Portable sulphotransferase domain determines sequence specificity of heparan sulphate 3-O-sulphotransferases

Tomio YABE*†, Deepak SHUKLA*‡, Patricia G. SPEAR‡, Robert D. ROSENBERG*§, Peter H. SEEBERGER† and Nicholas W. SHWORAK*§²

*Angiogenesis Research Center, Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA 02215, U.S.A., †Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A., ‡Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, IL 60611, U.S.A., and §Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

3-O-Sulphates are the rarest substituent of heparan sulphate and are therefore ideally suited to the selective regulation of biological activities. Individual isoforms of heparan sulphate D-glucosaminyl 3-O-sulphotransferase (3-OST) exhibit sequence-specific action, which creates heparan sulphate structures with distinct biological functions. For example, 3-OST-1 preferentially generates binding sites for anti-thrombin, whereas 3-OST-3 isoforms create binding sites for the gD envelope protein of herpes simplex virus 1 (HSV-1), which enables viral entry. 3-OST enzymes comprise a presumptive sulphotransferase domain and a divergent N-terminal region. To localize determinants of sequence specificity, we conducted domain swaps between cDNA species. The N-terminal region of 3-OST-1 was fused with the sulphotransferase domain of 3-OST-3_A to generate N1-ST3_A. Similarly, the N-terminal region of 3-OST-3_A was fused to the sulphotransferase domain of 3-OST-1 to generate N3_A-ST1.

Wild-type and chimaeric enzymes were transiently expressed in COS-7 cells and extracts were analysed for selective generation of binding sites for anti-thrombin. 3-OST-1 was 270-fold more efficient at forming anti-thrombin-binding sites than 3-OST-3_A, indicating its significantly greater selectivity for substrates that can be 3-O-sulphated to yield such sites. N3_A-ST1 was as active as 3-OST-1, whereas the activity of N1-ST3_A was as low as that of 3-OST-3_A. Analysis of Chinese hamster ovary cell transfectants revealed that only 3-OST-3_A and N1-ST3_A generated gD-binding sites and conveyed susceptibility to infection by HSV-1. Thus sequence-specific properties of 3-OSTs are defined by a self-contained sulphotransferase domain and are not directly influenced by the divergent N-terminal region.

Key words: anti-thrombin, anti-coagulant, gD viral glycoprotein, herpes simplex virus.

INTRODUCTION

Higher organisms express heparan sulphate (HS) proteoglycans on cell surfaces and within extracellular matrices. HS consists of a repeated disaccharide unit, GlcNAc α 1/4HexA β 1–4 (in which HexA represents L-iduronic or D-glucuronic acid), which contains *N*-acetyl, *N*-sulphate, *N*-unsubstituted and *O*-sulphate groups. In large part, the specific arrangement of sulphate moieties gives rise to distinct binding sequences that activate ligands, such as anti-thrombin (AT) and growth factors, and thereby regulate numerous biological processes including anti-coagulation, angiogenesis and susceptibility to pathogens (reviewed in [1–5]).

Modification of the co-polymer backbone is thought to occur primarily through an ordered series of reactions. Initially subsets of GlcNAc residues are converted to *N*-sulpho-D-glucosamine (GlcNS) by isoforms of HS *N*-deacetylase/*N*-sulphotransferase (NDST) [3,6–9]. The four NDST isoforms show distinct enzymic properties that, in part, might govern the final density of *N*-sulphation and production of rare *N*-unsubstituted GlcN residues [9–11]. Regions with a high density of GlcNS are then preferentially modified. A single isoform of HS C₅ epimerase converts occasional GlcA residues to IdoA [12,13]. The HS 2-sulphotransferase modifies IdoA residues at high frequency and GlcA residues at low frequency to produce L-iduronic acid 2-*O*-sulphate (IdoA2S) or 2-*O*-sulphated glucuronic acid (GlcA2S) respectively [14,15]. HS 6-*O*-sulphotransferase next converts

occasional glucosamine residues to 6-*O*-sulphated glucosamine [16]. Three isoforms are known with distinct specificities that are dependent, in part, on the specific epimer of an adjacent uronic acid [4]. Finally, the rarest HS modification, 3-*O*-sulphation of glucosamine, is added by the sequence-specific action of HS D-glucosaminyl 3-*O*-sulphotransferase (3-OST). There are at least five different isoforms, each of which is expressed in a distinctive pattern of cells and tissues [5]. Given these properties, the 3-OST multigene family is exquisitely suited to regulate HS biological diversity through the production of distinct fine structures.

For example, the isoform 3-OST-1 is rate-limiting for endothelial cell production of anti-coagulant HS (HS^{act}) [17–20]. The enzyme preferentially modifies a specific precursor sequence to create the AT-binding site -Glc(NS/Ac)6S-GlcA-GlcNS3S $\pm$ 6S-IdoA2S-GlcNS6S- [18,21,22] (in which GlcNS3S $\pm$ 6S represents *N*-sulpho-D-glucosamine 3-*O*-sulphate or *N*-sulpho-D-glucosamine 3,6-*O*-bisulphate). On binding this sequence, a conformational change is induced in AT that markedly enhances the rate at which AT neutralizes coagulation proteases. The central 3-*O*-sulphate (shown in bold type) is absolutely essential for both high-affinity binding and activation of AT [21,23,24].

In contrast, the 3-OST-3 isoforms exhibit a distinct biology owing to a very different specificity [25,26]. Modification of HS by 3-OST-3_A creates a heparin lyase-resistant tetrasaccharide with the novel sequence Δ UA2S-GlcNS-IdoA2S-GlcN3S $\pm$ 6S

Abbreviations used: 3-OST, heparan sulphate D-glucosaminyl 3-*O*-sulphotransferase; AT, anti-thrombin; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GlcNS, *N*-sulpho-D-glucosamine; HS, heparan sulphate; HS^{act}, HS with AT-binding sites; HSV-1, herpes simplex virus 1; IdoA2S, L-iduronic acid 2-*O*-sulphate; NDST, HS *N*-deacetylase/*N*-sulphotransferase; PAPS, adenosine 3'-phosphate 5'-phosphosulphate.

¹ Present address: Department of Molecular Microbiology and Immunology, University of Missouri-Columbia, M610 G, Medical Science Building, 1 Hospital Drive, Columbia, MO 65211, U.S.A.

² To whom correspondence should be addressed. Present address: Department of Medicine, Angiogenesis Research Center, HB7504, Dartmouth-Hitchcock Medical Center, One Medical Center Drive, Lebanon, NH 03756, U.S.A. (e-mail nicholas.shworak@dartmouth.edu).

(in which ΔUA represents $\Delta^{4,5}$ -unsaturated uronic acid) [25]. This structure might contribute to infection by herpes simplex virus type 1 (HSV-1). HSV-1 initially binds to cell-surface HS by interactions of the envelope glycoproteins gC and/or gB (reviewed in [27]). The interaction of an additional viral glycoprotein, gD, with one of several receptors then leads to viral entry through fusion of the virion envelope to the cell membrane (reviewed in [28]). Shukla et al. [29] have demonstrated that modification by 3-OST-3 enzymes selectively creates HS sites for binding gD and for initiating HSV-1 entry.

The determinants of 3-OST specificity are unknown. All 3-OST enzymes comprise a divergent N-terminal domain and a homologous C-terminal domain. The conserved region shows similarity to most sulphotransferases and thereby might constitute a functional sulphotransferase domain [5,19,30,31]. This assertion is supported by the crystal structure of the very similar and homologous sulphotransferase domain from NDST1 [32]. However, the N-terminal domain might additionally contribute to sulphotransferase specificity. The 3-OST divergent domain lacks similarity in primary structure but does show a conserved regional organization, with most enzymes having a type II integral membrane architecture [5]. The lone exception is 3-OST-1, which is an intraluminal resident protein [19]. Despite this difference, the divergent domain is always extremely enriched in Ser, Pro, Leu, Ala and Gly residues. Conservation of composition suggests an important functional role. This divergent region might conceivably directly influence isoform sequence specificity.

In the present work we investigate whether sequence-specific modification is directly dependent on the N-terminal region by constructing proteins chimaeric between the functionally distinct 3-OST-1 and 3-OST-3_A. The results indicate the 3-OST conserved region is a self-contained sulphotransferase domain encompassing determinants for both catalysis and sequence specificity.

EXPERIMENTAL

Plasmids and recombinant proteins

pcDNA3.1-based expression plasmids containing mouse 3-OST-1 (pCMV-3-OST) or human 3-OST-3_A (pcDNA3-JL3.3) were described previously [5,19]. Recombinant mouse 3-OST-1 and human 3-OST-3_A were purified from baculovirus expression systems [25,33] and were gifts from Dr Jian Liu (University of North Carolina, Chapel Hill, NC, U.S.A.). gD-1:Fc, a fusion of the gD ectodomain and the Fc of rabbit IgG, was purified from PEAK[®] (Edge Biosystems) cell transfectants, as described [29].

Construction of expression plasmids

pN3_A-ST1 was constructed by three-piece ligation. A 203 bp fragment, the distal N-terminal domain of 3-OST-3_A with 9 nt of 3-OST-1 and an *EarI* site, was PCR generated from pcDNA3-JL3.3 with 5'-dGGCGGCACAGAGAAAGCGCCTCCTGCACT-3' and 5'-dCTCTTCGTGTGGAACCTTCGTCCAGGAGCAGCGCCAGG-3' primers; the product was cloned into PCR-Script Cam (Stratagene). The insert was released as a *KasI/KpnI* fragment, then partial *EarI* digestion generated fragment 1, a 157-bp *KasI/EarI* fragment. Next a 1.4 kb fragment containing an *EarI* site preceding the sulphotransferase domain of mouse 3-OST-1 was generated from pCMV-3-OST by PCR with 5'-dGCCTCAGAAGCCATAGAGCCCACCGCAT-3' and 5'-dCTCTTCCACACAGCAGCTGCCACAGACCATCATCATTGG-3' primers; the product was cloned into PCR-Script Cam. Fragment 2 was then released as a 1.1 kb *EarI/XbaI* fragment. Fragments 1 and 2 were cloned into *KasI/XbaI*

cleaved pcDNA3-JL3.3 to generate pN3_A-ST1. pN1-ST3_A formation required silent mutations to introduce *BamHI* sites at the domain border. First a 1.1 kb fragment *HindIII* fragment from pCMV-3-OST was subcloned into pUC18 (GenBank[®] accession number L09136) to create a mutation template. A *BamHI* site was introduced with a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and two mutagenic primers, 5'-dCAGCATCCAATGGATCCACACAGCAG-3' and 5'-dCTGCTGTGTGGATCCATTGGATGCTG-3' (*BamHI* site underlined). Digestion with *BamHI* produced a 524 bp fragment, N1, containing the N-terminal domain of 3-OST-1. Next a 2.8 kb *SmaI* fragment was isolated from pcDNA3-JL3.3 and amplified by PCR with 5'-dCCTGGACGAAGGATCCAAGCAGCTGCC-3' (*BamHI* site underlined) and 5'-dGCGTCTTCTCCATGTGATCTG-3' primers; the 219 bp product was cloned into PCR-Script Cam. Digestion with *EcoRI* and *KpnI* released a 216 bp fragment that was cloned into an *EcoRI*/partly *KpnI*-cleaved pcDNA3-JL3.3 plasmid to reconstitute the 3-OST-3_A sulphotransferase domain. Finally, this last plasmid was digested with *BamHI* and the above N1 fragment was inserted to create pN1-ST3_A. The reading frame and entire coding region of all constructs were confirmed by sequencing.

Transient expression in COS-7 cells

COS-7 cells (A.T.C.C., CRL-1651) were grown in Dulbecco's modified Eagle's medium (Life Technologies) containing 10% (v/v) fetal bovine serum (FBS) (Life Technologies) with 100 µg/ml streptomycin and 100 i.u./ml penicillin at 37 °C under a humidified air/CO₂ (19:1) atmosphere. For each construct a six-well plate (Falcon) was seeded with 7.5×10^4 cells and grown for 2 days (60–90% confluence). Monolayers were washed with PBS, then 6 ml of serum-free medium containing 12 µg of construct DNA and 60 µl of GenePORTER (Gene Therapy Systems) was split between six wells. After growth for 3–5 h, 1 vol. of medium with 20% (v/v) FBS was added. After growth for 24 h, monolayers were washed with PBS and incubated with fresh medium for 36 h. Cells from a single plate were harvested by treatment with trypsin and washed with PBS; the pellet was snap-frozen in liquid nitrogen and stored at –80 °C. Extracts were generated by vortex-mixing pellets in 100 µl of ice-cold 0.25 M sucrose with 1% (v/v) Triton X-100, then removing the insoluble residue by centrifugation at 10 000 g for 10 min. For analysis of secreted sulphotransferase activity, the final growth incubation was conducted in a previously described serum-free medium [20], which was clarified by centrifugation and then frozen as above.

Preparation of [³⁵S]HS

Chinese hamster ovary (CHO)-K1 cells (a gift from Dr Lijuan Zhang, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.) were inoculated at 38 000 cells/cm² in a 75 cm² flask, then grown at 37 °C under air/CO₂ (19:1) in Ham's F-12 medium (Life Technologies) with 10% (v/v) FBS. At 2 days after inoculation, the monolayer was washed with PBS, then incubated for 16 h in 4 ml of Ham's F-12 medium with 50 mCi of carrier-free Na₂³⁵SO₄ (ICN) and 10% (v/v) dialysed FBS. Cell-surface [³⁵S]HS was then purified as described previously [18].

Formation of AT-binding sites (HS^{act} conversion assay)

To ensure that reaction conditions were suitable for both enzymes, we initially evaluated extracts from 3-OST-1, 3-OST-3_A and pcDNA3 transfectants and varied previous conditions

[18,20,34] to maximize the HS sulphotransferase activity of 3-OST compared with empty-vector controls. The resulting optimal conditions were applied to both the HS^{act} conversion assay and the detection of the HS sulphotransferase activity. The HS^{act} conversion reactions (50 μ l) contained 2 μ g of cell extract protein, 5×10^4 c.p.m. of [³⁵S]HS from CHO-K1 cells, 500 μ M adenosine 3'-phosphate 5'-phosphosulphate (PAPS; Sigma), 50 mM Mes, pH 7.0, 10 mM MnCl₂, 5 mM MgCl₂, 1.2 mg/ml BSA, 60 mM NaCl, 0.2 mg/ml glycogen and 1% (v/v) Triton X-100. Reactions were incubated at 37 °C for 2 h, then 1.25 μ l of 100 μ M AT was added per reaction. After incubation for 30 min at room temperature the amount of [³⁵S]HS that bound AT (HS^{act}) was determined by AT-affinity microchromatography [17]. Activity was calibrated against standards of purified recombinant 3-OST-1 [33]. One unit of activity generates a 0.5% increase in HS^{act} after 20 min incubation under standard reaction conditions [20].

Analysis of HS sulphotransferase activity in cell extract

Reactions were as described above except that the sulphate acceptor was 1.2 μ g of bovine kidney HS (ICN Biomedicals) and the sulphate donor was [³⁵S]PAPS (30 μ M, 10^6 c.p.m.). Reactions were incubated at 37 °C for 2 h, quenched at 100 °C for 10 min and centrifuged at 14000 *g* for 10 min. Supernatant was combined with 1 ml of UPAS [35] and incorporation into [³⁵S]HS was determined by analytic DEAE chromatography [35]. Activity was typically 40% higher for 3-OST transfectant extracts than for the empty-vector control. Activity was similarly determined for 10 μ l samples of protein-free medium.

HSV-1 entry and detection of cell-surface gD-1 binding

Determination of CHO transfectant susceptibility to HSV-1 entry has been described previously [29,36]. Detection of gD-1 receptors on transfectants with gD-1:Fc was as described previously [29].

RESULTS

Construction of chimaeric proteins between 3-OST-1 and 3-OST-3_A

We applied a domain-swap strategy (Figure 1) to evaluate whether the distinct sequence specificities of 3-OST-1 and

Figure 2 Selective synthesis of ligand-binding sites by 3-OST enzymes

(A) Selective synthesis of AT-binding sites was revealed by analysis *in vitro* of cell extracts from COS-7 transfectants. Selectivity was calculated as the HS^{act} conversion activity divided by the net HS sulphotransferase activity. HS^{act} conversion activity was determined by incubating cell extracts (2 μ g) with PAPS and [³⁵S]HS lacking 3-O-sulphates. The resulting HS forms with nascent AT-binding sites (HS^{act}) were quantified by AT-affinity microchromatography [18]. Extracts from pcDNA3.1 transfectants showed only background conversion activity. Cell extracts (2 μ g) were also incubated with [³⁵S]PAPS and HS; incorporated sulphate was then determined by DEAE chromatography and expressed as pmol of sulphate transferred from [³⁵S]PAPS to make [³⁵S]HS. Net HS sulphotransferase activity from 3-OST enzymes was determined as the extract activity minus the value for the empty-vector control. Results are mean $\pm$ S.E.M. for six independent transfections. (B) Cell surface gD-binding sites synthesized *in vivo* were determined for the indicated CHO transfectants. Monolayers were incubated with gD-1:Fc for 30 min, followed by fixation and incubation with secondary antibody conjugated to horseradish peroxidase. After incubation with substrate the reaction was quenched with 2 M HCl. The enzymic reaction product was detected by measuring A₄₁₀. Values were corrected for the background binding of gD-1:Fc to control pcDNA3 transfectants (A₄₁₀ of 0.393) and are shown as means $\pm$ S.D. for three determinations.

Figure 1 Mature structures of wild-type and chimaeric 3-OST enzymes

N-termini and C-termini are indicated. Met¹ to Glu¹⁴⁷ of human 3-OST-3_A was fused to Gly⁵³ to His³¹¹ of mouse 3-OST-1 to generate N3_A-ST1. Consequently, mature 3-OST-3_A and N3_A-ST1 contained a transmembrane domain and were predicted to localize to membranes of the *trans*-Golgi. Similarly, the entire N-terminal region of mouse 3-OST-1 (Met¹ to Asn⁵²) was fused to Gly¹⁴⁸ to Gly⁴⁰⁶ of human 3-OST-3_A to generate N1-ST3_A. However, mature 3-OST-1 and N1-ST3_A begin at His²¹ owing to signal peptidase cleavage of the 3-OST-1 leader sequence [19]. Thus mature 3-OST-1 and N1-ST3_A are intraluminal resident proteins and localization to the *trans*-Golgi is presumed to involve interaction with a retention protein [19].

Figure 3 Entry of HSV-1 into CHO cells is mediated only by 3-OST-3_A and N1-ST3_A

CHO cells were transfected with the indicated plasmids, then exposed to various concentrations of HSV-1(KOS)gL86, which expresses β -galactosidase from an insert in the viral genome. β -Galactosidase activity, a measure of viral entry, was quantified at 6 h after the addition of virus. Representative values from a single transfection show the amount of reaction product detected spectrophotometrically (A_{410}) as the mean from four wells per virus dose (PFU, plaque-forming units). Standard deviations were typically less than 15% of the mean. Comparable results were obtained in four independent transfection experiments.

3-OST-3_A were determined by their respective divergent N-terminal domains. Comparisons of 3-OST primary structures reveal a tightly demarcated border between the divergent region and the conserved C-terminal domain, which begins with an invariant glycine residue. This conserved span is considered the presumptive sulphotransferase domain of 3-OST enzymes [5]. Thus domain swaps conformed with this clearly defined border. The N-terminal region of 3-OST-1 was fused to the presumptive sulphotransferase domain of 3-OST-3_A to generate N1-ST3_A. Similarly, the N-terminal region of 3-OST-3_A was fused to the C-region of 3-OST-1 to generate N3_A-ST1. We deliberately avoided the addition of purification tags, which might have altered enzymic function. Each cDNA was subcloned into the pcDNA3.1 expression vector and sequence specificity was evaluated *in vitro* and *in vivo*.

Sequence specificity *in vitro* is unaltered by the divergent N-terminus

We compared the efficiency of wild-type and chimaeric enzymes *in vitro* towards generating AT-binding sites, the preferred product of 3-OST-1. COS-7 cells were transiently transfected with 3-OST expression vectors or the pcDNA3.1 control vector; cell extracts were then evaluated for HS^{act} conversion activity, which measures the 3-OST-dependent formation of AT-binding sites. Detection of even low activities was made possible by employing modified reaction conditions that provided for a 10-fold enhanced sensitivity, compared with earlier assay formats

[18,20,34]. Expression levels were inherently variable with transient transfections. To compensate for this, the HS^{act} conversion activity was standardized to the net increase in extract HS sulphotransferase activity (Figure 2A). Transfectional variation was also averaged out by performing several independent transfections. The sensitive assay conditions revealed, for the first time, that the 3-OST-3_A isoform was capable of generating AT-binding sites, albeit at a low activity (0.08 ± 0.02 unit/pmol of SO₄; Figure 2A). This result was validated by analysis of purified recombinant 3-OST-3_A [25], which showed a similar activity (0.14 ± 0.04 unit/pmol of SO₄, $n = 5$). [The low efficiency of purified recombinant 3-OST-3_A in forming AT-binding sites can be compensated for by mass action. Exhaustive modification of [³⁵S]HS with high enzyme levels maximally converts approx. 20% of HS chains to HS^{act}, which bind AT. A similar approx. 20% plateau is also attained by exhaustive modification of HS with purified 3-OST-1 (results not shown). Thus AT site formation by 3-OST-3_A is a detectable activity that modifies the same precursor population as 3-OST-1.] In contrast with 3-OST-3_A, extracts containing 3-OST-1 were 270-fold more active at forming AT-binding sites (Figure 2A). Again, the activity determined for purified recombinant 3-OST-1 enzyme (40 ± 5.7 units/pmol of SO₄, $n = 3$; results not shown) was similar to that obtained from cell extracts (21.80 ± 1.27 units/pmol of SO₄; Figure 2A). Most importantly, the high selectivity of the presumptive sulphotransferase domain of 3-OST-1 was unaltered by the N-terminal swaps (Figure 2A; compare 3OST1 with N3_A-ST1). The low selectivity of the 3-OST-3_A presumptive sulpho-

transferase domain was similarly unchanged (Figure 2; compare 3-OST-3_A with N1-ST3_A). Taken together, the above results suggest that enzymic determinants of sequence specificity *in vitro* are embodied within the 3-OST conserved C-terminal region.

Sequence specificity *in vivo* is unaltered by the divergent N-terminus

Wild-type and chimaeric enzymes were also examined by an assay *in vivo* that was dependent on the sequence-specific enzyme action of 3-OST-3 isoforms. Modification of HS by these isoforms creates binding sites for the gD envelope protein and renders CHO cells susceptible to infection by HSV-1 [29]. Thus CHO cells were transfected with 3-OST expression constructs and then exposed to a recombinant form of HSV-1; virus entry was monitored (Figure 3). The HS sulphotransferase activity of transfectant cell extracts was also measured, which confirmed that each 3-OST form was successfully expressed (compared with the empty-vector control, the activity of 3-OST transfectants was elevated by 30–80%). Despite successful expression of all constructs, cells became susceptible to HSV-1 entry only after the expression of constructs with the 3-OST-3_A presumptive sulphotransferase domain (Figure 3). Furthermore, the N-terminal domain swaps did not substantially alter the HSV-1 dose response. The formation of cell-surface gD-binding sites was also evaluated by incubating cells with gD-1:Fc. Figure 2(B) shows that substantial levels of cell-surface gD-binding sites were generated only when transfectants expressed the presumptive sulphotransferase domain of 3-OST-3_A. Thus 3-OST-3 sequence-specific properties stem from the conserved C-terminal region.

Domain swaps alter enzyme secretion

3-OST-1 is the only isoform that is not membrane-bound and can be secreted, presumably because the relevant Golgi retention mechanism, if any, is leaky [5,19,26]. Thus the domain swaps should alter cellular secretion. This possibility was tested by measuring the HS sulphotransferase activity within culture medium conditioned by transiently transfected COS-7 cells. Results were generated from three independent transfections and are expressed as fmol of SO₄ transferred from [³⁵S]PAPS to HS per µl of medium (means ± S.D.). For transfectants expressing type II integral membrane constructs the activity was comparable to background (pcDNA3.1, 17 ± 0.9; 3-OST-3_A, 18 ± 1.1; N3_A-ST1, 20 ± 2.7). In contrast, intraluminal resident constructs gave an activity in medium that was significantly greater than that of pcDNA3.1 [3-OST-1, 59 ± 6.6, *P* < 0.0004; N1-ST3_A, 26 ± 2, *P* < 0.004 (two-tailed Student's *t* test)]. Thus the N-terminal domain governs cellular retention.

DISCUSSION

Sulphotransferases (EC 2.8.2.), which make up a large superfamily, transfer a sulphuryl group from the donor PAPS to numerous acceptor molecules [37]. Multiple sequence alignments show that virtually all such enzymes share a 260–290-residue C-terminal presumptive sulphotransferase domain, with at least 25–30% similarity between the most functionally divergent members [5,19,31]. The greatest conservation is found in structures that bind and/or catalyse the obligate cofactor PAPS [31,38,39]. In contrast, specificity for the acceptor substrate must be determined by non-conserved residues. For the oestrogen sulphotransferase, residues defining acceptor specificity lie within the sulphotransferase domain [40]. However, this enzyme has

Figure 4 Comparison of sulphotransferase regions

Alignment of amino acid sequences of mammalian 3-OST and NDST isoforms. Consensus residues are indicated for each position where candidates exhibit identical or similar amino acids for all enzymes (black) or only NDST isoforms (grey). Residue numbers are given for each full-length enzyme. (A) Junction of 3-OST domains (J) and the comparable NDST region. Highlighted features include the 5'-phosphosulphate-binding loop (5'PSB) containing the conserved catalytic lysine residue (*). Cysteine residues that formed a disulphide bond are also indicated (J). (B) Two major divergent sulphotransferase regions that might contribute to enzymic sequence specificity. Prefixes indicate the organism of origin: h, *Homo sapiens*; m, *Mus musculus*; r, *Rattus norvegicus*; c, *Canis familiaris*; b, *Bos taurus*. Nucleotide sequences are from the GenBank[®] database with the following accession numbers: h3OST1, AF019386; m3OST1, AF019385; r3OST, AF177430; c3OST1, AJ388534 (partial sequence); h3OST2, AF105375; h3OST3_A, AF105376; h3OST3_B, AF105377; m3OST3_B, AF168992; h3OST4, AF105378; hNDST1, U36600; mNDST1, AF074926; rNDST1, M92042; bNDST2, AF064825; hNDST2, U36601; mNDST2, P52850; hNDST3, AF074924.

only a single domain [41] and so might not be predictive of the multidomain 3-OSTs. Consequently, we conducted domain swaps to determine whether the divergent domain directly affects the sequence specificity of the presumptive sulphotransferase domain.

Reciprocal swaps were constructed to maintain the clearly defined border between the divergent and conserved domains (Figure 4A, J). This site is only 13 residues from the conserved 5'-phosphosulphate-binding loop, which contains an essential catalytic lysine residue (Figure 4A, 5'PSB and asterisk respectively) [5,29,39,42]. Despite such close proximity to critical structures, the chimaeric enzymes were functional. We were able to quantify the sequence selectivity *in vitro* for both 3-OST isoforms by using an improved assay for HS^{act} conversion activity. This approach revealed that 3-OST-3_A was 270-fold less efficient at generating HS^{act} than 3-OST-1. (This value is similar to our earlier estimate of 300-fold. Because 3-OST-3_A activity was initially undetectable, the original estimate was determined by comparing 3-OST-1 activity with the assay detection limit [26]. For 3-OST-1 only approx. 40% of transferred sulphates

actually create HS^{act} [19,20], suggesting that only approx. 0.15 % of 3-OST-3_A-transferred sulphates should occur in AT-binding sites. Although this output seems low, substantial AT-binding sites might be generated in tissues with high enzyme expression.) Most importantly, the enzymic specificity of the sulphotransferase domain *in vitro* was quantitatively unaltered by the swaps. Sequence selectivity was also unaltered *in vivo*. Only constructs with the 3-OST-3_A sulphotransferase domain were capable of forming gD-binding sites and enabling the cellular entry of HSV-1. Combined, the results strongly indicate that the N-terminal domain does not directly alter enzymic substrate specificity. Thus the conserved C-terminal region of 3-OST enzymes is a functionally complete sulphotransferase domain that encodes the determinants for both catalysis and sequence specificity.

Our functional identification of the 3-OST sulphotransferase domain might serve to clarify the border between the two distinct activities of the NDST family. Given the high similarity between these homologous families, the domain margins might correspond tightly [5,19]. It has previously been suggested that the sulphotransferase domain of human NDST1 extends from Leu⁵⁵⁸ to the C-terminus [30,39]. However, X-ray crystallography of this region shows that ordered structure initiates at Asp⁵⁷⁹. By sequence alignment, this position is 20 residues away from the start of the 3-OST-1 sulphotransferase domain, Gly⁵³ (Figure 4A). However, the comparative three-dimensional distance must be much closer, because the NDST span contains a 16-residue cystine-bridged peptide loop (Figure 4A, from f to j) [32,43]. Thus Asp⁵⁷⁹ might be the actual start of the NDST sulphotransferase domain. This assertion is in part supported by an NDST1 Cys⁶⁰¹ → Ala mutation, which greatly decreases sulphotransferase activity with minimal effect on deacetylase function [43].

Comparison of 3-OST and NDST primary structures also reveals non-conserved spans that might confer sequence-specific properties [5]. Two such regions are particularly striking (Figure 4B). The structures are not only distinct between 3-OST and NDST families, but differences also occur between 3-OST isoforms. In the crystal structure of human NDST-1 [32], these two non-conserved regions lie on opposite faces of the cleft that binds HS. Moreover, one end of each sequence lies in close proximity to the 5' phosphosulphate of PAPS. Thus the location of these regions is ideal for restricting active-site access to distinct HS structures. However, full sequence-specific properties of 3-OST isoforms probably involves additional residues because the two regions cover only a portion of the HS-binding groove.

The N-terminal portions of many Golgi enzymes, including NDST1, contain information for appropriate subcellular targeting [44,45]. Although our experiments were not designed to examine subcellular targeting, the domain swaps suggest such a role, given that cellular secretion occurred only for enzymes containing the N-terminal domain of 3-OST-1. We have previously proposed that the divergent region might interact with other HS biosynthetic components and thereby restrict access of selective substrates to the sulphotransferase domain [5]. Thus the N-terminal region might indirectly control sequence specificity. From this perspective it initially seems surprising that the N1-ST3_A construct conveyed HSV-1 entry. Under the conditions used, the N1-ST3_A enzyme clearly had access to appropriate HS precursors to generate a gD-binding site. However, transient transfection results in unphysiologically high expression levels that might flood the Golgi and thereby mask any potential for selective targeting. Thus a more physiologically rigorous approach is necessary to test whether the N-terminal region can indirectly control sequence specificity.

In conclusion, the conserved C-terminal region is a portable and self-contained sulphotransferase domain, which directly determines the specificity of 3-OST isoforms. Having localized the critical region, future studies will be directed towards pinpointing critical residues that define the HS sequence specificity of 3-OST enzymes.

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