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The binding of the bone morphogenetic protein antagonist gremlin to kidney heparan sulfate: Such binding is not essential for BMP antagonism

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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 temporospatially 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.

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 Rider and Mulloy, 2010; Mulloy and Rider, 2015). 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 (Rider and Mulloy, 2010). In particular, mice homozygous for *Gremlin* gene knock-out show neonatal lethality due to severe defects in limb and kidney development (Khokha et al., 2003; Michos et al., 2004). 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 spon-

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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taneous fractures (Gazzerro et al., 2005), whereas its conditional deletion results in a transient increase of bone volume and mass due to increased osteoblast activity (Gazzerro et al., 2007).

Gremlin is also seen to be important in chronic diseases. In cancer, gremlin overexpression has been implicated in the progression of certain carcinomas (Mulloy and Rider, 2015). 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 (Davis et al., 2015). Gremlin is also re-expressed in adult organs during pulmonary hypertension and fibrotic diseases of the lung, eye, liver and kidney (Costello et al., 2010). 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 (Dolan et al., 2005). Moreover, heterozygous deletion of the *gremlin* gene attenuates the progression of nephropathy in diabetic mice (Roxburgh et al., 2009). By contrast, mice with transgenic overexpression of human gremlin in renal proximal tubular epithelial cells showed increased susceptibility to folic acid-induced nephrotoxicity (Droguett et al., 2014). In cell culture models of diabetic nephropathy, *gremlin* silencing reverses pro-fibrotic changes, whereas forced *gremlin* overexpression exacerbates injury (Zhang et al., 2013; Huang et al., 2013; Li et al., 2013). 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 (Rider and Mulloy, 2010; Mulloy and Rider, 2015). Gremlin is the only CAN known to bind with high affinity (K_d 47 ± 15 nM) and activate the VEGF receptor 2 (VEGFR2) thereby promoting angiogenesis (Mitola et al., 2010). Several CANs, including gremlin, bind strongly to the sulfated polysaccharides, heparin and heparan sulfate (HS), but not dermatan sulfate or chondroitin sulfate (Chiodelli et al., 2011; Tatsinkam et al., 2015). These two interactions are co-operative, in that gremlin binding to cell surface HS is required for its binding and activation of VEGFR2 (Chiodelli et al., 2011).

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 (Tatsinkam et al., 2015). 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 Rider and Mulloy (2010)). 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 ~0.45 M, compared to 0.8 M 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 (Tatsinkam et al., 2015). 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 (Chiodelli et al., 2011).

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 Mulloy and Rider (2006)) . 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 (Mulloy and Rider, 2006). In particular, disaccharide analysis of murine kidney HS reveals around 40% unsulfated disaccharides, 30% monosulfated disaccharides, 18% disulfated disaccharides and 11% trisulfated disaccharides (Nagamine et al., 2012). Amongst monosulfated disaccharides, 2-*O*-sulfated 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 20 kDa, is relatively low sulfated, established by 500 mHz ^{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 8 kDa, 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 (Rickard et al., 2003).

Wild-type murine gremlin bearing a C-terminal Myc tag was cloned and expressed in CHO-S, Chinese hamster ovary cells, as described elsewhere (Tatsinkam et al., 2015). Myc-tagged gremlin mutants, MGR5 and MGR6, both with reduced heparin binding affinity were similarly expressed (Tatsinkam et al., 2015). 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 (10 kDa 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 (Najjam et al., 1997).