We have identified in *Caenorhabditis elegans* a homologue of the vertebrate Crim1, *crm-1*, which encodes a putative transmembrane protein with multiple cysteine-rich (CR) domains known to have bone morphogenetic proteins (BMPs) binding activity. Using the body morphology of *C. elegans* as an indicator, we showed that attenuation of *crm-1* activity leads to a small body phenotype reminiscent of that of BMP pathway mutants. We showed that the *crm-1* loss-of-function phenotype can be rescued by constitutive supply of *sma-4* activity. *crm-1* can enhance BMP signaling and this activity is dependent on the presence of the DBL-1 ligand and its receptors. *crm-1* is expressed in neurons at the ventral nerve cord, where the DBL-1 ligand is produced. However, ectopic expression experiments reveal that *crm-1* gene products act outside the DBL-1 producing cells and function non-autonomously to facilitate *dbl/sma* pathway signaling to control body size.

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**Keywords:** *C. elegans; crm-1; Body size; BMP pathway**

### Introduction

Bone Morphogenetic Proteins (BMPs) control a variety of cellular processes required for proper differentiation of many organs in vertebrates (Yamamoto and Oelgeschlager, 2004). In *Caenorhabditis elegans*, the BMP-like ligand, DBL-1, is required for determining body size and male sensory organ patterning (Morita et al., 1999; Suzuki et al., 1999). In addition, conserved signaling molecules acting in the BMP pathway have been identified. They include the receptors, DAF-4 and SMA-6 (Estevez et al., 1993; Krishna et al., 1999), and the Smad proteins, SMA-2, SMA-3, and SMA-4 (Savage et al., 1996). Mutations in their encoding genes generate small body morphology and sensory organ patterning defects.

A number of previous studies have reported that BMP signaling is modulated by secreted proteins that bind BMPs in the extracellular space (Fig. 1A). For examples, Chordin, Noggin, Follistatin and Cerberus antagonize BMP signalling by directly association with BMP ligands (Smith and Harland, 1992; Sasai et al., 1994; Hemmati-Brivanlou et al., 1994). Some of these extracellular factors can also positively regulate BMP signalling. In *Drosophila*, short gastrulation (*sog*) mutant displays a dorsalization phenotype and *sog* acts against decapentaplegic (*dpp*) in the embryonic development (Ferguson and Anderson, 1992). On the other hand, *sog* is required for maximal *dpp* activity for the formation of dorsal most tissues (Decotto and Ferguson, 2001). Thus, *sog* functions both as an antagonist and an agonist of *dpp* in a context-dependent manner. However, no such positive or negative regulator of the *Bmp* signalling pathway has been identified in *C. elegans*. It is tempting to find out if such a regulator exists and whether it shares common property as its counterparts in other animal models.

CHORDIN and SOG proteins are characterized by their multiple cysteine-rich repeats (CR) (Sasai et al., 1994). The only CR domain-containing protein in *C. elegans* was first noted as a putative protein with similarity to the vertebrate CRIM1 (Cysteine-rich Motor Neuron 1) protein (Kolle et al., 2000). Overexpression of a truncated product of this *C. elegans* ortholog in *Xenopus* embryos can induce a secondary axis similar to that induced by *chordin* or *procollagen IIA* mRNA
injection (Larrain et al., 2000). Hence, this CRIM1 homologue is implicated in negative regulation of BMP signaling event. Consistent with this notion, overexpression of CRIM1 in human cells effectively reduces the secretion of BMP4 (Wilkinson et al., 2003). On the other hand, loss-of-function studies on Crim1 in zebrafish and mice are less conclusive. The elimination of the zebrafish Crim-1 activity shows defect in somite and vascular developments, but the phenotypes do not appear to be associated with an altered BMP level (Kinna et al., 2006). The disruption of Crim1 gene in mice results in a severe and complex phenotype affecting numerous organogenesis events, and there is no evidence of its role in early embryonic patterning (Pennisi et al., 2007). Although C. elegans crm-1 homolog has been identified, its expression and regulatory function in C. elegans development remains undefined.

In this paper, the phenotype of C. elegans crm-1 mutant in different genetic backgrounds is examined to evaluate its regulatory relationship with the BMP pathway. We provide genetic evidence that crm-1 facilitates BMP signaling in controlling body size. We showed that crm-1 acts along the BMP pathway and its activity is dependent on the presence of the DBL-1 ligand. crm-1 expression profile and the effect of ectopic crm-1 expression were also evaluated to establish a working model of CRM-1 function. We unambiguously demonstrate that CRM-1 functions non-autonomously to promote dbl-1 activity.

Materials and methods

C. elegans strains

Nematode strains were cultured and maintained according to the standard procedures described by Brenner (1974) or otherwise indicated. The following mutations were used: sma-4(e729), sma-6(e1482), sma-6(k7), lon-1(e185), sma-2(e502), dpy-2(e8), dbl-1(nk3), him-5(e1490) and crm-1(tm2218).

RNA interference of the crm-1 gene

Double-stranded RNA interference (RNAi) was performed by both dsRNA microinjection and bacterial feeding methods. For injection purpose, linearized plasmid of full-length EST (yk240a9) in pBluescript SK(−) was used for in vitro RNA synthesis. The complementary strands were annealed to generate dsRNA for microinjection (Fire et al., 1998). The dsRNAs were introduced into the gonad of young adult hermaphrodites at a concentration of 1–3 μg/μl with TE buffer as control. To generate a large number of animals for quantitative analysis, we adopted the bacterial feeding method. A SpeI to AgeI fragment of the crm-1 gene was released from the cDNA clone yk240a9 and subcloned into the L4440 vector. The plasmid was transformed into an RNAseIII-deficient E. coli strain HT115(DE3) (Timmons et al., 2001). The optimized bacterial feeding protocol for young adult hermaphrodites was described by Kamath et al. (2001) where empty vector harboring HT115(DE3) was used as control.

Body-length measurement and male tail phenotype characterization

L4 hermaphrodites grown at 20 °C were transferred to fresh NGM plates or RNAI-feeding bacteria plates. Five days later, 100 F1 adult hermaphrodites were randomly photographed under the 5× or 10× objectives with SPOT RT camera (Diagnostic Instruments). Their body length was measured through a scale adjustment with SPOT RT v3.4.5 software and was presented as mean length with standard error. The male tails were examined by Nomarski microscopy (N>50). Of the transgenic strains, only transformed worms carrying the markers were imaged and scored.

Hypodermal ploidy measurements

The intensity of DAPI staining of the hypodermal nuclei in the hermaphrodite was determined from video-microdensitometry as described by Lozano et al. (2006). Nuclear images were captured as described above. The images were analyzed in Adobe Photoshop 7.0. The C values of hypodermal nuclei were calculated by dividing their DAPI-based densitometric value by an average of the values from 10 ventral cord nuclei in the same animal. Twenty hypodermal nuclei were scored for each young adult worm, and 15–20 worms were scored to obtain the average ploidy of the hypodermis.

Gene reporter constructs

Two kilobases of the genomic sequence upstream of the putative start codons of crm-1a and crm-1b were cloned into pPD95.75 green fluorescence protein (gfp) reporter vector, respectively. crm-1a genomic fragment spanning from putative exon 1 to 3′UTR (from +5 bp to +7105 bp) was subsequently inserted after unc-54 3′UTR to generate perm-1a::gfp reporter. Genomic fragment from putative exon 1 of crm-1b to its 3′UTR (from +1 bp to +9605 bp) was cloned after unc-54 3′UTR to generate perm-1b::gfp. The promoter region

Fig. 1. Vertebrate Bmp4 can functionally substitute dbl-1. (A) A model of the BMP signaling pathway where cysteine-rich (CR) domain-containing proteins function as both positive or negative regulator. (B–D) dbl/sma pathway controls worm body length. (C) A dbl-1 mutant have shorter body as compared with a wild-type animal (B). (D) A dbl-1 mutant expressing Xenopus Bmp4 has its body length restored (D). Scale bar=100 μm.
driven by the 1.3 kb unc-47 transgenic line. Xenopus Bmp4 hermaphrodites have a body length ranging from 1.10 mm to mutant phenotype to wild-type (Fig. 1D). Healthy adult (injected at 200 ng/treated as the positive control. These same constructs were also employed expressing the crm-1 cDNA (a gift from H.F. Kung) or mouse Nodal cDNA (a gift from E.J. Robertson) was inserted between the 1.5 kb of the dbl-1 promoter and unc-54 3’UTR in the pPD95.75 vector. dbl-1(nk3) mutant animals were used as recipients in these rescue experiments. The dbl-1 (nk3) mutant animals carrying these XBmp4 and mouse Nodal cDNA transgenes were examined for transgene expression by RT-PCR and were monitored for their respective body lengths and male tail phenotypes.

To express the sma-4 cDNA specifically at the hypodermal layer, wild type and mutant animals were transformed with plasmid with a sma-4 3’UTR (yk193k06) driven by a one-kilobase dpy-13 promoter (Ko and Chow, 2002). To express crm-1 ectopically in GABergic neurons, crm-1(tm2218) mutant animals were transformed with a plasmid containing crm-1a cDNA (yk240a9) driven by the 1.3 kb unc-47 promoter (Eastman et al., 1999) to generate transgenic line wxEx65. The same unc-47 promoter was used to express dbl-1 cDNA (yk1350b03) ectopically in the dbl-1 (nk3) mutant to generate transgenic line wxEx67. The rescued crm-1 mutant by a 9.1 kb crm-1 genomic fragment carrying the entire crm-1 locus (wxEx66) and the rescued dbl-1 mutant by expressing the dbl-1 cDNA using the 1.5 kb dbl-1 promoter (wxEx68) were treated as the positive control. These same constructs were also employed (injected at 200 ng/ml) to overexpress crm-1 or dbl-1 in the wild type animal. At least three independent lines each with transmission efficiency above 60% were scored for each specific transgene activity and were subjected to crm-1 RNAi feeding.

Results

**dbl-1** is the functional homologue of vertebrate BMP4

Genetic screening in *C. elegans* for small body size mutants uncovered many sma alleles, which correspond to mutations in genes encoding components in the dbll/O signaling pathway (Savage-Dunn et al., 2003). The secreted ligand encoded by dbl-1 is expressed in the neurons along the ventral nerve cord and can trigger signaling events via the SMA-6/DAF-4 receptors and the Smad transducers molecules in the hypodermis (Morita et al., 1999; Savage et al., 1996; Yoshida et al., 2001; Wang et al., 2002). DBL-1 ligand was predicted to be a member of the gene family consisting of Vg1, DPP and BMP with its C-terminus most closely related to NODAL (Morita et al., 1999). The Fitch–Margoliash algorithm, however, suggests DBL-1 to have a closer resemblance to BMP4/2 (Suzuki et al., 1999). To resolve the functional equivalence of DBL-1 in vertebrates, we tested the ability of BMP4 and NODAL to rescue the dbl-1 mutant phenotype (Fig. 1C). Expressing a full-length *Xenopus Bmp4* cDNA with dbl-1 promoter reverted the small body mutant phenotype to wild-type (Fig. 1D). Healthy adult hermaphrodites have a body length ranging from 1.10 mm to 1.40 mm with a mean of 1.23 ± 0.06 mm (Fig. 1B and Table 1). These *Xenopus Bmp4*-rescued dbl-1 animals, however, displayed a Lon phenotype (1.39 ± 0.05 mm), a feature resembling animals over-expressing nematode *dbl-1* (1.40 ± 0.07 mm) (Table 1). Expression of the full-length mouse Nodal cDNA by the same promoter could not restore the body size of dbl-1 mutants (0.83 ± 0.04 mm). Therefore, the Bmp4 gene, like dbl-1, acts in a dose-dependent manner to control the body size.

When this Bmp4 transgene was introduced into sma-6(wk7) mutants, the mean body length remained at 0.81 ± 0.03 mm. As the ligand encoding transgene was actively transcribed as monitored by RT-PCR (data not shown), the differential effects in these two genetic backgrounds suggest that the vertebrate BMP4 also acts through the SMA-6 receptor, and DBL-1 is a functional equivalent of vertebrate BMP4 but not NODAL.

**crm-1** mutant animals display the small phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
<th>1.2</th>
<th>1.4</th>
<th>1.6</th>
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<td>-</td>
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<tr>
<td>crm-1(nk3)</td>
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<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>dbl-1(nk3)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bmp4 over-expressed in dbl-1(nk3)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Nodal over-expressed in dbl-1(nk3)</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>sma-6(wk7)</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>sma-6(e1822)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>smo-4 over-expressed in wild type</td>
<td>-</td>
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</tr>
<tr>
<td>smo-2(e502)</td>
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<tr>
<td>lon-1(e185)</td>
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</tr>
</tbody>
</table>

* Significant difference *p* < 0.001.
analyses. When *crm-1* activity was increased by introducing more copies of 9.1 kb *crm-1* wild-type genomic fragment with full activity, no increase of body length was observed (1.23 ± 0.08 mm). That means whereas the availability of other regulatory components is limiting in wild-type animals, an increase of the *crm-1* activity alone was not sufficient to extend the body length.

Since mutations in the *C. elegans* *dbl/sma* pathway also affects patterning of male-specific sensory rays and spicule formation in the male tail (Savage et al., 1996; Baird and Ellazar, 1999, Morita et al., 1999), we scored the *crm-1*(RNAi) and the *crm-1(tm2218)* population for male tail abnormality. None of these animals exhibited any defect in their male sex organs, indicating that *crm-1* may not be required in the *dbl-1/sma* signaling events in the male tail (data not shown).

**crm-1 controls body size via endoreplication**

*dbl-1* and other components in BMP pathway have been reported to control endoreplication in the hypodermis after the L4/adult moulting process (Flemming et al., 2000, Morita et al., 2002, Lozano et al., 2006). We sought to determine if the *crm-1* mutant exhibits a similar cellular defect, and similarities between *crm-1* and the *dbl/sma* mutants were found. Wild-type animals had a mean ploidy of 8.8±0.6C (Table 2). *sma* mutants, e.g., *sma-2*, had a mean ploidy of 5.7±0.6C (n=16, p=0.0005) while *dpy-2* acting independent of the *dbl* pathway had a normal hypodermal ploidy. Endoreplication of hypodermal nuclei was reduced in both *crm-1(tm2218)* animals (7.0±0.7C, n=19, p<0.0005) and *crm-1*(RNAi) animals (6.3±0.9C, n=20, p<0.0005). The respective reduction in ploidy suggests that *crm-1*, like the other components in the BMP pathway, controls body size via regulation of endoreplication in the hypodermis.

**crm-1 is acting upstream of the co-smad transducer encoded by *sma-4***

To further characterize the role of *crm-1* in the *dbl/sma* pathway, we used the body size phenotype in these mutant animals as an indicator and manipulated the *dbl-1* pathway activity in the presence or absence of *crm-1* to test for their regulatory relationship. We showed that overexpression of *sma-4* cDNA using a hypodermis-specific promoter from *dpy-13*
was sufficient to rescue the Sma phenotype in *sma-4* mutant (from 0.72±0.06 mm to 1.21±0.09 mm). When this transgene array was introduced into *dbl-1* null mutant, wild-type body length could not be restored (0.83±0.05 mm). Similarly, Wang et al. (2005) have shown that an integrated *sma-3* array cannot rescue the defect in *sma-6(wk7)* mutant. Hence, a functional ligand and its cognate receptors are required to initiate the downstream signaling event acting through these Smad molecules (Miyazono et al., 2005). Overexpression of the downstream transducer molecules is in vain without the initiating signal on the recipient cell surface. On the other hand, elevation of the downstream *sma-4* activity in wild-type background will generate transgenic animals with slightly increased body length (1.28±0.05 mm, \(p<0.01\)) (Table 1). Interestingly, when these transgenic animals were fed with bacteria expressing *crm-1 dsRNA*, no significant reduction of body length was observed (1.27±0.07 mm) (Table 1). The results imply that overexpression of *sma-4* is epistatic to *crm-1* depletion. The *Sma* phenotype of the *crm-1(RNAi)* animals could be masked by constitutively supplying *sma-4* function. Consistent with this notion, a similar result was obtained in *lon-1* mutant animals. Mutations of *lon-1*, a downstream target of the *dbl/sma* pathway, confers a long body phenotype (Morita et al., 2002). The *crm-1(RNAi)* impact on the body length could be nullified by the *lon-1(e185)* mutation and the *crm-1(RNAi); lon-1(e185)* double mutants displayed a Lon phenotype (Table 1).

Based on the above observation, *crm-1* is not only upstream of *sma-4* and probably other R-Smad proteins encoded by *sma-2* and *sma-3* (Savage et al., 1996). If so, the *crm-1* function would be irrelevant in the absence of these Smad components. Indeed, the mean body length of the *crm-1(RNAi); sma-2(e502)* double mutants (0.73±0.04 mm) was almost the same as that of *sma-2* mutants (0.72±0.03 mm) (Table 1).

**crm-1** function requires both the *dbl-1* ligand and the *sma-6* receptor

If *crm-1* acts upstream of the Smads, does it function via interaction with the ligand or the receptors? The *dbl-1* gene functions in a dose-dependent manner to control nematode body length (Morita et al., 1999; Suzuki et al., 1999). While the *dbl-1(nk3)* mutant with complete absence of ligand production displayed a mean body length of 0.82±0.03 mm, heterozygous *dbl-1(nk3)* animals had a mean length of 1.09±0.07 mm and transgenic animals over-expressing *dbl-1* had a mean length of 1.40±0.07 mm (Table 1). We showed that the *crm-1(RNAi)* effect is sensitive to the dose of the *dbl-1* ligand. *crm-1(RNAi)* in *dbl-1* (*nk3*) null mutants had no impact on the body length (0.82±0.04 mm). The mean length of the *crm-1(RNAi)* animals heterozygous of *dbl-1(nk3)* had a moderate decrease of body length (1.00±0.06 mm, \(p<0.001\)), while the same treatment reduced the body length more drastically from 1.40±0.07 mm to 1.17±0.10 mm in animals over-expressing *dbl-1*. The results suggest that *crm-1(RNAi)* can exert its impact on body length only when the DBL-1 ligands are present. Moreover, *dbl-1* overexpression could increase the body length of *crm-1(RNAi)* animals to 1.17±0.10 mm from 1.03±0.08 mm in a wild-type background (\(p<0.001\)). Therefore, *crm-1* function is certainly *dbl-1* activity-dependent. This mutual suppression effect of *dbl-1* overexpression and *crm-1(RNAi)* also argues that *crm-1* is acting in parallel to the ligand action.

Interestingly, this same *crm-1(RNAi)* effect was not observed in *Bmp4* over-expressing animals (1.38±0.05 mm with *crm-1(RNAi)* treatment versus 1.39±0.05 mm without treatment) (Table 1). Evidently, *CRM-1* is needed only to facilitate the activity of the *C. elegans* DBL-1, but not the vertebrate BMP4.

Whether it suggests existence of a species-specific molecular interaction, or additional auxiliary proteins are required for such interaction with vertebrate ligand, remains to be investigated. Nevertheless, increase of body length can be achieved by overproducing the ligand molecule in the absence of the CRM-1 agonist. This feature argues that receptors are not the limiting factor for controlling body length.

Similarly, we examined the *crm-1 RNAi* effect in two *sma-6* alleles, *wk7* and *e1482*. The *sma-6(wk7)* allele has a stop codon in front of the kinase domain and is a molecular null displaying a mean body length of 0.82±0.04 mm (Table 1) (Krishna et al., 1999). *crm-1(RNAi)* on these *sma-6(wk7)* animals generated no further shortening of body length (0.81±0.06 mm). In contrast, while the *sma-6(e1482)* allele with residual receptor activity displayed a weak *Sma* phenotype (0.89±0.03 mm) (Krishna et al., 1999), *crm-1(RNAi)* treatment significantly reduced the mean body length to 0.82±0.05 mm \((p<0.001)\), close to that of *sma-6* null. These combined results suggest that the activity of both *dbl-1* and *sma-6* is required for the wild type *crm-1* function.

If *crm-1* function is dependent on wild type copies of *dbl-1* and *sma-6*, a possible route of regulation could be through direct transcriptional activation of these two genes. Yet, when *dbl-1::gfp* and *sma-6::gfp* transgenic animals were subjected to *crm-1(RNAi)* treatment, no noticeable change of intensity nor change of expressing cells was observed (data not shown), suggesting that the agonistic interaction of *crm-1*, *dbl-1* and *sma-6* is probably at the post-transcriptional level.

crm-1 and *dbl-1* are co-expressed in the ventral nerve cord

To further distinguish whether *crm-1* intercept *dbl/sma* signaling via the ligand or the receptor, we examined the expression pattern of different *crm-1* transcripts using *gfp* gene reporters in transgenic animals. Among the five predicted *crm-1* transcripts, all of them except *crm-1b* share a common transcription initiation site as that for *crm-1a*. We constructed two genomic *gfp* reporters harboring the locus sequence to investigate their tissue expression patterns (Fig. 3A). In the transgenic animals carrying *pcrm-1a::gfp*, consistent signals were detected in neurons in the ventral nerve cord. By the positions and clustering of the cell body as well as the axonal outgrowth along the ventral nerve cord, *crm-1a* is found to be expressed in the DA neurons 2 to 7, the DB neurons 3 to 7 and additional neurons in the VA, VB and AS classes along the nerve cord (VNC) (Fig. 3B). Expression was
also observed in neurons around the pharynx (Fig. 3C). In the male tail, gfp signals in the RnA neuronal cells of sensory rays 2 and 4 (Fig. 3D) and the PVR neuron with the entire axonal process along the VNC were also observed (data not shown). crm-1b expression was active in the entire pharynx (Fig. 3E) and posterior gut (Fig. 3F). Since the body size defect of crm-1(tm2218) mutant could be successfully rescued by the genomic fragment used in pcrm-1a∷gfp reporter (mean length = 1.22±0.05 mm, p < 0.001) which excludes the production of crm-1b transcript, the expression pattern of crm-1a should account for the biological function defined by the crm-1 mutant.

dbl-1 is active in the VNC and has been detected in DA, DB, VA and VB neurons of transgenic animals carrying a rfp reporter driven by a 1.5 kb promoter sequence of dbl-1 (Morita et al., 1999; Suzuki et al., 1999) (Fig. 4C). In a transgenic animal co-transformed with perm-1a∷gfp and pdbl-1∷DsRed2(1.5k) reporters, overlapping expression of crm-1 and dbl-1 was found in most of the neurons and their processes (Fig. 4D), except a few scattered neurons, e.g., PVR neurons and some neurons of the AS class (data not shown). Hence, crm-1 and dbl-1 may function in the same cells.

crm-1 functions non-autonomously in facilitating dbl-1 activity

If crm-1 and dbl-1 are co-expressed in the same cells, how are they interacting with each other? Since neither crm-1 nor dbl-1 was expressed in the GABAnergic neurons along the VNC, these neurons would offer good ectopic expression sites for a functional assessment. The unc-47 promoter active in 26 GABAnergic neurons (Eastman et al., 1999, Cinar et al., 2005) showed neither overlapping expression with dbl-1 (Figs. 5B–D) nor crm-1 (Figs. 5F–H). When the dbl-1 cDNA was ectopically turned on with this unc-47 promoter in dbl-1 null mutant, transgenic animals with DBL-1 product made in these non-CRM-1-producing cells displayed a Lon phenotype (1.39 ± 0.07 mm, p < 0.001) (Fig. 5I), a feature resembling the dbl-1 overexpression lines (1.40 ± 0.07 mm). In addition, when the same unc-47 promoter was used to ectopically express crm-1a cDNA in the crm-1 mutant, the Sma body phenotype was significantly rescued (1.22±0.09 mm, p < 0.001). Since CRM-1 and DBL-1 can be ectopically produced in cells where they are not normally expressed and independently led to rescue of mutant phenotype when their locales of synthesis are dissociated, the CRM-1 facilitation of DBL-1 signaling probably occurs...
extracellularly where the CRM-1/DBL-1 physical interaction potentially takes place.

Discussion

crm-1 is a critical but not the limiting factor for body length determination

While there are plenty of reports on how the BMP signaling events are modulated by secreted proteins with CR domains, such a regulatory event has not been reported in C. elegans (Little and Mullins, 2006). In this paper, we present the first functional analysis of such a regulatory protein in C. elegans. Several lines of evidence suggest a facilitating role of CRM-1 on BMP signaling. Using body length as an assessment parameter, we showed that CRM-1 can enhance the BMP signalling, although this activity was not noted in male tail sensory organ patterning.

The small body phenotype of crm-1(tm2218) and crm-1(RNAi) animals confirmed that crm-1 is important in developing a normal body size. The severity of their phenotypes suggests that the crm-1 function, like that of the DBL-1 ligand and its receptors, is also dose-dependent. crm-1, however, is not the limiting factor that determines the body length in wild-type animals. While increase of crm-1 activity could not result in an increase of body length, elevated DBL-1 ligand production alone can do so. That means, crm-1 activity is in excess in wild-type animals and body length of the animals is primarily influenced by the ligand concentration. Furthermore, crm-1 mutant animals displayed a reduction of hypodermal DNA content due to a reduction of endoreplication in the hypodermal nuclei, as the other dbl/sma mutants did. Hence, both the gross body phenotype and the cellular defect consistently show that crm-1 is acting in the dbl/sma pathway.

crm-1 is a new player in the nematode BMP signalling

Many C. elegans mutants with a small body size are defective in component(s) of the BMP signaling pathway (Savage-Dunn, 2001). sma-9 encodes a Schnürr zinc finger transcription factor acting as a downstream component in DBL-1 signaling pathway (Liang et al., 2003). The serine-threonine kinase KIN-29 modulates TGF-β signaling to regulate body size. Genetically, kin-29 is epistatic to dbl-1 and acts upstream of lon-1 (Maduzia et al., 2005). In addition, the characterization of uncloned sma mutants and analysis of their interactions with existing components in the Sma/Mab pathway showed that sma-16, sma-17, sma-18 and sma-20 are epistatic to lon-1 and are probably acting further downstream in this pathway (Savage-Dunn et al., 2003). None of these new sma mutants reveals any modulating function on the extracellular ligand activity. Therefore, the crm-1 is the first regulator gene in C. elegans acting on the ligand/receptor level. The elucidation of its function may therefore shed light on the role of its orthologs in developmental events in other animal models.

Comparison of the body length of crm-1(RNAi) and dbl-1 mutant animals shows that severity of the phenotype of crm-1(RNAi) animals is weaker than that of dbl-1 null mutants. If dbl-1 function requires crm-1, their inequivalent phenotypes must be explained. One possible reason would be the ineffectiveness of knocking down the crm-1 gene primarily expressed in the neuronal cells more resistant to RNAi treatment (Kamath et al., 2001). In fact, RNAi treatments targeting dbl-1 and sma-6 genes gave similar weak loss-of-function phenotypes (data not shown). On the other hand, it is possible that the dbl-1 function is not entirely dependent on the crm-1 function. In the absence of crm-1 activity, residual dbl-1 signal can propagate and be sufficient to prevent an extreme reduction of body length as exhibited by null dbl-1 mutants. The independence of Xenopus Bmp4 from this crm-1 function certainly supports this second hypothesis. The potent frog BMP4 does not require the CRM-1 activity at all and is capable of generating long animals on its own. This result also suggests that the nematode BMP receptors, SMA-6 and DAF-4, are not the limiting factors restricting the increase of body length.
Novel mechanism of modulating DBL-1 signal by CRM-1

We showed by genetic approach that *crm-1* acts non-autonomously outside the expressing cell to promote the *dbl/sma* pathway activity possibly through its interaction with the DBL-1 ligand or/and its receptors. As a facilitator of DBL-1 signaling, CRM-1 may function in the ligand producing cells to facilitate the ligand secretion process, or it may associate with...
the ligand to enhance its presentation to the receptor. A third possibility is that CRM-1 act as a co-receptor to augment the ligand–receptor binding and thus strengthen the signalling event. It is well documented that SMA-6 receptors are synthesized in the pharynx, intestine, male tail and hypodermis (Krishna et al., 1999), but only its expression in the hypodermis is required for body size determination (Yoshida et al., 2001). Consistent with this notion, the DAF-4 receptor is required only in the hypodermis for determination of body length (Inoue and Thomas, 2000). The hypodermis is the crucial tissue required for body size regulation. When no crm-1 transcriptional activity was detected in the hypodermis, crm-1 unlikely functions at this receiving end of the signaling as a co-receptor.

Neurons along the ventral nerve cord serve as the source of the DBL-1 signal, which globally regulates the body size (Suzuki et al., 1999). Dual fluorescent reporter-labelling showed that both crm-1 and dbl-1 are expressed in the same cells along the VNC. If CRM-1 functions to facilitate DBL-1 ligand secretion in the same cell, ectopic expression of dbl-1 alone in GABAnergic neurons, which normally express neither crm-1 nor dbl-1, should not rescue dbl-1 mutant phenotype. However, such manipulation is sufficient to fully restore wild-type morphology in dbl-1 mutant and generates an extension of body length. Similarly, ectopic expression of crm-1 alone in these GABAnergic neurons can also rescue crm-1 phenotype. These results argue against that CRM-1 modulates the level of ligand production in the expressing cells.

Under these circumstances, we are left with the option that CRM-1 works extracellularly to control the DBL-1 activity (Fig. 6). That means, CRM-1 may enhance cell–cell communication events that are dependent on the secreted DBL-1 ligand and its cognate receptor function in the extracellular matrix. Many CR domain-containing proteins are secreted molecules that help to establish the BMP gradients in the extracellular space through physical association. Their activity can be further regulated by a class of metalloproteases, e.g., XOLLLOID that cleaves CHORDIN complexes at specific sites and generates protein fragments with intact BMP-binding CR domains (Larrain et al., 2000). A regulatory step of a similar nature may be required for CRM-1 function. In fact, CRM-1 proteins could interact with a variety of extracellular molecules in C. elegans, some of which are matrix-associated metalloproteases with regulatory activity (Li et al., 2004). Whether CRM-1 protein is cleaved and how it interacts with DBL-1 remain unknown. As an alternative, the gene products of isoforms, crm-1c and crm-1e, lacking any transmembrane domain could account for this mode of CRM-1 action in the extracellular matrix without invoking any processing step. Should these cDNA isoforms be available, ectopic expression of them in mutants will tell the functional roles of different isoforms apart.

On the other hand, we have not been able to demonstrate in our preliminary study any direct physical interaction of CRM-1 and DBL-1 in any in vivo or in vitro condition. The topology of the CRM-1 molecule, however, has been ascertained using N-terminal GFP fusion reportes. Both the signal peptide and transmembrane domain properties have been confirmed, suggesting CRM-1 to be localized on the membrane of the expressing cells. So far, this finding is consistent with our understanding about non-autonomous function of CRM-1.

Is the CRM-1 function conserved?

CRIM1 homologues have been identified in humans, mice, chickens, zebrafish and worms (Kolle et al., 2000). Knock-down of crim1 in zebrafish results in an expansion of the ventral mesoderm-derived posterior blood island and loss of pioneer muscle cells. The phenotypes are not consistent with a role of crim1 in the dorso-ventral patterning, an event requiring BMP signaling (Kinna et al., 2006). Disruption of crim1 gene expression in mice results in cerebral edema, eye, rib, kidney and skull defects (Pennisi et al., 2007). In the mouse embryo, expression of Crim1 is detected in the notochord, somites, floor plate, early motor neurons and interneuron of developing spinal chord (Kolle et al., 2000). These patterns indeed overlap spatially and temporally with that of Bmp4 and Bmp7, although information of their products co-localization is lacking. Our data from functional assay and expression analysis at single cell level argue strongly that CRM-1 products do have an agonistic role in BMP signalling. The demonstration of CRM-1 function in C. elegans as an extracellular modulator would allow us to formulate hypothesis and evaluate the impact of this modulator on ligand diffusibility and presentation, which can be experimentally tested. As a note of caution, because the effect of reducing CRM-1 activity in the C. elegans could not be recapitulated when the worm DBL-1 ligand was substituted by the Xenopus BMP4, species-specific interaction should be considered when molecular interaction is monitored.

In recent studies, a number of CR-containing proteins have been found to contain additional domains. For example, the Nel-like secreted protein (NELL) contains the EGF-like domain for binding protein kinase C and a TSP-1-like domain for heparin binding (Kuroda and Tanizawa, 1999; Kuroda et al., 1999). Xenopus Kielin and Drosophila Crossveinless 2 (Cv-2) contain the vWF-D domain at the C-termini to promote cell–cell interactions (Hunt and Barker, 1987). CRIM1 is the first CR domain-containing protein with an IGFBP domain. Many IGFbPs have been found to interact with IGF-I and/or IGF-II in vitro (Kim et al., 1997). Members of this protein

Fig. 6. A model of crm-1 function in the BMP signaling pathway in C. elegans. Both CRM-1 and DBL-1 are produced from the same neuronal cells. CRM-1 facilitates the presentation of DBL-1 in the extracellular space. The signaling event acting via the SMA-6/DAF-4 receptors is transduced into the hypodermis affecting its differentiation events.

family can modulate the binding of insulin to its corresponding receptors and inhibit insulin signalling (Yamanaka et al., 1997). Do CRM-1 and its vertebrate counterparts have the same property that can potentially integrate multiple pathways via its modular structure? Structural dissection of CRM-1 protein generated from different transcripts in worm should allow us to address such issue systematically. Nonetheless, RNAi knockdown specifically targeting the crm-1b transcript generates no phenotype or any effect on the body length (data not shown). This observation may simply be caused by the ineffectiveness of RNAi treatment using fragment spanned across the crm-1b specific region. Or, it is due to the lack of an appropriate biological assay to reveal the activity of this isoform. While this IGFBP domain may not be required for the modulation of the dbl/sma pathway to control body length, its specific expression in the pharyngeal bulb and gut dictated by an alternative promoter does suggest its role in a distinct developmental context. dbl signaling pathway has been implicated to function in the induced immune response via activation of lysozyme genes in the intestine (Mallo et al., 2002). The same innate immunity depends on the daf-2/daf-16 insulin-like growth factor pathway (Millet and Ewbank, 2004). Hence, we would not be surprised if this crm-1b encoded product is specifically required in the digestive tract to coordinate the activity of these two pathways. Certainly, pathogen infection and animal resistance assays will be useful to confirm this notion.

It is interesting to note that while early work often defined the CR-containing molecules as BMP antagonists, an emerging view is that a lot of these molecules have an agonistic effect on BMP signaling. For example, the Cv-2 gene product promotes the DPP signal at the CVs and the ends of LVs in Drosophila wing, although Cv-2 product is structurally similar to a BMP antagonizing factor, KIELIN (Conley et al., 2000; Matsui et al., 2000). Similarly, the mammalian Crossveinless 2 and a related molecule Kcp (aka Crim2) displayed a pro-BMP activity in mouse organogenesis (Ikeya et al., 2006). The chordin-related molecule encoded by Twisted gastrulation was found to exhibit antagonistic and agonistic effect on BMP signaling depending on its dosage manipulated by overexpression or depletion (Xie and Fisher, 2005). Crm-1 can indeed be an antagonist. Wilkinson et al. (2003) demonstrated that overexpression of the human CRIM1 with BMP4 in metanephric explant cultures resulted in a reduction of BMP secretion, thus concluding that CRIM1 antagonizes BMP function by blocking its cellular release. When crm-1 is found to be coexpressed with dbl-1 in nematode, a conserved mechanism in operation would have been expected. Nevertheless, crm-1 activity at the physiological level is clearly needed to enhance the BMP activity specifically to hypodermal differentiation. Moreover, crm-1 activity is dispensable for the male tail sensory ray patterning, suggesting a differential impact of this molecule in alternative biological processes. The difference in tissue requirement may not necessarily suggest any contradictory interpretation but simply highlights the variation of the dbl-1 signaling events during the ontogenic development of these animal models.

Acknowledgments

We thank members of Chow laboratory for helpful discussion throughout this work and comments on the manuscript. We are grateful to the Caenorhabditis Genetics Center for the nematode strains, to the National Bioresource Project, Japan, for providing the crm-1(tm2218) deletion strain, to A. Fire for the RNAi and the expression vectors, to Yuji Kohara for the cDNA and the mouse Nodal cDNA, respectively. This work was supported by HKUST6118/93M, HKUST6239/04M, HKU2/01C and HKU4/05C of Central Allocation Vote for Group Research, Research Grants Council, Hong Kong.

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