

BMP-binding modules in chordin: a model for signalling regulation in the extracellular space

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SUMMARY

A number of genetic and molecular studies have implicated Chordin in the regulation of dorsoventral patterning during gastrulation. Chordin, a BMP antagonist of 120 kDa, contains four small (about 70 amino acids each) cysteine-rich domains (CRs) of unknown function. In this study, we show that the Chordin CRs define a novel protein module for the binding and regulation of BMPs. The biological activity of Chordin resides in the CRs, especially in CR1 and CR3, which have dorsalizing activity in *Xenopus* embryo assays and bind BMP4 with dissociation constants in the nanomolar range. The activity of individual CRs, however, is 5- to 10-fold lower than that of full-length Chordin. These results shed light on the molecular mechanism by which Chordin/BMP complexes

are regulated by the metalloprotease Xolloid, which cleaves in the vicinity of CR1 and CR3 and would release CR/BMP complexes with lower anti-BMP activity than intact Chordin. CR domains are found in other extracellular proteins such as procollagens. Full-length *Xenopus procollagen IIA* mRNA has dorsalizing activity in embryo microinjection assays and the CR domain is required for this activity. Similarly, a *C. elegans* cDNA containing five CR domains induces secondary axes in injected *Xenopus* embryos. These results suggest that CR modules may function in a number of extracellular proteins to regulate growth factor signalling.

Key words: BMP, Chordin, Collagen, TGF β , Spemann organizer

INTRODUCTION

Recent work suggests that cell signaling in embryos is modulated by secreted proteins that bind to and inhibit growth factors in the extracellular space (Harland and Gerhart, 1997; Nieto et al., 1999). In *Xenopus*, dorsoventral patterning is regulated by the antagonistic interaction between Chordin and Bone Morphogenetic Proteins (BMPs). Chordin, a protein of 120 kDa secreted by Spemann's organizer (De Robertis and Sasai, 1996) binds to ventral BMPs in the extracellular space, preventing the interaction of BMPs with their receptors (Piccolo et al., 1996). The region in Chordin to which BMPs bind has not yet been identified.

This model of dorsoventral patterning has received strong support from genetic analyses in model organisms. In zebrafish, *chordino*, a mutant encoding a null allele of *chordin*, has a reduction of dorsal structures in ectoderm and mesoderm (Schulte-Merker et al., 1997) and genetically interacts with the dorsalizing mutant *swirl*, which is a BMP2 loss-of-function allele (Kishimoto et al., 1997). Double *chordino*; *swirl* loss-of-function mutants display a *swirl* dorsalized phenotype, suggesting that the sole function of Chordin is to inhibit BMP

signals (Hammerschmidt et al., 1996). In *Drosophila*, *short-gastrulation (sog)*, a *chordin* homologue (François and Bier, 1995; Holley et al., 1995), antagonizes *decapentaplegic (dpp)* and *screw*, which function as dorsoventral morphogens in the *Drosophila* embryo (Ferguson and Anderson, 1992; Neul and Ferguson, 1998; Nguyen et al., 1998) and are functionally homologous to vertebrate BMPs (Padgett et al., 1993; Holley et al., 1995). As is the case for *swirl* and *chordino*, *dpp* and *screw* are epistatic to *sog* in double mutants (Biehs et al., 1996; Holley et al., 1996; Neul and Ferguson, 1998), indicating that *Sog* is a dedicated antagonist of BMP signals.

Xolloid, an extracellular zinc metalloproteinase, cleaves Chordin at two specific sites (Piccolo et al., 1997). Cleavage of inactive Chordin/BMP complexes results in the reactivation of BMPs, which are then able to signal and ventralize *Xenopus* explants, providing a paradigm for how a protease can reverse an inhibitory interaction in the extracellular space (Piccolo et al., 1997). These results are in agreement with genetic studies in *Drosophila*, in which the product of *tolloid*, a *Xolloid* homologue, enhances the activity of *Dpp* (Ferguson and Anderson, 1992; Ashe and Levine, 1999) and can cleave *Sog* (Marqués et al., 1997). In zebrafish, loss-of-function mutations

in a *tolloid* homologue, *mini-fin*, cause excessive dorsal tissue to form in a manner consistent with an increased activity of Chordin (Connors et al., 1999). Despite intense biological interest, the molecular mechanism by which Chordin/BMP complexes are regulated by Tolloid proteases, including the properties of the cleavage products, remains unknown.

Chordin, like Sog in *Drosophila*, contains four cysteine-rich repeats (CRs) that share similarities, particularly in the spacing of their 10 cysteines, with many extracellular matrix (ECM) proteins, including fibrillar procollagens, thrombospondin and von Willebrand factor (François et al., 1994; Sasai et al., 1994). In the case of fibrillar procollagens I, II, III and V, which are the major components of the ECM of skin, bones and cartilage (Scriver et al., 1995), the CR domain is present in the NH₂-propeptide. It has recently been shown that BMP2 and TGFβ1 can bind to the procollagen IIA CR domain (Zhu et al., 1999), although no biological activity was demonstrated.

In the present study, we show that the biological activity of Chordin resides in the CRs, particularly in CR1 and CR3. However, we also show that these domains have BMP binding and biological activities that are 5-10 times lower than those of full-length Chordin. We propose that Xolloid inactivates Chordin by the production of fragments which, although still retaining binding activity, have less affinity for BMP than full-length Chordin. Finally, we show that type IIA procollagen containing a CR domain has dorsalizing activity in *Xenopus* assays. Therefore, procollagen IIA, which is expressed in the notochord and dorsal somites, could function as a regulatory molecule during dorsal development in vertebrates. Taken together, the results suggest that the Chordin CRs define a modular BMP-binding domain that may provide a paradigm for understanding the function of a number of extracellular proteins containing similar domains.

MATERIALS AND METHODS

Expression constructs and synthetic mRNA preparation

Full-length *chordin* (accession number AF096276) was cloned by low-stringency screening of a mouse 6.5 day embryo cDNA library prepared in this laboratory by Drs M. Blum and J. C. Izpisua-Belmonte. To construct the library, cDNA was directionally cloned into the *SacI-EcoRI* and *HindIII* sites of the λSHlox-1 vector (Novagen). The sequence of mouse Chordin has been reported by Pappano et al. (1998) and by Bachiller et al. (2000). The full-length cDNA was divided into three parts by making use of conveniently located internal restriction sites *BamHI* and *BglIII*. Each fragment was cloned into the pCS2 expression vector (Lee et al., 1995) carrying a mouse Chordin signal peptide without epitope tag.

PCR fragments of individual CRs (with 20 amino acids of flanking sequence at each end and *SacI* and *XbaI* cloning sites) were synthesized using the full-length mouse *chordin* cDNA as template and cloned into a pCS2 expression construct containing the mouse Chordin signal peptide and putative signal peptidase cleavage site (amino acids 1-32), followed by a Myc epitope tag (EQKLISEEDL) and a *SacI* cloning site. The primers used to amplify the individual repeats were:

CR1: F5'-GCCGAATTCCATGCCGAGCCTCCCGCCCCGC-CGGCCCCG-3', R5'-CTCTCTAGACTTGTCATCGTCGTCCTGTAGTCCCTTGGATACTCGAAGACCG-3'.

CR2: F5'-TGGAGCCCCAGTACCAGCCAAACATG-3', R5'-CTCTCTAGAGCCTTCTCCTGGCTCCAG-3'.

CR3: F5'-TGGAGCTCCCAGGCAGTAGAGACCTCC-3', R5'-TTATCTAGACCCAGGGCCCATCAGCC-3'.

CR4: F5'-TGGAGCTCGGGTCAGGGACTAATGCCAAG-3', R5'-TTATCTAGACTACTTGTCATCGTCGTCCTGTAGTCGCTC-CCTAGGAGTCTCCG-3'.

The full-length *Xenopus procollagen IIA* cDNA was excised with *EcoRI* from a pUC18 vector (a kind gift of Dr Francesco Ramirez) and ligated to pCS2. For the type IIA procollagen CR construct a PCR fragment (440 bp) containing the CR domain in a *SacI-XbaI* fragment was cloned into the same pCS2 expression vector used for the mouse Chordin CR domains and designated pCS2-*myc-Coll II-CR*. The primers used to amplify the collagen CR were: F5'-CGATGCCAAG-ATGAAGAAGAT-3', R5'-TTCTCTCTAGATCCTTGTTCACC-3'.

To prepare a collagen II construct mimicking the type IIB transcript lacking the CR (Ryan and Sandell, 1990), we used pCS2-*myc-Coll II-CR* as a template for deletion of amino acids D34 to S102 in *Xenopus* collagen II (Su et al., 1991) by a PCR procedure. The amplified fragment was digested with *EcoRI* and *XbaI* and cloned in a three-fragment ligation with a 4000 bp *XbaI-StuI* fragment of *Xenopus collagen II* and pCS2 vector cut with *EcoI* and *StuI*, to generate pCS2-*myc-procollagen IIB*. A similar ligation was carried out with the original pCS2-*myc-Coll-CR EcoRI-XbaI* fragment to generate pCS2-*myc-procollagen IIA*. This construct had the same activity as pCS2-*procollagen IIA* lacking the myc-tag in embryo injections and was used as the control in the experiments using *myc-procollagen IIB* mRNA. Both myc-tagged collagen constructs produced stable secreted proteins of the expected length when transfected into 293T cells. To prepare *C. elegans* CAA04886 expression vector the full-length cDNA, EST yk227e2, obtained from Dr Yuji Kohara (National Institute of Genetics, Japan) was subcloned into pCS2. Synthetic mRNAs for microinjection was prepared in all cases from pCS2-based vectors linearized with *NotI* and transcribed with SP6 Polymerase using the Message Machine kit (Ambion).

For *in situ collagen II* hybridizations, we used a 1200 nucleotide probe from the 3' region (Su et al., 1991), generated by linearizing pCS2-*AS-Coll II* with *XhoI* and transcribing with SP6 RNA polymerase. The *Xenopus chordin* probe was as described (Sasai et al., 1994).

Protein expression and quantitation

Human 293T cells were transfected at 70% confluence (Piccolo et al., 1997) with Myc-tagged CR1, CR2, CR3, CR4 or full-length mouse Chordin in pCS2 (15 µg of DNA per 10 cm Petri dish) in DMEM-10% fetal calf serum. 24 hours after transfection, a subconfluent cell monolayer was washed three times with Hank's balanced salt solution and cultured in serum-free Iscove's medium for 48 hours. Proteins secreted by 293T cells transfected with pCS2 without insert and cultured in the same way were used as a negative control throughout this study. The conditioned media were concentrated and the buffer exchanged to CMFM by Centriplus-30 (Amicon). To this end, samples were first concentrated 10 times and then diluted 10 times with CMFM; this procedure was repeated twice (Piccolo et al., 1997). Aliquots were snap frozen and stored at -80°C. Protein was quantitated on western blots using a range of concentrations of a standard protein (*Xenopus Myc-Chordin*, Piccolo et al., 1996) and an anti-Myc monoclonal antibody (Santa Cruz Biotech).

Immunoprecipitation

Mouse Chordin and CR proteins were incubated with BMP4 for 3 hours on ice in 100 µl of saline buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1.5 mM CaCl₂, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.1% Octylglucoside, 0.1% CHAPS, 5% glycerol and 0.1% BSA (IP buffer). A polyclonal anti-Myc antibody (Santa Cruz Biotech, lot no. C028, 5 µl of serum per reaction) was prebound to protein A Sepharose (Pharmacia) and 20 µl of resuspended beads were added to each reaction. In some experiments, a commercial

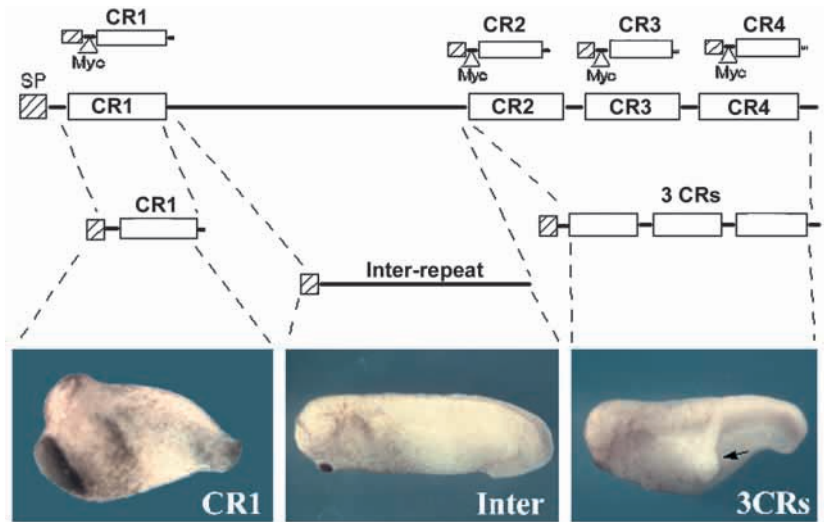


Fig. 1. The biological activity of Chordin resides in the cysteine-rich repeats (CRs). Mouse *chordin* was divided into three parts, each with a mouse Chordin signal peptide (SP). These vectors did not contain an epitope-tag. Synthetic mRNA from these constructs was injected ventrally into *Xenopus* embryos (100 pg mRNA/embryo). The individual CR constructs used in subsequent experiments are shown at the top, each with a mouse Chordin signal peptide followed by a Myc epitope tag.

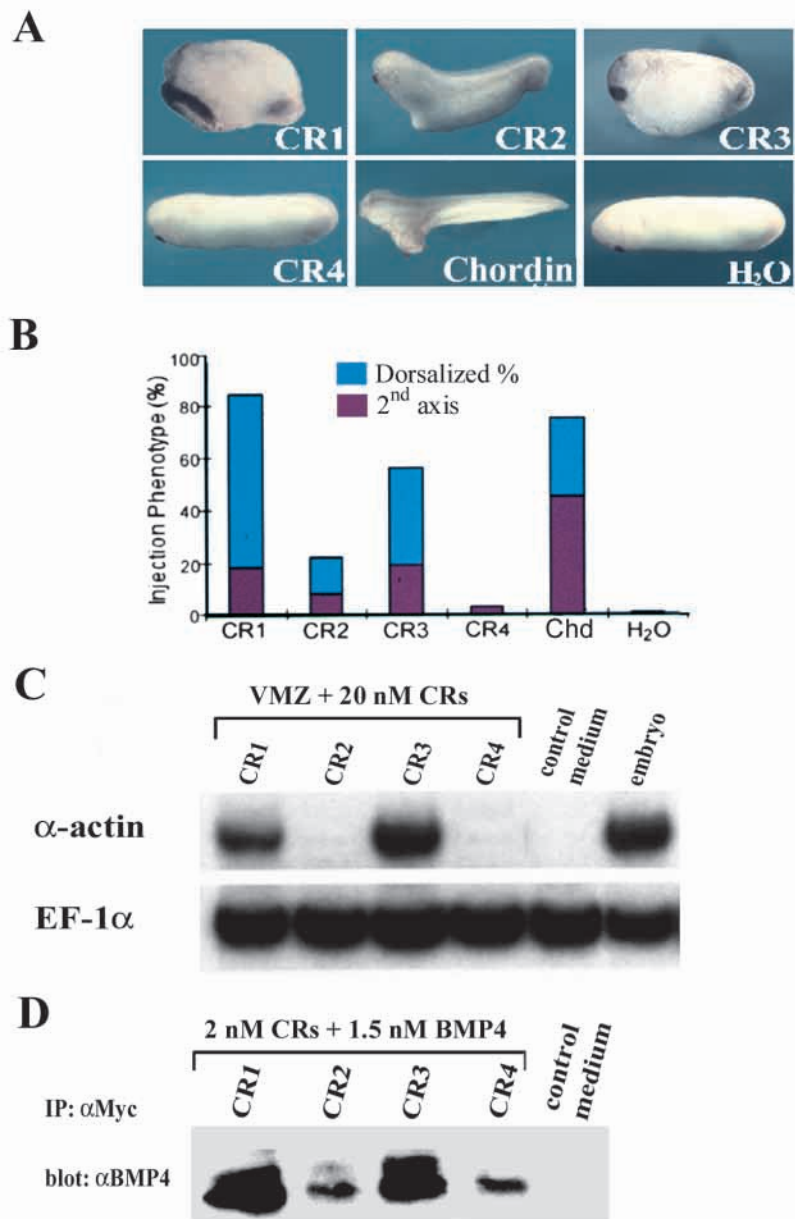
mouse BMPR-IA/Fc fusion protein (R&D Systems) was bound to BMP4 and competed with Chd or CR1 proteins and then bound to protein A Sepharose. After binding at 4°C for 2 hours in an Eppendorf tube with constant mixing, beads were pelleted at 14,000 revs/minute for 1 minute at 4°C, resuspended in precooled 1 ml of IP buffer as above, except for the omission of BSA and washed three times at 4°C (5 minute per wash). To elute BMP/CR complexes, anti-Myc beads were incubated in 20 μ l of 1 M NaCl, 0.15% SDS, 10 mM Tris-HCl pH 6.8 for 15 minutes at 37°C. After centrifugation, the supernatants were subjected to SDS gel electrophoresis under reducing conditions and BMP4 was detected with monoclonal antibody as described (Piccolo et al., 1996).

Embryological manipulations and RT-PCR analysis

Artificial fertilization and mRNA injection into *Xenopus* embryos were performed as described (Sasai et al., 1994). Ventral marginal zone explants (constituting 60° of the VMZ) from stage 10 embryos were prepared in LCMR (Piccolo et al., 1996), opened with an eyebrow knife, and immediately treated with LCMR containing various protein concentrations of the individual CR domains and of full-length Chordin. After overnight incubation at room temperature, the explants were transferred to 0.4 \times MMR.

Fig. 2. Individual CRs, particularly CR1 and CR3, exhibit dorsalizing activity and can bind BMP4 directly.

(A) Representative phenotypes after synthetic mRNA from individual CR constructs was injected ventrally into *Xenopus* embryos (100 pg per embryo). (B) Summary of the injection phenotypes from two independent experiments. The percentage of dorsoanteriorized embryos (short trunk, large head and cement gland) is shown in blue and that of embryos with secondary axes is indicated in red (number of embryos ranges between 35 and 83 embryos per sample). (C) RT-PCR analysis of dorsalization of ventral marginal zones (VMZs). VMZ explants were treated with 20 nM of each CR protein. α -Actin is a dorsal mesoderm marker and *elongation factor-1 α* (*EF-1 α*) was used as a measure of RNA recovery. (D) Western blot analysis of BMP4 (1.5 nM) bound to the individual CRs (2 nM) after immunoprecipitation with anti-Myc polyclonal antibody.



Immunostaining with the muscle marker 12-101 was as described (Gont et al., 1996). Expression of marker genes was assayed by RT-PCR at stage 27. The conditions and primer sequences were as described (Sasai et al., 1995; www.lifesci.ucla.edu/hhmi/derobertis/index.html).

RESULTS

The biological activity of Chordin resides in the cysteine-rich repeats

In the course of ongoing targeted mutation studies, mice homozygous for a truncated *chordin* allele lacking the three C-terminal CRs (CR2-4), but retaining CR1 and most of the inter-repeat region, were found to be viable hypomorphic mutants displaying only minor skeletal defects (D. B., J. Klingensmith, J. Rossant and E. M. D. R., unpublished results). This observation suggested that the *chordin* gene fragment retained partial biological activity, and provided the initial impetus for analyzing the function of the Chordin CR domains. A full-length mouse *chordin* cDNA was first subdivided making use of convenient restriction sites. As shown in Fig. 1, this yielded three constructs; one containing the N terminus plus the CR1 domain (a protein fragment very similar to one of the products generated by the digestion of Chordin by Xolloid; Piccolo et al., 1997; Scott et al., 1999), another containing exclusively the inter-repeat region, and lastly a fragment ("3 CRs") containing the CR2, CR3 and CR4 domains. Each construct carried the mouse *chordin* signal peptide to permit proper secretion.

Injection of full-length *chordin* mRNA induces secondary axes and dorsalizes the *Xenopus* embryo (Sasai et al., 1994), and we used this as our activity assay. Single ventral injections of synthetic mRNAs into *Xenopus* embryos showed that the inter-repeat region (constituting over half of the total protein) was devoid of activity, whereas the two constructs containing CRs had biological activity; CR1 generated principally embryos with increased dorsoanterior structures and the 3 CRs induced mainly secondary axes (Fig. 1). These results suggested that the biological activity of Chordin resides in the CR repeats.

CR1 or CR3 overexpression dorsalizes *Xenopus* embryos

To explore the function of individual CRs, each repeat was cloned in an expression vector encoding the mouse Chordin signal peptide followed by a Myc epitope-tag (Fig. 1, top row). When injected into single ventral blastomeres of 8-cell *Xenopus* embryos, 100 pg of CR1 or of CR3 mRNA had dorsoanteriorizing activity, whereas the same amount of CR2 had a much weaker effect and CR4 mRNA was devoid of activity (Fig. 2A,B).

Embryos injected with CR1 or CR3 mRNA (100 pg) were predominantly dorsalized (Fig. 2A,B), whereas full-length *chordin* mRNA injection (100 pg) gave rise to secondary axes at high frequency (Fig. 2A,B). One explanation for the tendency of overexpressed CR1 and CR3 to dorsalize the entire embryo instead of producing double axes could be that the repeats, with their smaller molecular mass, might diffuse further than the full-length protein in the embryo, rather than

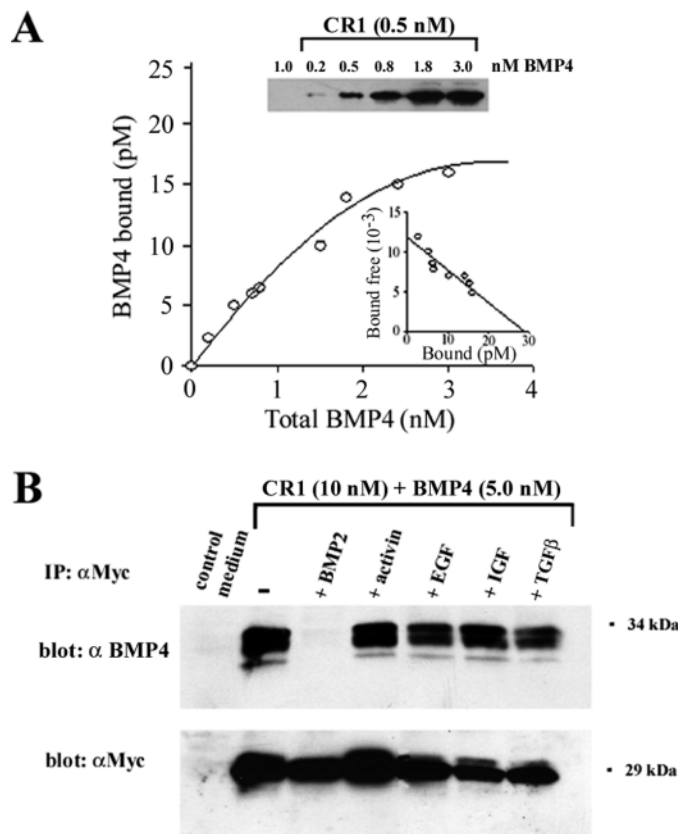


Fig. 3. Biochemical analysis of the binding of CR1 to BMP4. (A) Equilibrium binding of increasing concentrations of BMP4 to 0.5 nM CR1 protein. Two independent experiments were performed. Scatchard analysis (inset) yields a K_D of 2.4 nM. Immunoprecipitates were resolved in western blots, developed with anti-BMP4 monoclonal antibodies and quantitated with a Phosphorimager (B) The binding of CR1 to BMP4 can be competed by BMP2, but not by Activin, EGF, IGF or TGF β 1. 10 nM CR1 was incubated with 5.0 nM BMP4 and with a 10-fold molar excess of BMP2, Activin, EGF, IGF or TGF β 1. BMP binding was analyzed by immunoprecipitation with polyclonal anti-myc antibodies and western blot with a monoclonal anti-BMP4 antibody. To measure CR1 recovery after immunoprecipitation the same membrane was stripped and probed again with an anti-Myc monoclonal antibody.

having more localized effects at the site of ventral injection (secondary axes). Another explanation for the tendency of CR1 and CR3 to affect the entire embryo may be provided by the fact that the individual repeats are resistant to cleavage by the Xolloid protease in biochemical assays (data not shown).

The differences in activity observed between CR1/CR3 and CR2/CR4 injections could have been due to differences in translation efficiency of the different constructs. In order to exclude this, conditioned medium from 293T human kidney cells transiently transfected with each one of the Myc-tagged repeats were quantitated and tested in ventral marginal zone (VMZ) assays. VMZs were explanted at early gastrula and incubated in each CR protein preparation at 20 nM. As shown in Fig. 2C, α -actin mRNA, a marker for dorsal mesoderm, was induced in VMZ explants treated with CR1 or CR3 proteins, but not in explants treated with CR2, CR4 or control medium.

We conclude that the Chordin dorsalizing activity resides mainly in the CR1 and CR3 modules.

CR modules bind BMP4

To investigate whether individual CR domains can bind BMP4 directly, the same protein preparations tested above were used in co-immunoprecipitation assays. Equal concentrations (2 nM) of each CR protein were incubated with 1.5 nM BMP4, immunoprecipitated with anti-Myc polyclonal antibody and the amount of BMP4 bound to each CR was determined on western blots (Piccolo et al., 1996). Whereas all four CRs bound BMP4 to a detectable extent, CR1 and CR3 bound more effectively than CR2 and CR4 (Fig. 2D). Control medium from cells transfected with DNA vector alone was used as a negative control and found to be devoid of binding activity (Fig. 2D). We conclude from these results that the CRs, especially CR1 and CR3, can bind BMP4 directly and that the ability of individual CRs to bind BMP4 correlates with their dorsalizing activity (Fig. 2C,D).

For a more quantitative analysis of the binding affinity of CR1 and CR3, the immunoprecipitation binding assay was carried out with increasing concentrations of BMP4 (Fig. 3A). Scatchard analyses indicated that CR1 and CR3 bound BMP4 with a dissociation constant (K_D) in the nanomolar range ($K_D=2.4$ nM for CR1 and 2 nM for CR3, Fig. 3A and data not shown). This affinity is approximately one order of magnitude lower than the subnanomolar dissociation constant (0.3 nM) determined for the binding of full-length Chordin to BMP4 (Piccolo et al., 1996).

Chordin has been shown to preferentially bind BMPs (BMP2 and BMP4), but not other members of TGF β family such as Activin (Piccolo et al., 1996). To address the issue of the specificity of the interactions with isolated CRs, we competed the BMP4 binding to CR1 with a 10-fold molar excess of BMP2, activin,

EGF, IGF and TGF β 1. As shown in Fig. 3B, CR1 retains a similar specificity to that of Chordin, since only BMP2 effectively competed with BMP4 binding to CR1.

CR1 has less anti-BMP activity than Chordin

Since CR1 binds BMP4 with a lower affinity than full-length Chordin, we investigated whether CR1 had less anti-BMP dorsalizing activity than Chordin. VMZ explants were incubated with conditioned medium containing different concentrations of CR1 or Chordin proteins. As shown in Fig. 4A, the dorsal

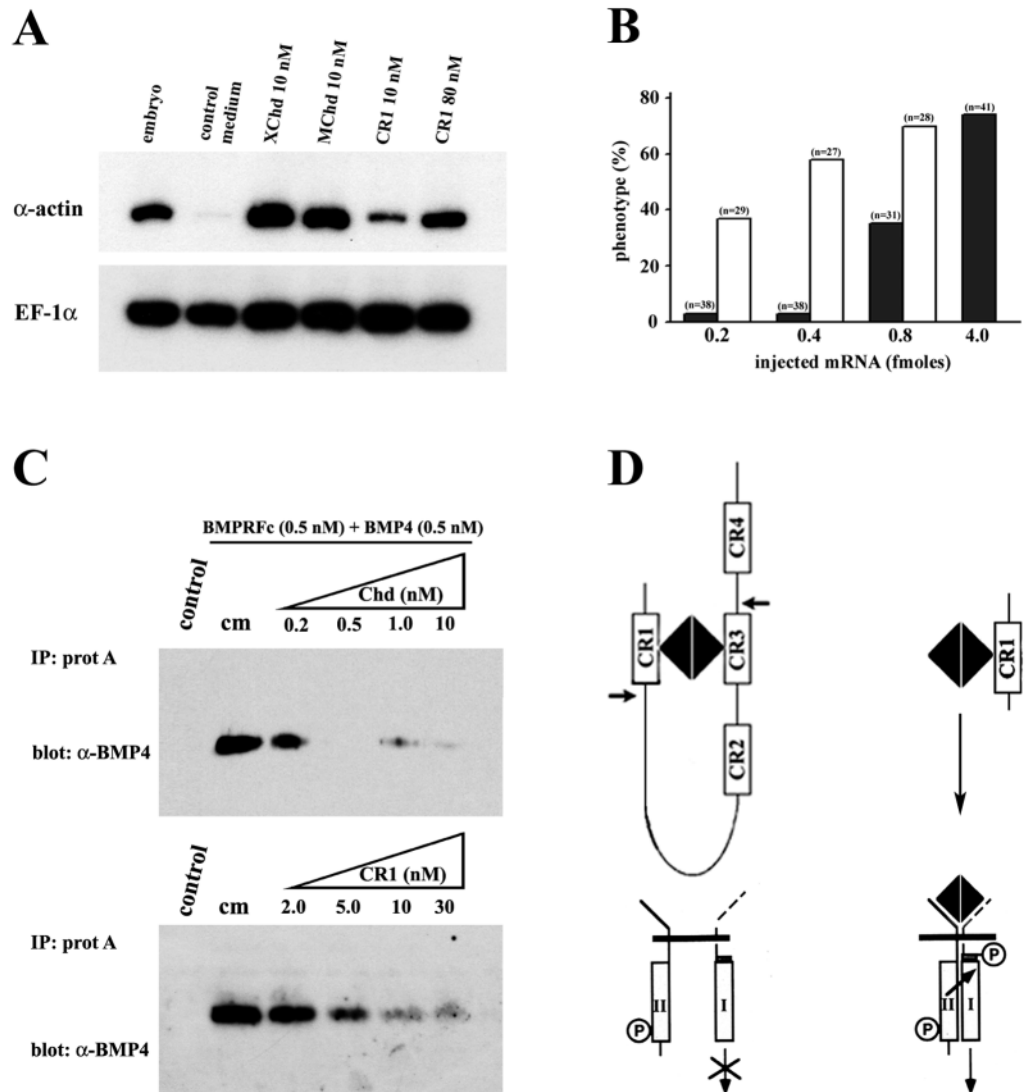


Fig. 4. CR1 has less activity than full-length Chordin. (A) RT-PCR analysis of dorsalization of ventral marginal zones (VMZ). VMZ explants were treated with 10 nM *Xenopus* Chordin (XChd) or Mouse Chordin (MChd) protein, and 10 or 80 nM CR1 protein. α -Actin is a dorsal mesoderm marker and *EF-1 α* was used as loading control. (B) Histogram showing the percentage of embryos with dorsalized phenotypes (either dorsalization of the entire embryo or secondary axes) after single ventral injections of equimolar amounts of synthetic mRNA for mouse *chordin* (open bars) or *CR1* mRNA (filled bars); full-length *chordin* is more active than *CR1*. (C) Binding of BMP4 to a BMPR-Fc fusion protein is competed more effectively by full-length *Xenopus* Chordin than by CR1 of mouse or *Xenopus* (not shown) origin. cm, conditioned medium control. (D) Hypothetical model showing that full-length Chordin binds BMP4 (one dimer per Chd monomer, Piccolo et al., 1996) with higher affinity (K_D 3×10^{-10} M) than CR1 alone (K_D 2.4×10^{-9} M). Chordin blocks signalling via BMP receptors more effectively than the individual CR repeats. The cleavage sites of Xolloid protease on its Chordin substrate are indicated by arrows.

equilibrium binding experiment was performed. Mouse BMPR/Fc fusion protein was bound to BMP4 in the absence or presence of increasing amounts of full-length Chordin or CR1 proteins and immunoprecipitated with protein A Sepharose. As can be seen in Fig. 4C, Chd at 0.2 nM competed over 50% of the binding, whereas 5 nM of CR1 was required for the equivalent degree of competition. This represents a 10-fold difference in biochemical affinity. CR1 proteins of either mouse or *Xenopus* origin behaved similarly in this assay (data not shown).

Taken together, the results show that CR1 is approximately 5-10 times less active than full-length Chordin in biological assays, a finding that correlates with the lower affinity of the CR1/BMP interaction in biochemical assays. Since the CR1 construct mimics one of the digestion products of the Xolloid metalloprotease (Piccolo et al., 1997; Scott et al., 1999), the results suggest that Xolloid digestion may regulate Chordin/BMP complexes by releasing proteolytic fragments with decreased anti-BMP activity, as indicated schematically in Fig. 4D.

Type IIA procollagen has dorsalizing activity

CR repeats of the type present in Chordin are found in many ECM proteins. To test whether our findings with Chordin could serve as a more general model for understanding the interaction of TGF β superfamily members with the ECM, we examined the function of procollagen IIA. Studies by Sandell and colleagues have shown that procollagen II is synthesized in two alternatively spliced forms, type IIA and IIB. Type IIA contains a 69 amino acid CR domain in its NH₂-propeptide that is specifically spliced out in the IIB form expressed in mature chondrocytes (Ryan and Sandell, 1990; Sandell et al., 1991, 1994; Ng et al., 1993).

From a developmental standpoint procollagen IIA is of particular interest, since it has long been used as a dorsal marker in early *Xenopus* development (Su et al., 1991). As shown in Fig. 5, *Xenopus procollagen II* is expressed in dorsal mesoderm, especially in the notochord and dorsal somites beginning at the early neurula stage. This expression overlaps in part with that of *chordin* and becomes more intense at stages in which *chordin* mRNA decreases and becomes restricted to the tailbud (compare Fig. 5D and D'). At these stages, all *Xenopus procollagen II* transcripts are of the IIA type containing the NH₂ terminal CR repeat; synthesis of type IIB starts only days later with the differentiation of mature cartilage (Su et al., 1991). Thus, *procollagen IIA* is expressed with a spatial distribution and timing consistent with a role in dorsal development of the embryo, perhaps replacing the activity of Chordin at later stages of *Xenopus* embryogenesis.

To determine whether the procollagen CR (cloned as a 115 amino acid fragment) had binding activity, the co-immunoprecipitation assay described above was utilized. We found that procollagen IIA CR bound BMP4 about as well as CR2 (Fig. 6A). We also analyzed the specificity of this binding and found that it was competed by BMP2 and TGF β 1, but not by activin, EGF and IGF (Fig. 6B). These results confirm the independent findings of Zhu et al. (1999), who have shown that human procollagen type IIA CR can bind BMP2 ($K_D=5.23$ nM) and TGF β 1 ($K_D=7.65$ nM).

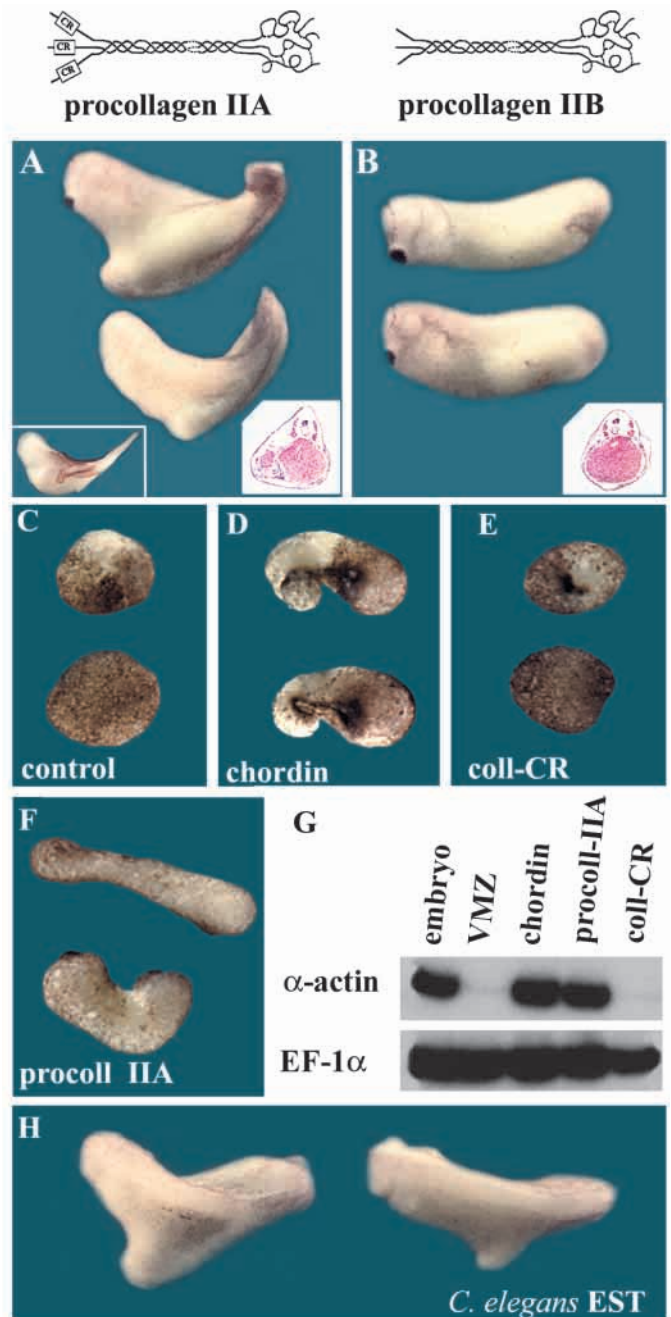


Fig. 7. *Xenopus* type IIA procollagen has anti-BMP activity. (A) Ventral injection of *Xenopus procollagen IIA* mRNA (400 pg) induces secondary axes (61%, $n=32$). Insets show that the secondary axes contain muscle stained with the MZ 12-101 mAb and also seen in the histological section. (B) Injection of a similar construct in which the CR domain was deleted to generate *procollagen IIB* does not induce twinning. (C-F) Dorsalization of ventral marginal zone explants. 8-cell embryos were injected twice ventrally with (C) H₂O, (D) *chordin* (100 pg mRNA/injection), (E) type IIA coll-CR (200 pg/injection) and, (F) full-length procollagen IIA (200 pg/injection). VMZ explants were excised at early gastrula and cultured until stage 27. (G) RT-PCR analysis of VMZ explants treated as above; the expression of the dorsal marker α -actin and EF-1 α were analyzed by RT-PCR. Note that full-length collagen, but not the CR domain alone, can dorsalize mesoderm. (H) Secondary axes caused by microinjection of *C. elegans* CAA94886 synthetic mRNA (800 pg) encoding a protein containing multiple CR repeats (58% axes, $n=31$).

These observations raise the possibility that CR domains could provide binding modules for a variety of members of the TGF β superfamily of growth factors. As shown in Fig. 6C, CR domains are present in several extracellular proteins and, except for 10 invariant cysteines and one tryptophan, as well as a few other conserved amino acids, differ significantly in amino acid sequence. Notable among these putative growth factor binding modules are Nel (Matsuhashi et al., 1995), Thrombospondin-1 (Bornstein, 1992), peroxidasin (Nelson et al., 1994) and *C. elegans* protein CAA94886 which was identified during the genome sequencing project as a hypothetical protein containing five CR domains (Wilson et al., 1994).

When synthetic mRNA for the *procollagen IIA CR* (*coll-CR*) domain was injected into *Xenopus* embryos, we failed to detect any phenotypes over a wide range of concentrations (Fig. 7E and data not shown). However, full-length *Xenopus* type IIA procollagen synthetic mRNA injected into single ventral blastomeres of 8-cell embryos (400 pg) induced secondary axes (Fig. 7A) containing muscle (marked by the monoclonal antibody 12-101, left inset) but lacking notochord (in histological sections, right inset). Presumably the injected full-length procollagen forms homotrimers that bring together three CR domains and this could increase the affinity for BMPs, as occurs with Chordin which has four CR repeats. This activity of *Xenopus* procollagen II requires the CR repeat, since a construct (see methods) mimicking type IIB procollagen (lacking the CR domain) was devoid of secondary axis inducing activity (Fig. 7B); the IIA and IIB constructs were secreted as stable proteins of the expected length in transfected 293T cells (not shown). To further test the effect of *Xenopus procollagen IIA* mRNA on mesoderm dorsalization, embryos were injected ventrally and VMZ explants analyzed. As shown in Fig. 7C-G, full-length *procollagen IIA*, but not the *coll-CR* construct, led to dorsal mesoderm formation, indicated by explant elongation and expression of α -actin mRNA. These effects were similar to those of *chordin* mRNA injection (Fig. 7D,G). We conclude that the type IIA procollagen has dorsalizing activity when overexpressed in vivo and that this activity resides in its CR domain.

To explore whether other CR-containing proteins might have similar biological activity in *Xenopus*, we injected full-length *C.elegans* CAA94886 synthetic mRNA (800 pg) encoding a protein containing 5 CRs (Wilson et al., 1994), which caused formation of secondary axes of the type induced by *chordin* or procollagen IIA (Fig. 7H).

DISCUSSION

The experiments presented here provide evidence that the biological activity of Chordin resides in the cysteine-rich repeats (CRs), particularly in CR1 and CR3. These repeats can dorsalize embryos and VMZ explants but, at equimolar levels, CR1 and CR3 have 5-10 times less activity than full-length Chordin. CR1 and CR3 repeats bind BMP4 with an affinity (K_D 2.4 and 2.0 nM, respectively) that is 7- to 8-fold lower than that of full-length Chordin ($K_D=0.3$ nM) (Piccolo et al., 1996). CR2 and CR4 have almost no biological activity

and bind BMP4 only very weakly (Fig. 2). The presence of CR1 and CR3 in the same molecule would provide a Chordin monomer with two binding sites for each BMP dimer (Fig. 4D). In addition, the presence of the less active CR2 and CR4 domains could cooperate by increasing the local concentration of BMP binding sites. In this way, if a BMP molecule were to dissociate from a CR, it would be more likely to be bound by an adjoining CR in the same molecule. By this proposed mechanism, a molecule with multiple CRs could bind BMPs with a higher affinity and with a lower off rate than those of its individual CR repeats. However, it is also conceivable that the function of Chordin CR2 and CR4 is to bind different members, as yet unknown, of the TGF β superfamily.

The Chordin/BMP pathway is regulated by the zinc metalloprotease Xolloid (Piccolo et al., 1997), a homologue of *Drosophila* Tolloid that regulates the activity of Sog (Marqués et al., 1997). Our observations begin to provide a molecular explanation for how Xolloid may regulate Chordin. Xolloid cleaves Chordin at two sites, which had been roughly mapped close downstream of CR1 and CR3 (Piccolo et al., 1997). Recently, the cleavage sites have been sequenced and found to correspond to conserved aspartic residues (Scott et al., 1999). The CR1 protein used in this study is very similar in length (only 8 amino acids shorter) to the fragment generated by metalloprotease cleavage in the N-terminal site of Chordin. We have shown that CR1 binds BMP4 with a lower affinity (8-fold lower), is less efficient in competing BMP4 binding to BMPR (10 times lower), and has less biological activity (5- to 10-fold lower) than full-length Chordin. It is conceivable that the Xolloid protease inactivates Chordin by the generation of smaller fragments that can still bind BMP and perhaps transport it. However, each of these binding modules alone would not have high enough affinity to compete (Fig. 4D) with the higher affinity of BMP for its cognate receptors, which is in the same range as that of full-length Chordin for BMP4 (Piccolo et al., 1996).

In *Drosophila*, elegant studies have shown that Sog not only inhibits Dpp signalling but is also able to enhance it at a distance (Zusman et al., 1988; Ferguson and Anderson, 1992; Ashe and Levine, 1999). This enhancement of BMP signals requires Sog diffusion (presumably carrying bound Dpp or Screw) and the activity of the Tolloid protease (Ashe and Levine, 1999). It has been suggested that cleavage products of Sog, or Sog fragments complexed with Dpp, could augment the binding of Dpp/Screw to its receptors (Bier, 1999). None of our Chordin constructs, including a series of carboxy-terminal protein truncations not described here, displayed ventralizing effects as would be expected if there were increased binding to receptors. Rather, the observation that Chordin fragments are either weakly dorsalizing or inactive in *Xenopus* assays tends to support the initial proposal by Holley et al. (1996). In this proposal, the diffusion of Chd/Sog complexed with BMP/Dpp contributes to the formation of morphogen gradients in which maximal levels of signalling are achieved by cleavage of the inhibitor and release of the active BMP signal.

The CR domains of Chordin define a novel protein module

for BMP binding. The cysteine-rich (CR) domains present in the NH₂-propeptide of *Xenopus* type IIA procollagen bind BMP4 with an affinity comparable to that of CR2, and this binding can be competed by BMP2 and TGFβ1 (Fig. 6A,B). The collagen IIA CR domain alone does not have biological activity in *Xenopus* assays (Fig. 7E,G), probably because of its low affinity for BMP (Zhu et al., 1999). However, we report here that full-length type IIA procollagen mRNA has dorsalizing (anti-BMP) activity. Type IIA procollagen is secreted as a homotrimer and the NH₂-propeptide remains attached to the collagen fibrils in the ECM (Zhu et al., 1999). This structure probably permits the association of the three CR domains and, as in the case of the multiple repeats of Chordin, may result in the stabilization of the interaction between BMP and the CR domain by increasing the local concentration of BMP4 binding sites. We have not yet been able to produce soluble trimeric procollagen IIA to test this proposition. In the case of collagen type I, a stable trimeric NH₂-propeptide is released after proteolytic maturation. This trimer is composed of two α1 chains containing a CR domain, and an α2 chain lacking it. This trimeric protein has been isolated in large amounts from fetal calf bone matrix and, interestingly, also from fetal calf serum in which it reaches a concentration of 106 μg/ml (Fisher et al., 1987). It would therefore be of interest to determine whether trimeric collagen NH₂-propeptides containing CRs are able to function as growth factor regulators.

CRs are present in many ECM proteins such as fibrillar procollagens (I, II, III and V), von Willebrand factor, thrombospondin 1 and 2 (Bornstein, 1992), *Drosophila* peroxidase (Nelson et al., 1994), the secreted proteins Nel, Nel-like-1 and Nel-like-2, which contain four CR repeats and six EGF repeats each (Matsushita et al., 1995; Watanabe et al., 1996) and the aforementioned *C. elegans* protein CAA94886 containing five CR repeats. Since these CR repeats vary widely in sequence, they might bind a wide range of TGFβ superfamily members. This is in fact likely in view of the observations that, whereas Chordin CR1 interacts with BMPs but not TGFβ1, procollagen IIA CR can interact both with BMP and TGFβ1 (Zhu et al., 1999; this study). It is attractive to propose that CRs, which are abundantly represented in the ECM of many tissues, may function as a sink for TGFβ superfamily members. These bound growth factors could be subsequently liberated by specific proteases, such as members of the Tolloid/BMP1 family, when required for tissue differentiation and homeostasis (Piccolo et al., 1997; Marqués et al., 1997; Goodman et al., 1998; Imamura et al., 1998).

The dorsalizing activity of *Xenopus* type IIA procollagen could be of functional importance during early development. This molecule is one of the classical markers used to identify dorsal regions during vertebrate development. In the frog embryo, it is strongly expressed in the notochord and somites at neurulation and tailbud stages (Su et al., 1991). In zebrafish, collagen II expression is very strong in posterior notochord, floor plate and hypochord, and its downregulation in the notochord is finely spatiotemporally regulated in a number of developmental mutations (Yan et al., 1995; Stemple et al., 1996). In mouse, procollagen II is strongly expressed in the notochord and sclerotomes (Cheah et al., 1991). A mouse mutant lacking the carboxy-terminus of collagen II shows a

failure of the notochord to degenerate and form the nucleus of the intervertebral discs (Aszódi et al., 1998). In future, mouse mutations targeting specifically the CR of procollagen II will be of great interest.

Because of its anti-BMP activity and expression pattern, type IIA procollagen is a good candidate to cooperate in the maintenance of dorsal values, particularly during later stages of development in which *chordin* mRNA levels decrease (Fig. 5). Although procollagen IIA forms homotrimers, other collagens exist in different combinations of chains (Scriver et al., 1995). By bringing together CRs with differential binding affinity, combinations of chains could provide a finely tuned extracellular system of growth factor regulators.

In conclusion, the large body of genetic data concerning the interactions between the Chordin/Sog, Dpp/BMP and Xolloid/Tolloid gene products has led to a useful paradigm of how cell signalling is modulated in the extracellular space. The present demonstration that this regulation works in part through binding to cysteine-rich domains in Chordin may provide mechanistic insights into how other ECM proteins with CR domains might function.

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