Isolation and Characterization of Low Sulfated Heparan Sulfate Sequences with Affinity for Lipoprotein Lipase*

Received for publication, May 16, 2006, and in revised form, June 16, 2006. Published, JBC Papers in Press, June 16, 2006, DOI 10.1074/jbc.M604702200

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Lipoprotein lipase (LPL), which is an important enzyme in lipid metabolism, binds to heparan sulfate (HS) proteoglycans. This interaction is crucial for several aspects of LPL function, such as intracellular/extracellular transport and high capacity attachment to cell surfaces. Retention of LPL on the capillary walls, and elsewhere, via HS chains is most likely affected by the quality and quantity of HS present. Earlier studies have demonstrated that LPL interacts with highly sulfated HS and heparin oligosaccharides. Since such structures are relatively rare in endothelial HS, we have re-addressed the question of physiological ligand structures for LPL by affinity purification of end-labeled oligosaccharides originating from heparin and HS on immobilized LPL. By a combination of chemical modification and fragmentation of the bound material we identified that the bound fraction contained modestly sulfated oligosaccharides with an average sulfation of one O-sulfate per disaccharide unit and tolerates N-acetylated glucosamine residues. Therefore LPL, containing several clusters of positive charges on each subunit, may constitute an ideal structure for a protein that needs to bind with reasonable affinity to a variety of modestly sulfated sequences of the type that is abundant in HS chains.

Heparan sulfates (HS) are important glycosaminoglycans (GAG) found on virtually all cell surfaces and in extracellular matrices. They have been shown to function as receptors for different proteins associated with cellular development and maintenance. HS is characterized by a long, linear backbone structure consisting of alternating glucuronic acid and N-acetylated glucosamine residues (for general overview, see Refs. 1 and 2). During biosynthesis this backbone is modified by N-deacetylation/N-sulfation of the glucosamine unit, C5-epimerization of the glucuronic to the iduronic acid residue, and O-sulfation of both monosaccharide units. Due to biosynthetic constraints not all sites are modified, and a large number of different sequences are produced. In the chains microdomains of different sulfation degree are apparent. Highly sulfated sequences with contiguous N-sulfated disaccharides (so called NS domains) are interspaced by moderately sulfated domains with alternating N-sulfated and N-acetylated disaccharides (NA/NS domains) and low sulfated domains with stretches of N-acetylated disaccharides (NA domains). The regulation of HS biosynthesis and the resulting distribution of domains within the chains are poorly understood, although an increasing body of evidence suggests a controlled expression of disaccharide content and HS epitopes in a tissue-specific way (3–5).

Some small proteins like fibroblast growth factors interact with short, highly sulfated NS domains (2, 6). For interaction under physiological conditions, however, many proteins need longer sequences of HS oligosaccharides, yet the number and length of NS domains, usually associated with a large potential to interact with proteins, seems to be limited (7). Instead, proteins may interact with composite sequences of high and low sulfated domains. Well suited for such a type of binding are proteins with several potential HS-binding sites in form of clusters of positive charges in oligomeric or multidomain proteins like lipoprotein lipase (LPL).

LPL is a crucial enzyme for hydrolysis and uptake of lipids contained in plasma lipoproteins (8). The enzyme is synthesized by parenchymal cells, mostly in adipose tissue, skeletal muscle, and heart, and is then transported through the endothelial monolayer to the luminal side of the vessel wall where the enzyme is retained on cell surfaces by interaction with HS proteoglycans. During transport, LPL is probably also associated with HS chains (9). Besides its potential role as carrier for LPL, HS has been suggested to act as a chaperone keeping the enzyme active during the passage from the site of synthesis to the site of action (9). Studies with cells deficient in HS showed, however, that HS is not required for the intracellular processing and secretion of LPL (10). Secretion of LPL to the culture medium from HS-deficient cells was even higher than from wild type cells, which is in line with that HS may also have a role as co-receptor in internalization and catabolism of LPL (11–14).

Based on homology, LPL has been modeled on the crystal structure of the related pancreatic lipase (15). This family of triglyceride lipases contain two domains with the catalytic site...
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located in a pocket of the N-terminal domain. In LPL, four clusters of basic amino acids are exposed on the opposite side of the molecule compared with the entrance to the catalytic site, and at least two, located in the N-terminal domain, seem to be important for HS binding (14–16, 17). Participation of positive regions also in the C-terminal domain of LPL have been demonstrated (17–19). Heparin fragments of 8–10 monomer units have been shown to bind to LPL and to displace the enzyme from endothelial sites, from synthetic heparin-covered surfaces and from surfaces covered by lipoprotein receptors (20–22).

Affinity purification of endothelial HS fragments with high affinity for LPL has revealed a 10-mer fragment with the unusual sequence of five di-O-sulfated and N-sulfated disaccharides (23). This type of sequence is common in heparin but is very rare in HS from tissues (4, 5), and we have attempted to identify common HS fragments with binding properties for LPL rather than the very rare, fully sulfated sequences that resemble heparin.

**EXPERIMENTAL PROCEDURES**

**Materials**—LPL was isolated from bovine milk and purified as described previously (24). HS from swine intestine was a gift from Dr. G. van Dedem, Diosynth, Oss, The Netherlands. HS from bovine aorta and kidney were received from Seikagaku and characterized as described (4). Heparin from bovine lung (The Upjohn Co.) was purified as described (25) and capsular polysaccharide from Escherichia coli K5 as described (26). Iduronate 2-sulfatase and iduronidase were purchased from Glyko (Upper Heyford, UK). Bio-Gel P-10, fine, was from Bio-Rad.

**Radiolabeling of Glycosaminoglycans**—Heparin and HS from bovine aorta and kidney were deacetylated by hydrazinolysis for 30 min at 96 °C in 1 ml of 3% (v/v) ethanolamine in 0.1M NaHCO₃, pH 9.0, 0.5M NaCl, for 4 h. After wash, the gel was stored in 10 mM BisTris, pH 6.5, 0.1 M NaCl, containing 1 mg of heparin/ml to protect the enzyme from inactivation. Previous studies have shown that, under the conditions used, the gel contains ~1 mg of LPL protein/ml, and the binding properties remain essentially unchanged for 2–3 weeks on storage at 4 °C (30, 31). The LPL-Sepharose beads were therefore used within 2 weeks after manufacturing, and all column runs were performed at 4 °C. Before application of the sample of heparin or heparin fragments, the LPL-Sepharose was washed with buffer (20 mM Tris, pH 7.4) containing 2 M NaCl to elute the protecting heparin before application of 5 volumes of equilibration buffer (20 mM Tris, pH 7.4, 0.15 M NaCl). Radiolabeled GAG or size-defined fragments were applied to LPL-Sepharose (1-ml column) in 0.5 ml of equilibration buffer. The sample was allowed to enter the gel, and the column remained with stopped flow for 20–30 min before elution was initiated. Four to five column volumes each of equilibration buffer and equilibration buffer containing increasing salt concentrations were applied, and the eluates were collected as 1-ml fractions and analyzed for radioactivity. Fractions with affinity-selected radiolabeled fragments were pooled and desalted on a PD-10 column in 0.2 M NH₄HCO₃ before further characterization. Affinity-selected pools were tested for rebinding capacity on the LPL column before and after chemical cleavage by adjusting the sample to the salt concentration of the equilibration buffer and then performing affinity chromatography as for the preparative separation.

**Competition Assay**—Competition of LPL binding to HS was performed by surface plasmon resonance on a Biacore 2000 instrument (Biacore, Uppsala, Sweden). Streptavidin was covalently immobilized to the dextran matrix of CM5 sensor-
chips using the procedure recommended by the manufacturer. Biotinylated HSPG (from cultured human umbilical endothelial cells (32), a kind gift from Dr L.-Å. Fransson, Lund University, Sweden), was bound to the immobilized streptavidin. The biotinylated HSPG increased the base-line level by 200–230 resonance units. Interaction of LPL (17 nM) with immobilized HSPG was monitored at a constant flow of 5 μl/min. For competition studies heparin, E. coli K5 polysaccharide, HS from swine intestine, or SAS fragments were mixed with LPL just before injection at the indicated concentrations. Experiments were done at 4 °C in 20 mM Hepes, pH 7.4, 0.15 M NaCl, 2 mg/ml bovine serum albumin, and 0.05% Triton X-100. After each injection the sensorchips were regenerated by injection of 10 ml of 1 M guanidinium chloride.

Characterization of Affinity-isolated SAS Fragments—For identification of the position of N-acetyl groups within the end-labeled, affinity-purified fragments, aliquots were deacetylated completely with hydrazine-hydrazine sulfate for 4 h at 96 °C as described above, desalted on a PD-10 column in 0.2 M NH₄HCO₃, and cleaved by deamination at pH 3.9. Alternatively, affinity-purified fragments were cleaved by deamination at pH 1.5 (33). The deamination products were analyzed for size by fractionation on a Superdex 30 column in 50 mM Tris, pH 8, 1 M NaCl and tested for affinity on the LPL column as described above.

Sequencing of Affinity Isolated NS Fragments—Partial sequences of NS domains were determined essentially as described (6, 34). Affinity-purified 8-mer N-sulfated [1-3H]-α-Man₉ end group-labeled fragments were fractionated by anion-exchange chromatography on a Propac column with a gradient of [NaCl] from 0 to 1.5 M at pH 3.0 with an increment of 10 mM/min. Individual charge homogeneous peaks were pooled and desalted on PD-10 columns before re-analysis on the anion-exchange column and partial deamination at pH 1.5. The deamination products were sequentially treated with iduronate-2-sulfatase and iduronidase, and the intermediate products of the sequencing reactions were separated on the anion-exchange column and their elution profiles compared with profiles of characterized structures (6, 34).

Analysis of GAGs—GAGs were quantified by colorimetric determination of hexuronic acid using the meta-hydroxy-diphenyl method (35) with glucuronic acid (GlcA) as a standard. A factor of 3 was arbitrarily employed to convert values to saccharide mass. The specific activity of different samples was determined colorimetrically and by scintillation counting an aliquot. Specific activities are expressed per mole of size-defined oligosaccharides or per mass of size heterogeneous poly- and oligosaccharides. As molar mass an average of 600 was assumed for an HS/heparin-disaccharide. Compositional analysis and quantification of samples used for competition assays were performed by complete digestion with heparin lyases and characterization by reversed phase ion pairing chromatography as described (5).

RESULTS

Binding of Heparin and HS Chains to LPL—Radiolabeled, 3H-acetylated heparin and HS chains from different origin (aorta, kidney, and intestine) bound to LPL immobilized on sepharose beads. The affinity of the different chains for LPL was estimated from the ionic strength needed for elution, reflecting the relative strength of the interaction of the different GAGs with the protein. While heparin chains bound to the immobilized enzyme and eluted at 0.5 M NaCl, HS chains eluted between 0.3 and 0.5 M NaCl (data not shown). Intestinal HS was preparatively isolated on the affinity column. Approximately one-third of the retained material eluted at 0.5 M NaCl, whereas two-thirds eluted at 0.3 M NaCl (Fig. 1A). After reapplication of the non-retained pool another ~45% was bound, with a majority of the reapplled chains (~40% of loaded material) eluted at 0.3 M NaCl, indicating a depletion of chains with higher affinity for LPL after the first separation (Fig. 1A).

Binding of Size-defined Heparin and HS Oligosaccharides to LPL—Size-fractionated [3H]heparin oligosaccharides in the size of 6–12-mer, prepared by partial deaminative cleavage, bound to the LPL column. The affinity for LPL was dependent on the size of the fragments, with a higher proportion of the heparin 12-mer oligosaccharide binding strongly (elution at 0.5

FIGURE 1. Affinity chromatography of HS chains and oligosaccharides on LPL-Sepharose. A, N-acetylated pig intestine HS (10,000 cpm) was loaded and allowed to equilibrate with the column before stepwise elution with a salt gradient (—in) as described under “Experimental Procedures”. Fractions were collected and the radioactivity determined by scintillation counting. The non-bound material (eluted with 0.15 M NaCl) was pooled and rechromatographed in the same way ( ). B, similar molar amounts of [3H]-labeled size-defined heparin 4-mer ( ), 6-mer ( ), 8-mer ( ), 10-mer ( ), and 12-mer ( ) oligosaccharide were affinity-chromatographed as described for A.
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m NaCl) as compared with smaller fragments (Fig. 1B). Oligosaccharide 8- and 10-mers eluted both at 0.5 and 0.3 m NaCl, whereas 6-mers eluted at 0.3 m. Heparin and heparin oligosaccharides are highly sulfated, and N-sulfated domains in HS are much less and more heterogeneously sulfated. Therefore, size-defined HS N-sulfate domain oligosaccharides were prepared by deacetylation of native intestinal HS and consecutive deamination at pH 3.9 to isolate the N-sulfated domains (NS). Such N-sulfated oligosaccharides of 8–10-mer size eluted at 0.3 m NaCl (data not shown). This finding suggested that the HS-NS are weaker binders, most likely due to lower sulfation than found in heparin oligosaccharides prepared from highly sulfated bovine lung heparin, which have a homogeneous O-sulfation pattern throughout the fragment (36).

**Binding of SAS Domains to LPL**—LPL contains several clusters of basic amino acids, which may cooperate to form an extended HS-binding site (15, 16, 37). Because single long NS domains are rare in HS chains isolated from tissues (7), we made an attempt to purify fragments that contain sulfation at the ends. Such SAS domains were produced by limited deamination at pH 1.5 to cleave HS chains at a few positions within N-sulfated sequences thereby resulting in oligosaccharides that carry O-sulfation also in the terminal disaccharides. The LPL-retained fraction of such fragments was eluted at 0.3 m NaCl and therefore had a similar binding affinity as purely N-sulfated HS oligosaccharides of the length 8–10-mer (Fig. 1B). A size-defined population of SAS fragments of ~8–16-mer was affinity-purified on the LPL column (Fig. 2A). Non-retained material, eluted with the equilibration buffer at 0.15 m NaCl, and retained material, eluted with 0.3 m NaCl, were recovered and pooled separately for further analyses. Upon reapplication to the LPL column, the non-retained fragments did not bind (Fig. 2B), indicating that this fraction was depleted of binding structures. The originally bound fragments, on the other hand, were essentially quantitatively retained on the column on reapplication and eluted at ≥0.3 m NaCl (Fig. 2C). As the population of fragments in the original SAS pool covered a broad size interval we analyzed the LPL-bound versus non-bound fragments for their apparent lengths. The non-bound and bound fragments showed an overlapping size distribution, excluding the possibility that size alone was the determining factor for binding to LPL (Fig. 2D), although the non-bound tended to be smaller than the bound fragments. The non-bound oligosaccharides represented a heterogeneous pool of approximate size ≤12-mers, whereas the LPL-retained fraction contained fragments in the size of ~8–18-mers. To identify other differences, the smallest size fragments (~8–10-mer) with capacity to bind LPL and the corresponding pool of non-binding fragments in the same size class were isolated for further analysis (fractions indicated with bar in Fig. 2D).

**Localization of N-Acetylated Glucosamines in SAS Fragments with High or Low Affinity for LPL**—N-Sulfated fragments have been postulated to be important sites for protein binding in general (2) and for LPL binding in particular (23). Yet, with the SAS domains we had isolated oligosaccharides with affinity for LPL that also contain N-acetylated residues. We therefore intended to identify where in the sequence these N-acetylated residues are tolerated. To pinpoint the location of the N-acetylated glucosamines within the smallest LPL-retained and non-retained oligosaccharides, we applied a scheme of selective deaminative chemical cleavages to both fractions. The cleavage

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**FIGURE 2. Size distribution and affinity properties of LPL affinity-selected SAS domains.** A, a pool of 3H-labeled SAS fragments (~8–16-mers) was affinity-purified on LPL-Sepharose. The fractions of bound (elution with 0.3 m NaCl; salt gradient indicated by —) and non-bound fragments (elution with 0.15 m NaCl) were pooled, respectively. B and C, non-bound (B) and bound (C) fragments were re-applied separately to the LPL column and eluted as before. D, the pools of non-bound (C) and bound (D) fragments were analyzed for size distribution by chromatography on Superdex 30. The smallest fragments of overlapping size were pooled for characterization (fractions marked with bar). The elution positions of standard, size-defined 3H-labeled oligosaccharides derived from heparin and from E. coli K5 polysaccharides prepared as described (44) are indicated by arrows and arrowheads, respectively. Heparin fragments are highly sulfated, whereas the bacterial saccharides are non-sulfated.
schemes produced unique fragments of each pool of oligosaccharides. To locate the first $N$-acetylated glucosamine residue relative to the $^3$H-labeled reducing end, the oligosaccharides were $N$-deacetylated followed by deaminative cleavage at pH 4. This cleavage procedure is selective for the glucosaminic linkage next to the newly created free amino group and will therefore cleave the oligosaccharide at the glycosidic bond neighboring the deacetylated glucosamine residue and consequently split the oligosaccharide into the $^3$H-labeled reducing end and the non-labeled non-reducing end. Upon separation only the labeled fragment is detected. As complementation, the position of the first $N$-sulfated glucosamine relative to the $^3$H-labeled end was identified by deamination at pH 1.5, which is selective for $N$-sulfated glucosaminidic linkages.

When the non-retained SAS fragments were cleaved by deamination at pH 1.5, a majority of the oligosaccharides (>50%) was cleaved into end-labeled tetrasaccharides or larger fragments (Fig. 3A). This indicated that the nearest $N$-sulfated glucosamine from the reducing end was in position five or even more distal, whereas the glucosamines closer to the reducing end in these fragments were $N$-acetylated. Some of the oligosaccharides (~20%) were fully $N$-acetylated oligosaccharide fragments and eluted unaffected by deamination at pH 1.5 in the original elution volume. The complementary cleavage at acetylated groups resulted mainly in disaccharides (Fig. 3C). This indicated that the nearest $N$-sulfated glucosamine residue in position 3 from the reducing end. The remainder of the cleavage products eluted as tetrasaccharides (Fig. 3C).

To test the remaining LPL binding capacity of the deaminated fragments, the originally LPL-bound oligosaccharides were reapplied onto the LPL-Sepharose column after chemical cleavage, as described under "Experimental Procedures" (Fig. 3E). The elution positions of standard oligosaccharides are as indicated in the legend to Fig. 2.

Partial Sequence Analysis of LPL-bound N-Sulfated Fragments—To determine the degree and distribution of $O$-sulfation in LPL-binding NS domains, $N$-sulfated oligosaccharides were isolated by LPL-affinity chromatography and sequenced by a recently developed sequencing technique (6, 38). To this end, a size-defined pool of $^3$H-end-labeled 8-mer NS oligosaccharides was isolated from bovine intestine HS. This pool of NS domains contained a broad range of oligosaccharides with variable degree of $O$-sulfation as analyzed by ion-exchange chromatography (data not shown). The fraction of these NS domains retained by LPL (eluted from LPL by 0.3 M NaCl) was characterized by separation on the anion-exchange column (Fig. 4A). The oligosaccharides eluted in a broad range at ~70–130 min corresponding to 8-mer oligosaccharides with at least three $O$-sulfate groups (Fig. 4A) as compared with...
mannitol-containing oligosaccharides were mainly of 2-, 4-, 3H-end-labeled 8-mer oligosaccharides. These [3H]anhydro-in peak P1 eluted at LPL. A pool of LPL affinity-purified HS-derived NS octasaccharides (eluted Lipoprotein Lipase-Heparan Sulfate Interaction first step in the sequencing strategy with exoenzymes (Fig. 4 chromatography and eluted with a salt gradient (H11011/H11011)-sulfate groups (Fig. 4 species affected by iduronate-2-sulfatase (H18528/H18528/H18528/H18528) as described under "Experimental Procedures."Species affected by iduronate-2-sulfatase (H18528/H18528/H18528/H18528) and iduronidase (H18528) and were filled circles representing non-assigned positions of C–E in inset B. Two disaccharides, 2a and 2b, eluted unaffected by the enzyme confirming the absence of a 2-O-sulfate group as already indicated by their elution behavior compared with known standards. Both 4-mers, on the other hand, shifted position to the extent as expected by the loss of one sulfate group. After 2-O-sulfatase treatment peak 4a did not contain any O-sulfate group and eluted at 13 min, while peak 4b still contained one O-sulfate group. In the following step an aliquot of the iduronate-2-O-sulfatase-treated samples was digested with iduronidase and separated on the anion-exchange column (Fig. 4E). Fragments susceptible for iduronidase digestion moved in the chromatogram due to the loss of one sugar unit from the fragment (peak identity marked by “-I”). This was manifest for the disaccharide species 2a, which was partially susceptible to iduronidase digestion creating a non-charged monosaccharide eluting at 2 min (denoted 2a-I). The two sulfated structures 4a-2S and 2b shifted position as consequence of the iduronidase treatment and co-eluted at 10 min (denoted 4a-I2S and 2b-I). The tetramer species 4b-2S was also affected by the iduronidase eluting as 4b-I2S at 25 min (Fig. 4E). Similarly as for the 4-mers also the 6-mers were susceptible for the iduronate-2-O-sulfatase treatment (6a-2S (elution at 35 min) and 6b-2S (at 52 min), shifted by ~15 min compared with the parent species 6a and 6b) (Fig. 4D) and thereafter for iduronidase (6a-I2S and 6b-I2S, shifted by 4 and 1 min compared with 6a-2S and 6b-2S, respectively) (Fig. 4E). Combined, the readout of the sequence analyses of these fragments suggested the presence of at least two different species of 8-mers with the indicated distribution of four O-sulfates (see inset in Fig. 4B). Important to note is that the average content of O-sulfate was only one per disaccharide unit in these 8-mers in contrast to the earlier suggested requirement of two O-sulfated groups in consecutive disaccharide units for binding of LPL (23).

FIGURE 4. Partial sequence analysis of NS domains capable of binding to LPL. A pool of LPL affinity-purified HS-derived NS octasaccharides (eluted with 0.3 m NaCl from the LPL column) (A) was separated by anion-exchange chromatography and eluted with a salt gradient (—) as described under “Experimental Procedures” (identical for all separations shown in Fig. 4, indicated only in A). Fractions were collected and the radioactivity of an aliquot determined by scintillation counting. Individual fractions from the affinity-retained material were pooled as indicated (P1, P2, and P3) and reanalyzed by anion-exchange chromatography (shown for P1 in B). The peak material corresponding to the least sulfated species (P1) was sequenced after partial deamination (C) by digestion with iduronate-2-O-sulfatase (D) and iduronate-2-O-sulfatase followed by iduronidase (E) as described under “Experimental Procedures.”Species affected by iduronate-2-O-sulfatase (–2S) and iduronidase (–I) are marked, respectively. The proposed sequences for fragments 8a and 8b, deduced from the analyses in C–E, are shown as an inset in B where open circles represent non-assigned positions of O-sulfates, and filled circles represent assigned positions of O-sulfates. Symbols above the line represent O-sulfated glucosamine residues, and symbols below the line represent O-sulfated glucosamine residues and symbols below the line represent O-sulfated glucosamine residues. The labeled end of the oligosaccharide is located to the right.

sequenced species (6, 34). The least sulfated, dominant species in peak P1 eluted at ~85–90 min corresponding to NS oligosaccharides with four O-sulfate groups (Fig. 4A, P1) and were retrieved for sequencing. The re-analysis of the isolated pool P1 by anion-exchange chromatography (Fig. 4B) indicated the presence of at least two main components, indicated as 8a and 8b in the chromatogram. This pool was partially deaminated as first step in the sequencing strategy with exoenzymes (Fig. 4C), thereby creating even-numbered fragments of the original 3H-end-labeled 8-mer oligosaccharides. These [3H]anhydromannitol-containing oligosaccharides were mainly of 2-, 4-, and 6-mer size, besides some remaining original 8-mers, all of which contained the original [3H]anhydromannitol in their NaB1H4-reduced ends (nota bene the complementary fragments of these 8-mer cleavage products are non-labeled and therefore not detected after fragmentation). Besides two minor components eluting at the position of the uncleaved starting material, the deamination resulted in five major fragments (Fig. 4C). Two disaccharides (2a, 2b) reflecting the non-O-sulfated and 6-O-sulfated variant of disaccharide IdoA/GlcA-aManR were identified based on their retention on the anion-exchange column. Similarly, two tetramers could clearly be distinguished with the structure IdoA/GlcA-2-SO3-GlcNS-IdoA/GlcA-aManR and IdoA/GlcA-2-SO3-GlcNS-IdoA/GlcA-aManR-6-SO3, respectively. Only one of the expected 6-mers appeared as a distinct peak (6a), whereas several minor fragments eluted at positions of three O-sulfated 6-mers, which reflects the heterogeneity in sequence of the starting material. Next an aliquot of the deamination products was treated by iduronate-2-sulfatase, which removes the 2-O-sulfate group on the iduronic acid residue positioned at the non-reducing end and the mixture was applied on the ion-exchange column (Fig. 4D). A loss of a sulfate group shifted the elution position of an oligosaccharide considerably (15–20 min in the applied gradient), while the loss of an uronic acid residue had much less impact on the elution behavior of the remaining fragment due to the protonated form of the carboxyl groups at the pH used. All fragments that originally contained a 2-O-sulfated uronic acid residue in the non-reducing end shifted position (indicated by “-2S” appended to the original peak identification) as compared with the deaminated sample in Fig. 4C. The two disaccharides, 2a and 2b, eluted unaffected by the enzyme confirming the absence of a 2-O-sulfate group as already indicated by their elution behavior compared with known standards. Both 4-mers, on the other hand, shifted position to the extent as expected by the loss of one sulfate group. After 2-O-sulfatase treatment peak 4a did not contain any O-sulfate group and eluted at 13 min, while peak 4b still contained one O-sulfate group. In the following step an aliquot of the iduronate-2-O-sulfatase-treated samples was digested with iduronidase and separated on the anion-exchange column (Fig. 4E). Fragments susceptible for iduronidase digestion moved in the chromatogram due to the loss of one sugar unit from the fragment (peak identity marked by “-I”).
Competition of LPL Binding to Endothelial HS—To test the effect of GAGs on LPL binding to cellular HSPG, different poly- and oligosaccharides were used in a competition assay applying plasmon resonance (Fig. 5). Heparin and HS chains competed well against LPL binding to umbilical cord endothelial cell HSPG, whereas non-sulfated E. coli K5 cell wall polysaccharide was ineffective. The size-defined (~12–16-mer) SAS oligosaccharides were charge-fractionated into increasingly sulfated pools I–IV and characterized for content of sulfation (Fig. 5A). The least sulfated fraction contained very few sulfate groups and had no effect on LPL binding. All sulfated fractions II–IV, on the other hand, were able to compete in the assay, indicating that not only heparin and heparin-like highly sulfated oligosaccharides but also SAS oligosaccharides with an average sulfation degree not larger than 1.5 sulfate groups per disaccharide, and HS chains are effective competitors for LPL binding to cellular HSPGs (Fig. 5B).

**DISCUSSION**

HS proteoglycans are important for the metabolism of blood lipids. LPL and other members of the same gene family (hepatic lipase and possibly endothelial) are bound to endothelial surfaces through interaction with HS proteoglycans (for review, see Refs. 8 and 39). In addition several of the apolipoproteins (apoB, apoE, and apoAV) have affinity for HS (39, 40). Interaction of the lipoprotein particle with endothelial HS causes retention of the lipoproteins in the neighborhood of the lipases and presentation to LDL receptors for internalization (8, 41). Heparin has been widely used to release LPL from the vascular endothelium into the circulating blood (8), and heparin oligosaccharides have been used to study the HS binding properties of LPL (20–22). Earlier studies, aimed to retrieve high affinity ligands for LPL isolated from endothelial HS, indicated that some very rare, highly sulfated heparin-like oligosaccharides interacted with high affinity with LPL (23) (Fig. 6A). Tissue HS is generally of much lower degree of sulfation than heparin (1, 2, 4), and LPL binding is therefore more likely dependent on features more common in HS. Our aim was therefore to analyze HS oligosaccharides for LPL binding.

Two approaches were taken: on the one hand, partial deaminative cleavage was used to cleave the long HS chains within their N-sulfated domains to create a mixture of fragments of...
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SAS-type (also containing some other combinations). On the other hand, purely N-sulfated oligosaccharides were retrieved by complete deacetylation followed by deamination. Two-thirds of the LPL-retained SAS domains in size ~8–10-mer turned out to be purely N-sulfated oligosaccharides, while the remaining third of the material probably contained a single N-acetylated glucosamine in position 3 from the reducing end (Fig. 6B). This fraction of the LPL-bound oligosaccharides was degraded into disaccharides by deacetylation/deamination pH 4 and into tetramers by deamination at pH 1.5. Considering the proportional amount of deacetylation/deamination pH 4-resistant (Fig. 3D) and deamination pH 1.5-sensitive oligosaccharides (Fig. 3C), there are only a few percent of fragments to be expected with N-acetylation in a different position than number 3 from the reducing end. The majority of the fully N-sulfated oligosaccharides with an O-sulfation degree of four per octasaccharide did bind to the enzyme with similar affinity (Fig. 4B). The heterogeneity of the isolated peaks P1–P3 indicates that, even though the number of sulfates is given, the exact sequence is of minor importance and many combinations can mediate binding to LPL. The partial sequence of P1 (Fig. 4B–E) indicates a mixture of at least two or three different sequences all containing four O-sulfate groups, i.e. an average of one/disaccharide (Fig. 6C). Consequently findings with either SAS domains or N-sulfated oligosaccharides indicated that the enzyme may bind to oligosaccharides with much less sulfation than the average heparin fragment of nature [IdoA2S-GlcNS6S]n identified as a rare, but strong, binding sequence from endothelial HS (23). SAS domains were also effective in competition of LPL for HSGP binding (Fig. 5B). Taken together these findings suggest that LPL binds to modestly sulfated sequences and can make use of general features of the HS chains with N-sulfated domains interrupted by low sulfated N-acetylated disaccharides.

Many studies with HS-binding proteins are performed with heparin, an HS-related GAG found intracellularly in connective tissue-type mast cells. HS chains, decorating essentially all cell surfaces, are much less sulfated than heparin (Fig. 5A). Furthermore, their sulfation is more uneven, resulting in patches with denser sulfation alternating with sequences with sparse sulfation. The large area of clusters of numerous basic residues in LPL would therefore be well suited to adapt to the great variability in the sulfation pattern of HS, providing numerous, but heterogeneous, binding sites able to retain the enzyme on the vascular wall. HS isolated from aorta, kidney, and intestine bound with similar affinity to LPL, although they are characterized by a different sulfation degree (4). When isolating purely N-sulfated fragments, the patch structure of HS is lost, and the majority of N-sulfated fragments are potentially too short, as longer N-sulfated fragments needed for binding to LPL are rare in HS (7). The low abundance of long, N-sulfated domains cannot explain the much larger fraction of HS chains that can bind to the enzyme. Indeed, an attempt to isolate high affinity HS oligosaccharides from endothelial cells resulted in a very small proportion of low affinity octasaccharides and high affinity decasaccharides with the heparin like composition of repetitive 2- and 6-O-sulfated disaccharides of the type [IdoA2S-GlcNS6S] (23).

Models of the LPL dimer in complex with heparin have been proposed (15, 19). Yet the exact combination of the dimer with its oligosaccharide ligand awaits further refinement. Independent of the model, the molecular dimensions of a highly sulfated heparin 10-mer oligosaccharide and HS chains would allow those ligands to get in contact with both subunits in the dimer. Neither shorter, highly sulfated HS-8-mers (23) nor the average HS-10-mer oligosaccharide (data not shown) seem to bind with an affinity as high as heparin or full-length HS chains. The fragments are eluted with 0.3 M NaCl, while some intact HS chains required >0.5 M NaCl for elution. This suggests that the shorter oligosaccharides are only sufficient to cover the heparin-binding sites on one of the LPL subunits. The combination of shorter sequences with relatively high sulfation alternating with sequences of low sulfation in an HS chain might guarantee possibilities for simultaneous interaction with several sites in both protein subunits in the LPL dimer and thereby result in high binding affinities. Similar properties for HS binding have been described for other extracellular effector molecules such as the multimeric cytokines interferon γ (42), platelet factor 4 (43), interleukin-8 (44), but also for the monomeric protein endostatin (7). For stabilization of LPL activity, shorter fragments like hexa- and octasaccharides are similarly effective as heparin, indicating that the trans-subunit binding is not necessary for stabilization but rather that fragments can bind to each of the subunits in the LPL dimer and thereby stabilize the active conformation (22).

The inactive LPL monomer has a much lower affinity for heparin and HS than the active, dimeric form (22). Inactive LPL is therefore the dominating form in the circulating blood, where it is attached to the lipoproteins and is thought to assist in directing the lipoproteins to receptor-mediated endocytosis in the liver (45). The efficient retrieval of LPL by the liver may be explained by the higher degree of sulfation in liver HS (5, 46). In accord with this, LPL is more easily released from extrahepatic tissues than from liver by the use of heparin oligosaccharides (47). In respect to atherosclerosis it is interesting to note that aortic HS has been shown to increase in sulfation and number of triple-sulfated disaccharide species which would favor strong binding to LPL (48). Several studies in animal models indicate that expression of LPL by resident macrophages in the artery wall aggravate the development of atherosclerotic plaques (49).

Acknowledgments—We thank Gunilla Pettersson for skillful technical assistance, Elin Thörnblom for initial experiments, and Ulf Lindahl for critical interest and stimulating discussion.

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