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The binding of human glial cell line–derived neurotrophic factor to heparin and heparan sulfate: importance of 2-*O*-sulfate groups and effect on its interaction with its receptor, GFRα1

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We report ELISA studies of the glycosaminoglycan binding properties of recombinant human glial cell line–derived neurotrophic factor (GDNF). We demonstrate relatively high affinity binding as soluble heparin competes with an IC_{50} of 0.1 μg/ml. The binding of GDNF to heparin is particularly dependent on the presence of 2-*O*-sulfate groups. Highly sulfated heparan sulfate is also an effective competitor for GDNF binding. We also show that heparin at low concentrations protects GDNF from proteolytic modification by an endoprotease and also promotes the binding of GDNF to its receptor polypeptide, GFRα1. In both of these actions, 2-*O*-desulfated heparin is less effective. Considered overall, these findings provide strong support for a hypothesis that the bioactivity of GDNF during prenatal development is essentially dependent on the binding of this growth factor to 2-*O*-sulfate-rich heparin-related glycosaminoglycan.

Key words: GDNF/GFRα1 receptor/glycosaminoglycan/heparan sulfate/heparin

Introduction

Glial cell-line derived neurotrophic factor (GDNF) is a disulfide-linked homodimer of 18–22-kDa *N*-glycosylated polypeptides (Lin *et al*., 1993). It is the prototypic member of a subfamily of four cytokines, the GDNF family ligands (GFLs), which also include artemin (Baloh *et al*., 1998), neurturin (Kotzbauer *et al*., 1996), and persephin (Milbrandt *et al*., 1998). The GFLs are in turn members of the larger family of transforming growth factor β–related cytokines (for review see Baloh *et al*., 2000). The GFLs possess a high degree of sequence homology and also share usage of the cell surface tyrosine kinase Ret as the transmembrane signaling element of their receptors (Baloh *et al*., 2000). In addition, GFL receptor complexes contain a ligand-binding, nonsignaling polypeptide, or GDNF family receptor (GFR) α. Albeit with some limited cross-talk, there appears to be a specific GFRα for each GFL, that for GDNF being GFRα1 (Baloh *et al*., 2000).

It is well established that GDNF exerts potent neuroprotective activity both *in vitro* and *in vivo*; indeed GDNF-expressing vectors have proven effective in protecting against neurodegeneration and promoting regeneration in murine (Choi-Lunberg *et al*., 1997) and primate (Kordower *et al*., 2000) models of Parkinson's disease, and clinical trials of recombinant human (rh) GDNF infusion for this condition are now under way with promising initial results (Major, 2002). However, the neuroprotective activity of GDNF is not limited to dopaminergic neurones, as numerous studies show beneficial effects in central and peripheral neurones and axons following challenge with various mechanical, ischemic, and neurotoxic insults. This raises the prospect of therapeutic applications of either gene-vector–expressed GDNF or recombinant GDNF in a wide range of nervous system injury or disease.

Gene deletion studies in mice reveal an essential, nonredundant role for GDNF in the development of the enteric nervous system (Moore *et al*., 1996; Pichel *et al*., 1996; Sanchez *et al*., 1996). *GDNF*$^{-/-}$ mice also show other selective neurone deficits in the trigeminal motor nucleus, spinal chord, and sensory and sympathetic ganglia. However, the most striking feature of the *GDNF*$^{-/-}$ phenotype is the complete absence of kidneys, which is presumably the major contributor to the early postnatal mortality seen in this phenotype. Similar phenotypes, involving renal agenesis and the absence of enteric neurones, also result from targeted disruption of the genes encoding GFRα1 (Cacalano *et al*., 1998; Tomac *et al*., 2000) and Ret (Schuchardt *et al*., 1994). These and other experimental observations suggest that high levels of GDNF expressed in the embryonic metanephric blastema serve to activate cells of the ureteric bud, which grows out from the preexisiting metanephric (Wolffian) duct. Cells at the tip of the bud express both GFRα1 and Ret, and contact with the blastema initiates subsequent events in kidney formation (reviewed by Schedl and Hastie, 2000).

Heparan sulfate (HS) is an acidic polysaccharide that is nearly ubiquitous on cell surfaces and in the extracellular matrix. It has been shown to bind to an increasing number of diverse cytokines. The biosynthesis of HS involves postincorporation modifications of hexoses in the nascent polysaccharide chains. Such modifications include the

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epimerization of glucuronate residues to iduronate and their possible subsequent *O*-sulfation at the C-2 position (reviewed by Lindahl *et al.*, 1994). This latter modification is catalyzed by a specific HS 2-*O*-sulfotransferase (HS2ST).

As part of a genetic screen approach to the study of embryogenesis, a mutation of the gene encoding HS2ST was recovered. Mice homozygous for disruption of the HS2ST gene exhibit a variety of developmental defects, but the most striking phenotype is absence of kidneys, recapitulating the phenotypes resulting from *GDNF*, *GFRα1*, and *cRet* gene disruption (Bullock *et al.*, 1998). These observations could be accounted for by hypotheses in which GDNF and 2-*O*-sulfated HSs interact only via some indirect mechanism involving intermediary factors. However, the simplest and most direct hypothesis is that the high levels of GDNF expressed in the metanephric blastema bind to HS in the local extracellular matrix or on cell surfaces. This retention would serve in a critical manner to maintain a high localized concentration of the cytokine required to promote invasion by the extending uretric bud. Such a direct binding hypothesis predicts that 2-*O*-sulfate groups are essential for the binding of GDNF to HS, such that HS lacking 2-*O*-sulfate groups is substantially less active in this regard. It would also predict that HS-bound GDNF remains biologically active by being readily available to its cell surface receptors. GDNF is known to be a heparin-binding cytokine, because during purification, GDNF bound to a heparin-Sepharose column and was eluted between 0.5 and 1.5 M NaCl (Lin *et al.*, 1994). However, up to now there has been no detailed examination of the affinity and specificity of the binding of GDNF to sulfated glycosaminoglycans and how this might affect its receptor engagement and stability.

We have established an enzyme-linked immunosorbent assay (ELISA) approach to investigate the binding of cytokines to glycosaminoglycans, involving capture on an immobilized heparin–protein conjugate. Using this approach we have found that interleukins (ILs) 2, 6, and 12 bind to heparin and HS (Najjam *et al.*, 1997, 1998; Hasan *et al.*, 1999; Mummery and Rider, 2000). In each case this binding is selective because it is undetectable with the cytokines TNF-α, IL-10, and IL-16 (unpublished data) and specific in that other sulfated glycosaminoglycans, particularly the chondroitin sulfates, are either poor or negligible competitors of heparin/HS binding (Najjam *et al.*, 1997; Hasan *et al.*, 1999; Mummery and Rider, 2000). Here we report the use of this ELISA approach to characterize the interaction between rhGDNF and heparin. We also investigate the effect of heparin binding on the interaction between GDNF and a soluble chimeric construct of its cognate receptor, GFRα1. These studies provide evidence for a major role for 2-*O*-sulfate groups in the binding of GDNF to heparin/HS and show that heparin-bound GDNF stills binds GFRα1 and is protected from proteolytic degradation.

Results

ELISA of rhGDNF clearly demonstrates strong binding to the heparin–bovine serum albumin (BSA) complex, but not to wells coated with mock-conjugated BSA (see Figure 1A).

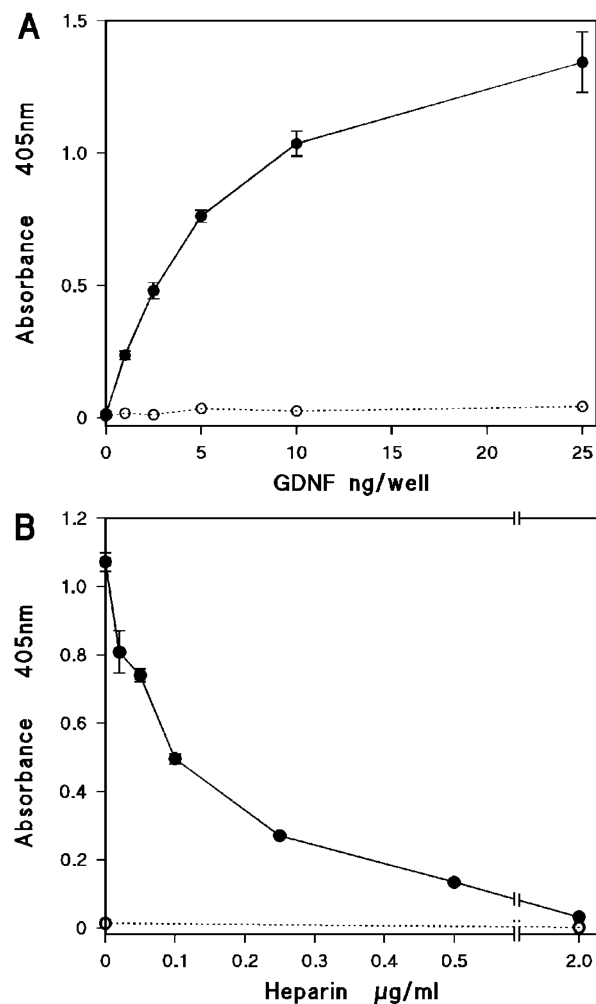

Fig. 1. ELISA of the binding of rhGDNF to immobilized heparin. (**A**) Dose-dependency of binding. (**B**) Competition with free heparin. Results shown are typical of three independent experiments. Each data point is the mean of four replicate wells in **A** and six replicates in **B**. Closed symbols represent binding in wells coated with heparin–BSA complex; open symbols denote binding to mock-conjugated BSA. Error bars indicate SEM and are omitted for mock-treated BSA values because they are less than the symbol diameter.

The binding is entirely reproducible and may be detected with great sensitivity, since 1 ng rhGDNF per well gives clear immunoreactivity. This binding to the heparin complex is also specific because parallel studies of IL-9 using the same batch of complex and second antibody failed to show binding above the background levels observed in wells coated with mock-conjugated BSA (unpublished data). It is noteworthy that in our previous use of this experimental approach to demonstrate the binding to heparin of IL-2 (Najjam *et al.*, 1997, 1998), IL-6 (Mummery and Rider, 2000), and IL-12 (Hasan *et al.*, 1999), the dose-dependent binding curves were all nearly linear. However, as may be seen in Figure 1A, the binding curve for rhGDNF is markedly exponential, with inflection of the curve between 5 and 10 ng/well; this is indicative of saturation of available binding sites.

To confirm that the rhGDNF binding to the heparin–BSA complex occurs via the glycosaminoglycan chains,

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we employed soluble heparin as a competitor. As seen in Figure 1B, increasing concentrations of soluble heparin causes displacement of the rhGDNF. This displacement is essentially complete at 2 μg/ml, with an IC_{50} of around 0.1 μg/ml.

Because soluble heparin is an effective competitor with the heparin-complex for GDNF binding, we examined the specificity of GDNF–glycosaminoglycan interactions by employing a range of heparin-related preparations in this competitive variant of the ELISA. As may be seen in Figure 2A, 2.0 μg/ml heparin again gives essentially complete inhibition of binding. By comparison, bovine kidney HS, although used at a fivefold higher concentration, is a weak inhibitor, reducing GDNF binding only by some 30%. In contrast, fucoidan, a sulfated polyfucose from marine algae, appears to be a more potent inhibitor than heparin because it provides complete inhibition at a concentration that with heparin would only provide some 60% inhibition (see Figures 1B and 2B). The three forms of chondroitin sulfate, A, B, and C, are only weak inhibitors. Statistical analysis of two independent experiments by analysis of variance with post hoc analysis establishes that bovine kidney HS and all forms of chondroitin sulfate give significant inhibition at the $p \leq 0.05$ level but fails to establish any significant difference between the effects of all four of these glycosaminoglycans.

To shed light on the structural basis of GDNF–heparin binding, we examined the potencies of various chemically modified bovine lung heparin preparations as inhibitors of binding. As may be seen in Figure 2B (columns a and b), the unmodified parental bovine heparin shows an inhibitory potential that is indistinguishable from that of porcine heparin (Figure 1B). However, *N*-desulfation removes inhibitory activity (Figure 2B, column c), although subsequent re-*N*-acetylation gives partial restoration of this activity (column d). Removal of 2-*O*-sulfates (column e) also results in a product that fails to compete for heparin binding. This particular finding is of considerable significance, because it is entirely consistent with the hypothesis discussed in the *Introduction*, which predicts an essential role for HS 2-*O*-sulfate groups in retaining GDNF at key microanatomical sites during kidney development. In contrast, 6-*O*-desulfated heparin remains a significant inhibitor, albeit with reduced potency compared to the parental, unmodified preparation. The two porcine intestinal HSs prove to be significant inhibitors (Figure 2B, columns g and h), with both providing more inhibition than is seen with bovine kidney HS (Figure 1B) and the more highly sulfated HSE giving virtually complete inhibition.

The structural characteristics of HSA and HSE were investigated by 500 mHz ^{1}H nuclear magnetic resonance (NMR), and the ratios of various substituents, as determined by integration of specific peak signals (Mulloy *et al.*, 1994; Yates *et al.*, 1996), are summarized in Table I. The 5th International Standard heparin (from porcine mucosa) was simultaneously analyzed as a control. As may be seen, HSE is extensively *N*-deacetylated and *N*-sulfated, to an extent approaching that seen in heparin. In contrast, in HSA only approximately half of the glucosamine residues remain in the unmodified *N*-acetyl form. All three preparations have similar ratios of 6-*O*-sulfate to *N*-sulfate. However the preparations differ markedly in the ratio of 2-*O*-sulfate to *N*-sulfate, that of HSA being considerably lower than that of heparin and less than 50% that seen with HSE. Given the markedly lower abundance of *N*-sulfate groups in HSA compared to the other preparations, it may be estimated that HSA has only around 25% of the 2-*O*-sulfate groups found in HSE.

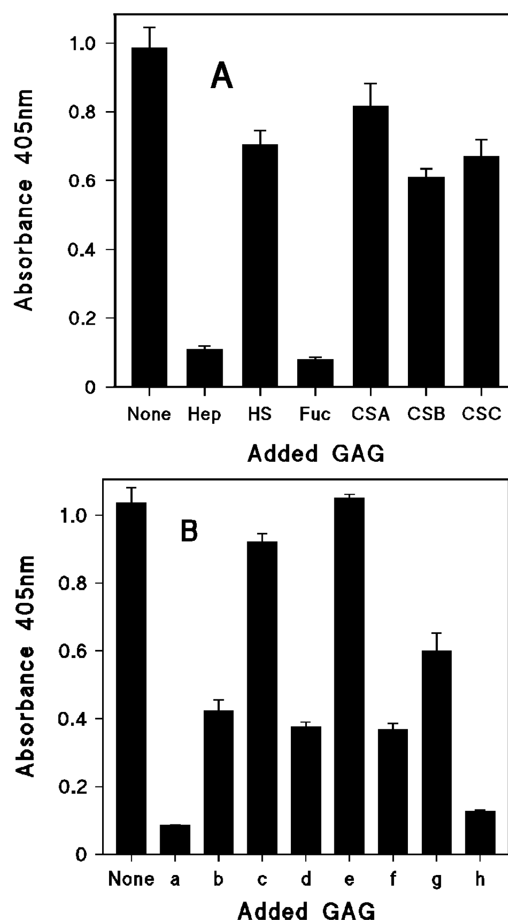

Fig. 2. Inhibition of binding of rhGDNF to heparin complex by glycosaminoglycans. RhGDNF, 10 ng/well, was preincubated for 30 min in the absence (None) or presence of various glycosaminoglycans as indicated. (**A**) Competition with different classes of glycosaminoglycan; Hep, heparin (2 μg/ml); HS, heparan sulfate from bovine kidney (10 μg/ml); Fuc, fucoidan (0.2 μg/ml). CSA, CSB, and CSC are, respectively, chondroitin sulfates A, B, and C and were all added at 10 μg/ml. (**B**) Competition with chemically modified heparins, and HSs; columns a and b, parental bovine heparin at final concentrations of 2 μg/ml and 0.2 μg/ml, respectively; c, *N*-desulfated heparin, d, *N*-desulfated, *N*-reacetylated heparin; e, 2-*O*-desulfated heparin; f, 6-*O*-desulfated heparin; g, HSA; h, HSE. Glycosaminoglycans, c–h, were all at a final concentration of 10 μg/ml. Each panel is a single experiment, representative of two carried out under identical conditions.

We also used our ELISA approach to investigate the ability of heparin-derived oligosaccharides to compete with GDNF binding to the heparin complex. As may be seen in Figure 3, under the conditions employed the oligosaccharide fragments show reduced activity. Even the largest fraction tested, the tetradecasaccharides, showed less activity than the intact heparin, and activity reduces stepwise with shorter fragments, such that the dodecasaccharide pools gives only

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Table I. Structural characteristics of heparan sulfates A and E determined by 500 mHz ^{1}H NMR spectroscopy

	Substituent ratios		
	N-acetyl/ *N*-sulfate	2-*O*-sulfate/ *N*-sulfate	6-*O*-sulfate/ *N*-sulfate
HSA	0.9	0.4	0.6
HSE	0.3	1.0	0.5
Heparin (5th International Standard)	0.2	0.7	0.6

The *N*-acetyl content was determined from the integral of the *N*-acetyl methyl signal, divided by three, *N*-sulfate from the integral of the GlcNS H2 signal, and 6-sulfate from the integral of the GlcNS,6S H6 signal.

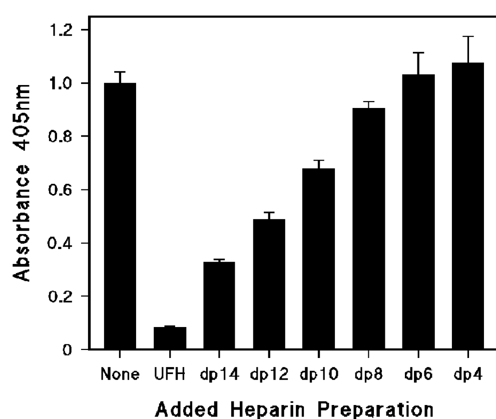

Fig. 3. Inhibition of binding of rhGDNF to heparin complex by heparin oligosaccharides. Heparin and heparin-derived oligosaccharides were employed at a concentration of 2 μg/ml. UFH, intact heparin; Dp, degree of polymerization, that is, number of hexoses present. Each column represents the mean of six replicate wells with error bars showing SEM. The results shown are for a single experiment representative of three.

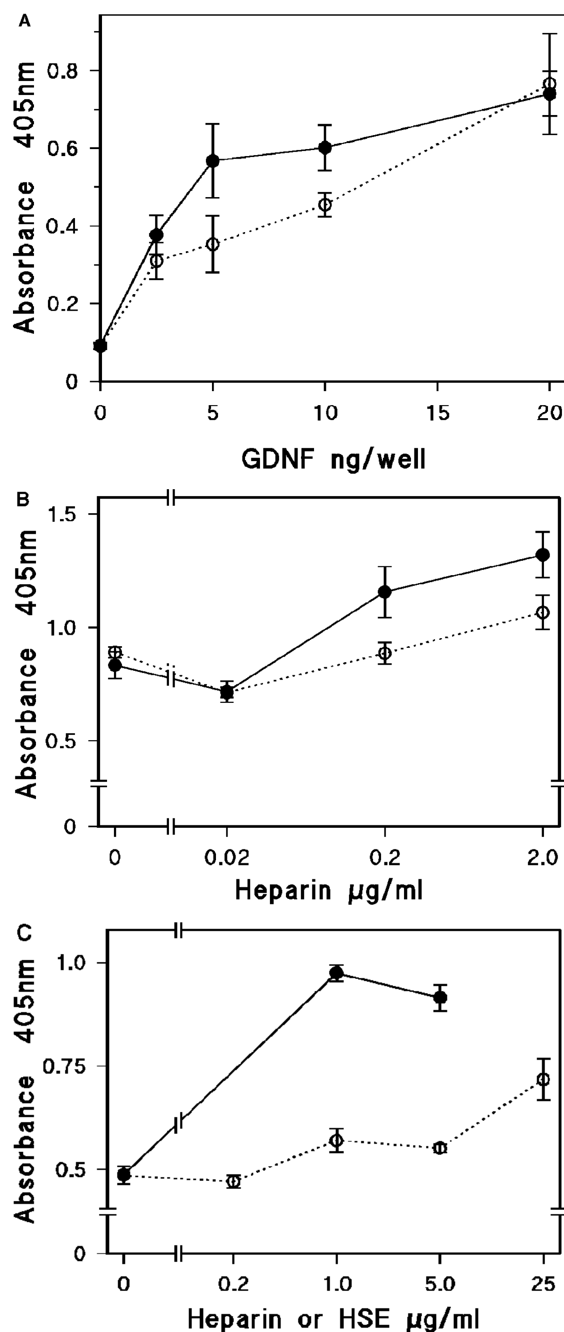

Fig. 4. Effect of heparin and 2-*O*-desulfated heparin on the binding of rhGDNF to immobilized chimeric GFRα1. (**A**) Binding of increasing amounts of rhGDNF in the presence (●) or absence (○) of 10 μg/ml heparin. (**B**) Binding of 10 ng/well rhGDNF in the presence of increasing concentrations of heparin (●) or 2-*O*-desulfated heparin (○). (**C**) Binding of 10ng/well rhGDNF in the presence of increasing concentrations of heparin (●) or heparin sulfate HSE (○). Each symbol is the mean of hextuplicate wells, and each panel shows the data from a single experiment representative of three or four.

around 50% inhibition of binding, and the hexasaccharides and below are inactive.

Where a cytokine is found to bind to glycosaminoglycan, a key question is how this might affect subsequent engagement of its cell surface polypeptide receptor. To address this issue we studied the binding of rhGDNF preincubated in the presence or absence of heparin to a recombinant chimera of the GDNF-specific receptor, GFRα1, captured via its Fc domain to ELISA well surfaces. As may be seen in Figure 4A, in the presence of heparin, rhGDNF not only continues to bind to the GFRα1 chimera but also, at subsaturating concentrations of GDNF, binding is in fact increased. This increase was consistently observed. In three independent experiments conducted under identical conditions, the binding of 5 ng/ml rhGDNF on preincubation with presence of 10 μg/ml heparin increased by 50–150%.

We next sought to compare the effectiveness of 2-*O*-desulfated heparin with intact heparin in increasing the binding of rhGDNF to the GFRα1 chimera. As may be seen in Figure 4B, heparin concentrations of 0.2 μg/ml and higher produce an increase in rhGDNF binding similar to that observed in Figure 4A. However, the 2-*O*-desulfated derivative is less effective in promoting binding. Over a series of four independent experiments, this was most

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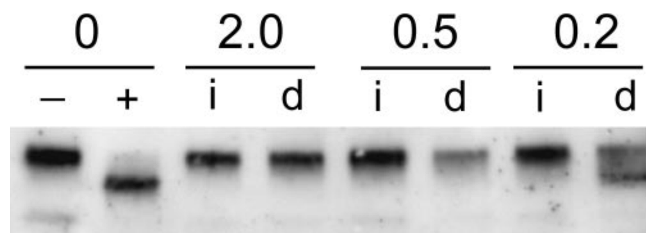

Fig. 5. Protection of rhGDNF from proteolysis by endoproteinase LysC. RhGDNF was incubated in the absence (0), or presence of either intact heparin (i) or 2-*O*-desulfated heparin (d). The figure shows the heparin preparation concentration in μg/ml. LysC was added to all sample incubations except the single track on the left (–). The intense band in this undigested track migrates at around 35 kDa. Results shown are for a single experiment representative of three.

clearly and entirely reproducibly seen at low heparin concentrations of heparin, because 0.2 μg/ml of the intact preparation gave 30–115% increases in GDNF binding, whereas the same concentration of the 2-*O*-desulfated product gave no reproducible increase.

We also sought to determine whether or not HS, like heparin, could potentiate the binding of rhGDNF to the GFRα1 chimera. The results of a single experiment, representative of a series of three, are shown in Figure 4C. Here heparin at 1 μg/ml is seen to double the amount of rhGDNF captured by the immobilized GFRα1 chimera. This increase is entirely consistent with the data shown in Figure 4A and B and appears maximal under the experimental conditions because there is no further increase with 5 μg/ml heparin. Equivalent concentrations of HSE give essentially no increase in GDNF–GFRα1 binding, however at 25 μg/ml HSE a 25% increase in binding is seen.

It has been shown for several other cytokines that binding to heparin provides protection from proteolytic degradation. As shown in Figure 5, digestion of rhGDNF with LysC *in vitro* resulted in the clipping of the GDNF polypeptide; as on western blotting, the GDNF immunoreactive band migrates marginally faster. Under conditions that gave essentially complete clipping of the polypeptide, concentrations of heparin as low as 0.2 μg/ml completely protected the cytokine from this degradation. However, 2-*O*-desulfated heparin was less active in this regard; as with 0.2 μg/ml the product migrates as a smeared doublet with substantial immunoreactivity migrating faster, like the clipped polypeptide, albeit with some unprocessed polypeptide remaining. In addition to demonstrating a protective effect, these experiments provide a demonstration, independent of ELISA, that heparin binds with high affinity to GDNF.

Discussion

We show here dose-dependent binding of rhGDNF to immobilized heparin. This binding is competed out by soluble heparin with an IC_{50} of 0.1 μg/ml. This value is similar to those we have previously obtained for fibroblast growth factor-2 (Najjam *et al.*, 1997) and IL-12 (Hasan *et al.*, 1999) using the same method and an order of magnitude smaller than the IC_{50} values of 2 and 5 μg/ml we similarly obtained for IL-6 (Mummery and Rider, 2000) and IL-2 (Najjam *et al.*, 1997), respectively. This indicates that compared to a range of other cytokines, rhGDNF has a relatively high affinity for heparin.

As well as soluble heparin, several other glycosaminoglycans compete for the binding of rhGDNF to the immobilized heparin complex. As in our previous studies of IL-2 (Najjam *et al.*, 1997) and IL-6 (Mummery and Rider, 2000), but not in the case of IL-12 (Hasan *et al.*, 1999), fucoidan is a more potent inhibitor than heparin. Fucoidan is highly sulfated, with the major disaccharide repeat unit being a trisulfated difucose, in which both 2C positions are sulfated (Chevolot *et al.*, 2001). In contrast to the unbranched chains of mammalian glycosaminoglycans, it also has a branched, comb-like structure (Patankar *et al.*, 1993). Thus fucoidan offers a high density of sulfate groups with particularly diverse spatial organizations, enabling it to furnish potential binding sites for clusters of basic residues on the surfaces of polypeptides.

When comparing the results obtained here with rhGDNF for glycosaminoglycan inhibition of binding with those obtained previously by the same method with other cytokines, subtle differences in the patterns of inhibition are evident. For instance, in contrast to the results obtained here for GDNF where chondroitin sulfates A, B, and C are all significant inhibitors, albeit with modest potency, in the case of IL-6, only chondroitin sulfate A provided significant inhibition of binding (Mummery and Rider, 2000), and with IL-12 only chondroitin sulfate B was a significant inhibitor (Hasan *et al.*, 1999). These variations indicate that these different cytokines have differences in specificity for the glycosaminoglycan structures with which they interact.

The use of chemically modified heparins and HSs again reveals unique aspects in the binding of GDNF to heparin. In contrast to the results with rhGDNF here, equivalent studies of IL-6, using the same modified heparin preparations, showed that 2-*O*-sulfated heparin was the most active competitor, and moreover that the various HSs used were essentially indistinguishable from each other as partial inhibitors (Mummery and Rider, 2000). In addition to revealing an essential role for 2-*O*-sulfates in the binding of GDNF to heparin, the present findings also indicate some involvement of 6-*O*-sulfates and *N*-sulfates. In the case the *N*-desulfated heparin, the partial recovery of inhibitory activity on re-*N*-acetylation was also seen in our studies of the heparin-binding properties of the HIV-1 envelope glycoprotein gp120. It may be accounted for either by the blocking of the positively charged, which would otherwise occur on the unsubstituted amino group of the *N*-desulfated glucosamine, or by the requirement for a bulk derivative group on the amino group, whether charged (sulfate) or uncharged (acetyl), to maintain an appropriate conformation within the heparin chain for binding (Rider *et al.*, 1994).

The nitrous acid cleavage method used to generate the heparin oligosaccharides used in this study cleaves the chains preferentially at *N*-sulfated glucosamine residues (Shively and Conrad, 1976). The inhibitory activity here appears particularly sensitive to this treatment, compared to previous results using the same oligosaccharides in the study of rhIL-12 (Hasan *et al.*, 1999). Together with the sensitivity of inhibitory activity to chemical desulfation, particularly 2-*O*-desulfation, and the observation also that the most active

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HS is the highly sulfated HSE preparation, this all suggests that highly sulfated domains within heparin/HS chains (Gallagher *et al.*, 1992) are important in GDNF binding.

Taken overall, our findings show that GDNF binds strongly to heparin/HS and that in this interaction 2-*O*-sulfate groups play a particularly prominent role. This provides strong biochemical support for a hypothesis that the critical role for 2-*O*-sulfated HS in the developmental activity of GDNF, demonstrated in the *HS2ST*$^{-/-}$ mouse (Bullock *et al.*, 1998), arises from the direct binding of GDNF to 2-*O*-sulfate-rich HS. How might such binding influence the bioactivity of GDNF? One important outcome of glycosaminoglycan binding, particularly for small proteins, is that the free diffusion of the protein away from sites of secretion will be slowed and restricted. Interestingly, in a study of the infusion of rhGDNF and other GFLs into rat brain, it was observed that coinfusion with heparin increased the volume of diffusion fivefold, and the authors speculated that this might arise through the blocking of the binding of the cytokine to sites in the extracellular matrix (Hamilton *et al.*, 2001). In kidney development, GDNF in the embryonic metanephric blastema functions as a chemoattractant factor (Schedl and Hastie, 2000), and it is entirely plausible that in the absence of high-affinity binding to HS, GDNF diffuses away, and thus its concentration falls below a critical threshold. A second mechanism to explain the dependence of GDNF activity on HS, for which we provide some *in vitro* evidence, is that glycosaminoglycan protects the cytokine from proteolytic degradation. We show here that 2-*O*-desulfated heparin is less effective in this regard.

Finally, we provide further evidence in support of a direct binding hypothesis to explain the dependence of GDNF bioactivity on intact heparin-like glycosaminoglycan. This is that heparin-bound GDNF still engages its cognate receptor polypeptide, GFRα1. Indeed, we report a modest potentiation of this first interaction in the GDNF signaling pathway. This effect is observed not only with heparin but also with a highly sulfated preparation of HS, albeit with the latter being less efficient in this regard.

The activity of GDNF in kidney development is especially sensitive to quantitative change, because unusually some 30% of mice heterozygotes for GDNF gene disruption show the absence of one or both kidneys (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). It could therefore be argued that the combined effect of the mechanisms revealed thus far for the involvement of HS in the biological functioning of GDNF might be adequate to explain the phenotype of the *HS2ST*$^{-/-}$ mouse. Nonetheless, it remains likely that there are additional mechanisms to be elucidated by further studies of effects of heparin and HS on GDNF signaling.

Materials and methods

Reagents and materials

Porcine intestinal mucosal heparin (sodium salt, grade I-A), chondroitin sulfates A–C, HS from bovine kidney, and fucoidan were all purchased form Sigma-Aldrich (Poole, Dorset, UK). Low and highly sulfated porcine intestinal mucosal HSs, HSA, HSE, and size-fractionated pools of heparin oligosaccharides obtained by nonexhaustive nitrous acid cleavage of porcine intestinal mucosal heparin, were obtained as described previously (Hasan *et al.*, 1999). Glycosylated rhGDNF and soluble recombinant rat GFRα-1/human IgG_1 chimeric protein, both expressed in a murine myeloma cell line, were purchased from R&D Systems Europe (Abingdon, Oxford, UK), as was goat polyclonal–rhGDNF. Murine anti-human IgG Fc ascites, alkaline phosphatase–conjugated rabbit anti-goat IgG, *p*-nitrophenol phosphate tablets, and endoproteinase LysC (E.C. 3.4.21.50) were purchased from Sigma-Aldrich. Preabsorbed alkaline phosphatase–conjugated rabbit anti-goat IgG was from Jackson Immunoresearch (West Grove, PA). NUNC Maxisorb 96-well ELISA plates were obtained from Life Technologies (Paisley, Scotland).

NMR spectroscopy

^{1}H NMR spectroscopy was performed using a Varian Unity 500 MHz spectrometer, with deuterated trimethylsilylpropionic acid as an external reference. The resulting spectra were all in agreement with those reported by Mulloy *et al.* (1994) and Yates *et al.* (1996).

Preparation and characterization of chemically modified heparins

The parent material for all modified heparins was bovine lung heparin from the 2nd International Standard heparin. *N*-desulfation of heparin and its subsequent re-*N*-acetylation were performed as described by Mulloy *et al.* (1994). Selective 2-*O*-desulfation and 6-*O*-desulfation, including re-*N*-sulfation of the latter to correct for a degree of *N*-desulfation, were performed as fully described elsewhere (Ostrovsky *et al.*, 2002). The extent of desulfation at specific positions was assessed by ^{1}H NMR spectroscopy. The intensity of the characteristic signal for H2 of *N*-sulfated glucosamine in the spectra of *N*-desulfated, re-*N*-acetylated heparin was below the limit of detection, indicating that the *N*-desulfation was at least close to completion. Likewise, the signal for H2 of 2-*O*-sulfated iduronate, which is prominent in the ^{1}H spectrum of the parent heparin, was undetectable in the spectrum of the 2-*O*-desulfated heparin. Similarly, the signals for H6 of the 6-*O*-sulfated glucosamine were undetectable in the 6-*O*-desulfated material. In the case of each of the eventual selectively desulfated products, the NMR spectra showed no detectable nonspecific desulfation.

Heparin-binding ELISA

A covalent heparin–BSA conjugate was synthesized by coupling the reducing ends of the heparin chains to the protein using sodium cyanoborohydride, as fully described elsewhere (Najjam *et al.*, 1997), except that 34 mg BSA (equivalent to 0.5 μmol) and 910 mg heparin ($\cong$ 75 μmol) were added to the reaction mixture. The resulting complex was separated from the unconjugated reactants by gel filtration on Sepharose 4B (Amersham Pharmacia Biotech, Little Chalfont, UK) (Najjam *et al.*, 1997). Mock-treated BSA was prepared with cyanoborohydride in the same way but in the absence of heparin with subsequent gel filtration.

ELISA wells were coated with either 5 ng heparin–BSA complex (as measured by BSA content) or mock-treated

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BSA and washed as described previously (Mummery and Rider, 2000). After blocking for 5 min with phosphate buffered saline (PBS) containing 1% (w/v) BSA, wells were washed three times with PBS, and rhGDNF diluted in PBS-BSA was added for 2 h at room temperature. In competitive experiments, rhGDNF was preincubated with glycosaminoglycans in PBS-BSA for 30 min before addition to the plate. Wells were washed three times with PBS containing 0.05% (v/v) Tween 20; anti-GDNF, diluted 1/200 in PBS-BSA, was added for 30 min. Following an additional three washes with PBS-Tween, alkaline phosphatase second antibody (Sigma) diluted 1/1000 in PBS-BSA was added. After 30 min, wells were washed three times with PBS-Tween and twice more in PBS. Substrate solution, 100 μl/well was added and after a suitable color development, typically around 30 min, the plate was read at 405 nm in an Emax plate reader (Molecular Devices, Sunnyvale, CA). All plate incubations were carried out at room temperature with gentle shaking, except for the final incubation with the substrate, which was carried out at 37°C with rapid shaking in microtitreplate incubator (Wellwarm 1, Labsystems, Cambridge, UK).

GDNF–GFRα1 binding ELISA

ELISA plate wells were coated overnight at 4°C with 100 μl mouse anti-human IgG ascites fluid diluted 1/200 in 30 mM sodium bicarbonate buffer, pH 8.5. After three washes in PBS, wells were blocked for 30 min with PBS-BSA at room temperature. After three washes with PBS, GFRα1 chimera, 20 ng/well, was added in PBS-BSA for 1 h before washing again in PBS. rhGDNF, preincubated in PBS-BSA in the absence or presence of the appropriate glycosaminoglycan, was then added and incubated for 90 min before more PBS washes. Anti-GDNF diluted 1/300 in PBS-BSA was added, and the remainder of the assay was conducted as described, except the alkaline phosphatase second antibody was from Jackson Immunoresearch and diluted 1/250.

LysC digestion of GDNF

Endoproteinase LysC was stored frozen at 0.1 μg/ml enzyme in 20 mM Tris–HCl buffer, pH 8.0, containing 0.05% 2-mercaptoethanol. Aliquots of 65 μl containing 100 ng GDNF and 5 μg BSA carrier protein in the same buffer but without mercaptoethanol were preincubated in the absence or presence of varying concentrations of bovine lung heparin or its 2-*O*-desulfated derivative for 30 min at lab temperature; then 5 μl of 15-fold diluted LysC stock solution was added. After incubation for 75 min at 37°C, reactions were stopped by the addition of concentrated sodium dodecyl sulfate–polyarcrylamide gel electrophoresis sample buffer, followed immediately by boiling for 3 min. Samples containing 15 ng GDNF were then separated on 14% acrylamide gels, which were overrun by 50% of the time needed for the tracker dye to reach the bottom of the gel.

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Abbreviations

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GDNF, glial cell line–derived neurotrophic factor; GFL, GDNF family ligand; GFR, GDNF family receptor; HS, heparan sulfate; HS2ST, heparan sulfate 2-*O*-sulfotransferase; IL, interleukin; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; rh, recombinant human.

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