Calpain-Cleaved Collapsin Response Mediator Protein-3 Induces Neuronal Death after Glutamate Toxicity and Cerebral Ischemia

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Collapsin response mediator proteins (CRMPs) mediate growth cone collapse during development, but their roles in adult brains are not clear. Here we report the findings that the full-length CRMP-3 (p63) is a direct target of calpain that cleaves CRMP-3 at the N terminus (+76 amino acid). Interestingly, activated calpain in response to excitotoxicity in vitro and cerebral ischemia in vivo also cleaved CRMP-3, and the cleavage product of CRMP-3 (p54) underwent nuclear translocation during neuronal death. The expression of p54 was colocalized with the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling-positive nuclei in glutamate-treated cerebellar granule neurons (CGNs) and in ischemic neurons located in the infarct core after focal cerebral ischemia, suggesting that p54 might be involved in neuronal death. Overexpression studies showed that p54, but not p63, caused death of human embryonic kidney cells and CGNs, whereas knock-down CRMP-3 expression by selective small interfering RNA protected neurons against glutamate toxicity. Collectively, these results reveal a novel role of CRMP-3 in that calpain cleavage of CRMP-3 and the subsequent nuclear translocation of the truncated CRMP-3 evokes neuronal death in response to excitotoxicity and cerebral ischemia. Our findings also establish a novel route of how calpain signals neuron death.

Key words: CRMP-3; excitotoxicity; cerebral ischemia; cerebellar granule neurons; siRNA; neuronal death

Introduction

Collapsin response mediator proteins (CRMPs) are important brain-specific proteins with distinct functions during development and under disease conditions. In developing neurons, CRMPs mediate growth cone collapse in response to repelling guidance cues, such as semaphorin 3A or lysophosphatidic acid (Arimura et al., 2000). Specifically, CRMP-2, expressed in growth cones, modulates axonal length through modifying microtubule assembly, actin filaments, and cytoplasmic flow (Bradke and Dotti, 1999, 2000; Inagaki et al., 2001; Fukata et al., 2002; Nishimura et al., 2003; Yuasa-Kawada et al., 2003; Arimura et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005). CRMP-4 has also been shown to regulate actin bundling (Rossenbroich et al., 2005). Although how CRMPs precisely modulate the cytoskeleton to induce growth cone collapse remains unclear, it is known that CRMP-2 acts as a common intracellular target by integrating both positive and negative effects on axonal extension (Fukata et al., 2002; Yuasa-Kawada et al., 2003).

In adult brain, the functions of CRMPs are less clear. The expression levels of CRMPs are generally decreased, but CRMP-3 was selectively expressed in the spinal cord, cerebellum, and, to a lesser extent, forebrain (Wang and Strittmatter, 1996). Members of the CRMP family may have distinct functions in diseased brains (Weitzauderfer et al., 2001; Suzuki et al., 2003). For example, CRMP-3 and CRMP-4 expression increased in Down syndrome fetal brains, whereas CRMP-2 expression was significantly decreased (Weitzauderfer et al., 2001). CRMPs may directly participate in axonal damage and neuronal death, as exemplified by the fact that expression of semaphorins and its downstream effector CRMP were associated with neuronal injury in epileptic brains, Alzheimer’s disease brains, and cerebral ischemia (Kee et al., 2001; Czech et al., 2004; Good et al., 2004). Antibodies and peptides against semaphorin 3A blocked CRMP-2 activities and neuronal death (Gagliardini and Fankhauser, 1999; Shirvan et al., 1999, 2000). Overexpression of CRMP-2 in mouse N2A neuroblastoma cells caused blebbing of the cytoplasm and cell death (Gu and Ihara, 2000). These findings are in sharp contrast to the fact that overexpression of CRMP-2 induced the formation of multiple axons of hippocampal neurons and was associated with neuronal survival (Inagaki et al., 2001). It is, therefore, important to investigate the role that each CRMP plays in adult disease brains.
Calpains play a central role in ischemic neuronal death (Roberts-Lewis and Siman, 1993; Lipton, 1999) by cleaving a large number of substrates, including cytoskeletal proteins such as spectrins and important regulatory proteins such as cyclin-dependent kinase-5 (Patzeke and Tsai, 2002). The activities of calpain are modulated by an endogenously expressed inhibitory protein, calpastatin, and can be inhibited by synthesized inhibitors ALLN [N-acetyl-Leu-Leu-Nle-CHO] and calpeptin. Expression of calpain inhibitor calpastatin is neuroprotective to several neurological diseases, such as Parkinson’s disease and cerebral ischemia (Lankiewicz et al., 2000; Crocker et al., 2003). Identification of targets of calpain in response to NMDA receptor activation will shed light on the molecular mechanisms of neuronal death.

In the present report, we specifically investigated the role of CRMP-3 during neuronal death caused by excitotoxicity and show that calpain directly targets CRMP-3 during neuronal death. Nuclear translocation of calpain-cleaved CRMP-3 signals neuronal death.

Materials and Methods

Materials. All chemicals and reagents, unless stated otherwise, were purchased from Sigma (Burlington, Ontario, Canada). Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Advanced Immunochemicals (Long Beach, CA). Antibodies to CRMP-1, CRMP-2, CRMP-3, and CRMP-4 have been described previously (Franken et al., 2003) and were used at 1:10,000 for Western blotting.

Cell cultures and treatment. Primary cultures of mouse (C57BL/6) cerebellar granule neurons (CGNs) were prepared from 6- to 9-d-old postnatal mice as described previously (Smith et al., 2003). Briefly, cerebella were explanted and cleaned free of meninges. Mechanical and enzymatic dissociation in a 0.025% w/v trypsin solution for 25 min followed. A trypsin inhibitor was then added to block the enzyme, and 0.05% w/v DNase was added to break DNAs from dead cells. A series of trituration and mild centrifugation steps were included to disperse the neurons before resuspension in medium and to remove undissociated debris before plating in Eagle’s minimum essential medium containing 0.8 mm glutamine, 27 mm glucose, 0.01% gentamycin, and 9% FBS to prevent glial cell proliferation. One set of samples on the membrane was subjected to SDS-PAGE, followed by Western blotting using the primary antibody against smooth muscle actin (Promega, Madison WI). Digestion was performed overnight at 37°C, after which the supernatant was collected and the gel bands were extracted with 50% methanol and 0.5% acetic acid. The extracts were combined with the digest solutions, concentrated to 10 µl, and analyzed by nanoHPLC-MS/MS using a Q-TOF Ultima (Waters, Milford, MA). The entire samples were injected onto a 0.3 × 5 mm C18 micro precolumn cartridge (Dionex/LC Packings, Sunnyvale, CA), which was then brought on-line with a 75 µm × 50 mm Picofrit nano-column (New Objective, Woburn, MA) packed with BioBasic C18 reversed-phase media. The peptides were separated using a gradient supplied by a Waters CapLC pump (5–40% acetonitrile, 0.2% formic acid in 25 min, ~300 nL/min flow rate). The mass spectrometer was set to automatically acquire dual mass spectrometric spectra on double-, triple-, and quadrupole-charged ions. Database searching was performed in batch mode using Mascot Daemon (Matrix Science, London, UK) against the NCBInr protein sequence database. All search results were confirmed manually.

Calpain cleavage of CRMP-3 in vivo. The calpain inhibitors ALLN (Calbiochem, La Jolla, CA) or calpeptin (Calbiochem) was added to CGN cultures at the specified concentrations for 30 min before the addition of glutamate. After 2–6 h, cells were lysed and collected for Western blotting or fixed for immunostaining. To demonstrate that the endogenous calpain cleaves CRMP-3, normal brain extracts were mixed with 5 mm calcium in the presence or absence of calpain inhibitors. The mixture was incubated at 4°C. After 1, 4, or 18 h, reactions were terminated by the addition of protein loading buffer. Equal amount of brain extract was subjected to SDS-PAGE and Western blotting to detect CRMP-3 cleavage.

Immunostaining and TUNEL labeling. Neuronal death was confirmed in fixed tissue by reaction for TUNEL (Roche Products, Welwyn Garden City, UK) or propidium iodide (PI) assay (Hou et al., 2002). Immunolabeling was performed using specific primary antibodies with a cyanine 3 (Cy3)-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) as described previously (Tu et al., 1998; Hou et al., 2002). The primary antibodies were used at the following concentrations: mitogen-activated protein (MAP-2), 1:100; CRMP-3, 1:10,000; and neuronal-specific nuclear protein (NeuN), 1:200. The secondary antibody (conjugated with Cy3) was used at 1:200. Finally, cells were mounted in medium containing 5 µg/mL Hoechst 33258 to stain the nuclei. Slides were dried at 4°C overnight and examined under a fluorescent microscope.

Western blotting. Protein at 10 µg was electrophoresed and then electroblotted onto a nitrocellulose membrane in transfer buffer exactly as described previously (Hou et al., 2002; Smith et al., 2003). The membrane was then probed with a primary antibody at 4°C overnight. After washing with 0.01% PBS, horseradish peroxidase-conjugated secondary antibody was applied to the membrane for 1 h at room temperature. Enhanced chemiluminescence detection of the target protein was performed using a LumiGlo substrate kit (KPLaboratories, Gaithersburg, MD) and x-ray film.

Mass spectrometry analysis of protein gel bands. The protein gel bands of interest were excised and automatically destained and digested using Investigator PrepLot (Genomic Solutions, Ann Arbor, MI). Briefly, destaining was achieved using a 1:1 ratio of 30 mm potassium ferricyanide and 100 mm sodium thiosulfate (Ghahrenghi et al., 1999). The gel bands were then washed with deionized water, shrunk with acetonitrile, and reswollen with 50 mm ammonium bicarbonate containing 200 ng of modified trypsin (Promega, Madison WI). Digestion was performed overnight at 37°C, after which the supernatant was collected and the gel bands were extracted with 50% methanol and 0.5% acetic acid. The extracts were combined with the digest solutions, concentrated to ~10 µl, and analyzed by nanoeHPLC-MS/MS using a Q-TOF Ultima (Waters, Milford, MA). The entire samples were injected onto a 3 × 5 mm C18 micro precolumn cartridge (Dionex/LC Packings, Sunnyvale, CA), which was then brought on-line with a 75 µm × 50 mm Picofrit nano-column (New Objective, Woburn, MA) packed with BioBasic C18 reversed-phase media. The peptides were separated using a gradient supplied by a Waters CapLC pump (5–40% acetonitrile, 0.2% formic acid in 25 min, ~300 nL/min flow rate). The mass spectrometer was set to automatically acquire dual mass spectrometric spectra on double-, triple-, and quadrupole-charged ions. Database searching was performed in batch mode using Mascot Daemon (Matrix Science, London, UK) against the NCBInr protein sequence database. All search results were confirmed manually.

Determination of calpain cleavage site on CRMP-3. His-tagged CRMP-3 was purified using a nickel column. After digestion with active calpain I or II, duplicate samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Arlington Heights, IL). One set of samples on the membrane was sub-
Projecting to Western blotting, and the other was subjected to N-terminal microsequencing by cutting out the bands corresponding to p63 and p54. Protein sequencing was performed at the National Research Council Biotechnology Research Institute Protein Sequencing Facility (Montreal, Quebec, Canada). The obtained N-terminal polypeptide sequence was analyzed using Proteinprospector (http://prospector.ucsf.edu) and then aligned with the mouse CRMP-3 sequence using Clustal W to identify the N-terminal sequence of the cleaved CRMP-3.

Small interfering RNA design and transfection. Several small interfering RNAs (siRNAs) targeted to various regions of the CRMP-3 mRNA were designed, synthesized commercially by Qiagen (Mississauga, Ontario, Canada), and used to reduce the expression of CRMP-3. The most effective siRNA that was capable of downregulating CRMP-3 was selected empirically through transfection into CGNs and Western blotting to detect the reduced expression of CRMP-3. The following duplex siRNA tagged with Alexa Fluor 546 targeted to the CRMP-3 mRNA sequence between nucleotides 860 and 881 nt were selected and synthesized: 5′-r(UUCUCCGAGUACUUGAGGC)d(TT)-3′-Alexa Fluor 546. A negative control siRNA to CRMP-3 targeted with Alexa Fluor 546 to the 3′ end was also synthesized: r(UUCUCCGAGUACUUGAGGC)d(TT)-Alexa Fluor 546-3′. The annealed double-stranded siRNAs at 0.3–1 μg/well (Falcon 24-well plate) were transfected into 7 DIV CGNs using FuGene 6.0 (Qiagen) transfection reagent kit exactly following the instructions of the manufacturer. After 2 d of transfection, CGNs were either fixed with 4% paraformaldehyde for morphological examination or collected for protein analysis by Western blotting.

Plasmid construction, purification, and transfection. The full-length CRMP-3 (p63) and the N-terminal truncated CRMP3 (p54) were amplified from the plasmid CRMP3-pBCKCMV as template using the primer pairs rCRMP3-pr14/rCRMP3-pr15 and rCRMP3-pr16/rCRMP3-pr15, respectively. These primer pairs contain sequences encoding the restriction sites KpnI and BamHI at the end. The primer sequences are as follows: rCRMP3-pr14, 5′-gggttacctgcttccacaggaag-3′; rCRMP3-pr15, 5′-ggggttacctgcttccacaggaag-3′; and rCRMP3-pr16, 5′-gggttacctgcttccacaggaag-3′.

The amplified fragments were then digested with KpnI and BamHI and subcloned into the KpnI/BamHI restriction sites of the plasmid pEGFP-C1 (Clontech, Palo Alto, CA), the plasmid was purified using the plasmid midi-preparation kit purchased from Sigma (Sigma-Aldrich, Oakville, Ontario, Canada) following exactly the protocol of the manufacturer. Purified plasmid at 10 μg was used to transfect HEK293 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Burlington, Ontario, Canada) as described previously (Hou et al., 2002; Smith et al., 2003). Neurons were also fixed in 4% formaldehyde and mounted in Dako (High Wycombe, UK) fluorescent mounting medium containing 5 μg/ml Hoechst 33258 for examination of cell morphology with a fluorescent microscope. Quantitative assessment of each treatment was made on each plate in at least three separate experiments per treatment.

Data analysis. Data were analyzed using Microsoft (Seattle, WA) Excel. Error bars in all graphs represent SD of at least three independent repeats. Statistical significance was determined by one-way ANOVA and an additional post hoc test for significant groups using Tukey's test. p < 0.05 was considered statistically significant.

Results

Calpain cleaves CRMP-3

CRMP-3 protein appeared as a single band of 63 kDa (p63) on Western blot (Fig. 1A, B). However, in glutamate-treated CGNs (Fig. 1A) and focal cerebral ischemic brains (Fig. 1B), a smaller band at 54 kDa (p54) appeared below p63, Treating CGNs with the NMDA receptor antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepta-5,10-imine maleate] prevented the appearance of p54 (Fig. 1A), which had been shown to protect neurons from glutamate-induced death (Lin et al., 2005). Although treating CGNs with semaphorin 3A significantly inhibited axonal outgrowth of CGNs, this treatment did not induce the production of the p54 band (Fig. 1A, last lane). To confirm that p54 was the breakdown product of p63, the following four experiments were performed. (1) For confirmation of the specificity of the CRMP-3 antibody, Western blotting was performed using CRMP-3 antibody against overexpressed His-tag purified CRMP-1, CRMP-2, CRMP-3, and CRMP-4 proteins. Only His-CRMP-3 cross-reacted with the antibody, confirming the specificity of CRMP-3 antibody (data not shown). (2) To eliminate the possibility that the double-banding pattern was the result of CRMP-3 phosphorylation, calf intestine phosphatase was used to treat CGN neuronal protein extract. No visible changes in the levels of p63 and p54 occurred (data not shown). (3) The p54 band was excised for mass spectrometry analysis, which confirmed that p54 was CRMP-3. An example of one of the mass
CRMP-3 could be detected in both samples, whereas the lower concentrations of active calpain I (0.001 U) failed to cleave CRMP-3 (Fig. 2C,D). ALLN and calpeptin completely prevented the cleavage of CRMP-3 by added calpain I (Fig. 2C). Calpain II was also able to cleave CRMP-3, albeit at a relatively higher concentration compared with calpain I (data not shown). Fourth, for N-terminal sequencing, bacterial-expressed His-tagged CRMP-3 was purified using a nickel column, and the purified protein was cleaved with calpain I. The cleavage product was transferred onto a PVDF membrane, and the p54 band was excised for N-terminal microsequencing, which confirmed the cleavage site on CRMP-3 as shown in Figure 1D. Collectively, these data demonstrate that CRMP-3 is a direct target of calpain and that p54 is a cleavage product of p63.

### Nuclear localization of CRMP-3 after excitotoxicity and MCAO

Glutamate-induced death of CGNs was preceded by the appearance of neurite shortening, followed by the condensation of the nucleus and neuronal death. We examined CRMP-3 expression during this death process using immunostaining. As shown in Figure 3A, CRMP-3 was exclusively expressed in the cytoplasm of untreated control CGNs. After 4 h exposure to glutamate, most of the CRMP-3 staining appeared either around or in the nucleus (Fig. 3B, arrows). Pretreatment of CGNs with calpain inhibitors ALLN or calpeptin prevented the nuclear accumulation of CRMP-3 (Fig. 3C) and protected CGNs against glutamate toxicity (Fig. 3D).

To begin to understand the significance of CRMP-3 nuclear translocation, double labeling for CRMP-3 and TUNEL was performed on CGNs treated with glutamate. Interestingly, nuclear CRMP-3 staining colocalized with those nuclei positive for TUNEL staining, suggesting that the nuclear translocation of the CRMP-3 is associated with neuronal death (Fig. 3E,F). To further characterize nuclear CRMP-3, subcellular fractionation was performed, and the fractions were analyzed by Western blotting (Fig. 3G,H). Surprisingly, only p54, but not the full-length CRMP-3 (p63), was detected in the nuclear fraction derived from CGNs treated with glutamate (Fig. 3G) and ischemic brains (Fig. 3H). The nuclear fractions were free from cytosolic protein contaminations as confirmed by the lack of GAPDH on Western blot. Histone H1 was used as nuclear protein loading control in Figure 3, G and H. To quantitatively characterize CRMP-3 nuclear translocation, the relative intensities of nuclear CRMP-3 band were measured against histone H1 band using densitometry measurement. Nuclear p54 level was almost fivefold higher in glutamate-treated CGNs and ischemic brains compared with the non-ischemic (contralateral) side of the brain (Fig. 3I). These data indicate that CRMP-3 undergoes nuclear translocation during neuronal death.

Because glutamate-mediated excitotoxicity is a major cause of neuronal death after cerebral ischemia (Hou and MacManus, 2002), we examined changes in CRMP-3 expression in mouse brains subjected to MCAO. As shown in Figure 3J–M, CRMP-3 was expressed only in the cytoplasm of normal neurons located in the non-ischemic (contralateral) side of the brain (Fig. 3J), and no TUNEL-positive nuclei were detected (Fig. 3K–M). In contrast, on the ischemic side of the brain, CRMP-3 expression was mostly associated with the nuclei having condensed morphology and, at the same time, being TUNEL positive after reperfusion for 20 h (Fig. 3N–Q) or 24 h (Fig. 3R–U). These data demonstrate that nuclear translocation of CRMP-3 is associated with ischemia-induced neuronal death.
Downregulation of CRMP-3 protects CGNs from glutamate toxicity

To further investigate the role of p54 in neuronal death, specific siRNA to CRMP-3 was designed and used to downregulate CRMP-3 expression to see whether the reduction in CRMP-3 provides neuronal protection. As described in Materials and Methods, control siRNA and siRNA specific to CRMP-3 (siRNA-1 and siRNA-2) were commercially tagged with fluorescent Alexa Fluor 546 and transfected into cultured CGNs (Fig. 5D–F). CRMP-3 expression was downregulated more than fivefold by siRNA-1 compared with the control siRNA-treated CGNs and nontransfected CGNs (Fig. 5A,B). A second siRNA sequence to CRMP-3, siRNA-2, was less effective compared with siRNA-1 (Fig. 5A, lane 3). The effect of siRNA-1 on the reduction of CRMP-3 expression was selective because the expression levels of other CRMPs (i.e., 1, 2, and 4) were not affected (Fig. 5C). The reduced expression of CRMP-3 in the cytoplasm of neurons were confirmed by immunostaining for CRMP-3 (Fig. 5G–I). After 2 d transfection with siRNA, neurons were treated with glutamate (50 μM). Viable neurons were detected using CFDA and quantified using a plate reader (Fig. 5J). siRNA alone did not induce neuronal death (Fig. 5J). After 4 h treatment with 50 μM glutamate, CGNs transfected with siRNA-1 had significantly more viable neurons compared with glutamate-treated CGNs either transfected with the control siRNA or without transfection (Fig. 5J). Together, these experiments demonstrate that CRMP-3 plays a role in glutamate-induced neuronal death.

Collectively, these data led us to propose a working model in that Ca^{2+}-activated calpain cleaves CRMP-3. Truncated CRMP-3 (p54) translocates into the nucleus to cause neuronal death (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). In addition, evidence based on the series of loss-and-gain of function studies described here also suggests that the full-length CRMP-3 plays a distinct role from other family members such as CRMP-2, in that, when overexpressed in neurons, unlike CRMP-2, CRMP-3 does not cause the growth of multiple axons.

Discussion

The present study focused on the role of CRMP-3 and demonstrated for the first time that CRMP-3 is a direct target of calpain and that the cleavage product of CRMP-3 functions as a positive injury signal to cause neuronal death after cerebral ischemia and excitotoxicity. To the best of our knowledge, this is the first report describing such a novel property of CRMP-3.

Excitotoxicity through NMDA receptor-mediated intracellular calcium influx has been regarded as the major cause of toxicity to neurons after cerebral ischemia (Hou and MacManus, 2002; Bano et al., 2005). Early response to excitotoxicity of cultured neurons is axonal retraction, followed by, over time, neuronal death. Accumulating evidence suggests that intracellular signaling pathways are important contributing factors to transmit injury signals from distal...
Figure 3. Altered expression of CRMP-3 during neuronal death. A–C, CGNs were treated with glutamate (Glut) (50 μM) in either the absence (B) or presence (C) of calpain inhibitor ALLN (10 μM). Untreated CGNs were used as a negative control (Ctl) (A). ALLN protected CGNs against glutamate toxicity (C). After 4 – 6 h of treatment with 50 μM glutamate, cells were either fixed and subjected to immunostaining for CRMP-3 (A–C) or double stained with TUNEL (E, F). Note that glutamate-treated CGNs had shorter neurites and higher levels of CRMP-3 immunostaining in areas in and around the nucleus (B) compared with those in the untreated and ALLN-protected neurons. Blue in A–C indicates Hoechst 32558-positive nuclei. Neuroprotection by calpain inhibitors was also quantified using PI staining as described in Materials and Methods (D). G–I, Nuclear fractions from glutamate-treated CGNs (G) and ischemic brains (H) were prepared as described in Materials and Methods and were probed with antibodies to CRMP-3. Histone H1 and GAPDH were also probed as loading controls for nuclear and cytosolic fractions, respectively. Total protein extracts were also used as positive controls. A clear p54 band appeared in the nuclear fraction of glutamate-treated CGNs (G) and ischemic brains (H). The intensity of each band was quantified using densitometry and plotted in I. Data represented the average of at least three repeats, with error bars representing the SD. Statistical analysis in D and I was performed using Student’s t test, and significant groups were
Figure 4. Overexpression of p54 induces cell death. A, Plasmid constructs of EGFP-tagged full-length CRMP-3 (pEGFP-p63), p54 (pEGFP-p54), and EGFP vector control (pEGFP) were transfected into HEK293 cells. The overexpressed proteins were detected by Western blotting (B). EGFP fusion protein with p63 and p54 had a molecular weight of 98 and 89 kDa, respectively (B). Proteins from HEK293 and PC12 cells were subjected to Western blotting to confirm that they did not express endogenous CRMP-3 (C). Cultured HEK293 cells were transfected with 10 μg of plasmids. After 2 d, cells were fixed and viewed under a fluorescent microscope. EGFP vector construct expressed EGFP in both the nucleus and the cytoplasm. In contrast, EGFP-p63 only expressed in the cytoplasm (E), whereas EGFP-p54 expressed mostly in the nucleus (F). Overexpression of p54 (pEGFP-p54) caused nuclear condensation (F), which overlapped with TUNEL positivity (F’ and F’’). The number of TUNEL-positive cells were counted and plotted in G. Scale bar, 50 μm. **p < 0.01 by one-way ANOVA and post hoc Tukey’s test. H–M are CGNs infected with lentiviral constructs. Lentiviral vector expressing EGFP, EGFP-p63, and EGFP-p54 were made as described in Materials and Methods and were used to infect 7-d-old mature postmitotic CGNs for 72 h. Cell were examined live under a fluorescent microscope, followed by fixation with fresh Formalin and TUNEL staining (J, K, M). The number of TUNEL-positive cells were counted and plotted in N. Statistical analysis was performed using one-way ANOVA, and significant groups were identified by Tukey’s post hoc analysis. *p < 0.01.

The mechanism of CRMP-3-induced neuronal death is still not clear but is obviously involved in the nuclear translocation of calpain-cleaved CRMP-3. Inhibitors to caspases failed to prevent the breakdown of CRMP-3, suggesting that CRMP-3 is specifically cleaved by calpain. Calpain cleaves many important signal transduction molecules (Tompa et al., 2004). It is perhaps not surprising that calpain targets CRMP-3 during neuronal death. However, it is important to note that the cleaved product of CRMP-3 propagates a death signal by spatial translocation into the nucleus. We also attempted to examine p54 nuclear translocation by constructing a C-terminally tagged CRMP-3. However, such attempts have failed because tagging CRMP-3 at the C terminus with either six histidines or EGFP rendered the fusion protein unstable (data not shown), confirming published findings (Yuasa-Kawada et al., 2003). Although the present study did not examine how p54 was translocated into the nucleus, searches for a typical nuclear localization signal in adult neurons in response to excitotoxicity.

Axons to the cell body to cause neuronal degeneration (Raff et al., 2002; Aarts and Tymianski, 2004; Perlson et al., 2004). In the present study, we clearly demonstrate that, in response to glutamate toxicity and cerebral ischemia, the expression pattern of CRMP-3, normally in the cytoplasm of neurons, was altered in dying neurons and CRMP-3 cleavage is correlated with neuronal death. Calpain-cleaved CRMP-3 was translocated into the nucleus (Fig. 3). Neuroprotection by NMDA receptor antagonist MK-801 and calpain inhibitors prevented CRMP-3 cleavage, nuclear translocation, and neuronal death. Collectively, these studies show that CRMP-3 has a unique role in functioning as a retrograde-transported positive in-
The tissue-selective expression of CRMP family members in the adult nervous system argues for possible distinct functions of CRMPs that may not only be limited solely to growth cone collapse (Luo et al., 1993; Wang and Strittmatter, 1996). Indeed, the present study, for the first time, demonstrated that CRMP-3 had a completely different role compared with CRMP-2. In contrast to CRMP-2, one of the most well studied members of CRMPs, neither the overexpression of CRMP-3 nor the downregulation of CRMP-3 by siRNA affected neuronal survival and the morphology of the dendrites of mature neurons. However, downregulation of the full-length CRMP-3 expression rendered neurons more resistant to excitotoxicity-induced death, suggesting that CRMP-3 plays an important role in neuronal susceptibility to excitotoxicity. These observations are in sharp contrast to the role of CRMP-2 in that its overexpression promotes neurite elongation and axon induction by modifying actin filaments, microtubules, and cytoplasmic flow (Inagaki et al., 2001; Fukata et al., 2002; Nishimura et al., 2003; Arimura et al., 2004; Yoshimura et al., 2005). CRMP-2 overexpression transformed the established dendrites to become an axon without affecting the total number of processes emerging from the cell body (Arimura et al., 2004). Overexpression of CRMP-2 also accelerates nerve regeneration (Pasterkamp et al., 1998a; Suzuki et al., 2003), suggesting that CRMP-2 is associated with neuronal survival.

Under the normal physiological conditions, CRMPs are downstream of semaphorin 3A receptors, which encompass neuropilin, plexin, and L1 subunits and associated tyrosine kinases and act as cytosolic mediators for axonal guidance by collapsing growth cones. Importantly, under the conditions of neuronal injury and recovery, inhibitory factors secreted from the scar tissues, such as semaphorins, Nogo, MAG, oligodendrocyte myelin glycoproteins, and myelin-associated chondroitin sulfate proteoglycans, form major impediments to the successful repair of CNS connections (Pasterkamp et al., 1998a,b; Sandvig et al., 2004; Domeniconi and Filbin, 2005) by either inducing neuronal death or preventing the formation of new axonal connections, possibly through CRMP-mediated axonal retraction and death. Evidence from the present study showing that CRMP-3 was indeed part of the death response to excitotoxicity, together with recent demonstrations that semaphorin and neuropilin expression are associated with neuronal death, including cerebral ischemia (Shirvan et al., 1999, 2000; Fujita et al., 2001; Kee et al., 2001; Zhang et al., 2001; Beck et al., 2002; Good et al., 2004), lend additional support to the suggestion that CRMPs may be potential targets for therapeutic modulation to facilitate neuroprotection and regeneration.

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