Molecular organization of the interferon γ-binding domain in heparan sulphate

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Interferon (IFN)-γ, in common with a number of cytokines or growth factors, strongly interacts with heparan sulphate (HS). It has been shown previously that one of the C-terminal basic clusters of amino acids (a regulatory element of IFN-γ activity) is involved in this interaction. The structural organization of the HS domain that binds to human IFN-γ has been investigated here. IFN-γ-affinity chromatography of HS oligosaccharides released by either enzymic or chemical cleavage showed that the binding site is not found in a domain that is resistant to either heparinase or heparitinase or exclusively N-sulphated or N-acetylated. This led us to take a 'footprinting' approach in which HS was depolymerized in the presence of IFN-γ and the cytokine-protected sequences were separated from the digested fragments. Using this strategy we consistently isolated an IFN-γ-protected domain (IPD; approx. 10 kDa) which displayed the same affinity as full-length HS for the cytokine. Treatment of IPD with either heparinase or heparitinase strongly reduced its affinity, confirming that the high-affinity binding site encompassed a mixture of HS structural domains. Patterns of depolymerization with either enzymic or chemical agents were consistent with IPD being composed of an extended internal domain (approx. 7 kDa) which is predominantly N-acetylated and GlcA-rich, flanked by small N-sulphated oligosaccharides (mainly hexa- to octasaccharides). This is the first description of an HS protein-binding sequence with this type of molecular organization. Furthermore, using a cross-linking strategy, we demonstrated that one HS molecule bound to an IFN-γ dimer. Together these results lead us to propose a novel model for the interaction of HS with a protein, in which two sulphated terminal sequences of the binding domain interact directly with the two IFN-γ C-termini and bridge the two cytokine monomers through an internal *N*-acetyl-rich sequence.

INTRODUCTION

Interferon γ (IFN-γ), the most recent IFN class to be discovered, was first recognized on the basis of its antiviral activity [1]. This highly pleiotropic cytokine plays a central role in modulating nearly all phases of inflammatory and immune responses [2,3]. It also displays antiproliferative activities and regulates a great number of molecules of the pericellular space, including cell-surface components and extracellular matrix molecules [4,5].

The X-ray-crystal structure of recombinant human IFN-γ has recently been solved. This 143-amino acid cytokine was shown to be a compact globular homodimer, in which the two subunits are intertwined [6]. Whereas the N-terminal coordinates have been clearly determined, the C-terminal side of IFN-γ gives weak electronic density and therefore seems to be a very flexible region which does not adopt a rigid conformation [6,7]. Nevertheless, because of the head to tail organization of the dimer the N- and C-termini of the two opposite polypeptide chains are close to each other [6]. Several studies using limited enzymic digestion, monoclonal antibodies, synthetic peptides or CD spectra analysis have established that the N-termini are essential to the structural integrity of IFN-γ and are directly involved in receptor recognition [8–10]. The functional role played by the C-termini has also been widely studied, but this has revealed a much more complex picture. A great number of C-termini-shortened IFN-γ have been prepared. Whereas it was reported that IFN-γ lacking 15 [11], 21 [12] or even 23 [13] C-terminal amino acids is still active, the majority of studies demonstrate a sharp decline in activity as 11 [14] or more [15] C-terminal residues are removed, strongly suggesting that the integrity of the C-termini is critical for biological activity. Monoclonal antibodies directed against this domain of the cytokine have been shown to be both inhibitory [16] and without effect [17]. In a more extensive study, the activity of IFN-γ lacking 4, 5, 6, 8, 9, 10, 11, 14 or 18 C-terminal amino acids was measured. It was found that if a limited number of amino acids are removed, the activity is greatly enhanced. Maximal increase in activity was observed with the deletion of 9 or 10 residues, but the removal of 14 or 18 amino acids resulted in loss of biological activity [18]. These studies suggested a central role for the basic sequence KRKR (amino acids 128–131), as the beginning of its removal correlates with the reduction of activity [18,19], and it has been postulated that this domain is a regulatory element of the cytokine [18]. Finally, synthetic peptides spanning the C-terminal domain of the cytokine competed with IFN-γ for binding to the cloned and purified receptor, but not when intact cell membranes were used [20], suggesting that the C-terminus binds to other cell-surface molecules [21,22].

Proteoglycans are ubiquitous cell-surface components and are also found in the extracellular matrix and basement membrane [23,24]. It has been shown that IFN-γ binds to heparan sulphate (HS) proteoglycan with high affinity (K_d approx. 10^{-9} M) [25]. HS, in which the binding site has been located, has been shown to provide a local concentration of the cytokine in the cellular environment [25,26]. The amino acids of IFN-γ that bind to HS

Abbreviations used: IFN-γ, interferon γ; IPD, interferon-protected domain; GAG, glycosaminoglycan; HS, heparan sulphate; *DP*, degree of polymerization; UA, uronic acid; ΔUA, unsaturated uronic acid; GlcA, glucuronic acid; IdoA, iduronic acid; GlcNAc, *N*-acetylglucosamine; $GlcNSO_3$, *N*-sulphated glucosamine; BS^3, bis(sulphosuccinimidyl)suberate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imine; S-NHS, *N*-hydroxy-sulphosuccinimide.

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are located in the C-terminal domain of the cytokine, within a consensus sequence for heparin recognition, and interestingly involve mainly the KRKR sequence [27]. Given the importance of HS in modulating the activities of a number of other heparin-binding growth factors [24,28,29], and the data accumulated on the role of the KRKR domain in IFN-γ activity, it could be reasonably postulated that IFN-γ activity is also regulated by HS [30]. Preliminary data have shown that once bound to IFN-γ, HS modulates the extent of the proteolytic cleavage of the C-termini and thus leads to an increase or a decrease in biological activity. The observation that native IFN-γ is variously processed at the C-terminal extremities, and lacks 9–16 amino acids with up to at least six different C-termini, reinforces the idea that binding to HS could be a mechanism for regulation of the cytokine activity [4,31].

HS polysaccharides are highly complex and structurally diverse molecules [32], and it has not been established whether the binding to IFN-γ requires a specific structure along the glycosaminoglycan (GAG) chain or whether it is due simply to the anionic character of HS. The fact that other polyanionic GAG chains such as chondroitin sulphate or dermatan sulphate do not bind to IFN-γ [26] supports the former hypothesis and the present paper describes work undertaken to characterize the HS binding domain for the cytokine. We found that it is a complex structure composed of two sulphated sequences linked together by an extended *N*-acetyl-rich domain. In agreement with data from HS/IFN-γ cross-linking studies, the results suggest that sequences of this type function by bridging two IFN-γ monomers.

MATERIALS AND METHODS

Materials

Heparin, HS (from bovine intestinal mucosa; 15–16 kDa), heparinase (EC 4.2.2.7), heparitinase (EC 4.2.2.8), chondroitinase ABC and papain were from Sigma. Heparin molecules of defined molecular mass were kindly given by Dr. M. Petitou (Sanofi Recherche). Affi-Gel 10, Bio-Gel P-6, Bio-Gel P-10, PAGE system and chemicals came from Bio-Rad. Sepharose CL-6B, DEAE-Sephacel and an FPLC system were purchased from Pharmacia. Bis(sulphosuccinimidyl)suberate (BS^3), 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imine (EDC) and *N*-hydroxysulphosuccinimide (S-NSH) were obtained from Pierce. All cell-culture reagents were from Gibco, and D-[1-^{3}H]glucosamine (3.7 Ci/mmol) was from Amersham. Human recombinant IFN-γ was a gift from Roussel Uclaf.

Metabolic labelling and purification of HS

Confluent human skin fibroblasts were incubated for 72 h in Dulbecco's modified Eagle's medium/10% fetal calf serum containing 10 μCi/ml [^{3}H]glucosamine, after which the medium was removed and the cells were treated with trypsin. Trypsin-treated cells and medium were pooled, centrifuged and the supernatant was loaded on to a DEAE-Sephacel column (0.9 cm × 5 cm) equilibrated in 20 mM sodium phosphate/0.15 M NaCl buffer, pH 6.8. A linear gradient from 0.15 to 0.8 M NaCl in 200 ml was applied at 0.2 ml/min, using an FPLC system. The HS proteoglycan peak (eluted at approx. 0.5–0.6 M NaCl) was dialysed against distilled water, dried under vacuum, sequentially digested with chondroitinase ABC (1 unit/ml in 100 mM Tris/acetate buffer, pH 8) and papain (1 mg/ml in 100 mM sodium acetate/5 mM EDTA/5 mM cysteine, pH 6.5), and the digest was repurified on a small DEAE-Sephacel column.

Chemical and enzymic depolymerization of HS

Low-pH HNO_2 deaminative cleavage was performed as described previously [33]. Briefly equal volumes of 0.5 M H_2SO_4 and 0.5 M $Ba(NO_2)_2$ were mixed at 4 °C and centrifuged to remove the Ba_2SO_4 precipitate. Then 100 μl of the supernatant (1 M HNO_2, pH 1.5) was mixed with the HS sample previously dried under vacuum, and incubated for 15 min at room temperature. The reaction was stopped with 50 μl of 1 M Na_2CO_3.

Heparinase digestion of HS was performed in 50 mM Tris/HCl buffer/100 mM NaCl, pH 7.2, at 30 °C, and heparitinase in 50 mM Tris/HCl buffer, pH 7.6, at 35 °C [34]. Depolymerization was generally carried out overnight in the presence of carrier unlabelled GAGs (heparin for heparinase and HS for heparitinase, both at 1 mg/ml), using 10–25 Sigma units/ml (approx. 15–40 Seikagaku m-units/ml where 1 m-unit is defined as the amount of enzyme that cleaves 1 nmol of susceptible linkages/min). This range of concentrations should result in the cleavage of linkages that represent the primary specificities of these enzymes only [35]. The reaction was followed in parallel by monitoring the increase in absorbance at 232 nm. In some experiments IFN-γ was mixed with HS before the addition of enzyme.

Strong-anion-exchange HPLC of disaccharides

Disaccharide composition was analysed by HPLC on a ProPac PA1 analytical strong-anion-exchange column (4 mm × 25 mm; Dionex). After equilibration in mobile phase (double-distilled water adjusted to pH 3.5 with HCl) at 1 ml/min, samples were injected and disaccharides eluted with a linear gradient of NaCl (0–1 M over 45 min) in the same mobile phase. The eluate was monitored in-line for UV absorbance (A_{232} for unlabelled disaccharides) and radioactivity (Radiomatic Flo-one/Beta A-200 detector). The elution position of specific disaccharides was established by comparison with authentic standards.

IFN-γ-affinity column and affinity chromatography

Packed and water-washed Affi-Gel 10 (1 ml) was mixed with 1 ml of 5 mM Tris/HCl, pH 6.8, containing 0.5 mg of IFN-γ and 1 mg of heparin. The twofold excess of heparin in the coupling buffer was added to protect the heparin-binding region of the cytokine C-terminus which contains three lysine and five arginine residues [36] and is likely to react with the Affi-Gel matrix. The low concentration of Tris in the coupling buffer was calculated to be sufficient to reduce the very high binding capacity of the matrix, in order to minimize the number of linkages to IFN-γ. The mixture was rotated end-over-end for 16 h at 4 °C. The Affi-Gel was then packed in a small column, extensively washed with 50 mM Tris/HCl (pH 6.8)/2 M NaCl. The column was stored in 10 mM Tris/HCl, pH 6.8, containing 10 μg/ml heparin and 0.02% NaN_3. Before chromatography the IFN-γ-affinity matrix was washed with 5 ml of 2 M NaCl and then equilibrated in 10 mM Tris/HCl, pH 6.8 (running buffer). Samples (200 μl in running buffer) were loaded on the column and eluted with a step gradient (0–1 M) of NaCl in 100 mM steps. Five fractions of 1 ml were collected for each NaCl concentration.

IFN-γ/HS cross-linking

IFN-γ (250 ng) was mixed with various concentrations of HS and then incubated for 30 min at 4 °C with 2 mM EDC (an aminocarboxy heterobifunctional cross-linker). S-NHS (1 mM)

was added to the reaction mixture to enhance the yield of EDC coupling. In some experiments, IFN-γ was also incubated for 15 min at room temperature in 20 mM Hepes with 2 mM BS[3] (an aminoreactive homobifunctional cross-linker). The reactions were stopped with 2 × SDS PAGE sample buffer, and the products of the cross-linking experiments were analysed by SDS/PAGE (15% gel).

Molecular-sieving chromatography

Bio-Gel P-6, P-10 and Sepharose CL-6B columns (1 cm × 100 cm) were run at 4 ml/h in 0.5 M NH_4HCO_3, and 1 ml fractions were collected. For Sepharose CL-6B chromatography, Blue Dextran (200 kDa) and ^3H_2O were included in each run to monitor void and total volume. Approximate mean molecular-mass values were determined by calculating the $K_{av.}$ values and using the calibration described by Wasteson [37].

Competition experiments

IFN-γ (0.3 μM) was allowed to equilibrate for 1 h at room temperature with labelled HS (10000 c.p.m.), in the presence of heparin (62 μM) of different molecular masses (approx. 1.8, 4.5, 9, 12.5 and 21 kDa). The incubation mixtures were then filtered rapidly through a nitrocellulose membrane in a vacuum-assisted dot-blot apparatus. The membrane was then washed twice with 200 μl of buffer (10 mM Tris/HCl, pH 6.8) and the radioactivity bound to the blotted cytokine was counted after solubilization of the nitrocellulose dots in 500 μl of DMSO.

RESULTS

Preparation of IFN-γ-affinity matrix

IFN-γ coupling to the Affi-Gel matrix was performed in the presence of heparin in order to protect the heparin/HS-binding site of the cytokine. After an overnight coupling reaction with 500 μg of IFN-γ, 50 μg of protein was detected in the column washes, indicating a coupling efficiency of 90%. When heparin was run on the column it was eluted between 0.5 and 1 M NaCl (results not shown). On the other hand, using the same buffer conditions, IFN-γ was eluted from a commercial heparin-affinity column (heparin–ultrogel; IBF Biotechnics) in the same concentration range (approx. 0.7 M NaCl; results not shown). These similarities between the two systems indicated that critical amino acids had not been blocked during the coupling of IFN-γ to the Affi-Gel matrix, and that unmodified heparin/HS-binding domains were available on the affinity column. The affinity column gave identical results for at least 20 runs.

HS fractionation on IFN-γ-affinity matrix

HS metabolically labelled with [^{3}H]glucosamine and purified from cultured human skin fibroblasts was applied to the IFN-γ-affinity column and eluted stepwise with increasing NaCl concentrations (from 0 to 1 M) in 100 mM steps. All the applied material bound to the column, and four main peaks were eluted at 0.3, 0.4, 0.5 and 0.6 M NaCl (Figure 1a). The profile obtained indicates that the HS chains are not homogeneous with respect to their cytokine-binding properties, presumably because of differences in structure between chains. Fibroblast HS has been described as having an alternating arrangement of N-sulphated domains ranging from hexasaccharides [degree of polymerization (*DP*)6, approx. 1.5 kDa] to approximately tetradecasaccharides (*DP*14 approx. 4 kDa), spaced apart by longer predominantly N-acetylated sequences (from 7 to 10 kDa). These two different HS

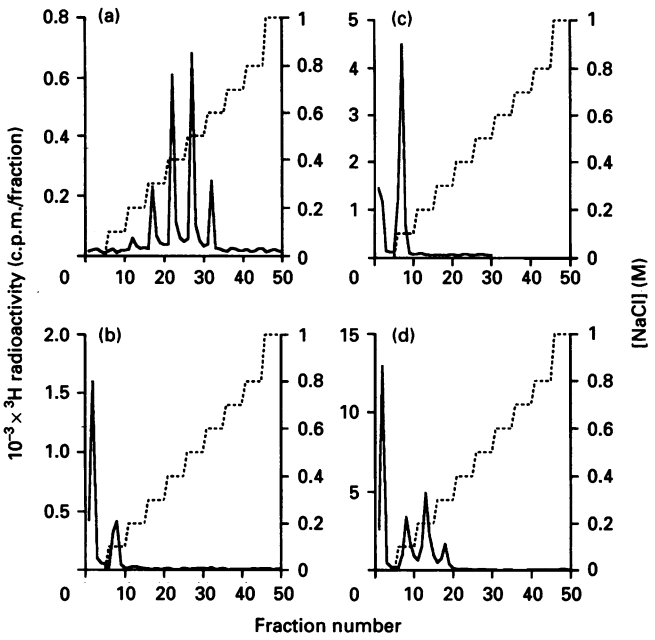

Figure 1 Fractionation of full-length HS on an IFN-γ-affinity column

Metabolically labelled fibroblast HS was applied to an IFN-γ-affinity matrix and eluted with a step gradient of NaCl from 0 to 1 M (....), in 10 mM Tris/HCl, pH 6.8, buffer. For each step, five 1 ml fractions were collected. (**a**) Intact HS; (**b**) low-pH HNO_2-depolymerized HS; (**c**) heparinase-depolymerized HS; (**d**) heparitinase-depolymerized HS.

structural domains are particularly sensitive to heparinase and heparitinase respectively [38]. Although the fine structure varies from one HS to another, the structural model described by Turnbull and Gallagher [38] was found to be consistent with structural analyses performed on the HS described here (results not shown). In addition, in initial studies the fibroblast HS described by Turnbull and Gallagher [38] and the HS described above gave identical profiles for IFN-γ affinity (results not shown).

Depolymerization of HS with HNO_2 (which cleaves HS at N-sulphated glucosamine residues [33]) led to an almost complete loss of affinity, with the majority of the material failing to bind to the column, and the remaining material (25%) being eluted with 100 mM NaCl (Figure 1b). Since N-sulphated glucosamine residues are generally clustered in sulphated domains, this suggests that these N-sulphated regions may be involved in recognition of the cytokine. In addition, the N-acetylated sequences, which are resistant to HNO_2, clearly displayed no significant affinity for IFN-γ. Consistent with this hypothesis, the digestion of HS with heparinase [which cleaves at IdoA(2S) residues within the N-sulphated domains] also led to an almost complete loss of affinity (Figure 1c). Therefore intact heparinase-sensitive domains were prepared (by heparitinase depolymerization of HS) and run on the affinity column (Figure 1d). As expected a large part of the applied material failed to bind to the column [heparitinase, which under the conditions used here cleaves primarily at GlcA residues found mainly in unsulphated N-acetylated sequences, depolymerizes a large proportion of the chain (60–70%) to disaccharide products], but the remaining bound material was eluted in the range 100–300 mM NaCl. N-Sulphated oligosaccharides (*DP*2 to *DP*12) obtained by heparitinase depolymerization of HS were also separated by molecular sieving on a Bio-Gel P-6 column [38] and run

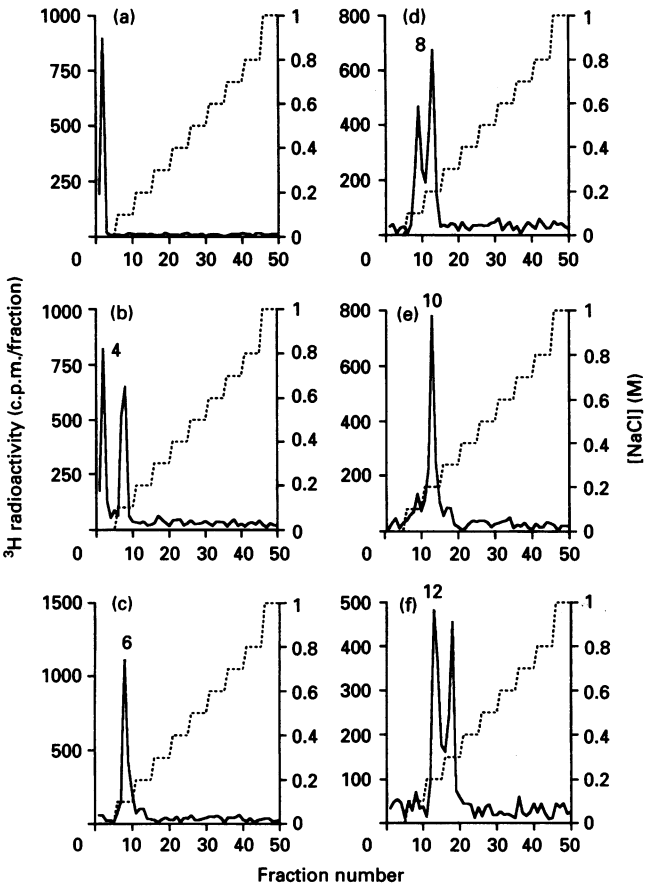

Figure 2 Fractionation of heparitinase-resistant oligosaccharides on IFN-γ-affinity columns

HS was first depolymerized with 10 units/ml heparitinase to generate the N-sulphated (heparitinase-resistant) domains. The resulting oligosaccharides (from di- to dodeca-saccharides) were then separated by molecular sieving on a Bio-Gel P-6 column as described [36], freeze-dried, resuspended in the affinity-chromatography buffer, and run individually on the IFN-γ matrix. (**a**) Disaccharide, (**b**) tetrasaccharide, (**c**) hexasaccharide, (**d**) octasaccharide, (**e**) decasaccharide, (**f**) dodecasaccharide.

individually on the IFN-γ-affinity matrix (Figures 2a–2f). Although the affinity increased with increasing length, none of these oligosaccharides displayed high affinity for IFN-γ, the largest ones tested (*DP*12) being eluted with 0.2–0.3 M NaCl. Therefore the high-affinity binding site is not contained exclusively in sequences that are resistant to either heparinase and heparitinase and appears to overlap domains that are sensitive to both these enzymes. Nevertheless, N-sulphated domains probably represent an important part of the binding site as they still display significant affinity for the cytokine (Figures 1d and 2a–f) whereas the N-acetylated domains do not (Figures 1b and 1c).

Preparation of an IFN-γ-binding domain

In view of the apparent complexity of the IFN-γ-binding site in HS, we decided to employ a 'footprinting' approach in order to isolate binding sequences. HS was depolymerized with heparitinase in the presence of an equimolar amount of IFN-γ (5 μM) in order to protect the bound part of the molecule. As a control, HS was also digested under the same conditions in the absence of IFN-γ. The digests were then analysed by Bio-Gel

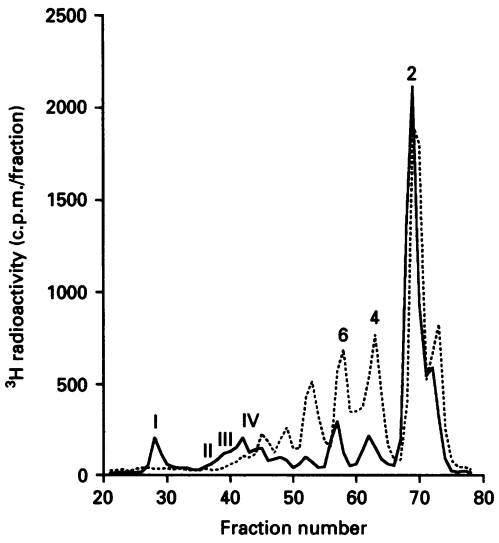

Figure 3 Purification of an IFN-γ-protected domain (IPD)

HS was digested for 4 h at 30 °C with 25 units/ml heparitinase in the absence (....) or presence (——) of an equimolar concentration of human IFN-γ. The digests were then run separately on a Bio-Gel P-10 column at a flow rate of 4 ml/h in 0.5 M NH_4HCO_3. One ml fractions were collected and a small aliquot of each was used for liquid-scintillation counting.

P-10 chromatography (Figure 3). Heparitinase digestion of HS led to oligosaccharides ranging from *DP*2 to *DP*14.

When IFN-γ was mixed with HS before digestion, additional peaks (designated I, II, III and IV) were obtained (Figure 3). These peaks were collected, freeze-dried, resuspended in affinity-column buffer and individually run on the IFN-γ-affinity column. Peak I produced an affinity profile almost identical with the intact chains, with major peaks at 400 and 500 mM NaCl, and minor ones at 300 and 600 mM NaCl (see Figure 9a). This peak thus appears to represent a high-affinity cytokine-binding domain. In contrast, the other peaks (II, III and IV) were eluted at much lower NaCl concentrations (100–300 mM NaCl; results not shown). These results were reproduced consistently three times with HS originating from two different fibroblast donors. Peak I was then designated the IFN-γ-protected domain (IPD) and subjected to further characterization.

Initial characterization of the IPD

Oligosaccharides obtained by low-pH HNO_2 or heparinase depolymerization of IPD and the parent HS were compared by Bio-Gel P-6 molecular sieving (Figures 4a–4b). As expected IPD was sensitive to both HNO_2 and heparinase, and the level of breakdown by the former was more extensive in IPD than in HS. A striking depletion of large fragments in IPD was observed compared with HS, and a higher amount of disaccharides (35% for IPD compared with 18% for HS) indicated a higher level of contiguous sequences of N-sulphated disaccharides (Figure 4a). The frequency (f) of N-sulphation in IPD and HS can be evaluated from the profiles in Figure 4(a) by using the equation:

$$f = \Sigma H_i/n_i,$$

where H_i is the percentage of total ^{3}H radioactivity in peak i and n_i is the number of disaccharide repeat units in that peak. We

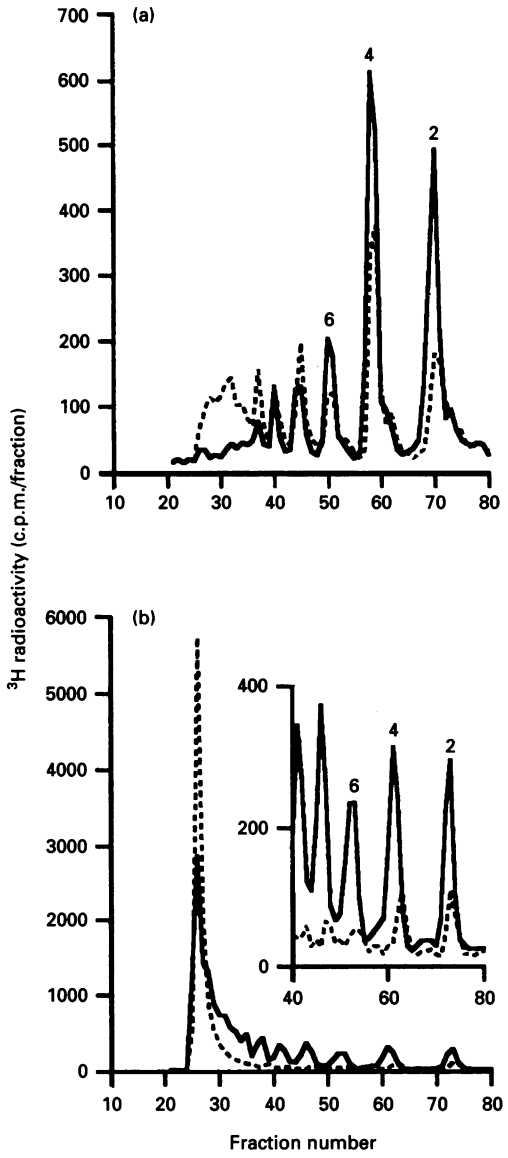

Figure 4 Patterns of depolymerization of the IPD and HS by low-pH HNO_2 or heparinase

IPD (——) and HS (....) were depolymerized by either (**a**) low-pH HNO_2 or (**b**) heparinase (10 units/ml). The resulting oligosaccharides were separated on a Bio-Gel P-6 column in 0.5 M NH_4HCO_3 at 4 ml/h. Fractions eluted in the void volume of the heparinase digest column (**b**) were pooled and freeze-dried for further analysis. The inset shows the cleavage profile with an expanded scale.

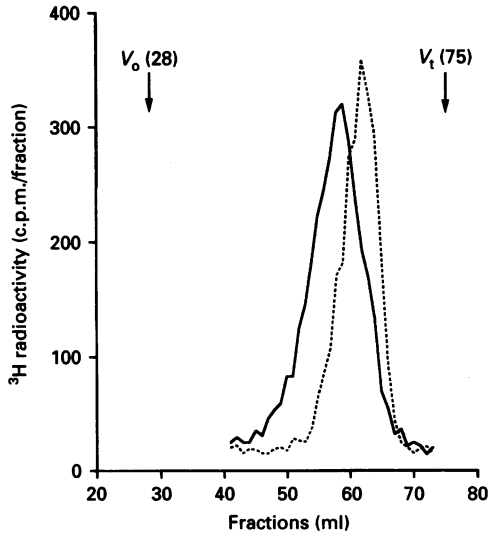

Figure 5 Molecular-mass determination of the IPD

IPD (——) was mixed with Blue Dextran and ^3H_2O (void-volume V_o and total-volume V_t determination), and run at 4 ml/h on a Sepharose CL-6B column (1 cm × 100 cm) equilibrated in 0.5 M NH_4HCO_3. The heparinase-resistant IPD (void-volume material of Figure 4b) was also run under the same conditions (....). The molecular masses were then estimated from the standard curves of Wasteson [37].

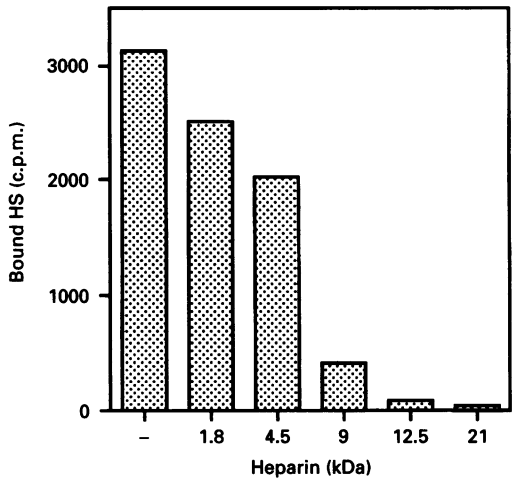

Figure 6 Competition between HS and heparin of different molecular masses for binding to IFN-γ

IFN-γ and labelled HS (10000 c.p.m.) were co-incubated in the absence (—) or presence of heparin molecules of different molecular masses (1.8, 4.5, 9, 12.5 or 21 kDa). After 1 h of incubation the amount of HS bound to the cytokine was determined by filtration through nitrocellulose membrane. No more than 50 c.p.m. were measured when IFN-γ was omitted.

found that 61 % of IPD disaccharides are N-sulphated (compared with 42 % for HS), and hence 39 % are N-acetylated. Heparinase-sensitive disaccharides are also more abundant in IPD than in HS (Figure 4b inset), demonstrating that IPD is enriched in sulphated disaccharides containing IdoA(2S) residues compared with the parent HS molecule.

Both IPD and the IPD heparinase resistant domain (void-volume material of Figure 4b) were sized by molecular sieving on Sepharose CL-6B. The average molecular masses of these two preparations were estimated to be approx. 10 and 7 kDa respectively (Figure 5). These data are consistent with a structure for IPD in which the smaller oligosaccharides released from IPD by heparinase are located at each end of the fragment, although it does not exclude the possibility that they are positioned together at a single end. The molecular mass of the intact chain was also estimated to be approx. 40 kDa on the basis of Sepharose CL-6B chromatography (result not shown). Although IPD is a quite large fragment, we were unable to find a smaller fragment that still bound to IFN-γ with high affinity. Since IFN-γ is a homodimer ([6] and see below), we hypothesized that the length of IPD is a necessary characteristic that allows simultaneous binding to two IFN-γ monomers.

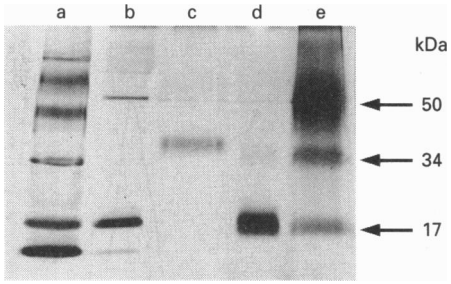

Figure 7 SDS/PAGE analysis of HS/IFN-γ cross-linking

IFN-γ was either cross-linked to itself with BS^3 (2 mM; 15 min at room temperature) or to HS with S-NHS/EDC (1 mM/2 mM; 30 min at 4 °C). Lane a, molecular-mass markers (96, 64, 45, 31, 17 and 14 kDa); lane b, uncross-linked IFN-γ; lane c, BS^3-treated IFN-γ; lane d, S-NHS/EDC-treated IFN-γ; lane e, S-NHS/EDC-treated IFN-γ in the presence of a 10-fold excess of bovine intestinal mucosal HS.

Competitive binding

In order to obtain greater insight into the fragment length necessary for optimal binding, we carried out competition experiments in which heparin molecules of different molecular mass were allowed to compete with labelled HS for binding to IFN-γ. As shown in Figure 6 only heparin molecules with molecular masses greater than 9 kDa were able to compete fully with HS for binding to IFN-γ. Heparin with a molecular mass of 9 kDa significantly inhibited (85 %) the binding of labelled HS to IFN-γ, whereas smaller fragments (4.5 and 1.8 kDa) were only capable of a small degree of inhibition. These results confirm that a relatively large sequence (approx. 10 kDa) represents the minimum length required to bind the cytokine strongly.

IFN-γ/HS cross-linking experiments

We first investigated the dimeric organization of IFN-γ by cross-linking IFN-γ polypeptides using an aminoreactive cross-linker (BS^3). On denaturing SDS/PAGE, IFN-γ migrated as a monomer of 17 kDa (Figure 7, lane b). When the cytokine was treated with BS^3 before electrophoresis, a 34 kDa band was observed (lane c), demonstrating that the molecule was in the dimeric form. In contrast, EDC/S-NHS (an aminocarboxy heterobifunctional cross-linker) did not cross-link the two IFN-γ monomers (lane d), presumably because there is no appropriate juxtapositioning of an amino group next to a carboxyl group between the interacting cytokine monomers. IFN-γ was then treated with EDC/S-NHS in the presence of various concentrations of unlabelled bovine intestinal mucosal HS in order to cross-link the uronic acid carboxyl groups in HS with amino groups of the cytokine. The material obtained (in addition to minor bands of 17 and 34 kDa) showed an apparent molecular mass of approx. 50 kDa. This molecular mass is consistent with that expected for an IFN-γ dimer (34 kDa) plus one HS molecule (15–16 kDa). This molecular mass was found even with a large molar excess of HS (10-fold; lane e), indicating that only one HS molecule binds to two IFN-γ monomers.

Domain organization of IPD

The 10 kDa IPD and the 7 kDa heparinase-resistant domain of IPD were depolymerized by heparitinase and separated on a Bio-Gel P-6 column (Figure 8). The 7 kDa domain of IPD was highly susceptible to heparitinase cleavage, and gave almost entirely

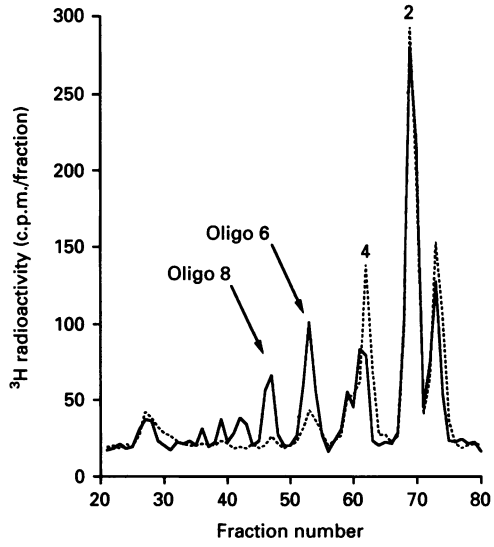

Figure 8 Pattern of depolymerization of IPD and IPD heparinase-resistant domain by heparitinase

IPD (——) and heparinase-resistant domain of IPD (....) were digested with 10 units/ml heparitinase. The products were then resolved by Bio-Gel P-6 column chromatography at 4 ml/h in 0.5 M NH_4HCO_3. One ml fractions were collected, and a small aliquot of each was used for radioactivity determination. Fractions corresponding to oligo 6 and oligo 8 (arrows) were pooled and freeze-dried for further analysis.

Table 1 Disaccharides released from IPD by heparitinase

IPD was treated with heparitinase and the resulting oligosaccharides were separated by chromatography on Bio-Gel P-6 (Figure 8, solid line). The disaccharide peak was pooled, freeze-dried and its composition analysed by strong-anion-exchange HPLC as described in the Materials and methods section.

Disaccharide	Disaccharides (%)	Elution time (min)
ΔHexUA-GlcNAc	57.8	9.9
ΔHexUA-GlcNAc(6S)	3.9	17.3
ΔHexUA-GlcNSO$_3$	31.4	15.4
Unknown*	6.9	19.8

* The structure of this peak is not known but is likely to be a monosulphated disaccharide.

disaccharide (67 %) and tetrasaccharide (26 %) products (Figure 8). Analysis of the profile showed that 82 % of the linkages were cleaved. This indicates that this region is highly enriched in GlcA-containing disaccharides, the majority of which are likely to be N-acetylated. In contrast, the intact IPD was less susceptible to heparitinase (64 % of linkages cleaved), producing di- and tetra-saccharides as well as some larger resistant oligosaccharides (Figure 8, see below). Analysis of the composition of the disaccharides released by heparitinase from intact IPD (Table 1) showed a high content of unsulphated N-acetylated disaccharides (ΔUA-GlcNAc; 58 %), with the remainder being predominantly N-sulphated disaccharides without O-sulphate groups (ΔUA-GlcNSO$_3$; 31 %). Since these disaccharides must all have been released from the heparinase-resistant 7 kDa domain (see below), this confirms the enrichment of N-acetylated disaccharides in this region. It is also apparent that this domain contains very few O-sulphate groups [ΔUA-GlcNAc(6S) only constitutes about

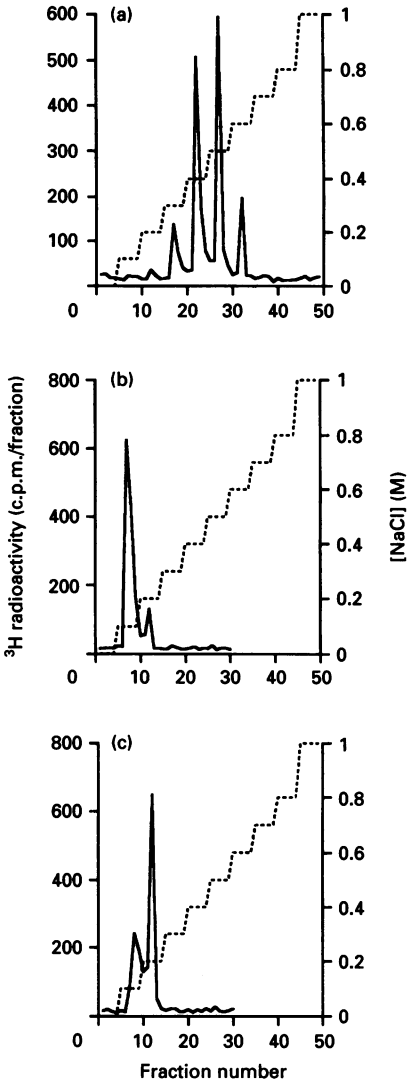

Figure 9 IFN-γ-affinity chromatography of IPD and IPD heparitinase-resistant domains

IPD (**a**) and IPD heparitinase-resistant domains (see oligos of Figure 8) oligo 6 (**b**) and oligo 8 (**c**) were loaded on the IFN-γ-affinity matrix and eluted with a step gradient of NaCl (....) in 10 mM Tris/HCl, pH 6.8, buffer. One ml fractions were collected and radioactivity was counted by liquid scintillation (——).

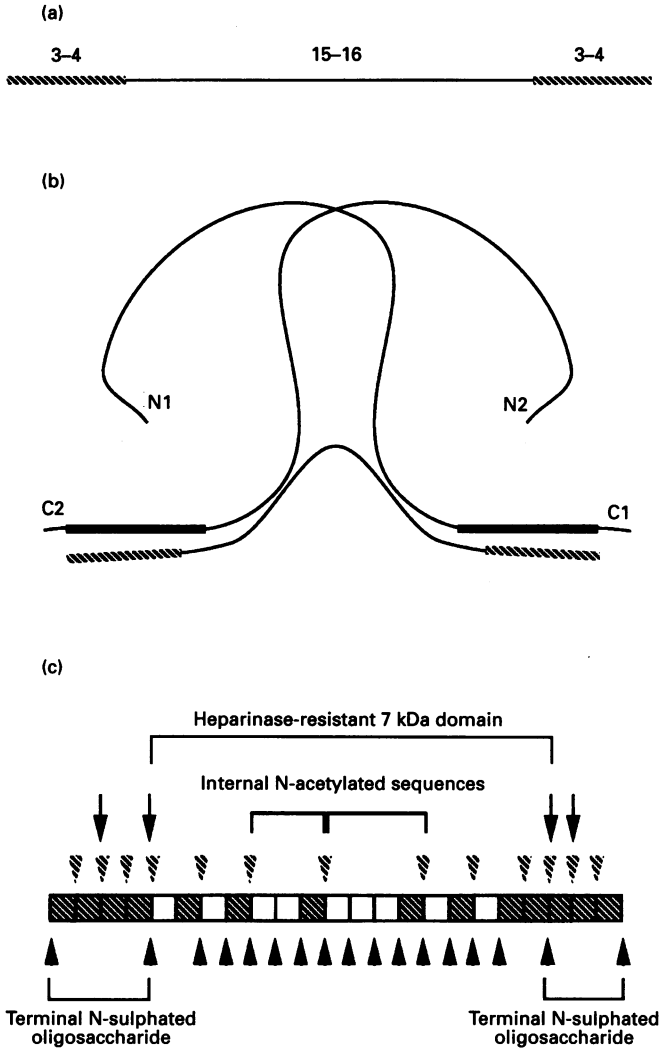

Figure 10 A model for the HS/IFN-γ interaction

The high-affiity IFN-γ-binding domain in HS (**a**) is composed of two small terminal N-sulphated domains (oligo 6 and oligo 8) three to four disaccharides in length linked together by an extended *N*-acetyl-rich domain (7 kDa; approx. 15–16 disaccharides). In this model, the N-sulphated domains form at least part of the HS sequence responsible for binding the two cytokine C-termini (**b**) and effectively bridge the two IFN-γ monomers by virtue of the intervening *N*-acetyl-rich domain. The consensus sequences for heparin/HS binding of the IFN-γ C-termini are shown in bold. A speculative model of the average sequence of IPD is shown in (**c**). The predicted cleavage patterns for HNO_2 (hatched arrowhead), heparinase (arrow) and heparitinase (arrowhead) are based on the structural analyses described in this paper. The two terminal N-sulphated oligosaccharides are depolymerized to disaccharides with HNO_2 and are also sensitive to heparinase, but resistant to heparitinase. The internal N-acetylated domain is depolymerized to di- and tetra-saccharides by heparitinase, and contains two N-acetylated sequences three to four disaccharides in length. The arrowheads at the termini of the sequence denote the cleavage of this fragment from HS by heparitinase. ▨, N-Sulphated disaccharide; □, N-acetylated disaccharide.

4% of the disaccharide products, and no 2-O-sulphated disaccharides were detected].

Most significantly, treatment of intact IPD with heparitinase gave two major heparitinase-resistant peaks corresponding to hexasaccharides (oligo 6) and octasaccharides (oligo 8) which were not present in the internal domain (Figure 8). These fragments must represent the predominant N-sulphated sequences which are cleaved from IPD (and partially degraded) by heparinase treatment. The loss of these fragments, which are probably the terminal sequences of IPD, results in a shift in molecular mass from 10 to 7 kDa (Figure 5). The small amount of higher-molecular-mass material, including that in the void volume (fractions 25–28), probably results from incomplete digestion of IPD. Both the oligo 6 and oligo 8 fractions were degraded completely to disaccharides by HNO_2, demonstrating that they are composed of contiguous sequences of N-sulphated disaccharides (results not shown). In addition they were susceptible to heparinase cleavage, with oligo 8 giving mainly tetrasaccharide products, and oligo 6 a mixture of tetra- and disaccharides (results not shown). The affinity of the oligo 6 and oligo 8 sequences for IFN-γ was also tested. They displayed only weak affinity for the cytokine (Figure 9), similar to that observed for the same-sized fragments released by heparitinase from intact HS (see Figure 2). Furthermore, the 7 kDa heparinase-resistant

domain also displayed only weak affinity for IFN-γ with material eluted at 0.1 and 0.2 M NaCl (results not shown). It is thus apparent that IPD consists of a number of regions with distinct structure, which, although displaying only weak individual affinities for IFN-γ, in combination appear to act synergistically to support high-affinity binding.

Overall, a detailed analysis of the data described above on the initial structural characterization of IPD indicates that its average structure is consistent with two terminal N-sulphated hexa-/octa-saccharides linked together by an extended (7 kDa) internal domain. The latter contains predominantly N-acetylated disaccharides, is GlcA-rich, and contains on average two hexa-/octa-saccharide N-acetylated sequences in addition to approximately four tetrasaccharides of general structure UA-GlcNAc-UA-GlcNSO$_3$. Assuming an average of 450 Da per disaccharide, this putative internal domain will be composed of around 15–16 disaccharides. We propose a speculative model sequence for IPD (Figure 10), in which the predicted average distribution and content of linkages cleaved by HNO_2, heparinase and heparitinase are shown on the basis of data from the experimental depolymerization profiles. The precise distribution of N-sulphated disaccharides within the 7 kDa internal domain is speculative, but plausible, and is consistent with our data. A novel model for the interaction of IPD with IFN-γ is also shown (Figure 10). We propose that the flanking N-sulphated oligosaccharides (and possibly additional adjacent internal sequences within IPD) participate directly in the interaction of HS with the two IFN-γ C-termini, thus bridging the two cytokine monomers through an internal *N*-acetyl-rich domain.

DISCUSSION

Proteoglycans are ubiquitous cell-surface components and are also found in the extracellular matrix and in basement membranes. They are involved in a broad range of activities and some of their proposed functions are likely to depend on interactions between the polyanionic domains in the GAG chains and other molecules [24]. GAGs are known to bind to many proteins, but it is not completely understood whether or not the anionic charge density itself accounts for some of these binding capacities or whether GAGs contain some specific structural sequences for protein binding. The first evidence of a specific GAG–protein interaction was the characterization of a pentasaccharide-binding site for antithrombin III in heparin, which contains a unique 3-O-sulphated glucosamine residue [39]. The characterization of GAGs and the elucidation of specific sequences along the polysaccharide chains have become a central issue in the understanding of their functions. HS is probably the most complex of the GAGs in terms of structural diversity, with an apparently unique design in which clusters of N-sulphated glucosamine and iduronate residues containing the majority of the O-sulphate groups are separated by low-sulphated predominantly N-acetylated domains [32,38]. These different structural domains seem to be designed to specifically bind a number of unrelated proteins. For example, HS is known to play an important role in the extracellular storage, stabilization and/or activation of growth factors and cytokines [29], and specific oligosaccharides that display high affinity for basic fibroblast growth factor [40] and mediate its activities [41,42] have recently been characterized.

In this study we have isolated and undertaken preliminary characterization of the HS domain from fibroblast HS proteoglycan which binds to IFN-γ. Although it has been shown that N-sulphated glucosamine-containing disaccharides are involved in the recognition of the cytokine [26], it appears from the present study that the binding site is not simply contained within an N-sulphated IdoA-repeat-type domain (i.e. heparitinase-resistant; Figures 1 and 2). As the binding is also abolished by heparinase treatment, the individual HS depolymerization enzymes (lyases) cannot be used to isolate the complete binding site as a resistant fragment.

This led us to adopt a 'footprinting' approach whereby HS was cleaved in the presence of IFN-γ, and the cytokine-protected domain separated from the other depolymerized fragments by Bio-Gel P-10 molecular sieving (Figure 3). The yield of IPD was very low (3.5%). As we had approximately equimolar amounts of IFN-γ and HS and as IPD and HS are 10 and 40 kDa respectively, one binding site per molecule would give a theoretical yield of 25% for IPD. This suggests that the binding site is present at a frequency of less than one per chain. However, the dynamic equilibrium between bound and free HS would probably enable some of the binding domains to be degraded. Owing to the use of quite a high enzyme concentration (25 units/ml, at least five times the concentration required for complete depolymerization of HS alone), we cannot exclude the possibility that heparitinase may have competed with IFN-γ for binding to HS and that some of the binding sites may thus have been cleaved by the enzyme. This enzyme concentration was found to be necessary, as lower concentrations gave rise to much larger 'protected' fragments (from 10 to 20 kDa depending on the enzyme concentration used). In these preliminary assays we failed to produce domains smaller than 10 kDa that still displayed the same affinity for the cytokine as full-length HS (results not shown). Therefore the 10 kDa fragments may represent the minimum requirement for high-affinity binding to IFN-γ (see below). Cross-linking experiments demonstrated that one small bovine intestinal mucosa HS chain (15 kDa) bridged two IFN-γ C-termini (Figure 7). This is consistent with IPD being a large fragment, although it is possible that longer HS chains containing appropriate binding domains could bind more than one IFN-γ homodimer. However, it is noteworthy that the affinity of the protected 10 kDa fragment is apparently equal to that of the 40 kDa intact chains. This provides clear evidence that the additional chain length does not confer the increase in affinity that would be expected if the intact chains were binding to multiple IFN-γ homodimers.

Our initial studies on the molecular organization of the protected IFN-γ-binding site suggest that it is composed of a large 7 kDa internal domain (which is resistant to heparinase cleavage) flanked by two small heparitinase-resistant sequences, predominantly three to four disaccharides in length. On treatment with HNO_2, these two hexa- and octa-saccharides gave rise only to disaccharide products (results not shown), indicating that they consist of contiguous sequences of N-sulphated glucosamine-containing disaccharides. We cannot yet exclude the possibility that these N-sulphated oligosaccharides are located together exclusively at one end of the IPD sequence. However, we believe that the type of arrangement shown in the model in Figure 10 is the most plausible, on the basis of what is known about HS structure and the symmetrical folding on the two IFN-γ monomers. In contrast with HS, in which the N-sulphated domains (sometimes described as heparin-like domains) are spaced apart by extended low-sulphated *N*-acetyl-rich regions, heparin contains over 80% of its glucosamines in the N-sulphated form and high levels of both 2-O- and 6-O-sulphation [43]. The fact that heparin fragments at least 10 kDa in size are required to compete effectively with HS for binding to IFN-γ (Figure 6) suggests that two small adjacent N-sulphated oligosaccharides are unable to bind strongly to the cytokine and support the molecular organization proposed for IPD in our model (Figure

10). In addition, the protection against heparitinase digestion of the N-acetylated domain by IFN-γ also favours the view that it is located internally and thus likely to be brought into close contact with the cytokine (see below). Overall, the heparinase-resistant domains of HS consist of fragments in the molecular mass range 7–15 kDa. Although this is long enough to link the two IFN-γ C-termini, they display only very low affinity for the cytokine (Figure 1c). Therefore the organization of IPD we have proposed in this study (i.e. two terminal N-sulphated sequences, which probably constitute at least part of the sequences that bind directly to the cytokine, linked together by a GlcA- and *N*-acetyl-rich domain) appears to be absolutely required for efficient binding. Simultaneous binding at two separate sites presumably has a strong synergistic effect on the affinity of the interaction.

The putative internal domain of IPD (7 kDa fragment) is around 16 disaccharides in length (13–16 nm), and this appears to be longer than the length necessary to link directly the two C-termini of the cytokine [6]. This leads us to propose the model depicted in Figure 10, in which the 7 kDa fragment forms a loop in order to present the correct sulphated terminal domains to the C-termini and allow efficient binding. This loop has been juxtaposed closely to the two IFN-γ monomers to account for the fact that it has been protected by the cytokine from heparitinase degradation. It presumably interacts with the cytokine, although the binding is likely to be relatively weak.

In conclusion, we have found that high-affinity binding to IFN-γ involves a specific domain within HS. This affinity seems to be determined not only by distinct sequences that directly interact with IFN-γ itself but also by the general organization of the binding site, in which the spacing apart of two sulphated domains by a GlcA- and *N*-acetyl-rich sequence appears to be a distinctive and important feature. More work is required to define the precise sequences within IPD that mediate the direct and presumably principal interactions with the IFN-γ C-termini. However, it is noteworthy that this type of molecular organization has not previously been observed for a protein-binding sequence in HS. The structures of previously elucidated high-affinity protein-binding sites have been much smaller and mainly N-sulphated sequences, namely the structurally complex antithrombin III-binding pentasaccharide [39], and the seven-disaccharide structure from HS, Oligo-H, which has high affinity for basic fibroblast growth factor [40]. In addition, our results give some functional significance to the domain model of HS proposed by Turnbull and Gallagher [38]. Our data show that, at least in some cases (such as IFN-γ), the chain organization of HS, and in particular the ordered spacing of sulphated domains, relates critically to protein-binding characteristics. As a number of HS-binding cytokines/growth factors are dimeric, the methodology described here may prove useful for isolating such binding domains. Finally, detailed structural characterization of the IFN-γ-binding site that we have isolated is now in progress, and the results should help us to design specific oligosaccharides that modulate the biological activities and availability of IFN-γ.

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REFERENCES

1 Wheelock, E. F. (1965) Science **149**, 310–311
2 Farrar, M. A. and Schreiber, R. D. (1993) Annu. Rev. Immunol. **11**, 571–611
3 De Mayer, E. and De Mayer-Guignard, J. (1992) Curr. Opin. Immunol. **4**, 321–326
4 Trinchieri, G. and Perussia, B. (1985) Immunol. Today **6**, 131–136
5 Jimenez, S. A., Freundlich, B. and Rosenbloom, J. (1984) J. Clin. Invest. **74**, 1112–1116
6 Ealick, S. E., Cook, W. J., Vijay-Kumar, S. et al. (1991) Science **252**, 698–702
7 Grzeiek, S., Döbeli, H., Gentz, R., Garotta, G., Labhardt, A. M. and Bax, A. (1992) Biochemistry **31**, 8180–8190
8 Magazine, H. I., Carter, J. M., Russel, J. K., Torres, B. A., Dunn, B. M. and Johnson, H. M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1237–1241
9 Hogrefe, H. H., McPhie, P, Bekisz, J. B. et al. (1989) J. Biol. Chem. **264**, 12179–12186
10 Arakawa, T., Horan, T. P., McGinley, M. and Rohde, M. F. (1990) J. Interferon Res. **10**, 321–329
11 Rose, K., Sinoma, M. G., Offord, R. E., Prior, C. P., Otto, B. and Thatcher, D. R. (1983) Biochem. J. **215**, 273–277
12 Sakaguchi, M., Honda, S., Ozawa, M. and Nishimura, O. (1988) FEBS Lett. **230**, 201–204
13 Luck, S. K. S., Jay, E. and Jay, F. T. (1990) J. Biol. Chem. **265**, 13314–13319
14 Leinikki, P. O., Calderon, J., Luguette, M. H. and Schreiber, R. D. (1987) J. Immunol. **139**, 3360–3366
15 Arakawa, T., Hsu, Y.-R., Parker, C. G. and Lai, P.-H. (1986) J. Biol. Chem. **261**, 8534–8539
16 Seelig, G. F., Wijdenes, J., Nagabhushan, T. N. and Trotta, P. P. (1988) Biochemistry **27**, 1981–1987
17 Favre, C., Wijdenes, J., Cabrillat, H., Djossou, O., Banchereau, J. and De Vries, J. E. (1989) Mol. Immunol. **26**, 17–25
18 Döbeli, H., Gentz, R., Jucker, W., Garotta, G., Hartmann, D. W. and Hochuli, E. (1988) J. Biotechnol. **7**, 199–216
19 Lundell, D., Lunn, C., Dalgarno, D. et al. (1991) Protein Eng. **4**, 335–341
20 Griggs, N. D., Jarpe, M. A., Pace, J. L., Russell, S. W. and Johnson, H. M. (1992) J. Immunol. **149**, 517–520
21 Wetzel, R., Perry, L. J., Veilleux, C. and Chang, G. (1990) Protein Eng. **3**, 611–623
22 Carter, J. M., Torres, B. A., Russell, J. K., Johnson, H. M. and Dunn, B. M. (1986) Fed. Proc. Fed. Am. Soc. Exp. Biol. **45**, 1546
23 Gallagher, J. T. (1989) Curr. Opin. Cell Biol. **1**, 1201–1218
24 Jackson, R., Busch, S. J. and Cardin, A. D. (1991) Physiol. Rev. **71**, 481–539
25 Lortat-Jacob, H., Kleinman, H. K. and Grimaud, J.-A. (1991) J. Clin. Invest. **87**, 878–883
26 Lortat-Jacob, H. and Grimaud, J.-A. (1992) Biochim. Biophys. Acta **1117**, 126–130
27 Lortat-Jacob, H. and Grimaud, J.-A. (1991) FEBS Lett. **280**, 152–154
28 Ruoslahti, E. and Yamaguchi, Y. (1991) Cell **64**, 867–869
29 Flaumenhaft, R. and Rifkin, D. B. (1992) Mol. Biol. Cell **3**, 1057–1065
30 Lortat-Jacob, H. and Grimaud, J. A. (1991) Cell. Mol. Biol. **37**, 253–260
31 Curling, E. M. A., Hayter, P. M., Baines, A. J. et al. (1990) Biochem. J. **272**, 333–337
32 Gallagher, J. T., Turnbull, J. E. and Lyon, M. (1992) Int. J. Biochem. **24**, 553–560
33 Shively, J. E. and Conrad, H. E. (1976) Biochemistry **15**, 3932–3942
34 Lohse, D. L. and Linhardt, R. J. (1992) J. Biol. Chem. **267**, 24347–24355
35 Desai, U. M., Wang, H. M. and Linhardt, R. J. (1993) Arch. Biochem. Biophys. **306**, 461–468
36 Rinderknecht, E., O'Connor, B. H. and Rodriguez, H. (1984) J. Biol. Chem. **259**, 6790–6797
37 Wasteson, A. (1971) J. Chromatogr. **59**, 87–97
38 Turnbull, J. E. and Gallagher, J. T. (1991) Biochem. J. **273**, 553–559
39 Lindahl, U., Thunberg, L., Backstrom, G., Riesenfeld, J., Nordling, K. and Bjork, I. (1984) J. Biol. Chem. **259**, 12368–12376
40 Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C. and Gallagher, J. T. (1992) J. Biol. Chem. **267**, 10337–10341
41 Walker, A., Turnbull, J. E. and Gallagher, J. T. (1994) J. Biol. Chem. **269**, 931–935
42 Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U. and Rapraeger, A. C. (1993) J. Biol. Chem. **268**, 23906–23914
43 Gallagher, J. T. and Walker, A. (1985) Biochem. J. **230**, 665–674