Defining the Interleukin-8-binding Domain of Heparan Sulfate*

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Interleukin-8, a member of the CXC chemokine family, has been shown to bind to glycosaminoglycans. It has been suggested that heparan sulfate on cell surfaces could provide specific ligand sites on endothelial cells to retain the highly diffusible inflammatory chemokine for presentation to leukocytes. By using selectively modified heparin and heparan sulfate fragments in a nitrocellulose filter trapping system, we have analyzed sequence requirements for interleukin-8 binding to heparin/heparan sulfate. We demonstrate that the affinity of a monomeric interleukin-8 molecule for heparin/heparan sulfate is too weak to allow binding at physiologically ionic strength, whereas the dimeric form of the protein mediates binding to two sulfated domains of heparan sulfate. These domains, each an N-sulfated block of 6 monosaccharide units, are contained within an 22–24-mer sequence and are separated by a region of ≤14 monosaccharide residues that may be fully N-acetylated. Binding to interleukin-8 correlates with the occurrence of the di-O-sulfated disaccharide unit -IdoA(2-OSO3)-GlcNSO3(6-OSO3)-. We suggest that the heparan sulfate sequence binds in horseshoe fashion over two antiparallel-oriented helical regions on the dimeric protein.

Glycosaminoglycans (GAGs), i.e. the linear, sulfate-substituted carbohydrate constituents of proteoglycans, are ubiquitous components of cell surfaces. They are generally believed to exert their biological functions by interacting with proteins. Heparin and heparan sulfate (HS) are sulfated GAGs of alternating hexuronic acid and glucosamine residues that have been shown to bind a variety of enzymes, cytokines/growth factors, and extracellular matrix molecules (for review, see Ref. 1). Such interactions may be highly specific, as illustrated by heparin/HS and antithrombin. A defined pentasaccharide sequence in the GAG chain binds antithrombin and thus strongly promotes its function as an inhibitor of the serine proteases acting within the coagulation cascade (2). Other proteins, such as histones, bind GAGs due to their overall basic character, without any apparent need for a specific saccharide sequence. Several attempts have been made to identify minimal consensus sequences in proteins required to bind heparin or HS (3, 4). Clusters of basic amino acid residues may provide binding sites that are either located within a short linear peptide stretch or composed of residues that occur topologically close together, but on different peptide loops (for review, see Ref. 5).

Sulfate groups on the GAG chains have been identified as important determinants of protein-binding sites. They are introduced during the process of polymer modification, through which the initial (GlcUAβ1,4-GlcNAc1,4)n polysaccharide chain is transformed into the final product (6). While each reaction, catalyzed by a distinct enzyme, generates the substrate for subsequent reactions, these are generally incomplete, which leads to progressive structural heterogeneity. Due to modulated activity of the GlcNAc N-deacetylase/N-sulfotransferase, i.e. the enzyme that initiates polymer modification in HS and heparin biosynthesis, variable proportions of GlcNAc units escape N-deacetylation/N-sulfation. These residual N-acetylated units are few and isolated in heparin and are more abundant and typically arranged in consecutive sequence in HS. The N-sulfate groups are prerequisite to substrate recognition by the GlcUA 5-epimerase and the O-sulfotransferases that catalyze the subsequent modification reactions. Consequently, heparin shows a high proportion of IdeA-containing, O-sulfated disaccharide units and a relatively homogeneous overall sulfation pattern, whereas HS is composed of alternating sulfated and nonsulfated sequences of variable length (7, 8). A typical HS chain will thus contain essentially unmodified regions of up to 8–10 consecutive GlcUA-GlcNAc disaccharide units. Clearly, the occurrence of such regions will profoundly influence the mode of interaction with proteins.

Multidomain interaction between HS and proteins has so far received little attention. Such interaction would apply in particular to oligomeric proteins composed of monomers carrying single “heparin-binding sites.” To study such a system, we have chosen the heparin-binding cytokine interleukin-8 (IL-8). IL-8 is a proinflammatory cytokine of the CXC chemokine family that forms noncovalently linked dimers with a characteristic appearance (9). Crystallographic and NMR studies have provided insights into the shape of the molecule, which consists of a flat array of a β-pleated sheet with the α-helices of the two monomers arranged as antiparallel rods on top of this sheet (10–12). The heparin-binding sites have been tentatively localized to the exposed positively charged residues on the top of these helices (13, 14).

The results of this study define constraints critical to the interaction of a HS chain with the IL-8 dimer. A minimal sequence of 18–20 monosaccharide units is required to span the two saccharide-binding sites on the dimer. The corresponding peptide-binding regions may be separated by a nonsulfated intervening stretch composed of up to 7 GlcUA-GlcNAc disaccharide units.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IL-8 was expressed in Escherichia coli and purified as described previously (15), except that a final step of affinity chromatography on heparin-agarose was added. Twenty mg of lyophilized IL-8 were dissolved in 10 ml of phosphate-buffered saline.

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† The abbreviations used are: GAGs, glycosaminoglycans; HS, heparan sulfate; IL-8, interleukin-8; aMan, 2,5-anhydromannitol.
and passed over a 2-ml column of heparin-agarose (Sigma, type I) equilibrated in the same buffer. The column was developed with a linear salt gradient ranging from 0.15 to 2 M NaCl in 20 mM phosphate buffer, pH 8.2. The protein eluted as a single major peak at around 0.6 M NaCl. The pooled fractions were dialyzed against four changes of 50 mM ammonium acetate and lyophilized. The size-defined fragments were further purified by gel chromatography on a Partisil-10 SAX column (4.6 × 250 mm) and Whatman No. 3MM filter paper were purchased from Whatman Ltd. (Maidstone, Kent, Great Britain). NaB₃H₄ (24–28 Ci/mmol) and [³H]acetic anhydride (500 mCi/mmol) were obtained from Amersham International (Buckinghamshire, United Kingdom). Hydrazide hydrate was purchased from Fluka (Buchs, Switzerland). All other reagents were of highest grade available.

**Radiolabeling of Glycosaminoglycans**—Heparin was N-³H-acetylated at free amino groups as described (18) to a specific activity of 20,000 dpm of ³H/µg of hexuronic acid (–0.2 × 10⁶ dpm/µmol of heparin). Bovine kidney HS (0.5 µg) was partially N-deacetylated by hydrazinolysis for 30 min at 96 °C in 1 ml of hydrazide hydrate (–30% water) and 1% hydrazine sulfate (19). The product was N-³H-acetylated with 2.5 mCi of [³H]acetic anhydride essentially as described (18) and extensively dialyzed against 1 M sodium acetate, water, and 0.25 mM HIO₄ to eliminate any hydrazides found (20) and finally against water. A specific activity of –0.4 × 10⁶ dpm/µg of uronic acid was achieved (corresponding to an average of –4.6 × 10⁶ dpm/µmol of HS based on an estimated molecular mass of 11.5 kDa for the HS chains labeled).

**Chemical Modification of Heparin**—Selective chemical desulfation of heparin was performed as described previously (21) (see Table I). A preferentially 6-O-desulfated sample obtained by treatment with dimethyl sulfoxide/methanol (9:1, v/v) for 2 h at 93 °C was used as starting material for chemical depolymerization as described below.

**Chemical Depolymerization of Polysaccharides**—Limited deamidation of native and modified heparin was performed as follows to create size-defined fragments. The cleaved chains at N-sulfate residues were separated by gel chromatography on a column (1 × 146 cm) of Bio-Gel P-10 in 0.5 M NH₄HCO₃. Preferentially 6-O-desulfated heparin was cleaved at some of the N'-unsubstituted amino groups of glucosamine residues that had been created by the preceding desulfation step. A 10-ng sample was dissolved in water, and the solution was adjusted to pH 4.0 with CH₃COOH and rendered 0.1 M in NaNO₂ (final volume, 1 ml). The sample was incubated on ice for 30 min. The reaction was terminated, and the sample was ³H-labeled as described above. All O-desulfated heparin fragments were re-N-sulfated as described (21) before final separation by gel chromatography on Bio-Gel P-10. The specific activities of the ³H-labeled modified heparin fragments ranged between 0.25 and 0.5 × 10⁶ dpm/µmol of oligosaccharide.

**Enzymatic Depolymerization of Polysaccharides**—Size-defined HS fragments were produced enzymatically. Ten µg of N-³H-acetylated bovine kidney HS (specific activity, 0.4 × 10⁶ dpm/µg of uronic acid, –4.6 × 10⁶ dpm/µmol of HS) were digested with 0.04 units of heparinase II in 200 µl of 5 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl, and 0.1 mg/ml bovine albumin at 37 °C. The digestion was stopped by heating at 97 °C for 20 min. The fractions were separated on a Superose 12 column (in 50 mM Tris, pH 7.5, 1 mM NaCl, and effluent fractions were analyzed by scintillation counting and pooled as indicated.

**RESULTS**

**Minimal Size of Heparin Oligosaccharide Binding to IL-8**—The smallest oligosaccharide fragment able to bind to IL-8 was identified by in-solution binding studies using radiolabeled size-defined heparin fragments. Under physiological buffer conditions, the smallest fragment with appreciable affinity for IL-8 was a 18-mer (Fig. 1A). The interaction between heparin and IL-8 was previously shown to be of relatively low affinity, with a Kᵟ of –6 × 10⁻⁸ M (26), and we therefore considered the possibility of a charge-dependent interaction involving a shorter saccharide sequence that had escaped detection due to the ionic conditions of the assay. Indeed, washing of the nitrocellulose filter at lower ionic strength (10 mM phosphate buffer, pH 7.0, 150 mM NaCl, and 0.1 mg/ml bovine albumin) was able to competitively displace labeled heparin for binding to IL-8 at physiological ionic strength. The smallest oligosaccharide that inhibited the binding of labeled heparin for 50% or more was a 6-mer (Fig. 1B).

**Sulfate Dependence of Heparin Binding to IL-8**—The importance of different sulfate groups in the binding of heparin-related saccharides to IL-8 was evaluated using a competition assay with variably modified heparin chains. Full-length radiolabeled heparin was allowed to interact with IL-8 in the presence of unlabeled native or selectively modified heparin (Fig. 2 and Table I). Native bovine lung heparin showed the highest inhibitory capacity, above that of any of the modified heparins. A drop in competition effectiveness by a factor of –10 was seen with two of the selectively monodesulfated species, i.e., the selectively N-desulfated and the O-2-desulfated preparations, whereas the preferentially O-6-desulfated preparation was...
Heparin (3H) labeled fragments were recovered together with the protein from the Samples were applied to nitrocellulose membranes under suction. Retained fragments were recovered together with the protein from the membrane and quantified by scintillation counting as described under “Experimental Procedures.” B, shown is the competition between [3H]heparin (~17 pmol) and unlabeled size-defined heparin oligosaccharides (100 μg) for binding to IL-8 (500 pmol). Incubation was done in 200 μl of phosphate buffer, pH 7.4, containing 0 (●) or 140 (○) mM NaCl for 1 h at room temperature. Samples were applied to nitrocellulose membranes under suction. Repeated experiments were performed as described in the legend to Fig. 1B at the indicated concentrations of modified heparin preparations. The competitors used were heparin (●), N-desulfated/N-acetylated heparin (○), 2-O-desulfated heparin (▲), preferentially 6-O-desulfated heparin (▼), N-/2-O-desulfated heparin (●), N-/6-O-desulfated heparin (▼), extensively 2-O-/6-O-desulfated heparin (●), and N-/2-O-/6-O-desulfated heparin (▼). See Table I for specification of heparin modifications.

![Graph A](image1)

**Fig. 1.** Binding of heparin fragments to IL-8. A, identical amounts (~17 pmol) of [3H]heparin from bovine lung were incubated with IL-8 (500 pmol) in 200 μl of 10 mM phosphate buffer, pH 7.4, containing 0 (●) or 140 (○) mM NaCl for 1 h at room temperature. Samples were applied to nitrocellulose membranes under suction. Re- tained fragments were recovered together with the protein from the membrane and quantified by scintillation counting as described under “Experimental Procedures.” B, shown is the competition between [3H]heparin (~17 pmol) and unlabeled size-defined heparin oligosaccharides (100 μg) for binding to IL-8 (500 pmol). Incubation was done in 200 μl of phosphate buffer, pH 7.4, containing 140 mM NaCl.

![Graph B](image2)

**Fig. 2.** Effect of variably modified heparins on the binding of [3H]heparin to IL-8. Incubations were performed as described in the legend to Fig. 1B at the indicated concentrations of modified heparin preparations. The competitors used were heparin (●), N-desulfated/N-acetylated heparin (○), 2-O-desulfated heparin (▲), preferentially 6-O-desulfated heparin (▼), N-/2-O-desulfated heparin (●), N-/6-O-desulfated heparin (▼), extensively 2-O-/6-O-desulfated heparin (●), and N-/2-O-/6-O-desulfated heparin (▼). See Table I for specification of heparin modifications.

slightly less efficient (it should be noted that chemical 6-O-desulfation is accompanied by partial 2-O-desulfation). The more completely 2,6-O-desulfated sample was a hundredfold less efficient than native heparin. The remaining preparations, i.e. the N-/2-O-, N-/6-O-, and completely N-/2-O-/6-O-desulfated samples, were all ineffective as competitors. These results indicate that N-, 2-O-, and 6-O-sulfate groups all contribute appreciably to IL-8 binding.

**Affinity Fractionation of Partially Desulfated Heparin Oligosaccharides—**To further define the role of O-sulfate groups, 3H-labeled 18-mers derived from selectively 0-desulfated heparins (see “Materials”) were affinity-fractionated, and the disaccharide composition of IL-8-bound fractions was compared with that of the corresponding unfractionated material. Application of a preferentially 6-O-desulfated 18-mer to this procedure resulted in retention of 6% of the material on the nitrocellulose filter, complexed with IL-8, whereas the 2-O-desulfated fraction yielded 15% of IL-8-bound material.

These bound fragments were dissociated from nitrocellulose-adsorbed protein by sodium chloride and desalted. In analytical assays, aliquots of the bound fractions were compared with the respective unfractionated starting material for binding to IL-8. Both preparations, independent of the type of modification, showed increased binding to IL-8 as compared with the unfractionated starting material. Under similar interaction conditions, 1.8 pmol of unmodified heparin, 0.8 pmol of 2-O-desulfated, and 0.3 pmol of 6-O-desulfated heparin were bound to IL-8. From the recovered, once IL-8-bound pools, 1.4 pmol of the selectively 2-O-desulfated and 0.9 pmol of the correspondingly preferentially 6-O-desulfated preparations were sequestered on the filter in a second binding experiment. The preselected 2-O-desulfated sample thus approached the binding ability of unmodified heparin.

A compositional analysis of the IL-8-bound fractions showed only marginal differences in the overall degree of sulfation and distribution of O-sulfate groups compared with the corresponding unfractionated materials (Fig. 3 and Table II). The bound material derived from a 2-O-desulfated 18-mer was thus abundantly 6-O-sulfated, but low in 2-O-sulfate groups, whereas that recovered from a 6-O-desulfated 18-mer showed the reverse proportions. However, both fractions showed significantly increased amounts of the minor 2,6-di-sulfated disaccharide representing -IdceA(2-OSO₃)-GlcNSO₃(6-OSO₃) structures in the intact polysaccharide; this increase was particularly conspicuous for the 6-O-desulfated material. The amounts of the di-O-sulfated disaccharide unit approached 1 mol/mol of either type of IL-8-bound 18-mer (i.e. 11% of the total disaccharide units), and it therefore seems reasonable to conclude that this structure is of importance to the interaction between IL-8 and heparin or HS. While most of the additional 2-O- and 6-O-sulfate groups, located on mono-O-sulfated disaccharide units, are mostly likely redundant for IL-8 binding, we cannot exclude that selected residues contribute to the interaction.

**Domain Structure of IL-8-binding Heparan Sulfate—**The interaction studies using native or modified heparin oligomers demonstrated that the IL-8 dimer may be spanned by exclusively N-sulfated, extended saccharide sequences. However, since the physiological GAG ligand for IL-8 at the cell surface is presumably HS rather than heparin, we proceeded to study HS-derived IL-8-binding oligosaccharides, with the explicit aim of assessing to what extent such species may contain N-acetylated domains. N-[3H]Acetylabeled kidney HS was first degraded by limited digestion with heparinase to cleave glucosaminidic linkages between GlcNSO₃(±6-OSO₃) and IdceA(2-OSO₃) units, thus generating HS oligomers with internal (labeled) N-acetylated sequences flanked on both sides by N-sulfated, presumably IL-8-binding regions (Fig. 4A). After size separation, a fraction corresponding to a heparin ~20–24-mer was selected for binding to IL-8 using the nitrocellulose trapping system. Following incubation with the protein, the bound (~10% of added [3H under conditions of low-salt wash) and unbound fractions were recovered and treated exhaustively with nitrous acid at pH 1.5. The resulting labeled, exclusively N-[3H]acetylated fragments were separated by gel chromatography (Fig. 5). The IL-8-unbound fraction yielded a spectrum of variously sized oligomers, with a predominant component larger than a heparin 12-mer or a K5 16-mer (see legend to Fig. 5). This component was largely lacking in the deamination products from the IL-8-bound fraction. Instead, these products showed a larger proportion of smaller labeled oligosaccharides, which ranged in size from heparin 4- to 12mers. This result indicates that an IL-8-binding region in a HS...
TABLE I
Preparation and composition of modified heparins

| Preparation          | Treatment of native heparin | Sulfate groups/ disaccharide unit | Distribution of sulfate groups (N- Sulfates, 2-O-Sulfates, 6-O-Sulfates) | Major disaccharide |
|----------------------|-----------------------------|----------------------------------|-------------------------------------------------------------------------|-------------------|
| Native               | None                        | None                             | 2.7 >95 80 88                                                           | 6S                |
| N-Desulfated/N-acetylated | Me₃SO/H₂O, 90 min, 50 °C   | N-Acetylation                     | 1.6 0 76 85                                                             | 2S NS             |
| 2-O-Desulfated       | Alkalai/lyophilization      | N-Sulfation                      | 1.9 >95 1 88                                                             | 2S NAc            |
| Preferentially 6-O-desulfated | Me₃SO/MeOH, 2 h, 93 °C     | N-Sulfation                      | 1.7 >95 61 10                                                            | NAc               |
| N/-2-O-Desulfated    | Alkalai/Me₃SO/MeOH, 90 min, 50 °C | N-Acetylation                  | 1.1 0 17 88                                                              | NS                |
| N/-6-O-Desulfated    | Me₃SO/MeOH, 2 h, 93 °C     | N-Acetylation                     | 0.7 0 61 10                                                              | NS                |
| Extensively 2-O/6-O-desulfated | Me₃SO/MeOH, 8 h, 93 °C | N-Sulfation                      | 1.3 >95 31 3                                                             | 2S NAc            |
| N/-2/O-6/O-Desulfated | Me₃SO/MeOH, 8 h, 93 °C     | N-Acetylation                     | 0.3 0 31 3                                                               | NS                |

a Bovine lung heparin was selectively modified as described under "Experimental Procedures."b Values are average number of sulfate groups/single disaccharide unit as calculated from compositional analysis.c This was calculated from compositional analysis. The proportions of disaccharide units carrying the indicated sulfate groups are expressed as percent of total disaccharide units.d [3H]Acetyl-labeled kidney HS was incubated with various amounts of enzyme in the presence or absence of IL-8 as described in the legend to Fig. 6. Gel chromatography of the digests obtained in the absence of cytokine indicated a fairly homogeneous distribution of oligosaccharides, with peak elution positions shifting corresponding to the amounts of enzyme added (Fig. 6B). Cleavage patterns in the presence of IL-8 appeared biphasic, such that a minor fraction was protected from extensive degradation while the majority of labeled material was degraded, in fact more extensively than the corresponding material in the absence of IL-8 (Fig. 6A). To characterize the two pools, unprotected and protected digests (Fig. 6C) were subjected to deaminative cleavage of the N-sulfated disaccharides, followed by separation of the HNO₂-resistant [3H]acetyl-labeled species on a sizing column. Whereas the size

The heparin-derived 18-mers were [3H]-end-labeled. For compositional analysis, further [3H] label was introduced through reduction of disaccharide deamination products with NaB₃H₄ (see "Experimental Procedures"). The compositional data obtained after separation of the disaccharides are expressed as percent of total disaccharide units (average of three independent determinations). The non-O-sulfated disaccharides to the sum of GlcUA-GlcNSO₃ and -IdceA-GlcNSO₃ sequences in the intact 18-mers. 2-O-desulfated disaccharides to the sum of GlcUA-GlcNSO₃(6-OSO₃) and -IdceA-GlcNSO₃(6-OSO₃) structure.

TABLE II
Disaccharide composition of affinity-selected modified heparin fragments

| Preparation          | Disaccharide composition | % |
|----------------------|--------------------------|---|
| Heparin 18-mer, 2-O-desulfated | Non-O-Sulfated, 2-O-Sulfated, 6-O-Sulfated, 2,6-O-Sulfated | |
| Unfractionated       | 6 2 89 4                 |   |
| IL-8-bound (15%)     | 11 3 79 8                |   |
| Heparin 18-mer, preferentially 6-O-desulfated | 44 51 4 1 | |
| Unfractionated       | 34 53 6 7                |   |

a The heparin-derived 18-mers were [3H]-end-labeled. For compositional analysis, further [3H] label was introduced through reduction of disaccharide deamination products with NaB₃H₄ (see "Experimental Procedures"). The compositional data obtained after separation of the disaccharides are expressed as percent of total disaccharide units (average of three independent determinations). The non-O-sulfated disaccharides to the sum of GlcUA-GlcNSO₃ and -IdceA-GlcNSO₃ sequences in the intact 18-mers. 2-O-desulfated disaccharides to the sum of GlcUA-GlcNSO₃(6-OSO₃) and -IdceA-GlcNSO₃(6-OSO₃) structure.

The oligosaccharides analyzed were unfractionated and 2-O-desulfated (A and B, respectively) and preferentially 6-O-desulfated (C and D, respectively) 18-mers. Peaks were identified by reference to the elution position of standard disaccharides as follows: peak 1, GlcUA-aManR(6-OSO₃); peak 2, GlcUA-aManR(6-OSO₃); peak 3, IdceA-aManR(6-OSO₃); peak 4, IdceA(2-OSO₃)-aManR; peak 5, IdceA(2-OSO₃)-aManR. Peaks marked with a bracket under an asterisk indicate tetrasaccharides, partly due to “anomalous” ring contraction (24), and were not included in the quantification of disaccharides presented in Table II.

The oligosaccharides correspond to the sum of GlcUA-GlcNSO₃ and -IdceA-GlcNSO₃ sequences in the intact 18-mers. 2-O-desulfated disaccharides to the sum of GlcUA-GlcNSO₃(6-OSO₃) and -IdceA-GlcNSO₃(6-OSO₃) structure.
of the IL-8-protected fragment (Fig. 6, C, pool I; and D) is clearly larger than a standard 20-mer, degradation by HNO2 at pH 1.5 results in fragments of various sizes from 4-mers up to ~16-mers, in accord with the notion that a sequence of up to ~7 contiguous N-acetylated disaccharide units may be inserted between the sulfated IL-8 monomer-binding regions. In contrast to these fragments, the prominent fraction of low-molecular mass degradation products (pool II), which presumably reflected facilitated enzymatic attack of unprotected stretches of GlcNAc-containing saccharides, was clearly smaller (Fig. 6E) than pool I material and was fairly similar to the total pool created in the IL-8-free digest (Fig. 6F). Both these preparations were completely degraded to short oligosaccharides by HNO2.

DISCUSSION

A number of cytokines and chemokines, including granulocyte/macrophage colony-stimulating factor (27), interleukin-3 (28), IL-8 (9), macrophage inflammatory protein-1β (29), and platelet factor-4 (22, 30), have been shown to bind to proteoglycans/GAGs. While the functional implications of these interactions are not fully understood, it has been proposed that the proteoglycans may act as storage sites for the small highly diffusible molecules or aid in their presentation to receptors (28, 31–34). A model study involving various chemokines and heparin suggested that GAG chains might provide specificity to chemokine action also in vivo (26), yet little information has been available regarding the structural properties of these chains as required to mediate protein binding.

One aim of this study was to define the role of sulfate substituents in GAG binding to IL-8 as reflected through the interaction with fully sulfated, 3H-labeled heparin. The interpretation of such experiments is complicated by the potential redundancy of sulfate groups in saccharide sequences shown to be involved in binding. A striking example is the interaction of basic fibroblast growth factor with a fully sulfated heparin hexasaccharide region, in which only one out of two or three IdceA 2-sulfate groups and none of the GlcN 6-sulfate residues were found to be essential for binding (35, 36). One approach to the analysis of sulfate requirement is to apply selectively desulfated heparin derivatives in competition experiments with fully sulfated, radiolabeled heparin and the protein ligand. Preferential removal of N-, 2-O-, or 6-O-sulfate groups was thus found to appreciably impede the interaction with IL-8, suggesting that all types of sulfate substituents contribute to binding. This conclusion was verified in direct binding experiments utilizing partially O-desulfated, 3H-labeled heparin 18-mers. Preferential 2-O- and 6-O-desulfation both yielded products from which minor fractions could be sequestered by binding to IL-8. While the overall degree of sulfation of each IL-8-binding species did not differ significantly from that of the corresponding parent preparation, the two binding fractions differed markedly in composition from each other (Table II). Clearly, it must be assumed that neither all the IdceA 2-O-sulfate groups present in the IL-8-binding fraction derived from the preferentially 6-O-desulfated heparin 18-mer nor all the GlcN 6-O-sulfate groups in the corresponding species obtained following preferential 2-O-desulfation could be essential for the interaction. However, both fractions of binding components were appreciably enriched in the minor di-O-desulfated -IdceA(2-OSO3)-GlcNSO3(6-OSO3)- disaccharide unit; this finding applied in particular to the preferentially 6-O-desulfated material (Table II). We conclude that the 2-O-
and 6-O-disulfated disaccharide structure promotes binding. On the other hand, each 18-mer contains two binding sites for IL-8 (see below), whereas neither of the two IL-8-binding fractions contained more than 1 mol of di-O-sulfated disaccharide unit/mol of 18-mer. Therefore, it must be assumed that some of the mono-O-sulfated disaccharides may substitute for a “missing” di-O-sulfated disaccharide unit. The precise and optimal arrangement of variously sulfated sugar units in the IL-8-binding site remains to be defined.

The interaction of heparin with IL-8 is relatively weak (26) and was found in this study to require fragments $\geq$18-mers for measurable binding under physiological ionic conditions (Fig. 1). However, under conditions of reduced ionic strength, a distinct 8-mer lower size limit for binding was detected using the nitrocellulose filter trapping assay. Considering the cleavage specificity of the nitrous acid reaction used to randomly depolymerize the heparin starting material, the actual protein-binding site might be as small as a pentasaccharide sequence within the recovered 8-mer (cf. the analogous antithrombin-binding site in heparin (37)). Accordingly, heparin oligosaccharides $<$10-mers could efficiently compete with full-sized radiolabeled heparin for binding to IL-8 (Fig. 1). Taken together with the known propensity of IL-8 to form dimers (12, 38), these findings are readily interpreted in terms of a trimeric complex involving an extended sequence that spans two small binding sites, one on each IL-8 monomer. Indeed, dimerization of IL-8 upon binding to heparin was recently demonstrated (39).

The physiological saccharide ligand for the IL-8 dimer presumably is not heparin, but rather HS proteoglycans at the surface of the vascular endothelium (32, 34). Witt and Lander (26), using affinity co-electrophoresis, noted that HS bound to IL-8 with about the same affinity as heparin having a much higher overall degree of sulfation. This finding was attributed to the orientation of the Glu-63 residues in the IL-8 homodimer,
Eventually, E. coli K5 oligosaccharides, which could promote chemotraction by enhancing the local concentration of IL-8, either by retaining the chemokine on the HS chain or by protecting it from rapid degradation by the released protease (46, 47). It has been noted that whereas HS appears to preferentially bind the dimeric form of IL-8 under physiological conditions (39), monomeric IL-8 has been claimed to be fully active in eliciting a functional response (38, 48). The IL-8/HS interaction may therefore primarily serve to retain the cytokine at its site of production and secretion.

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