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Developmental Expression Pattern

Expression and regulation of *Xenopus CRMP-4* in the developing nervous system

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ABSTRACT The collaspin response mediator proteins (CRMPs) are a family of cytosolic phosphoproteins which play a critical role in the establishment of neuronal polarity and growth cone guidance. Here, we describe the temporal and spatial expression of *CRMP-4* during early *Xenopus* embryogenesis. *CRMP-4* transcripts were first detected by whole mount *in situ* hybridization at the end of gastrulation in the prospective neuroectoderm. During open neural plate stages, *CRMP-4* was expressed broadly throughout the anterior neural plate and in the three bilateral stripes of the posterior neural plate where primary neurons arise. The expression in the territories of primary neurogenesis prefigures that of the post-mitotic neuronal marker *N-tubulin*. At tadpole stages, expression was maintained throughout the central nervous system and in the retina of the eye. Consistent with the observed expression, *CRMP-4* transcripts are positively regulated by X-ngnr-1 and negatively by Notch signaling. The observed expression and regulation of *CRMP-4* differ from that of the *CRMP-2*, which is induced by the events of neural induction.

KEY WORDS: *Xenopus, primary neurogenesis, neurogenin, CRMP, Notch*

The collapsin response mediator proteins (CRMPs) (also known as TOAD (turned on after division), Ulip (unc-33 like protein) and DRP (dihydropyrimidinase family) are a conserved family of cytosolic phosphoproteins highly expressed in the nervous system (Wang and Strittmatter, 1996). Even though the CRMPs exhibit more than 60% amino acid identity to the amidohydrolase family, they do not possess enzymatic activity (Wang and Strittmatter, 1997).

The first CRMP was identified as an intracellular mediator of semaphorin/collapsin growth cone collapse (Goshima *et al.*, 1995). However, numerous studies have demonstrated that the activities of CRMPs are not restricted to this repulsive guidance cue and participate in a broad spectrum of additional activities, with function being dependant on the specific interaction with various protein partners (Arimura *et al.*, 2004). CRMP-2 participates in LPA-induced growth cone collapse and regulates axonogenesis through the binding of tubulin heterodimers (Inagaki *et al.*, 2001; Fukata *et al.*, 2002). CRMP-2 also contributes to the establishment of neuronal polarity through the association with Numb and promoting Numb-mediated endocytosis of the neuronal cell adhesion molecule L1 (Nishimura *et al.*, 2003).

CRMP proteins are also targets of a variety of protein kinases. CRMP-2 and CRMP-4 were identified as brain-specific substrates for glycogen synthase kinase 3 (GSK3) and during growth cone collapse, phosphorylation by Rho-associated kinase inhibits microtubule assembly and Numb-mediated endoyctosis (Arimura *et al.*, 2005; Yoshimura *et al.*, 2005; Cole *et al.*, 2006). Recently, CRMP-2 was identified as a negative regulator of p53 and it has been suggested to play a role in the regulation of proliferation (Llanos *et al.*, 2006; Tahimic *et al.*, 2006). Moreover, the CRMPs may contribute to the pathogenesis of specific neurodegenerative disorders (Charrier *et al.*, 2003).

Presently, we describe the expression analysis of *CRMP-4*, during early *Xenopus* embryogenesis. *CRMP-4* is expressed in the differentiating primary neurons and later expression is maintained throughout the central nervous system and in the eye. Correspondingly, *CRMP-4* is positively regulated by X-ngnr-1 and negatively regulated by the Notch pathway.

Abbreviations used in this paper: CRMP, collapsin response mediator protein; DRP, dihydropyrimidinase; GSK3, glykogen synthase kinase 3; TOAD, turned on after division; Ulip, unc-33 like protein.

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Results and Discussion

Xenopus CRMP-4 was identified in a microarray screen aimed at identifying genes induced in dissociated ectodermal explants by the neuronal determination transcription factor, X-ngnr-1. Comparison of the predicted amino acid sequence revealed *Xenopus* CRMP-4 is 90% identical to the corresponding human and mouse sequences. Lower identity was observed between *Xenopus* CRMP-2 (75%) and other mammalian CRMP members (71-74%) (Fig. 1). The Cdk5 priming and GSK3 phosphorylation sites identified in the mammalian CRMP-2 are conserved (Uchida *et al*., 2005; Yoshimura *et al*., 2005).

The expression of *CRMP-4* during early *Xenopus* embryogenesis was investigated by RT-PCR analysis with RNA isolated from various stages of development (Fig. 2A). Zygotic transcripts of *CRMP-4* were detected at low levels by the end of gastrulation, increased during neurula stages and were maintained at constant levels throughout tailbud stages and later development.

As shown in Figure 2B and 2C, transcripts are first detected at stage 11.5 by whole mount *in situ* hybridization, broadly throughout the prospective neural ectoderm. At stage 12.5, *CRMP-4* is enriched in the anterior neural plate and two longitudinal stripes flanking the midline (Fig. 2D). As development proceeds, the posterior expression becomes more prominent and *CRMP-4* is strongly detected throughout the territories of primary neurogenesis including the medial, intermediate and lateral stripes, where motor-, inter- and sensory neurons will later differentiate, respectively (Fig. 2E-G) (Chitnis *et al*., 1995). This pattern of expression is similar to that of proneural transcription factors and prefigures that of the neural specific β-tubulin (N-tubulin), which marks terminally differentiated neurons (Oschwald *et al*., 1991). Transcripts are still detected broadly throughout the anterior neural plate and in a lateral group of cells associated with the trigeminal ganglia. Neuronal differentiation in the anterior neural plate is delayed until tadpole stages, thus in contrast to *CRMP-4*, known proneural genes are absent from this territory (Papalopulu and Kintner, 1996). This restricted expression in the posterior neural plate contrasts the panneural expression of *Xenopus* CRMP-2 (Kamata *et al*., 1998).

During neurula and early tailbud stages, *CRMP-4* transcripts are found throughout the central nervous system including the developing brain and neural tube, as well as the eye (Fig 2I-K). As shown by the transversal section, transcripts of *CRMP-4* are present primarily in the subventricular and outer marginal layers of the neural tube (Fig 2L), where cells that initiate differentiation and post-mitotic cells are localized, respectively. Additionally, in the anterior ventrolateral region of the embryo, a scattered ring of cells expresses *CMPR-4*. A transversal section demonstrates that these are isolated cells lying immediately below the ectoderm (Fig. 2M). These *CRMP-4* expressing cells are maintained through tailbud stages but disappear at tadpole stages (compare Fig. 2K and N). This punctuate pattern is similar to that of *Xphox2A* and *XHand2* and has been suggested to be progenitors of smooth muscle cells or pericytes of the forming vasculature (Smith *et al*., 2000; Talikka *et al*., 2004). Interestingly, we have also observed a similar punctuate expression by other proneural genes such as *X-MyT1* (data not shown) and *N-tubulin* (Fig 2P) suggesting they are neuronal cells. At tadpole stages (Fig. 2N), *CRMP-4* expression is maintained in the CNS, the eye, as

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XCRMP4   1MS_YQGKKNIPRITSDRLLIKGGRIVNDDQSFYADIYMEDGLIKQIGDNLIVPGGVKTIE
hCRMP4   1""_""""""""""""""""""""""""""""""""""""""""""""""""""""""""""
hCRMP2   1""_""""""""""""""""""K""""""""""""""""""""""E""""""""""""
XCRMP2   1MSG""""""""""""""""""""K""""""""H""""""""""""""E""""""""""""

XCRMP4   60ANGKMVIPGGIDVHTHLQMPYRGMTTVDDFFQGTKAALAGGTTMIVDHVIPEPEASLTEA
hCRMP4   60""""""""""""""""F""""K""""""""""""""""""""""""I""""V""""S"""""
hCRMP2   60"HSR""""""""""""RF""""DQ""""SA""""""""""""""""""I""""V""""GT""LA"
XCRMP2   61"H"R""V""""""""""CF"K"CN""VSS""""L""""""""""""""""I""""V""""GS""LS"

XCRMP4  120LEKWREWADGKTCCDYSLHVDITHWSDSVKQEVETLVKQKGVNSFMVYMAYKDMYQMSNT
hCRMP4  120Y""""""""""S""""A""""""""N""""""""QN"I"D""""""""""""""""L""V"""
hCRMP2  120FDQ""""""""S"S""""""""""SE"HKGIQE"M"A""""DH""""""L""""F""RF"LTDC
XCRMP2  121FDQ""""""""A"S""""""""""""E"HKG"QE"M"A""""DH""""""L""""""""Q""LTDS

XCRMP4  180ELYEIFTFLGGLGAIAQVHAENGDIIAQEQNRMLELGITGPEGHVLSRPEELEAEAVFRA
hCRMP4  180""""""""C""E""""""""""""""""""""""T""""M""""""""""""""""""""""""
hCRMP2  180 QI""VLSVIRDI""""""""""""""""E""Q"I"D""""""""""""""""V""""""N"""
XCRMP2  181 QI""V"SVIRDI""""E""""""""V""E""F"I""Q""""""""""""""""V""""""N"S

XCRMP4  240ITIASQTNCPLYVTKVMSKSSVDLISQARKKGYVVFGEPITASLGTDGTHYWSKNWAKAA
hCRMP4  240""""""""""""""""""""AA""""""""""N""""""""""""I""""""""""""""""
hCRMP2  240""""N""""""""I""""""""AEV"A""""""T""Y""""""""""""S""""""""""""
XCRMP2  241""""SN""""""""""""""""""AEV"A""""""A""Y""""""""""""S""""""""""""

XCRMP4  300AFVTSPPLSPDPTTPDYINSLLASGDLQVTGSAHATFSTAQKAIGKDNFTLIPEGTNGIE
hCRMP4  300""""""""""""""""""""""""""""LS""""C""""""""""""""""A""""""""V"
hCRMP2  300""""""""""""""""""FL""""SC""""""""""C""N""""""V""""""""""""""T"
XCRMP2  301""""""""""""S""""FL""""SC""""""C""""C""""N""""""V""""""V""""""""A"

XCRMP4  360ERMSVIWDKSVATGKMDENQFVAVTSTNAAKIFNLYPRKGRIAVGSDSDLVIWDPDAVKI
hCRMP4  360""""""""""A""""""""""""""""""""""""""""""""""S""""""""""""""""""
hCRMP2  360""""""""""A"V""""""""""""""""""""""V""""""""""""""A""""""""S""T
XCRMP2  361""""IV""RA"V""""""""""""""""""""""""""""""""""""""A""""""""NLRT

XCRMP4  420VSAKSHHSAAEYNIFEGMELRGAPLVVICQGKIMMEDGTLHATQGTGRFIPCSPFPDYVY
hCRMP4  420""""N"Q""""""""""""""""""""""""""""L""""N""V""""A""""""""""S""""
hCRMP2  420I""""T"N"SL""""""""""C""S""""""S""""VL""""""V"E"S""Y""RK""""F""
XCRMP2  421I""""T"N"NV""""""""""C""S""""""S""""VL"E""""""E"S""Y""RK""""""""
                                                509   514  518  522
XCRMP4  480KRIKARTKMAELHAVPRGMYDGPVHDLASTPKAGTPAGSTKGSPTKQ_TA_PVRNLHHSG
hCRMP4  480""""""R""""D""""""""""""""F""TT""""G""""""AR""""RP_NP_""""""Q""
hCRMP2  480""""""SRL""""RG""""L""""""CEVSV""""TV""""S"A"T""A""QAP_""""""Q""
XCRMP2  481""""""SRL""""RG""""L""""""CEVSV""""V""""S"A"T""A""_P"Q"I""""Q""
                                    555
XCRMP4  538FSLIGNQADESGVRSASRRIVAPPGGRSNITSLS
hCRMP4  538""""S"T"V""_""""""K""""""""""""""""
hCRMP2  539""""S"A"I"DNIP"RTTQ""""""""""A""""""G
XCRMP2  540""""S"A"V"DNIP"RTTQ""""""""""A""""""G
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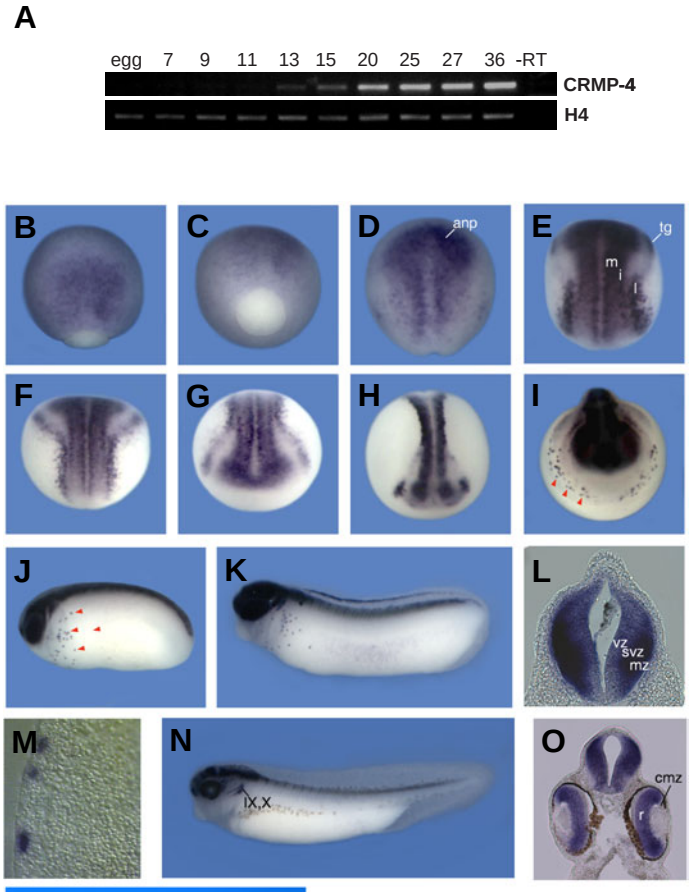
Fig. 1. Amino acid alignment of the predicted open reading frame (ORF) of *Xenopus* CRMP-4 and CRMP-2, with the corresponding human sequences. *Identical amino acids are indicated by ditto (") marks. Phosphorylation sites known for the mammalian CRMP-2 are shaded in gray and include the Cdk5 priming site (S522) for GSK3b phosphorylation (T509, T514 and S518), as well as the CRMP-2 Rho-associated kinase target site (S555) (Arimura* et al*., 2000, Uchida* et al*., 2005, Yoshimura* et al*., 2005).*

well as in the cranial ganglion IX (glossopharyngeal) and X (vagus ganglion) cells. Consistent with the exclusion of *CRMP-4* from the proliferating cells of the neural tube, staining in the eye is found in the central and marginal zones of the retina and is absent from the ciliary marginal zone where proliferating retinoblasts are found (Fig. 2O).

The regulation of *CRMP-4* was studied in ectodermal explants (animal caps) from blastula stage embryos; these explants are normally fated to become epidermal tissue, but can be converted to derivatives of all three germ layers. The animal blastomeres of two-cell stage *Xenopus* embryos were injected bilaterally with mRNA encoding putative regulators and animal caps were dissected at blastula stage. Total RNA was isolated at stage 14 and analyzed by RT-PCR. As shown in Fig. 3A, animal caps neuralized with the BMP inhibitor Noggin exhibited strong induction of *NCAM* as compared with uninjected animal caps, but no influence on *CRMP-4* expression was observed. This is in contrast to the regulation of the closely related *CRMP-2*, which is activated by Noggin (Figure 3A and Kamata *et al.*, 1998). The neuronal determination factor X-ngnr-1 robustly induced the activation of both *CRMP-4* and *CRMP-2*. The positive regulation of *CRMP*-2 by X-ngnr-1 is similar to the activation of other panneural genes such as *Nrp-1* and *NCAM* (Klisch *et al.*, 2006).

The regulation of *CRMP-4* was also investigated in whole embryos. mRNAs encoding putative regulatory factors were injected into one blastomere of two-cell stage embryos together with *LacZ* mRNA to localize the distribution of the injected mRNA. Consistent with the results from the animal cap assay, in whole embryos, X-ngnr-1 ectopically activated *CRMP-4* within the neural and non-neural ectoderm (100%, n=50; Fig. 3B). In addition to its proneural activity, X-ngnr-1 activates the Notch pathway in the neighboring cell, thereby restricting the number of cells that undergo neuronal differentiation. Overexpression of the intracellular domain of Notch1 receptor (Notch-ICD), which functions as a constitutively active form of Notch



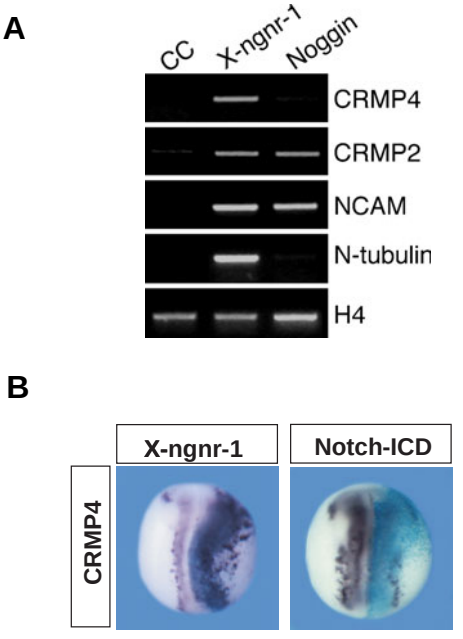


Fig. 2 (Left). Expression of *Xenopus CRMP-4*. (A) *Temporal expression of* CRMP-4 *determined by RT-PCR at various stages of* Xenopus *development. The ubiquitous marker Histone H4 served as a loading control. (B-O)* Xenopus *embryos at the indicated stages were analyzed by whole mount* in situ *hybridization using a digoxigenin-labelled* CRMP-4 *antisense probe.* **(B)** *Stage 11.5 d.,* **(C)** *stage 11.5, veg.,* **(D)** *stage 12.5 d.,* **(E)** *stage 14 d.,* **(F)** *stage 15 d.,* **(G)** *stage 15 ant.,* **(H)** *stage 16 d-ant.,* **(I)** *stage 19 ant.,* **(J)** *stage 24,* **(K)** *stage 28,* **(L)** *transversal section of (K) through the spinal chord,* **(M)** *transversal section of (K) showing through the punctuate anterior-lateral cells,* **(N)** *stage 37, lat.,* **(O)** *transversal section of (N) through the midbrain region.* **(P)** *N-tubulin antisense probe, stage 27. anp, anterior neural plate; ant, anterior; cmz, cilliary marginal zone; d, dorsal; i, intermediate; l, lateral; mz, marginal zone; m, medial; r, retina; svz, subventricular zone; tg, trigeminal placode; veg, vegetal; vz, ventricular zone; IX, glossopharngeal ganglion; X, vagus ganglion. Red arrowheads indicate scattered cells.*

Fig. 3 (Right). Regulation of *CRMP-4*. (A) Xenopus *embryos were injected bilaterally with* Noggin *(50 pg) or* X-ngnr-1 *(25 pg) and animal caps excised at the blastula stage. When sibling embryos reached stage 14, the caps were collected, total RNA isolated and RT-PCR analysis performed.* Histone H4 *served as a loading control.* **(B)** *Whole mount* in situ *hybridization of stage 14 embryos injected with sense RNA encoding* X-ngnr-1 *(25 pg) or* Notch-ICD *(50 pg) in one blastomere at the two-cell stage.*

(Coffman *et al.*, 1993), inhibits *CRMP-4* expression on the injected side (97%, n=57; Fig. 3B).

Taken together, the temporal expression pattern observed in the territories of primary neurogenesis, as well as the regulation of *CRMP-4* by Notch signaling and X-ngnr-1, support an early role for CRMP-4 in neuronal precursor cells as they initiate differentiation during primary neurogenesis. Both expression and regulation of *CRMP-4* differ from that of *CRMP-2*, which is induced by the events of neural induction. It will be of interest to elucidate the function of CRMP-4 during the differentiation of primary neurons in *Xenopus,* as the CRMP family has primarily been characterized during neuronal maturation in mammalian systems.

Experimental Procedures

Xenopus *CRMP-4*

CRMP-4 was identified in a *Xenopus* cDNA library obtained from the German Resource Center for Genome Research (IMAGE ID: 4408246, Accession number BC082618). The *Xenopus CRMP-4* cDNA clone contained 1909 bp insert comprising 146 bp of 5'-UTR, 1713 bp of coding sequence and 50 bp of 3'-UTR in pCMVSport6.

Xenopus *embryo collection and whole mount* in situ *hybridization*

Xenopus laevis embryos were obtained by HCG induced egg-laying, dejellied in 2% cysteine pH 8.0, washed and cultured in 0.1X MBS. Embryos were fixed in MEMFA at the desired stage according to Nieuwkoop and Faber (1967). The spatial expression patterns were determined by whole mount *in situ* hybridization (Harland, 1991) using a DIG labelled antisense probe. XCRMP-4pCMVSport6 was linearized with EcoRI and transcribed with T7 polymerase. Embryos were embedded in gelatine and 30 μm sections were prepared using a vibratome.

Microinjection of embryos

Capped mRNA for microinjections were prepared by *in vitro* transcription (mMessage-mMachine™ Ambion) and purified over an RNeasy column (Qiagen). Embryos were injected in one or both blastomere of the two-cell stage with the indicated amount of RNA: 50 pg *Noggin* (Smith *et al.*, 1993), 50 pg *Notch-ICD* (Coffman *et al.*, 1993), 25 pg *X-ngnr-1* (Ma *et al.*, 1996). As a lineage tracer, 50 pg nuclear *lacZ* mRNA was coinjected (Chitnis *et al.*, 1995).

Animal cap assay and RT-PCR

Two-cell stage embryos were injected bilaterally, animal caps dissected from stage 8-9 embryos and cultured until sibling controls reached stage 14. Total RNA was extracted from the various embryonic stages or animal caps (Qiagen RNeasy Kit) and cDNA prepared using random hexamer primers and MuLV reverse transcriptase (Perkin–Elmer). PCR was performed with Taq polymerase using the following gene specific oligonucleotide primer pairs:

Histone H4 (26 cycles)
forward: 5´-CGGGATAACATTCAGGGTATCACT-3´
reverse: 5´-ATCCATGGCGGTAACTGTCTTCCT-3´
NCAM (32 cycles)
forward: 5´-CACAGTTCCACCAAATGC-3´
reverse: 5´-GGAATCAAGCGGT5ACAGA-3´ (Hemmati-Brivanlou and Melton, 1994).
N-tubulin (28 cycles)
forward: 5´-ACACGGCATTGATCCTACAG-3´
reverse: 5´-AGCTCCTTCGGTGTAATGAC-3´ (Good *et al.*, 1989).
CRMP-2 (30 cycles)
forward: 5´-GGAGAACATGGTTCACACTA-3´
reverse: 5´-TGCAGCATTTGTACTGGTGAC-3' (Kamata *et al.*, 1998).
CRMP-4 (28 cycles)
forward: 5´-GGAACATTGGCGAGAGAGAAC-3´
reverse: 5´-GTTGTCTCCAATCTGCTTGAT-3´.

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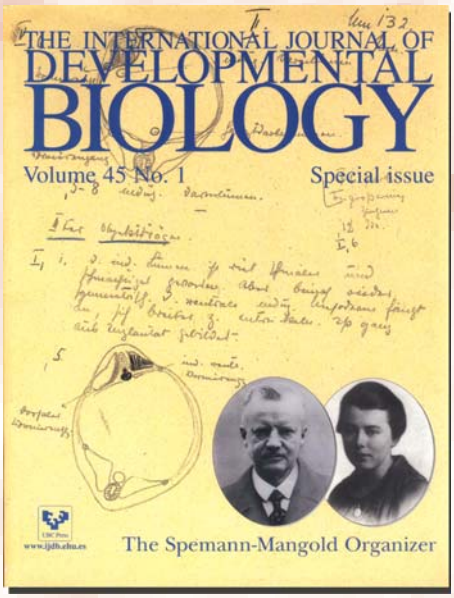

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