Heparin/Heparan Sulfate Biosynthesis

PROCESSIVE FORMATION OF N-SULFATED DOMAINS

Received for publication, February 29, 2008, and in revised form, May 14, 2008. Published, JBC Papers in Press, May 16, 2008, DOI 10.1074/jbc.M801652200

Pernilla Carlsson, Jenny Presto, Dorothe Spillmann, Ulf Lindahl, and Lena Kjellén
From the Department of Medical Biochemistry and Microbiology at Uppsala University, SE-751 23 Uppsala, Sweden

Heparan sulfate (HS) proteoglycans influence embryonic development as well as adult physiology through interactions with various proteins, including growth factors/morphogens and their receptors. The interactions depend on HS structure, which is largely determined during biosynthesis by Golgi enzymes. A key step is the initial generation of N-sulfated domains, primary sites for further polymer modification and ultimately for functional interactions with protein ligands. Such domains, generated through action of a bifunctional GlcNAc N-deacetylase/N-sulfotransferase (NDST) on a [GlcUA-GlcNAc]n substrate, are of variable size due to regulatory mechanisms that remain poorly understood. We have studied the action of recombinant NDSTs on the [GlcUA-GlcNAc]n precursor in the presence and absence of the sulfate donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS). In the absence of PAPS, NDST catalyzes limited and seemingly random N-deacetylation of GlcNAc residues. By contrast, access to PAPS shifts the NDST toward generation of extended N-sulfated domains that are formed through coupled N-deacetylation/N-sulfation in an apparent processive mode. Variations in N-substitution pattern could be obtained by varying PAPS concentration or by experimentally segregating the N-deacetylation and N-sulfation steps. We speculate that similar mechanisms may apply also to the regulation of HS biosynthesis in the living cell.

HS is synthesized in the Golgi network through complex, concerted action of several distinct enzymes (Fig. 1) (2). First, a glucuronic acid-galactose-galactose-xylose (GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-) “linkage tetrasaccharide” is formed that will connect the HS chain proper with a serine unit of a proteoglycan core protein. After the addition of a GlcNAc residue, polymerization proceeds through transfer of alternating units of GlcUA and GlcNAc catalyzed by complexed EXT1 and EXT2 polymerases. The nascent [4GlcUAβ1-4GlcNacα1-]n polysaccharide chain is modified by N-deacetylation and N-sulfation of selected GlcNAc units, effected by a family of bifunctional N-deacetylase/N-sulfotransferase (NDST) enzymes. N-Sulfated glucosamine (GlcNS) units are typically found in contiguous stretches (NS domains) separated by non-modified N-acetylated regions (NA domains). Often, regions of alternating GlcNS and GlcNAc units (NA/NS domains) surround the NS domains (2). Subsequent modifications are largely confined to the NS domains. These include epimerization of GlcUA units into l-iduronic acid catalyzed by C5-epimerase and O-sulfation at different positions generated by 2-O-, 6-O-, and 3-O-sulfotransferases. The association of all modifications with N-sulfated regions demonstrates the key importance of the NDSTs in designing HS structure. Except for the C5-epimerase and the 2-O-sulfotransferase, which are encoded by single genes in mammals, the modification enzymes occur in several isoforms (4). Four NDSTs (NDST1–4) have been identified, NDST1 and NDST2 being the most widely distributed (5).

The mechanisms behind the non-random distribution of N-acetylated and N-sulfated disaccharide units in HS are unknown. The N-deacetylation and N-sulfation reactions could be experimentally segregated using a heparin-producing mastocytoma microsomal system (6); only after identification of bifunctional NDSTs was it realized that both processes should be attributed to a single enzyme (7–9). In the present study, recombinant NDSTs were used to define the mode of enzyme action, in the absence and presence of the sulfate donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS). N-Deacylation of a fully N-acetylated precursor polysaccharide in the absence of PAPS was found to be an essentially stochastic process generating a limited proportion of randomly distributed, N-unsubstituted glucosamine (GlcNH2) residues. By contrast, the addition of PAPS converted the enzyme to a virtually processive mode of action, resulting in formation of extended sequences of consecutive, N-sulfated disaccharide units. Our findings thus suggest that key structural features of HS chains are essentially established during HS biosynthesis (2) but may also be modified through postbiosynthetic action of recently identified endosulfatasases (3).
may be ascribed to mechanisms inherent to the NDST enzymes.

**EXPERIMENTAL PROCEDURES**

**cDNA Construct and Transfection**

**His-tagged NDST1**—Full-length cDNA of wild type mouse NDST1 cloned in pBluescript SK vector was used as template in a PCR to introduce an enterokinase cleavage site followed by a His6 tag in the C-terminal end of the NDST1 protein. Forward and reverse primers were 5’-ctctcaagcacctggtg-3’ and 5’-ggagattctagttcatcgtcatcgtccctgtgctctgagctctccgcagcca-3’ (His tag in bold), respectively. The PCR product was cleaved with BglII and EcoRI and used to replace the corresponding sequence of wild type NDST1 in pBluescript SK. The resulting NDST1-His sequence was then cloned into expression vector pBud4.1CE (Invitrogen) using XhoI and NotI.

**His6-tagged NDST2**—Full-length cDNA of wild type mouse NDST2 cloned in pUC119 vector was used as template in a PCR to introduce an enterokinase cleavage site followed by a His6 tag in the C-terminal end of the NDST2 protein. Forward and reverse primers were 5’-ctcaggaacgaagccccct-3’ and 5’-tgtctagttcatcgtcatcgtccctgtgctctgagctctccgcagcca-3’ (His tag in bold), respectively. The PCR product was cleaved with XbaI and Kpn21 and used to replace the corresponding sequence of wild type NDST2 in pUC119. The resulting NDST2-His sequence was then cloned into expression vector pCDNA3 using XbaI and EcoRI.

The His constructs were transfected into HEK-293 cells using Lipofectamine™ 2000 (Invitrogen). Stable clones were selected at a high concentration of Zeocin (400 µg/ml, Invitrogen) for NDST1 or G418 (800 µg/ml, Invitrogen) for NDST2 in Dulbecco’s modified Eagle’s medium (Statens Veterinärmedicinska Anstalt) containing 2 mM l-glutamine (Statens Veterinärmedicinska Anstalt), 10% fetal calf serum (Invitrogen), 2.5 µg/ml amphotericin B (Fungizone®, Invitrogen), 60 µg/ml penicillin G, and 50 µg/ml streptomycin (PeSt, Statens Veterinärmedicinska Anstalt) and were maintained in the same medium containing 200 µg/ml Zeocin for NDST1-expressing cells and 200 µg/ml G418 for NDST2-expressing cells.

**Purification of Recombinant NDSTs**

Transfected NDST1- and NDST2-expressing cells, respectively, from four confluent T175 cell flasks were washed in phosphate-buffered saline and solubilized in 1.5 ml of solubilization buffer (20 mM Tris-HCl, pH 7.5, 0.8% Triton X-100, 0.3 M NaCl, 1 mM Pefabloc (Roche Applied Science), 10 µg/ml pepstatin A (Sigma-Aldrich)). Cell debris was removed by centrifugation, and the supernatant was applied to 60 l of Talon metal affinity resin (BD Biosciences) and incubated end-over-end for 2 h at 4 °C. The resin was then washed twice with each of three buffers (buffer 1: 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM NaCl, 1 mM imidazole; buffer 2: 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM NaCl; buffer 3: 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.3 mM NaCl) and finally eluted with 2 x 125 µl of elution buffer (50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 150 mM imidazole). Glycerol was added to a final concentration of 25% by dialysis of the eluate against 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.15 M NaCl, 25% glycerol. The purified recombinant proteins were stored at −20 °C.

N-Deacetylase activities of NDST1 and NDST2, measured as described previously (8), were 700 and 400 cpm/µl, respectively. Briefly, the enzymes were incubated with 10,000 cpm of N-[3H]acetyl-labeled *Escherichia coli* K5 capsular polysaccharide for 30 min at 37 °C in 0.2 ml of 50 mM MES, pH 6.3, 10 mM MnCl₂, 1% Triton X-100, and 0.25 µg/ml Polybrene. The released [3H]acetate was quantified in a biophase scintillation counting system (8).

**Isolation of 14C-labeled K5 Polysaccharide**

K5 polysaccharide was metabolically labeled and isolated essentially as described previously (10). Further purification of the 14C-labeled K5 polysaccharide included β-elimination with 0.5 M NaOH, 18 h, 4 °C, and neutralization by HCl followed by treatment with 12 units Benzonase (Merck) for 2 h in 37 °C and
purification on a Sep-Pak Plus C-18 cartridge (Waters) primed in methanol. Finally, the polysaccharide was applied to DEAE-Sephacel, equilibrated in 50 mM Tris-HCl, pH 8.0, washed in 50 mM NaAc, pH 4.0, and eluted in 0.5 M NaCl. The purified $^{14}$C-labeled K5 polysaccharide, molecular weight $\sim 100,000$ and specific activity $35 \times 10^3$ cpm/µg, was desalted on PD-10 column (GE Healthcare) before it was used in the enzyme incubations.

**Incubations of E. coli K5 Polysaccharide with NDSTs**

Equal amounts of NDST1 and NDST2, based on His tag content as assayed by Western blotting using penta-His-antibody (Qiagen), were used in the incubations with $^{14}$C-labeled K5 capsular polysaccharide in 50 mM MES, pH 6.3, 1% Triton X-100, 10 mM MnCl$_2$, and 0.25 µg/ml Polybrene, in the presence or absence of PAPS. Standard incubations of 100 µl contained $^{14}$C-labeled K5 substrate (20,000 cpm) and enzyme corresponding to an N-deacetylase activity of 700 cpm (see above), with or without PAPS (Sigma-Aldrich; 0.1 µl if not otherwise indicated) and were maintained at 37 °C for different periods of time. Additions of enzyme and PAPS were renewed each hour. Incubations with PAP were performed as described above, using PAP (Sigma-Aldrich) instead of PAPS.

**Analysis of Products**

Enzymatically modified K5 polysaccharide was examined for occurrence and distribution of GlcNH$_2$ and N-sulfated GlcNS residues by selective deaminative cleavage. Samples were treated with nitrous acid at pH 3.9 or at pH 1.5 to achieve chain cleavage at GlcNH$_2$ and GlcNS residues, respectively, as described previously (11). Deamination products were separated by gel chromatography on Superdex 30 (GE Healthcare) in 0.5 M NH$_4$HCO$_3$ at a flow rate of 0.7 ml/min. Fractions were analyzed by scintillation counting. The extent of N-deacetylation or N-sulfation was calculated from the relative proportions of the various even-numbered oligosaccharide deamination products.

NS domains were prepared as described (12). Briefly, samples of modified K5 polysaccharide were first chemically N-deacetylated by treatment with 70% (w/v) aqueous hydrazine (Fluka) containing 1% (w/v) hydrazine sulfate at 96 °C for 4 h (13) and were then cleaved by treatment with nitrous acid at pH 3.9. Products were separated by gel chromatography as described above.

**RESULTS**

The *E. coli* K5 capsular polysaccharide has the same [4GlcUA$\beta_1$-4GlcNAc$\alpha_1$-]$_n$ structure as the unmodified endogenous substrate that NDST enzymes will encounter in the Golgi compartment of the cell. We applied metabolically radiolabeled K5 polysaccharide as target for recombinant NDSTs and aimed at elucidating the mode of action of the enzyme under different conditions of PAPS access. Recombinant NDSTs with an enterokinase-sensitive C-terminal His tag were produced in HEK 293 cells. The proteins are retained in the Golgi compartment through their membrane-spanning domain, and the cells were therefore solubilized in detergent-containing buffer before purification by Talon metal affinity chromatography. To ascertain that enzyme activity was not affected by the added His tag, N-deacetylase activity was measured before and after cleavage of purified NDSTs with enterokinase. As shown in supplemental Fig. 1, removal of the His tag did not affect enzyme activity. For further experiments, the enzymes were used without removal of the tag.

**Polymer Modification by NDST1 and NDST2 in the Absence or Presence of PAPS—**$^{14}$C-labeled K5 polysaccharide was incubated with NDST1 or NDST2 in the absence of PAPS, and the products were cleaved at the generated N-unsubstituted GlcNH$_2$ units by deamination at pH 3.9. Gel chromatography of both samples revealed a disperse population of oligosaccharides, with tetramers and hexamers as the predominant species (Fig. 2, A and B). Notably, very small amounts of disaccharides were observed after cleavage, indicating that the enzyme rarely attacked GlcNAc residues in neighboring disaccharide units. The distribution of variously sized oligosaccharides pointed to an essentially random mode of enzyme attack for both NDST1 and NDST2.

Inclusion of PAPS in the incubations enabled the enzymes to perform both N-deacetylation and N-sulfation and resulted in a dramatically altered modification pattern (Fig. 2, A and B). Products were essentially resistant to deamination at pH 3.9, pointing to the absence of N-unsubstituted GlcNH$_2$ residues (data not shown). By contrast, deamination at pH 1.5 generated disaccharides as the predominant cleavage product, indicating degradation of consecutive N-sulfated disaccharide units. Access to PAPS thus altered the target selection pattern of the NDST enzyme, from random N-deacetylation to concerted N-deacetylation/N-sulfation of adjacent disaccharide units, as required to generate NS domains. Moreover, a greater proportion of the substrate was subject to modification, amounting to $\sim 65$% N-sulfation in the presence of PAPS and either NDST1 or NDST2, as compared with $\sim 40$% N-deacetylation in the absence of the sulfate donor (calculated from the chromatograms in Fig. 2, A and B). PAPS apparently affected the two NDST enzymes in a similar fashion and to the same extent. For further characterization of the influence of PAPS on enzyme action, we chose to study NDST2.

The effect of PAPS on the course of NDST action appeared strongly associated with the sulfate donor function but could conceivably also reflect a regulatory influence of the nucleotide portion. To test this alternative, 0.1 mM PAP rather than PAPS was included in the incubations, and the distribution of N-unsubstituted residues was determined. Judging from the increased susceptibility of the incubated polysaccharide to deamination at pH 3.9, PAP appeared to stimulate N-deacetylation (Fig. 2D). However, the same oligosaccharides were seen as after incubation in the absence of PAP, without any significant amounts of disaccharides. Binding of the nucleotide per se thus does not seem to modulate NDST action in a qualitative sense.

**Processive Formation of NS Domains—**To determine the size distribution of the NS domains generated by NDST2 in the presence of PAPS, the remaining unmodified GlcNAc units in the $^{14}$C-labeled K5 substrate were deacetylated through hydrazinolysis and cleaved by reaction with nitrous acid at pH 3.9. Gel chromatography of the products showed extended NS.
domains, composed of ≥8 disaccharide units (Fig. 3). Hardly any oligosaccharides of smaller size (<8-mer) were obtained, suggesting that sulfation occurred in long, continuous stretches with only very few GlcNAc residues remaining unmodified between the NS domains. Such domains could be formed either by processive action of the NDST along the polysaccharide chain or by random, but efficient, coupled N-deacetylation and N-sulfation, eventually yielding extended N-sulfated stretches.

To distinguish between these alternatives, incubation of the labeled substrate with NDST2 and PAPS was conducted for different periods of time, and the products were cleaved at N-sulfated residues by deamination at pH 1.5. The procedure resulted in progressive formation of disaccharides with increasing incubation time but gave very small amounts of larger oligosaccharides (Fig. 4), pointing to an essentially processive mode of N-sulfation.

Effects of PAPS on NDST Target Pattern—Authentic HS species contain NS domains and unmodified NA domains but also contain substantial proportions of NA/NS domains composed of alternating N-acetylated and N-sulfated disaccharide units (14). In our experimental setting, extended NS domains domi-
nated the N-sulfation pattern of the substrate when PAPS was included in the incubations (Figs. 2–4). We next decided to test whether altering the concentration of PAPS would influence the domain structure. At the lowest concentration tested, 0.1 μM, no N-sulfation of the substrate was detected (Fig. 5). When increased to 1 μM, di- and tetrasaccharides as well as larger cleavage products were obtained after treatment of the substrate with nitrous acid at pH 1.5 (Fig. 5). At a PAPS concentration of 5 μM, the relative abundance of consecutive N-sulfated disaccharide units in the substrate was higher, as shown by the increased amounts of disaccharides obtained after nitrous acid treatment (Fig. 5). Incubations in the presence of 5 and 10 μM PAPS gave identical results (data not shown). These data suggest that the N-substitution pattern of HS may be regulated through variation in PAPS concentration during biosynthesis.

Since the enzyme attacked the substrate in an apparently random mode in the absence of PAPS, we wanted to see how uncoupling of the N-deacylation and N-sulfation reactions would influence N-substitution patterns. 14C-Labeled K5 polysaccharide was therefore incubated with NDST2 for 1 h in the absence or presence of PAPS followed by incubation for an additional hour with 0.1 mM PAPS. The formation of N-unsubstituted and N-sulfated GlcN units was monitored as before after selective deaminative cleavage of the polysaccharide. As in previous experiments, incubation without PAPS resulted in the generation of N-unsubstituted GlcNH₂ residues dispersed along the chain and separated by one, two, or more N-sulfated disaccharide units (Fig. 6A). An additional hour in the presence of PAPS only marginally lowered the amounts of N-unsubstituted residues in the substrate (Fig. 6A). However, during this second hour, N-sulfation had occurred, as indicated primarily by the formation of di- and tetrasaccharides after nitrous acid cleavage at pH 1.5 (Fig. 6B) but also by the appearance of extended saccharide sequences resistant to deamination at pH 3.9 (fraction emerging between 38 and 41 ml in Fig. 6A). Taken together, these findings suggest that preformed N-unsubstituted GlcNH₂ units are not preferred targets for N-sulfation as compared with GlcNAc residues that undergo concerted N-deacylation/N-sulfation. In chains containing such GlcNH₂ units the concerted, presumably processive modification reactions will primarily involve extended, uninterrupted N-acetylated regions but may then entail N-sulfation also of adjacent GlcNH₂ units (see scheme in Fig. 6).

**DISCUSSION**

Current information regarding HS structure points to extensive variability regarding level of sulfation as well as domain organization (14–16). Such variability applies to samples derived from different tissues, cell types, or developmental stages of a particular species, whereas variation between individuals is low (at least in inbred mice (15)). These and similar observations support the view that HS biosynthesis is subject to stringent control. So far, little is known about the mechanisms of regulation. The lack of a template, such as DNA in protein synthesis, suggests that regulation is exerted largely through the availability of various HS biosynthetic enzymes with different activities and substrate specificities. The concerted action of these membrane-bound enzymes would in turn be controlled by their organiza-
Processive Formation of Heparan Sulfate N-sulfated Domain

The observation of extended NS domains being generated by unaided NDST action again raises the crucial question of regulation. We presently showed that lowering PAPS concentration led to a relative decrease in disaccharide deamination products, suggesting that formation of NS domains had subsided (Fig. 5). More dramatic effects were noted by experimentally segregating NDST action in the absence and presence of PAPS, on the same polysaccharide substrate (Fig. 6). Although NS domains were generated in the presence of PAPS, saccharide regions containing the scattered GlcNH₂ residues introduced during preceding incubation in the absence of the sulfate donor remained essentially unaffected.

Several reports including immunolocalization studies (22, 34, 35) indicate that HS biosynthesis in several cell types occurs proximal to the trans Golgi/trans Golgi network, where chondroitin sulfate is synthesized and PAPS transporters are believed to reside (36, 37). Hence, the first encounter between NDST enzymes and the growing HS chain may occur in Golgi subcompartments, where PAPS is absent or present at low concentrations. Notably, studies of NDST mutant cells suggested that N-deacetylation and N-sulfation can occur independently; overexpression in 293 cells of NDST1 lacking N-sulfotransferase activity resulted in elevated HS N-sulfation with no concomitant increase in GlcNH₂ residues (38). The more random modification in the absence of PAPS followed by the apparently processive synthesis of NS domains in the presence of the sulfate donor could thus represent one means to generate the cell specific domain structure of HS.

REFERENCES

1. Bulow, H. E., and Hobert, O. (2006) Annu. Rev. Cell Dev. Biol. 22, 375–407
2. Esko, J. D., and Lindahl, U. (2001) J. Clin. Investig. 108, 169–173
3. Lamanna, W. C., Kalus, L., Padva, M., Baldwin, R. J., Merry, C. L., and Dierks, T. (2007) J. Biotechnol. 129, 290–307
4. Kusche-Gullberg, M., and Kjellen, L. (2003) Curr. Opin. Struct. Biol. 13, 605–611
5. Grobe, K., Ledin, J., Ringvall, M., Holmborn, K., Forsberg, E., Esko, J. D., and Kjellen, L. (2002) Biochim. Biophys. Acta 1573, 209–215
6. Hook, M., Lindahl, U., Hallen, A., and Backstrom, G. (1975) J. Biol. Chem. 250, 6065–6071
7. Kjellen, L., Pettersson, I., Unger, E., and Lindahl, U. (1992) Adv. Exp. Med. Biol. 313, 107–111
8. Pettersson, I., Kusche, M., Unger, E., Wlad, H., Nylund, L., Lindahl, U., and Kjellen, L. (1991) J. Biol. Chem. 266, 8044–8049
Processive Formation of Heparan Sulfate N-sulfated Domain

9. Wei, Z., Swiedler, S. J., Ishihara, M., Orellana, A., and Hirschberg, C. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3885–3888
10. Roman, E., Roberts, I., Lidholt, K., and Kusche-Gullberg, M. (2003) Biochem. J. 374, 767–772
11. Shively, J. E., and Conrad, H. E. (1976) Biochemistry 15, 3932–3942
12. Kreuger, J., Prydz, K., Pettersson, R. F., Lindahl, U., and Salmivirta, M. (1999) Glycobiology 9, 723–729
13. Shaklee, P. N., and Conrad, H. E. (1984) Biochem. J. 217, 187–197
14. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) J. Biol. Chem. 271, 17804–17810
15. Ledin, J., Staatz, W., Li, J. P., Gotte, M., Selleck, S., Kjellen, L., and Spillmann, D. (2004) J. Biol. Chem. 279, 42732–42741
16. Lindahl, B., Eriksson, L., and Lindahl, U. (1995) Biochem. J. 306, 177–184
17. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471
18. McCormick, C., Duncan, G., Goutsos, K. T., and Tufaro, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 668–673
19. Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., Lidholt, K., Lindahl, U., and Kusche-Gullberg, M. (2000) EMBO Rep. 1, 282–286
20. Lidholt, K., Kjellen, L., and Lindahl, U. (1989) Biochem. J. 261, 999–1007
21. Presto, J., Thuveson, M., Carlsson, P., Busse, M., Wilén, M., Eriksson, I., Kusche-Gullberg, M., and Kjellén, L. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 4751–4756
22. Pinhal, M. A., Smith, B., Olson, S., Aikawa Ji, J., Kimata, K., and Esko, J. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 30, 30
23. Casu, B., and Lindahl, U. (2001) Adv. Carbohydr. Chem. Biochem. 57, 159–206
24. Kreuger, J., Matsumoto, T., Vanvildemeersch, M., Sasaki, T., Timpl, R., Claesson-Welsh, L., Spillmann, D., and Lindahl, U. (2002) EMBO J. 21, 6303–6311
25. Murphy, K. J., Merry, C. L., Lyon, M., Thompson, J. E., Roberts, I. S., and Gallagher, J. T. (2004) J. Biol. Chem. 279, 27239–27245
26. Riesenfeld, J., Hook, M., and Lindahl, U. (1982) J. Biol. Chem. 257, 421–425
27. Aikawa, J., Grobe, K., Tsujimoto, M., and Esko, J. D. (2001) J. Biol. Chem. 276, 5876–5882
28. Forsberg, E., Peijler, G., Ringvall, M., Lunderius, C., Tomasini-Johansson, B., Kusche-Gullberg, M., Eriksson, I., Lind, J., Hellman, L., and Kjellén, L. (1999) Nature 400, 773–776
29. Humphries, D. E., Wong, G. W., Friend, D. S., Gurish, M. F., Qiu, W. T., Huang, C., Sharpe, A. H., and Stevens, R. L. (1999) Nature 400, 769–772
30. Kusche-Gullberg, M., Eriksson, I., Pikas, D. S., and Kjellén, L. (1998) J. Biol. Chem. 273, 11902–11907
31. Ledin, J., Ringvall, M., Thuveson, M., Eriksson, I., Wilen, M., Kusche-Gullberg, M., Forsberg, E., and Kjellén, L. (2006) J. Biol. Chem. 281, 35727–35734
32. Riesenfeld, J., Hook, M., and Lindahl, U. (1982) J. Biol. Chem. 257, 7050–7055
33. Escobar Galvis, M. L., Jia, J., Zhang, X., Jastrebova, N., Spillmann, D., Gottfridsson, E., van Kuppevelt, T. H., Zcharia, E., Vlodavsky, I., Lindahl, U., and Li, J. P. (2007) Nat. Chem. Biol. 3, 773–778
34. Crawford, B. E., Olson, S. K., Esko, J. D., and Pinhal, M. A. (2001) J. Biol. Chem. 276, 21538–21543
35. Nagai, N., Habuchi, H., Esko, J. D., and Kimata, K. (2004) J. Cell Sci. 117, 3331–3341
36. Kamiyama, S., Sasaki, N., Goda, E., Ui-Tei, K., Saigo, K., Narimatsu, H., Iigami, Y., Kannagi, R., Irimura, T., and Nishihara, S. (2006) J. Biol. Chem. 281, 10945–10953
37. Kamiyama, S., Suda, T., Ueda, R., Suzuki, M., Okubo, R., Kikuchi, N., Chiba, Y., Goto, S., Toyoda, H., Saigo, K., Watanabe, M., Narimatsu, H., Iigami, Y., and Nishihara, S. (2003) J. Biol. Chem. 278, 25958–25963
38. Bengtsson, J., Eriksson, I., and Kjellén, L. (2003) Biochemistry 42, 2110–2115