Evidence That Collapsin Response Mediator Protein-2 Is Involved in the Dynamics of Microtubules*

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Collapsin response mediator protein-2 (CRMP-2) is a member of the CRMP/TOAD/Ulip/DRP family of cytosolic phosphoproteins involved in neuronal differentiation and axonal guidance. CRMP-2 mediates the intracellular response to collapsin 1/semaphorin 3A, a repulsive extracellular guidance cue for axonal outgrowth. The mutation of unc-33, a Caenorhabditis elegans homolog of CRMP-2, results in abnormality of microtubules in neurites, but the mechanism of CRMP-2 action remains to be clarified. Here, we report that overexpression of human CRMP-2 in Neuro2a cells, a mouse neuroblastoma cell line, results in blebbing of the cytoplasm. Furthermore, some cells exhibited intranuclear inclusions, which were labeled with antibodies to CRMP-2 and tubulin. CRMP-2 was found to be associated with microtubule bundles in the spindles at the metaphase and in the midbodies at the late telophase in mitotic cells. Thus, it is most likely that failure of complete disassembly of the spindle microtubules during mitosis is responsible for the formation of these intranuclear inclusions. We suggest that CRMP-2 functions by regulating the dynamics of microtubules.

The development of the nervous system requires the growth of neuritic processes and their guidance toward the appropriate targets with which they must establish accurate synaptic contacts. Collapsin response mediator proteins (CRMPs)† are a family of cytosolic phosphoproteins involved in neurite outgrowth and axonal guidance (1). Their expression and phosphorylation are spatially and temporally regulated during development (2–4). Immunocytochemical studies showed that CRMPs are distributed in the lamellipodia and filopodia of the growth cone, the shaft of axons, and the neuronal cell body (2, 3, 5). CRMP-2 (also referred to as TOAD-64, Turned on after Division, 64 kDa (2); Ulip2, or UNC-33-like phosphoprotein-2 (3); DRP-2, dihydropyrimidinase-related protein-2 (6)) is the member most widely expressed within the nervous system. The protein has been reported to mediate semaphorin III/D-induced growth cone collapse through a signal transduction cascade involving G-protein (5). CRMP-2 has a sequence homologous to the product of unc-33, a nematode gene required for appropriately directed axonal extension (7). Mutations of unc-33 result in abnormal outgrowth of axons, leading to severely uncoordinated movements in Caenorhabditis elegans (8). Neurites in the mutant showed significant defects in microtubule organization; a superabundance of microtubules was found in sensory dendrites, and some of these microtubules were larger than normal in diameter, and some formed hooks or multiple tubules. These findings suggest that the axonal guidance defects are a consequence of cytoskeletal, in particular, microtubular, abnormalities and that the product of unc-33 should be involved in appropriate organization of microtubules in neurites. A straightforward interpretation is that UNC-33 is a microtubule-associated protein that should control the assembly or stability of microtubules in vivo. However, no distinct cytoskeletal association of CRMP-2 has thus far been recognized.

CRMP-2 may also be involved in the process of neurodegeneration. Highly phosphorylated forms of CRMP-2 were shown to be associated with neurofibrillary tangles in Alzheimer’s disease brains (9, 10). Semaphorin III/D and CRMP-2 have also been reported to mediate neuronal death in a dopamine-induced apoptotic cell model (11).

To study the functions of CRMP-2, especially its possible effects on the cytoskeleton, we established a regulable expression system for hCRMP-2 in Neuro2a (N2a) cells, a mouse neuroblastoma cell line, using the edysone-inducible mammalian expression system. In this system, the extent of CRMP-2 expression can be regulated by varying the dose of the inducer, ponasterone A. The potential toxic effects of overproduced CRMP-2 may thus be avoided, and changes attributable to CRMP-2 itself may be assessed more accurately. Here, we show evidence suggesting that CRMP-2 affects microtubule dynamics: overexpression of hCRMP-2 in N2a cells induced blebbing of the cytoplasm (and subsequent apoptosis of the cells); acetylated tubulin and CRMP-2 were occasionally deposited as intranuclear inclusions; and CRMP-2 was associated with microtubule bundles in the spindles at the metaphase and in the midbodies at the late telophase in mitotic cells.

EXPERIMENTAL PROCEDURES

Generation of Stable N2a Cell Lines with Inducible CRMP-2 Expression—A pTARGET-hCRMP-2 construct expressing hCRMP-2 was prepared by polymerase chain reaction as described before (10). The cDNA fragment encoding the complete sequence for hCRMP-2 was then cut with EcoRI and NotI from the plasmid and inserted into the pIND(SP1) vector. The new construct was named pIND(SP1)-hCRMP-2.

A founder N2a cell line stably transfected with pYGIXR (Zeocin-resistant), which constitutively expresses a functional edysone receptor, was established as described (12). The founder cells were then transfected with pIND(SP1)-hCRMP-2 to generate double-stable transfectants expressing hCRMP-2. Pooled clones were established by selection using 0.4 mg/ml Zeocin and 1.0 mg/ml G418. Monoclones were then
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RESULTS

Inducible Expression of CRMP-2 in N2a Cells—To study the biological functions of CRMP-2 in the cell, we employed an edysone-inducible system to obtain regulable expression of hCRMP-2 in N2a cells. To assess inducible CRMP-2 expression, the lysate of a representative clone was analyzed by Western blotting. As shown in Fig. 1, expression of CRMP-2 was induced by ponasterone A in a dose-dependent manner. Very low levels of CRMP-2 were found to be expressed in N2a cells in the absence of ponasterone A. This level of expression was the same as that in nontransfected cells and was thus considered representative of the level of endogenous CRMP-2. With increasing dose of ponasterone A, the expression levels of CRMP-2 increased, but with >1 μM ponasterone A the expression became saturated at a level that was about 7-fold that of the endogenous level. In the case of tau, a microtubule-associated protein, no saturation of inducible expression was observed up to 5 μM ponasterone A (data not shown), probably because high levels of CRMP-2 are toxic to the cell; the cells expressing higher levels of CRMP-2 started to die (see below).

Overexpression of CRMP-2 Results in Blebbing of the Cytoplasm and Subsequent Death of N2a Cells—The effects of CRMP-2 overexpression in N2a cells were first examined under a phase-contrast microscope. After 10–12 h of induction with 5 μM ponasterone A, some cells became rounded and detached from the tissue culture plate. Blebbing of the cytoplasm could be observed in some cells at this stage, and cell death occurred after prolonged induction with ponasterone A. About 15% of the cells died after 24 h of induction with 5 μM ponasterone A, as judged by trypan blue staining.

Immunostaining using antibodies to CRMP-2 showed that blebbing of the cytoplasm appeared only in those cells that expressed higher levels of CRMP-2 (Fig. 2A). The levels of inducibly expressed CRMP-2 varied even among the cells of a monoclonal origin, and its levels in the dying cells were well above those in the morphologically normal ones. If the mean level of CRMP-2 induced by 2 μM ponasterone A is assumed to be 7-fold that of the endogenous levels, much higher levels must have been expressed in the dying cells. Most of the cells showing cytoplasmic blebbing had intact nuclei, suggesting that alteration of the cytoskeleton occurred before the condensation of nuclei (Fig. 2B).

Cell death was demonstrated by MTT assay and trypan blue exclusion. The cell viability decreased depending on the dose of ponasterone A, becoming significant with >2 μM ponasterone A (Fig. 2C). Although differentiated N2a cells were used in these studies, in our hands, only about 80% of the cells were differentiated by treatment with 5 mM Bt2cAMP for 24 h, as judged by generation of neurite-like processes. The remaining undifferentiated cells still underwent division, and the cell numbers increased significantly after 2 days (Fig. 2D). Trypan blue staining showed no obvious effect of Bt2cAMP treatment on the cell viability.
CRMP-2 expression with 0, 1.0, 2.0, or 5.0 μM ponasterone A for 24 h and immunolabeled with C4G, a monoclonal antibody to CRMP-2, followed by incubation with FITC-conjugated goat anti-mouse IgG (A). The nuclei were visualized with Hoechst 33258 (B) as described under ‘Experimental Procedures.’ The arrow in each panel indicates a cell expressing high levels of CRMP-2 and blebbing without any obvious changes in the nucleus. C, cell differentiation was induced with 5 mM Bt2cAMP and CRMP-2 expression with 0, 1.0, 2.0, or 5.0 μM ponasterone A for 3 days. The viability of the cells on day 3 was determined using an MTT assay. The data are the means ± S.D. (bars) from 16 cultures. The viability of the cells not subjected to ponasterone A treatment (0 μM) was regarded as 100%. D, cell differentiation was induced with 5 mM Bt2cAMP and CRMP-2 expression with 5.0 μM ponasterone A, and the cell numbers were counted every 24 h following trypan blue staining. The data are the mean ± S.D. (bars) from four independent cultures. Statistics were performed by one-way analysis of variance followed by Bonferroni’s multiple comparison test (C) and Student’s t test (D). Values significantly different from nontreated cells are labeled as follows: *, p < 0.01; ***, p < 0.0001.

Survival of N2a cells, as reported by others (12). In contrast, the fraction of cells that did not exclude trypan blue increased significantly upon treatment with ponasterone A, reaching 50–60% after 5 days of treatment. Thus, the decreased cell viability was largely due to cell death, although inhibition of cell proliferation by CRMP-2 might also contribute to some extent. There was no significant change in the viability of mock-transfected cells by ponasterone A treatment (data not shown).

Association of CRMP-2 with Microtubule Bundles—The distribution of CRMP-2 in N2a cells was examined by immunofluorescence before and after induction by ponasterone A, using C4G, a monoclonal antibody to CRMP-2. As reported by others, CRMP-2 is diffusely distributed in the cytoplasm. This distribution was distinct from that of microtubules, actin filaments (Fig. 3A), or neurofilaments (data not shown) in N2a cells. However, in some N2a cells overexpressing CRMP-2, the monoclonal antibody also labeled some distinct structures, i.e. the spindles at the metaphase and the midbodies at the late telophase in mitotic cells. These structures are characterized by the presence of microtubule bundles. Colocalization was confirmed by double labeling with the antibodies to α-tubulin and CRMP-2 (Fig. 3B).

Intranuclear Inclusions of CRMP-2 and Tubulin in N2a Cells—We found that intranuclear inclusions were formed in a small proportion of N2a cells (~2%) overexpressing CRMP-2. In such cells, stronger immunostaining of CRMP-2 was found in the inclusions than in the cytoplasm. These inclusions had a regular outline, and most appeared globular and were 1–2 μm in diameter (Fig. 4A). An antibody to α-tubulin strongly stained these intranuclear inclusions (Fig. 4B). The inclusions were quite compact and replaced the chromatin, as seen in Hoechst dye-negative areas in the nucleus (Fig. 4C). No correlation was observed between the formation of intranuclear inclusion and the blebbing of cytoplasm or the condensation of chromatin. Sometimes the inclusions appeared as tapered to curly thick...
fibers continuous with the cytoplasmic microtubules (Fig. 4B), suggesting that they originate from the microtubule bundles formed during mitosis. This view was further supported by an observation that very few inclusions appeared in the N2a cells differentiated by Bt,cAMP.

An antibody to acetylated-tubulin weakly stained the microtubules in the cytoplasm. However, it strongly labeled the intranuclear inclusions, suggesting that the deposits are composed mainly of the mature form of tubulin (Fig. 4C).

**DISCUSSION**

The CRMP/TOAD/Ulip/DRP family of cytosolic proteins play important roles in neuronal differentiation and axonal guidance. The lack of sequence homology between the CRMP/UNC-33 family and other proteins in data bases indicates that CRMPs may have a unique and as yet poorly understood molecular mechanism of action.

Our results point to the involvement of CRMP-2 in the regulation of microtubule dynamics. First, CRMP-2 was associated with bundled microtubules in N2a cells. In this respect, CRMP-2 differs from classical microtubule-associated proteins, which are associated with individual microtubules. In vitro assembly/disassembly cycles of microtubules revealed no direct association of CRMP-2 (data not shown). CRMP-2 may be involved in the interaction between microtubules or may help link the microtubules to other cellular components. All members of the CRMP family have a basic region, adjacent to a differentially phosphorylated region, in the carboxy-terminal portion of the molecule (10). The basic region may be responsible for direct or indirect binding to the microtubules, which is likely regulated by phosphorylation of the adjacent region. It has been reported that CRMPs exist as (hetero)tetramers (13), a good candidate for a cross-linker of individual microtubules. Second, overexpression of CRMP-2 led to blebbing of the cytoplasm of N2a cells. This event is believed to be due to cytoskeletal alterations affecting the dynamics of actin or defective interactions between microfilaments and microtubules. Finally, overexpression of CRMP-2 in N2a cells induced depolymerization of tubulin, in particular, its stable form, as intranuclear inclusions. Acetylation of α-tubulin is a relatively slow enzymatic reaction that occurs only on microtubules and not on free tubulin molecules. The reaction is rapidly reversed when tubulin molecule depolymerizes (14). Thus, the presence in abundance of acetylated tubulin in the intranuclear inclusions indicates that they originate from stable microtubules. The stabilizing effect of overexpressed CRMP-2 on bundles of microtubule structures likely results in the failure of disassembly of spindles during cell division. The present finding may thus explain, in part, some observations in the unc-33 mutant of C. elegans, in which the neurites contain a great abundance of microtubules (8).

CRMP-2 has been reported to be necessary for G-protein-mediated transduction of an extracellular collapsin signal into growth cone collapse (5). However, the observed G-protein-independent effects of pertussis toxin on neurite growth and growth cone morphology (15) suggest that CRMP might also be a component of a non-G-protein-dependent signaling pathway. Alterations of the cytoskeletal network have been proposed to be involved in changes of the growth cone morphology in response to extracellular cues. The actively extended tips of the growth cone are filled with a population of dynamic actin filaments. Microtubules are found in abundance in the central domain of the growth cone, with some of them extending to the base of the filopodia at the leading edge; they play important roles in the maintenance and regulation of the growth cone remodeling by forming highly stable cross-linked bundles (16, 17). In N2a cells, CRMP-2 partially overlaps with filamentous actin in the spreading cytoplasm (see Fig. 3A). However, no apparent change of organization of actin filaments was found in the cells overexpressing CRMP-2. We suggest that one pathway of CRMP-2 mediated-collapsing activity is through its regulation of microtubule reorganization. A cascade of kinase activities may be involved in the collapsin signaling pathway, in which Rac1 regulates the organization of actin filaments (18), and phosphorylation of CRMP-2 may destabilize microtubule bundles in the growth cone. On the other hand, the stabilizing effect of CRMP-2 on the specific microtubule arrangements in the axon shaft contributes to its maintenance. In support of this assumption, the phosphorylation level of CRMP-2 in the brain under development is much greater than that of inducibly expressed CRMP-2 in N2a cells (9, 10).

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