Antibody-based assay for N-deacetylase activity of heparan sulfate/heparin
N-deacetylase/N-sulfotransferase (NDST): novel characteristics of NDST-1 and -2

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A new assay was developed to measure the N-deacetylase activity of the glucosaminyl N-deacetylase/N-sulfotransferases (NDSTs), which are key enzymes in sulfation of heparan sulfate (HS)/heparin. The assay is based on the recognition of NDST-generated N-unsubstituted glucosamine units in Escherichia coli K5 capsular polysaccharide or in HSs by monoclonal antibody JM-403. Substrate specificity and potential product inhibition of the NDST isoforms 1 and 2 were analyzed by comparing lysates of human 293 kidney cells stably transfectected with mouse NDST-1 or -2. We found HSs to be excellent substrates for both NDST enzymes. Both NDST-1 and -2 N-deacetylated heparan sulfate from human aorta (>0.6 sulfate groups/disaccharide) with comparable high efficiency, apparent Km values of 0.35 and 0.76 μM (calculation based on [HexA] being lower (representing a higher affinity) than those for K5 polysaccharide (13.3 and 4.7 μM, respectively). Comparison of various HS preparations and the unsulfated K5 polysaccharide as substrates indicate that both NDST-1 and -2 can differentially N-sulfate polysaccharides already modified to some extent by various other enzymes involved in HS/heparin synthesis. Both enzymes were equally inhibited by N-sulfated sequences (≥6 sugar residues) present in N-sulfated K5, N-deacetylated N-sulfated HS, and heparin. Our primary findings were confirmed in the conventional N-deacetylase assay measuring the release of [3H]-acetate of radiolabeled K5 or HS as substrates. We furthermore showed that NDST N-deacetylase activity in crude cell/tissue lysates can be partially blocked by endogenous HS/heparin. We speculate that in HS biosynthesis, some NDST variants initiate HS modification/sulfation reactions, whereas other (or the same) NDST isoforms later on fill in or extend already modified HS sequences.

Key words: biosynthesis/heparan/heparin/N-deacetylase/sulfotransferase

Introduction

Heparan sulfate proteoglycans (HSPGs) constitute a major class of proteoglycans found in the extracellular matrix, especially in basement membranes, and at the cell surface, associated with the cell membrane (David, 1993; Gallagher, 1989; Lindahl et al., 1994). HSPGs have been implicated in development, renal ultrafiltration, cell growth, cell adhesion, cell recognition, and cell migration (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Filmus, 2001; Kanwar et al., 1991; Vlodavsky et al., 1996; Couchman and Woods, 1996; Walz et al., 1997; Higashiyama et al., 1993). Many of these biological activities of HSPGs are due to interactions between the heparan sulfate (HS) polysaccharide side chains and a variety of proteins, which include extracellular matrix molecules, enzymes, enzyme inhibitors, growth factors, and other cytokines (Lindahl et al., 1994; Jackson et al., 1991; Kjellen and Lindahl, 1991; Bernfield et al., 1992, 1999). Some of these interactions depend on defined sulfation patterns within given sequences of sugar residues in HS (see references in Spillmann and Lindahl, 1994; Salmivirta et al., 1996 for a general discussion).

The biosynthesis of HS involves the formation of a non-sulfated (GlcAβ1,4 → GlcNAcα1,4)n precursor polysaccharide, which concomitantly with polymerization undergoes a series of polymer-modification reactions. These reactions start with N-deacetylation/N-sulfation of N-acetylgalactosamine (GlcNAc) residues, followed by C5 epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA) units, and finally by O-sulfation at various positions (Lindahl et al., 1994). Structural analysis of HS preparations has revealed that the modifications tend to colocalize in block sequences, separated by relatively unmodified domains (Schmidt et al., 1992; Lyon et al., 1994; Turnbull and Gallagher, 1990; Lindblom et al., 1991; Maccarana et al., 1996). The extent of biosynthetic modification, affecting the number, length, and substitution patterns of the modified domains as well as their position along the HS chain, may differ among cell types (Kato et al., 1994), alter during proliferation (Schmidt and Buddecke, 1990), and change as a result of cell transformation (Pejler and David, 1987; Iozzo, 1989). This clearly indicates that the biosynthetic machinery involved in HS modification is tightly regulated; however, the underlying mechanisms are largely unknown.

The substrate specificity of GlcA C-5 epimerase and O-sulfotransferases dictates the presence of N-sulfate groups, pointing to a key role for the glucosaminyl N-deacetylase/
Development of the ELISA-based N-deacetylase assay

K5 polysaccharide substrate is incubated with a cell lysate overexpressing NDST-1 or -2. In due course, the enzyme removes acetyl groups from GlcNAc residues of the K5 polysaccharide but is not able to catalyze N-sulfation of the formed free amino groups due to the absence of the natural sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which disintegrates quickly in crude tissue extracts and is not added to the reaction mixture. The number of enzymatically N-deacetylated GlcN residues in K5 is a measure of NDST activity and is quantified in a capture ELISA assay using immobilized JM-403 to bind NDST-modified K5 and biotinylated JM-403 to develop the ELISA.

The major characteristics of the ELISA-based N-deacetylase assay are shown in Figure 1 using crude cell lysates of human kidney 293 cells stable transfected with mouse NDST-1 (clone 11) or NDST-2 (clone S5). Figure 1A depicts a protein concentration range of the cell lysates (30 min 37°C, 25 μg K5 substrate/ml), showing that both NDST-1 and -2 dose-dependently increase the ELISA signal as a measure for N-deacetylation. Above 50 μg protein/ml NDST-1 signal is not linear anymore, probably due to the saturation of the ELISA read-out system (maximal signal is 3). Wild-type 293 cells demonstrate a low NDST polysaccharides. The possible significance of these findings is discussed in the context of the biosynthesis of HS and heparin.

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activity compared to both transfected cell lines. No signal was found in the absence of Mn$^{2+}$ or K5 polysaccharide substrate or by omission of immobilized JM-403 in the wells. Figure 1B shows that the amount of product formed is linear with time for 30 min, for both NDST-1 and -2 (50μg protein/ml, 25μg K5 substrate/ml). Linearity is lost after longer incubation periods, especially for NDST-1. Therefore, we performed all experiments for 30 min at 37°C. To determine substrate concentrations at which enzyme saturation was reached, N-deacetylation was performed using a fixed concentration of enzyme (50μg protein/ml) and various K5 concentrations. As can be seen in Figure 1C, for both NDST-1 and -2, saturation is reached at ~66μM HexA, which corresponds to 25μg K5/ml. Although the $V_{\text{max}}$ of both isoforms differ, the $K_m$ values are in the same range as calculated from the Lineweaver-Burke plot (Figure 1D), namely, 13.3μM for NDST-1 and 4.7μM for NDST-2 (calculation based on [HexA]). These data show the ELISA to be linear within a given time and enzyme concentration and to reach saturation by increasing the amount of K5 substrate.

**HS as substrate for NDST-1 and -2**

To investigate substrate specificity, both NDST enzymes were also tested with two different HS preparations. A low-sulfated HS (HS-II, ~0.6 sulfate groups/disaccharide, isolated from human aorta) and a commercially obtained HS isolated from bovine kidney (HSBK, ~0.85 sulfate groups/disaccharide) were used. Because both HS preparations contain a few free amino groups and thus are bound by mAb JM-403, chemical N-acetylation of these sites was performed before abrogating JM-403 reactivity. Figure 2 shows in a dilution range N-deacetylation reactivity of NDST-1 and -2, using K5, N-acetylated HS-II and N-acetylated HSBK as substrates. Both enzymes N-deacetylate HS-II equally effective. In contrast to this, the ELISA signals using HSBK as substrate are much lower.

We previously showed the binding of JM-403 with various HS preparations to be influenced by sulfation (van den Born et al., 1995). So, a lower ELISA signal using HSBK as a substrate (in comparison with HS-II) does not necessarily identify HS-II as a better substrate. Despite this restriction, the experiment clearly shows that already sulfated HS polysaccharides can be substrates for NDST-1 and -2. Besides that, these data show differences in substrate specificity between both NDST isoforms. Both enzymes N-deacetylate HS-II equally effective, whereas NDST-1 is superior with K5 as substrate and NDST-2 is superior with HSBK as substrate. These data suggest NDST-2 to be more tolerant with respect to sulfation of the substrate, which is compatible with the finding that this isoform is highly expressed in cells producing heparin or HS with heparin-like domains (Kusche-Gullberg et al., 1998; Toma et al., 1998).

The $K_m$ values of both NDST isoforms were determined using various HS-II substrate concentrations. The saturation curve of both enzymes is shown in Figure 3A, and in Figure 3B the corresponding Lineweaver-Burke plots appear. This revealed $K_m$ values of 0.35μM for NDST-1 and 0.76μM for NDST-2 (on [HexA] basis), both of which are much lower (representing a higher affinity) than the corresponding values using K5 polysaccharide as substrate. These data show that HSs can serve as substrates for NDST-1 and -2, and that the affinity of both NDST variants for HS-II is much higher than for the K5 polysaccharide. To strengthen these conclusions, we performed a validation experiment in the conventional N-deacetylase assay using equal amounts radiolabeled K5 or radiolabeled HSBK as substrates. Incubation was done at 0.5mg protein/ml for 30 min. Results are shown in Table I.

Using $^3$H-K5 as substrate NDST-1 has a higher reactivity than NDST-2; using $^3$H-HSBK as substrate both enzymes are equally effective. In line with the $K_m$ values found for both substrates, more $^3$H-acetate is liberated by both enzymes using $^3$H-HSBK as substrate. The higher affinity of both NDST enzymes for HS than for K5 is also shown by inhibition experiments using unlabeled K5 (Table I). Clearly, competition was possible using $^3$H-K5 as the substrate. However, using $^3$H-HSBK as substrate, no competition was achieved, indicating a clear preference of both NDST isoforms for HS.

The finding that HSs are excellent substrates for both NDST enzymes could also be visualized by immunofluorescence on tissue sections. The normal staining pattern of

**Fig. 2.** Substrate specificity of both NDST enzymes. Increasing amounts of NDST-1 (A) and -2 (B) were added to samples containing 25μg/ml of K5 polysaccharide (■), N-acetylated HS-II (○), or N-acetylated HSBK (▲). Results are expressed in absorption units at 450 nm.

**Fig. 3.** Determination of the $K_m$ values of NDST-1 and -2 using N-acetylated HS-II as substrate. (A) Increasing concentrations of N-acetylated HS-II (on HexA basis) were added to samples with a constant concentration of 6μg/ml NDST-1 (●) or NDST-2 (○). Results are expressed in absorption units at 450 nm. (B) The corresponding Lineweaver-Burke plots revealed $K_m$ values of 0.35μM for NDST-1 (●) and 0.76μM for NDST-2 (○).
HS in the rat kidney by mAb JM-403 is shown in Figure 4A. The glomerular basement membranes were clearly stained by this mAb. Preexposition of sequential tissue sections by NDST-1 or -2, created HS staining in various tubular basement membranes that were negative under normal conditions (Figure 4B). This indicates N-deacetylation of GlcNAc units in HS isoforms that normally do not express unsubstituted GlcN units. Figure 4C shows loss of JM-403 staining after HS N-acetylation of the section; however, some background staining (due to the N-acetylation of the section) can be seen in the brush borders of the proximal tubuli. If, after N-acetylation of the section, the tissue is exposed to either of the NDST enzymes, JM-403 staining reappears due to the generation of free amino groups by N-deacetylation (Figure 4D). No clear differences could be observed between both NDST isoforms.

**N-sulfated polysaccharides as inhibitors of NDST-1 and -2**

In the experiments with HS as substrates, we observed that the ELISA signals both for NDST-1 and -2 fall down at increasing HS substrate concentrations (at 25 μg HS/ml we found a reduction of ~50% compared to 2.5 μg HS/ml). This finding suggested NDST inhibition, presumably by the N-sulfated domains. Therefore, in the next series of experiments we tested whether both NDST enzymes were subject to product inhibition. To this end, increasing concentrations of N-sulfated polysaccharides were added to samples with a constant NDST and K5 or HS-II substrate content. Results (Table II) show that both NDST-1 and -2 can be inhibited effectively by N-sulfated K5, N-deacetylated N-resulfated HS (N-sulfated HS), by low-molecular-weight heparin, N-sulfated heparin oligosaccharides (obtained after N-deaceylation of heparin followed by nitrous acid deamination at pH 3.9), and by heparin-derived octa- and hexasaccharides. These compounds did not interfere with the binding of JM-403 and NDST-treated substrates as carefully controlled in ELISA experiments. In general, higher amounts of polysaccharides are needed to inhibit both NDST enzymes using HS-II as substrate, even though enzyme and substrate concentrations are lower as compared to K5 substrate conditions. This again underlines the higher affinity of both enzymes for HS-II. It is obvious that N-sulfation in itself is sufficient to inhibit both NDST enzymes, as can be seen by the inhibition of N-sulfated K5, which neither contains O-sulfates nor IdoA units.

To analyze the minimal length of N-sulfated oligosaccharides that inhibit the NDST enzymes, heparin-derived N-sulfated deamination products of different length were prepared by nitrous acid deamination at pH 3.9 of N-deacetylated low-molecular-weight heparin. After reduction with NaBH₄ the resulting oligosaccharides were separated by gel filtration on a Biogel P-10 column. Fractions were then analyzed for their inhibitory capacity of both NDST isoforms.

### Table I. Substrate specificity of NDST-1 and -2 measured in the conventional ³H-radiolabeled N-deacetylase assay (10,000 cpm substrate and 500 μg protein/ml)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Released ³H-acetate (cpm)</th>
<th>% Inhibition by the addition of</th>
<th>100 μg/ml K5</th>
<th>2.5 mg/ml K5</th>
<th>100 μg/ml heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>K5 substrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDST-1</td>
<td>426 (51)</td>
<td>0</td>
<td>51</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>NDST-2</td>
<td>276 (47)</td>
<td>0</td>
<td>100</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>HS-BK substrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDST-1</td>
<td>781 (116)</td>
<td>0</td>
<td>8</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>NDST-2</td>
<td>792 (86)</td>
<td>0</td>
<td>0</td>
<td>89</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as means (SD) of five independent determinations. Inhibition of N-deacetylase activity is performed by competing with unlabeled K5 (100 μg/ml and 2.5 mg/ml) or by product inhibition using unlabeled heparin (100 μg/ml).
From Figure 5 it becomes clear that the larger the N-sulfated oligosaccharides, the more effective the NDST inhibition. No obvious differences could be found between NDST-1 and -2. It is suggested that, using HS-II as the substrate, the small (<10 sugar residues) N-sulfated oligosaccharides did not inhibit very well, but this might be related to different assay conditions using K5 as substrate versus HS-II as substrate (see legends to Figure 5). To verify the major conclusion that both NDST variants can be effectively inhibited by N-sulfated polysaccharides, we performed experiments in the conventional N-deacetylase assay using 3H-K5 and 3H-HSBK as the substrates (10,000 cpm, NDST-1 and -2 at 500 μg/ml). In a representative example we added 100 μg low-molecular-weight heparin/ml as inhibitor to the samples. Results are shown in Table I. Both enzymes were inhibited in the same order of magnitude, and no clear differences between both substrates were observed (inhibition between 72% and 94%). These data confirm our data in the new ELISA-based assay and suggest NDST inhibition by N-sulfated domains to be a regulatory mechanism during HS/heparin synthesis.

**Table II.** Inhibition of N-deacetylase activity of NDST-1 and -2 by N-sulfated polysaccharides (IC₅₀ is given in ng/ml)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K5 substrateᵃ</th>
<th>N-Ac HS-II substrateᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-sulfated K5</td>
<td>144 31</td>
<td>567 257</td>
</tr>
<tr>
<td>N-sulfated HS-BK</td>
<td>122 68</td>
<td>308 190</td>
</tr>
<tr>
<td>LMW heparin</td>
<td>455 520</td>
<td>470 346</td>
</tr>
<tr>
<td>N-sulfated LMW heparin oligo’s</td>
<td>712 693</td>
<td>1107 607</td>
</tr>
<tr>
<td>Heparin octa</td>
<td>2800 1324</td>
<td>13,750 11,350</td>
</tr>
<tr>
<td>Heparin hexa</td>
<td>9750 7450</td>
<td>45,000 36,000</td>
</tr>
</tbody>
</table>

ᵃK5 polysaccharide at 25 μg/ml; NDST-1 and -2 at 25 μg/ml.
ᵇN-Ac HS-II at 2.5 μg/ml; NDST-1 and -2 at 6 μg/ml.

From Figure 5 it becomes clear that the larger the N-sulfated oligosaccharides, the more effective the NDST inhibition. No obvious differences could be found between NDST-1 and -2. It is suggested that, using HS-II as the substrate, the small (<10 sugar residues) N-sulfated oligosaccharides did not inhibit very well, but this might be related to different assay conditions using K5 as substrate versus HS-II as substrate (see legends to Figure 5). To verify the major conclusion that both NDST variants can be effectively inhibited by N-sulfated polysaccharides, we performed experiments in the conventional N-deacetylase assay using ³H-K5 and ³H-HSBK as the substrates (10,000 cpm, NDST-1 and -2 at 500 μg/ml). In a representative example we added 100 μg low-molecular-weight heparin/ml as inhibitor to the samples. Results are shown in Table I. Both enzymes were inhibited in the same order of magnitude, and no clear differences between both substrates were observed (inhibition between 72% and 94%). These data confirm our data in the new ELISA-based assay and suggest NDST inhibition by N-sulfated domains to be a regulatory mechanism during HS/heparin synthesis.

**Influence of endogenous HS/heparin on NDST N-deacetylase activity**

Because endogenous HS/heparin that potentially could inhibit NDST activity might be present in crude tissue/cell lysates, we compared NDST activity from various sources before and after removal of endogenous HS/heparin by DEAE-Sepharose chromatography. Using 50 mM Tris, 200 mM NaCl, pH 7.4, conditions, radiolabeled HS effectively binds to the column, whereas almost all NDST-1 and -2 (present in the transfected 293 cells) is retained in the effluent (data not shown). Figure 6 shows the ELISA signals of various cell/tissue lysates at 100 μg protein/ml using K5 polysaccharide as substrate. In almost all lysates (except the kidney) the ELISA signal increases after removal of endogenous glycosaminoglycans (mean increase ~38%). Although we realize that the DEAE procedure might also remove other, nonglycosaminoglycan/proteoglycan charged molecules, these data suggest that a variable pool of endogenous HS/heparin in crude cell/tissue lysates may interfere with quantitative analysis of NDST activity.

![Fig. 5. NDST-1 and -2 inhibition by nitrous acid (pH 3.9) deamination products of N-deacetylated low-molecular-weight heparin. A 1.5-mg sample of N-deacetylated low-molecular-weight heparin was subjected to nitrous acid degradation at pH 3.9 followed by gel filtration of the fragments on a Bio-Gel P-10 fine column as described under Materials and methods. Effluent fractions were analyzed for inhibition of NDST-1 (closed circles) and NDST-2 (open circles) in the ELISA-based N-deacetylase assay using K5 polysaccharide (A) or N-acetylated HS-II (B) as substrates (K5 substrate at 25 μg/ml, NDST-1 and -2 at 25 μg/ml, inhibitor fractions 1:40; HS-II substrate at 2.5 μg/ml, NDST-1 and -2 at 6 μg/ml, inhibitor fractions 1:80). Under these conditions, the ELISA signal for both NDST-1 and -2 is around 1.0 and maximal inhibition by the heparin-derived oligosaccharides is around 90%. The column was calibrated by dextran blue (V₀) and NaB₃H₄ radiolabeled deamination products of rat glomerular HS obtained after nitrous acid at pH 1.5. Based on the elution pattern of those oligosaccharides, the elution positions in the chromatogram of di-, tetra-, hexa-, octa-, deca-, and dodecasaccharides and larger oligomers were estimated. Results are expressed as percentage inhibition of the ELISA signal without inhibitor.](https://academic.oup.com/glycob/article-abstract/13/1/556004/31145555/2019)
Discussion

This study describes the development of a new assay for the measurement of N-deacetylase activity of NDST, a key enzyme in the HS/heparin biosynthesis. The assay is based on the recognition of N-deacetylated GlcN units in enzymetreated K5- and HS-polysaccharides using mAb JM-403. We previously showed that antibody binding requires N-unsubstituted GlcN residues in HS preparations and in (chemically or enzymatically) N-deacetylated K5 polysaccharide (van den Born et al., 1995). Because the same enzyme normally will also N-sulfate the GlcN units, the assay was carried out in the absence of the sulfate donor PAPS to uncouple the two catalytic activities. We thus could show that our new assay qualitatively meets the available assay but differs in various aspects. The sensitivity of the conventional N-deacetylase assay is directly correlated to the specific activity of the radiolabeled polysaccharide substrate and the amount of substrate added to each tube. The sensitivity of the new ELISA-based assay is dependent on ELISA conditions, such as coating conditions of the immobilized JM-403, on substrate concentration, and on the detection system (JM-403-biotin-streptavidin-PO-color reaction). This makes it difficult to conclude about superiority of one of the assays. From Figure 1 is becomes clear that lyase concentrations $<100 \mu g/ml$ can be detected, which suggests superior sensitivity of the new assay. $K_m$ values can be calculated by both assays, although our $K_m$ values using K5 as the substrate are somewhat lower (representing higher affinity) than found by others (Orellana et al., 1994; Bame et al., 1991b; Navia et al., 1983). At present we do not have a satisfactory explanation for these differences, which, however, might be related to the type of assay used. Based on these data we are not able to conclude superiority of one of the assays with regard to $K_m$ values.

Regarding the $V_{max}$, it is clear that the conventional assay allows proper calculation of this parameter, whereas this is not possible or is very difficult in the ELISA-based assay, because it is not easy to translate an A 450 signal into acetyl units removed from the substrate polysaccharide. In this respect the conventional assay is superior. Besides these kinetic parameters, there are some other differences between both assays. In the ELISA-based assay there is no need for radiolabeled substrates. Moreover, the new assay is carried out in microtiter plates, which enables handling of large amounts of samples at one occasion.

In conclusion, which assay should be chosen depends on the problem to be studied. When elaborate kinetic data on the NDST enzymes are required, the conventional assay is favorable; on the other hand, when qualitative characteristics of the NDST enzymes will be studied (especially in many, small samples), we clearly prefer the ELISA-based assay. This new assay was used to study two major questions concerning the biosynthesis of HS/heparin, namely, the substrate specificity of the NDST-1 and -2 variants, and their sensitivity to product inhibition by HS-related carbohydrates.

Experiments using various polysaccharides as substrates (K5 versus two HS preparations) revealed differences between both NDST isoforms. Deacetylation of K5 is better performed by NDST-1 than by NDST-2, although the $K_m$ values are in the same micromolar range. Apparently, the ELISA signal (as a measure for the $V_{max}$) of NDST-1 is higher (Figure 1C and D). On the contrary, using HSBK as the substrate, NDST-2 is superior. With HS-II substrate, both NDST enzymes are equally effective, with lower $K_m$ values than for K5 (indicating a higher affinity) and higher ELISA signals. Thus, both NDST-1 and -2 N-deacetylate HS much more efficiently relative to the unsulfated K5 polysaccharide, and NDST-2 tolerates more substrate sulfation than NDST-1.

From the experiments we got the impression that the degree of N-sulfation is proportional to the affinity of the polysaccharide for the NDST enzymes. Thus, the more N-sulfated, the higher affinity. If a polysaccharide is partly N-sulfated, the enzyme bind to these N-sulfated domains with high affinity and starts to further N-deacetylate adjacent N-acetylated GlcN units. This idea is supported by our finding of lower $K_m$ values (representing higher affinity) of both NDST isoforms for HS compared to K5.

These findings raise some fundamental questions about the involvement of NDST in the sequence of HS/heparin modification reactions. It is generally assumed that the initiating N-deacetylation/N-sulfation of the HS/heparin modification cascade is carried out by NDST-1 and/or -2. Recently, however, a third and a fourth NDST variants are described (Aikawa et al., 2001; Aikawa and Esko, 1999). We thus speculate NDST-1 and -2 also to be involved in later HS/heparin modification reactions. Several findings hint in this direction. First, our data indicate that HS is a superior substrate (compared to K5) for both NDST-1 and -2. Second, HS analysis of our 293 cells transfected with NDST-1 or -2 revealed an increase in N-sulfation without apparent changes in O-sulfation (Cheung et al., 1996; Pikas et al., 2000). This would be very unlikely when NDST-1 or -2 action precedes C-5 epimerization and O-sulfation. It suggests that deacetylation by NDST-1 or -2 occurs to some extent after C-5 epimerization and O-sulfation. Third, the primary sequence of the four NDST enzymes differ in their N-terminal region, which contain the Golgi retention signals (Aikawa et al., 2001; Aikawa and Esko,
of the Golgi complex and thus are involved in various stages in the trans-Golgi network (Humphries et al., 1997), which might suggest NDST-1 involvement after HS synthesis/modification by enzymes located in a more proximal part of the Golgi complex.

Besides NDST heterogeneity, various 6-O and 3-O sulfotransferase isofoms have recently been found (Shwora et al., 1999; Habuchi et al., 2000). All of these findings are compatible with different enzyme machineries located in separate Golgi compartments in a single cell; some NDST isofoms (-1 and -2) also located more distally. On the other hand, structural HS/heparin analysis of cells deficient for one of the NDST enzymes, revealed a lower percentage of IdOA units and/or reduced O-sulfation (Ishihara et al., 1992; Bame et al., 1991a; Ringvall et al., 2000; Toyoda et al., 2000), which is more compatible with early NDST involvement. Obviously, more work is needed to establish the localization and possible cooperation between the various NDST enzymes and the other HS/heparin synthesizing enzymes. More insight into these processes will be provided by detailed analysis of HS produced in animals with targeted disruption of the various NDST isofoms, which were described recently (Ringvall et al., 2000; Forsberg et al., 1999; Humphries et al., 1999).

Regarding the inhibition experiments, it is clear that N-deacetylase activity of NDST-1 and -2 is blocked by N-sulfated HS-like polysaccharides. This might indicate that accumulation of HS/heparin in the Golgi compartments decreases NDST activity. This type of product inhibition is a general phenomenon in enzyme kinetics and tightly regulates enzyme activity between narrow borders. This indicates that N-deacetylase catalytic activity is primarily governed by the NDST content relative to the polysaccharide concentration. The fact that low-molecular-weight heparin and heparin-related oligosaccharides inhibited the NDST enzymes less effective is in all likelihood related to chain length. The shorter the oligosaccharide, the less effective inhibition of the NDST enzyme. This is clearly illustrated in Figure 5.

This study furthermore shows that NDST N-deacetylase activity in crude cell/tissue lysates can be subject to product inhibition due to the presence of a variable pool of endogenous HS/heparins. It thus seems to be necessary to remove endogenous HS/heparins before quantitation of NDST N-deacetylase activity. Because different experimental/pathological conditions may influence the content and composition of endogenous HS/heparins, differences in NDST N-deacetylase activity that have been found in the past in unpurified lysates (Unger et al., 1991; Kofoed-Enevoldsen, 1992; Kofoed-Enevoldsen et al., 1993; Bourin, 1997) should be interpreted with caution.

Materials and methods

Glycosaminoglycans

The Escherichia coli K5 capsular polysaccharide, with the same (GlcA-GlcNAc)ₙ structure as the nonsulfated HS/heparin precursor polysaccharide (Vann et al., 1981), and heparin-derived hexa- and octasaccharides (obtained after heparinase treatment) were kindly provided by Dr. G. van Dedem (Organon, Oss, Netherlands). N-sulfated K5 (N-sulfation ~100%) was a gift of Dr. B. Casu (Instituto di Chimica e Biochimica G. Ronzoni, Milan, Italy). Low-molecular-weight heparin (Lovenox) was commercially obtained from Rhône-Poulenc Rorer (Collegeville, PA). Heparan sulfate isolated from bovine kidney was obtained from Seikagaku (Tokyo). HS (preparation HS-II) isolated from human aorta essentially according to Iverius (1971), was provided by M. Salmivirta (Uppsala University, Sweden).

Chemical modifications of polysaccharides

N-acetylation of HS was performed by treatment with acetic anhydride as described by Höök and colleagues (1982). N-deacetylated heparin was prepared by hydraduloylsis as described previously (van den Born et al., 1995). Deamination products obtained after degradation of 1.5 mg of N-deacetylated low-molecular-weight heparin by nitrous acid, pH 3.9 (cleaves the polysaccharide chain at N-unsubstituted GlcN residues; Shively and Conrad, 1976) followed by reduction with NaBH₄ were analyzed by gel chromatography on a column (1 × 140 cm) of Bio-Gel P-10 fine (Bio-Rad Laboratories, Hercules, CA) in 0.5 M ammonium hydrogen carbonate, eluted at a rate of 3.6 ml/h. Effluent fractions of 0.9 ml were collected and analyzed for hexuronic acid by the carbazole reaction (Chen et al., 1989), and tested in the ELISA-based N-deacetylase assay as inhibitors of either NDST enzyme (see later description).

Cell and tissue lysates

As a source of NDST-1 and -2 cell lysates we used human kidney 293 cells stably transfected with the mouse transcript coding for NDST-1 (clone 11) and -2 (clone S5), respectively. The characterization of the NDST-1 and -2 transfected cells and impact on HS synthesis have been published before (Cheung et al., 1996; Pikas et al., 2000). Both clones displayed a comparable ~10-fold overexpression of NDST-1 and -2, respectively, as assessed by northern blotting and reverse transcription polymerase chain reaction. Lysates were made by homogenizing cells from culture or tissues on ice in 5 ml/g tissue of 50 mM Tris, pH 7.4, containing 2 mM ethylenediamine tetra-acetic acid, 1% Triton X-100 and a cocktail of protease inhibitors (Complete, Boehringer Mannheim, Germany). The homogenates were centrifuged for 15 min at 13,000 rpm at 4°C; the supernatant was collected and diluted 1:1 in glycerol and stored in ~20°C until use. Protein concentration of the cell/tissue lysates varied between 2–13 mg/ml as determined by the method of Lowry et al. (1951).

To remove endogenous HS/heparin, cell/tissue lysates were applied to DEAE ion exchange resins in 50 mM Tris–HCl, 200 mM NaCl, pH 7.4. The efficiency of HS/heparin binding to the column was controlled by adding 3H-acetylated HS (from rat glomeruli) to the samples. Under these conditions all 3H-HS effectively bound to the
column. The effluent was collected and desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) in 50 mM Tris buffer, pH 7.4, containing 10 mM MnCl₂, 1% Triton X-100, and protein inhibitors and was compared in the NDST ELISA with the untreated extracts.

Conventional N-deacetylase assay

The conventional N-deacetylase assay, based on the liberation of ³H-acetate from radiolabeled K5 or HS isolated from bovine kidney, was performed as described previously (Navia et al., 1983). The 293 cell lysates at a protein concentration of 0.5 mg/ml were incubated in the presence of 10,000 cpm of ³H-K5 or ³H-HS for 30 min at 37°C. Specific activity of both polysaccharides is ~100,000 cpm/µg (dry weight).

ELISA-based N-deacetylase assay

The new N-deacetylase assay is based on the recognition of N-deacetylated K5 or HS by mAb JM-403, which we characterized previously (van den Born et al., 1995). Binding of this mAb is essentially dependent on the presence of N-unsubstituted GlcNH₃⁺ units. Samples containing NDST activity were incubated during 30 min at 37°C with K5 (25 µg/ml) or N-acetylated HS (2.5 µg/ml) in 80 µl buffer containing 50 mM MES, 1% Triton X-100 in the presence of 10 mM MnCl₂. After 30 min the reaction was stopped by the addition of 40 µl stop solution containing 50 mM Tris, 450 mM NaCl, pH 9.5. Due to this addition the pH rose to 7.0 and the final NaCl concentration became 150 mM, which stops NDST enzyme activity and makes the solution suitable for antibody binding.

Of each sample, 100 µl is transferred to the wells of a polystyrene flat-bottom microtiter plate (NUNC Maxisorp, Gibco, Breda, Netherlands) in which mAb JM-403 has been immobilized before (overnight 1:2000 dilution of JM-403 in phosphate buffered saline [PBS], followed by 2 h blocking of aspecific binding sites by PBS-1% gelatin). NDST-exposed K5 or HS will bind to the immobilized antibody. After 1 h the wells were washed with PBS containing 0.05% Tween-20 (PBS-T) and incubated with a 1:1000 dilution of biotinylated JM-403 in 100 µl PBS-1% gelatin. Biotinylation of JM-403 was done with biotin-hydrazide according to the instructions of the manufacturer (Pierce, Rockford, IL) after acid oxidation of JM-403 with 20 mM sodium periodate. After washing with PBS-T, the ELISA was developed for 1 h by the addition of 100 µl of a 1:1000 dilution of peroxidase conjugated streptavidin (Amersham, Piscataway, NJ) in PBS-1% gelatin. After washing, 100 µl/well of 3,5,3',5' tetramethylbenzidine substrate solution was added (SFRI Laboratories, Berganton, France). The reaction was stopped after 15 min by adding 100 µl 2 M H₂SO₄ to each well, and the absorbance at 450 nm was measured. Enzyme inhibition was performed by the addition of N-sulfated K5, N-sulfated HS, and N-sulfated heparin-derived oligosaccharides. Percentage inhibition was calculated as \[ 1 - (A_{450 \text{ with inhibitor}}/A_{450 \text{ without inhibitor}}) \times 100. \]

IC₅₀ (ng inhibitor/ml) is defined as the concentration of inhibitor that gives 50% inhibition in the ELISA system.

Immunohistology

To visualize whether HS in vivo is a suitable substrate for NDST-1 and -2, rat kidney cryostat sections of 2 µm were used. Immunofluorescence staining with mAb JM-403 was performed as described (van den Born et al., 1995). In some experiments the sections were N-acetylated for 10 min at room temperature by 0.25% acidic anhydride in 0.5 M borate buffer, pH 9.0, to abolish JM-403 staining. After this eventual pretreatment and prior to the JM-403 incubation, sections were incubated by the cell lysates of the 293 cells transfected with NDST-1 or -2. 250 µg protein/ml. Staining was visualized using a Zeiss Axioskop microscope equipped with epi-illumination.

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Abbreviations

ELISA, enzyme-linked immuno sorbent assay; GlcA, glucoronic acid; HS, heparan sulfate; HSBK, heparan sulfate isolated from bovine kidney; HSPG, heparan sulfate proteoglycan; IdoA, iduronic acid; mAb, monoclonal antibody; NDST, heparan sulfate/heparin N-deacetylase/N-sulfotransferase; PAPS, phosphoadenyl 5’-phosphosulfate; PBS, phosphate buffered saline.

References


