Characterization of heparan sulphate 3-O-sulphotransferase isoform 6 and its role in assisting the entry of herpes simplex virus type 1

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Heparan sulphate (HS) 3-O-sulphotransferase transfers sulphate to the 3-OH position of the glucosamine residue of HS to form 3-O-sulphated HS. The HS modified by 3-O-sulphotransferase isoform 3 binds to HSV-1 (herpes simplex virus type 1) gD (envelope glycoprotein D), and the resultant 3-O-sulphated HS serves as an entry receptor for HSV-1. In the present paper, we report the isolation and characterization of a novel HS 3-O-sulphotransferase isoform, designated HS 3-O-sulphotransferase isoform 6 (3-OST-6). Mouse 3-OST-6 gene was identified in the EST (expressed sequence tag) database and cloned into pcDNA3.1/Myc-His vector. A CHO (Chinese-hamster ovary) cell line that stably expresses 3-OST-6 (3OST6/CHO cells) was prepared. The disaccharide analysis of the HS isolated from 3OST6/CHO cells revealed that 3-OST-6 exhibits HS 3-O-sulphotransferase activity. Furthermore, 3OST6/CHO cells were susceptible to infection by HSV-1, but not by other alphaherpesviruses examined, suggesting that 3-OST-6 produces a specific entry receptor for HSV-1. Our results indicate that a new member of 3-OST family generates an entry receptor for HSV-1. The findings add to the growing body of evidence that HSV-1 entry is mediated by 3-O-sulphated HS generated by multiple members of 3-O-sulphotransferases.

Key words: heparan sulphate, heparin, herpes simplex virus (HSV), sulphotransferase, viral entry.

INTRODUCTION

Heparan sulphate (HS) is a highly sulphated and unbranched polysaccharide. The disaccharide repeats of HS are GlcA (glucuronic acid) or IdoA (iduronic acid) and glucosamine with various sulphations (structures shown in Figure 1). HSs are widely present on the surface of mammalian cells and in the extracellular matrix. HSs are involved in many biological processes, including blood coagulation, viral infections, embryonic development, wound healing and tumour growth suppression [1–6]. The biosynthesis of HS occurs in the Golgi apparatus. After the polysaccharide backbone is synthesized, it is subjected to various modifications. These modifications include N-deacetylation and N-sulphation of glucosamine, C_5-epimerization of GlcA to form IdoA residues, 2-O-sulphation of GlcA or IdoA residues, as well as 6-O-sulphation and 3-O-sulphation of glucosamine residues. The enzymes that carry out the modifications have been cloned, including HS C_5-epimerase, N-deacetylase/N-sulphotransferase, 2-O-sulphotransferase, 6-O-sulphotransferase and 3-O-sulphotransferase [7].

HS 3-O-sulphotransferase (3-OST) transfers sulphate from PAPS (adenosine 3′-phosphate 5′-phosphosulphate) to the 3-OH position of glucosamine residue to form the 3-O-sulphated HS as illustrated in Figure 1. Six different isoforms of 3-OST have been identified (3-OST-1, -2, -3A, -3B, -4 and -5). Among them, 3-OST-3A and 3-OST-3B have nearly identical amino acid sequences in the sulphotransferase domain and generate the same 3-O-sulphated disaccharides [8,9]. These isoforms share greater than 60 % identity in the sulphotransferase domain and exhibit distinct tissue expression patterns [10,11]. In addition, the isoforms recognize the unique saccharide sequences around the modification site [10,12]. The substrate specificity of 3-OST-1, 3-OST-3 and 3-OST-5 has been studied extensively, and is presented in Figure 1. The resultant 3-O-sulphated HS generated by different isoforms display distinct biological functions. For instance, the HS modified by 3-OST-1 and by 3-OST-5 contain anticoagulant activity, whereas the HS modified by 3-OST-3 and 3-OST-5 serve as entry receptors for HSV-1 (herpes simplex virus type 1) [9,13–15].

HSV-1 belongs to the alphaherpesviruses subfamily of the herpesvirus family. Infections with HSV-1 are highly prevalent in humans and cause localized mucocutaneous lesions and encephalitis in rare cases [16,16a]. It has been known that the cell-surface HS plays an important role in assisting HSV-1 attachment to host cells [17], as well as in inducing viral entry into the target cells [6,18]. The attachment process primarily involves the interaction between HS and the virion envelope gC (glycoprotein C) and/or gB [19]. Specific sulphated saccharide structures probably contribute to the binding of HS and gC [20]. Following the attachment, HSV-1 enters the target cells by interacting with

Abbreviations used: AnMan3S, 2,5-anhydromannitol 3-*O*-sulphate; AnMan6S, 2,5-anhydromannitol 6-*O*-sulphate; AnMan3S6S, 2,5-anhydromannitol 3,6-*O*-disulphate; AT, antithrombin; BHV-1, bovine herpesvirus; CHO, Chinese-hamster ovary; Con A, concanavalin A; EST, expressed sequence tag; gB (etc.), envelope glycoprotein B (etc.); GlcA, glucuronic acid; HS, heparan sulphate; HSV-1, herpes simplex virus type 1; HVEM, herpesvirus entry mediator; IdoA, α-iduronic acid; IdoA2S, L-iduronic acid 2-*O*-sulphate; MTN, Multiple Tissue Northern; ORF, open reading frame; 3-OST, HS 3-O-sulphotransferase; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; PRV, pseudorabies virus; RPIP-HPLC, reverse-phase ion-pairing HPLC.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number AY574375.

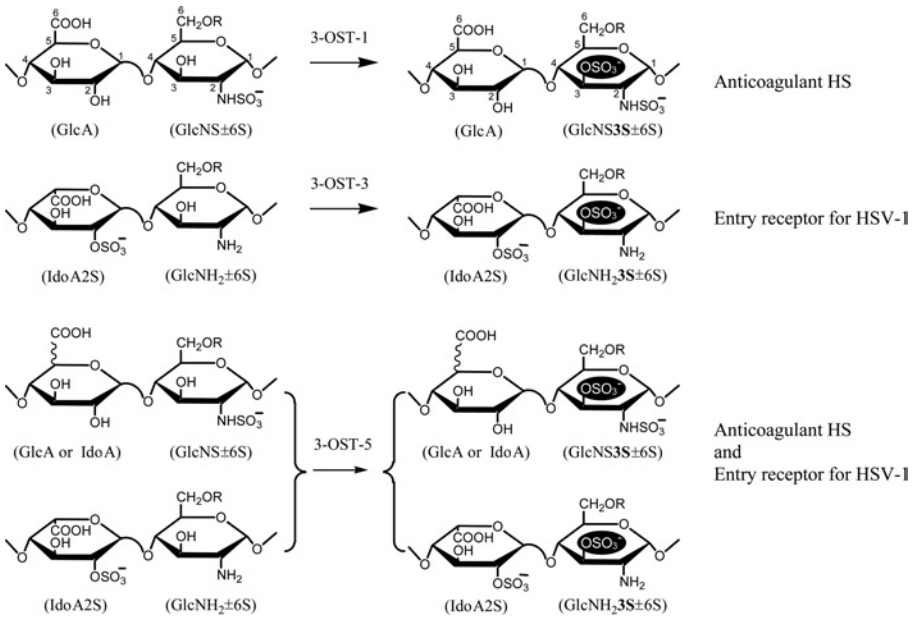

Figure 1 Substrate specificity of 3-OSTs and the structure of HS disaccharide repeating unit

3-OST-1 transfers sulphate to the 3-OH position of a glucosamine N-sulphate (GlcNS ± 6S) unit that is linked to a GlcA unit to form the 3-O-sulphated HS containing GlcA-GlcNS**3S** ± 6S, which is an anticoagulant HS. 3-OST-3 transfers sulphate to the 3-OH position of an N-unsubstituted glucosamine unit ($GlcNH_2$ ± 6S) that is linked to an IdoA2S unit to form the 3-O-sulphated HS containing IdoA2S-$GlcNH_2$**3S** ± 6S, which is an entry receptor for HSV-1. Both 3-OST-3A and 3-OST-3B have nearly identical amino acid sequences in the sulphotransferase domain, and sulphate identical disaccharides. For clarity, 3-OST-3 represents both 3-OST-3A and 3-OST-3B, unless specified. 3-OST-5 transfers sulphate to the 3-OH position of a modified glucosamine unit ($GlcNH_2$ ± 6S or GlcNS ± 6S) that is linked to various GlcA or IdoA/IdoA2S units. The HS modified by 3-OST-5 displays anticoagulant activity and serves as entry receptor for HSV-1. The numbers indicate the positions of saccharide unit. The 3-O-sulphation by 3-OSTs is shaded and indicated in bold. R represents a proton (-H) or a sulphate group ($-SO_3$).

specific cell-surface entry receptors to establish the infection. Three families of HSV-1 entry receptors are present on the target cell surface and bind to viral envelope gD [21]. HVEM (herpesvirus entry mediator) and nectin-1 represent two families of those receptors, which belong to the TNFR (tumour necrosis factor receptor) family and the immunoglobulin superfamily respectively [22,23]. The 3-O-sulphated HS, which is generated by 3-OST-3 and 3-OST-5, represents the third family of HSV-1 entry receptor [9,14]. This receptor is unique as it is a polysaccharide and contains a specific saccharide structure. Our research attention has been focused on the identification of the role of 3-O-sulphated HS in assisting the entry of HSV-1 and the enzymes involved in the biosynthesis of this subtype of HS. We have identified the structure of a gD-binding octasaccharide from 3-OST-3-modified HS [24]. Supporting the role of 3-O-sulphated HS in assisting HSV-1 entry, two putative 3-O-sulphated HS binding pockets on gD were observed in the co-crystal of gD and HVEM [25]. One of the putative 3-O-sulphated HS binding sites is located at the N-terminal of gD. The virus carrying gD mutations in this N-terminal domain is unable to infect the cells expressing 3-OST-3, confirming the location of a 3-O-sulphated-HS-binding site as predicted by the crystal structure [26].

In the present paper, we report that a novel isoform of 3-OST, designated 3-OST-6, generates a HSV-1 entry receptor. The HS isolated from stably transfected 3OST6/CHO (Chinese-hamster ovary cell line that stably expresses 3-OST-6) cells contains the characteristic gD-binding disaccharides, IdoA2S-AnMan3S (where IdoA2S is L-IdoA 2-*O*-sulphate and AnMan3S is 2,5-anhydromannitol 3-O-sulphate) and IdoA2S-AnMan3S6S (where AnMan3S6S is 2,5-anhydromannitol 3,6-O-disulphate). Accordingly, we also found that 3OST6/CHO cells are susceptible to HSV-1 infection. The newly identified enzyme provides an additional tool for understanding the relationship between the saccharide sequence and the biological functions of HS. The results suggest that a series of 3-OST isoforms synthesize the unique gD-binding 3-O-sulphated HS, demonstrating the important and complex roles of HS in promoting HSV-1 infection.

MATERIALS AND METHODS

Materials

$Na_2{}^{35}SO_4$ was purchased from ICN. Human AT (antithrombin) was from Cutter Biological (Berkeley, CA, U.S.A.). A truncated form of HSV-1 gD, gD-1 (306t), and monoclonal anti-gD (DL6) were gifts from Dr Cohen and Dr Eisenberg of the University of Pennsylvania (Philadelphia, PA, U.S.A.). The preparations of plasmid expressing human 3-OST-5 (pcDNA3.1-3OST5) and human 3-OST-5 stable expression CHO (3OST5/CHO) cells have been described elsewhere [9,27].

Cell culture and viruses

CHO cells were maintained in exponential growth by subculturing twice a week at 37 °C under 6 % CO_2 humidified atmosphere. CHO cells were grown in Ham's F-12 medium (Invitrogen) supplemented with 10 % (v/v) foetal bovine serum (JRH Biosciences, Lenexa, KS, U.S.A.). 3OST5/CHO cells were maintained in Ham's F-12 medium supplemented with 10 % (v/v) foetal bovine serum and 1 mg/ml Geneticin (G418 sulphate, Invitrogen). Recombinant β-galactosidase-expressing viruses, HSV-1(KOS)gL86, HSV-1(KOS)tk12, HSV-2(333)gJ-, gH-negative PRV(Kaplan) (pseudorabies virus) and BHV-1(Cooper) (bovine herpesvirus), were cultured as described elsewhere [28].

Isolation of 3-OST-6 cDNA

Screening the GenBank® database with human 3-OST-3 amino acid sequence, an exon coding for the catalytic domain of 3-OST-6 was identified from a human genomic clone with the accession no. AE006640.1. After further probing the EST (expressed sequence tag) database with the deduced partial amino acid sequence of human 3-OST-6, its mouse homologue (with 94 % identity with 3-OST-6 amino acid sequence) was found (accession no. AI447860). The insert of this EST clone was fully sequenced for both strands, revealing a complete ORF (open reading frame). The insert was then cloned into a pcDNA3 vector using *Eco*RI/*Xba*I sites (pcDNA3-3OST6).

Expression of 3-OST-6

Preparation of Myc-tagged 3-OST-6 expression vector

We introduced Myc and His_6 tags at the C-terminal of 3-OST-6 by subcloning 3-OST-6 cDNA into pcDNA3.1/Myc-His vector (Invitrogen). The ORF of 3-OST-6 was amplified from pcDNA3-3OST6 by PCR using the 5′-specific primer, 5′-GAGCTCG-GATCCACTAGTAACGGC-3′; and the 3′-specific primer, 5′-AA-ATTTCTCGAGGACCCAGCCAAAGTCCTG-3′ (*Xho*I site underlined). The PCR product was subcloned into pcDNA3.1/Myc-His using *Eco*RI/*Xho*I sites. The coding region was completely sequenced for both strands (pcDNA3.1-3OST6-Myc-His).

Preparation of 3-OST-6 stable expression cells (3OST6/CHO)

The expression plasmid pcDNA3.1-3OST6-Myc-His was transfected into wild-type CHO cells using LIPOFECTAMINE 2000 (Invitrogen) following the manufacturer's protocol. At 6 days after transfection, the cells were trypsinized and transferred into a 48-well plate at a concentration of 0.5 cells per well. The cells were grown in Ham's F-12 medium containing 10 % (v/v) foetal bovine serum and 1 mg/ml Geneticin at 37 °C under 6 % CO_2 for 2–3 weeks until confluence was attained. The clone with the highest expression level of 3-OST-6 was identified by Western blot analysis.

Characterization of 3-OST-6 activity

In vitro assay for 3-OST-6 activity

Approx. 3×10^6 3OST6/CHO cells were mixed with 100 μl of cold 0.25 M sucrose containing 1 % (v/v) Triton X-100. The insoluble residues were removed after centrifuging at 10000 ***g*** for 10 min. The HS sulphotransferase activity was determined by incubating the cell extract with 1 μg of unlabelled HS (from bovine kidney), 4×10^7 c.p.m. of [^{35}S]PAPS in 50 μl of a buffer containing 50 mM Mes, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 1 % (v/v) Triton X-100 (pH 7). The reaction was incubated at 37 °C for 1 h and was then subjected to a 200 μl DEAE-Sepharose column to purify [^{35}S]HS. The resultant [^{35}S]HS was subjected to the disaccharide analysis as described below.

Metabolically labelling 3OST6/CHO cells and the preparation of [^{35}S]HS

CHO-K1 and 3OST6/CHO cells were grown to 60–70 % confluence in T-75 flasks. Cells were then incubated with 5 ml of growth medium containing 1 mCi/ml of $Na_2{}^{35}SO_4$ for 10 h at 37 °C. The [^{35}S]HS was purified using DEAE chromatography.

Disaccharide analysis of [^{35}S]HS

The [^{35}S]HS isolated from 3OST6/CHO cells was degraded with nitrous acid at pH 1.5, followed by reduction with sodium borohydride [29]. The disaccharides were resolved by a C_{18} reverse-phase column (0.46 cm × 25 cm) (Vydac) under the RPIP-HPLC (reverse-phase ion-pairing HPLC) condition [30]. The identities of the disaccharides were determined by co-eluting with appropriate ^{3}H-labelled and ^{35}S-labelled disaccharides [30].

Western blot analysis

Approx. 300 μg of cell extract was resolved on SDS/13 % PAGE and was transferred on to a nitrocellulose membrane (Amersham Biosciences). Biotinylated protein molecular-mass markers (1 μg) (Amersham Biosciences) were used as the molecular-mass standards, and the markers were probed by avidin–horseradish peroxidase (Amersham Biosciences). Myc-tagged 3-OST-6 protein was detected by using mouse anti-Myc antibody (Invitrogen) followed by horseradish-peroxidase-conjugated anti-mouse IgG secondary antibody (Amersham Biosciences). ECL® (enhanced chemiluminescence) Western blotting detection reagents (Amersham Biosciences) were used to induce chemiluminescence, and the blot was exposed to X-ray film (Kodak) for 15 min.

Determination of the bindings of 3-O-sulphated HS to AT and HSV gD-1

Determination of the binding of HS to AT

The AT binding of HS isolated from 3OST6/CHO cells was performed by using an AT/Con A (concanavalin A)–Sepharose approach [13]. Briefly, [^{35}S]HS (100000 c.p.m.) was incubated in 150 μl of a buffer containing 10 mM Tris/HCl, 150 mM NaCl, 1 μM dextran sulphate, 1 mM Ca^{2+}, Mg^{2+} and Mn^{2+}, and 0.1 mg/ml AT (pH 7.5), at room temperature (25 °C) for 30 min. The solution was mixed with the prewashed Con A–Sepharose (60 μl of 1:1 slurry) and agitated at room temperature for 1 h. The gel was then washed three times with 1 ml of a buffer containing 10 mM Tris/HCl, 0.0004 % (v/v) Triton X-100 and 150 mM NaCl (pH 7.5). The HS was eluted from the gel using 1 ml of a buffer containing 10 mM Tris/HCl, 1000 mM NaCl and 0.0004 % (v/v) Triton X-100 (pH 7.5).

Determination of the binding of gD to HS

The assay for determining the binding of 3-O-sulphated HS to gD was carried out by an immunoprecipitation procedure using anti-gD monoclonal antibody [14]. The [^{35}S]HS (100000–200000 c.p.m.) was incubated in 50 μl of a buffer containing 50 mM Tris/HCl, 150 mM NaCl and 0.01 % (v/v) Triton (pH 7) (binding buffer), and 2 mg/ml of gD at room temperature for 30 min. The anti-gD monoclonal antibody DL6 (5 μl) was added and incubated at 4 °C for 1 h followed by the addition of Protein A–agarose gel (80 μl of 1:1 slurry) and agitated at 4 °C for an additional 1 h. The gD-binding HS was eluted from the gel with 1 ml of 1 M NaCl in the binding buffer.

HSV entry assay

Viral entry assays were based on the quantification of the activity of β-galactosidase from the recombinant HSV viruses in which the expression of β-galactosidase is turned on upon viral entry into mammalian cells [14,22,23]. Briefly, wild-type CHO-K1, 3OST5/CHO and 3OST6/CHO cells were plated (2–4×10^4 cells/well) in 96-well culture dishes for 14 h before the infection by recombinant HSV-1 (KOS). In separate experiments, other members of the alphaherpesvirus subfamily expressing β-galactosidase, including HSV-2(333), PRV (gH^-) and BHV-1, were tested for their entry into 3OST6/CHO cells. Wild-type CHO

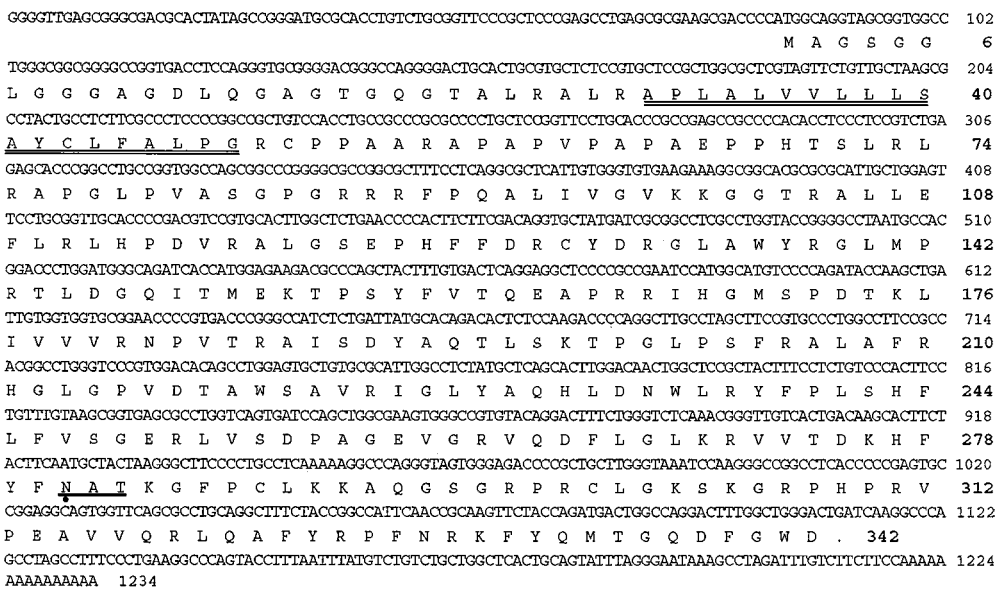

Figure 2 Nucleotide and deduced amino acid sequences of mouse 3-OST-6

The single predicted membrane-spanning domain and a potential N-linked glycosylation site are indicated by the double underlining and by the underlining with a ● below the glycosylated asparagine residue respectively.

cells and the CHO cells expressing nectin-1 were used as positive and negative controls respectively throughout the study [23]. Cells (post-infection) were assayed for the activity of β-galactosidase [15].

Cell–cell fusion assay

The assay was described in a report by Pertel et al. [31]. Briefly, subconfluent wild-type CHO-K1 'effector cells' in a six-well dish were transfected with plasmids expressing HSV-1 glycoproteins: gB (pPE98), gD (pEP99), gH (pEP100) and gL (pEP101) and T7 RNA polymerase (0.5 μg/well of each plasmid). In a separate six-well dish, CHO K-1 'target cells' were transfected with 3-OST-5 or 3-OST-6 plasmids (1.0 μg/well) and vectors expressing luciferase under the control of the T7 promoter. The total amount of DNA transfected was kept constant at 2.5 μg/well by balancing with the vector plasmid (pcDNA3.1). LIPOFECTAMINE reagent (Invitrogen) in Opti-MEM (Gibco) was used for all transfections. After incubation at 37 °C in 5 % CO_2 for 16 h, the mixture was removed. Ham's F-12 containing 10 % (v/v) foetal bovine serum was added, and the cells were incubated for an additional 4 h. Cells were detached subsequently using trypsin-EDTA. The effector and target cells were mixed at a 1:1 ratio before plating on to 24-well plates. The luciferase activity was quantified using the Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.), and the cell–cell fusion microimages were captured 24 h after the mixing. Cell fusion was visualized by microscopy following Giemsa staining. Microscopy was performed using $\times$ 20 objective of the inverted microscope (Zeiss, Axiovert 100M). The Slidebook version 3.0 (Intelligent Imaging Innovations, Cambridge, MA, U.S.A.) was used for recording the images.

Northern blot analysis

The coding sequence of 3-OST-6 was used as a template to prepare the ^{32}P-labelled probe using the Random Primer Kit (Stratagene) and [α-^{32}P]dCTP to hybridize the Mouse MTN (Multiple Tissue Northern) blot (Clontech). The hybridization was carried out in a standard hybridization buffer [5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution is 0.2 mg/ml Ficoll 400/0.2 mg/ml polyvinylpyrrolidone/0.2 mg/ml BSA) and 0.5 % SDS] at 68 °C overnight, and the blot was washed twice with 2 $\times$ SSC containing 0.05 % (w/v) SDS at room temperature for 30 min), followed by washing twice with 0.1 $\times$ SSC containing 0.1 % (w/v) SDS at 50 °C for 40 min. The membrane was exposed to X-ray film (Kodak) for 5 days.

RESULTS

Isolation of the cDNA encoding 3-OST-6

Screening the non-redundant database of GenBank® with the deduced amino acid sequence of human 3-OST-3 (accession no. NM_006042), we identified an exon coding for the catalytic domain of a novel 3-OST. Probing the EST database further with the deduced partial sequence of the novel human sulphotransferase, a mouse homologue (with 94 % identity in amino acid sequence) was identified from an EST cDNA clone with accession no. AI447860. This protein contains a complete ORF encoding a protein with 342 amino acid residues, and was designated 3-OST-6 (Figure 2). It has a single potential N-glycosylation site with the predicted molecular mass of 37 417 Da. The deduced amino acid sequence has 87, 63 and 61 % identity with m3-OST-3 and m3-OST-5 and m3-OST-1 in the sulphotransferase domain respectively. The amino acid sequence alignment of 3-OST-1, -3A, -5 and -6 is shown in Figure 3. Putative PAPS-binding sites were also found in 3-OST-6, based upon the consensus sequences (Figure 3) [10,32].

Determination of the sulphotransferase activity of 3-OST-6

Because 3-OST-6 has high identity with 3-OST-1, 3-OST-3 and 3-OST-5, we decided to examine its ability to generate

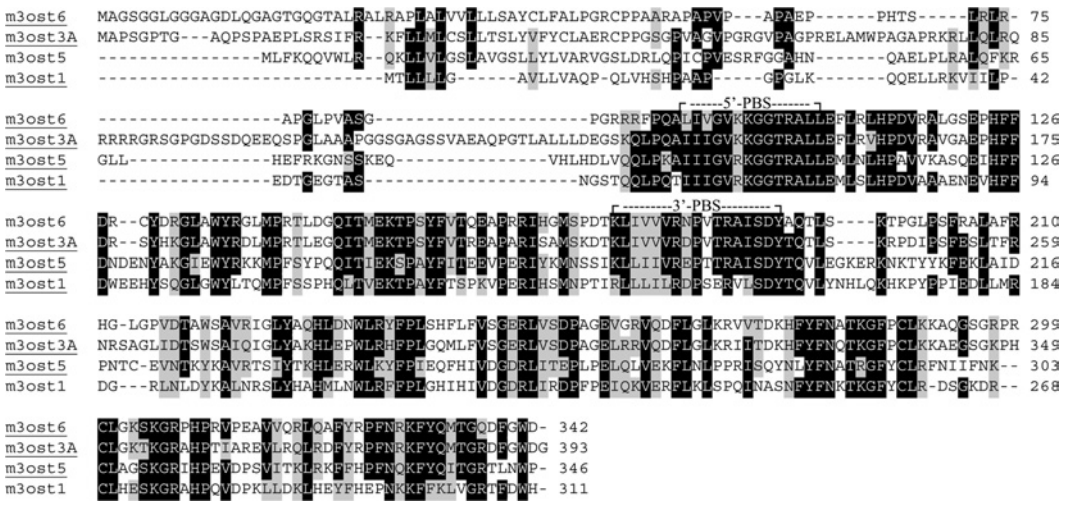

Figure 3 Multiple amino acid sequence alignment of mouse 3-OST-6 with mouse 3-OST-1, 3-OST-3A and 3-OST-5

The alignment was performed by using the BioEdit program. Introduced gaps are shown as dashes, and aligned amino acids are boxed and shaded black for identical residues and dark grey for similar residues. 5′-PBS represents the putative domain that binds to the 5′-phosphate of PAPS, and 3′-PBS represents the putative domain that binds to the 3′-phosphate of PAPS.

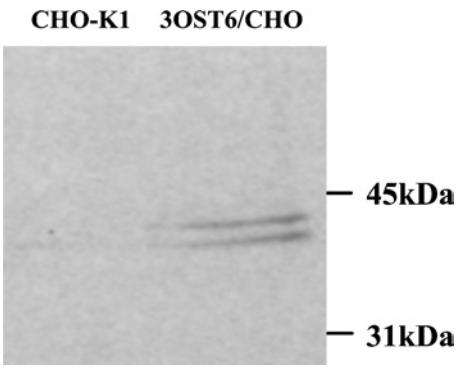

Figure 4 Western blotting analysis of 3OST6/CHO cell extract

3OST6/CHO and CHO-K1 cell extracts were resolved on SDS/13 % PAGE. Myc-tagged 3-OST-6 was detected by incubating the blot with mouse anti-Myc antibody and then horseradish-peroxidase-conjugated anti-mouse IgG secondary antibody. The apparent molecular masses of the two bands were approx. 38 kDa and 41 kDa. The predicted molecular mass of 3-OST-6–Myc/His is 37.4 kDa. Molecular masses of standards are given to the right of the gel.

3-O-sulphated HS in transfected cells. Initially, we transiently expressed 3-OST-6 (pcDNA3-3OST-6) in COS-7 cells, however, we failed to detect any 3-OST activity in the transfected cells using an *in vitro* assay (results not shown). To overcome this problem, a CHO cell line with stable expression of 3-OST-6 (3OST6/CHO cells) was prepared, where a Myc-tag was introduced to the C-terminus of the protein for monitoring the level of expression of 3-OST-6.

Western blot analysis using anti-Myc antibody was performed on the lysates obtained from 3OST6/CHO cells and non-transfected CHO cells (CHO-K1 cells, as a negative control) (Figure 4). We observed two protein bands in the lysate of CHO/3OST6 cells at the molecular masses of 38 and 41 kDa (Figure 4), whereas no signals were detected in the non-transfected CHO cells. The protein band with lower molecular mass was very close to the predicted molecular mass (37.4 kDa) of 3-OST-6. The protein band with higher molecular mass probably represented the 3-OST-6 with glycosylation or other post-translational modifications.

To characterize substrate specificity of 3-OST-6, we analysed the structure of the HS isolated from 3OST6/CHO cells by conducting the disaccharide analysis. The 3OST6/CHO cells were metabolically labelled with $Na_2{}^{35}SO_4$, and the [^{35}S]HS was isolated. The resultant [^{35}S]HS was subjected to nitrous acid degradation at pH 1.5, followed by sodium borohydride reduction to prepare ^{35}S-labelled disaccharides (the schematic representation of the disaccharide analysis of [^{35}S]HS illustrated in Figure 5C). The ^{35}S-labelled disaccharides were resolved by RPIP-HPLC, and the chromatograms are shown in Figure 5. Comparing the profiles of the degraded [^{35}S]HS that was isolated from CHO-K1 cells (Figure 5A), we found two additional ^{35}S-labelled disaccharides in the HS from 3OST6/CHO cells (Figure 5B). Those two ^{35}S-labelled disaccharides were 3-O-sulphated disaccharides with the structures of IdoA2S-AnMan3S (eluted at 36.5 min) and IdoA2S-AnMan3S6S (eluted at 69.5 min) by co-eluting with the disaccharide standards [30]. A large peak of ^{35}S-labelled disaccharide with a structure of IdoA2S-AnMan6S (AnMan6S is 2,5-anhydromannitol 6-O-sulphate) was also observed (eluted at 56–58 min) both in the samples from CHO-K1 and 3OST6/CHO. The presence of ^{35}S-labelled IdoA2S-AnMan6S is expected because it is a common disaccharide in HS. We should also observe IdoA-AnMan6S after nitrous acid (pH 1.5) degradation of [^{35}S]HS, which is a monosulphated disaccharide from nitrous acid degraded polysaccharide. To identify this monosulphated disaccharide requires a different HPLC condition. The HPLC condition used in the present study is designed to identify both disulphated and trisulphated disaccharides [30]. This condition provides the best resolution among disulphated and trisulphated disaccharides. However, it does not provide the best resolution for monosulphated disaccharides. These results suggest that the HS isolated from 3OST6/CHO cells contain 3-O-sulphated HS, therefore 3-OST-6 encodes a protein with the anticipated 3-OST activity. Unlike previously reported 3-OST isoforms, we were unable to detect the 3-OST activity in the lysate of 3OST6/CHO cells by an *in vitro* assay, suggesting a unique biochemical property of 3-OST-6. In a parallel control experiment, we readily observed the activity of 3-OST-5 in the extract of 3OST5/CHO

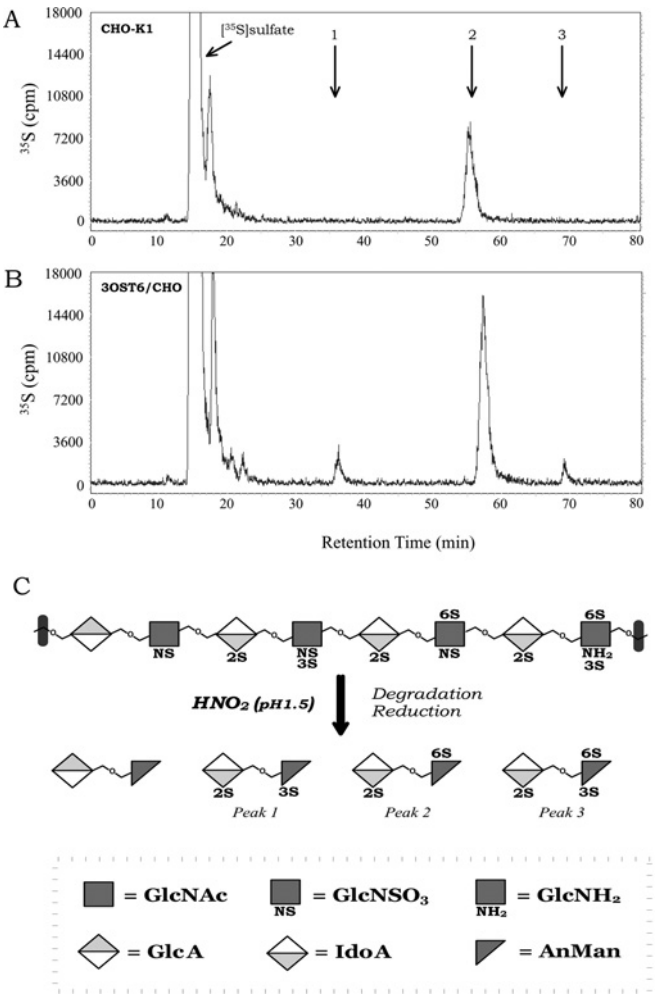

Figure 5 RPIP-HPLC chromatograms of the disaccharide analysis of HS isolated from 3OST6/CHO cells

[^{35}S]HS was extracted from CHO-K1 (**A**) and 3OST6/CHO (**B**) cells after incubating them with $Na_2{}^{35}SO_4$. [^{35}S]HS was then depolymerized by nitrous acid at pH 1.5, followed by sodium borohydride reduction. The resultant ^{35}S-labelled disaccharides were resolved on RPIP-HPLC. The elution positions of disaccharides are indicated by arrows, where arrow 1 represents IdoA2S-AnMan3S, arrow 2 represents IdoA2S-AnMan6S, and arrow 3 represents IdoA2S-AnMan3S6S. (**C**) The reaction used for degrading HS with nitrous acid. Nitrous acid (at pH 1.5) reacts with an N-sulphated glucosamine ($GlcNSO_3$) unit to form 2,5-anhydromannitol (AnMan). The identities of the resultant disaccharides were determined by co-eluting with disaccharide standards on RPIP-HPLC.

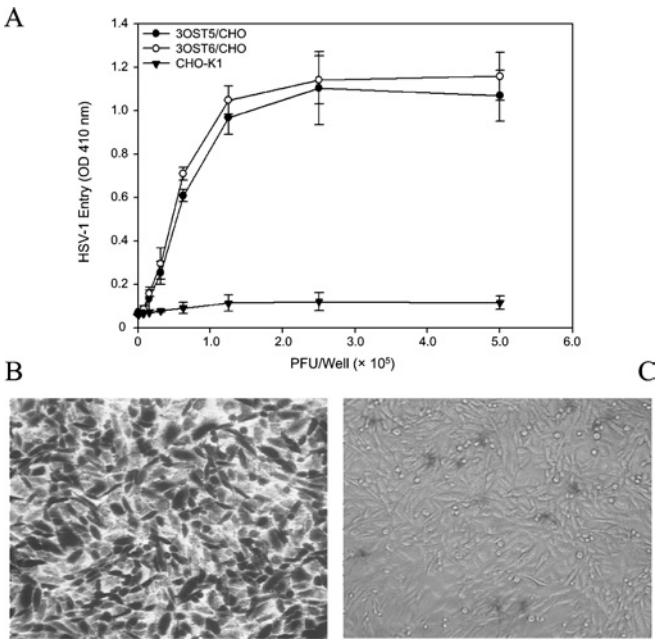

Figure 6 Entry of HSV-1 into 3OST6/CHO cells and CHO-K1 cells

(**A**) As indicated, 3OST6/CHO, 3OST5/CHO and CHO-K1 cells were infected with several PFUs (plaque-forming units)/well of HSV-1(KOS-gL86). The cells were lysed for the quantification of β-galactosidase activity as a measure of viral entry. Attenuance at 410 nm (OD 410 nm) of ONPG (*o*-nitrophenyl β-D-galactopyranoside) reaction products were plotted against PFUs used. 3OST6/CHO (**B**) and CHO-K1 (**C**) cells were exposed to KOS-gL86 at 100 (PFUs)/cell. At 6 h later, the cells were washed, fixed and incubated with X-gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) to identify infected cells (dark cells).

Table 1 Binding of 3OST6/CHO HS to gD and AT

Binding of the HS and gD was determined by incubating modified [^{35}S]HS with gD followed by immunoprecipitation using anti-gD monoclonal antibody (DL6) to precipitate the complex of [^{35}S]HS and gD. Results are means $\pm$ S.D. Binding of HS to AT was determined by incubating modified [^{35}S]HS and AT by using an AT/ConA–Sepharose gel as described in the Materials and methods section. The control was the [^{35}S]HS extracted from CHO-K1 cells labelled with $Na_2{}^{35}SO_4$.

	Binding to gD (%)	Binding to AT (%)
Control	3.8 ± 0.9 ($n = 2$)	0.5 ± 0.1 ($n = 3$)
HS from 3OST6/CHO	8.8 ± 2.0 ($n = 2$)	0.8 ± 0.2 ($n = 3$)
HS from 3OST5/CHO	7.3 ($n = 1$)	4.1 ± 1.2 ($n = 3$)

cells by using this *in vitro* assay (results not shown). It is important to note that IdoA2S-AnMan3S and IdoA2S-AnMan3S6S are characteristic gD-binding HS disaccharides [14] (structures shown Figure 1).

The activity of 3-OST-6 in assisting HSV-1 entry and cell–cell fusion

Non-transfected CHO cells are resistant to HSV-1 infection. However, introduction of the cDNA that allows the cells to synthesize an entry receptor renders the susceptibility of CHO cells to HSV-1 infection. A significant increase in β-galactosidase activity was observed in 3OST6/CHO cells infected by HSV-1, compared with that of CHO-K1 cells (Figure 6A). Furthermore, the level of β-galactosidase was comparable with the HSV-1-infected cells that stably express 3-OST-5 (3OST5/CHO) (Figure 6A). 3-OST-5 is known to generate an entry receptor for HSV-1 [9]. We also compared the activity of β-galactosidase *in situ*. As expected, nearly all of 3OST6/CHO cells were susceptible (Figure 6B, dark cells) to the entry of HSV-1. Clearly, no dark cells were observed in CHO-K1 cells infected with the same HSV-1 (Figure 6C). Our results suggest that 3-OST-6 renders the susceptibility of CHO cells to HSV-1 infection. A direct binding between 3-OST-6-modified HS to gD was also detected (Table 1). As expected, the HS from 3OST6/CHO cells has an approx. 2.3-fold increase in binding to gD as compared with the HS from CHO-K1; a similar increase was observed for the HS from 3OST5/CHO cells. Taken together, these observations are consistent with the conclusion that 3-OST-6 generates HSV-1 entry receptor and that the entry mediating activity of 3-OST-6 is very similar to that of 3-OST-5.

In separate experiments, we also examined the activity of 3-OST-6 in assisting the entry of other members of alphaherpesvirus

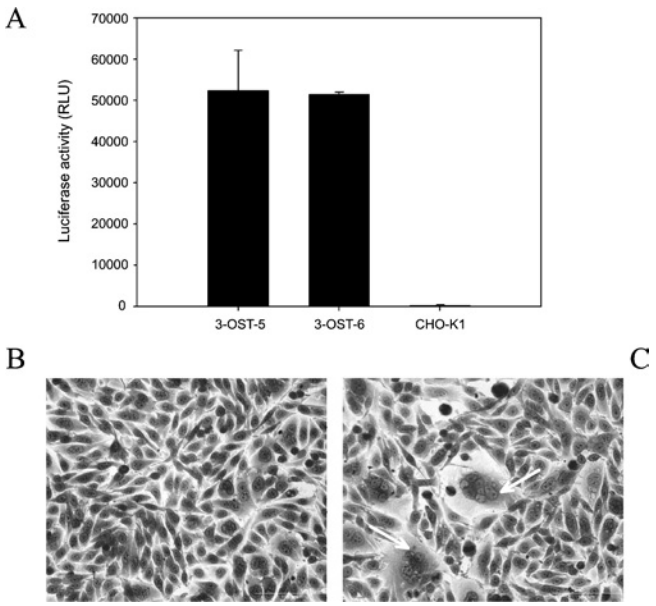

Figure 7 Cell–cell fusion mediated by 3-O-sulphated HS

(**A**) Cell fusion is dependent on expression of 3-OST-6. CHO-K1 cells were used as effector and target cell. Effector cells were transfected with plasmid expressing HSV-1 glycoproteins and luciferase reporter plasmids. Target cells were transfected with T7 RNA polymerase and the plasmid expressing 3-OST-5 or 3-OST-6. Luciferase activity was measured 24 h after mixing and co-cultivating the effector and target cells. The luciferase activity is from one experiment performed in triplicate. (**B**) and (**C**) are the microscopic images for the observation of HSV-1 glycoprotein-induced cell fusion. CHO-K1 effector cells were transfected with HSV-1 glycoproteins (gB, gD, gH and gL). The target CHO-K1cells were transfected either with an empty vector (**B**) or with the plasmid expressing 3-OST-6 (**C**). Cells were fixed and then stained with Giemsa. Shown are the photographs of representative cell monolayers taken after 24 h. Arrows (in **C**) indicate the polykaryocyte cells.

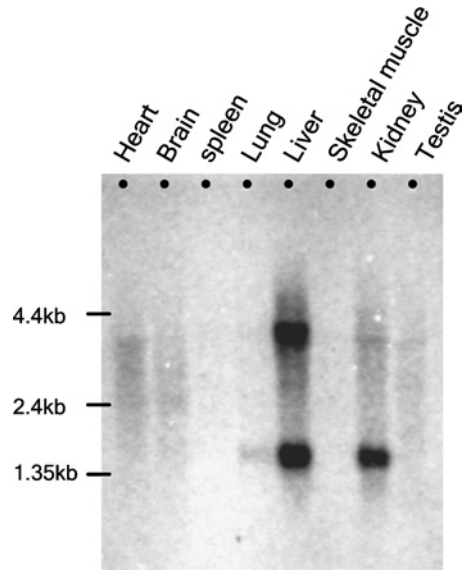

Figure 8 Expression of 3-OST-6 mRNA in mouse tissues

A mouse MTN blot was hybridized with mouse 3-OST-6 cDNA probes labelled with [^{32}P]dCTP under the conditions described in the Materials and methods section. Sizes (in kb) are indicated to the left of the blot.

subfamily, including HSV-2, PRV and BHV-1. We found that, very much in line with previously characterized 3-O-sulphated HS generated by 3-OST-3 and 3-OST-5 [9,14], the cells expressing 3-OST-6 are resistant to the infection by these other members of the alphaherpesvirus subfamily (results not shown).

To strengthen further the conclusion that 3-OST-6-modified HS provides an entry receptor for HSV-1, we examined whether 3-OST-6-expressing cells (target cells) could induce fusion with the cells that express HSV-1 envelope proteins essential for viral fusion (effector cells). This cell–cell fusion assay mimics the fusion of HSV-1 to the target cells, and the fused cells are readily quantified with an appropriate reporter gene and visualized under a microscope. In the assay, the target cells also carried an inducible luciferase as a reporter gene that is activated upon the fusion with effector cells to permit quantification of the cell–cell fusion [15,31]. As shown in Figure 7(A), we found that CHO cells transiently transfected with 3-OST-6 efficiently fused with the effector cells expressing gB, gD, gH and gL, as determined by the elevated level of luciferase. The fusion between cells was very similar to that observed with similarly transfected 3-OST-5 cells, which served as a positive control for the fusion experiment [34]. The cell–cell fusion mediated by 3-OST-6 was also visualized. Cells expressing 3-OST-6 resulted in polykaryocyte formation, an indication of cell–cell fusion (Figure 7C), whereas no polykaryocytes were observed using CHO-K1 as target cells (Figure 7B). These data also suggest that 3-O-sulphated HS can potentially mediate viral spread among neighbouring cells.

3-OST-6 does not generate AT-binding HS

The binding of the HS from 3OST6/CHO to AT was also determined to evaluate the substrate specificity of 3-OST-6, as the saccharide sequences for binding to gD (serving as HSV-1 entry receptor) and AT (having anticoagulant property) are distinct. The results from this experiment allowed us to identify whether 3-OST-6 is more similar to 3-OST-3 or to 3-OST-5 (as shown in Figure 1). We observed only 0.8 % of 3OST6/CHO HS bound to AT, which is very close to that of the HS isolated from non-transfected CHO cells (0.5 %) (Table 1). In contrast, the binding of 3OST5/CHO HS to AT was 7.6-fold higher than the that of the HS from non-transfected CHO cells control sample, which is consistent with the conclusion that 3-OST-5 generates AT-binding sites [9]. The results from AT-binding assay suggest that 3-OST-6 does not generate AT-binding sites. Therefore the substrate specificity of 3-OST-6 and 3-OST-5 is distinct.

Tissue distribution of 3-OST-6

Northern blot analysis was carried out on a mouse MTN blot using 3-OST-6 probe. We detected that 3-OST-6 is expressed primarily in liver with sizes of $\sim$1.6 kb and $\sim$3.6 kb, and in kidney with a size of $\sim$1.6 kb, and is also expressed at lower levels in heart, brain, lung and testis (Figure 8).

DISCUSSION

HSV-1 utilizes distinct families of cell-surface molecules as entry receptors for infection via an unknown mechanism [18]. One of the families is 3-O-sulphated HS, which interacts with gD with a binding affinity in the micromolar range [6]. The 3-O-sulphated HS is biosynthesized by specific 3-OSTs. In the present study, we report the isolation and characterization of a novel 3-OST cDNA, namely 3-OST-6. To our knowledge, the biochemical functions and enzymic activity of this gene have not been reported. The cDNA was identified by homology

search using the amino acid sequence of 3-OST-3, a known sulphotransferase that synthesizes an entry receptor for HSV-1 [14]. We utilized a similar approach to successfully identify 3-OST-5 [9]. Our results demonstrated that 3-OST-6 cDNA encodes a protein that has 3-OST activity, and synthesizes an entry and cell–cell fusion receptor for HSV-1, but not for other subtypes of the alphaherpesvirus family. The isolated cDNA encodes a type II membrane-bound protein, which is consistent with the structural features found in all previously characterized 3-OST isoforms, including 3-OST-1 [a secreted enzyme, containing a signal peptide (12-amino-acids long)], 3-OST-2, 3-OST-3 and 3-OST-5 [8,9]. The full-length cDNA sequence for 3-OST-4 has not been published. As expected, the HS isolated from 3OST6/CHO cells binds to gD and contains two characteristic 3-O-sulphated disaccharides (IdoA2S-AnMan3S and IdoA2S-AnMan3S6S), suggesting that 3-OST-6 sulphates similar disaccharides to those sulphated by 3-OST-3 (for substrate specificity of 3-OST-3, see Figure 1). It should be noted, however, that we do not know whether the saccharide sequences of the gD-binding sites in 3-OST-3-modified HS and those in 3-OST-6-modified HS are identical. We also identified the human 3-OST-6 from a genomic clone with accession number AE006640.1, because the gene encodes a protein having 94% sequence identity with mouse 3-OST-6.

One of the reasons for successfully characterizing the sulphotransferase activity of 3-OST-6 was that we obtained a CHO cell clone that stably expresses 3-OST-6 gene. It is very interesting to note that we were unable to detect the activity of 3-OST-6 in the cellular extract using an *in vitro* assay, which has been successfully used to characterize all previously reported 3-OSTs [9,12,33]. This observation suggests two possible explanations: either 3-OST-6 activity is unstable under *in vitro* experimental conditions, or the enzyme sulphates a very unique HS substrate that is absent in the HS utilized for the *in vitro* assay. (HSs from three different sources, including the HS from bovine kidney, from non-transfected CHO cells and from mouse L-cells, were examined. None of these HSs is a substrate of 3-OST-6.) The physiological functions of 3-O-sulphated HS, generated by 3-OST-3, 3-OST-2 and 3-OST-6, are not known despite the fact that it serves as an entry receptor for HSV-1. It is known that the 3-O-sulphated HS biosynthesized by 3-OST-1 serves as an anticoagulant on the surface of endothelial cells, although 3-OST-1-modified HS is not an entry receptor for HSV-1 [14,27,38]. The 3-O-sulphated HS biosynthesized by 3-OST-5 display the activities in anticoagulation and in assisting HSV-1 entry [9]. Reports suggest that 3-O-sulphated HS could be involved in numerous biological functions, and 3-OST genes are present among the animal kingdoms from *Caenorhabditis elegans* and *Drosophila* to humans [10,34]. Edge and Spiro [35] reported that decreased levels of IdoA-AnMan3S and IdoA2S-AnMan3S in HS isolated from the glomerular basement membrane were observed in diabetic patients. Since IdoA2S-AnMan3S is the product of 3-OST-3, 3-OST-2, 3-OST-6 or 3-OST-5, and IdoA-AnMan3S is the product of 3-OST-5, these results suggest that the specific 3-O-sulphated HS might contribute to defects in the anionic filtration barrier in diabetic patients. The expression of 3-OST-2 in rat pineal gland is daylight-sensitive and is regulated by the β-adrenergic signalling pathway [36]. In addition, a lower expression of 3-OST-2 was discovered in various human cancers, suggesting potential roles of 3-OST-2 in controlling the transformation of normal cells to cancer cells [37].

The wide host range of HSV infection is possibly because of the ability of the virus to use multiple cell-surface molecules, including 3-O-sulphated HS, for entry [21]. The discovery of 3-OST-6 as an enzyme for the biosynthesis of a gD receptor adds to the growing body of evidence that several members of 3-OST family generate such receptors. Collectively, these members are expressed in a wide variety of human cell types and tissues (Table 2). Thus wide expression of the enzymes raises the possibility that 3-O-sulphated HS is present in many cell types, and could potentially play a significant role in HSV-1 entry and spreading during primary or disseminated diseases. The disseminated form usually involves the liver, adrenal gland and lung. It is very interesting that 3-OST-6, along with 3-OST-3, is expressed in the liver. We reported recently that co-expression of 3-OST isoforms can actually enhance HSV-1 entry and cell fusion, potentially by enhancing the gD-binding sites within HS chains [15]. It raises the possibility that co-expression of 3-OST-6 and 3-OST-3 could also enhance the susceptibility of liver cells for HSV-1. This possibility would require further experiments to confirm.

Similarly, the inability of 3-O-sulphated HS to mediate HSV-2 entry raises the possibility of mechanistic differences between the entry of HSV-1 and -2, where HSV-2 infections commonly occur in genital tissues. The differences could be at the level of receptor recognition or at a subsequent step during the entry. In either case, our observation suggests that receptors could play a role in deciding the tissue tropism. More conclusive studies on the cellular expression of 3-OSTs and their significance in cell types that are natural hosts to HSV-1 are needed to test this possibility. Such studies will be beneficial not only for understanding the pathogenic effects of the virus, but also to dissect it from HSV-2 pathogenesis.

Table 2 Tissue distributions of 3-OST isoforms and the biological functions of 3-O-sulphated HS

Tissue distributions of human 3-OST-1, -2, -3A, -3B and -4 were reported by Shworak et al. [10]. Tissue distribution of human 3-OST-5 was reported by Xia et al. [9] and Mochizuki et al. [11]. 3-OST-3A and 3-OST-3B have nearly identical amino acid sequences in the sulphotransferase domain, and sulphate the same disaccharides as described in Figure 1 [10,12]. 3-OST-4 sulphates the same disaccharides as 3-OST-3A [39]. 3-OST-2 sulphates the GlcN residue that links to an IdoA2S residue as well as the GlcN residue that links to a GlcA2S residue [12]. For clarity, the complete substrate specificity of 3-OST-2 is not shown in Figure 1. The activities of 3-OST-2 and 3-OST-4 in assisting the entry of HSV-1 were reported by Shukla and Spear [6].

3-OSTs isoform	Tissue distribution	Known properties
3-OST-1	Heart, brain, lung, kidney	Anticoagulant HS
3-OST-2	Brain	HSV-1 entry
3-OST-3A	Heart, placenta, lung, liver, kidney	HSV-1 entry
3-OST-3B	Heart, brain, lung, liver, kidney	HSV-1 entry
3-OST-4	Brain	HSV-1 entry
3-OST-5	Brain, spinal cord, skeletal muscle	Anticoagulant HS and HSV-1 entry
3-OST-6	Liver, kidney	HSV-1 entry

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