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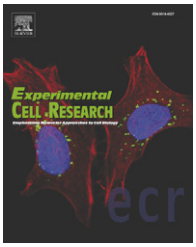

Research Article

Characterization of the role of full-length CRMP3 and its calpain-cleaved product in inhibiting microtubule polymerization and neurite outgrowth

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ABSTRACT

Collapsin response mediator proteins (CRMPs) are key modulators of cytoskeletons during neurite outgrowth in response to chemorepulsive guidance molecules. However, their roles in adult injured neurons are not well understood. We previously demonstrated that CRMP3 underwent calcium-dependent N-terminal protein cleavage during excitotoxicity-induced neurite retraction and neuronal death. Here, we report findings that the full-length CRMP3 inhibits tubulin polymerization and neurite outgrowth in cultured mature cerebellar granule neurons, while the N-terminal truncated CRMP3 underwent nuclear translocation and caused a significant nuclear condensation. The N-terminal truncated CRMP3 underwent nuclear translocation through nuclear pores. Nuclear protein pull-down assay and mass spectrometry analysis showed that the N-terminal truncated CRMP3 was associated with nuclear vimentin. In fact, nuclear-localized CRMP3 co-localized with vimentin during glutamate-induced excitotoxicity. However, the association between the truncated CRMP3 and vimentin was not critical for nuclear condensation and neurite outgrowth since over-expression of truncated CRMP3 in vimentin null neurons did not alleviate nuclear condensation and neurite outgrowth inhibition. Together, these studies showed CRMP3's role in attenuating neurite outgrowth possibility through inhibiting microtubule polymerization, and also revealed its novel association with vimentin during nuclear condensation prior to neuronal death.

Introduction

Collapsin response mediator proteins (CRMPs), consisting of five highly related members (CRMP1 to 5), are homologues of Unc33 whose mutation in *Caenorhabditis elegans* causes an ubiquitous impairment in the formation of neural circuits and severely uncoordinated locomotion [1,2]. During rodent development, CRMPs are involved in neurite path-finding in response to semaphorin-mediated growth cone collapse through neuropilins and neuropilin co-receptors [3–6]. Activated by the upstream serine/threonine protein kinases, CRMPs directly modulate the integrity of cytoskeletons to affect growth cone morphology and neurite outgrowth [7]. Structurally, although all CRMPs contain a sequence conserved domain similar to dihydropyrimidinase (DHP), it has been shown that CRMPs have no DHP enzymatic activity, rather they have acquired a regulatory function [8,9].

CRMP2 is the most intensely studied amongst all CRMPs. Expressed abundantly in growth cones and distal parts of the

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growing axons, CRMP2 modulates neurite length through direct binding to tubulin and promoting microtubule polymerization [10–12]. Over-expression of CRMP2 promotes neurite genesis in cultured hippocampal neurons [13]. In addition to regulating microtubule assembly, CRMP2 is also involved in modifying actin filaments, microtubule cytoplasmic flow and polarized Numb-mediated endocytosis of proteins such as L1 [14]. CRMP4 has also been shown to promote F-actin bundling *in vitro* [15]. The *in vivo* functions of CRMP family members remain less clear. Recent genetic studies showed that CRMP1 regulates neuronal migration by mediating reelin signaling. Deletion of CRMP1 during mouse development caused severe retardation of radial migration [16] and impaired long-term potentiation and impaired spatial learning and memory [17]. Similarly, deletion of CRMP3 also negatively affects hippocampal CA1 dendritic organization and plasticity [18] suggesting that CRMPs are important in maintaining cognitive functions.

Indeed, increasing number of studies also shed light on the role of CRMPs during brain injury. Insults to rodent brain elicit increased expression of CRMPs' indicating their involvements in response to brain injury [7,19–21]. For example, CRMP2 expression was up-regulated during the early stages of dopamine-induced neuronal apoptosis [22]. CRMP3 and CRMP4 expression levels were also increased in Down Syndrome fetal brains, whereas CRMP2 expression was significantly decreased [23]. In a rat hypoglossal nerve injury model, only CRMP1, CRMP2, and CRMP5 expression were increased, but not CRMP3 and CRMP4 [24]. It, therefore, appears that each CRMP family member functions in a context-dependent manner which remains to be investigated.

Most importantly, our recent studies showed that CRMPs are targets of calcium-activated calpain in ischemic mouse brain [7,19,25]. We provided biochemical evidence to show that calpain-cleaved CRMP3 product with a molecular weight of 54 kDa (hence p54) translocated into the nucleus to modulate neuronal death both during glutamate excitotoxicity *in vitro* and cerebral ischemic *in vivo* [25]. Moreover, calpain-mediated degradation of CRMPs occurs during oxidative stress *in vitro* and brain trauma *in vivo* [26–28]. Recent studies also showed that developmental processed CRMP2 undergoes nuclear translocation to inhibit neurite outgrowth [29]. However, how CRMP and its cleavage product translocate into the nucleus and what nuclear proteins CRMPs interact with remain unclear. Based on sequence comparison, it has been indicated that CRMPs lack a classical nuclear translocation signal in their polypeptide sequences. Nevertheless, all CRMPs share several conserved domains such as the D domain. These information led us to design several cDNA constructs expressing each domain of CRMP3 and to investigate whether a particular CRMP3 domain is necessary for nuclear translocation. Moreover, we also investigated the role of both the full-length and the calpain-cleaved CRMP3 in modulating adult neurite outgrowth and provided evidence to show vimentin as a nuclear binding partner with truncated CRMP3.

Materials and methods

All chemicals and reagents, unless stated otherwise, were purchased from Sigma Chemical Co. (Burlington, Ontario, Canada). Antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Advanced Immunochemicals (Long Beach, CA). Antibodies to CRMP3 were a generous gift from Dr. J. Kappler [25]. Detection of Western blots utilized ECL-Plus reagents from Amersham-Pharmacia biotech Inc. (Piscataway, NJ). Nuclear pore blocking polypeptide-conjugated to a leader sequence for increased permeability to neurons (AAVALLPAVLLAL-LAPVQRKRQKLMP) was commercially synthesized and HPLC purified by GeneScript Co. (Piscataway, NJ).

Cell cultures

All procedures using animals were approved by the local Animal Care Committee. Primary cerebellar granule neurons (CGNs) were cultured as previously described [30]. 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, at the end of which a trypsin inhibitor was 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 prior to resuspension in medium and to remove undissociated debris prior to plating in Eagle's minimum essential medium containing 0.8 mM glutamine, 27 mM glucose, 0.01% gentamycin, 9% FBS and supplemented with K^+ to a final concentration of 23 mM. Cells were plated onto 24-well dishes containing poly-lysine coated coverslips at a density of 6×10^5 per well. After approximately 18 h, cytosine β-D-arabinofuranoside (AraC) was added to a final concentration of 5 μM, to prevent glial cell proliferation. Human embryonic kidney 293 cells (HEK293) cultures were prepared exactly as described previously [31].

Sub-cellular fractionation

The general procedure for sub-cellular fractionation was as previously described [32]. Specifically, nuclear fractions were obtained using the following method: cells were collected into a 15 ml tube and spun at 1500 rpm for 5 min at 4 °C in a bench top centrifuge. Cell pellet was transferred into a 1 ml Eppendorf tube and washed briefly in buffer I (containing 2 mM KH_2PO_4, pH 6.55; 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.5 mM phenylmethylsulfony flouride). The pellet was finally resuspended in 3 ml of Buffer I containing 10% Triton X-100 and left on ice for 10 min. After centrifugation at 3000 rpm at 4 °C for 3 min, the cell pellet was resuspended in 3 volumes of Buffer II (containing 20 mM Hepes pH 7.9, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM henylmethyl-sulfony flouride). Following incubation with Buffer II on ice for 30 min, the nuclear lysate was spun at 55,000 rpm for 10 min at 4 °C. The supernatant contained soluble nuclear fractions. The pellet was sonicated for 15 s in Buffer II and spun at 55,000 rpm for 10 min at 4 °C. This supernatant contained proteins from the insoluble nuclear fraction.

EGFP pull-down assay

HEK293 cells at 75% confluency were transfected with the following plasmids: pEGFP, pEGFP-p63, pEGFP-p54 using Lipofectamine 2000. After 3 days of transfection, nuclear proteins were isolated and subjected to immunoprecipitation using DYNAL beads/streptavidin-mouse anti-EGFP (Dynal Inc) or Protein G beads (Invitrogen) following the manufacturers' instructions. After overnight incubation and extensive washing, proteins were