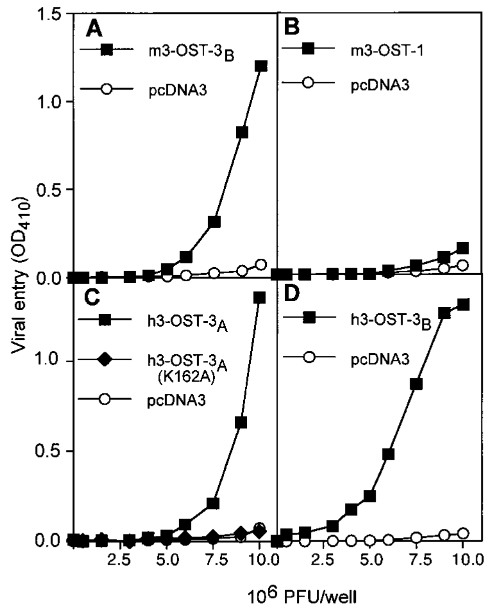

Figure 3. Entry of HSV-1 into CHO Cells Mediated by Murine or Human 3-OST-3_B or 3-OST-3_A

CHO cells transfected with plasmids expressing the murine or human forms of the enzymes indicated or the enzymatically inactive mutant h3-OST-3_A(K162A), or with the empty vector pcDNA3, were exposed to various concentrations of HSV-1(KOS)gL86, which expresses β-galactosidase from an insert in the viral genome immediately upon entry into cells. β-galactosidase activity was quantitated as a measure of viral entry at 6 hr after the addition of virus. The values shown represent the amount of reaction product detected spectrophotometrically (OD410).

3-OST-3 activity, as was confirmed experimentally (Table 1). Second, in contrast to the results obtained with m3-OST-3_B, expression of HveC rendered all the cell lines susceptible to HSV-1 entry (Figure 4A), indicating that presence of HS was not required for HveC-dependent entry. The presence of HS to which HSV-1 could bind via gB or gC clearly enhanced viral entry, however. The ability of HS to enhance the susceptibility resulting from HveC expression depended on the overall level of sulfation (wild-type was greater than pgsE-606 cells) and on 2-*O*-sulfation of hexuronic residues (pgsF-17 cells), consistent with previous studies suggesting that these groups are important for HSV-1 binding to cells (Herold et al., 1996; Feyzi et al., 1997). Third, we have reproducibly found in multiple experiments that HveC can confer greater susceptibility to HSV-1 entry than does m3-OST-3_B. In the case of HveC, new gD-binding receptors are overexpressed from the transfected plasmid, whereas, in the case of m3-OST-3_B, the enzyme may modify endogenous molecules to generate receptors for virus. If so, the levels of endogenous receptor would govern the degree of susceptibility to infection that could be attained.

HSV-1 Entry Mediated by 3-OST-3 Depends on Modified HS as a gD Receptor

Several lines of evidence demonstrated that HSV-1 entry mediated by mouse or human 3-OST-3 is dependent on gD and on the generation of gD-binding sites in HS.

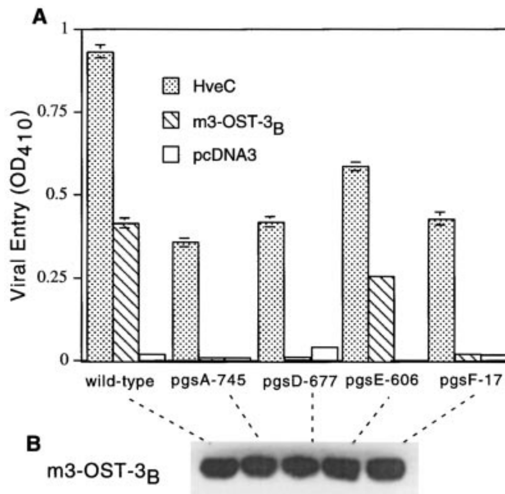

Figure 4. HSV-1 Entry Activity of m3-OST-3_B Depends on Presence of Cell Surface HS and on 2-*O*-Sulfation of the HS

(A) Wild-type CHO cells or CHO cell mutants (Figure 2D) were transfected with plasmids expressing HveC or m3-OST-3_B (Myc-tagged) or with the empty vector pcDNA3. The cells were then exposed to HSV-1(KOS)gL86 at various input doses and β-galactosidase was quantitated after 6 hr, as described in Figure 3, as a measure of viral entry. The results shown (means of triplicate determinations plus standard deviation) are for 7.5×10^5 pfu/well, an amount of virus within the linear range of the dose response curve for all samples. (B) Extracts were prepared from replicate cultures of the transfected cells described in (A) for detection of m3-OST-3_B (Myc-tagged) expression by Western blot, using an anti-Myc mAb.

First, a mutant of HSV-1(KOS) that has a single amino acid substitution at position 27 in gD (Dean et al., 1994) was unable to infect cells expressing m3-OST-3_B (data not shown). Other phenotypes caused by this mutation (Rid1 mutation) include loss of ability to infect HveA-expressing cells (Montgomery et al., 1996) and acquired ability to infect cells expressing HveB (Warner et al., 1998).

Second, both membrane-bound and soluble gD competed with virus for the receptors generated by 3-OST-3.

Table 1. Disaccharide Compositions of 3-OST-3_A-Modified HS from Wild-Type CHO Cells and 2-OST-Deficient pgsF-17 Cells

	Source of HS[a]		
Disaccharides	Wild Type (pmol)	pgsF-17 (pmol)	Wild Type: pgsF-17
IdoA2S-[^{35}S]aManol3S[b]	6.7	0.3	22:1
IdoA2S-[3-^{35}S]aManol3S6S[b]	5.1	0.2	26:1
IdoA-[3-^{35}S]aManol3S6S	0.2	1.4	1:7
GlcA-[3-^{35}S]aManol3S6S	Not detected	0.2	—

[a] [^{35}S]HS was prepared by incubating unlabeled HS (1 μg) from wild-type CHO cells and pgsF-17 cells with purified 3-OST-3_A (60 ng) in the presence of [^{35}S]PAPS (20×10^6 cpm, 10 μM). The [^{35}S]HS was degraded with nitrous acid at pH 1.5 followed by a reduction with sodium borohydride. The [^{35}S]disaccharides were isolated by Biogel P-2 and analyzed on reverse phase ion pairing HPLC. The identities of the [^{35}S]disaccharides were determined by coelution with [^{3}H]disaccharide standards.

[b] The characteristic disaccharides resulting from 3-OST-3_A modification and degradation.

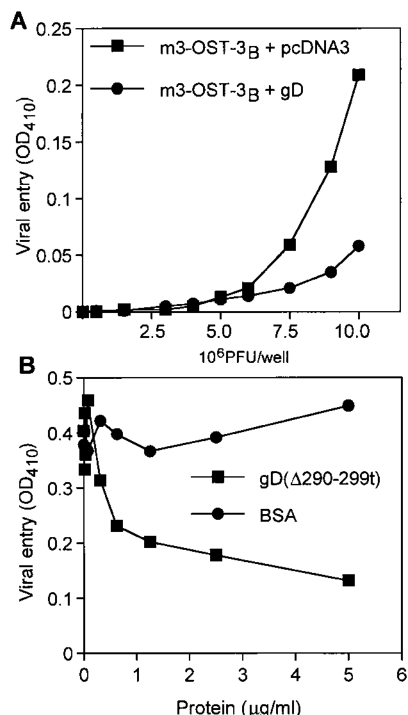

Figure 5. HSV-1 gD Competes with Virus for Receptors Generated by m3-OST-3_B

(A) CHO cells were transfected with a m3-OST-3_B-expressing plasmid mixed in 1:4 ratio with a gD-1-expressing plasmid or the empty vector pcDNA3. The transfected cells were then exposed to HSV-1(KOS)gL86 at the input doses indicated and viral entry quantitated after 6 hr, as described in Figure 3.

(B) CHO cells transfected with a plasmid expressing m3-OST-3_B were exposed to HSV-1(KOS)gL86 (7.5×10^5 pfu/well) that had been preincubated with gD-1(Δ290–299t), a soluble form of gD-1 shown to block the infection of cells via other gD-binding receptors (Whitbeck et al., 1997), or with BSA, at the concentrations indicated. Viral entry was quantitated at 6 hr as described above.

Coexpression of HSV-1 gD with m3-OST-3_B in CHO cells made the cells more resistant to HSV-1 entry (Figure 5A). Cellular expression of gD has been shown to interfere with HSV-1 entry only when the form of gD expressed by the cell is capable of interacting with the gD receptors (HveA, HveB, or HveC) available for viral entry (Mauri et al., 1998; Geraghty et al., unpublished data). Also, a soluble truncated form of gD, shown previously to block HSV-1 entry into cells expressing HveA (Whitbeck et al., 1997), inhibited infection of CHO cells transfected with m3-OST-3_B (Figure 5B).

Third, binding of gD to 3-OST-3-modified HS, but not to the enzymes themselves, could be demonstrated. HS extracted from wild-type CHO cells was left unmodified or sulfated by incubation in vitro with 3-OST-1 or h3-OST-3_A and tested for binding to gD in coimmunoprecipitation experiments. Table 2 shows that the fraction of input labeled HS bound to immunoprecipitated gD-1(306t) was enhanced almost 10-fold for HS modified by incubation with 3-OST-3_A. Modification by 3-OST-1 did not significantly enhance the binding to gD-1(306t). The HS from pgsF-17 cells, after incubation with 3-OST-3_A, bound to gD-1(306t) with about the same efficiency as unmodified HS from CHO cells.

Table 2. The Binding of Enzyme-Modified HS to gD-1

	[^{3}H]HS Bound to gD-1 (306t) (%)[b]	
[^{3}H]HS[a]	Wild Type	pgsF-17
Unmodified HS	1.7 ± 0.9 (n = 5)	0.1 ± 0.1 (n = 2)
3-OST-1-modified HS[c]	3.4 ± 1.6 (n = 2)	ND
3-OST-3_A-modified HS[d]	14.8 ± 1.7 (n = 6)	1.6 ± 0.7 (n = 2)

[a]Metabolically labeled [^{3}H]HS from wild-type CHO or pgsF-17 cells was incubated with [^{35}S]PAPS and purified m3-OST-1 (60 ng) or h3-OST-3_A (60 ng).

[b]The binding of the modified or unmodified [^{3}H/^{35}S]HS to gD-1(306t) was determined by incubating mixtures with the anti-gD antiserum R7 (or the anti-gD monoclonal antibody DL6, 5 μl) to form an immune complex. The percentage of input HS bound to gD-1(306t) was calculated based on the input and eluted ^{3}H counts. Data are expressed as the mean ± SD. ND, not determined.

[c]The sulfated structure generated is -GlcA-GlcNS±6S<u>3S</u>-.

[d]The sulfated structure generated is -IdoA2S-GlcNH$_2$±6S<u>3S</u>-.

Affinity coelectrophoresis (Lee and Lander, 1991) was performed to estimate dissociation constants for the gD–HS interactions. [^{35}S]HS was purified from CHO cells, modified by incubation with purified h3-OST-3_A in vitro and then fractionated by coimmunoprecipitation with gD-1(306t), yielding HS$^{gD\text{-}Act}$ in the bound fraction and HS$^{gD\text{-}Inact}$ in the unbound fraction. The fraction of labeled HS in the bound fraction was similar to that shown in Table 2. The two preparations of HS were then electrophoresed in an agarose gel through zones containing gD-1(306t) at various concentrations. The migration of HS$^{gD\text{-}Act}$ was retarded by the presence of gD-1(306t), with the degree of retardation dependent on the gD-1(306t) concentration (Figure 6A), whereas the migration of HS$^{gD\text{-}Inact}$ was much less affected (data not shown). From these data the K_D for interaction of HS$^{gD\text{-}Act}$ with gD-1(306t) was calculated to be 2 μM, whereas the K_D for HS$^{gD\text{-}Inact}$ was 43 μM or greater (Figure 6B), considering that the removal of HS$^{gD\text{-}Act}$ from the HS$^{gD\text{-}Inact}$ fraction was undoubtedly not complete.

Efforts to demonstrate direct interactions between 3-OST-3 and gD were unsuccessful. In ELISA assays no binding of purified gD-1(306t) to purified h3-OST-3_A was evident, whereas binding to purified HveC was readily detected in control experiments (data not shown).

Finally, experiments were done to determine whether heparinases could remove from 3-OST-3-expressing cells the receptors that bind gD and mediate viral entry. Wild-type CHO cells or pgsA-745 cells were transfected with plasmids expressing m3-OST-3_B or h3-OST-3_A or with control plasmid, and the wild-type cells were later incubated with a mixture of heparinases I and III, which cleave HS at different sites (Figure 2D) to liberate the majority of HS chains from the cell surface, or incubated in buffer without the heparinases. The cells were then incubated with a gD hybrid protein consisting of the ectodomain of gD fused to the Fc of rabbit IgG (gD-1:Fc). Figure 7A shows that expression of either 3-OST-3 caused enhanced binding of gD-1:Fc to the untreated CHO cells (but not to the pgsA-745 cells) and that this enhanced binding was eliminated by pretreatment of the wild-type cells with heparinases I/III. Figure 7B shows that, for CHO cells expressing m3-OST-3_B, treatment with heparinases I/III almost completely eliminated

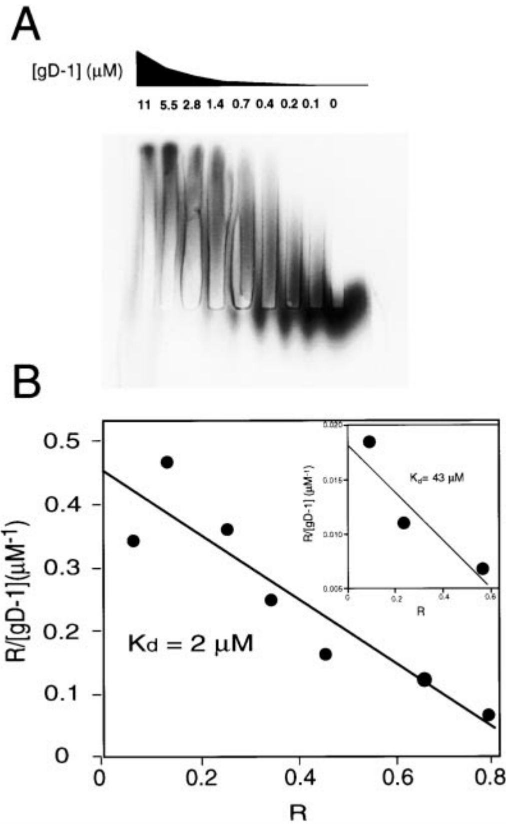

Figure 6. Binding Constant (K_D) for Interaction between h3-OST-3_A-Modified HS and gD-1 as Determined by Affinity Coelectrophoresis

(A) Autoradiograph of the agarose gel in which [^{35}S]HS purified by coprecipitation with gD-1(306t) (HSg$^{D\text{-}Act}$) was electrophoresed through zones containing gD-1(306t) at the concentrations indicated. Approximately 50,000 cpm (1.3×10^{-15} mol) of [^{35}S]HS$^{gD\text{-}Act}$ was loaded in each separation zone.

(B) Plot of R/[gD-1]$_{total}$ versus R, where the retardation coefficient $R = (M_0 - M)/M_0$. M_0 is the migration of free [^{35}S]HS, and M is the observed migration of [^{35}S]HS in the presence of gD-1(306t). Assuming that [^{35}S]HS and gD-1 form a 1:1 complex and gD-1 is in great excess, this plot should yield a straight line with a slope of $-1/K_D$ according to the Scatchard equation. The main plot (linear coefficient value = 0.89) shows the results of the analysis done with [^{35}S]HS$^{gD\text{-}Act}$ (A), whereas the inset (linear coefficient value = 0.92) is for the analysis done with [^{35}S]HS$^{gD\text{-}Inact}$ (HS that failed to coprecipitate with gD-1[306t]).

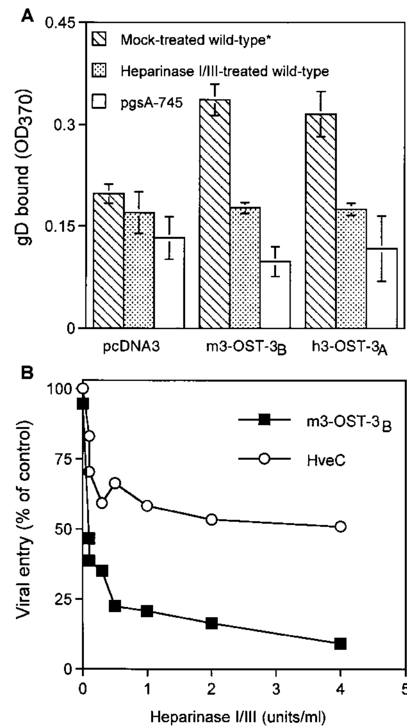

Figure 7. Enzymatic Removal of 3-OST-3-Modified HS from Cells Removes gD-Binding Receptors and Renders the Cells Resistant to HSV-1 Entry

(A) Wild-type CHO cells or pgsA-745 cells transfected with plasmids expressing m3-OST-3_B or h3-OST-3_A or with the empty vector pcDNA3 were treated with heparinase I/III (4 U/ml of each) or were incubated with the enzyme buffer but without added enzyme. The cells were then washed and incubated with gD-1:Fc for 30 min, followed by fixation and incubation with a secondary antibody and a horseradish peroxidase detection system. The values shown (means of triplicate determinations plus standard deviations) represent the amount of reaction product detected spectrophotometrically (OD_{370}). In this experiment (samples indicated by the asterisk) and two others, the difference in amount of gD-1:Fc bound to 3-OST-3-expressing cells and control cells was statistically significant (Student's t test, $p < 0.001$).

(B) CHO cells transfected with plasmids expressing m3-OST-3_B or HveC were treated with heparinases I/III at the concentrations indicated (total units present in 1:1 mixtures) or were incubated with the enzyme buffer but without added enzymes. The cells were then exposed to HSV-1(KOS)gL86 at 7.5×10^5 pfu/well and viral entry was quantitated 6 hr later, as described in Figure 3. Entry into the heparinase I/III-treated cells is presented as a percentage of the entry into mock-treated cells.

susceptibility to HSV-1 entry, whereas for cells expressing HveC, this treatment reduced susceptibility to HSV-1 infection by only 50%.

Discussion

The surprising finding is that specific sites in HS can serve as gD-binding receptors for HSV-1 entry into cells. These sites are of unknown size but must include one of the disaccharides, -IdoA2S-GlcNH$_2$3S6S- or -IdoA2S-GlcNH$_2$3S-. This conclusion is based on evidence that 3-OST-3s capable of introducing the final modification characteristic of these disaccharides can convert resistant cells to susceptibility to HSV-1 entry, that ability of the 3-OST-3s to confer susceptibility depends on presence of the appropriate HS substrate and on enzymatic activity, that gD receptors are generated by the action of these enzymes, and that these gD receptors can be removed from cells by the action of heparinases.

The previously identified gD-binding receptors for wild-type HSV-1 are HveA (TNFRSF14) and HveC (nectin 1 or Prr1), both cell surface proteins (Montgomery et al., 1996; Geraghty et al., 1998). The apparent diversity of functional gD receptors, which include proteins from two different families and specific sites in HS, is astonishing. In all three cases, the dissociation constants for interactions with soluble gD are in the range of 2–3 ×

10^{-6} M (Willis et al., 1998; Krummenacher et al., 1999; this study). It should be noted that gD does not normally mediate the binding of virus to cells. This role is taken by gB and/or gC (Herold et al., 1991, 1994). The binding of virus to cell surface HS clearly does not require the same modifications as are introduced by 3-OST-3s to generate gD binding activity, because unmodified HS expressed by CHO cells is fully functional for HSV-1 binding but not for entry (Shieh et al., 1992). It seems likely that the role of gD is, through its interactions with any one of several specific cell surface molecules, to activate the fusogenic activity of other viral glycoproteins, gB and/or gH–gL. Probably, gD is not itself a fusogen, based on findings with other alphaherpesviruses that absence of gD can be compensated by second-site mutations resulting in recovery of some viral infectivity (Schmidt et al., 1997; Schroder et al., 1997).

The results presented here raise the possibility that HSV-1 entry into cells could be mediated entirely by HS, provided the appropriate sites for virus binding and gD binding were present in the HS. Alternatively, HveA or HveC could serve as the operative gD-binding receptor. HveA (Montgomery et al., 1996), HveC (Lopez et al., 1995; Geraghty et al., 1998), and the 3-OST-3s (Shworak et al., 1999) are all expressed in a variety of human tissues and cell types, making it difficult to predict which is the operative gD receptor for any given cell type in vivo. Also, we cannot rule out the possibility that additional unidentified cell surface receptors participate in HSV-1 entry. If these putative additional receptors exist, it seems unlikely that they mediate the binding of virus to cells, but they could interact with viral ligands made accessible after interaction of the virus with HS, 3-*O*-sulfated HS, or one of the other gD-binding receptors.

Wild-type strains of HSV-1 and HSV-2 seem to be equivalent in usage of HveA or HveC for entry, but they differ in usage of the receptors generated by 3-OST-3. Mouse melanoma cells expressing 3-OST-3 in the absence of HveA or HveC are significantly more susceptible to HSV-1 strains than to HSV-2 (D. S. and P. G. S., unpublished results), suggesting that expression of these enzymes could predispose human cells to HSV-1 infection. While the clinical manifestations of disease caused by HSV-1 and HSV-2 can be indistinguishable, there are differences in epidemiology and pathogenesis of disease caused by the two serotypes (Lafferty et al., 1987; Corey and Spear, 1988). HSV-1 is the usual cause of adult sporadic encephalitis, keratitis, and oral mucocutaneous lesions, whereas HSV-2 is more likely to cause genital lesions, meningitis, and neonatal infections. A factor contributing to these differences could be preference for different receptors and perhaps different cell types.

Our results demonstrate that human and mouse 3-OST-3s can modify HS to provide sites for the binding of HSV-1 gD and that presence of these sites is associated with susceptibility of the cells to viral entry in the absence of any known protein gD-binding receptors. Given that these enzymes are widely expressed in human tissues (Shworak et al., 1999), and also in mouse tissues (D. S. and P. G. S., unpublished results), the ability of 3-OST-3 to mediate HSV-1 entry may partially explain the very broad host range of HSV-1 with respect to cell type and species. HSV-2 also has a broad host range, suggesting that other isoforms of the 3-OSTs should be tested for their ability to mediate entry of HSV-1 and HSV-2. Finally, the fact that cells expressing 3-OST-3 are more susceptible to HSV-1 than to HSV-2 provides a basis for investigating differential susceptibility of human cell types to HSV-1 and HSV-2 and potential contribution of this differential susceptibility to patterns of disease.

Experimental Procedures

Cells and Viruses

Wild-type CHO cells and the mutant cell lines pgsA-745 (Esko et al., 1985), pgsD-677 (Lidholt et al., 1992), pgsE-606 (Bame and Esko, 1989; Aikawa and Esko, 1999), and pgsF-17 (Bai and Esko, 1996) were grown in Ham's F12 medium supplemented with 10% fetal bovine serum. Wild-type virus strains used were HSV-1(KOS), HSV-1(Patton), HSV-1(F), and HSV-2(333). Mutant strains included HSV-1(KOS)Rid1 and Rid2 (Dean et al., 1994). Recombinant viruses carrying the *lacZ* gene of *E. coli* and capable of expressing β-galactosidase as a reporter of entry included HSV-1(KOS)gL86 (Montgomery et al., 1996), HSV-1(KOS)tk12, and HSV-1(KOS)Rid1-tk12 (Warner et al., 1998).

Antibodies and Purified Recombinant Proteins

Antibodies used included rabbit antiserum R7 specific for HSV gD (Isola et al., 1989), the anti-gD mAb DL6 (Isola et al., 1989), and an anti-Myc mAb (cat. no. R950-25, Invitrogen). Purified recombinant proteins included m3-OST-1 secreted from L cells (Liu et al., 1996), a secreted *N*-truncated form of h3-OST-3_A (Liu et al., 1999a), and two secreted *C*-truncated forms of gD-1, designated gD-1(306t) and gD-1(Δ290–299t) (Nicola et al., 1996). The latter three proteins were produced in insect cells from baculovirus vectors and purified as described in the references cited. The hybrid protein gD-1:Fc was produced by CHO cells transfected with plasmid pBG64 and purified from the medium (Ham's F12 supplemented with 3% fetal bovine serum depleted of IgG) by protein G–Sepharose chromatography.

Screening Assay for Plasmids Encoding HSV-1 Entry Proteins

A 15.5 days gestation mouse embryonic cDNA expression library (GIBCO-BRL) was screened for plasmids capable of rendering transfected CHO cells susceptible to HSV-1 entry, essentially as described previously (Montgomery et al., 1996). A single plasmid, designated pDS43, was obtained. Nucleotide sequence of the insert was determined by the University of Chicago Cancer Research Center Sequencing Facility, using a UBI Prism 377 DNA sequencer. The sequence of the insert was deposited in GenBank (Accession No. AF168992).

Plasmids

Plasmid pDS43/*myc*-His was generated by in-frame fusion of the C terminus of the m3-OST-3_B open reading frame, through conversion of the stop codon to a BamHI site, with the Myc-His tag from the plasmid pcDNA3.1/*myc*-His (Invitrogen). Plasmid pBG64 has the ectodomain of HSV-1(KOS) gD (amino acids 1–343) fused in frame to the Fc region of a rabbit IgG heavy chain and cloned into pcDNA3. Plasmids described elsewhere include those expressing m3-OST-1 (Shworak et al., 1997), h3-OST-3_A, and h3-OST-3_B (Liu et al., 1999b), pBG38 expressing HveC (Geraghty et al., 1998), and pRE4 expressing HSV-1 gD (Cohen et al., 1988).

Enzymatic Modification of HS and Analysis of Products

Purified enzymes (m3-OST-1 or h3-OST-3_A) or extracts of COS-7 cells (Shworak et al., 1997) expressing various forms of 3-OST-3 were incubated with HS prepared from wild-type CHO cells or pgsF-17 cells and [^{35}S]PAPS. The reaction conditions, detailed in the text, ensure the modification of all potential 3-*O* sulfation sites recognized by each enzyme. In some cases the enzyme-modified or unmodified HS was then incubated with nitrous acid at pH 1.5 and 0°C for 30 min and reduced with sodium borohydride (0.05 M) under alkaline conditions at 50°C for 30 min (Liu et al., 1999b). The nitrous acid–degraded HS products were resolved by chromatography on BioGel

P-2. The disaccharides (about 60% of total loaded ^{35}S counts) were subfractionated on reversed-phase ion pairing HPLC with appropriate disaccharide standards (Liu et al., 1999a).

Entry Assays

Entry assays were based on quantitation of β-galactosidase expressed from the viral genome or by CHO-IEβ8 cells in which β-galactosidase expression is inducible by HSV infection (Montgomery et al., 1996). Cells were transiently transfected in six-well dishes, using Lipofectamine (GIBCO-BRL) with plasmids expressing one of the entry mediators or control plasmid, pcDNA3, at 1.5 to 2.0 μg per well in 1 ml. At 24 hr after transfection, the cells were replated in 96-well tissue culture dishes (2–4 $\times$ 10^4 cells/well) at least 16 hr prior to infection. Cells were washed and exposed to virus in 50 μl of phosphate-buffered saline (PBS) containing glucose and 1% calf serum (PBS-G-CS) for 6 hr at 37°C before solubilization in 100 μl of PBS containing 0.5% NP-40 and the β-galactosidase substrate, o-nitro-phenyl β-D-galactopyranoside (ONPG, 3 mg/ml). The enzymatic activity was monitored by spectrometry at several time points after the addition of ONPG to define the interval over which the generation of product was linear with time (Dynatech ELISA reader or a Spectromax 250). Results are presented as the means of triplicate or quadruplicate determinations. Assays for inhibition of infection by soluble gD were similar, except that dilutions of virus were mixed with gD-1(Δ290–299t) or BSA and incubated for 30 min at 37°C prior to addition of the mixtures (50 μl/well) to washed cell monolayers.

gD Interference Assay

Wild-type CHO cells were cotransfected, using Lipofectamine (GIBCO-BRL), with m3-OST-3_B expression plasmid (pDS43/*myc*-His) and gD expression plasmid (pRE4 for HSV-1 wild-type gD) or control plasmid (pcDNA3), in 1:4 (m3-OST-3_B :gD or control) ratio, in 6-well dishes (2 μg of plasmid DNA total/well). After 24 hr the transfected cells were replated in 96 well plates and, 24 hr later, were exposed to various doses of β-galactosidase-expressing HSV-1. Six hours later, viral entry was quantitated as described above.

Western Blot

For Western blot analyses, cells transfected with pDS43/*myc*His or control plasmid pcDNA3 were lysed in sample buffer for SDS-PAGE. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane that was blocked with 5% skim milk (w/v) in PBS and 0.05% (w/v) Tween 20 (PBS-T) for 1 hr at room temperature. The blot then was incubated sequentially with the anti-Myc antibody diluted in 1% (w/v) BSA in PBS-T and horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). Bound antibodies were visualized by the enhanced chemiluminescence (ECL) system (Amersham).

Binding of gD-1 to 3-*O*-Sulfated HS

Metabolically labeled [^{3}H]HS (2–4 $\times$ 10^6 dpm, 1.2–2.5 pmol) from wild-type CHO cells or pgsF-17 cells was incubated with [^{35}S]PAPS (10 μM, 20 $\times$ 10^6 dpm) and 60 ng of purified m3-OST-1 or h3-OST-3_A. The average number of 3-*O*-sulfate groups introduced per HS molecule from wild-type CHO cells was 8 for 3-OST-1 and 14 for h3-OST-3_A, based on the ^{35}S count transferred to the HS, given the specific activities of [^{35}S]PAPS (18 Ci/mmol) and [^{3}H]HS (727 Ci/mmol) and assuming a molecular weight of 81,000 for HS. Each preparation of enzyme-modified or unmodified HS (1–2 $\times$ 10^5 dpm of ^{3}H) was mixed with gD-1(306t) (10 μg) in 50 μl of binding buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl and 0.01% Triton X-100) for 30 min at room temperature. The anti-gD antiserum R7 (or the anti-gD monoclonal antibody DL6) (5 μl) was added for incubation at 4°C for 1 hr, followed by addition of protein A–agarose gel (80 μl of 1:1 slurry) and agitation at 4°C for an additional hour. The protein A–agarose gel was washed with binding buffer and the bound HS eluted with 1 ml of binding buffer containing 1 M NaCl.

For affinity coelectrophoresis (Lee and Lander, 1991), metabolically labeled [^{35}S]HS was modified by incubation with purified h3-OST-3_A as above except that unlabeled PAPS (10 μM) was the sulfate donor. The molecules of modified [^{35}S]HS capable of binding gD-1 ([^{35}S]HS$^{gD\text{-}Act}$) were collected by coprecipitation with gD-1(306t) as described above. The molecules that failed to coprecipitate were designated [^{35}S]HS$^{gD\text{-}Inact}$, although this fraction probably contained some gD-1-binding material. Purified gD-1(306t) was cast in low melting point 1% agarose gel (GIBCO) in a buffer containing 125 mM sodium acetate, 50 mM 3-(N-morpholino)-2-hydroxypropane-sulfonic acid, and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (pH 7.0) at nine different concentrations for [^{35}S]HS$^{gD\text{-}Act}$, ranging from 0 to 11 μM, and at four different concentrations for [^{35}S]HS$^{gD\text{-}Inact}$, ranging from 0 to 83 μM, in each separation zone. Approximately 50,000 cpm of [^{35}S]HS$^{gD\text{-}Act}$ or [^{35}S]HS$^{gD\text{-}Inact}$ was loaded in each separation zone and electrophoresis was performed at 400 mA for 2.5 hr in a cold room with circulated cold water. The gels were dried and analyzed on a Beta-scope 603 (Betagen) to determine the migration of [^{35}S]HS. The [^{35}S] intensity was plotted against the migration distance through the separation zone to define the distance migrated in the presence or absence of gD-1(306t).

Detection of gD-1 Receptors on Cells

CHO cells and pgsA-745 cells were transfected with pDS43 or pcDNA3 and plated in 96-well plates as described above. Some cultures were treated with heparinases as described below prior to the assay. After washing the cells and blocking with PBS containing 3% BSA for 30 min, the cells were incubated with purified gD-1:Fc (1 μg/ml) in 1% BSA-PBS for 45 min at room temperature. The cells were then washed and fixed with 2% formaldehyde and 0.2% gluteraldehyde in PBS. Cell-bound gD-1:Fc was detected by sequential incubations with biotin-conjugated goat anti-rabbit IgG antibody (Sigma), Amdex streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech, Inc.), and the peroxidase substrate 3,3′,5,5′-tetramethyl benzidine (Sigma) in 50 mM phosphate-citrate buffer. Product was quantitated by use of a Spectromax 250 ELISA reader to measure OD_{370}.

Heparinase Treatment

Heparinase I (heparin lyase I; heparinase; EC 4.2.2.8) and heparinase III (heparitin sulfate lyase III; heparitinase, EC 4.2.2.8) were obtained from Sigma. Transfected cells plated in 96-well dishes were washed twice with PBS lacking magnesium and calcium ions, overlayed with several dilutions of heparinase I and heparinase III or PBS alone, and incubated at 30°C for 2 hr. The cells were then washed with PBS and used for viral entry assays or for quantitation of gD-1:Fc binding to the cells.

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