The Binding of the Bone Morphogenetic Protein Antagonist Gremlin to Kidney Heparan Sulfate: Such Binding is not Essential for BMP Antagonism.

Arnold Junior Tatsinkam[a,b], Naomi Rune[a], Joy Askew[a,c], Jill T. Norman[d], Barbara Mulloy[a] and Christopher C. Rider[a,e]

[a] Centre for Biomedical Sciences, Royal Holloway University of London, Egham Hill, Egham, Surrey, TW20 0EX, UK. [b] Present address: Department of Pathology, Anatomy, and Cell Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA. [c] Present address: The Rayne Institute, St. Thomas' Hospital, Kings College, London, SE1 7EH, UK. [d] UCL Centre for Nephrology, University College London, Royal Free Campus, Rowland Hill Street, London NW3 2PF, UK.

[e] Corresponding author: email; c.rider@rhul.ac.uk

Abstract

Gremlin-1, a bone morphogenetic protein (BMP) antagonist, has essential roles in kidney and limb bone development, and is important in chronic diseases including tissue fibrosis. It also functions as an activating ligand of the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2), and binds strongly to the sulfated polysaccharide, heparin. Here we investigated the extent to which gremlin binds to the related polysaccharide heparan sulfate (HS), which unlike heparin is widely distributed spread within tissues. We determined that both highly sulfated HS and kidney HS are able to partially compete for the binding of heparin to gremlin, whereas low sulfated HS is a poor competitor. In further investigations of the interaction between gremlin and HS, we found that wild-type gremlin is able to bind broadly across the various regions of kidney in an HS-dependent manner, with particularly intense binding to tubular structures in the renal cortex. In a model of chronic kidney disease, fibrotic changes in the kidney result in a loss of gremlin binding sites. Gremlin mutants with reduced affinity for heparin showed negligible binding under the same conditions. These mutants nonetheless remain functional as BMP antagonists on C2C12 myoblastic cells transfected with a Smad 1 reporter gene construct. Overall our findings indicate that on secretion, gremlin will bind to HS structures on the cell surface and in the extracellular matrix, thus providing for a localised reservoir which can modulate BMP activity in a temparospatially restricted manner. Although binding of heparin/HS to gremlin has been shown elsewhere to be necessary for gremlin activation of VEGFR2, this does not appear to be essential for BMP antagonism by gremlin. Thus these sulfated polysaccharides differentially regulate the activities of gremlin.

Keywords

Gremlin; heparan sulfate; bone morphogenetic protein; kidney; renal cortex.

Abbreviations

BMP, bone morphogenetic protein; CAN, Cerberus and DAN; CKD, chronic kidney disease; DAN, differentially screening-selected gene abberative in neuroblastoma; FGF, fibroblast growth factor; GAG, glycosaminoglycan; HS, heparan sulfate; MGR, mutant gremlin; TGFβ, transforming growth factor beta; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

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1. Introduction

The secreted glycoprotein gremlin, also known as gremlin-1 and Drm (down regulated by *mos*), is one of seven members of the CAN (Cerberus and DAN) family of bone morphogenetic protein (BMP) antagonists. The CANs share considerable structural homology, most notably within their TGF-β superfamily cystine-knot domains, and function as high affinity ligands for BMPs, blocking subsequent BMP receptor engagement, and signalling [for reviews see 1,2]. Since the BMPs have various roles in cell differentiation, organogenesis and developmental morphogenesis, their antagonists including the CANs, are involved in the regulation of these processes [1]. In particular, mice homozygous for *Gremlin* gene knock-out show neonatal lethality due to severe defects in limb and kidney development [3,4]. The role of gremlin in regulating BMP activity in skeletal development is further demonstrated by manipulation of its expression level under the control of the osteocalcin promoter. Transgenic overexpression of gremlin results in reduced bone density and a high rate of spontaneous fractures [5], whereas its conditional deletion results in a transient increase of bone volume and mass due to increased osteoblast activity [6].

Gremlin is also seen to be important in chronic diseases. In cancer, gremlin overexpression has been implicated in the progression of certain carcinomas [2]. Most recently, aberrant overexpression of gremlin in the intestinal epithelia was shown to drive the formation of ectopic adenomatous crypts which give rise to mixed cell morphology intestinal and colonic polyps [7]. Gremlin is also re-expressed in adult organs during pulmonary hypertension and fibrotic diseases of the lung, eye, liver and kidney [8]. Chronic kidney disease (CKD) characterised by renal fibrosis, of which diabetic nephropathy is a major and increasingly common cause, is a progressive disease leading ultimately to renal failure. Expression of gremlin is upregulated in diabetic nephropathy [9]. Moreover, heterozygous deletion of the *gremlin* gene attenuates the progression of nephropathy in diabetic mice [10]. By contrast, mice with transgenic overexpression of human gremlin in renal proximal tubular epithelial cells showed increased susceptibility to folic acid-induced nephrotoxicity [11]. In cell culture models of diabetic nephropathy, *gremlin* silencing reverses pro-fibrotic changes, whereas forced *gremlin* overexpression exacerbates injury [12-14]. Taken overall, these findings strongly indicate a causative role for gremlin in CKD.

A number of the CAN family BMP antagonists interact with various non-BMP protein ligands and thereby have roles beyond the regulation of BMP signalling [1,2]. Gremlin is the only CAN known to bind with high affinity (K_d 47 ± 15nM) and activate the VEGF receptor 2 (VEGFR2) thereby promoting angiogenesis [15]. Several CANs, including gremlin, bind strongly to the sulfated polysaccharides, heparin and heparan sulfate (HS), but not dermatan sulfate or chondroitin sulfate [16, 17]. These two interactions are co-operative, in that gremlin binding to cell surface HS is required for its binding and activation of VEGFR2 [16].

In our previous studies of the heparin binding properties of gremlin we employed a docking simulation to predict a large discontinuous heparin binding site comprising 12 arginine and lysine residues, 11 of which were distributed in 3 basic sequence clusters, residues 86-91, 145-148 and 166-177 [17]. We verified this prediction by generating and expressing a panel of six mutant gremlin-Myc tagged (MGR) variants, all bearing a C-terminal Myc tag, in which different combinations of predicted contact residues were substituted with amino acids lacking basic sidechains. To minimise the possibility of disrupting the cystine-knot fold we employed residues found at these positions in DAN and Cerberus, two CANs which we have predicted not to have affinity for heparin (see Supplementary Material of [1]). Of this panel, MGR5 and MGR6, were found to be expressed at levels comparable with that of wild-type gremlin-Myc. Both of these MGRs showed markedly reduced affinity for heparin, being eluted from an immobilised heparin column at NaCl concentrations of ~0.45M, compared to 0.8M NaCl required for the wild-type gremlin-Myc. However in an ELISA, both mutants were found to bind similar amounts of BMP-4 as wild-type gremlin. Furthermore low molecular weight heparin neither promoted nor inhibited the binding of gremlin binding to BMP-4 [17]. Taken together these data suggest that binding to heparin does not affect the ability of gremlin to bind BMPs. This is in direct contrast to gremlin-VEGFR2 interactions in which heparin or HS have an essential role [16].

Although heparin is a convenient experimental representative of the heparin/HS class of glycosaminoglycans (GAGs), it has a restricted physiological distribution, being normally localised within the cytoplasm granules of mast cells, and is also subjected to high levels of sulfation and epimerisation during its biosynthesis [reviewed briefly in 18]. By comparison, HS is nearly ubiquitously distributed in the extracellular matrix (ECM) and on cell surfaces, being a product of apparently all animal cells which possess a Golgi apparatus. HS occurs in lower sulfated forms and therefore in general, interacts more weakly with basic residue clusters on proteins [18]. In particular, disaccharide analysis of murine kidney HS reveals around 40% unsulfated disaccharides, 30% monosulfated disaccharides, 18% disulfated disaccharides and 11% trisulfated disaccharides [19]. Amongst monosulfated disaccharides, 2-*O*-sulphated uronic-*N*-acetylglucosamine is notably less abundant in the kidney than in the HS of other tissues, comprising only 0.2% of total disaccharides. Overall these analyses reveal an *N*-acetyl/*N*-sulfate ratio of 1.1, and a 2-*O*-sulfate/*N*-sulfate ratio of 0.4.

In the present study we sought to investigate the interaction between gremlin and HS, in order to determine whether this might localise secreted gremlin within tissue microcompatments. Because of the emerging pathological importance of gremlin in chronic kidney disease we chose to focus on the kidney. We also sought to examine how HS binding might affect the activity of gremlin as a BMP antagonist, in order to determine whether or not the HS binding of gremlin might either block or facilitate its interactions with BMPs.

2. Materials and Methods

2.1 Materials

Mammalian-expressed murine gremlin with a C-terminal 10X-His tag, human BMP-4, biotinylated anti-human BMP-4, biotinylated goat polyclonal anti-murine gremlin, 9E10 Mab and streptavidin-alkaline phosphatase were purchased from R&D Systems (Bio-Techne), UK. NUNC Maxisorb 96-well ELISA plates were obtained from Life Technologies, UK. Unfractionated sodium heparin from porcine intestinal mucosa (Grade 1-A, H-3393) and bovine kidney heparan sulfate were obtained from Sigma Aldrich, UK. HS1 and HS2 were fractionated from porcine intestinal mucosa. HS1 (formerly designated HSA), M_r 20kDa, is relatively low sulfated, established by 500mHz ^{1}H NMR spectroscopy to have an N-acetyl/N-sulfate ratio of 0.9, and a 2-O-sulfate/N-sulfate ratio of 0.4, whereas HS2 (formerly HSE), M_r 8kDa, is more highly sulfated with an *N*-acetyl/*N*-sulfate ratio of 0.3, and a 2-*O*-sulfate/*N*-sulfate ratio of 1.0 [20].

Wild-type murine gremlin bearing a C-terminal Myc tag was cloned and expressed in CHO-S, Chinese hamster ovary cells, as described elsewhere [17]. Myc-tagged gremlin mutants, MGR5 and MGR6, both with reduced heparin binding affinity were similarly expressed [17]. Both of these proteins carry the amino acid substitutions K145M, K147A, and K148Q in cluster 2 of the heparin binding site. In addition, MGR5 is mutated in cluster 3 (R172L, K174E and R177L), whereas MGR6 has substitutions in cluster 1 (K90H and R91W). For use in cellular assays of BMP-4 antagonist activity (see subsection 2.3), conditioned supernatants were concentrated 20-fold in Amicon Ultra-15 disposable centrifugal units (10kDa cut-off: Merck Millipore, Germany).

2.2 Competitive ELISA

A covalent heparin-bovine serum albumin (BSA) complex, together with mock-conjugated BSA, was prepared by reductive end-coupling as previously described [21]. Heparin-binding ELISAs were conducted as described elsewhere [22] except that wells were coated by incubating overnight on a rotating platform at 4^{o}C with either 5ng heparin-BSA complex, as measured by protein content, or the same quantity of mock-treated BSA. After washing three times in phosphate-buffered saline (PBS), wells were blocked in PBS containing 2% (wt/vol) BSA for 1h at room temperature. Wells were then washed thrice in PBS containing 0.05% (wt/vol) BSA, hereafter referred as washing buffer. Aliquots (100µl) of conditioned supernatants from cells transfected to express gremlin-Myc, diluted as required in washing buffer, were preincubated for 15min at room temperature in washing buffer in the presence and absence of selected GAGs before addition to the wells for 90min incubation. After further washing as before, wells were incubated with primary antibody, biotinylated goat anti-murine

gremlin diluted 1/250 for 90min. Wells were then washed as before, and streptavidin-alkaline phosphatase diluted 1/1000 in washing buffer, was added for a further 30min. Finally, after one wash in PBS containing 0.05% (vol/vol) Tween 20, and two washes in PBS, wells were developed with p-nitrophenylphosphate substrate (Sigma-Aldrich).

2.3 Cellular Assays of BMP-4 Activities

Murine myoblastic C2C12 cells were grown in DMEM (GlutaMAX-1, Life Technologies) supplemented with 10% (vol/vol) heat-inactivated foetal bovine serum from the same supplier. Cells were sub-cultured at around 80% confluence by trypsinisation, and used within 20 passages of the original frozen stock. Cells were transiently transfected with 12XSBE-Oc-Luc-pGL3 reporter plasmid kindly provided by Dr Zhao Ming, University of Texas Health Science Center, USA) [23, 24] using either Lipofectamine 2000 (Life Technologies) for 24h (Figure 3 panel A), or Fugene 6 (Promega, UK) for 4h (Figure 3 panel B), according to the respective manufacturer's protocols, and then grown overnight in 24-well plates before BMP-4 and gremlin addition. Where both BMP-4 and gremlin were employed, these were pre-incubated together at room temperature for 45min prior to addition to the wells. Cells were harvested after 24h of cytokine/antagonist exposure, lysed and assayed for luciferase activity using a Luciferase Assay System kit (Promega) following the supplier's protocol. Luciferase activity was measured in a GloMax luminometer (Promega) calibrated and set to a 10sec integration period with a delay of 2sec before each reading.

2.4 Immunohistochemistry

Kidneys were bisected along the transverse axis and fixed in 4% (vol/vol) paraformaldehyde (PFA) (Sigma Aldrich) in PBS containing 0.9mM $CaCl_2$ and 0.49mM $MgCl_2$ overnight at 4^{o}C with gently agitation. Tissues were washed thrice in PBS, dehydrated through graded ethanol to xylene and embedded in paraffin wax. Sections (5µm), were cut using a Leica Jung CM3000 microtome and stored at room temperature. Immediately prior to staining, sections were deparaffinized using Histoclear (Scientific Laboratory Supplies) and rehydrated using sequential ethanol dilutions. Sections were then blocked for 1 hour in PBS containing 1% (wt/vol) bovine serum albumin (BSA; PBS-BSA) (Melford, UK) at room temperature. After three washes in PBS sections were incubated overnight at 4^{o}C with conditioned gremlin-Myc supernatants diluted 3-fold in PBS in a humidified chamber. Following a further washing in PBS, sections were incubated with anti-Myc murine monoclonal antibody 9E10 diluted 1/1000 in PBS-BSA for 90min at room temperature, and washed in PBS. Sections were finally incubated in goat anti-murine Cy3 (Life Technologies) and 4'6-diamino-2-phenylindole (Sigma Aldrich) both diluted 1/1000 (vol/vol) in PBS for 1h at room temperature. Some sections were digested with 0.25 units/ml heparitinase 1 (Sigma Aldrich) in PBS for 1h at 37^{o}C prior to incubation with wild-type gremlin supernatant. Heparitinase activity was confirmed by staining for 2 h at 4^{o}C with FITC-conjugated 3G10 monoclonal antibody helpful to indicate what this antibody

recognises (Seikagaku Corp, Japan) diluted 1/1000 in PBS-BSA. Slides were mounted in FluorSave (Calbiochem) and examined on an Olympus IX81/FV1000 confocal fluorescent microscope.

2.5 Experimental Nephrotoxicity

Chronic kidney disease was induced in adult male C57Bl/6 mice by intra-peritoneal injection of folic acid as previously described [25]. Kidneys were harvested at 2 days to 12 weeks after treatment and processed as described in Section 2.4. Control groups of 4 animals of vehicle alone-treated animals and experimental groups of 6 animals were used at each time point, with a minimum of two kidney sections stained for each individual.

3. Results

To explore the binding to various HSs of wild-type gremlin, and its mutants with reduced affinity for heparin, we employed a competitive ELISA in which the proteins were pre-incubated with soluble heparin or HS before adding to wells coated with a capture layer of a heparin-BSA complex. We have previously used this approach in HS binding studies of other cytokines including glial cell line-derived neurotrophic factor (GDNF) [19] and interleukin-12 [26]. As in our previous study of gremlin [17], both the wild-type gremlin and the two mutants studied here bind strongly heparin-coated wells in the absence of competitor, although in this study higher absorbances were obtained with the wild-type (Figure 1). This is indicative of the wild-type protein binding to more sites than the two mutants. Figure 1A shows that the binding of wild-type gremlin to the heparin-BSA- coated wells is almost totally out-competed by pre-incubation with 50μg/ml soluble heparin. This is also the case with the gremlin mutants MGR5 and MGR6. However, pre-incubation with the same concentration of the relatively low sulfated HS1 results in no significant competition with any of the three proteins. By contrast, the more highly sulfated HS2 significantly inhibits binding of all three proteins, although to a markedly lesser degree than observed with heparin, In particular inhibition of binding of MGR6 was weaker than for either wild-type protein or MGR5. Similar but weaker partial competition is seen with kidney HS, and in the particular experiment shown here there is no significant competition of MGR6 binding.

Further insight into the ability of heparin and HS to compete for gremlin binding with the immobilised heparin-BSA complex is provided by examining the concentration dependence of these effects (Figure 1B). Soluble heparin is a strong competitor with concentrations as low as 10μg/ml provided maximal competition of binding to the coated wells, with only a residual binding of around 10% remaining with all three gremlin variants. From the competition curves, IC_{50} values of around 3μg/ml for wild-type gremlin and MGR5 and 6μg/ml for MGR6 may be estimated. By contrast, kidney HS was a much weaker competitor, giving a maximum inhibition binding of only around 60% with wild-type gremlin and MGR5 at the highest concentration employed (50μg/ml), with IC_{50} values of 20μg/ml and 30μg/ml, respectively. Inhibition of MGR6 binding was even weaker with in the case of the experiment shown in Panel B, the maximal inhibition of binding attained was ~20%.

Having established that wild-type gremlin interacts with soluble HS, albeit with weaker binding than to heparin, we next investigated whether gremlin and the two mutants would be able to bind to HS chains of proteoglycans within tissues. Because of the pathological importance of gremlin in renal fibrosis, we focussed on the kidney. Since this organ comprises a number of different cell types, it was of interest to determine whether or not gremlin binding might be selective for particular microcompartments within this complex tissue. Therefore paraffin-embedded sections of mouse kidney were incubated with wild-type gremlin-Myc conditioned cell culture supernatants. As may be

seen in Figure 2A, following such treatment there is extensive Myc-immunoreactivity across the kidney, with more intense staining of the cortex compared to the medulla. At higher magnification the cortical staining is seen to be strongest in the tubular structures (Figure 2B). The specificity of this staining is established in Figure 2C, in which only minimal background staining binding is observed when the gremlin-Myc conditioned supernatant was replaced by conditioned supernatant from mock-transfected cells. Likewise in Panel D, only background binding occurs in a section incubated with gremlin-Myc supernatant but developed with an isotype-matched (IgG_1) antibody in place of the anti-Myc Mab. Substitution of the wild-type gremlin-Myc with either MGR5 (Figure 2E) or MGR6 (Figure 2F) also resulted in only background binding of the anti-Myc antibody, indicating that under these experimental conditions, little or no binding of these mutants is detectable within the kidney. This marked difference in the binding of the wild-type gremlin and the two mutants occurs despite their similar expression levels in the conditioned supernatants, as previously demonstrated in a Myc-capture ELISA [17]. Binding is also reduced to background levels by preincubation of gremlin-Myc supernatant with soluble heparin (100μg/ml), an observation entirely consistent with effective competition for the heparin/HS binding site on wild-type gremlin (Figure 2G). Treatment of sections with heparitinase I prior to incubation with wild-type gremlin-Myc, also abolished staining, indicating that it is dependent on the presence of intact HS chains (Figure 2H). Following heparitinase I treatment, there is strong staining of the sections with 3G10, a Mab specific for the stubs of HS chains remaining after such treatment (Panel I), confirming efficacy of HS chain digestion.

In kidney sections from animals treated with folic acid to induce nephrotoxicity, areas of markedly reduced tubular gremlin binding were observed as early as 2 days after injection (Figure 2J). These areas of negligible staining appear to correspond to glomeruli (Figure 2J), and expanded progressively with time (Figure 2K), such that 12 weeks after injection they occupied the major area of the cortex (Figure 2L). This is further illustrated by the respective surface plots of fluorescent intensity (Supplementary Figure S1). The regions of low gremlin binding corresponded to areas of tubular injury and disruption. No areas of reduced gremlin-Myc binding were observed in control mice treated with vehicle only (data not shown).

We previously demonstrated that, compared to wild-type gremlin-Myc, MGR5 and MGR6 have markedly reduced affinities for heparin [17]. However, in an ELISA in which these three gremlin variants were captured on a surface of immobilised 9E10 anti-Myc Mab, all bound comparable amounts of BMP-4. Moreover we showed that addition of soluble low molecular weight heparin to this assay neither inhibited nor potentiated the binding of BMP-4 by wild-type gremlin [17]. Thus binding of heparin does not appear to affect the interaction between gremlin and one of its major cytokine ligand. However these particular experiments were conducted with isolated proteins level *in vitro*. They are therefore unable to preclude the possibility that interactions of gremlin with cell surface-localised proteoglycan HS might affect the ability of the antagonist to regulate BMP signaling

at the plasma membrane of the cell where both HS proteoglycans and BMP receptors are present as membrane intercalated macromolecules. To compare the antagonist activity of the gremlin mutants with intact gremlin we employed C2C12 cells transiently transfected with the 12XSBE-Oc-Luc-pGL3 reporter plasmid previously developed for the assay of BMP activity [23, 24]. This plasmid contains the gene encoding firefly luciferase under the control of the osteocalcin promoter, preceded by 12 copies of a Smad 1 protein binding element. As may be seen in Figure 3A and B, exposure of these cells to recombinant human BMP-4 results in potent induction of luciferase expression. This induction increases progressively over the range 0-10nM BMP-4 (data not shown). Using sub-maximal BMP-4 concentrations, 3-5nM, we found that commercial, purified recombinant gremlin-His was able to substantially reduce the induction of luciferase when used in molar excess (Figure 3B). Concentrated wild-type gremlin-Myc supernatant was also able to significantly inhibit the luciferase expression in a concentration-dependent manner (Figure 3A), although there was some inter-experiment variation in the magnitude of this inhibition (Figure 3 A and B). Similarly concentrated conditioned supernatants of the MGR5 and MGR6 (Figure 3A and B respectively) also showed significant inhibition, indicating that these too are active BMP-4 antagonists at the cellular level. However as indicated in Figure 3A and B, it was noted that over a series of experiments the mutant gremlin-Myc proteins were less inhibitory than the wild-type protein. This was despite the Myc-capture ELISA and Western blotting studies showing comparable levels of gremlin immunoreactivity in all the conditioned supernatants employed here [17].

4. Discussion

An increasing number of secreted proteins involved in cellular signalling are known to bind to heparin and HS, leading to a growing interest in the role of these GAGs in signal pathway activity. In this context, the BMPs represent a particularly interesting case for investigation because not only are a high proportion of BMPs known to be heparin binding proteins, but so too are the majority of their specific protein antagonists [1]. This is the case for both the antagonist gremlin [16, 17] and its BMP ligands including BMP-4. These are small soluble glycoproteins which would be expected to be readily diffusible on secretion. However their association of HS chains on cell surfaces and in the extracellular matrix would restrict diffusion and thereby serve to localise their biological activities close to the sites of secretion.

These interactions raise a number of questions regarding how heparin-related GAGs within the tissues might affect BMP signalling. A fundamental issue here is the extent to which binding interactions with heparin, a highly sulfated variant of this class of GAG but of restricted cellular distribution, also occur with HS, which due to its wide cellular distribution is of greater physiological and pathological relevance [18]. A complication with HS, is that although it is generally less sulfated than heparin, a broad spectrum of HSs with widely varying levels of sulfation and other modifications occur [18]. However both heparin and HS possess alternating sulfated and unsulfated domains along their GAG backbones.

We show here that the three HS variants used, including kidney HS, are weak competitors for the binding of gremlin to immobilised heparin, compared to soluble heparin itself. HS2 and kidney HS gave similar levels of competition and were more effective than the low sulfated HS1. These observations fit the general pattern for proteins binding to heparin and HS, that interactions with the latter GAG are weaker compared to those with the highly sulfated heparin. However our data reveal subtle differences in HS interactions with gremlin compared to those of GDNF and IL-12, which we have previously subjected to similar investigation. In the case of GDNF, both HS1 and HS2 competed more strongly than kidney HS [20], whereas with IL-12, neither kidney HS nor HS1 provided any significant competition, but HS2 did [26]. Such variation indicates that the heparin/HS binding specificity of gremlin is different from that of either of these two proteins. Indeed, we found that the binding of GDNF to heparin has an unusual dependence on 2-*O*-sulfates. By contrast Chiodelli et al. [16] have shown that gremlin binding shows more equal dependence on 2-*O*-, 6-*O*- and *N*- sulfation. Taken overall, gremlin fits the paradigm for heparin/HS binding, that although in general higher levels of sulfation tend to promote greater binding, each protein preferentially recognises differing GAG chain structures.

Since kidney comprises several differentiated cell types organised into a complex tissue architecture, kidney HS has considerable structural variation with an intricate distribution pattern across its cellular

compartments. Van den Born et al. [27] employed a panel of anti-HS monoclonal antibodies with differing structural specificities to demonstrate that this is indeed the case. Thus, monoclonal antibody 3G10, which is comparatively insensitive to HS chain structural variations as it recognises the HS chain stubs remaining after heparitinase digestion, stains strongly all extracellular structures throughout kidney sections following treatment with this enzyme. By contrast 10E4, which recognises mixed N-acetylated and N-sulfated domains, specifically stains Bowman's capsule and the basal laminae of vascular smooth muscle cells, whereas 865, which is specific for unsulfated domains, preferentially stains the glomerular basement membrane [27]. Variation in HS chain structure will affect the binding of proteins, as has shown to be the case with the alternative complement pathway regulatory proteins, properdin and Factor H. In proteinuric models, both of these proteins bind to the luminal side of renal tubular epithelial cells, but to different HS epitopes [28]. Our immunohistochemical studies here show that wild-type gremlin binds to HS in a widely distributed manner throughout the kidney, but preferentially within the tubular compartment of the kidney cortex. This implies that on secretion of gremlin within the kidney, the protein will tend to associate with the HS on adjacent cell surfaces especially in the tubular compartment, thereby restricting its diffusion within the organ. Gremlin expression is undetectable in normal kidney, but is observed in cellular glomerular crescents and in tubular epithelial cells in diabetic nephropathy [29]. However our observations in the model of nephrotoxicity employed here, consistent with a large body of data showing over-expression of heparanase-1 in renal fibrosis and a loss of tubular HS [reviewed in 30], suggest that gremlin re-expressed in CKD will tend not to associate with areas subjected to fibrotic change, presumably allowing it to have longer range biological activity.

The prototypic examples of heparin binding cytokines are fibroblast growth factors (FGFs) -1 and -2. Here the activities of both are dependent on the presence of heparin or HS. A likely explanation for this arises from the ability of heparin/HS to dimerise FGFs, thus stabilising the active FGF/FGF receptor complex [31]. Several of the BMPs have been established as heparin/HS binding cytokines, but the role of cell surface HS in BMP signalling is uncertain due to somewhat conflicting results obtained thus far [32, 33]. Most of the BMP antagonist proteins also bind to heparin/HS, including gremlin [2, 16-17]. However the functional consequences of such interactions in terms of BMP signalling regulation are poorly understood, although in the case of gremlin, its secondary function as an agonist of VEGFR2 requires the presence of heparin/HS [16].

We have sought to investigate the role of heparin/HS in gremlin's function as a BMP antagonist, in part by generating gremlin mutants with reduced affinity for heparin [17]. Here we show by immunohistochemistry that two such mutants, MGR5 and MGR6, demonstrate negligible affinity for kidney HS, since under conditions in which wild-type gremlin binds broadly across kidney sections in an HS-dependent manner, binding of the two mutants fails to generate staining above background levels. However we also show here that in a well-established cellular bioassay of gremlin based on

the myoblastic cell line, C2C12, which is known to possess cell surface HS [34], that the mutants MGR5 and MGR6 are active as BMP antagonists, implying that HS binding is not essential for BMP antagonist activity. Thus overall we conclude that the role of HS is to localise secreted gremlin, with no apparent role in activating or promoting its BMP antagonist activity. We did however note that the gremlin mutants showed somewhat reduced antagonistic activity compared to the wild-type protein. This modest effect may arise from both BMPs and wild-type gremlin binding to cell surface HS, concentrating both of them in the same location as the BMP receptors. Accordingly, gremlin variants with lower HS affinity and thereby reduced cell surface binding, would thus be less likely to encounter and bind BMP.

Overall our findings indicate that secreted gremlin, by binding to cell surface HS, will exert its BMP antagonist activity in a localised manner within a complex organ such as the kidney. On the completion of development, gremlin expression falls to undetectable levels in the mature, healthy kidney but there is re-expression with the onset of pro-fibrotic changes. Our observations suggest that with the loss of HS from fibrotic areas, gremlin would no longer be restricted in its diffusion by association with this GAG.

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Figure legends

Fig. 1. Competition for binding of wild-type and mutant gremlins to immobilised heparin. A: Effect of preincubation of gremlins with soluble heparin and heparan sulfates, Wild-type (WT) and mutant gremlins were pre-incubated with soluble heparin, HS1, HS2 or kidney HS, all 50µg/ml, before adding to heparin coated wells. Solid bars, wild-type gremlin; hatched bars, MGR5, and open bars MGR6. Results shown are for a single experiment representative of 2-4 independent determinations, with each bar representing the mean of 4 individual wells, and error bars showing ±SEM. Prior to normalisation, the mean absorbance readings in the absence of competitor were 1.55, 1.03 and 0.95 for wild-type, MGR% and MGR6 respectively. Statistical analysis was by one way ANOVA with Bonferroni post hoc test. Significant difference from control value in the absence of GAG is indicated by * $p < 0.05$, ** $p < 0.0001$. B: Effect of increasing concentrations of soluble heparin (solid symbols and continuous connector line) and kidney HS (open symbols and dashed connector). □,■ wild type gremlin; Δ,▲ MGR5; and, ○,● MGR6. Each data point represents the mean of quadruplicate determinations. Prior to normalisation, the mean absorbance readings in the absence of competitor were 1.65, 0.72 and 1.04 for wild-type, MGR% and MGR6 respectively.

Fig. 2. Immunofluorescence of wild-type gremlin-Myc binding in the adult mouse kidney. A and B: sections were incubated with supernatant from wild-type Myc-gremlin transfected cells followed by Mab 9E10; primary antibody binding was visualised with Cy3-conjgated goat and murine IgG. A: longitudinal section of the kidney at low magnification showing the medulla (central) and cortex (peripheral) viewed at low magnification (x4 objective). B-I: higher magnification of kidney cortex in A. C: conditioned supernatant from mock transfected cells used to replace gremlin-Myc supernatant. D: section incubated with gremlin-Myc followed by isotype-matched IgG control to replace anti-Myc 9E10. E: section incubated with MGR5-Myc replacing wild-type gremlin-Myc. F: section incubated with MGR6-Myc. G: Wild-type gremlin-Myc supernatant was preincubated with heparin (100µg/ml) prior to addition to the section. H and I: sections were incubated with heparitinase I prior to wild-type gremlin-Myc incubation (Panel H), or incubation with FITC-conjugated Mab 3G10. All sections were counterstained with 4'6-diamino-2-phenylindole (DAPI) to visualise cell nuclei. Panels J, K and L show kidney sections, incubated as described for Panels A and B, from folic acid-treated animals, 2 days, 2 weeks and 12 weeks after injection, respectively. Scale bars: 100µm.

Fig. 3. Luciferase reporter assay of BMP-4 activity in the absence and presence of gremlin variants. C2C12 cells were transiently transfected with the 12XSBE-Oc-Luc-pGL3 reporter construct in which luciferase expression is under the control of 12-tandem repeats of a phospho-SMAD protein binding element. Cells were incubated for 24hr in the presence or absence of BMP-4 and gremlin variants as indicated, immediately followed by cell harvesting and lysis. Lysates were then assayed for luciferase activity. A; BMP-4 activity in the absence and presence of wild-type gremlin-Myc (WT-Myc) and

MGR5, both added as concentrated conditioned supernatants in the volumes indicated. 0 indicates no additions. B; BMP-4 in the absence and presence of wild-type gremlin-Myc and MGR6, added as above, or commercial recombinant His-tagged gremlin (WT GREM1-His). The results shown are for single experiments representative of at least 2 independent experiments. All bars are the means of triplicate determinations, with error bars showing SEM. Checkered columns, no BMP-4 or antagonist; solid columns, BMP-4 alone added; unfilled columns, BMP-4 plus wild type gremlin-Myc; hatched columns, BMP-4 plus MGR5; stippled column, BMP-4 plus MGR6; and cross-hatched columns BMP-4 plus commercial wild type gremlin-His. Statistical analysis was by Kruskal-Wallis non-parametric one-way ANOVA with post-hoc LSD. Significant difference from BMP-4 alone (filled columns) is indicated by * $p < 0.05$, ** $p < 0.01$,*** $p<0.001$.