Acta Biochim Biophys Sin 2012, 44: 450–454 | © The Author 2012. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gms017.
Advance Access Publication 16 March 2012

Short Communication

Cep70 promotes microtubule assembly *in vitro* by increasing microtubule elongation

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Microtubules are dynamic cytoskeletal polymers present in all eukaryotic cells. In animal cells, they are organized by the centrosome, the major microtubule-organizing center. Many centrosomal proteins act coordinately to modulate microtubule assembly and organization. Our previous work has shown that Cep70, a novel centrosomal protein regulates microtubule assembly and organization in mammalian cells. However, the molecular details remain to be investigated. In this study, we investigated the molecular mechanism of how Cep70 regulates microtubule assembly using purified proteins. Our data showed that Cep70 increased the microtubule length without affecting the microtubule number in the purified system. These results demonstrate that Cep70 could directly regulate microtubule assembly by promoting microtubule elongation instead of microtubule nucleation.

Keywords Cep70; centrosome; microtubule assembly; tubulin

Received: November 7, 2011 Accepted: January 4, 2012

Introduction

The centrosome, composed of a pair of barrel-shaped centrioles surrounded by electron-dense pericentriolar material (PCM), is the major microtubule-organizing center in animal cells [1,2]. Microtubule organization at the centrosome involves distinct processes such as nucleation, anchoring, and release of microtubules [3]. Nucleation of microtubules from the centrosome is mediated by γ-tubulin ring complexes (γTuRCs), consisting of γ-tubulin and γ-complex proteins (GCPs) [4]. After being recruited to the centrosome, γTuRC promotes the polymerization of α/β-tubulin subunits into microtubule polymers [5]. During interphase, the centrosome nucleates and anchors the radial array of microtubules with minus ends embedded in the centrosome and the dynamic plus ends extend toward the cell cortex [2]. The radial microtubule array can be remodeled to noncentrosomal array to meet different needs of cells. In neurons and polarized epithelial cells, many microtubules are released from centrosomes and reorganized into nonradial arrays that project into neurite or away from the apical face of the cell [6–9]. The microtubule-organizing ability of the centrosome depends on accurate control of the anchoring and release of the nucleated microtubules [10].

Cep70, a centrosomal protein of 70 kDa, was initially described in a proteomic study of human centrosome [11]. Recently, the biological function of Cep70 in different species is beginning to be unraveled. In zebrafish embryo, Cep70 has been characterized as a mediator of ciliogenesis, which has an essential role in defining the correct patterning of the body [12]. In addition, the Chlamydomonas procentriole protein CRC70, a member of the conserved Cep70 family, acts as a scaffold for the assembly of the centriole precursor [13]. In our previous study, Cep70 has been shown to participate in microtubule assembly and mitotic spindle orientation in mammalian cells [14]. In this study, we sought to investigate the mechanism by which Cep70 regulated microtubule assembly. The results demonstrate that Cep70 could regulate microtubule assembly by promoting microtubule elongation instead of microtubule nucleation.

Materials and Methods

Materials

Ex Taq, T4 DNA ligase, restriction enzymes of *Bam*HI and *Sal*I were purchased from TaKaRa (Dalian, China). Ampicillin and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from Sangon Biotech (Shanghai, China), anti-MBP (maltose binding protein) antibody and Coomassie Brilliant Blue R250 were from Sigma-Aldrich (St Louis, USA). Horseradish peroxidase-conjugated anti-mouse antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, USA). Plasmid sequencing was performed in Invitrogen (Carlsbad, USA). DNA

Marker III was from Tiangen Biotech (Beijing, China), and prestained protein ladder was from Fermentas (Shenzhen, China).

Construction of plasmid pMALp2T-Cep70

Cep70 fragment was amplified from 293T complimentary DNA (cDNA) library by polymerase chain reaction (PCR), with the following primers: forward, 5′-GGATCCATGTTT CCGGTAGCCC-3′ and reverse, 5′-GTCGACATGATTT GATTTGTTTTCAGTAT-3′. The PCR fragment was digested with *Bam*HI and *Sal*I, and then inserted between the *Bam*HI and *Sal*I sites of pMALp2T vector, in which the factor Xa cleavage site of pMALp2 was replaced with a thrombin recognition site, and pMALp2 was obtained from New England BioLabs (Ipswich, USA). The sequence was confirmed by sequencing, which is identical to the National Center for Biotechnology Information reference sequence of human Cep70 (NM_024491.2).

Purification of MBP-Cep70 fusion protein

To purify MBP-Cep70 fusion protein, the recombinant plasmid of pMALp2T-Cep70 was transformed into BL21(DE3) strain of *Escherichia coli*. The condition to express the MBP-Cep70 fusion protein was optimized when culturing at 18°C for 16 h with 0.1 mM IPTG. Big batch of bacterial culture was prepared for protein expression. The pellet and the supernatant were obtained after sonication of the bacteria. The supernatant was incubated with the amylose resin (New England BioLabs), and proteins were purified according to the manufacturer's instruction. Bovine brain tubulin (microtubule-associated protein-free, >99% pure) was obtained from Cytoskeleton Inc. (Denver, USA).

In vitro tubulin polymerization assay

Spectrophotometer cuvettes contained a solution consisting of 100 mM PIPES, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM $MgSO_4$, and 1 mM GTP (guanosine triphosphate) (pH 6.8). The cuvettes were kept at room temperature before the addition of purified tubulin and Cep70, then transferred to a temperature-controlled spectrophotometer (Amersham Biosciences, Piscatway, USA) at 37°C. Tubulin polymerization into microtubules was monitored by measuring the changes of absorbance at 350 nm wavelength as described previously [15].

Analysis of fluorescent microtubules

The fluorescent microtubules biochem kit (Cytoskeleton, Inc.) was used according to the instructor's manual. Briefly, rhodamine tubulin (5 mg/ml) was diluted with unlabeled tubulin (5 mg/ml) to reach 0.15 labeling ratio, then incubated with MBP-Cep70 or MBP at 37°C for 20 min. The polymerized tubulin was removed from 37°C, and added taxol/microtubule buffer solution, resulting in a population of taxol stabilized microtubules. The morphology of microtubules was examined with an Axio Observer A1 fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany).

Western blot analysis

Proteins were resolved by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked in Tris-buffered saline containing 0.2% Tween 20% and 5% fat-free dry milk and incubated first with anti-MBP antibody (1:2000) and then with horseradish peroxidase-conjugated anti-mouse antibody (1:5000). Specific proteins were visualized with enhanced chemiluminescence detection reagent (Pierce, Rockford, USA).

Results

Construction of the bacterial expression plasmid pMALp2T-Cep70

To construct the pMALp2T-Cep70 plasmid, we first employed PCR technique to amplify Cep70 fragment. The agarose gel electrophoresis showed a clear and specific band of around 1.8 kb [**Fig. 1(A)**]. After double enzymes' digestion, the resulting PCR fragment was inserted into pMALp2T vector. The results of colony PCR and double-restriction enzyme identification indicated that the Cep70 fragment was inserted into pMALp2T vector [**Fig. 1(B,C)**]. The exact insertion of Cep70 gene was confirmed by DNA sequencing. The data showed that the bacterial expression plasmid of the MBP-Cep70 protein was constructed successfully.

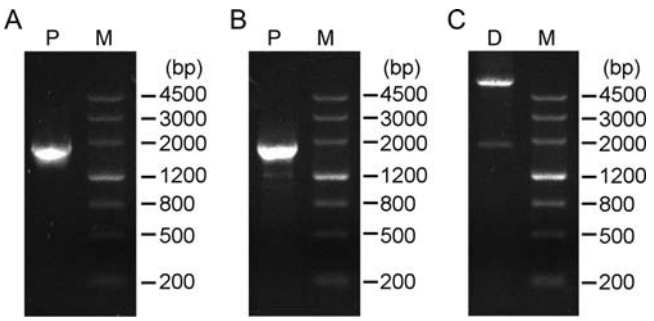

Figure 1 Construction of the bacterial expression plasmid of the MBP-Cep70 fusion protein (A) PCR amplification of the *Cep70* gene including *Bam*HI and *Sal*I restrict enzyme sites (P: PCR product, M: marker). (B) Electrophoretic analysis of recombinant pMAL2T-Cep70 by colony PCR (P: PCR product, M: marker). (C) Recombinant plasmid pMAL2T-Cep70 was digested with *Bam*HI and *Sal*I enzymes (D: digested *Cep70* gene and plasmid fragment, M: marker).

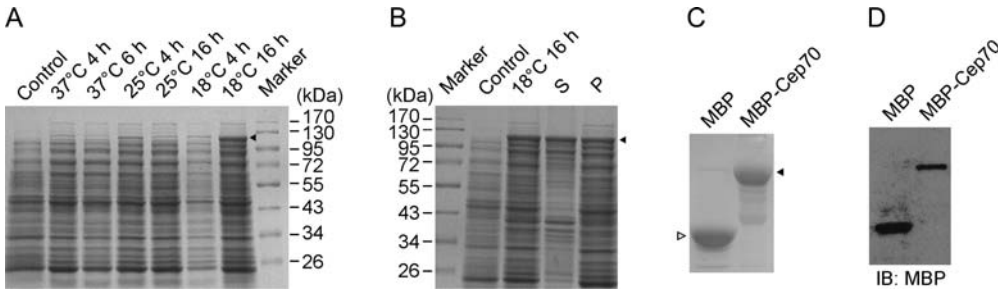

Figure 2 Purification of MBP-Cep70 from bacteria using the amylase resin (A) Optimizing the expression condition of MBP-Cep70 fusion protein (Control: total cellular extracts from BL21 strain of *E. coli* containing pMAL2T-Cep70 before induction). (B) SDS−PAGE analysis of the expression of MBP-Cep70 fusion protein (Control: total cellular extracts before induction; S: the supernatant of ultrasonic disintegrated cellular extracts; P: the inclusion body of ultrasonic disintegrated cellular extracts). (C) After purification with the amylose resin, MBP and MBP-Cep70 were examined by SDS−PAGE. Arrowhead and open arrowhead indicate MBP-Cep70 and MBP, respectively. (D) Purified MBP and MBP-Cep70 were examined by western blot with anti-MBP antibody.

Purification of MBP-Cep70 from bacteria using the amylose resin

The BL21(DE3) strain of *E. coli* was used to express MBP-Cep70 fusion proteins or MBP alone. As shown in **Fig. 2(A)**, the optimal culture condition was the induction at 18°C for 16 h with 0.1 mM IPTG. The results of SDS−PAGE showed that MBP-Cep70 fusion protein is equivalent in pellet and supernatant, and the fusion protein is suitable for purification [**Fig. 2(B)**]. Both the MBP and MBP-Cep70 fusion proteins were purified with the amylose resin [**Fig. 2(C)**]. To further confirm the expression of MBP-Cep70, the purified proteins were examined by western blot with anti-MBP antibody. As shown in **Fig. 2(D)**, the anti-MBP antibody specifically recognized MBP and MBP-Cep70. These data demonstrate that MBP-Cep70 fusion protein is purified from bacteria with the amylose resin.

Effect of Cep70 on microtubule assembly *in vitro* examined by the light scattering assay

Our previous finding has shown that Cep70 is involved in microtubule assembly. However, it is unknown as to whether Cep70 regulates microtubule assembly directly or via other centrosomal proteins. To answer this question, we examined the effect of purified Cep70 on microtubule assembly *in vitro* by measuring changes in the turbidity produced upon tubulin polymerization into microtubules. As shown in **Fig. 3**, Cep70 could increase tubulin turbidity in a concentration-dependent manner. These results indicate that Cep70 could promote tubulin polymerization into microtubules directly.

Fluorescence microscopic analysis of the morphology of microtubules assembled in the presence of Cep70

To gain more mechanistic insight into the role of Cep70 in regulating microtubule assembly, we studied the morphology of microtubules assembled *in vitro*. Purified MBP or

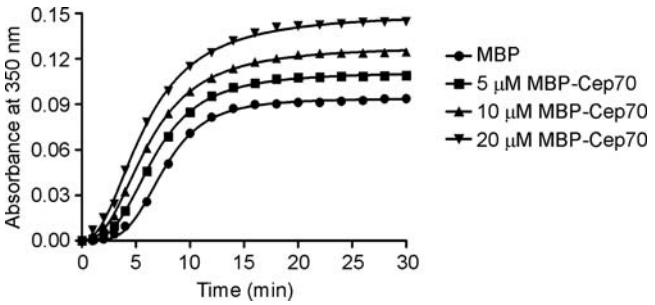

Figure 3 Effect of Cep70 on microtubule assembly *in vitro* examined by the light scattering assay Effects of MBP or 5, 10, or 20 μM purified MBP-Cep70 on tubulin polymerization *in vitro*, reflected by the changes in absorbance at a 350 nm wavelength at 37°C.

MBP-Cep70 fusion protein was incubated with rhodamine-labeled tubulin at 37°C for 20 min, and the polymerized microtubules were stabilized with taxol. The morphology of microtubules was examined with a fluorescence microscope. Compared with the control group, MBP-Cep70 could increase microtubule length in a concentration-dependent manner [**Fig. 4(A,B)**]. However, there was no significant difference in microtubule number in the two groups [**Fig. 4(C)**]. These data indicate that Cep70 enhances microtubule elongation instead of microtubule nucleation.

Discussion

Microtubules are hollow tubes assembled from α/β-tubulin heterodimers. Microtubule assembly, consisting of microtubule nucleation and elongation, is exquisitely modulated by a complex network of cellular factors [5,16]. Microtubule nucleation, the critical step in microtubule assembly, is initiated by γTuRC from the centrosome [17]. By interacting with γ-tubulin and recruiting γTuRC to the centrosome, many centrosomal proteins such as pericentrin, centrosome and Golgi localized protein kinase

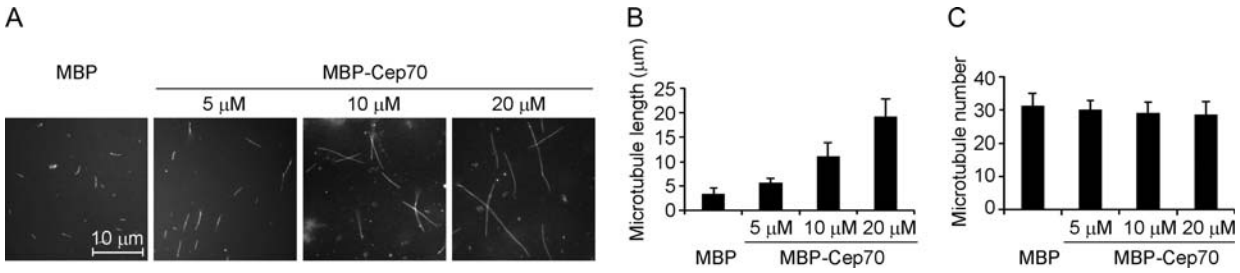

Figure 4 Fluorescence microscopic analysis of the morphology of microtubules assembled in the presence of Cep70 (A) Rhodamine-labeled tubulin was incubated with MBP or 5, 10, or 20 μM purified MBP-Cep70 at 37°C for 20 min. After the polymerized microtubules were stabilized with taxol, the morphology of microtubules was examined with an Axio Observer A1 fluorescence microscope. (B) Experiments were performed as in panel A, and the microtubule length was calculated. (C) Experiments were performed as in panel A, and microtubule number was counted.

N-associated protein (CG-Nap), centrosomal protein ninein-like protein (Nlp), and neural precursor cell expressed developmentally down-regulated protein 1 (NEDD1), are involved in microtubule nucleation [18–21]. In addition, classical microtubule-associated proteins (MAPs), such as MAP1, MAP2, and tau bind to the outside of microtubules to stabilize microtubules and promote microtubule elongation [22,23].

In our previous work, cells transfected with Cep70 small interfering RNAs (siRNAs) showed retardation in microtubule nucleation, as evidenced by the formation of smaller asters. However, microtubule elongation was not significantly affected in Cep70 depleted cells [14]. In this study, we demonstrated that the purified protein of Cep70 accelerated tubulin polymerization into microtubule by promoting microtubule elongation. This action of Cep70 is distinct from most other centrosomal proteins, which could regulate microtubule assembly by exerting on the microtubule nucleation process. In addition, the role of Cep70 *in vitro* is clearly different from its action in cells. It is possible that Cep70 regulates microtubule nucleation in cells by interacting with γ-tubulin or other centrosomal proteins. Moreover, Cep70 does not affect the microtubule elongation in cells, suggesting that Cep70 may facilitate microtubule polymerization after microtubule nucleation, which is consistent with our *in vitro* results. It will be important to investigate in the future whether Cep70 associates with MAPs to regulate microtubule elongation in cells.

It is worthy of note that, in the *in vitro* tubulin polymerization assay, microtubule regrowth is a collective result of microtubule polymerization at the plus end and microtubule depolymerization at the minus end. Our studies showed that Cep70 promoted microtubule polymerization by increasing microtubule elongation. However, it is possible that Cep70 functions in inhibiting microtubule depolymerization but not accelerating its polymerization. It will be important to explore whether Cep70 acts in microtubule depolymerization in further studies, which may provide additional insight into the mechanism of how Cep70 regulates microtubule assembly.

Accumulating evidence has implicated that Cep70 might participate in cellular processes linked to the centriole. Depletion of Cep70 in zebrafish with antisense morpholino oligonucleotides reduces the length of axoneme [12]. Knockdown of CRC70 produces flagella-less cells and inhibits the recruitment of other centriolar components [13]. Cilia and flagella are specialized organelles protruding from the cell surface. They consist of a basal body and a protruding body named the axoneme. The centrioles of the centrosome are similar to the basal bodies of cilia or flagella that have important roles in cell signaling and development [24]. Therefore, it will be interesting to study the potential role of Cep70 in centriole-associated cellular activities.

Funding

This work was supported by grants from the National Natural Science Foundation of China (30825022) and the National Basic Research Program of China (2010CB912204).

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