

**Molecular Characterization of CRMP5, a Novel Member of the
Collapsin Response Mediator Protein Family**

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Abstract

The CRMP (collapsin response mediator protein) family, a family of cytoplasmic proteins predominantly expressed in the developing nervous system, is thought to play key roles in the growth cone guidance. CRMP was first isolated as a factor required for collapsin-1 (Semaphorin3A)-mediated signaling. Four members (CRMP1-4) of the family have been demonstrated to form hetero-multimeric structures through mutual associations. In this study, a novel member of this family, CRMP5, was cloned by the yeast two-hybrid method, and characterized from various aspects.

A search for molecules topographically expressed in the embryonic chick retina using Restriction Landmark cDNA Scanning (RLCS) was performed to identify molecules implicated in regional specificity in the retina and in the topographic retinotectal projection. Among a number of molecules thus isolated, CRMP3 was identified as being asymmetrically expressed along the nasotemporal (anteroposterior) axis, although this expression pattern was transient during retinal development.

To identify CRMP3-interacting molecules, I performed a yeast two-hybrid screen of a mouse brain cDNA library using chick CRMP3 as bait. In this screening, sixty-one clones, mainly containing CRMP1, 3, 4 and dihydropyrimidinase (DHPase), were isolated. Among them, I found a novel clone which is homologous to the CRMP isoforms already known. I termed this molecule CRMP5. This protein consists of 564 amino acids and has a calculated molecular mass of 61,516 Da. When poly (A)⁺ RNA was analyzed, two bands of 4.8 and 5.2 kb, probably derived from a difference in poly (A) addition sites, were detected. CRMP5 bears several consensus sequences for phosphorylation sites that are conserved among the family, indicating that CRMP5 is also a phosphoprotein as are the other CRMP isoforms. CRMP5 shares relatively low amino acid identity with the other CRMPs (49-50%) and also with DHPase (51%), though CRMP1-4 exhibit higher identity with each other (68-75%). An unrooted phylogenetic tree of CRMP isoforms suggested that CRMP5 might be classified to a subfamily distinct from the

four other CRMP members. This notion was supported by the genomic structure of CRMP5 because the exon-intron organization is not completely conserved with that of CRMP1 and CRMP2, or DHPase either. To determine the chromosomal location of the mouse CRMP5 gene, fluorescence *in situ* hybridization was performed using a mouse CRMP5 genomic DNA fragment as a probe. The CRMP5 gene was mapped to chromosome 5 B1, a region that shares homology with human chromosome 7q. Recently, chromosomal locations of the human CRMP family (CRMP1-4) and DHPase genes were determined. These results indicate that CRMPs loci are widely dispersed throughout the human and mouse genome.

To reveal the expression profile of mouse CRMP5, I performed Northern blot analysis and *in situ* hybridization. Northern blot analysis of various mouse tissues indicated that CRMP5 mRNA is expressed in the central nervous system but not in non-neural tissues. The CRMP5 expression profile during development resembled that of both CRMP1 and CRMP4, in that the expression level peaked in the first postnatal week and markedly decreased in adulthood. To reveal in more detail the expression patterns in mice, *in situ* hybridization analysis was performed on developing embryonic sections. CRMP5 was expressed specifically in the nervous system, and signals were detected from the retina, olfactory epithelium, spinal cord, dorsal root ganglion, sympathetic ganglion, intestinal nerve, and brain with especially strong signals in the neocortex. This expression pattern is almost identical to that of CRMP4.

In contrast to the rapid progress in identification and characterization of the axon guidance molecules and their receptors, much remains to be explored about the intracellular mechanism by which signals are transduced into the eventual response of the growth cone. It has been reported that CRMPs form hetero-tetrameric structures. Consistently, I have identified other CRMPs by a two-hybrid screen using CRMP3 as mentioned above. Therefore, I tested the associations between CRMP5 and all CRMP isoforms. In the yeast two-hybrid system, CRMP5 interacted with each CRMP member with high affinity, except for CRMP1 and CRMP5. The same result was obtained from immunoprecipitation assays using an epitope-tagged expression system in COS-7 cells. Next, I tested

the interaction between the CRMP isoforms in all combinations by immunoprecipitation. CRMP3 showed very low association with CRMP1, CRMP3 and CRMP4, similar to that between CRMP5 itself. This means that endogenous CRMP tetramers are composed in combinations of 1/2/4, 2/3/5 and 2/4/5. CRMP5 mRNA was upregulated as was CRMP4 mRNA in PC12h cells treated with NGF, suggesting that CRMP4 and CRMP5 are responsible for the neurite extension. Consistent with this observation, CRMP5 expression increases when the neural network forms during development. CRMP complexes with different isoforms may exert distinct intracellular signalings from the extracellular signal, and explain the variegated responses of axons from different origins.

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Abbreviations

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
contig	group of overlapping clones
CRMP	collapsin response mediator protein
DMEM	dulbecco's modified eagle medium
DHPase	dihydropyrimidinase
DIG	digoxigenin
DRP	dihydropyrimidinase related protein
DRG	dorsal root ganglion
<i>En</i>	embryonic day <i>n</i>
EST	expressed sequence tag
FCS	fetal calf serum
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HS	horse serum
kb	kilobase(s)
kDa	kilodalton
NBT	4-nitroblue tetrazolium chloride
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
<i>Pn</i>	postnatal day <i>n</i>
PND	paraneoplastic neurological disease
RACE	rapid amplification of cDNA ends
RLCS	restriction landmark cDNA scanning
RT-PCR	reverse transcriptase-polymerase chain reaction
Sema3A	semaphorin-3A
TOAD64	turned on after division
Ulip	<i>unc33</i> -like phosphoprotein

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Chapter I

General introduction

Introduction

It is now established that growing axons respond to a complex balance of guidance cues, attractive or repulsive factors acting at short or long range. Well-known guidance cues are collapsins/semaphorins, netrins and ephrins (Tessier-Lavigne et al., 1996). The semaphorins form a large group with at least 30 members (Chen et al., 1997). They serve as repulsive guidance cues, influencing growth cone guidance not only in a contact-dependent way but also from a distance (Messersmith et al., 1995; Shepherd et al., 1997; Varela-Echavarria et al., 1997). Using a COS cell expression cloning approach, a transmembrane protein called neuropilin-1 was identified as a collapsin-1/semaphorin-3A (Sema3A) receptor (He et al., 1997; Kolodkin et al., 1997). Both neuropilin-1 and Sema3A knockout mice exhibited defasciculation and spreading of cranial nerves over a large area (Kitsukawa et al., 1997; Taniguchi et al., 1997). DRG growth cones from the neuropilin-1 knockout mice did not collapse in response to Sema3A, proving that the receptor neuropilin is necessary for inducing collapse (Kitsukawa et al., 1997).

In contrast to the rapid progress in the identification and characterization of axon guidance molecules and their receptors, much remains to be explored about the intracellular mechanism by which signals are transduced into the eventual response of the growth cone. CRMP62 has since been identified as an intracellular protein required for collapsin-1/Sema3A signaling (Goshima et al., 1995).

Identification of CRMP as a collapsin response mediator protein

CRMP was first identified as a factor required for the collapsin-1 signaling cascade, thus it was termed Collapsin Response Mediator Protein (Goshima et al., 1995). Goshima et al. used a *Xenopus laevis* oocyte expression system to identify molecules involved in collapsin signaling, with collapsin-induced inward currents as a barometer, and found a novel protein, CRMP62. The necessity of CRMP62 for collapsin signaling was demonstrated when the introduction of anti-CRMP62 antibody into DRG neurons blocked collapsin-induced growth cone collapse. CRMP62 is a 62 kDa protein, composed of 572 amino acids, and has no signal peptide or membrane-spanning domain, suggesting that it is an intracellular protein. CRMP shares homology with the *Caenorhabditis elegans unc-33* gene. Unc-33 mutant shows uncoordinated movement and defects in neuronal connectivities that result from abnormal axonal guidance (Hedgecock et al., 1985; Li et al., 1992). Therefore, CRMP62 is most likely a functional mammalian homolog of the *C.elegans unc-33* gene product and plays important roles in axonal guidance.

CRMP family proteins

To date, four CRMP members have been identified from several animal species and variously designated as TOAD/Ulip/DRP. Rat TOAD64 was identified as a developmentally regulated neuronal protein by two-dimensional gel analysis, because its expression was upregulated seven-fold between E14 and E21 (Minturn et al., 1995 a, b). TOAD64 is exclusively expressed in embryonic postmitotic neurons, and its name stands for turned on after division. Mouse Ulip (Ulip-1)

was identified as a neuronal phosphoprotein and its phosphorylation state is altered in nerve growth factor (NGF)-treated PC12 cells (Byk et al., 1996). Based on sequence similarity with the nematode *unc-33* gene and its highly phosphorylated state *in vivo*, it was named *unc-33* like phosphoprotein. Human dihydropyrimidinase (DHPase) related protein (DRP) was cloned on the basis of its sequence similarity with DHPase. DHPase is an enzyme that catalyzes the hydrolysis of a variety of 5, 6-dihydropyrimidines as well as hydantoins and succinimides (Hamajima et al., 1996). Extended screening further identified four CRMP isoforms in chick, mouse, rat and human (Wang and Strittmatter, 1996; Byk et al., 1998). The many names of the CRMP isoforms are summarized in Table I-1. In this thesis, I use the name CRMP because it seems the most functional.

All four CRMPs are expressed exclusively in the developing nervous system, though CRMP2 is also found in lung (Wang and Strittmatter, 1996) and CRMP4 in heart and adult testis (Wang and Strittmatter, 1996; Kato et al., 1998). It was demonstrated that the expression of individual CRMPs is differentially regulated (Wang and Strittmatter, 1996; Byk et al., 1998). CRMP1 and CRMP4 show expression profiles peaking in the first postnatal week and markedly decreasing in adulthood (Wang and Strittmatter, 1996; Byk et al., 1998). In contrast, CRMP2 is expressed at high levels continuously in adulthood, as is CRMP3 but only in the cerebellum (Wang and Strittmatter, 1996; Byk et al., 1998). Some CRMP isoforms are also expressed in neuron-related cell lines. For example, CRMP2 mRNA and protein are upregulated in parallel to the course of retinoic acid-induced neuronal differentiation and neurite extension in P19 mouse embryonic carcinoma cells (Minturn et al., 1995). CRMP4 mRNA is also upregulated in some neuroblastoma cell lines by retinoic acid treatment (Gaetano et al., 1997). In PC12 rat pheochromocytoma cells, NGF stimulates the expression of CRMP4, which parallels the course of neurite extension (Byk et al., 1998). Even

in adult rats, when the sciatic nerve was crushed, CRMP2 mRNA was re-expressed in corresponding motor neurons in the ventral horn of the spinal cord (Byk et al., 1995). These observations suggest that CRMPs compose a protein family required for neurite extension and implicated in axonal guidance.

It was reported that CRMPs form heterotetramers by mutual association (Wang and Strittmatter, 1997). This was demonstrated by the yeast two-hybrid method, an *in vitro* binding assay, and the purification of native CRMPs (Wang and Strittmatter, 1997). The observation is similar to that for DHPase in liver, which forms tetramers by self-association (Kikugawa et al., 1994), but CRMPs bear no DHPase activity (Goshima et al., 1995; Byk et al., 1996; Wang and strittmatter., 1997). This finding is very interesting because it suggests that CRMP functions in tetramers *in vivo*.

Table I-1. Nomenclature of the CRMP

CRMP	Ulip	DRP	others
CRMP1	Ulip3	DRP1	
CRMP2	Ulip2	DRP2	TOAD64, CRMP62
CRMP3	Ulip4	DRP4	
CRMP4	Ulip1	DRP3	
CRMP5			
	Ulip5		DHPase

Chapter II

Cloning of cDNA encoding mouse CRMP5 and chromosomal mapping

Introduction

In contrast to the rapid progress in the identification and characterization of axon guidance molecules and their receptors, much remains to be explored about the intracellular mechanism by which signals are transduced into the eventual response of the growth cone. Since chick CRMP62 (now designated CRMP2) was first isolated as a factor required for collapsin-1 (Sema3A)-mediated signaling (Goshima et al., 1995), a new family of cytoplasmic proteins that may be involved in signal transduction for semaphorins, has been identified through the work of several groups (Quinn et al., 1999). They are independently referred to as CRMP (collapsin response mediator protein; refs. Goshima et al., 1995; Wang and Strittmatter, 1996; Yoshida et al., 1998; Kamata et al., 1998ab), TOAD (turned on after division; Minturn et al., 1995ab), Ulip (Unc-33-like phosphoprotein; Byk et al., 1996, 1998; Gaetano et al., 1997) and DRP (DHPase related protein; Hamajima et al., 1996). To date, four different CRMP (TOAD/Ulip/DRP) genes have been described in chick, rat, mouse and human, all expressed exclusively in the developing nervous system (Wang and Strittmatter, 1996; Byk et al., 1998); exceptionally, CRMP2 mRNA is also detectable in lung (Wang and Strittmatter, 1996), and CRMP4 in heart and adult testis (Byk et al., 1996; Kato et al., 1998).

We searched for topographically expressed molecules in the embryonic chick retina, using a cDNA subtraction technique and a novel cDNA display system called Restriction Landmark cDNA Scanning (RLCS) (Yuasa et al., 1996; Suzuki et al., 1996) to identify molecules which are involved in regional specificity in the retina, including those responsible for the topographic retinotectal projection. Among a number of molecules thus isolated, CRMP3 was included in those asymmetrically expressed along the nasotemporal (anteroposterior) axis, although this expression pattern was transient during the retinal development.

In the present study, I performed a yeast two-hybrid screen of a mouse brain cDNA library using chick CRMP3 as bait to identify CRMP3-interacting molecules, and found a novel CRMP. This molecule, which I refer to as CRMP5, was nearly equally divergent from other CRMP members and DHPase. This chapter describes the identification of mouse CRMP5, a fifth member of the CRMP family, as a CRMP3-interacting molecule and determination of the chromosomal location of the mouse CRMP5 gene by fluorescence *in situ* hybridization.

Materials and Methods

Yeast Two-hybrid Screening

The cDNA fragment encoding the full-length coding region of chick CRMP3 (GenBank Accession Number AF249294) was amplified by PCR with specific primers containing restriction sites using the isolated cDNA clone of cCRMP3 as a template. After confirmation of the sequence identity, this fragment was inserted into the *EcoRI* and *BamHI* sites of the bait vectors, pBTM116 (kindly provided by Drs. P. Bartel and S. Fields) containing the LexA-coding sequence and pGBT9 (Clontech, Palo Alto, CA) containing a GAL4 DNA-binding domain, to generate pLexA-cCRMP3 and pGAL4BD-cCRMP3, respectively. pLexA-cCRMP3 was then transformed into *Saccharomyces cerevisiae* strain L40 which harbors reporter genes *HIS3* and *LacZ* under the control of upstream LexA-binding sites. The library screening was performed as described (Fields and Song, 1989; Kawachi et al., 1999). Approximately 1.0×10^6 clones were screened using a mouse 17-day embryo MATCHMAKER cDNA library (Clontech). Positive clones were selected with 1 mM 3-aminotriazole on plates lacking leucine, tryptophan and histidine.

To confirm the positive interaction using another two-hybrid system, each positive clone thus obtained was again transformed into yeast strain SFY526 (Clontech) harboring the reporter gene *LacZ* together with pGAL4BD-cCRMP3, and assayed for β -galactocidase activity according to the manufacturer's protocol (MATCHMAKER Library User Manual; Clontech). The nucleotide sequences of positive clones thus isolated were determined on both strands.

cDNA Cloning of the 5' -Portion of CRMP5

To obtain a longer 5'-flanking region, 5'-rapid amplification of cDNA ends (5'-RACE) was performed using total RNA prepared from embryonic day 17 (E17)

mouse brain. Briefly, total RNA was reverse-transcribed with ThermoScript reverse transcriptase (Gibco BRL, Rockville, MD) in the presence of 0.6 M trehalose (Wako, Osaka, Japan) (Carninci et al., 1998). After two rounds of PCR, amplified DNAs were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and subjected to sequence analysis.

Chromosome Preparation and Fluorescence in Situ Hybridization

The direct R-banding FISH method was used for chromosomal assignment of the mouse CRMP5 gene. Preparation of R-banded chromosomes and FISH were performed as described (Matsuda et al., 1992; Matsuda and Chapman, 1995). Mitogen-stimulated splenocyte culture was synchronized by thymidine blockage, and the incorporation of 5-bromodeoxyuridine during the late replication stage was made for differential replication staining after the release from excessive thymidine. R-band staining was performed by exposure of chromosome slides to UV light after staining with Hoechst 33258. The chromosome slides were hardened at 65°C for 2 hr and then denatured at 70°C in 70% formamide in 2 x SSC and dehydrated in a 70 – 85 – 100% ethanol series at 4°C.

Genomic fragments covering the mouse CRMP5 gene were screened from the mouse genomic library using the *SaI* (linker site) - *NcoI* cDNA fragment (nucleotide residues -42 - 249) as a probe. One of the genomic fragments (22kb long) was labeled by nick translation with biotin 16-dUTP (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. The labeled DNA fragment was ethanol-precipitated with ten times volume of mouse Cot-1 DNA (Gibco BRL) and then denatured at 75°C in 100% formamide. After hybridization, the slides were washed with 50% formamide in 2 x SSC at 37°C for 20 min, 2 x SSC, and 1 x SSC for 20 min each at room temperature. They were incubated with

Cy2-labeled streptavidin (Amersham Pharmacia Biotech, Tokyo, Japan) at a 1 : 500 dilution in 1% BSA, 4 x SSC for 1 hr at 37°C. The slides were washed with 4 x SSC, 0.1% Nonidet P-40 in 4 x SSC, and 4 x SSC for 10 min each on the shaker, and then stained with 0.75 µg/ml propidium iodide. Nikon filter sets B-2A (Fig. II-4, A and C) and UV-2A (Fig. II-4, B) were used for observation. Kodak Ektachrome ASA100 films were used for microphotography.

Results

Yeast Two-hybrid Screening for CRMP3-interacting Molecules

To elucidate the signal transduction cascade of CRMPs upstream and downstream, I attempted to identify CRMP3-interacting molecules by the yeast two-hybrid screening of a mouse embryo cDNA library using chick CRMP3 as bait (pLexA-cCRMP3). Sixty-one clones were isolated by surveying approximately 1.0×10^6 transformants. Sequence analysis showed that CRMP1, CRMP3, CRMP4 and the mouse homologue of DHPase were included among them: six clones for CRMP1, one clone for CRMP3, 29 clones for CRMP4, and six clones for DHPase. This result supports the notion that CRMP isoforms associate with one another (Wang and Strittmatter, 1997). The sequence of mouse DHPase, determined for the first time in the present study, shared 94 and 88% amino acid identity with rat and human DHPase, respectively (GenBank Accession Number: AF249296).

Besides the already identified CRMP isoforms described above, I found one clone, 1-8-7, encoding a novel protein sequence that shows significant but relatively low homology with CRMP1-4 and DHPase. Interaction of this novel CRMP isoform with chick CRMP3 was verified by the two-hybrid method using another bait construct pGAL4BD-cCRMP3 (data not shown). I here designate this new

CRMP as CRMP5. Moreover, two clones with no homology to these CRMP isoforms, which are supposed to be novel CRMP-interacting molecules, were also identified (to be reported elsewhere).

Molecular Cloning and Sequence Analysis of the Full-length CRMP5

The CRMP5 cDNA clone, 1-8-7, contained a putative open reading frame (ORF) that was in-frame with the GAL4 activation domain sequence and therefore expected to be expressed as a fusion protein. Because clone 1-8-7 included no in-frame stop codon located upstream of the first methionine (nucleotides 1-3 in Fig. II-1), I performed 5'-RACE to identify further the upstream sequence. By a reverse transcriptase reaction with trehalose (Carninci et al., 1998), I obtained longer RACE-PCR products and found two in-frame stop codons in clone 4-1. Thus, I concluded that the initially isolated CRMP5 cDNA clone, 1-8-7, has the full-length ORF. In Fig. II-1, the contig nucleotide sequence composed of 1-8-7 and 4-1, together with the deduced amino acid sequence, is presented.

My search of the mouse EST database identified 12 overlapping mouse cDNAs matching the 3'-noncoding region of 1-8-7. The resultant contig sequence had an additional poly(A) signal that is located about 400 bp downstream of the nucleotide sequence shown in Fig. II-1 (data not shown; for GenBank Accession Numbers, see the legend). Therefore, two types of CRMP5 mRNA transcripts that differ only in the length of the 3'-noncoding region are probably generated.

I screened EST databases for homologues of CRMP5 in other species. This analysis identified six human EST sequences (GenBank Accession Numbers N51749, AA350414, AA058664, AA351100, AA488145, and AI36969) showing significant, although still partial, homology with mouse CRMP5; the N-terminal (corresponding to amino acid residues 1 – 184 of mCRMP5) and C-terminal (residues 482 – 564) amino acid sequences were predicted from the EST clones.

Compared with mouse CRMP5, the deduced amino acid identity was 88% for the N-terminal part and 98% for the C-terminal part (Fig. II-2).

Amino Acid Sequence Comparison between CRMP5 and the Other CRMP Isoforms

The CRMP5 cDNA encoded a protein of 564 amino acids whose calculated molecular mass is 61,516 Da. It shares approximately 50% amino acid identity both with the members of the CRMP family (CRMP1-4; 49-50%) and with DHPase (51%) through the entire length (Fig. II-3A). Because the already known CRMP family members exhibit approximately 70% identity with each other (68-75%), CRMP5 might be categorized to another subfamily (Wang and Strittmatter, 1996; Byk et al., 1998). As shown in Fig. III-3B, a phylogenetic tree of CRMP isoforms indicated that CRMP5 is relatively close to DHPase and suggested that CRMP1-4 diverged first from a common ancestor with DHPase, followed after a lag by CRMP5.

The amino acid sequences of CRMP isoforms were aligned (Fig. II-5). The N-terminal three-quarters is more conserved than the C-terminal region. When CRMP5 is compared with the other CRMPs, it is evident that some amino acid substitutions are identical or similar to those of DHPase, together with the gap at the N-terminal region. On the other hand, the sequence at aligned positions 54-57 is absent, and the C-terminal region after 530 is present, similar to other CRMP members. It is notable that some regions (at aligned positions 279-298, 313-339, 508-536 and 572-576) were different from all the other CRMP isoforms including DHPase.

CRMP isoforms are known as phosphoproteins (Byk et al., 1996, 1997) and bear several consensus sequences for phosphorylation sites that are conserved among the members. CRMP5 contains 17 such potential phosphorylation sites (Fig. II-1); a single potential site for tyrosine phosphorylation, two potential sites

for protein kinase A, six sites for protein kinase C (PKC), and eight sites for casein kinase II. Among them, three PKC target sites are unique to CRMP5 (see legend to Fig. II-1). This suggests that the function of CRMP5 could also be regulated by phosphorylation, specifically and/or commonly.

Recently, it has been proposed that the amidohydrolases including DHPase share the same active site architecture (Holm and Sander, 1997). Notably, four histidine residues (at aligned positions: 77, 79, 202 and 258) and one aspartatic acid residue (at position 336) involved in metal-binding are highly conserved (Fig. II-5). Because CRMP5 has substitutions in two histidine residues, it is unlikely that it bears DHPase activity just as the other CRMP members do not (Goshima et al., 1995; Byk et al., 1996; Wang and Strittmatter, 1997).

Chromosomal localization of mouse CRMP5 gene

The chromosomal location of the mouse CRMP5 gene was determined by direct R-banding fluorescence *in situ* hybridization using a mouse CRMP5 genomic DNA fragment as probe. The CRMP5 gene was localized to the R-positive B1 band of chromosome 5 (Evans, 1996; Somssich and Hameister, 1996) (Fig. II-4).

Discussion

CRMPs are a family of cytoplasmic proteins predominantly expressed in the developing nervous system that have attracted attention because they might be key molecular components in shaping neural networks, although conclusive evidence about their functions remains to be obtained (Quinn et al., 1999; Mueller, 1999). To date, four CRMP members have been identified from several animal species and variously designated as TOAD/Ulip/DRP. Although individual

members show somewhat different expression patterns in the nervous system (Wang and Strittmatter, 1996), CRMPs are, as a whole, expressed in almost all the developing neurons peaking in the first postnatal week with a marked decrease in adulthood, except for CRMP2 and partly CRMP3.

While searching for topographically expressed molecules in the embryonic chick retina using the RLCS technique (Suzuki et al., 1996), I found that CRMP3 was expressed asymmetrically along the nasotemporal axis in the retina, although this expression pattern was transient during development. Analysis of the expression patterns of chick CRMP1-4 revealed that cCRMP1, cCRMP2, and cCRMP4 were expressed in a relatively similar pattern, whereas cCRMP3 was differentially expressed in the developing chick nervous system (unpublished observations), intimating its unique function in the process of neural network formation.

I have therefore attempted to screen CRMP3-interacting molecules using the yeast two-hybrid system to reveal the signal transduction cascade of CRMP3 upstream and downstream. In this study, I identified a novel CRMP, CRMP5. In previous studies using the yeast two-hybrid system with CRMP1-3 as bait, an *in vitro* binding assay, and also purification of native CRMPs, CRMPs were suggested to form tetrameric structures as in the case of DHPase (Wang and Strittmatter, 1997; Kikugawa et al., 1994). Therefore, it was expected that CRMP isoforms would be included in positive clones.

Mouse CRMP5 cDNA encoded a protein with 564 amino acids nearly identical in size to the other CRMP members. Amino acid sequence identity between CRMP5 and the four other CRMPs was significantly low (~50%) compared with that between CRMP1-4 (68-75%) and nearly equally divergent from that of DHPase (51%). A phylogenetic tree also showed that CRMP5 is significantly distant from CRMP1-4 and close to DHPase, suggesting that CRMP5 might be

classified to another CRMP subfamily. Here, it should be noted that CRMP isoforms more closely resemble each other in the N-terminal three-quarters, through which mutual association occurs (Wang and Strittmatter, 1997). However, when human and mouse CRMP5 sequences were compared, the C-terminal portion was found to be more conserved than the N-terminal region, suggesting a specific role for the C-terminal region of CRMP5.

Recent studies on the genomic structure of human CRMP1 and CRMP2 revealed that these genes consist of 14 exons and that the exon-intron organizations are completely conserved (Torres and Polymeropoulos, 1998; Kitamura et al., 1999). On the other hand, the human DHPase gene is composed of 10 exons and only five introns are shared by CRMP1, CRMP2 and DHPase (Torres and Polymeropoulos, 1998; Kitamura et al., 1999; Hamajima et al., 1998). I have cloned a fragment of the mouse CRMP5 gene. I found no intron insertions at the positions that correspond to intron 1 and intron 2 seen in the CRMP1 and CRMP2 genes but found an intron at the position that corresponds to intron 1 in the human DHPase gene together with an unique intron in the 5'-noncoding region (unpublished data). This finding again supports the view that CRMP5 might be classified to a subfamily distinct from the four other CRMP members. However, my low-stringency screening of a P1 mouse brain cDNA library and search of mouse and human EST databases for CRMP5-related molecules yielded no additional cognates, suggesting the absence of related subfamily members (data not shown).

Recently, chromosomal locations of the human CRMP family (CRMP1-4) and DHPase genes were determined: CRMP1/DRP1 at 4p15 - 4p16.1, CRMP2/DRP2 at 8p21, CRMP3/DRP4 at 10q25.2 - 10q26, CRMP4/DRP3 at 5q32, and DHPase at 8q22 (Kamata et al., 1998; Hamajima et al., 1996, 1998; Honnorat et al., 1999; Matsuo et al., 2000) (Table II-1). On the other hand, mouse CRMP2

and CRMP3 genes have been reported to be located at the central region of chromosome 14 and distal end of chromosome 7, respectively (Kamata et al., 1998; Quach et al., 2000). In this study, I demonstrated that the mouse CRMP5 gene maps to chromosome 5 B1, a region that shares homology with human chromosome 7q (DeBry and Seldin, 1996). These results indicate that the CRMP and DHPase loci are dispersed throughout the human and mouse genome.

Fig. II-1. Nucleotide and deduced amino acid sequences for mouse CRMP5. The predicted amino acid sequence is shown by the one-letter code below the nucleotide sequence. The original cDNA clone, 1-8-7, isolated by two-hybrid screening, starts at position -42. The upstream sequence (-443 to -9) is derived from clone 4-1 obtained by 5'-RACE. A putative polyadenylation signal is shown in bold. By searching the mouse EST database, 12 overlapping sequences were found to match the 3' untranslated region of CRMP5; GenBank Accession Numbers AA268030, AI509437, W40685, W54057, AA008915, W33434, W41424, AA032969, W78280, W77101, AA048239 and AA051700. An additional polyadenylation signal was found in the resultant contig, located about 400 bp downstream of the 3' end of the sequence shown here (data not shown). Consensus sequences for various phosphorylation sites are underlined: for protein kinase A (double solid line), protein kinase C (solid line), casein kinase II (dashed line), and protein tyrosine kinases (bold line). Potential phosphorylation sites conserved between CRMP5 and CRMP1-4 are S7, T77, T211, S219, S356 and S549; conserved sites between CRMP5 and some of CRMP1-4 are S108, S252, T458, S466, Y472 and S538; conserved sites between CRMP5 and DHPase are S108, T211, T229 and S356; CRMP5-unique sites are T54, T162 and T330. GenBank Accession Number for mouse CRMP5: AF249295.

CTAGTTCACCATGGACACGAAAGGACGCTGAGAAATGGCCCTTAGATGCTGGGGGTGTGAGGCTCTGGGGCCATGCCCC -361
 TGTCCTTCTGAGACCCGGGGCTGCAAAAGGACCTGTGGGATTTGAGTCCCAAGGCGGGGGCAACCGGGAGGACCGGGGGCTAC -271
 TGTCGGGGCTGAGCTAAGCTGCTAGCTGAGGGTGGGAGACCTTGGGGGGAGCCCTGGGATCGGTGATGGCCAGCGGGCTCCCGG -181
 CCCCACCCCTGTCCCAACCCCGCTGCGACTCGGGACTCGGCTCCAGGGGGGCAAGGCGCAACCGAGAGAGCGGGCTAGCAGCCGCT -91
 CCGCGCCCTGTCAACCGGCACTACCGGGGGGGGAGACCCAGAGACCCAGCGGAGCCGCAATCGCATCATCTGAGGACAGAAC -1
 ATGCTTGGCAATTCAGCCAGGTGGGGATTCATCAAGGGAGGCAAGGTTGGTGAAGATGACITGTACCCAGCGAGGAGGCTTACATC 90
 M L A N S A S V R I L I K G G K V V N D D C T H E A D V Y I 30
 GAGAGCGGCTCATCCAGCAGGTGGGGCTGAGCTCATGATCCCGGGTGGGGCAAGGTGATGACCGCAACCGGAACTGGTGTATCCCG 180
 E S G I I Q Q V G R E L M I P G G A K V I D A T G K L V I P 60
 GAGAGGATGACACCGACCCACTTCCACAGACCTTCATGAAACCCACTGTGTGGAGACTTCTAACATGGGACCAAGCGGGCCCTC 270
 G G I D T S T H F H Q T F M N A T C V D D F Y H G T K A A L 90
 GTTGGAGGCAACCATGATCGGTCACTCTGCGACAGGAGGACTTCCCTGTGGAGGCTATGAGAGTGCAGGGCTCTAGCT 360
 V G G T T M I I G H V L P D K E T S L V E A Y E K C R A L A 120
 GACCCCAAGGCTGCTGTGACTATGCCCTCCATGTGGGGTCACTTGGTGGGCAACCAAGGTGAAAGCAGAAATGGAGACACTGGTGGG 450
 D P K V C C D Y A L H V G I T W W A P K V K A E M B T L V R 150
 GAGAGGGGGTCAACTCTCCAGATGTCATGACTACAGGACTTGTACTGCTTCCAGACAGTGAAGTGTACCAAGTGTTCATGCC 540
 E K G V N S F Q M F M T Y K D L Y M L R D S E L Y Q V P H A 180
 TGCAAGCAGTGGGGGATCCCGGGTCCAGCTGAAATGGAGAGCTGGTGGCTGAGGGTCTAAGGAGGCTCTAGACTTGGGATC 630
 C R D I G A I P R V H A E N G E L V A E G A K E A L D L G I 210
 ACAGGGCCAGAGGATCGAGATCGCCATCCAGAGGAGCTGGAAGCAGAGCCACTCAAGGTGTCAACCATGCAAAACCGGACTCAC 720
 T G P E G I E I S H P E B L E A E A T H R V I T I A N R T H 240
 TGTCOCATCACTGGTCAATGTGTAGTATCTCAGCTGGTGTATTCATTCAGCTGCTAAGTCAAGGGAGGTTGGTCAAGCGAG 810
 C P I Y L V N V S S I S A Q D V I A A A K M Q G K V V L A E 270
 ACCACCAAGCACAACCCACTCCAGGGCTTGCATCTACCTCAGGACTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG 900
 T T N A H A T L T G L H Y Y H Q D W S H A A A Y V T V P P L 300
 AGACTGACCAACACCTTCCACCTACCTCATGAGCCCTGCGCCATGACACTTGAACATGTGGCATTCGATCAACCGGCAATTCAC 990
 R L D T N T S T Y L M S L L A N D T L N I V A S D H R P P T 330
 ACAAGGACAGAGCCATGGGCAAGGAGACTTCACTAAGATCCCGCCAGGTTGGAGGGTGTACAGGATCGAATGAGGGTGGTGGGG 1080
 T K Q K A M G K E D F T K I P H G V S G V Q D R M S V V W E 360
 AGGGGGTGGTGGAGGAAAGATGGATGAGAAACCGTTTGGGGGGTCAACAGTTCACAGCAGGACAGATCTCAACCGTATCTCGC 1170
 R G V V G G K M D E N R F V A V T S S N A A K I L N L Y P R 390
 AAGGGGGTATCATCCCGGAGCGGAGCTGTGTGTGTGGGAGCGGAGCCAGGACCATCTCAGCCAGCCAGCGGTTGAG 1260
 K G R I I P G A D A D V V W D P E A T K T I S A S T Q V Q 420
 GGTGGAGCTCAACTGTATGAGACATGGCTGCCAGGTTGACCGCTGGTCAACATCAGCGGGAGGAGTGGTGTATGAGAAAGG 1350
 G G D P N L Y E N M R C H G V P L V T I S R G R V V Y E N G 450
 GTCCTCATGTGTGAGAGGCGACTGGCAAGTCTGTTCCTCGGGTCTTCCAGACATTTGTCTAAGAGGCTGGTACAGAGAGAG 1440
 V F M C A E G T G K P C P L R S F P D I V Y K K L V Q R E K 480
 AACTAAAGTGGGGAGTGGAGCGGACTCCCTACCTAGGGGATGTGGCATTTGTTGTCACCCCTGGGAAAGAGATGGGACACCA 1530
 T L K V R G V D R T P Y L G D V A I V V H P G K K E M G T P 510
 TTGGAGACAGCCCAACCGGAGTCAACCGGCAAGGGGATGGGGCACTTCATGAAATCCAGTTTCAGGCTCTCTGGTTCAGATC 1620
 L A D T P T R P V T R H G G M R D L H E S S F S L S G S Q I 540
 GATGACCGTTCCAAAGCGGGCTCAGCTGGGTCCTTGGCGCCCTGGAGGCGGTCGAGCGGTTATTTGGTAAAGGCTCTGACCGCC 1710
 D D H V P K R A S A R I L A P P G G R S S G I W 564
 CCAACCTCAACAGTGGGATGCTAAACCAACACCTGCTGCAATGTTCCAGCGAGGCTGCAAGTGGTGGGAGGGTTGGGCAAGGCA 1800
 GTTCTCCACCGGTTGGGGCTCTAGGACAGGAGGAGGCTCCCTTGTCTGAAAGTGTTCAGGGGGCTTGTATCCACCCCTAACAGGCA 1890
 CTTTGAAGCTGTCCCTGCT 1980
 TGCTAGGACTGGGGCTAAACCTGCTTCAACATGACTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2070
 CCAACCCAGAGGTTGTCCAGGACCTTTAGCAATGGTGTGTTTATAAGTAAAGGGCTTCCCGCTTTCATCCCTTAGGCGCCATGGTGAAT 2160
 TCCCAAGTCAAGAGTGTCAACTCCAGCCATCCAGCCATCCAGCCATCCAGCCATCCAGCCATCCAGCCATCCAGCCATCCAGCCATCC 2250
 TCAGACACCCCGGGTGTGGCTGTGCTTGGGACCCCTGTGCTTCTGCTAGGCTTCTTCATTCCTAACCCAGCCCTTGGGAGTCCCA 2340
 GGCCCGGAGGGTAGCTGGGTGGGCAITGGCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT 2430
 GGTGTGACTGTGTGTGCAACCTGTGGAAATAGGGTGTGTGTGTGACTAGCATAGCATAGAAATCTGGAAATAGATTTGGTTGTAGGA 2520
 GATGCTGACTCAACCAACAGCAGTACAGGGCAGGAGGGGGGAGGGTGGAGCCCTGGGCGCTTCTTCATTCCTAACCCAGGGTCTTT 2610
 TGCTTCTGTACCGTCCCTCCCTATCCACACCTCTGCCACCCCTCCCTACTTCCCTACATCAAAACATGCTGGATGCTGACCCCTG 2700
 AAAACCTCAGGGCCAGTCTCTGCTCCGGGTTAOCAGGGCTTAACCCACATCCACACCCCTCATAGCCCAACACAGCTTCCATATACTG 2790
 CCCCCTGCTGGCAAGGCTAGCTACTCTCTCTGGTGTAGTAAAGGCTCTACTGCTGAGAAAGAGGAGCTGGGGGTTGGGGG 2880
 TTGCTAGGCAACCGGAGGAGGGCTTCAACCCAGAGGCTAGCTGTAGATCCACCCCACTGACAGGCTGCTATGAAATCTGTG 2970
 GCACTAAGCTCTCTTGAAGACTGGTGGGACCGGGCTTTTGAAGCTCTTGAAGCTCTTGAAGCTCTTGAAGCTCTTGAAGCTCTTGAAG 3060
 GCTCAGCTGTCTGCTTGTGATAGCTCATCCCGGGCTCTTTGGCAAGTGTCTCACTTTTGTGGAGCTCCACAACTGTGAGG 3150
 GCTTCCAGAGCTTGTGACTGACCCCTGACGCTTTTGTGCTGCTTGGTTCATCAAGAGGCTCAGCTCTCACTGAGTGAAGGTAAC 3240
 TCCAGGGCTCTCTGAGACTGGTGGGCTTACACTGTCCCGCTTTCAGATAAACCTGAGGCGCCCAAGGTTTGGGCAACAGAA 3330
 ATGCTGTCCATGACTCTCTCATGGCTGGCGTCAACCTAGCTGTGGAAACAGTGTGTGATGATGTGAGGCGCCACCGAGGTTT 3420
 AACAGAGAGGAGTCTTCTTCTGGGGGGTGTGCTGCTTGTGCTTGTGCAAGCGGATTTATCTTGTGATGACTGACTCATGTTG 3510
 AGCTGAGCTAGAAAGTGTAGGAGCTGGTGGGCTTACACTGTCCCGCTTTCAGATAAACCTGAGGCGCCCAAGGTTTGGGCAACAGAA 3600
 AGGACTGAGTAGCCCTGGAAACAGTGGTATGACCTAGAGGTCACAGGATCATAAACAGTGAAGCTGAGCGGTGTATCTCTCTT 3690
 CTCTCTACT 3780
 AATCTTCT 3870
 TAGCTATGACTCATCTTTGCTATTTCTGCGCTTGTGGCTTGTGGCTTGTGGCTTGTGGCTTGTGGCTTGTGGCTTGTGGCTTGTGGCT 3960
 AGGAGGGCCACTGGAGTGGGGCAGAGGGCTTTACCTCTCTAATGCCCTCCACTTGAAGTGTGATCCTGAGTGGTGGAGGAGG 4050
 CCGTACAGGGGCGACTGTGGGGCTTCACTTCACTCAACCCCGCTTCCACAGACCCAGCCCAACCCCTTTCATAGCGCTGTGCTCAC 4140
 CCAGGACTTCTAGATGGAGGCTTAGAGCCCAACCCCGCTCACTCCCTGTGTCCACTCCCTGCGCCATGAGCAGGCTCCCAACCC 4230
 CATCCAGGTTGGGCTGCCAGCTGGAGGAAAGGGTCTCTGGTGGCGGATGCACTCTCAAACTGCTTGTACTGCTGAAAT 4320
 AUNCACTGTGTCTTCCCTTAAAAAATAAAAAAAAAAAAAA 4361

Fig. II-2. Amino acid sequence comparison between mouse and human CRMP5. The N- and C-terminal amino acid sequences of human CRMP5 were deduced from the contigs assembled from the six EST sequences (GenBank accession numbers N51749, AA350414, AA058664, AA351100, AA488145, and AI36969). Identical amino acids in the two sequences are indicated by asterisks, and conservative substitutions by colons and dots as described (Hamajima et al., 1998). The broken line indicates the unidentified middle part of human CRMP5. The middle part of mouse CRMP5 is also represented by a dotted line.

mCRMP5 1 MLANSASVRILIKGGKVVNDDC THEADVYIESGIIQQVGRELMI PGGAKV 50
hCRMP5 MLANSASVRILIKGGKVVNDDC THEADVYIENGGIIQQVGRELMI PGGAKV

mCRMP5 51 IDATGKLVIPGGIDTSTHFHQTFMNATCVDDFYHGTKAALVGGTTMI IGH 100
hCRMP5 IDATGKLVIPGGIDTSTHXHQTFMNATCVDDFYHGTKAALVGGTTMI IGH

mCRMP5 101 VLPDKETSLVEAYEKCRALADPKVCCDYALHVGITWWAPKVKAEMETLVR 150
hCRMP5 VLPDKETSLVDAYEKCRGLADPKVCCDYAIHVKITLWAPEMKAVLETMLL
*****:*****:*****:*** **:*:*:* :***:

mCRMP5 151 EKGVNSFQMFMTYKDLMLRDSELYQVFHACRDIGAI PRVHAENGELVAE 200
hCRMP5 KKGIKSLEMFMTYKDLMLRVSELYQVLHACKDI-----
::*:*:*:***** *****:***:***

mCRMP5 451 VFMCAEGTGKFCPLRSFPDIIVYKLVQREKTLKVRGVDRTPYLGDVAIVV 500
hCRMP5 -----LKVRGVDRTPYLGDVAIVV
*****:***

mCRMP5 501 HPGKKEMGTPLADTPTRPVTRHGGMRDLHESSFSLSGSQIDDHVPKRASA 550
hCRMP5 HPGKKEMGTPLADTPTRPVTRHGGMRDLHESSFSLSGSQIDDHVPKRASA

mCRMP5 551 RILAPPGRRSSGIW 564
hCRMP5 RILAPPGRRSSGIW

Fig. II-3. Similarities between mouse CRMP isoforms. A, amino acid identity between the indicated pairs of sequences. The percentage was calculated using Clustal W Software. B, an unrooted phylogenetic tree of CRMP isoforms. The relationship among CRMP isoforms was represented in a tree by the Drawtree method (Phylip software package).

A

	CRMP5	CRMP1	CRMP2	CRMP3	CRMP4	DHPase
CRMP5	100	49	50	50	49	51
CRMP1		100	75	68	73	51
CRMP2			100	75	75	52
CRMP3				100	69	51
CRMP4					100	52
DHPase						100

B

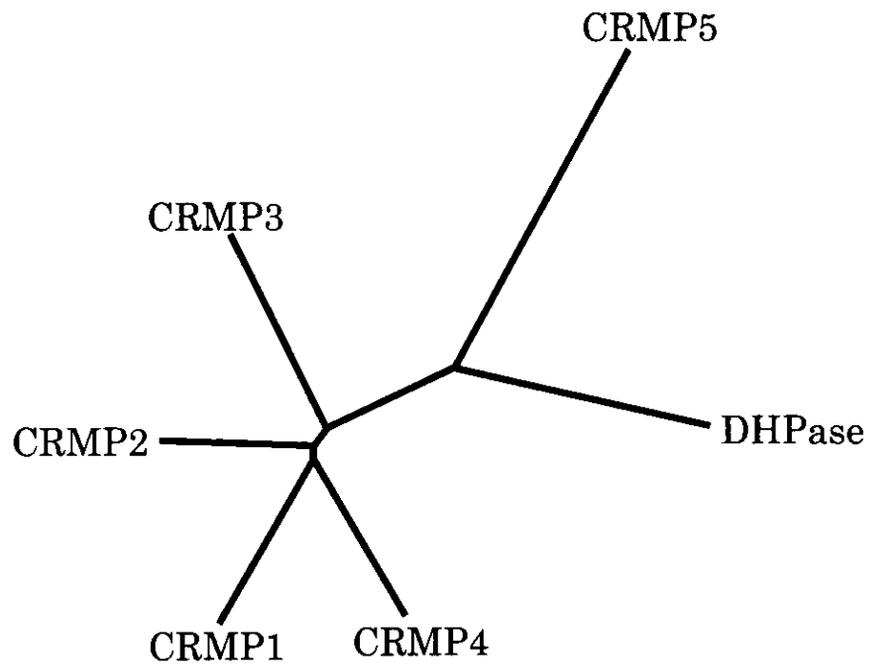


Fig. II-4. Chromosomal localization of the mouse CRMP5 gene. The localization of the CRMP5 gene to mouse R-banded chromosomes was analyzed using a biotinylated genomic DNA fragment. The signals are localized to chromosome 5 B1 (arrows). The metaphase spreads were photographed with a Nikon B-2A filter (A and C) and UV-2A filter (B).

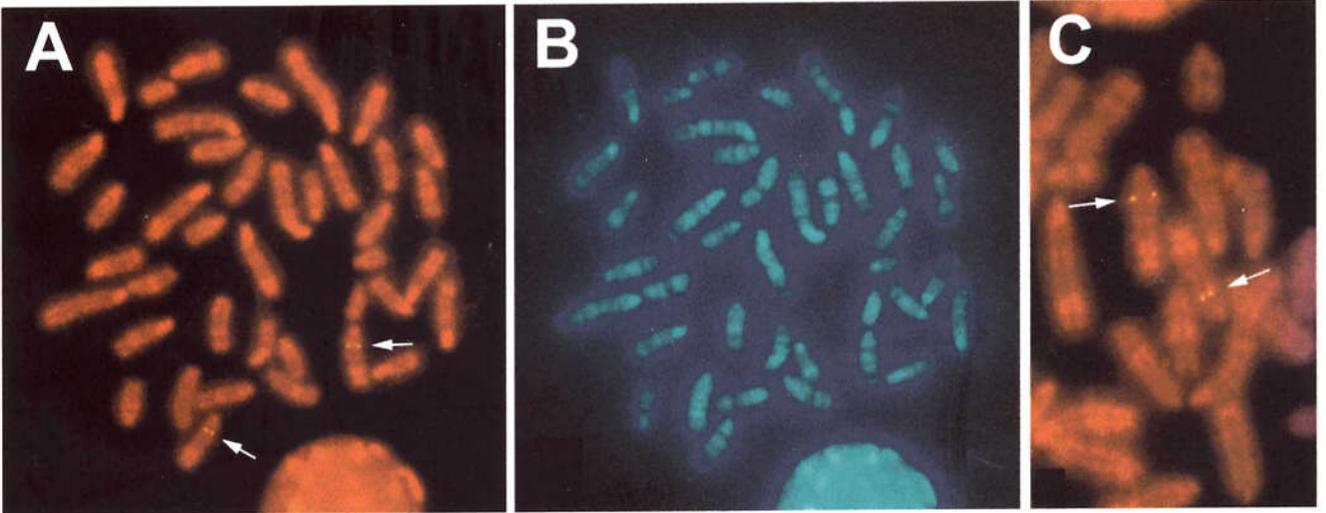


Table II-1. Chromosomal locations of CRMP family genes

	human	mouse
CRMP1	4p15-4p16.1	-
CRMP2	8p21	14 central region
CRMP3	10q25.2-10q26	7 distal end
CRMP4	5q32	-
CRMP5		5 B1 region
DHPase	8q22	-

Fig. II-5. **Amino acid sequence comparison of mouse CRMP5 with other CRMP isoforms.** The alignment of mouse CRMP isoforms was performed using the Clustal W software. Identical amino acids in more than four sequences are indicated by bold letters in dark shaded boxes, and homologous amino acids in light shaded boxes. The DHPase residues involved in metal-binding are indicated with closed arrowheads (histidine) and an open arrowhead (aspartatic acid). Sequences of mouse CRMPs and DHPase are available from GenBank under the following accession numbers: CRMP1/Ulip3, Y09080; CRMP2/Ulip2, Y10339; CRMP3/Ulip4, Y09079; CRMP4/Ulip1, X87817; CRMP5, AF249295; DHPase, AF249296.

I have found several amino acid substitutions in CRMP cDNA clones which I isolated and used for the study. In CRMP1/Ulip3, the replacement of isoleucine with threonine at codon 338 (I338T) and glutamate with lysine at 520 (E520K) was detected. Similarly, the following substitutions were found: in CRMP2/Ulip2, P11R, A208E, and P257S; and in CRMP3/Ulip4, D125E, G126R, V354G, and S372L.

10 20 30 40 50 60

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

70 80 90 100 110 120

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

130 140 150 160 170 180

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

190 200 210 220 230 240

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

250 260 270 280 290 300

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

310 320 330 340 350 360

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

370 380 390 400 410 420

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

430 440 450 460 470 480

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

490 500 510 520 530 540

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

550 560 570

CRMP1/Ul1p3
 CRMP2/Ul1p2
 CRMP3/Ul1p4
 CRMP4/Ul1p1
 CRMP5
 DHPase

Chapter III

**Expression profile of CRMP5 and association preferences
between CRMP family members**

Introduction

CRMP2 was first identified as an intracellular factor required for collapsin-initiated signaling (Goshima et al., 1995), but the cellular signaling process has not been elucidated. It was proposed that collapsin signaling is also mediated by a pertussis toxin (PTX) -sensitive G-protein coupled receptor, because PTX diminishes the collapsin induced growth cone collapse as well as the anti-CRMP antibody (Goshima et al., 1995). However, G-protein-independent effects of PTX on neurite growth and growth cone morphology (Kindt and Lander, 1995) suggest that CRMP2 is a component of a non-G-protein dependent signaling pathway (Wang and Strittmatter, 1996). Overall, there is no interpretation of CRMP functions at the molecular level.

It was found that CRMP forms hetero-tetrameric structures by mutual association by yeast two-hybrid assay, *in vitro* binding assay and the biochemical purification of native CRMPs (Wang and Strittmatter, 1997). In the two-hybrid analysis, the CRMP2-CRMP3 association was ten-fold higher than that of CRMP2-CRMP2, suggesting that CRMPs preferentially form hetero-oligomers (Wang and Strittmatter, 1997). Purified CRMPs from bovine brain migrate as a tetramer during size exclusion chromatography indicating that CRMP forms a tetramer *in vivo* as does DHPase (Wang and Strittmatter, 1997; Kikugawa et al., 1994). These observations suggest that CRMPs act in heterotetramers *in vivo*.

CRMPs are exclusively expressed in the nervous system when the neuronal network forms and markedly decrease in adulthood (Byk et al., 1998; Wang and Strittmatter, 1996; Minturn et al., 1995). CRMP2 mRNA continues to be expressed in large amounts in adulthood and CRMP3 mRNA is detected from both young and adult cerebellum (Wang and Strittmatter, 1996; Byk et al., 1998).

P19 mouse embryonic carcinoma cells express CRMP2 mRNA in response

to retinoic acid (Byk et al., 1998). Similarly, it was reported that in response to retinoic acid, CRMP4 mRNA is upregulated in some neuroblastomas (Gaetano et al., 1997). In PC12 cells, the expression of CRMP4 mRNA is upregulated during NGF-stimulated neuronal differentiation which is involved in the formation of neurite-like processes (Byk et al., 1998). These results suggest that CRMPs function in neuronal differentiation and neurite extension.

In this chapter, I describe the CRMP5 expression profile and the association between CRMP isoforms in all combinations. CRMP5 showed a developmentally regulated and nervous system-specific expression profile. CRMP5 associated with all other CRMPs, except CRMP1. Association assays revealed that each CRMP has a specific preference. Furthermore, the amount of CRMP5 mRNA was examined in PC12h cells, before and after the NGF-induced neuronal differentiation.

Materials and Methods

Northern Blot Analysis

Total RNA was prepared from various mouse tissues at postnatal day 0 (P0), and from whole embryos at E11, E13, E15, whole heads at E17, and whole brains at P0, P3, P7, P14 using ULTRASPECT™ RNA (Biotecx laboratories, Houston, TX). As for the staging of mice, the day on which a vaginal plug was detected was considered E0, and the day of birth, P0. Poly(A)⁺ RNA was isolated from E17 whole brains using Dynabeads Oligo d(T)₂₅ (DYNAL, Oslo, Norway). RNA separated on a 1% agarose gel containing 7.4% formaldehyde was transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech). The blot was prehybridized at 68°C in a solution containing 5 x SSC, 0.1% SDS, 2% blocking solution (Roche Molecular Biochemicals, Mannheim, Germany) and 50% formamide. Hybridization was carried out overnight at 68°C in the same solution containing the DIG-11-UTP-labeled antisense riboprobe transcribed from the *ApaI*-*SpeI* cDNA fragment (nucleotide residues 1,951 - 2,828: noncoding probe) of CRMP5 (clone 1-8-7) (for nucleotide numbers, see Chapter II, Fig. II-1), and detected using anti-DIG-alkaline phosphatase-conjugated antibody according to the manufacturer's instructions (Roche). To verify the amount of RNA loaded, the membrane was re-hybridized with DIG-labeled cRNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In Situ Hybridization

In situ hybridization was performed using DIG-riboprobes transcribed from the cDNA fragment (nucleotide residues 286 - 2,066) of Ulip1/CRMP4 (GenBank Accession Number X87817), and the *Sall* (linker site) - *BamHI* cDNA fragment (-42-1652: coding probe) of CRMP5 (clone 1-8-7) as templates (Yuasa et

al., 1996). E17 mouse embryos were fixed in 4% paraformaldehyde at 4°C overnight. Cryosections were made at 14 µm. Hybridization was carried out overnight at 50°C in 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 0.6 M NaCl, 0.1% SDS, 1 x Denhardt's solution, 50% formamide, 400 µg/ml yeast tRNA, and 10% dextran sulfate, followed by incubation with anti-DIG alkaline phosphatase-conjugated antibody. Excess antibody was washed away and color substrates (NBT and BCIP) were added to develop in the dark.

Binding Assay Using the Two-hybrid System

To examine mutual associations among the CRMP family members and DHPase, DNA fragments covering the coding regions of mouse CRMP1-5 and DHPase were amplified by PCR using specific primers from the respective clones or RT-PCR (for CRMP2) to make restriction sites for cloning. After confirmation of the sequence, these fragments were inserted into the *EcoRI* and *SaII* sites of the pGBT9 bait vector. The CRMP1 and CRMP5 fragments were inserted also into the *EcoRI* and *XhoI* sites of the pACT2 prey vector (Clontech). The bait and prey constructs in various combinations were transformed into yeast strain Y190 (Clontech) harboring reporter genes *HIS3* and *LacZ*, and transformants were cultivated on plates lacking leucine and tryptophan. Assay was performed as described (Kawachi et al., 1999). For quantitative analysis of the association, a liquid β-galactosidase assay was performed using ONPG as substrate according to the manufacturer's protocol (Yeast Protocols Handbook; Clontech).

Immunoprecipitation and Western Blotting

Myc-CRMPs, FLAG-CRMPs and FLAG-DHPase expression vectors were constructed in the following way: Oligo nucleotides encoding c-myc or FLAG peptide including the first methionine were ligated to the mammalian expression

vector pcDNA3.1 (Invitrogen, Carlsbad, CA), which was digested with *NheI* and *EcoRI*, to yield pcDNA-myc and pcDNA-FLAG, respectively. Full-length CRMP1-5 and DHPase cDNA were cut out from the various pGBT9 bait vectors with *EcoRI* and *SaII*, and inserted into pcDNA-myc or pcDNA-FLAG digested with *EcoRI* and *XhoI*. Myc-CRMPs and FLAG-CRMPs/FLAG-DHPase thus prepared were co-transfected into COS-7 cells using LIPOFECTAMINE PLUS™ Reagent (Gibco BRL). At 48 hr after transfection, cell lysates were prepared by sonication in RIPA buffer (50 mM Tris-HCl (pH 7.6), 1 mM DTT, 1% Triton X-100, 10 µg/ml leupeptin (Sigma, Saint Louis, Missouri), 10 µg/ml pepstatin A (Sigma), and 1 mM phenylmethoyl sulfonylfluoride), followed by centrifugation at 12,000 x g for 20 min. The lysates were incubated with agarose beads conjugated with 9E10 anti-myc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 hr with agitation. The beads were collected by low-speed centrifugation and washed four times by resuspension in 1 ml of RIPA buffer. Proteins eluted from the beads with sample buffer were subjected to SDS-PAGE, and analyzed by immunoblotting with M2 anti-FLAG monoclonal antibody (Sigma) or 9E10 anti-myc monoclonal antibody (Santa Cruz Biotechnology). To check the expression levels of FLAG-CRMPs and FLAG-DHPase, cell lysates were also analyzed using M2 anti-FLAG monoclonal antibody.

Quantitative RT-PCR

PC12h cells were grown in DMEM supplemented with 10% horse serum and 5% fetal calf serum. For NGF treatment, cells were starved of serum for 24h, and NGF added at a final concentration of 100nM. Twelve hours after the addition, cells were collected and total RNA was extracted as described above. First strand cDNA synthesized from 5µg of total RNA using superscript II (Life Technologies) with oligo dT primers was subjected to quantitative PCR.

Quantitative PCR was performed using the following sets of primers. For CRMP1: CCAGGCAGACGTTAGTTCCA and CCACCAGGAACAATCAGGTT; CRMP4: CATTCGCTCCACCTGATCTG and CCGAGGAATGTTCTTCTTGC, CRMP5: GAGGGTGCTAAGGAGGCTCT and CCACCTTCCCTTGCATCTTA; GAPDH: ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA. PCRs were performed using PLATINUM Quantitative PCR SUPERMIX-UDG (Gibco BRL) containing 0.5x green dye fluorescence (SYBR Green I; Applied Biosystems Inc.) with a rotor gene 2000 (CORBETT RESERCH) according to the manufacturer's instructions. Results are of three independent experiments.

Results

Expression of CRMP5 mRNA

The CRMP family is known to be expressed in the nervous system, while DHPase is present in liver and kidney (Hamajima et al., 1996). Because CRMP5 is almost equally divergent from the other CRMPs and DHPase, I next addressed the issue of whether CRMP5 expression is neural tissue-specific. Northern blot analysis using P0 mouse tissues clearly showed that CRMP5 mRNA is present only in the brain (Fig. III-1A). Next, the expression profile of CRMP5 mRNA during development was examined. CRMP5 mRNA was already expressed at E11, as early as CRMP4/Ulip1 (data not shown), peaking in the first postnatal week when the formation of the neural network and its refinement culminates, and subsequently declining to a lower level during the second week (Fig. III-1B). By longer exposure, another minor hybridization band with an estimated mRNA size of 10.7 kb was detected. The hybridization band of about 4.2kb is likely to be an artifact caused by overlap with 28S ribosomal RNA. These results suggest

that CRMP5 expression is neural tissue-specific and developmentally regulated as already reported for some CRMPs (Goshima et al., 1995; Wang and Strittmatter, 1996; Kamata et al., 1998ab; Minturn et al., 1995ab; Byk et al., 1996, 1998; Gaetano et al., 1997). The size of the mRNA for CRMP5 detected in Fig. III-1A and B was 5.0 kb but when poly(A)⁺ RNA was hybridized, the 5.0 kb band for CRMP5 turned out to be double bands of 4.8 kb and 5.2 kb (Fig. III-1C). When the expression level of CRMP5 mRNA was compared with that of CRMP4 (Byk et al., 1996) by Northern blot hybridization, it was found to be lower by approximately one order of magnitude, and this difference was retained during development up to adulthood (data not shown).

To elucidate the expression pattern of CRMP5, I conducted *in situ* hybridization on sections of E17 embryos with probes for CRMP5 and CRMP4. This analysis demonstrated that CRMP5 (Fig. III-2, A-E) is expressed selectively in the nervous system with almost the same expression pattern as CRMP4 (Fig. III-2, F-H). In these experiments, CRMP5 mRNA was not detected outside of the nervous system (Fig. III-2A). In the brain at E17, CRMP5 and CRMP4 were restricted to postmitotic neural cells (Fig. III-2, A-C and F). Both CRMP5 and CRMP4 were prominent in the neocortex and also detected in the olfactory epithelium. In the retina, these CRMPs were strongly expressed in the postmitotic layer which contains retinal ganglion cells, but undetected in the outer layer (Fig. III-2, D, G). As shown in Fig. III-2 (E and H), CRMP5 and CRMP4 displayed an almost identical expression pattern in the mantle layer of the spinal cord and dorsal root ganglion and sympathetic ganglia (data not shown). Overall, I conclude that CRMP5 is indeed a neural-specific CRMP.

Association between CRMP5 and the Other CRMP Isoforms

DHPase is well known to form tetramers by self association (Kikugawa et

al., 1994), and the CRMPs are also shown to interact with one another and form tetrameric structures (Wang and Strittmatter, 1997). Although CRMPs have no detectable DHPase activity, they are able to interact with the DHPase monomer weakly (Wang and Strittmatter, 1997). Because CRMP5 was originally identified by its ability to interact with chick CRMP3 and has sequence homology with the other CRMP isoforms, it was expected that this protein would also interact with itself and with some other isoforms. Therefore, I tested for interaction with the other CRMP isoforms using the two-hybrid system.

I first performed this analysis using CRMP5 in the prey vector and various mouse CRMPs and DHPase in the bait vector (Fig. III-3A). Interestingly, CRMP5 interacted with itself, CRMP2-4, and DHPase, but not with CRMP1 (Fig. III-3A). I verified the result for CRMP5 and CRMP1 by conducting experiments in the reverse, namely with CRMP1 in the prey vector and various CRMPs in the bait vector. As shown in Fig. III-3B, CRMP1 did not associate with CRMP5 and DHPase, but did associate with CRMP1-4, reproducing the first result. To quantitate these interactions, β -galactosidase liquid assays were performed with the indicated combinations (Fig. III-3C). CRMP5 interacted preferentially with CRMP2 (422 ± 26 units), CRMP3 (469 ± 25), and CRMP4 (311 ± 30), weakly with CRMP5 (14 ± 0.7) and DHPase (5 ± 0.3), and not at all with CRMP1 or control empty vector (Fig. III-3C).

To further verify the interaction observed in the two-hybrid assay (Fig. III-3), co-immunoprecipitation experiments were conducted. Myc-tagged CRMP5 and FLAG-tagged CRMPs or DHPase were co-transfected into COS-7 cells, and cell lysates were immunoprecipitated using agarose beads conjugated with 9E10 anti-myc monoclonal antibody. FLAG-CRMP2, 3, 4, and 5 and DHPase but not FLAG-CRMP1 were co-immunoprecipitated with myc-CRMP5 (Fig. III-4). The amount of FLAG-CRMP2-4, and DHPase associated with myc-CRMP5 was 10 to

20 fold that of FLAG-CRMP5. Consistent with the two-hybrid assay (Fig. III-3C), associations between CRMP5 and CRMP2-4 were much tighter than the association between CRMP5 itself, but that between CRMP5 and DHPase was significantly strong for some reason. When Myc-CRMP5 was not co-transfected, FLAG-tagged CRMPs were not immunoprecipitated with anti-Myc agarose. In these experiments, the amount of Myc-CRMP5 binding to anti-Myc agarose was nearly equal among the cell lysates (*middle pannel*), and FLAG-CRMPs and -DHPase expression levels in the cell lysates were also comparable (*lower pannel*).

Because the immunoprecipitation assay using COS-7 cells completely reproduced the result obtained with the yeast two-hybrid system, the interactions between CRMP isoforms in all combinations were examined by immunoprecipitation (Table III-1). From the amount of precipitated FLAG-CRMPs, I divided the isoforms into two groups, those that show a tight association like between CRMP5 and CRMP2-4 (see Fig. III-4) and those that show no or a very weak association like between CRMP5 and CRMP1 and CRMP5-CRMP5. This analysis revealed that each CRMP differs in its affinity for other isoforms. For example, CRMP3 strongly associated with CRMP2 and 5 but not with CRMP1, 3, or 4 (Table III-1). There is an apparent preference of association between CRMP family members. A tight association was not observed with the following combinations; CRMP1-CRMP3, CRMP1-CRMP5, CRMP3-CRMP3, CRMP3-CRMP4, CRMP5-CRMP5 (Table III-1). These findings suggest that, contrary to the view that all CRMP isoforms interact, preferences between the family members do exist (Wang and Strittmatter, 1997).

CRMP5 mRNA Expression in PC12h Cells

It was reported that NGF-treated differentiated PC12 cells exhibit a

marked upregulation of CRMP4 mRNA (Byk et al., 1998). PC12 cells normally show a rounded form, and stimulation with NGF induces a neuron-like structure with process extension. Similarly, some neuroblastomas and P19 mouse embryonic carcinoma cells begin to express CRMP4 and CRMP2, respectively, in response to stimulation with retinoic acid (Gaetano et al., 1997; Minturn et al., 1995). These patterns of expression seem to parallel the course of neuronal differentiation and neurite extension. To investigate whether CRMP5 is also concerned in neuronal differentiation and subsequent neurite extension, a quantitative RT-PCR was performed using NGF-stimulated PC12h cells (Fig. III-5A). PC12h cells unexpectedly expressed all CRMPs including CRMP5 without NGF stimulation (data not shown). Expression of CRMP5 mRNA was upregulated 1.7 fold 12h after the addition of NGF, but the amounts of CRMP1 and GAPDH (internal control) mRNA were unaltered. The expression of CRMP4 mRNA was also upregulated consistent with results previously obtained by Northern blot analysis (Byk et al., 1998). The amounts of CRMP1 and CRMP5 mRNA were of the same order of magnitude after the NGF treatment. When the amount of CRMP5 mRNA was compared with that of CRMP4, it was found to be lower by one order of magnitude. This result is similar to that obtained from Northern blot analysis, suggesting that the balance of CRMP4 and CRMP5 expression was conserved both *in vivo* and in PC12h cells. Twelve hours after the addition of NGF, PC12h cell showed a neural morphology with neurite-like process extensions (Fig. III-5B). These results suggest that CRMP5 also contributes to the neuronal differentiation and process formation cooperating with other CRMPs.

Discussion

Northern blot analysis detected three bands. The main 4.8-kb and 5.2-kb bands, probably derived from a difference in poly(A) addition sites, represent CRMP5 mRNA. The 10.7-kb band presumably corresponds to a heterogeneous RNA that contains intronic sequences. However, the possibility that a related protein is encoded by this mRNA species can not be excluded. Northern blot analysis of various tissues of P0 mice indicated that CRMP5 mRNA is only expressed in the brain, not in non-neural tissues like the lung (CRMP2) and heart and testis (CRMP4) (Wang and Strittmatter, 1996; Byk et al., 1996; Kato et al., 1998). The CRMP5 expression profile during development resembled the CRMP1 and CRMP4 profiles, because the expression level peaked in the first postnatal week and markedly decreased in adulthood (Wang and Strittmatter, 1996; Byk et al., 1998). In contrast, CRMP2 continues to be expressed in large amounts in adulthood, as does CRMP3 but only in the cerebellum (Wang and Strittmatter, 1996; Byk et al., 1998).

In situ hybridization analyses showed that CRMP5 is expressed exclusively in the nervous system, with almost the same expression pattern as CRMP4 in the brain. This profile was also similar to that of CRMP2 expression analyzed by immunohistochemistry in mouse brain sections at E16.5 (Kamata et al., 1998). In the spinal cord, the expression pattern of CRMP5 resembled that of CRMP1, CRMP2 and CRMP4 (Fig. III-2 and ref. Wang and Strittmatter, 1996). These CRMPs were expressed throughout the mantle layer in the developing spinal cord, whereas CRMP3 was localized to the dorsal matrix layer (Wang and Strittmatter, 1996). Taken together with the developmental profiles obtained by Northern hybridization, the results suggested that the expressional regulation of CRMP1, CRMP4 and CRMP5 is common and that of CRMP2 and CRMP3 is

rather unique. It is thus conceivable that CRMP5 functions in cooperation with other CRMPs in the nervous system during development.

CRMP5 could be assigned to another family as mentioned above. However, I concluded that CRMP5 is a member of the CRMP for the following reasons, 1) it is expressed exclusively in the nervous system, 2) it associates with other CRMPs, and 3) it probably lacks DHPase activity as do CRMP1-4 because a zinc ion binding site that is requisite for enzymatic activity is not conserved.

The yeast two-hybrid analysis and immunoprecipitation analysis using COS-7 cells revealed that the CRMPs form multimeric structures, in which, CRMP5 interacts with CRMP2, CRMP3, and CRMP4, but interestingly, not with CRMP1. CRMP5 also interacts with itself, but this interaction was very weak compared with that between CRMP5 and CRMP2-4. It is noted that CRMP5 does not interact only with CRMP1, because this is the first study to show no interaction between the CRMP family members. Therefore, I tested the interaction between CRMPs in all combinations by immunoprecipitation analysis (Table III-1). This analysis clearly showed that in addition to CRMP5-CRMP1 and CRMP5-CRMP5, there are certain combinations that exhibit very weak or no interactions. It has been proposed that CRMPs favor heterophilic binding over homophilic binding (Wang and Strittmatter, 1997). But in my immunoprecipitation analysis, CRMP1, 2, and 4 each showed a significantly tight association with itself (Table III-1). This indicates that a preference is exhibited by each CRMP, independent of the heterophilic or homophilic binding form. From these results, it is conceivable that possible CRMP complexes are composed from 1/2/4, 2/3/5 and 2/4/5. A CRMP can strongly interact with another only in these combinations. Overlaps of these CRMPs expression were observed in some regions during development (Wang and Strittmatter, 1996), suggesting that such combinations of CRMP actually function *in vivo*. The results suggest that

CRMP oligomers are classifiable into two populations: those that contain CRMP1 and those that contain CRMP5.

The expression of CRMP5 as well as CRMP4 mRNA was upregulated in NGF-treated PC12h cells (Fig. III-5). It was suggested that CRMP5 is responsible for neurite extension like CRMP4. In contrast, the mRNA expression of CRMP1 was not altered after the NGF treatment (Fig. III-5) similar to CRMP2 (Byk et al., 1998). In NGF-stimulated PC12 cells, the amount of CRMP3 mRNA was rather low (Byk et al., 1998). It should be noted that PC12h cells normally express all CRMP isoforms. Together with the results from the association experiments, it was suggested that in NGF-treated PC12h cells, CRMP5 and CRMP4-containing CRMP complexes are increased in abundance in contrast to complexes containing CRMP1. The increase might be required for neuronal differentiation involving neurite extension. It was reported that the phosphorylated state of CRMP4 is altered in NGF-treated PC12 cells (Byk et al., 1996). Therefore, neuronal differentiation might be regulated not only by the expression level, but also depending on the phosphorylated state of the CRMPs. Certain combinations of CRMP with phosphorylated components might exist even in a single neuron *in vivo*, which may provide for distinct functions in the formation of neural networks.

Unraveling the roles common to all CRMPs regardless of the composition of a complex and/or characteristics of each member through identification of binding partner(s) should lead to a better understanding of the basic principle for establishing complicated but extremely precise patterns of neuronal connectivity. It was shown that CRMP1-3 can associate with DHPase but the interaction is very weak compared with that between DHPases (Wang and Strittmatter, 1997). In this context, the finding that CRMP5 shows significant interaction with DHPase is interesting, although why it does is not clear at present. However, it

should be noted that interaction between the two would not occur *in vivo* because of the difference in expressed tissues.

Finally, it should be pointed out that almost all the developing output neurons (projection neurons) including semaphorin-insensitive cells apparently express several combinations of CRMPs. For example, retinal ganglion cells, which do not respond to semaphorins (Takahashi et al., 1998), express all the CRMPs. This suggests that their roles are not restricted to the semaphorin-initiated signal transduction cascade in growth cones as was first proposed (Goshima et al., 1995) but extend to more general processes underlying neural network formation in the entire nervous system. CRMP3 is recognized by anti-CV2 autoantibodies which are present in patients with paraneoplastic neurological disease (Honnorat et al., 1999). Furthermore, CRMP2 is associated with the neurofibrillary tangles observed in Alzheimer's patients, and Sema3A and CRMP2 are synchronously induced in apoptosis destined neurons (Yoshida et al., 1998; Shirvan et al., 1999). These results suggest that CRMPs are also involved in neuritic degeneration under not only developmental but also morbid conditions.

In summary, CRMPs might be key molecular components in shaping neural networks, but conclusive evidence as to their functions remains to be obtained. To reveal the molecular roles of CRMPs, it is necessary to know more about the molecules with which they interact. The identification and characterization of these molecules is required.

Fig. III-1. Northern blot analysis of CRMP5. Expression of CRMP5 mRNA was analyzed using DIG-labeled CRMP5-noncoding probe. A, the tissue distribution of CRMP5 mRNA was analyzed using total RNA prepared from various tissues of P0 mice (10 μ g each): lane 1, whole brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, stomach; lane 6, spleen; lane 7, kidney; lane 8, intestine; lane 9, muscle. The same membrane was re-hybridized with the DIG-labeled GAPDH probe (lower panel) to verify the amount of RNA loaded in each lane. The positions of size markers (in kb) are indicated on the left. B, the developmental time course of CRMP5 mRNA expression was analyzed using total RNA (10 μ g each): lane 1, E11 whole embryo; lane 2, E13 whole embryo; lane 3, E15 whole embryo; lane 4, E17 head portion; lane 5, P0 whole brain; lane 6, P3 whole brain; lane 7, P7 whole brain; lane 8, P14 whole brain. C, RNA blot hybridization using poly(A)⁺ RNA (1 μ g) prepared from the whole brains of E17 mouse embryos. Two transcripts were seen at 4.8 kb and 5.2 kb (arrowheads).

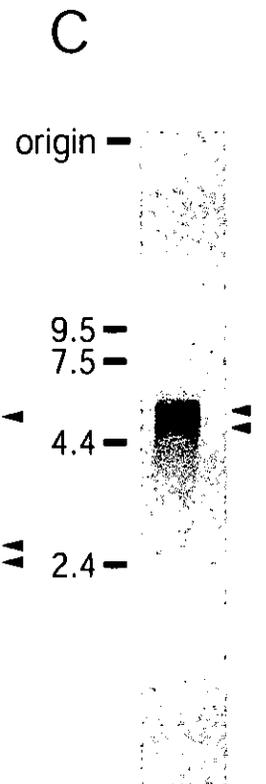
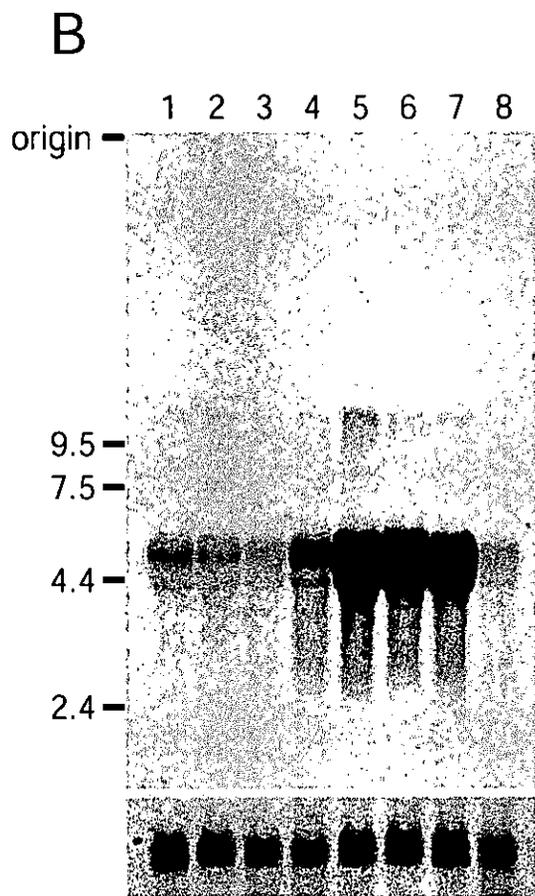
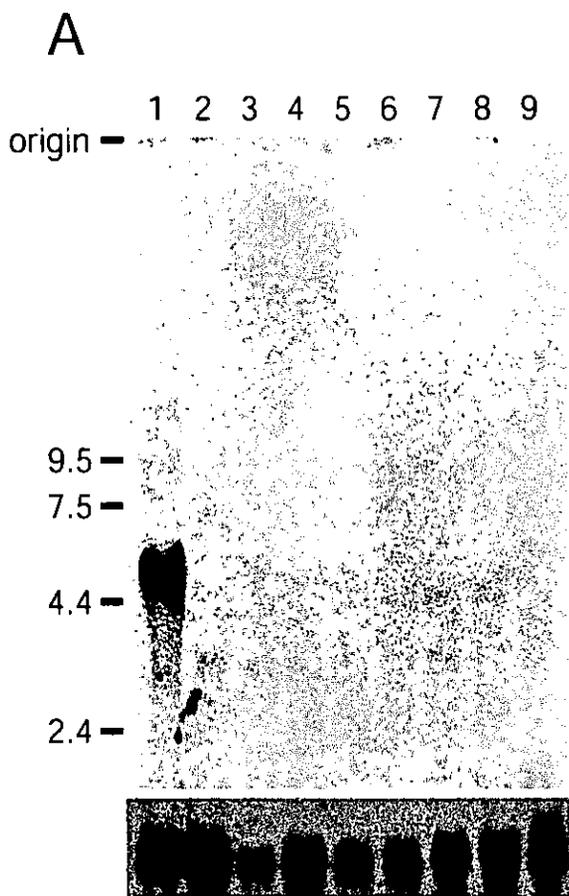


Fig. III-2. Expression pattern of CRMP5 and CRMP4 mRNA in the central and peripheral nervous system of E17 mouse embryos. CRMP5 (A-E) and CRMP4 (F-H) mRNA localization was detected by in situ hybridization with DIG antisense riboprobes derived from the coding region of each CRMP. A, sagittal sections of the whole mouse embryo. CRMP5 mRNA was detected in the brain, spinal cord (SC) and olfactory epithelium (OE), but not in heart (He), liver (Li) and lung (Lu). B, parasagittal section of the head. C and F, horizontal sections of the head at the level of the eye. D and G, high magnification views of the eye shown in C and F. E and H, transverse sections of the spinal cord. Control hybridization experiments using the sense riboprobes on adjacent sections gave no specific signals. Scale bars, 1 mm (A, B, C and F); 200 μ m (D, E, G and H).

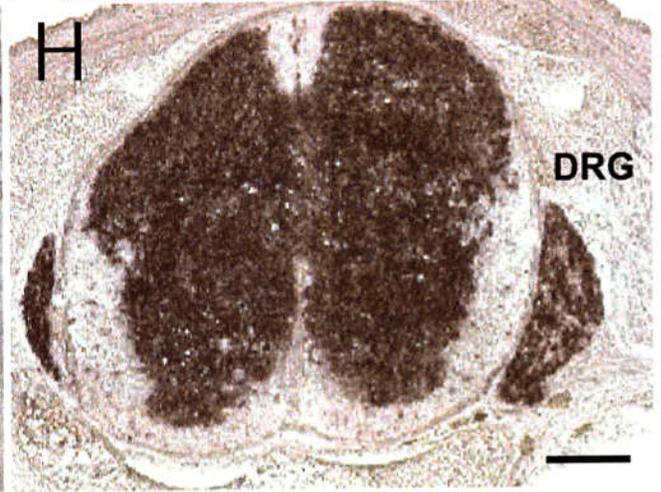
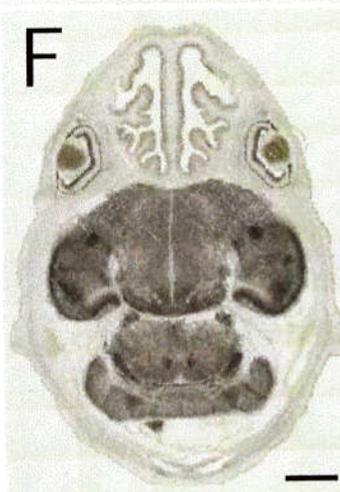
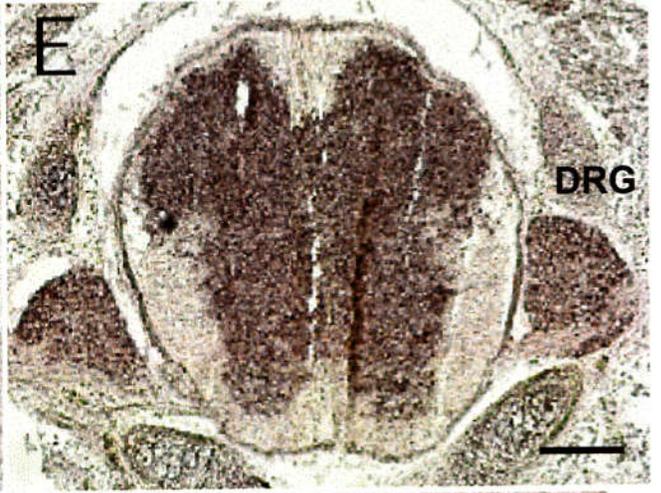
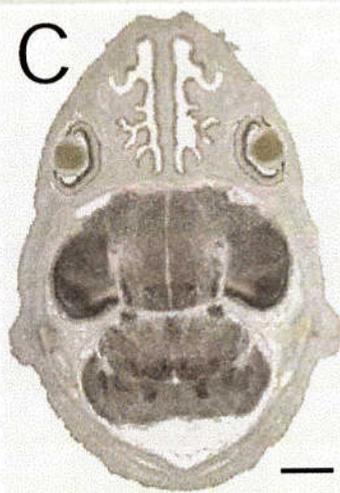
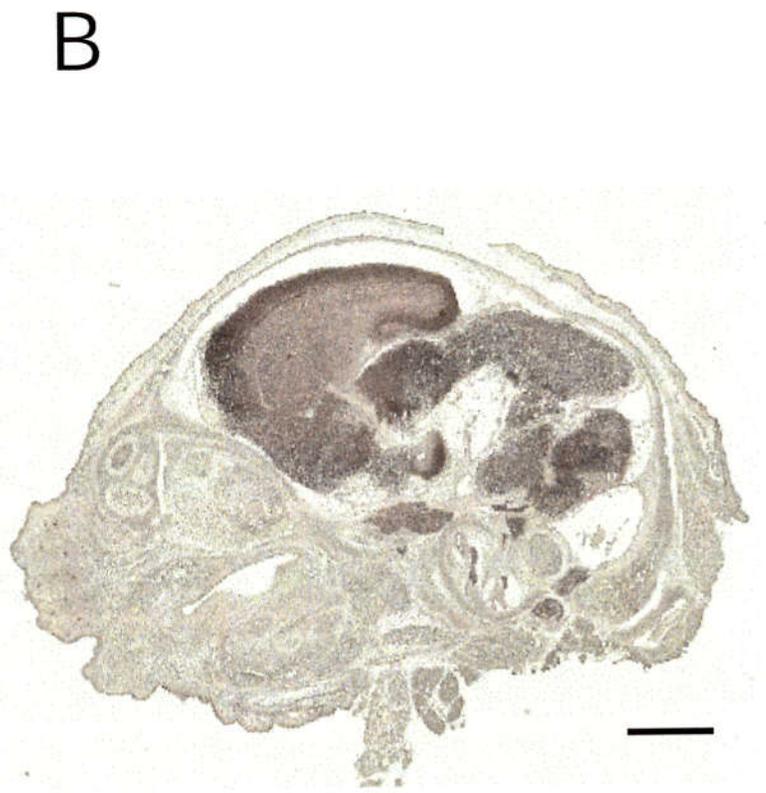
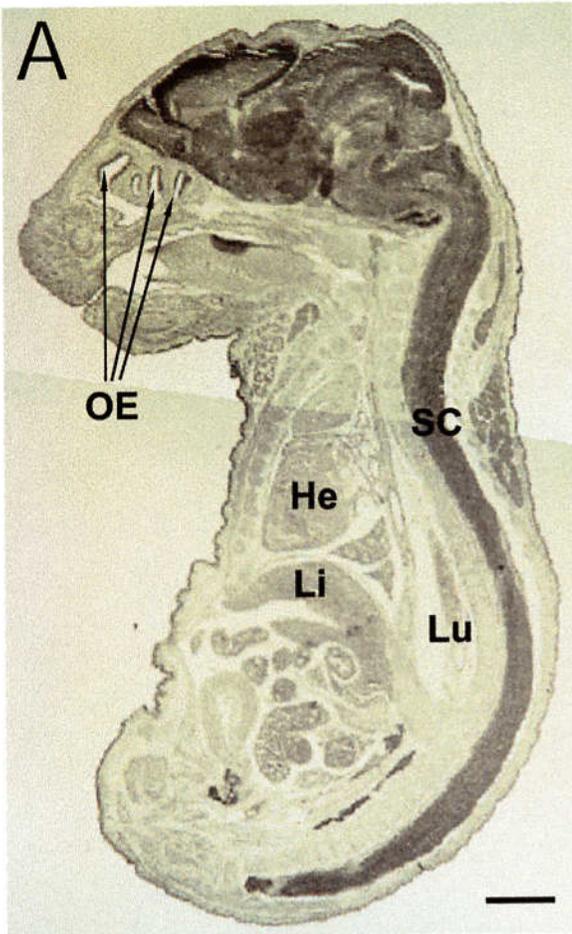


Fig. III-3. Interaction between mouse CRMP1-5 and DHPase in the yeast two-hybrid system. Various CRMP-expressing bait constructs were tested for interaction with the prey, CRMP5 (A) and CRMP1 (B), by induction of reporter gene, HIS3. A and B, individual yeast transformants were streaked on plates lacking leucine, tryptophan and histidine with 50 mM 3-aminotriazole to suppress leaky growth. Plates were incubated at 30°C for 3 days. Numbers in the figure correspond as follows: 1, CRMP1; 2, CRMP2; 3, CRMP3; 4, CRMP4; 5, CRMP5; 6, DHPase; 7, empty pGBT9 vector as a negative control. C, β -Galactosidase activity was measured in triplicate using *o*-nitrophenyl β -D-galactopyranoside as substrate. Colonies were selected on the plates lacking leucine and tryptophan and cultured in the selective liquid medium. Data are the mean from three independent colonies with standard errors (bars). The standard errors were less than 10% for values >1.

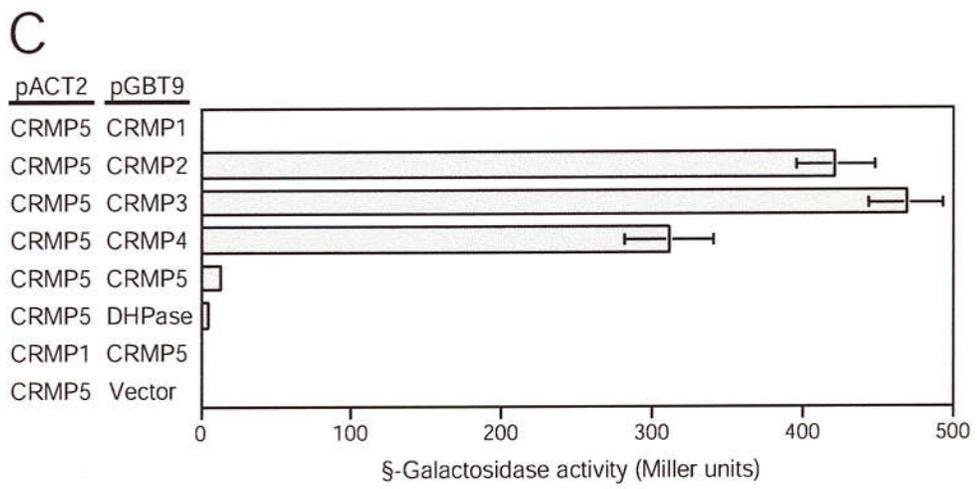
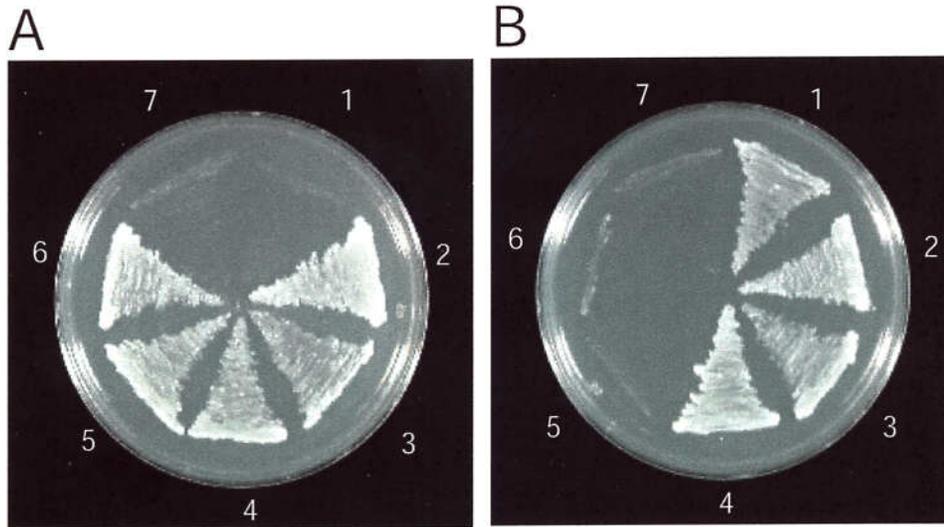


Fig. III-4. Co-immunoprecipitation of CRMP5 with the other CRMP isoforms. *In vivo* binding of myc-tagged CRMP5 to FLAG-tagged CRMPs or DHPase was analyzed. Lysates from COS-7 cells transfected with FLAG-CRMPs or FLAG-DHPase, together with (+) or without (-) myc-CRMP5, were incubated with 9E10 (anti-myc monoclonal antibody)-conjugated agarose. After washing, proteins retained on the beads were analyzed by SDS-PAGE and immunoblotting with M2 anti-FLAG monoclonal antibody (upper panel) or 9E10 anti-myc monoclonal antibody (middle panel). Lysates were analyzed by immunoblotting with M2 anti-FLAG monoclonal antibody to confirm that expression levels of FLAG-CRMPs and FLAG-DHPase were comparable (lower panel).

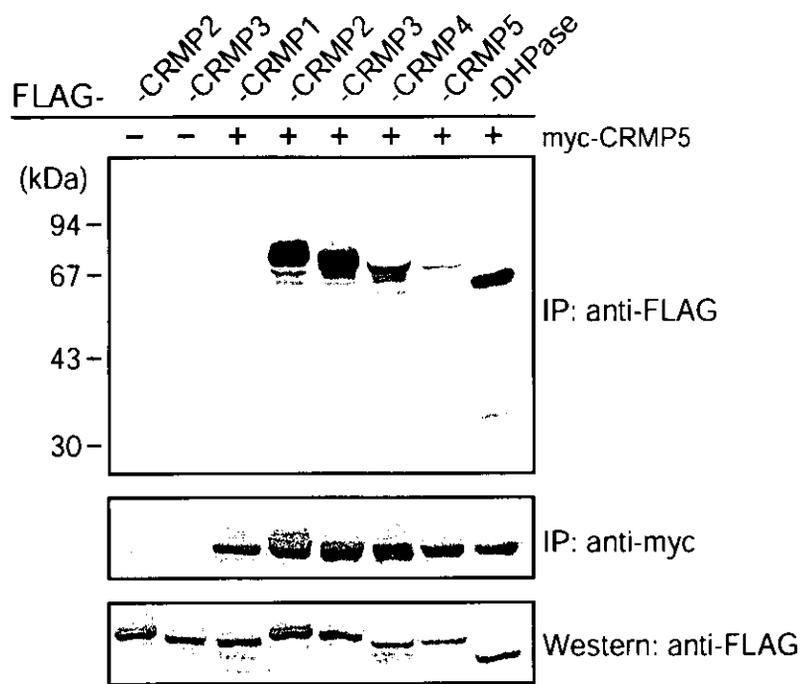


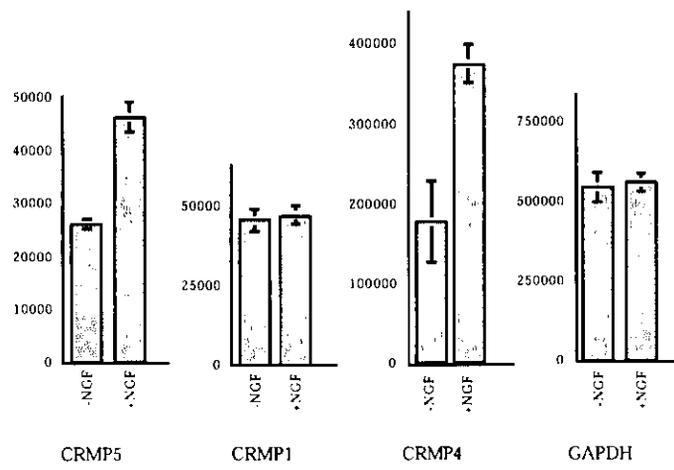
Table III-1. Preferences for mutual association

	CRMP1	CRMP2	CRMP3	CRMP4	CRMP5
CRMP1	○	○	×	○	×
CRMP2	○	○	○	○	○
CRMP3	×	○	×	×	○
CRMP4	○	○	×	○	○
CRMP5	×	○	○	○	×

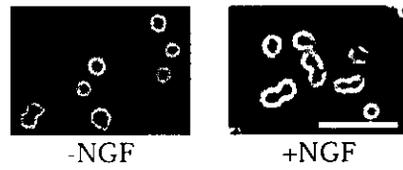
Association between the CRMP isoforms in all combinations was evaluated by immunoprecipitation assay as described in Fig. III-4. The intensity of each association is represented. (○) indicates a tight association and (×) indicates no or a very weak association.

Fig. III-5. CRMP5 mRNA is upregulated in NGF-stimulated PC12h cells. A, The amount of CRMP5 mRNA was measured by quantitative RT-PCR before and after the NGF treatment. PC12h cells were serum starved for 24h prior to NGF (100nM) stimulation. At 12h after the addition of NGF, cells were collected, total RNA was extracted, and quantitative RT-PCR was performed with the specific primers described in materials and methods. The expression of CRMP5 mRNA was upregulated 1.7 fold in NGF-treated cells although the amount of internal control GAPDH mRNA was not altered. The expression of CRMP4 mRNA was also upregulated, but that of CRMP1 was not altered. Data are from three independent experiments with standard errors (*bars*) and numbers on the left show the copy number per 10ng total RNA. B, PC12h cells were photographed before (-NGF) and after (+NGF) the NGF treatment (12h). *Scale bar*, 20 μ m.

A



B



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