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REVIEW ARTICLE

Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists

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The BMPs (bone morphogenetic proteins) and the GDFs (growth and differentiation factors) together form a single family of cystine-knot cytokines, sharing the characteristic fold of the TGF-β (transforming growth factor-β) superfamily. Besides the ability to induce bone formation, which gave the BMPs their name, the BMP/GDFs display morphogenetic activities in the development of a wide range of tissues. BMP/GDF homo- and hetero-dimers interact with combinations of type I and type II receptor dimers to produce multiple possible signalling complexes, leading to the activation of one of two competing sets of SMAD transcription factors. BMP/GDFs have highly specific and localized functions. These are regulated in a number of ways, including the developmental restriction of BMP/GDF expression and through the secretion of several specific BMP antagonist proteins that bind with high affinity to the cytokines. Curiously, a number of these antagonists are also members of the TGF-β superfamily. Finally a number of both the BMP/GDFs and their antagonists interact with the heparan sulphate side chains of cell-surface and extracellular-matrix proteoglycans.

Key words: bone morphogenetic protein (BMP), cytokine, growth and differentiation factor (GDF), heparan sulphate (HS), morphogen, transforming growth factor-β (TGF-β) superfamily.

BMPs (BONE MORPHOGENETIC PROTEINS) AND GDFs (GROWTH AND DIFFERENTIATION FACTORS): SEQUENCES AND STRUCTURES

In the present review, the BMPs are taken as encompassing the cytokines with either BMP or GDF nomenclatures. As may be seen in Figure 1, these two nomenclature systems overlap, and do not signify separate cytokine lineages; indeed in many instances the BMP/GDF designations are redundant. Jointly this is a family of some 20 highly related cytokines within the larger TGF-β (transforming growth factor-β) superfamily. The BMPs were originally isolated in the search for the regenerative molecules within bone matrix (reviewed in [1]). On peptide sequencing and gene cloning, the BMPs were found to possess the seven characteristically spaced cysteine residues indicative of the cystine-knot motif of the TGF-β superfamily. The exception among the BMPs is the product of the *BMP-1* gene, which is orthologous to the tolloid gene in *Drosophila*. The encoded product is a protease, and structurally quite unrelated to TGF-β, which exerts its pro-BMP activity by cleaving the BMP antagonist chordin, and also by releasing BMPs from their latent pre-protein complexes [2]. The GDFs were identified later, in the search for additional members of the TGF-β superfamily [3,4].

Solved high-resolution three-dimensional structures are found in the PDB for BMPs 1, 2, 3, 6, 7 and 9, and for GDF-5 (Supplementary Table S1 available at http://www.BiochemJ.org/bj/429/bj4290001add.htm). The structure of BMP-1 confirms it as a zinc endopeptidase with a structure related to astacin and the tolloid-like metalloproteinases [5]. All of the other structures are cystine knots of the TGF-β type, which may be described as "a narrow eight-membered ring comprising two intra-chain disulphide bonds, with a third cystine passing through the ring" [6] (see Figure 2A for the structure of a typical cystine knot). The two long sequences between Cys (1) and Cys (2), and Cys (5) and Cys (6) (Figure 1A), form slightly curved parallel finger-like β-sheet structures, extending away from the knot in the opposite direction to the N- and C-terminal sequences. The sequence between Cys (3) and Cys (4) contains a loop of variable size and an α-helical region. The analogy of a left hand is often used for convenience of description, with the cystine knot at the palm and the α-helix and pre-helix loop referred to as the wrist [6]. In these structures, most of the N-terminal sequence upstream of the cystine knot is missing, presumably due to conformational flexibility; this is particularly significant for BMP-6 and BMP-7 (Figure 1A).

Dimerization takes place with the 'wrist' region of one monomer tucked into the concave face of the fingers of the other (Figure 2B). Cys (4) of each monomer forms a disulfide bond, bringing the two cystine knots of the monomers close together.

The seven cysteine residues typical of the TGF-β motif are not completely conserved in the BMP family. The normal tandem pair of Cys (4) and Cys (5) are only present as a single cysteine in the case of GDF-3, GDF-9 and BMP-15; as Cys (4) is usually involved in the interchain disulfide bridge covalently linking the two chains of TGF-β type dimers, it is likely that this feature will be lacking in these three cytokines. GDF-3, as well as myostatin and GDF-11, have an additional cysteine residue in the N-terminal region upstream of Cys (1). The latter two cytokines have a second additional cysteine residue positioned immediately after Cys (1). As TGF-β2 also possesses two additional cysteine residues in equivalent positions, and these are known to form a disulfide bridge [7], an equivalent Cys–Cys-enclosed loop may be expected in myostatin and GDF-11.

Abbreviations used: ActR, activin A receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPR, BMP receptor; Dan, differential screening-selected gene aberrative in neuroblastoma; CAN, Cerberus and Dan; Dpp, decapentaplegic; EMT, endothelial-to-mesodermal transition; FSTL, follistatin-like; GDF, growth and differentiation factor; HS, heparan sulphate; LRP, low-density-lipoprotein-receptor-related protein; PRDC, protein related to Dan and Cerberus; R-SMAD, regulatory SMAD; TGF-β, transforming growth factor-β; TSG, twisted gastrulation; USAG-1, uterine sensitivity-associated gene-1.

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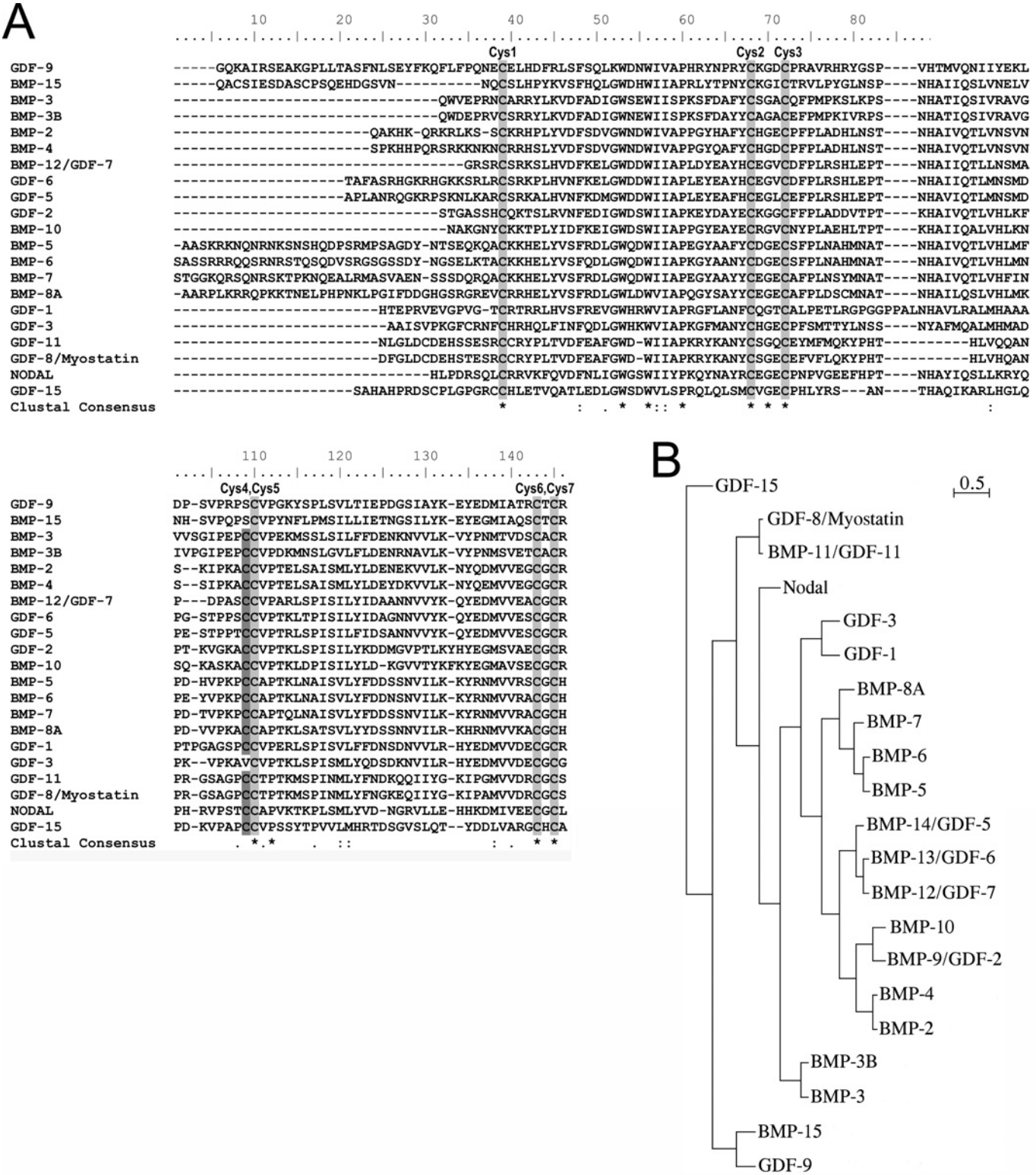

Figure 1 Alignment and phylogenetic tree for the BMP/GDF group of murine cytokines

(**A**) Alignment generated using the ClustalW multiple sequence alignment program and (**B**) phylogenetic tree generated using the UPGMA clustering algorithm, and visualized using the NJplot program for the murine BMPs/GDFs. UniProt accession numbers (except where indicated) are as as follows: BMP-2, P21274; BMP-3, P97737; BMP-4, P21275; BMP-5, P49003; BMP-6, NCBI accession number AAB18235; BMP-7, P23359; BMP-8, P34821; BMP-9/GDF-2, Q9WV56; BMP-10, Q9R229; BMP-11/GDF-11, Q9Z1W4; BMP-12/GDF-7, NCBI accession number NP_03855S; BMP-15, Q9Z0L4; GDF-1, NCBI accession number NP_032133; GDF-3, Q07104; GDF-5, P43027; GDF-6, P43028; GDF-8/myostatin, NCBI accession number NP_034964; GDF-9, Q07105; GDF-15, NCBI; accession number NP_035949.

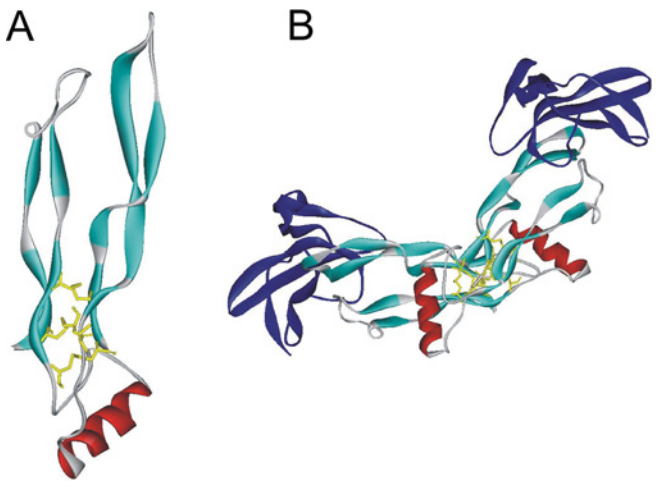

Figure 2 Structure of BMP-7

(**A**) Ribbon representation of the BMP-7 monomer (PDB code 1BMP). The stylised 'hand' consists of two finger-like double strands upwards of the cystine knot, with the loop and helix forming the wrist. (**B**) A similar representation of the BMP-7 dimer, complexed with the type II receptor ActRII (PDB code 1LX5). The BMP-7 dimer, stabilized by a disulphide bond, is formed by the facing 'palms' of the two hands, with fingers facing in opposite directions; their tips and 'knuckles' (shown in purple) interact with the type II receptor. Each type II receptor interacts with a single BMP monomer, but the type 1 receptor requires the dimer, binding to the more central area of the dimer.

Within the BMP family, there is a considerable degree of sequence similarity, especially within the cystine-knot domain. A particularly strongly conserved sequence located midway between Cys (1) and Cys (2) can be represented as: G/K-W-$X_{1/2}$-W-I/V-I/V-A/S-P (where X is any residue). This occupies the tip region of the first β-strand loop, and is an important region for receptor contact (see below).

The BMP/GDFs, like other TGF-β cytokines, are generated from much larger precursor proteins. The mature cytokines are cleaved from the N-terminal pro-regions after dimerization, and in almost all cases the mature BMP dimer is secreted from the cell without its pro-region. However, in GDF-8 (myostatin) and GDF-9, the pro-region remains associated with the dimeric ligand after secretion; in GDF-8 this pro-region inhibits binding to the receptor, but this is not the case for GDF-9 [8]. BMP-7 is also secreted as a complex of the mature protein still associated with its pro-domain despite proteolytic cleavage [9,10].

Some BMPs are glycosylated. There is a conserved N-glycosylation sequence (NXS/T) in the pre-helix loop of the BMP-2/4 and -5, -6, -7 and -8 groups, and also in GDF-3. Glycosylation of BMP-6 at this site (Asn^{73}) has been found to be essential for recognition by activin receptor type I, but not by BMPR (BMP receptor)-1A and B [11].

Biological function and signalling

The hallmark of the BMPs is their ability to induce bone formation *in vivo* by promoting osteoblast differentiation. This can be measured in cell culture by the transdifferentiation of myoblastic cell lines, such as C2C12, to an osteoblastic phenotype, with the down-regulation of muscle-type marker proteins and up-regulation of osteoblastic markers, such as alkaline phosphatase (see [12] for a review on early studies on the functional activity of the BMPs). Unsurprisingly, gene knockout of BMP expression often results in skeletal abnormalities; however, BMP-3 appears in fact to be an antagonist of this archetypal BMP activity [13]. In addition to their effects within skeletal tissue, BMPs have additional and quite distinct developmental activities in other tissues. For instance, homozygous gene deletion of BMP-7 expression results in perinatal lethality due to severe renal dysplasia [14,15]. This phenotype is not observed with other BMP/GDF-knockouts, thereby revealing a unique and critical role for BMP-7 in kidney development. As further examples of the distinctive roles of BMPs among more recent work, BMP-9 has been shown to be regulator of hepatic glucose homoeostasis [16] and BMP-10 has been demonstrated to have an essential role in the regulation of embryonic cardiomyocyte proliferation during heart development [17]. In addition, the two close paralogues GDF-9 and BMP-15 are tightly restricted in their expression to the male and female germ cells where they have roles in fertility [18]. Overall the BMP family has a wide range of morphogenetic and developmental activities in many tissues. Some of these roles are shared among several family members, others are restricted to a few and yet others are unique to individual cytokines. One question we address in the present review is how such a homologous family of cytokines exerts such a diverse range of activities.

The signalling mechanisms of the BMP family have been extensively studied and reviewed previously [19]. Like other members of the TGF-β cytokine superfamily, the cell-surface receptors are heterodimers. Although there is clearly some selectivity as to which ligands are able to signal via which heterodimer combinations, promiscuity is also evident. Of the seven type I TGF-β superfamily receptors, six [ALK (activin receptor-like kinase)-2–ALK-7] are employed by one or more of the BMPs. Likewise three [ActR (activin receptor) IIA, ActRIIB and BMPRII] of the five type II receptors have been implicated in BMP signalling. Our current understanding of receptor usage by the individual BMPs has been reviewed previously [20]. Among the complicating issues is the unknown extent to which different type I and type II receptor polypeptide combinations can give rise to functional signal-transducing complexes. Furthermore, in the physiological context, BMP heterodimers exist. As the assembled cytokine–receptor complex is envisaged to have two separate type I–type II receptor dimers, each interacting largely with only a single subunit of the cytokine dimer [19], the existence of biologically active BMP heterodimers increases the number of functional ligand–receptor complexes which might be assembled.

Several crystal structures of BMP–receptor complexes have been solved, including those of the ternary complexes of BMP-2 with BMPR1A and ActRII [21,22]. Although contact between BMP and the receptors is close and extensive, the two receptor dimers do not interact with each other on the outside of the cell. The type II receptor binds to a single BMP monomer (at the knuckle region on the convex side of the fingers; Figure 2B), but the type I receptor requires the BMP dimer, as it interacts with several amino acid residues near the interface between the two monomers. From structural information gathered so far, it seems that the geometry of the BMP–receptor complex is similar regardless of the individual identity of the BMP or receptor, so that changes in amino acids at the BMP–receptor interface must be the dominant influence in the variations of affinity noted between BMPs and their receptors [21,22]. It follows that the engineering of mutant BMPs to alter relative receptor affinities is possible, and even single amino acid changes have been shown to alter the receptor affinities of BMP-3 [23]. Likewise, the single amino acid change L51P in BMP-2 causes deficient receptor type I binding, so that this mutant becomes a receptor-inactive inhibitor of the BMP antagonist noggin [24]. Conversely, the single residue Arg^{57} of GDF-5 confers receptor specificity for BMPR1B [25].

BMP engagement by active receptor complexes leads to phosphorylation and subsequent nuclear uptake of R-SMAD (regulatory SMAD) transcription factors [19]. There are two sets of R-SMADs: SMAD-1, -5 and -8, and SMAD-2 and 3. These two sets of R-SMADs counter each other's activities. Examples of BMPs activating SMAD-1, -5 and -8 include BMP-2, -4, -5, -6 and -7, whereas SMAD-2 and -3 are activated by others including GDF-1, BMP-3, BMP-11 and GDF-8. Although close paralogues within the BMP family tend to give rise to phosphorylation of the same R-SMAD set, this is not universally the case. Notably, whereas GDF-9 leads to SMAD-1, -5 and -8 phosphorylation, BMP-15 gives rise to SMAD-2/3 phosphorylation. The phosphorylation of particular R-SMADs is determined by the type I receptor involved, with ALK-2, -3 and -6 being specific for SMAD-1, -5 and -8, whereas ALK-4, -5 and -7 exhibit specificity for SMAD-2 and -3 [20].

In essence therefore the canonical pathway of BMP signalling is a binary mechanism, in which one of two alternative sets of R-SMADs is activated [19]. At this simplistic level it is not possible to explain how the various individual BMP cytokines have such myriad and distinct activities. There are however several mechanisms which do provide some explanations for this. First, expression of the BMPs shows tight control in terms of tissue location and developmental stage. A notable example, as mentioned above, is the restriction of GDF-9 and BMP-15 to male and female germ cells [18]. Secondly, the individual receptor polypeptides also show tightly regulated expression patterns. Thirdly, many observations show that the BMPs function in a highly localized paracrine manner. One reason for this is that, like TGF-β, although the mature proteins are small and diffusible, they are initially secreted as membrane-bound pro-proteins from which they require release by specific proteolysis. Finally, there are a number of specific, high-affinity antagonist proteins, as discussed below. The activity of a BMP at a given tissue microcompartment will therefore depend not solely on its own expression, but also the presence or absence of particular antagonists.

BMP ANTAGONISTS

Among the high-affinity BMP antagonists are follistatin and its paralogues FSTL1 (follistatin-like 1) and FSTL3 [26,27]. These proteins share multiple copies of a characteristic ten-cysteine-containing domain with a characteristic fold [28]. Remarkably, other BMP antagonists are distant members of the TGF-β superfamily, sharing the same cystine-knot domain as their BMP cytokine ligands [29]; these are referred to as the CAN [Cerberus and Dan (differential screening-selected gene aberrative in neuroblastoma)] family of proteins.

Follistatin and FSTL proteins

Follistatin was originally characterized in the 1980s as an inhibitor of the pituitary FSH (follicle-stimulating hormone) secretion present in ovarian follicular fluid (reviewed in [30]); it was thus the first identified TGF-β superfamily antagonist protein. Its main mode of action is via its very high affinity for activin, a TGF-β cytokine outside the BMP family [31]. Estimates of the dissociation constant for this interaction, in the 0.03–0.3 nM range, confirm this, but binding between BMPs (including BMP-4, -5, -6, -7 and -15 and myostatin) and follistatin, have also been shown, albeit at lower affinities in the nanomolar range [32–34]. It is now clear that follistatin has roles beyond the reproductive system [30] and among more recently studied roles are those in skin and hair follicle development [35], muscle hypertrophy [36] and as an adipokine [37]. The FSTLs have been less extensively investigated, but FSTL1 has been proposed to be an immunoregulator. Both pro-inflammatory activity, up-regulating interferon-γ expression in experimental arthritis [38], and immunomodulatory activity, down-regulating pro-inflammatory cytokines, such as interleukin-6, interleukin-17A and interferon-γ in allograft tolerance [39], have been proposed. The balance of these apparently contradictory activities may depend on the particular cytokine milieu that pertains in different pathophysiological situations. Comparison of the cytokine binding specificity of follistatin with FSTL3 reveals some differences, with the former, but not the latter, showing BMP-6 and -7 compete for activin binding [34].

Cerberus

Cerberus was originally isolated as a product of the Spemann organizer in *Xenopus*, where it was able to induce ectopic heads, and duplicate hearts and livers in embryos [40], as well as antagonise the signalling of BMP-4, Nodal and Wnt proteins [41]. In mouse the Cerberus orthologue remains a marker of tissue-specifying anterior patterning [42], but it is no longer essential for head development, possibly due to redundancy of the mammalian factors regulating the anterior–posterior axis [43–45].

Chordin

Chordin was also originally characterized in studies of the products of the Spemann organizer. It is an outlier of the CAN family by having polypeptides possessing four cysteine-rich domains, rather than just one. Moreover these domains are a variant of the TGF-β motif, in having ten conserved cysteines [46]. These domains are possessed by a large number of proteins, including several closely related paralogues [47]. Among these are neuralin, chordin-like protein-1 and ventroptin, with three cysteine-rich domains, and crossveinless-2, with five such domains, both of which are BMP antagonists [48]. By contrast, KCP (kielin/chordin-like protein), with 18 of these domains functions as an enhancer of BMP signalling [49].

Coco

Coco is a *Xenopus* protein important during embryogenesis in establishing both the anterior–posterior [50] and left–right [51] axes.

Dan

Dan is one of the founder members of the CAN antagonist family. It is important in the patterning of the avian inner ear [52] and shows highly selective expression patterns in developing murine forebrain [53] and axon tracts [54]. Bioassays on *Xenopus* embryos indicate it is a more effective antagonist of GDF-5 compared with BMP-4 and -7 [54].

Gremlin

The gene encoding gremlin, now more precisely termed gremlin-1, was first identified as *drm* (down-regulated by the oncogene mos). It is highly expressed in non-dividing terminally differentiated cells, including neurons [55]. Gremlin-1 is increasingly being implicated in chronic fibrotic diseases. In the organogenesis of kidney and lung, endothelial cells are derived by transdifferentiation from mesodermal cells, a process driven by BMPs. It is now emerging that in chronic diseases of

these organs, fibrosis occurs via the reverse of this process, i.e. EMT (endothelial-to-mesodermal transition). The re-expression of gremlin-1 in pathological states is now seen to be a key step in driving EMT in both kidney [56,57] and lung [58,59]. Fibrosis in these organs is chronic, progressive and currently irreversible, and can lead ultimately to their failure. Gremlin-1 is similarly implicated in the ophthalmologic diseases, vitreoretinopathy and glaucoma [60,61]. In addition, gremlin-1 is highly expressed in the stromal cells of a wide range of carcinomas, implying that it is an important component of the cancer-cell niche [62]. More recently, gremlin-1 has been identified as a potent angiogenic factor due to its ability to induce the expression of angiopoietin-1 in endothelial cells [63].

Despite its compact size, gremlin-1 has been reported to be a multifunctional protein by binding avidly not only to BMPs, but also to the Slit proteins 1 and 2 [64]. This behaviour is shared with Dan. The Slits are repellent axon guidance cues, and inhibitors of leucocyte chemotaxis. They are critical in the development of lung, kidney and mammary gland, and are structurally quite unrelated to BMPs. Gremlin-1 binds Slits at a site distinct from its BMP-binding site, and strongly potentiates the inhibitory activity of Slits on leucocyte chemotaxis [64].

Noggin

Noggin was the first-characterized of several proteins, secreted by the Spemann organizer of the early *Xenopus* embryo, that function as developmental morphogens by inducing anterior markers. It was shown to bind with high affinity to human BMP-4 with a K_d of 19 pM, and also to BMP-2 and -7 [65]. The high resolution crystallographic structure of noggin complexed to BMP-7, the first of any BMP antagonist, confirmed that noggin has the typical fold of the TGF-β superfamily [66]. This also revealed that antagonism occurs by the β-strand loops of noggin being longer than those of the BMPs, and bending over the tips of their shorter counterparts on the cytokine, thereby blocking the receptor-binding sites [66,67].

It is clear that the fine control of BMP activity resulting from the balanced expression of the BMPs and noggin is important not only within the skeleton, but also in the morphogenesis of a number of organs including heart [68], pituitary [69], prostate [70] and thymus [71]. Noggin has activities of potential significance in regenerative medicine, such as the promoting the formation of oligodendrocytes [72], and neural precursors [73,74], including dopaminergic neuronal precursors in embryonal stem cell cultures [75]. It also expands neural stem cell numbers in the adult hippocampus *in vivo* [76]. In bone metastases of prostrate and breast cancer, noggin expression is associated with an osteolytic rather than osteoblastic behaviour [77], as might be predicted from its BMP antagonist activity.

PRDC (protein related to Dan and Cerberus)

PRDC (also sometimes referred to as gremlin-2 due to its close similarity to gremlin, with which it shares 69 % amino acid identity in the cysteine-rich motif sequence) was first isolated in a gene-trap screen for developmentally important genes [78]. PRDC has since been shown to be a potent antagonist of BMP-2 and BMP-4, and to bind to these cytokines with high affinity [79]. The same study also showed PRDC to be expressed in a number of tissues, with the highest levels of mRNA in ovary, brain and spleen. More recently, the ability of PRDC to antagonize BMP-2 activity and participate in the regulation of osteogenesis *in vitro* has been confirmed [80].

Sclerostin

Sclerostin, encoded by the gene *SOST*, was originally identified in a search for the gene mutated in sclerosteosis, a progressive inherited condition characterized by skeletal overgrowth which is most pronounced in the skull [81]. As the expression of sclerostin is highly localized to osteoblasts and osteocytes, it offers an attractive target for therapies aimed at promoting bone deposition as reviewed elsewhere [82]. Indeed rodent studies on both normal and osteoporotic animals have shown that neutralizing sclerostin antibodies can promote bone deposition [83–85]. Sclerostin is an antagonist of BMPs, binding to them with high affinities, K_d of 1.0–3.5 nM, [86]. An interesting aspect of the bone antagonist properties of sclerostin is its ability to form also a high affinity complex with noggin (K_d of 2.9 nM), which thereby neutralizes the BMP-antagonistic activity of both proteins [87]. However, sclerostin also modulates bone deposition by inhibiting the canonical Wnt signalling pathway. Sclerostin binds with high affinity to the Wnt co-receptors, LRP (low-density-lipoprotein-receptor-related protein)-5 and -6, thereby inhibiting Wnt signalling [88,89]. Thus sclerostin appears to be especially effective in inhibiting bone deposition by modulating two independent signalling pathways. Recent structural studies suggest that the LRP-binding site in sclerostin is within an unusual additional loop comprising part of the polypeptide sequence lying between the two β-strand fingers characteristic of the TGF-β family protein fold [85]. This additional loop replaces the short α-helix found in most TGF-β family structures.

TSG (twisted gastrulation)

Vertebrate TSG, the orthologue of *Drosophila* counterpart, has been shown by genetic studies to be a BMP antagonist that functions as a morphogen [90–93]. In addition to its interaction with chordin, TSG interacts, with crossveinless-2, a chordin homologue. Although the latter is usually considered a BMP antagonist [48], it appears to exert either pro- or anti- BMP activities, depending on the developmental context [94]. Thus the modulation of BMP activity by TSG may involve a complex regulatory network which has yet to be fully elucidated.

USAG-1 (uterine sensitivity-associated gene-1)

USAG-1 (also known as Wise) was identified as an mRNA up-regulated in uterine glandular epithelial cells with the onset of pregnancy [95]. Like sclerostin, USAG-1 is both a BMP antagonist [96] and also a modulator of Wnt signalling, binding to the Wnt co-receptors LRP-5 and -6 [89]. These two distinct functions have been shown to arise from two separate binding sites [97]. USAG-1 has been strongly implicated in the progression of renal fibrosis in experimental models of chronic kidney disease [96].

STRUCTURES OF THE CYSTINE-KNOT BMP ANTAGONISTS

The cystine-knot-containing BMP antagonists in the human genome are made up of: (i) two multi-domain proteins, TSG and chordin; (ii) the non-standard cystine-knot protein noggin; and (iii) the CAN family [29]. Of these, the CAN family resemble the BMPs themselves, with an eight-membered cystine knot, two fingers and a wrist region. Experimentally determined structures are listed in Supplementary Table S2 available at http://www.BiochemJ.org/bj/429/bj4290001add.htm.

The cystine knot of TSG has a nine-membered ring, that of chordin has a ten-membered ring, whereas noggin has a slightly

different ten-membered ring. There are no solved structures for TSG and chordin, but a chordin family member from Zebrafish, crossveinless-2, has been crystallized in complex with human BMP-2. Binding to BMP-2 takes place through a von Willebrand type C domain, which wraps round one BMP monomer like a paper clip, obscuring both type I and type II receptor sites ([98]; PDB code 3BK3).

Noggin is a cystine-knot dimer with a ten-membered knot, showing the same topology as the BMP eight-membered ring knot, but having a long N-terminal sequence with some helical regions disposed along the length of the two fingers. The crystal structure of noggin in complex with BMP-7 shows that noggin has a different 'head-to-head' mode of dimerization compared with BMP-7, resulting in a longer dimer ([99]; PDB code 1M4U) than BMP-7 and able to form a clamp-like structure round it. The type II receptor site of BMP-7 is obscured by the tips of the noggin fingers and a 'clip' region near the N-terminus fastens round the Type I receptor site of each BMP-7 monomer.

Solution structures have been determined for the CAN family member sclerostin, both human ([85]; PDB code 2K8P) and murine ([100]; PDB code 2KD3). As expected, the cystine knot is eight-membered, with an extra disulfide bond attaching the two fingers together (Figure 3A; see also the Supplementary text and Supplementary Table S3 available at http://www.BiochemJ.org/bj/429/bj4290001add.htm). Sclerostin has no helical segment in the wrist, only a long and disordered loop; it also has a marked positively charged stripe along its long axis which acts as a heparin-binding site (Figure 3B), though it has also been identified as a possible binding site for the Wnt co-receptor LRP-5 [100]. Similarity between sclerostin and the other CAN proteins is sufficiently high to allow the use of sclerostin as a basis for modelling the structures of human forms of USAG-1, Cerberus, Coco, Dan, PRDC and gremlin (Figure 3C). In addition, the extra disulfide bond at the fingertips is an aid to sequence alignment (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/429/bj4290001add.htm).

Functional roles of BMP antagonists

Some insight into the functional roles and importance of this antagonist family can be gained from genetic manipulation of their expression *in vivo*. Table 1 shows the results of such studies on mammalian development. For Cerberus, the lack of phenotypic consequences of gene deletion is probably indicative of functional redundancy amongst the antagonists. This may also limit the outcomes of loss of expression for other family members. Support for this notion comes from the observation that the chordin/noggin double-knockout is more severe than the single deletions, resulting in embryonic lethality with severe forebrain defects and perturbation of the left–right axis in cardiac development [101]. With USAG-1 and sclerostin, the reported outcomes of gene knockout are restricted to the mineralized tissues, teeth and bones respectively. With others, there are more widespread defects, reflecting the broader morphogenic patterning roles of the BMPs. Thus modulating their activities through altering the expression levels of their antagonists has more widespread morphogenic effects. Interestingly, gene knockout of chordin causes gross defects in the patterning of head and neck tissues, whereas loss of noggin mostly affects trunk and limb development. In two instances, the gene deletion experiments shown in Table 1 reflect rare naturally occurring mutations of the genes in human genetic diseases. Thus mutations of the noggin gene are found in some cases of FOP (fibrodysplasia ossificans progressiva), a severe and progressive ossification of muscles

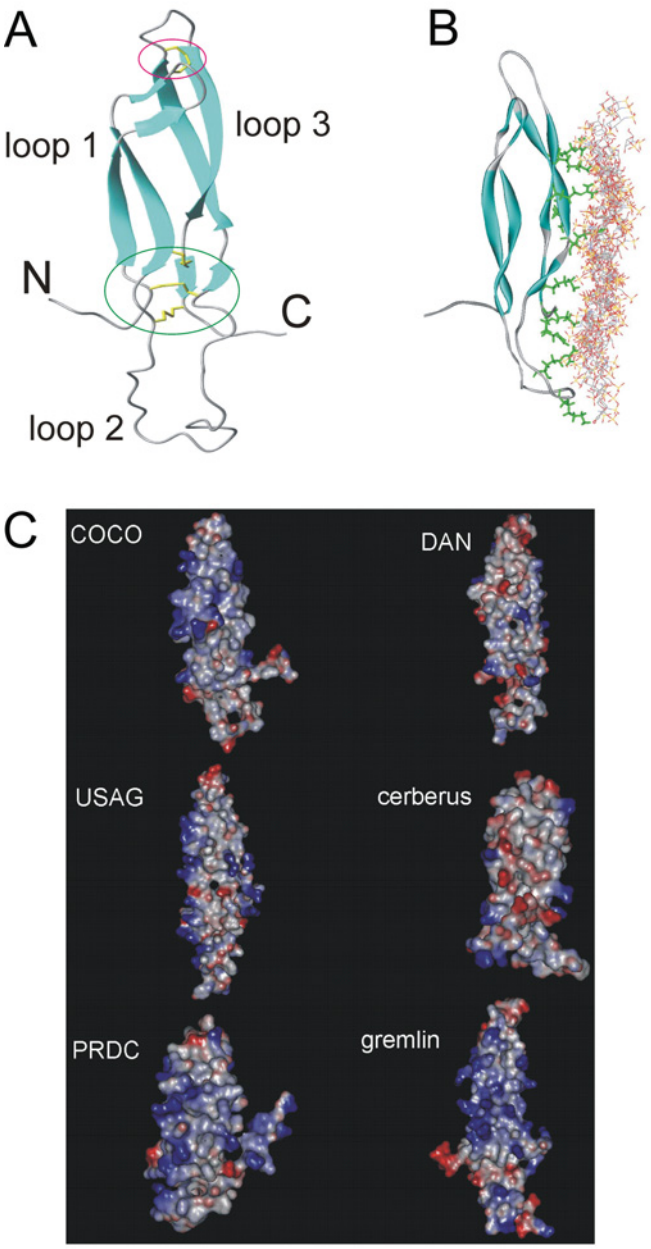

Figure 3 Structures of BMP antagonists

(**A**) Ribbon representation of the CAN family BMP antagonist sclerostin (the first of 38 structures in the NMR ensemble; PDB 2K8P). Long, disordered N- and C-terminal sequences are not shown. As with BMP-7 (Figure 2) the structure is stabilized by the cystine knot (circled in green), but in the case of the CAN family, the tips of loops 2 and 3 are also joined by a disulfide bond (circled in pink). Loop 2 does not contain a helix, and is relatively flexible in its conformation. (**B**) The same structure of sclerostin, turned through about 90°, with the arginine and lysine residues of the heparin-binding site in stick representation, interacting with heparin (shown as finer lines) as predicted using a published protocol [128]. The ten lowest energy complexes are shown. The localization of the heparin-binding site was confirmed by mutagenesis [85]. (**C**) Molecular models of the CAN family of BMP antagonists, based on simlarity with sclerostin (PDB code 2K8P). Details of the modelling protocol can be found in the Supplementary text and Supplementary Table S3 available at http://www.BiochemJ.org/bj/429/bj4290001add.htm. Each model is shown in approximately the same orientation, fingers upwards, with the pattern of basic residues predicted to interact with heparin/HS facing outwards, for all of the CAN family except Dan and Cerberus. These two proteins have fewer basic surfaces and are not predicted to have any marked affinity for heparin (see the Supplementary text for details of the prediction protocol). The homology models are, inevitably, all of similar topology, with differences in the conformations of loop 2, and the N- and C-terminal sequences accounting for the apparent differences in shape. A three-dimensional interactive structure for this Figure is available at http://www.BiochemJ.org/bj/429/bj4290001add.htm.

Table 1 Effects of genetic manipulation of *in vivo* expression of mammalian BMP antagonists

Antagonist	Genetic manipulation	Resulting phenotype
Follistatin	Homozygous gene deletion	Perinatal mortality due to respiratory difficulties, defects in multiple tissue [129]
	Conditional homozygous gene deletion in ovarian granulosa cells	Fewer and smaller litters [130]
FSTL-3	Homozygous gene deletion	Changes in glucose and fat homoeostasis, including increased insulin secretion and larger pancreatic islets with increased β-cell numbers [131]
	Conditional overexpression in gonads	Reduced fertility in both sexes [132]
Cerberus	Homozygous gene deletion	Wild-type [43-45]
Chordin	Homozygous gene deletion	Embryonically/perinatally lethal: multiple defects in patterning of head and neck tissues, and cardiovasculature [133]
Gremlin	Homozygous gene deletion	Neonatal lethality: bilateral kidney agenesis, absence of lung septa, gross skeletal defects in the limbs [134,135]
	Conditional homozygous deletion in bone	Developmentally transient increase in bone mass [136]
	Conditional overexpression in bone	Decreased body weight, decreased bone density, spontaneous fractures, tooth fragility, decreased osteoblast numbers [137]
Noggin	Homozygous gene deletion	Neonatal lethality (defects in neural tube growth, patterning and closure), gross skeletal defects in trunk and limbs; cartilage overgrowth [138]
	Conditional overexpression in bone	Smaller body size, reduced bone density, spontaneous fractures [139–141]
SOST/Sclerostin	Homozygous gene deletion	Increased bone mineral density, volume and strength [142]
	Conditional overexpression in bone	Reduced trabecular bone volume, reduced bone strength [86]
TSG	Homozygous gene deletion	Variable: dwarfism, poor survival, delayed ossification and reduced bone density, defects in lymphoid development [143]; developmentally transient decrease in trabecular bone volume [144]; reduced bone density due to enhanced osteoclastogenesis [145]
USAG-1	Homozygous gene deletion	Supernumerary teeth [146]

and joints [102], and mutations of the sclerostin gene result in hereditary hyperostosis [103,104].

Several reasons may be advanced to explain the range of differing phenotypes that emerge from these genetic studies. First, as many of the studies cited in Table 1 report, the antagonists vary considerably not only in the cell types in which they are expressed, but also in the developmental stages at which expression occurs. Both the locational and temporal regulation of endogenous antagonist expression will clearly have profound importance in tissue patterning and development. Secondly, the antagonists may show selective specificity towards the individual BMPs. Where studied, it is emerging that the individual antagonists tend to show promiscuity in terms of binding with several different BMP ligands. For instance, sclerostin binds to BMP-2, -4, -5 -6 and -7 with similar kinetics, and with high affinities in the 1.0–3.5 nM range [86]. However, such information is very limited, so the extent of cross-binding of the antagonists to different BMPs and *vice versa* is far from known. Even less certain is whether the binding of an antagonist to a BMP necessarily results in blocking the subsequent binding to all of the cognate receptors, and therefore inhibition of signalling activity. Thirdly, the antagonists may have cellular functions beyond binding to BMPs. For instance, sclerostin has been shown to bind to the Wnt co-receptors LRP-5 and LRP-6, and to function as an antagonist of the canonical Wnt signalling pathway [88], activities shared with USAG-1 [89]. This provides sclerostin and USAG-1 with a second cellular signalling pathway by which they can reduce bone mass and density. The extent to which other members of this family are Wnt as well as BMP antagonists is not known. Finally, these proteins do not necessarily antagonize BMP activity in all developmental and cellular contexts. For instance, as noted above, noggin and sclerostin bind to each other with high affinity and this duplex is unable to bind BMP-6. These observations support a model in which BMPs, noggin and sclerostin are in competition with each other to form binary complexes such that, where they are co-localized, noggin and sclerostin will neutralise each other's BMP antagonist activities, thereby facilitating BMP activity [87]. Moreover TSG and chordin are part of a complex regulatory switch mechanism which can transform TSG from a BMP antagonist to an agonist [105]; one proposed model is that BMPs, TSG and chordin form a ternary complex, resulting in strong blockade of BMP activity. However, chordin can be cleaved at several sites by the zinc metalloproteinase, tolloid/BMP-1. There is some evidence that TSG promotes the degradation of the chordin fragments, leading to release of the BMP–TSG binary complex. This is permissive to BMPR engagement and thus signalling. However, where chordin expression remains high, the binary complex will be captured by intact chordin again resulting in continued antagonism [105]. This intricate regulatory switching of TSG activity depending on the developmental context may go some way to explaining why the three groups reporting on the TSG gene knockout effect have reported variable outcomes (Table 1).

These genetic insights into the functional roles of BMP antagonists are inevitably limited, especially where embryonic or perinatal lethality arises. Studies on the expression of the antagonist, and the consequences of adding recombinant exogenous antagonist, specific antibodies or interfering RNA to cells and tissues *in vitro* have provided further insight into the function of these proteins. A considerable amount of information on biological activities of the individual antagonists has now been amassed.

BMPs and HS (heparan sulfate)

As cytokines regulating cell differentiation and functioning as morphogens, the BMPs must act in a highly restricted, localized manner. Yet, once released by proteolytic cleavage from their large membrane-bound precursor proteins, they are small, readily diffusible glycoproteins. So how can juxtacrine activity be achieved? One mechanism for restricting diffusion is for the mature BMP to remain associated with its larger pro-domain. This has been established in the case of BMP-7, and moreover the pro-domain anchors BMP-7 within the extracellular matrix through binding to fibrillin-1 [10]. A second, and more widely established mechanism is the binding of the mature, released cytokine itself to the highly acidic HS glycosaminoglycan found on the cell surface and in the extracellular matrix. Several BMPs have been found to bind at physiological ionic strength and pH to HS and its more experimentally amenable variant, heparin. BMP-2 and -4 both interact in this way, binding via clusters of basic

residues located in their short unstructured N-terminal sequences, upstream of their cysteine-rich domains. In both cases this binding has been shown to be functionally important in restricting activity locally [106,107]. The *Drosophila* orthologue of vertebrate BMP-2 and -4 is the morphogen Dpp (decapentaplegic) and this is a particularly well-studied instance of morphogen whose activity is dependent on interaction with HS. Dpp is a key factor defining the anterior–posterior axis in the developing wing. Like its mammalian counterparts, Dpp binds to heparin [108], and has a cluster of basic residues near its N-terminus that serves as the binding site [109]. It has now been clearly established that the concentration gradient of Dpp, which forms at the anteroposterior boundary across the wing, arises through transport of Opp from its sites of secretion by the HS proteoglycans dally and dally-like, which are both members of the cell-surface glypican family [110]. As vertebrates show strong conservation of not only the Dpp-like BMPs, but also the glypicans, it is entirely reasonable to expect that similar mechanisms of BMP gradient formation occur in higher organisms too. This is of pathological relevance as mutations of the glypican-3 gene give rise to Simpson–Golabi–Behmel syndrome, an X-linked condition characterized by overgrowth in multiple tissues. The limb and skeletal defects in mice cross-bred to be both glypican-$3^{+/-}$ and BMP-$4^{+/-}$ (i.e. heterozygous deficient) are more severe than those seen in the two singly heterozygous states [111]. Moreover, in micromass cultures of mesenchymal cells from developing chick-wing limb buds, overexpression of syndecan-3, a further cell-surface HS proteoglycan, inhibits BMP-2 induction of chondrogenic differentiation [112]. Such studies indicate a functional involvement of HS proteoglycans in BMP signalling and strongly implicate defects in this mechanism in the pathogenesis of Simpson–Golabi–Behmel syndrome.

Quite how HS affects BMP-2 and -4 activity remains unclear, as apparently contradictory observations have been reported. For instance in studies of the osteogenic transdifferentiation of C2C12 cells, a well-established cellular assay of BMP activity, some workers have reported that exposure of cells to chlorate, a competitive inhibitor of glycosaminoglycan sulfation, and enzymic digestion of cell-surface HS both increase BMP-2 activity, whereas addition of soluble exogenous heparin (2 μg to give 2 mg/ml) is inhibitory [113]. By contrast another group working on the same cellular bioassay reports that exogenous heparin in the range of 2–20 μg potentiates BMP-2 signalling and that digestion of cell-surface HS has no effect [114,115]. It may be possible to reconcile these findings by a model in which BMP-2 and -4 are able to bind to cell-surface HS, which may on the one hand promote signalling, by presenting the BMP to its receptors, and on the other hand facilitate internalization [113] and therefore degradation. The balance of the two activities may depend on precise experimental and physiological conditions. Further study is required to clarify the effects of HS on BMP-2 and -4 activity reported in these, and other similar, studies [116–118]. Such further investigation is potentially of applied significance as, *in vivo,* heparin and heparin-containing scaffolds promote BMP-2 induced ectopic bone production [115,119], an outcome of therapeutic potential in difficult-to-heal fractures and orthopaedic procedures.

Interaction with heparin/HS is not confined to the Dpp-like BMPs. BMP-5, -6, -7 and -8, comprise a second subfamily which show close homology with the *Drosophila* morphogen, Gbb (glass-bottomed boat). Compared with the Dpp-type BMPs this subfamily all possess considerably longer N-terminal sequences upstream of their TGF-β-type cystine-knot domains and in these longer sequences the basic residues arginine and lysine, which are key components of the heparin/HS-binding sites (reviewed in [120]), show a rather scattered distribution. Despite this, BMP-7 has also been shown to bind to heparin and HS. Exogenous soluble heparin, heparinase digestion and chlorate treatment all inhibit BMP-7 signalling in C2C12 cells, consistent with cell-surface HS serving a co-receptor function [121].

BMP antagonists and HS

An intriguing finding is that it is not just the BMPs, but also BMP antagonists that bind to heparin and HS. For instance chordin binds to heparin at physiological pH and ionic strength, via binding sites in at least three of its four cysteine-rich domains. In tissue sections, chordin shows selective binding to HS on the cell surface, but not to that of the extracellular matrix. This binding restricts the diffusion of chordin, participates in its cellular uptake and potentiates BMP antagonism [122].

Follistatin is well established as a protein with high affinity for heparin and HS. Both crystallographic and mutational studies have established that the heparin-binding site on follistatin is a sequence rich in basic amino acids located within the first follistatin-like domain [28,123]. Studies of the follistatin splice variants 228 and 315 have shown that despite earlier conclusions, these two isoforms have similar affinities for heparin, provided this is measured at physiological ionic strength. However, at elevated ionic strength, follistatin-315 has much reduced affinity for heparin, although this is restored by binding to activin, a TGF-β superfamily cytokine [124]. These findings imply that the follistatin-315–ligand complex may compartmentalize differently in the tissues compared with the free antagonist, through higher affinity to HS chains. With follistatin-288, binding to myostatin has also been shown to increase the latter's affinity for heparin as conformational changes result in the exposure of a new basic surface patch [125]. Overall these two studies raise the prospect of complex interactions between follistatin, BMPs and HS.

Noggin binds strongly to heparin and HS, and, at least *in vitro*, is retained on cell surfaces by binding to cell-surface HS proteoglycans [126]. This binding can be markedly reduced by deletion of residues 133–144, which include eight basically charged amino acids. This heparin-binding sequence lies just N-terminal to the cystine-knot domain. Interestingly this is in a similar position to the heparin-binding site in BMP-2 and -4. A mutant noggin with the heparin-binding site deleted retains apparently unaffected binding affinity for BMP-4 and has antagonist activity comparable with that of wild-type noggin [127]. That study provided further evidence that noggin binds to the highly sulfated, so-called S domains, of HS [99]. The crystallographic structure of noggin shows that in the noggin dimer, the two heparin-binding sites come into proximity with each other in an exposed position well removed from the BMP-binding surfaces [99].

More recently the CAN family antagonist sclerostin has been shown to bind to heparin and cell-surface HS [85]. The heparin/HS-binding site in this instance is principally composed of basic residues lying within the second β-stranded finger-like loop. These residues make up a positively charged surface exposed on one face of the protein. The homology models of some, but not all, of the other CAN family members also have a linear basic patch along one of the 'fingers' of the structure, a characteristic of a heparin-binding site, the two exceptions being Cerberus and Dan (Figure 3C). An established molecular modelling protocol [128] indicates that the contrast is considerable; those CAN proteins that have a potential heparin-binding site are predicted to bind strongly to heparin or HS, and the other two are predicted to have little or no affinity. The functional significance of heparin binding in this series of proteins is uncertain but offers an extra

way in which HS proteoglycans may modulate the activity of the BMP/GDFs.

Overall much remains to be determined about the involvement of heparin/HS glycosaminoglycans in the potentially complex interactions between BMPs, their cell-surface receptors and their antagonist proteins. Moreover, we do not know whether heparin/HS promotes BMP signalling or alternatively promotes antagonist activity. Indeed, it is quite possible that as different pericellular environments will vary in terms of the qualitative and quantitative availability of the various agents involved in this process both these opposing outcomes may occur, depending on the developmental context.

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multimedia online data

SUPPLEMENTARY ONLINE DATA

Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists

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Table S1 Experimentally solved structures for the BMP/GDF family of cytokines

All proteins are human except where indicated. Sequences refer to UniProt accession numbers. ECD, extracellular domain; IPA, isopropyl alcohol; MPD, 4*S*-2-methylpentane-2,4-diol; v., variant.

Protein, PDB code and reference	Oligomeric state	Sequence and mutations (UniProt accession numbers)	Ligand
BMP-1			
3EDG [1]	Monomer	C-terminal 201 residues of P13497	Zn; acetate
3EDH [1]	Monomer	C-terminal 201 residues of P13497	Zn; acetate; DMSO
BMP-2			
3BMP [2]	Dimer	C-terminal 114 residues of P12643	MPD
1ES7 [3]	Dimer	C-terminal 116 residues of P12643	BMPR1A
1REW [4]	Dimer	C-terminal 114 residues of P12643	BMPR1A
1REU [4]	Dimer	C-terminal 103 residues of P12643	MPD
2GOO [5]	Dimer	C-terminal 114 residues of P12643	BMPR1A-ECD; ActRII-ECD
2H62 [6]	Dimer	C-terminal 116 residues of P12643	BMPR1A; ActR2B
2H64 [6]	Dimer	C-terminal 116 residues of P12643	BMPR1A; ActR2B
3BK3 [7]	Dimer	C-terminal 114 residues of P12643; F41M, Y91M	Crossveinless-2 (from Zebrafish)
2QJ9 [8]	Dimer	283–397 of P12643	Human BMPR1A v. B1
2QJB [8]	Dimer	283–397 of P12643	Human BMPR1A v. 1A/1B
2QJA [8]	Dimer	283–397 of P12643	Human BMPR1A v. B12
BMP-3			
2QCQ [9]	Dimer	C-terminal 110 residues of P12645	
BMP-6			
2R52 [10]	Dimer	375–513 of P22004	IPA
2R53 [10]	Dimer	375–513 of P22004	IPA
2QCW [9]	Dimer	C-terminal 132 residues of P22004	
BMP-7			
1BMP [11]	Dimer	C-terminal 139 residues of P18075	
1M4U [12]	Dimer	C-terminal 139 residues of P18075	Noggin
1LXI [13]	Dimer	C-terminal 139 residues of P18075	
1LX5 [13]	Dimer	C-terminal 139 residues of P18075	ActRII
BMP-9			
1ZKZ [14]	Dimer	320-429 of Q9UK05	
GDF-5			
2BHK [15]	Dimer	C-terminal 120 residues of P43026	IPA
1WAQ [16]	Dimer	387–501 of P43026	MPD
3EVS [17]	Dimer	387–501 of P43026	BMPR-1B (mouse)

MODELLING OF THE CAN FAMILY OF BMP INHIBITORS

The lowest energy member of the ensemble of NMR structures (PDB code 2K8P), human sclerostin, was used as a template for homology modelling of the other human CAN family members. All of these proteins consist of a folded cystine-knot motif, with long N- and C-terminal tails. Only the core sequences, from Cys (1) to a few residues past Cys (8) [Cys (9) for Dan] were modelled, as the structure of the tails was not defined in 2K8P. Loop 2 of the cystine knot in 2K8P is also variable in structure. Sequence accession numbers (NCBI unless indicated) were: USAG, Q6X4U4.2 (UniProt); Cerberus, O95813.1; Gremlin, AAF06677.1; DAN, BAA92265.1; PRDC, NP_071914.3; and Coco, NP_689867.1, and the sequence alignments used are summarized in Supplementary Figure S1. Using the alignment given in [20], each individual CAN sequence was aligned in pair-wise mode with the 2K8P sequence and four homology models were generated using the Modeler module of InsightII (Accelrys). Models were visualized in Discovery Studio 2.5 (Accelrys).

DOCKING CALCULATIONS FOR HEPARIN OLIGOSACCHARIDES AND THE CAN FAMILY MEMBERS

Docking calculations were performed as described previously [18,21] for two heparin pentasaccharides (with iduronate in the $^{1}C_{4}$ and $^{2}S_{0}$ conformations) with flexible exocyclic bonds and for a

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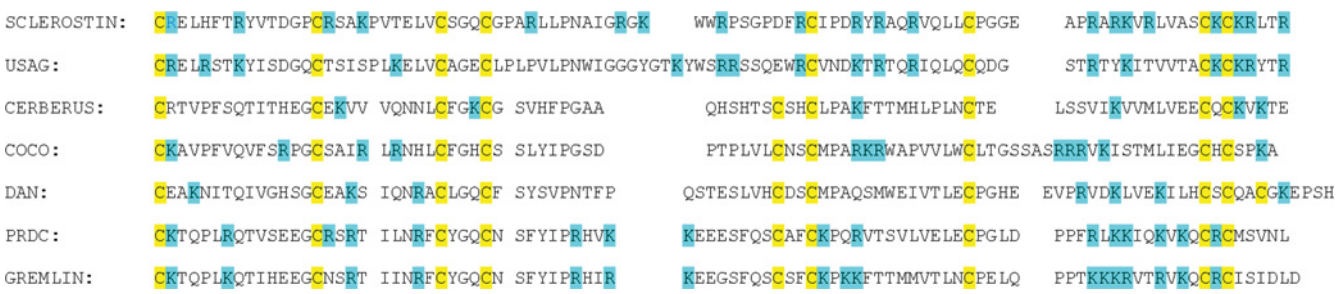

Figure S1 Alignment of the core cystine knot domains of the human CAN family BMP antagonists [20]

See the text for details of the accession numbers used. Cysteine residues are highlighted with a yellow background; arginine and lysine residues are highlighted with a blue background.

Table S2 Experimentally solved structures for the BMP antagonists

Sequences refer to UniProt accession numbers; VWC, von Willebrand factor type C.

Protein, PDB code and reference	Organism	Oligomeric state	Sequence and mutations	Ligand
Noggin				
1M4U	Human	Dimer	C-terminal 206 residues of Q13243	BMP-7
Crossveinless-2				
3BK3 [7]	Zebrafish		1st VWC domain, 27–94 of Q53734	BMP-2
Sclerostin				
2K8P [18]	Human (NMR structure)		25–213 of Q9BQB4	
2KD3 [19]	Mouse		59–167 of Q99P68	

Table S3 Calculated intermolecular energy (IE) of binding for heparin oligosaccharides with human CAN family BMP antagonists

Protein	IE for endecamer	IE for $^{1}C_{4}$ pentasaccharide	IE for $^{2}S_{0}$ pentasaccharide	Main site of interaction
Sclerostin	−5581	−2962	−2947	Loop 3 and loop 2
USAG-1	−4806	−2569	−2623	Loop 3 and loop 2
Cerberus	−577	−503	−494	
COCO	−4263	−2449	−2507	Loop 3
DAN	+587	+185	+277	
PRDC	−4805	−2619	−2599	Loop 3
Gremlin	−5358	−3155	−3222	Loop 3

rigid heparin endecamer model. One of the four homology models for each of the CAN family members was docked with all three oligosaccharides. The intermolecular energies (IE) of binding for the lowest energy docked complex in each case are summarized in Supplementary Table S2. These energy values are not precise but provide a useful, if approximate, measure of potential affinity; lower values indicate favourable binding conditions. Note the contrast between values for the two proteins Cerberus and Dan with the other members of the CAN family.

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