Heparan Sulfate Mimicry

A SYNTHETIC GLYCOCONEJGATE THAT RECOGNIZES THE HEPARIN BINDING DOMAIN OF INTERFERON-γ INHIBITS THE CYTOKINE ACTIVITY

Received for publication, July 15, 2005, and in revised form, August 29, 2005 | Published, JBC Papers in Press, September 9, 2005, DOI 10.1074/jbc.M507729200

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Cell-associated heparan sulfate (HS) is endowed with the remarkable ability to bind numerous proteins. As such, it represents a unique system that integrates signaling from circulating ligands with cellular receptors. This polysaccharide is extraordinary complex, and examples that define the structure-function relationship of HS are limited. In particular, it remains difficult to understand the structures by which HS interact with proteins. Among them, interferon-γ (IFNγ), a dimeric cytokine, binds to a complex oligosaccharide motif encompassing a N-acetylated glucosamine-rich domain and two highly sulfated sequences, each of which binds to one IFNγ monomer. Based on this template, we have synthesized a set of glycoconjugate mimetics and evaluated their ability to interact with IFNγ. One of these molecules, composed of two authentic N-sulfated octasaccharides linked to each other through a 50-Å-long spacer termed 2O₃₆₀ displays high affinity for the cytokine and inhibits IFNγ-HS binding with an IC₅₀ of 35–40 nM. Interestingly, this molecule also inhibits the binding of IFNγ to its cellular receptor. Thus, in addition to its ability to delocalize the cytokine from cell surface-associated HS, this compound has direct anti-IFNγ activity. Altogether, our results represent the first synthetic HS-like molecule that targets a cytokine, strongly validating the HS structural determinants for IFNγ recognition, providing a new strategy to inhibit IFNγ in a number of diseases in which the cytokine has been identified as a target, and reinforcing the view that it is possible to create “tailor-made” sequences based on the HS template to isolate therapeutic activities.

Current research increasingly implicates heparan sulfate (HS), a highly sulfated glycosaminoglycan present in the extracellular matrix and at the cell surface, in a plethora of phenomena that include cell proliferation, cell adhesion, matrix assembly, chemoinattraction, inflammation, immune response, development, lipid metabolism, angiogenesis, wound healing, and viral attachment (1, 2). Mechanistically, this extensive functional repertoire often relies on the ability of HS to recognize diverse proteins, the conformation, stability, local concentration, or biological activities of which are modified by the interaction (3–7).

Consistent with its wide protein binding activity, HS is structurally complex. It is composed of strongly anionic domains enriched in N-sulfated glucosamines and iduronic acids, typically 3–8 disaccharides long (referred to as NS or heparin-like domains), that bear a variable number of O-sulfate moieties and are hypervariable in sequence. These domains are separated by relatively regular regions encompassing a larger area that contain predominantly N-acetylated glucosamine and glucuronic acid domains (NA domains) and mixed NA/NS regions that make the transition between NA and NS domains (8). It has been thought that specific information for protein recognition resided within the NS domains in HS and, indeed, a large number of “heparin-binding proteins” interact with such structures. These include, for example, fibroblast growth factors (9), stromal cell-derived factor-1 (10), herpes simplex virus type 1 glycoprotein D (11), or antithrombin III (12). However, of the large number of heparin-protein complexes that can be experimentally demonstrated, the latter (heparin-antithrombin III) is the only one to date for which a specific sequence has been formally defined (13) and reproduced by chemical synthesis (14). Importantly, this achievement led to the development of an approved drug against deep venous thrombosis.

Interferon-γ (IFNγ), a multifunctional T cell-secreted cytokine (15, 16), has been identified as a heparin-binding protein some years ago (17). IFNγ and growth factors (such as fibroblast growth factors) belong to distinct groups in regard to their regulation by HS. In particular, HS does not promote IFNγ association to its cell surface receptor (IFNγR), and IFNγR does not bind to HS.2 IFNγ binding to HS was found to control the blood clearance, subsequent tissue targeting, and local accumulation of the cytokine. It also regulates IFNγ activity by a unique mechanism involving a controlled processing of the carboxyl-terminal peptide (18, 19).

In contrast to the above mentioned heparin-binding proteins, IFNγ does not interact with isolated NS domains. The binding requires a larger sequence that encompasses an internal NA domain flanked at both sides by two NS domains (20). In such a structure the two external NS regions are believed to interact with the two carboxyl-terminal sequences of an IFNγ dimer and bridge the two IFNγ monomers by virtue of the internal domain (Fig. 1). Because of the structural heterogeneity of HS, such complex binding motif is obviously impossible to obtain in pure form and in a large quantity from natural sources. We thus used a chemical approach that has been recently developed (21) to obtain compounds that would mimic the IFNγ binding site with the goal of validating the model of the IFNγ-HS complex initially proposed and obtaining material in homogeneous form and a large amount.

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2The abbreviations used are: HS, heparan sulfate; IFNγ, interferon-γ; IFNγR, IFNγ receptor; NA, N-acetylated glucosamine- and glucuronic acid-containing (region); NS, N-sulfated glucosamine- and iduronic acid-enriched (region); RU, resonance units.

* This work has been supported by the Commissariat à l’Energie Atomique, the CNRS (Contrat Physique et Chimie du Vivant), the Université Paris Sud, and la Région Rhône-Alpes (Programme Emergence). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡The on-line version of this article (available at www.jbc.org) contains supplemental Fig. 1 (H NMR spectra of glycoconjugates 2O, 2O, and 2O) and supplemental Fig. 2 (1H-1H COSY and 1H-13C HSQC [heteronuclear single quantum coherence] of compound 2O).

1H NMR spectra of glycoconjugates 2O, 2O, and 2O. The on-line version contains supplemental Fig. 1 (1H-1H COSY and 1H-13C HSQC spectra of glycoconjugates 2O, 2O, and 2O) and supplemental Fig. 2 (1H-1H COSY and 1H-13C HSQC spectra of compound 2O).

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A set of compounds in which authentic NS domains, ranging from tetrasaccharides to octasaccharides, linked to poly(ethylene glycol)-based spacers of different lengths that would mimic the internal NA domain was produced by chemical synthesis, and the ability of the compounds to interact with IFNγ was analyzed. One of these molecules inhibited the IFNγ/heparin interaction with an IC₅₀ of 35–40 nM. Importantly, this compound also inhibited the binding of IFNγ to IFNγR and, consequently, the biological activity of the cytokine. These results provide a new strategy to inhibit IFNγ in a number of diseases in which this cytokine has been identified as a target and strongly validate the model proposed for the IFNγ-NS complex.

**EXPERIMENTAL PROCEDURES**

**IFNγ Production and Characterization**—Human IFNγ cDNA was cloned into a pET11α expression vector (Novagen) and used to transform *Escherichia coli* strain BL21 Star DE3 (Invitrogen). Cells were grown at 37 °C in Luria broth medium containing 100 μg/ml ampicillin and induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 5 h. Purification from inclusion bodies was performed as described (22) with slight modifications. Briefly, inclusion bodies were solubilized in 6 M guanidine HCl, and the protein was refolded by dilution to 0.2 mg/ml into 50 mM phosphate buffer and 0.5 M guanidine HCl, pH 7. IFNγ was purified by ion exchange (Mono S HR 5/5 column) and gel filtration (Superdex 75 column) chromatography (Amersham Biosciences) and stored frozen in 10 mM Tris and 10 mg/ml mannitol, pH 6.8. Purified material was characterized by mass spectrometry and amino-terminal sequencing and quantified by amino acid analysis. Biological activities of IFNγ (antiviral activity and up-regulation of HLA-DR antigen; see below) were found to be identical to that of commercially available IFNγ (Promega), and both samples were used in this study.

**Synthesis of Oligosaccharides and Glycoconjugates**—The general approach used to synthesize HS oligosaccharides and glycoconjugate mimetics is illustrated in Fig. 2 and briefly described here (a fully detailed description of each synthetic step can be found in Ref. 21). A disaccharide building block with orthogonal allyl and para-methoxybenzyl protecting groups on the anomic and 4' position, respectively, was first prepared as described (23, 24). The allyl group was cleaved (25) followed by the activation of the anomeric position in the form of a methyl triflate to give the desired tetrasaccharide with total 2T₅,2H₁₁O₁₀ (where T, H, and O stand for tetrasaccharide, hexasaccharide, and octasaccharide, respectively, and 2 indicates that two oligosaccharides are linked through a spacer having n equal to 5, 10, or 32 ethylene glycol repeats). All samples were sterilized by filtration through 0.22-μm filters, quantified as described (28), and analyzed by polycrylamide gel electrophoresis analysis (29).

**Assay**—Soluble IFNγR (R&D Systems) at 250 μg/ml in 20 mM phosphate buffer, pH 6, was reacted for 20 min in the dark at 4 °C, with 10 mM ethanolamine (1 M), pH 8.5. Typically, this procedure allowed coupling 2000–2500 resonance units (RU) of streptavidin on both flow cells. Remaining activated groups were blocked with 50 μl of ethanolamine (1 M), pH 7.4. For binding assays, IFNγ, either alone or in combination with oligosaccharides, was simultaneously injected over both negative control and heparin surfaces for 5 min at 25 °C and 50 μl/min. The heparin surface was regenerated with a 250 μl pulse of 1.25 M NaCl.

**RESULTS**

**Design, Synthesis, and Characterization of the Glycoconjugates**—Our previous studies (20) suggested that in the IFNγ-NS complex two NS
domains directly interact with two IFN-γ carboxyl termini and bridge the two cytokine monomers through an internal NA domain (Fig. 1).

Although the atomic structure of IFN-γ has been solved by x-ray crystallography, the organization of the carboxyl termini, which are believed to be intrinsically disordered, is unknown downstream of the residue Ala-123 (32). Because the exact distance that spans the two heparin binding domains is thus not defined, we prepared synthetic glycoconjugates in which poly(ethylene glycol)-based spacers of different lengths were used to link chemically obtained NS domains. For that purpose, a disaccharide building block was first prepared and converted into either a disaccharide donor or a disaccharide acceptor, which were condensed together to give a tetrasaccharide (T). A similar strategy was used to prepare hexasaccharides and octasaccharides (Fig. 2A). The glycoconjugates 2T₉, 2T₁₀, 2T₃₂, 2H₅, 2H₁₀, 2H₃₂, 2O₅, 2O₁₀, and 2O₃₂ were obtained through conjugation of the oligosaccharides onto α,ω-bis-thio-poly(ethylene glycol) spacers of different lengths (Fig. 2B). ¹H NMR and heteronuclear single quantum coherence spectra were in accordance with the expected structures (data not shown). PAGE analysis (Fig. 3) showed that synthetic tetrasaccharides, hexasaccharides, and octasaccharides (lanes 2, 4, and 6) have the same migration pattern as the corresponding heparin-derived oligosaccharides (lanes 1, 3, and 5) but, in contrast to the natural size-purified mixtures, are homogenous. Conjugation to poly(ethylene glycol) linkers was clearly evidenced by the shift in migration observed for 2O₅ (Fig. 3, lane 7), 2O₁₀ (lane 8), and 2O₃₂ (lane 9), each of which migrated at the position of natural octadecasaccharides and larger. Similar migration changes were observed for tetrasaccharide and hexasaccharide conjugation (data not shown). In some case, conjugated oligosaccharides migrated as apparent doublets on the gel. However, because NMR analyses of these molecules were consistent with single structures, this result should not be caused by sample heterogeneity (see supplemental data, available in the on-line version of this article).

Inhibition of the IFN-γ-Heparin Interaction by the Glycoconjugates—To investigate the ability of the 12 different synthetic compounds to interact with IFN-γ, an inhibition assay was set up in which the cytokine, either alone or coincubated with each of the 12 molecules (at 37.5, 75, or 150 nM) to be analyzed, was injected over both a heparin-functionalized sensor chip and a streptavidin sensor chip used as a control surface. Injection of IFN-γ over the heparin surface produced at equilibrium a binding response of 180 RU, whereas a response of 5 RU was observed over the streptavidin surface (data not shown). Results showed (Fig. 4) that whereas the tetrasaccharides and hexasaccharides were completely inactive, the octasaccharide slightly prevented the IFN-γ-heparin binding (no more than 20% inhibition was observed with the highest dose of octasaccharide), indicating that by themselves these oligosaccharides did not display significant affinity for the cytokine. Conjugation of tetrasaccharides or hexasaccharides to the different poly(ethylene glycol) linkers did not lead to significant inhibition activity (25–30% of inhibition at 150 nM; Fig. 4C). In contrast, octasaccharides conjugated to spacers with 5, 10, or 32 poly(ethylene glycol) repeats (2O₅, 2O₁₀, or 2O₃₂) clearly inhibited the binding of IFN-γ to heparin, the most active molecule (2O₁₀) displaying a 50% inhibition at ~35–40 nM (Fig. 4A). These results strongly suggest that the glycoconjugates function by bridging the two IFN-γ monomers and that proper spacing of the oligosaccharides are important for the binding process. Similar experiments...
The 2O10 Glycoconjugate Displays Anti-IFNγ Activity—In view of the above data, we investigated whether 2O10 would inhibit IFNγ activity. We thus measured the ability of 2O10 to prevent the induction of the HLA-DR antigen by IFNγ, using Colo 205 cells. We found that optimum HLA-DR antigen was observed 72 h after the initial stimulation with 5 ng/ml of IFNγ, and these conditions were used in the following experiments. Cells were treated with IFNγ that was preincubated with a range of concentration of 2O10 (0–25 µg/ml), and HLA-DR expression was quantified 72 h later. Our data (Fig. 6A) showed that 2O10 clearly displayed anti-IFNγ activity, with an IC50 close to 1.5 µg/ml (250 nM). Analysis of all the other glycoconjugates demonstrated that their anti-

bilibed the ectodomain of the IFNγR on a Biacore sensor chip and analyzed the ability of the synthetic oligosaccharides to inhibit the IFNγ-IFNγR interaction. IFNγ was preincubated with increasing concentrations of the different molecules to be analyzed and then injected over the IFNγR surface (Fig. 5). The first two sets of sensorgrams show that unconjugated oligosaccharides (Fig. 5A) and conjugated tetrasaccharides (Fig. 5B) had no or minimal effect on the IFNγ-IFNγR interaction. Conjugated hexasaccharides (2H5, 2H10, and 2H32) yielded a modest inhibition (Fig. 5C) as did the conjugated octasaccharides 2O5 and 2O32 (Fig. 5, D and E), whereas the 2O10 had a marked effect on the binding process (Fig. 5F). The 2O10 compound activity is similar to that of a 6-kDa heparin fragment.

FIGURE 2. Oligosaccharides and glycoconjugates synthesis. A, synthesis of the protected tetrasaccharides, hexasaccharides, and octasaccharides. A single disaccharide building block was converted into a disaccharide acceptor (arrow a) or a donor (arrow b) and then condensed together (2 + 2) to give a tetrasaccharide (arrow c). This tetrasaccharide was similarly transformed into either a tetrasaccharide donor (arrow d) or acceptor (arrow b) that were then converted, using a 2 + 4 or 4 + 4 strategy (arrow c), into the expected hexasaccharide and octasaccharide. These compounds were treated as described under “Experimental Procedures” to give the non-conjugated glycoconjugates. The sulfated and benzylated tetrasaccharides, hexasaccharides, or octasaccharides were conjugated to bis-thio-poly(ethylene glycol) linkers of different lengths (n = 5, 10, or 32) under UV light irradiation (arrow d). The nine benzylated glycoconjugates thus obtained were debenzylated after oxidation of the thioether linkages to give the awaited nine deprotected glycoconjugates (arrow e).

FIGURE 3. PAGE analysis of oligosaccharides. Heparin-derived (size-purified mixture) or synthetic oligosaccharides (0.5 µg each) were run through a 30% polyacrylamide gel and stained with azure A. Lane 1, heparin-derived tetrasaccharide; lane 2, synthetic tetrasaccharide; lane 3, heparin-derived hexasaccharide; lane 4, synthetic hexasaccharide; lane 5, heparin-derived octasaccharide; lane 6, synthetic octasaccharide; lane 7, 2O5; lane 8, 2O10; lane 9, 2O32; lane 10, heparin-derived tetadesaccharide; lane 11, hexadesaccharide; lane 12, octadesaccharide.

performed with 15- and 5-kDa heparin indicated that 20 and 130 nM were required, respectively, to produce 50% inhibition (data not shown).

The Glycoconjugates Inhibit the IFNγ-IFNγR Interaction—A number of studies demonstrated that the IFNγ carboxyl termini are critical for bioactivity (33). In particular, it has been suggested that residues 128–132 (Lys-Arg-Lys-Arg-Ser) within this domain of the cytokine are required for receptor binding, and a model has been proposed where this basic cluster is located near an acidic patch of the IFNγR (32). We thus investigated whether the synthetic oligosaccharides would inhibit the binding of the cytokine to its receptor. For that purpose, we immo-
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IFNγ activity closely match their ability to inhibit the IFNγ-IFNγR interaction (Fig. 6B).

**DISCUSSION**

Structural heterogeneity of HS is the basis of the many functions these molecules fulfill, in particular through their unique ability to interact with a large array of proteins (34). Despite increased interest in the field, progress has been hampered by the extraordinary complexity of HS. In particular, the characterization of the protein-HS interface and the isolation of the corresponding binding domains are very difficult, an aspect particularly prominent in the case of IFNγ, the binding site of which is not simply contained within a single structural domain of the polysaccharide (20).

In this context, we chose a chemical strategy to investigate the general motif organization that IFNγ recognizes on HS. According to the model of the IFNγ-HS complex (see Fig. 1) that was used as a working hypothesis, different heparin-like oligosaccharides were synthesized and linked to each other using molecular spacers of distinct lengths. Binding studies first pointed out that properly spaced interacting sequences acted in a concerted manner to form a functional unit, because none of the unconjugated oligosaccharides displayed efficient binding activity. Optimal activity was observed when a spacer of 10 poly(ethylene glycol) repeats (~50 Å-long) was introduced between the two NS domains. Interestingly, the last two defined residues (Ala-123) in the IFNγ crystallographic structure are at a distance of 23 Å. The 50-Å spacer should thus function by optimally presenting the two binding NS domains to each other using molecular spacers of distinct lengths. Binding studies first pointed out that properly spaced interacting sequences acted in a concerted manner to form a functional unit, because none of the unconjugated oligosaccharides displayed efficient binding activity. Optimal activity was observed when a spacer of 10 poly(ethylene glycol) repeats (~50 Å-long) was introduced between the two NS domains. Interestingly, the last two defined residues (Ala-123) in the IFNγ crystallographic structure are at a distance of 23 Å. The 50-Å spacer should thus function by optimally presenting the two binding NS domains to each other using molecular spacers of distinct lengths.
number of diseases characterized by pro-inflammatory and autoimmune Th1 response (36). These include autoimmune pathologies such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, insulin-dependent diabetes mellitus, psoriasis, and alopecia areata (37) or chronic inflammatory bowel diseases such as Crohn disease and ulcerative colitis (38). IFNγ is also a key contributor to hyperoxia-induced lung injury (39) and, because of its ability to induce a major histocompatibility complex in many tissues, is associated with graft rejection (40). This cytokine is thus considered as an attractive target for the treatment of such diseases, and strategies have been developed to antagonize its biological activity. So far these strategies have focused on the use of soluble IFNγR (41, 42) or neutralizing antibodies, and in some cases these were found to offer substantial clinical benefits (37, 43). Humanized anti-IFNγ is also currently evaluated in Crohn disease (44). On the other side, administration of a HS binding cationic peptide (derived from the IFNγ carboxyl terminus) has delayed the time of rejection in a mouse model of allogenic skin flap transplantation (45). Most interestingly, the 2O10 molecule described here inhibits the binding of IFNγ to both HS and IFNγR, the two known ligands of the cytokine. Such a molecule should thus inhibit both bioactivity and local concentration within tissue and therefore should compare very favorably with neutralizing antibodies that have been selected for their ability to inhibit IFNγ bioactivity but not binding to HS.

There is enormous potential for the development of heparin-like structures as drugs for a range of diseases in addition to the current antithrombotic target (46, 47). Based on the 2O10 scaffold, our future work will investigate whether such type of molecules could be of some interest in pathologies for which IFNγ has been identified as a target.

Acknowledgments—We thank Jean-Pierre Andrieu for amino-terminal sequencing and amino acid analysis, Bernard Dublet for mass spectrometry, and Romain Vivès for reading of the manuscript.

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FIGURE 6. Synthetic oligosaccharides and glycoconjugates inhibit the IFNγ activity. A, monolayers of Colo 205 cells were treated with IFNγ (5 ng/ml), preincubated with a range of concentrations of 2O10 (0–25 μg/ml), and HLA-DR expression was quantified 72 h later. B, monolayers of Colo 205 cells were treated with IFNγ (5 ng/ml) that was preincubated or not with each of the 12 synthetic molecules (25 μg/ml), and HLA-DR expression was quantified 72 h later. Results are expressed as a percentage of inhibition. T, tetrasaccharide; H, hexasaccharide; O, octasaccharide.
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