

Chordin-like CR domains and the regulation of evolutionarily conserved extracellular signaling systems

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Abstract

In fruit flies as well as in humans the Short gastrulation (Sog)/Chordin protein functions as an antagonist of the signaling of decapentaplegic (Dpp)/bone morphogenetic protein (BMP) in the extracellular space. Such antagonism inhibits Dpp/BMP signaling by blocking its binding to the receptor. Modulation of Dpp/BMP signaling is phylogenetically conserved and is a key step for the establishment of the dorso-ventral axis in vertebrates and invertebrates. Molecular studies have shown that the inhibitory activity of Chordin on BMP resides in specific cysteine-rich (CR) domains. Interestingly, Chordin-like CR domains are present in a growing number of extracellular proteins, several of which appear to be involved in BMP signaling regulation. We review here the conservation of the Chordin and Sog proteins, and in particular their functional domain, the CR domain. We discuss how the study of CR domains may provide a general mechanism for the regulation of growth factor signaling in the extracellular space. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Bone morphogenetic protein; Chordin; Transforming growth factor β ; Cyr61; Nov; CCN; Connective tissue growth factor; Cysteine-rich motor neuron; Neuralin; Crossveinless; Kielin; Procollagen; von Willebrand factor; Insulin-like growth factor binding protein

1. Introduction

As an increasing amount of data is generated through the sequencing of complete genomes, molecular biologists are offered new ways of addressing fundamental biological problems such as the conservation of molecular mechanisms during evolution. In this context, we can directly search for conserved genes throughout gene databases. Strikingly, entire networks of genes are preserved among the various Phyla, allowing one to extrapolate at which point in evolution an ancestral gene has appeared. The study of dorso-ventral patterning provides a good example of how a complete gene network can be conserved in both vertebrates

and invertebrates. This mechanism determines where the dorsal and ventral tissues will develop in the embryo (reviewed by De Robertis and Sasai, 1996). In vertebrates, it involves the antagonism between the growth factor BMP (Bone Morphogenetic Protein) and the secreted protein Chordin that binds directly to BMP and thereby prevents the activation of the BMP receptor (Sasai et al., 1995; Piccolo et al., 1996). In the absence of repression BMP binds to its cognate receptor, activating signal transduction and the expression of downstream target genes (Massagué and Chen, 2000). Genes homologous to BMP and Chordin, designated *Dpp* (*Decapentaplegic*) and *sog* (*short gastrulation*), were identified initially by genetic studies in the fruit fly *Drosophila melanogaster* (François et al., 1994; Holley et al., 1995). Other factors are also highly conserved. One of them is Tolloid, a protease that cleaves and inactivates Chordin/Sog (Piccolo et al., 1997; Marqués et al., 1997). Another factor is Twisted Gastrulation (Tsg), a BMP-binding molecule that both cooperates and competes with Chordin/Sog products after Tolloid/Xolloid cleavage (Oelgeschläger et al., 2000; Ross et al., 2001; Chang et al., 2001; Scott et al., 2001).

Despite the conserved molecular mechanisms, a major difference is observed between vertebrates and invertebrates. Indeed, the BMP/Dpp gradient is inverted, with the

Abbreviations: BMP, bone morphogenetic protein; Alk2, activin receptor-like kinase 2; Amn, Amnionless; CCN, Cyr61, CTGF and Nov proteins; CHL, chordin-like protein; CR, cysteine-rich domain; CRIM-1, cysteine-rich motor neuron 1; CTGF, connective tissue growth factor; Cv, Crossveinless; Dpp, decapentaplegic; ECM, extracellular matrix; EGF, epidermal growth factor; GI, gene identification number; IGF-1/2, insulin-like growth factor 1 or 2; IGFBP, insulin-like growth factor binding protein; Nel, neural tissue EGF-like containing protein; NELL, Nel-like protein; kDa, kilodalton; Sog, short gastrulation; TGF- β , transforming growth factor β ; Tsg, twisted gastrulation; TSP, thrombospondin; vWF, von Willibrand factor

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low BMP/Dpp activity required for neural induction being attained dorsally in vertebrates and ventrally in invertebrates. The molecular studies support the proposal by Geoffroy Saint-Hilaire, who first suggested that an inversion of the dorso-ventral axis from a conserved body plan had occurred between vertebrates and invertebrates (De Robertis and Sasai, 1996).

Here we review the conservation of the BMP/Dpp antagonism by Chordin/Sog proteins, particularly through the study of their functional domains, the Cysteine-rich Repeats or CR domains. The specific amino acid motifs present in the CR domains are analyzed in an evolutionary perspective. The functional implications of the presence of CR domains in many extracellular proteins is explored. Finally, we discuss how the study of CR domain provides a general mechanism for the regulation of growth factor signaling in the extracellular space.

2. Chordin/Sog proteins are highly conserved throughout evolution

Chordin in vertebrates and Sog in *Drosophila* are secreted molecules of 950–1000 amino acids (François et al., 1994; De Robertis and Sasai, 1996). They share four highly conserved cysteine-rich (CR) repeats ranging from 60 to 80 amino acids in length, one located in the N-terminus (CR-1) and the three others located in tandem at the C-terminus (CR-2–4, Fig. 1A). The repeats have ten cysteines with a conserved spacing pattern (Fig. 1A). Typically, the Sog/Chordin CR domain contains a conserved CXXCXC motif in the middle and a CCXXC motif in the C-terminus (Fig. 1A). In this nomenclature 'X' can be any amino acid. In addition, a glycine and a tryptophan residue located between the first two cysteines are highly conserved (Fig. 1A). It is important to note that the CRs of Sog/Chordin are part of the canonical von Willebrand factor-C domain that can be found in many databases such as Pfam (<http://www.sanger.ac.uk/Pfam/>).

Phylogenetic analysis of human, mouse, chicken, *Xenopus* and zebrafish Chordin proteins and of *Drosophila* Sog reveal a higher degree of conservation for each specific CR among the different species, than that of different CRs in a particular Sog/Chordin protein within the same species (Fig. 1B). For example, human CR-1 is more similar to *Drosophila* CR-1 than to human CR-2. As expected, the *Drosophila* CRs are more divergent than their vertebrate counterparts (Fig. 1B). This sequence conservation is also reflected in the function of individual CRs. Indeed, in vitro experiments have shown that all four CRs of mouse Chordin are able to bind BMP-4 at a detectable level (Larraín et al., 2000), but the binding of CR-1 or CR-3 to BMP-4 has higher affinity than CR-2 or CR-4. In accordance with these different biochemical properties, only CR-1 and CR-3 present the same inducing properties as Chordin when injected into *Xenopus* embryos (Larraín et al., 2000).

However, the biological activity of individual CR-1 and CR-3, as well as their affinity to BMP, is ten fold lower than the affinity of full-length Chordin for BMP, suggesting a cooperativity in the function of the multiple CR domains present in the full-length protein.

The regulation of Sog/Chordin activity has been extensively studied in *Drosophila*, zebrafish, *Xenopus* and mouse, in particular its inactivation after cleavage by the metalloprotease Xolloid or Tolloid (Piccolo et al., 1997; Marqués et al., 1997; Blader et al., 1997; Scott et al., 1999). It has been shown that such a cleavage releases BMP from an inhibitory Sog/Dpp or Chordin/BMP complex. It has also been demonstrated that this cleavage occurs at two sites, resulting in proteolytic fragments containing the individual CR-1, CR-4, and a longer product containing both CR-2 and CR-3 (Fig. 1A). In view of the biochemical activities of these cleavage products, and the degree of conservation observed among different species, it is tempting to suggest a conserved function for these domains in the regulation of the BMP signals.

3. Chordin-like CRs are present in many extracellular proteins

A variety of extracellular proteins contain domains similar to Chordin CRs, including most isoforms of fibrillar Procollagens (types I, II, III and V), and the members of the Nel family (Matsushashi et al., 1995; Watanabe et al., 1996). More recent studies have led to the characterization of novel extracellular proteins that contain large number of CRs such as CRIM-1, Kielin, Crossveinless-2 and Neuralin/CHL (Kolle et al., 2000; Matsui et al., 2000; Conley et al., 2000; Coffinier et al., 2001; Nakayama et al., 2001). The CRs of these proteins share amino acid sequence similarity with Chordin CRs (Fig. 2). This similarity is reflected in the spacing of the cysteines and in the presence of the CXXCXC and CCXXC motifs (Fig. 2). As depicted in Fig. 3, some of these extracellular proteins contain exclusively Chordin-like CR domains whereas others combine CRs and other domains such as epidermal growth factor-like (EGF), Thrombospondin-1 (TSP), von Willebrand factor D (vWF-D) and insulin-like growth factor binding protein-like domains (IGFBP). We discuss below the structural organization of these proteins and highlight their potential functions in modulating growth factor signaling.

3.1. Procollagen IIA

Collagen II is the major component of the cartilage extracellular matrix (ECM). After secretion, procollagen molecules self-assemble forming triple helical structures forming fibrils, and the C-terminal and N-terminal propeptides are excised by specific metalloproteases (Scriver et al., 1995). Type II Procollagen is expressed as two differentially spliced forms during chondrogenesis. Type IIB Procollagen is produced by mature chondrocytes, whereas Type IIA is

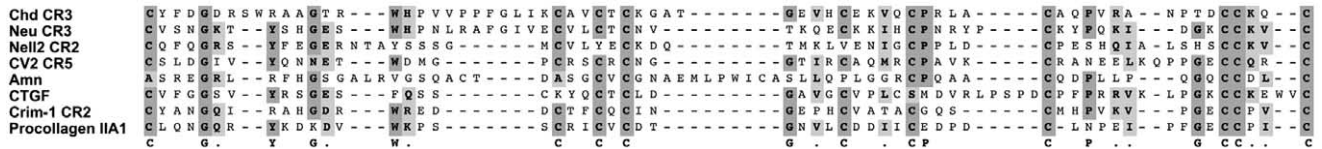


Fig. 2. CR-containing proteins show sequence similarities with Chordin CRs. Alignments of human Chordin CR-3 compared to divergent CRs. Gene identification numbers are: NP034026 (Chordin), AF385714 (Neuralin), Q61220 (NELL2), AAG01337 (Cv-2), AF320619 (Amn), P29268 (CTGF), AAF34410 (CRIM-1) and NP112440 (Procollagen IIA).

isoform devoid of the CR does not have this activity (Larraín et al., 2000). These results could be of functional importance in cell signaling during development, since *Procollagen IIA* is strongly expressed in the notochord, hypochord, floor plate and somites of *Xenopus*, mouse and zebrafish embryos (Su et al., 1991; Ng et al., 1993; Yan et al., 1995). Type IIA procollagen is therefore a good candidate to cooperate with other secreted anti-BMP molecules such as Chordin in the maintenance of dorsal territories, as well as in the induction and differentiation of the skeleton.

Procollagen II CR is most related to Chordin CR2, which is known to have low affinity for BMP4 (Larraín et al., 2000). This is interesting in view of the fact that Procollagen IIA also binds to TGF- β 1, raising the possibility that CR domains could provide binding modules for a variety of TGF- β superfamily members. Furthermore, Procollagen I, III and V also contain a CR domain, suggesting a general model of modulation of TGF- β signaling in the ECM.

3.2. Neuralin/Chordin-like

Neuralin/Chordin-like (CHL), a novel secreted protein, was cloned by its similarity to Chordin and is composed of three CRs domains without any additional recognizable Pfam protein domains (Coffinier et al., 2001; Nakayama et al., 2001). Neuralin/CHL binds BMP-4, as well as BMP-5, BMP-6, TGF- β 2 and TGF- β 1 (Nakayama et al., 2001). Like *chordin*, *neuralin/CHL* mRNA microinjection in *Xenopus* embryos results in strong dorsalization, suggesting anti-BMP activity (Coffinier et al., 2001; Nakayama et al., 2001). It remains to be determined whether the three CRs of Neuralin/CHL have specific binding activities for either BMPs or other TGF- β superfamily members.

Despite similar activities, Neuralin/CHL and Chordin have very different expression patterns in the early mouse embryo. *Neuralin/CHL* is expressed in the neural plate at a time in which *chordin* expression is restricted to the node and the notochord (Bachiller et al., 2000; Coffinier et al., 2001). Later during development, Neuralin/CHL is expressed in neural tissue derivatives and in many mesenchymes, including the forming axial skeleton (Coffinier et al., 2001; Nakayama et al., 2001). Thus, Neuralin/CHL may function as a modulator of the BMP signaling in the neural plate and in bone formation, as well as in other processes.

3.3. Kielin

Kielin is a novel secreted protein isolated from *Xenopus*

that contains 27 adjacent CR domains (Matsui et al., 2000). Kielin also contains an N-terminal TSP domain and a C-terminal vWF-D domain. Although it has not yet been shown biochemically whether Kielin binds BMP, microinjection of *Kielin* mRNA can dorsalize ventral mesoderm explants. In contrast to Chordin, Kielin can not neuralize ectoderm, suggesting that its activity is different from that of the Chordin CRs. During embryogenesis, Kielin is expressed in midline structures such as floor plate and the notochord (Matsui et al., 2000). Considering this expression pattern and its inductive activity, it seems likely that Kielin plays a role in dorso-ventral patterning.

3.4. Crossveinless-2

In *Drosophila*, genetic analyses have identified an additional CR-containing molecule, Crossveinless-2 (Cv-2). The *cv-2* gene encodes a secreted protein that contains five adjacent CR domains and a partial vWF-D domain at the C-terminus (Conley et al., 2000). Crossveinless-2 mutants are characterized by the loss of two crossveins in the *Drosophila* wing. This is reminiscent of phenotypes caused by decreasing Dpp/BMP signals, suggesting that Cv-2 is a critical player in this process. The data support the view that the wild-type *cv-2* gene product increases Dpp signals rather than inhibiting them. Thus, Cv-2 is the first CR-containing molecule proposed to act as a pro-BMP.

Another *Drosophila* mutation with the same defects in wing vein patterning is the *crossveinless* gene (*cv-1*), first identified many years ago (Bridges, 1920). Although *cv-1* and *cv-2* loss-of-function produce the same phenotype, genomic sequencing revealed that *cv-1* encodes a second *twisted gastrulation* gene in *Drosophila*, designated *Tsg-2* (Fly Base ID: FBgn0000394; Ross et al., 2001). As mentioned earlier, Tsg is one of the conserved components of the dorso-ventral patterning system, and one that can have either pro- and anti-BMP activity. Taken together, the data suggest that Cv-2 and Tsg-2 work together to attain the maximal Dpp/BMP activity required for the formation of the *Drosophila* crossveins. The relationship between CRs and Tsg homologues may be more general than initially expected.

3.5. Amnionless

The *amnionless* (*amn*) gene was first identified in mouse by insertional mutagenesis, which resulted in a failure of gastrulation. The *amn* gene is essential for the correct devel-

opment of the embryo and formation of trunk mesoderm (Tomihara-Newberger et al., 1998). Positional cloning led to the characterization of a novel gene specifically expressed in an extra-embryonic tissue, the visceral endoderm. (Kalantry et al., 2001). The *amn* gene encodes a new transmembrane protein (Fig. 3) with no known motif except for a CR domain located in the middle of the extracellular

domain (Kalantry et al., 2001). The Amn CR domain is divergent from the Chordin CRs and lacks certain conserved amino acids (Fig. 2). It is tempting to speculate that Amn interacts with BMPs secreted in the space surrounding of the embryo. In fact, *amn* transcript is expressed on the apical region of the visceral endoderm (Kalantry et al., 2001) where it might interact with BMPs expressed by the visceral

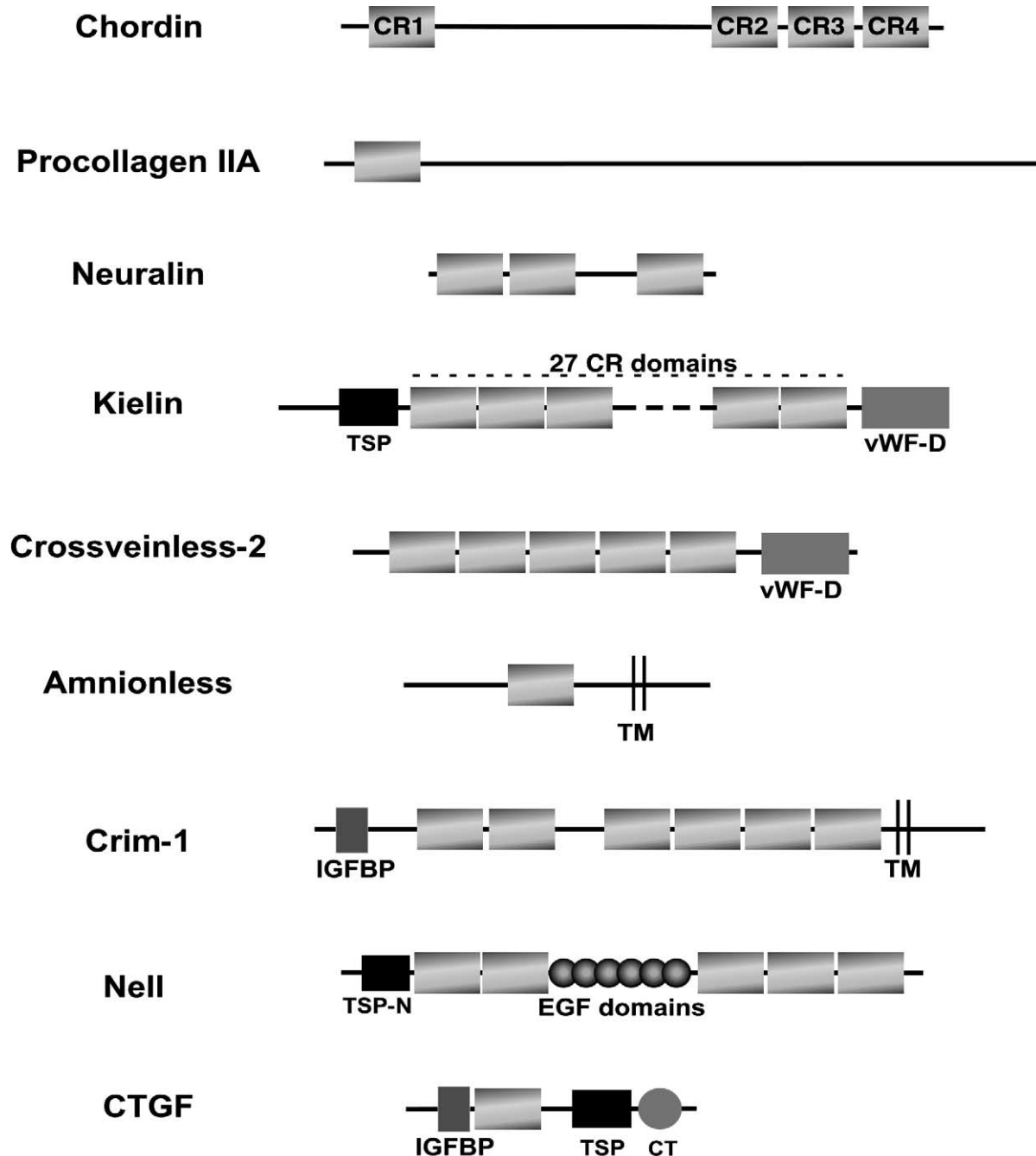


Fig. 3. Chordin-like CRs are present in many extracellular proteins. Schematic comparison of the CRs present in Chordin with the ones present in procollagen IIA, Neuralin, Kielin, Crossveinless-2, Amnionless, CRIM-1, NELL, and Connective tissue growth factor (CTGF). NELL protein contains an N-terminal thrombospondin (TSP-N) domain and six epidermal growth factor (EGF) domains. Kielin and CTGF also have a thrombospondin (TSP-1) domain. Kielin and Crossveinless-2 present similarity to von Willebrand factor D (vWF-D) domain. CRIM-1 and CTGF have an Insulin-like growth factor binding-like (IGFBP) domain. CRIM-1 and Amnionless contain a transmembrane (TM) sequence. CTGF has a C-terminal cysteine knot (CT). All these domains can be identified using the Pfam database.

endoderm itself or with BMP2 and BMP7 secreted from maternal tissues (Zhang and Bradley, 1996; Coucouvanis and Martin, 1999). Furthermore, the Amn phenotype is reminiscent of mutants lacking Type I BMP receptor Alk2, which is also expressed by the visceral endoderm (Gu et al., 1999). Altogether, these data suggest that Amn could act as regulator of the BMP pathway in the visceral endoderm to direct growth and patterning of the mammalian embryo.

3.6. Cysteine-rich *Motor Neuron protein 1: CRIM-1*

CRIM-1 encodes a putative transmembrane protein with a short cytoplasmic domain (Kolle et al., 2000). Its extracellular region contains six CR domains with best similarity to Procollagen IIA CR and Chordin CR-2. In vivo assays performed in *Xenopus* embryos have shown that overexpression of the *Caenorhabditis elegans CRIM-1* homologue can induce secondary axes of the type induced by Chordin and procollagen IIA (Larraín et al., 2000). *CRIM-1* is expressed in the notochord, somites, floor plate, and early motor neurons and interneurons of the developing spinal chord (Kolle et al., 2000).

Vertebrate CRIM-1 contains an IGF binding protein (IGFBP) domain in the N-terminus (Fig. 3) that is highly homologous to the IGFBP-7 protein, which is known to bind both IGF-1 and IGF-2 with high affinity. Interestingly, the nematode CRIM-1 (GI Z71178) does not contain an IGFBP-like domain, despite a general amino acid identity of 45% with its vertebrate counterparts (Kolle et al., 2000). This suggests that the IGFBP domain may have been acquired by vertebrate organisms as an adaptation of the protein in the course of the evolution. The association during evolution of diverse functional domains within the same protein, in what has been termed the ‘Rosetta Stone’ model, is an indicator of function in common biochemical pathways (Marcotte et al., 1999). The example of vertebrate CRIM-1 may indicate that signaling by IGF and BMP/TGF- β act in concert during development. A similar association between

IGFBP and CR domains is also seen in the CCN family of secreted proteins such as CTGF (Fig. 3).

3.7. The *Nel* family

The founding member of the *Nel* family of proteins was isolated from chick as a protein strongly expressed in neural tissue and containing EGF-like domains (Matsuhashi et al., 1995). *Nel* encodes a transmembrane protein of 93 kDa consisting of a TSP domain, two Chordin-like CRs domains, and five EGF-like domains followed by three additional CRs (Matsuhashi et al., 1995). Two additional *Nel*-like secreted proteins (NELL) have been isolated in human and rat (Watanabe et al., 1996; Kuroda et al., 1999). The expression pattern of NELL-1/2 in rat brain, particularly that of NELL-2 in the hippocampus, suggests a potential role for these molecules in neuronal plasticity (Kuroda et al., 1999). Chick *Nel* is strongly expressed in neural tissues such as brain, spinal chord and dorsal root ganglia of early embryos (Matsuhashi et al., 1995). Proteins from the *Nel* family have conserved numbers of CR domains; the number of EGF-like domains varies between 5 and 6. The mammalian NELL proteins lack the transmembrane domain (Fig. 3). Thus, NELL should be secreted in the extracellular space, as suggested by transfection into COS cells (Kuroda et al., 1999). In addition, NELL proteins contain a domain homologous to the N-terminus of Thrombospondin (Kuroda et al., 1999). TSP-1 domains are involved in the interaction with cell surface molecules such as heparan sulfan proteoglycans (Bornstein, 2001). Thus, the combined presence of the CR, TSP-1 and EGF-like domains make *Nel*/NELL good candidates to modulate the interaction between growth factors and their receptors in the ECM. To date, the ability of *Nel* proteins to bind TGF- β factors remains uncharacterized.

3.8. CTGF and the CCN family

The CCN family, named after *CYR61* (cysteine-rich protein), *CTGF* (connective tissue growth factor) and *NOV* (nephroblastoma overexpressed gene), consists of

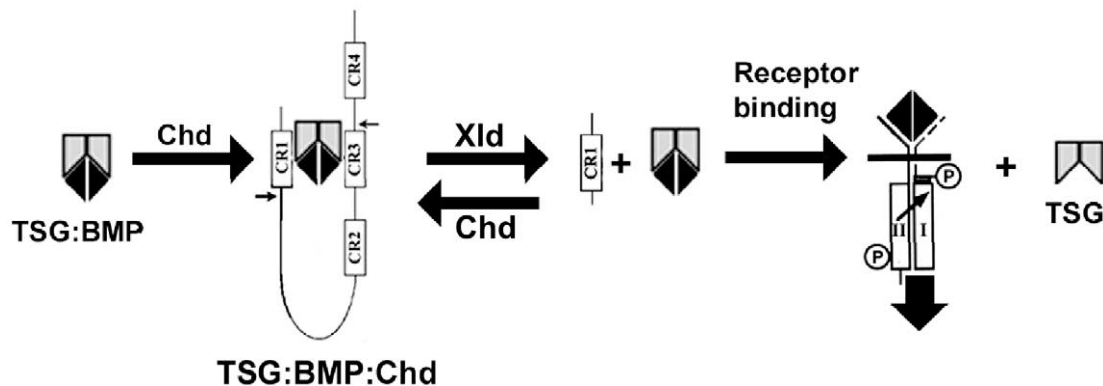


Fig. 4. The dorso-ventral gradient of bone morphogenetic protein is regulated by Chordin, CRs, Xolloid and Twisted gastrulation that work in a common biochemical pathway. Chd, Chordin; TSG, Twisted gastrulation; BMP, bone morphogenetic protein; Xld, Xolloid; CR1–4, Chordin cysteine repeat 1–4. Activated receptors become phosphorylated (circled P); relaying the signal intracellularly (downward arrow). See Section 4 for details.

six highly related secreted proteins. The CCN proteins contain a conserved modular organization composed of an IGFBP domain, a CR domain, a TSP domain and a cysteine knot (CT) at the C-terminus (Moussad and Brigstock, 2000; Perbal, 2001). The CR domain of CCN proteins has distinctive features with respect to the consensus discussed in Section 2. Like Chordin CRs, the CTGF CR is about 64 amino acids long and contains 10 cysteines. However, the conserved tryptophan residue is replaced by a phenylalanine and the motifs CXXXXC and CCXXC are replaced by CXXXXC and CCXXXXC, respectively. It will be interesting to see whether these particularities of the CCN CR alter its binding activities.

Interestingly, the modular architecture of the CCN members makes them good candidates for a molecules that combine modulation of growth factor signaling via binding and sequestration of ligands, as well as signaling as putative receptor-mediated ligands. These multiple potential functions of CCN proteins are reflected in the pleiotropic action of CTGF. CTGF has been shown to have ECM remodeling activities in many different physiological events, from embryogenesis and implantation to wound healing. In these processes, CTGF expression has been consistently shown to be stimulated by TGF- β and to induce collagen synthesis.

4. The Chordin system: multiple players regulate CR activity

In the preceding sections we have described multiple types of proteins containing CR domains. In some of these proteins, CR domains are associated to other functional domains such as IGFBP, vWF-D, EGF or TSP (Fig. 3). The various proteins can present different binding activities toward members of the TGF- β superfamily. Although most CR-containing proteins appear to act as BMP antagonists, some molecules such as Cv-2 are pro-BMP candidates. Understanding the function of CR domains could lead to fresh insights on the regulation of the TGF- β pathway.

In this context, analysis of Sog/Chordin in *Drosophila*, *Xenopus*, zebrafish and mouse provides a model system to study how CRs regulate BMP metabolism (Fig. 4). As mentioned earlier, Chordin activity is regulated by the Tolloid/Xolloid metalloprotease. In *Xenopus*, Xolloid cleaves Chordin at two conserved aspartic acids (Scott et al., 1999) just after CR-1 and CR-3 (Fig. 1A). However, the cleavage products can still bind BMP4, albeit with 5–10 fold lower affinity (Larraín et al., 2000). While biochemical and genetic analyses have provided strong support for a role for Xolloid in dorso-ventral patterning, it remained unclear how BMP is released from the CR domain after cleavage (Piccolo et al., 1997).

The characterization of a new player, Twisted Gastrulation (Tsg), has shed light on this issue. Tsg was originally characterized in the fly as a mutation lacking the embryonic

tissue that requires the highest level of Dpp/BMP signaling, the amnionserosa (Mason et al., 1994). Tsg homologues have been cloned in zebrafish, *Xenopus*, mouse and human. Tsg is a secreted protein able to bind BMP and Chordin (Oelgeschläger et al., 2000). Interestingly, the binding site for BMP resides in the N-terminal part of Tsg. Sequence comparisons have shown that this region shares similarities with the C-terminal half of Chordin-like CRs, particularly in the spacing of the last cysteines, prompting researchers to ask whether Tsg was a BMP-binding protein (Oelgeschläger et al., 2000).

Experiments in *Drosophila*, zebrafish and *Xenopus* have suggested that Tsg could play a dual role in BMP signaling. Depending on the presence of Tolloid/Xolloid, it would act as pro-BMP or as an anti-BMP (Oelgeschläger et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). First, Chordin becomes a better BMP antagonist in the presence of Tsg, through the formation of ternary complex (Chordin:BMP:Tsg). Second, after cleavage of Chordin by Xolloid BMP is released bound to TSG (BMP:Tsg binary complex). Tsg dislodges BMP from the Chordin CR, allowing the efficient transfer of BMP to the receptor and signaling (Fig. 4).

The fact that the N-terminus of Tsg is sufficient for BMP binding and that Tsg only contains half a Chordin-like CR suggests that the structural information for BMP binding resides in fewer amino acids than predicted from the entire CR or vWF-C conserved domains. This possibility is strengthened by the fact that in mouse the CRs of Chordin as well as those of Neuralin are always encoded in two exons with conserved intron positions (Coffinier et al., 2001).

Finally, the occurrence of molecules combining different domains such as IGFBP and CRs in vertebrate CRIM-1 might further modulate the cellular response to combinations of growth factors. Indeed, the growing family of CR-containing proteins underscores the multiple opportunities for regulation of signaling in the extracellular space, leading to new models for the regulation of cell signaling during development and homeostasis.

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