
Different Location of Katanin P60 Effect on Cellular Microtubule Cutting

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Abstract: Katanin is a heterodimeric hydrolase belonging to the AAA protein family and its function is cutting microtubules. It plays an important role in cell division, neural development, cell migration, and the formation of motor organelles. Katanin has two subunits including katanin p80 and p60. This research is aimed to study the effect of different location of katanin p60 on cellular microtubule cutting. First, katanin p60 gene was amplified by PCR, and the eukaryotic recombinant plasmid GFP-katanin p60 and GFP-katanin p60^{MIS} were constructed. The recombinant plasmid was identified by enzyme digestion and sequencing. The expression of katanin in RFL-6 cells was detected by western blot analysis. And, the distribution of katanin p60 in RFL-6 cells and its effect on microtubule were further examined. The results showed that the GFP-katanin p60 and GFP-katanin p60^{MIS} recombinant plasmids were successfully constructed. By western blot analysis, GFP-katanin p60 and GFP-katanin p60^{MIS} recombinant plasmids could be successfully expressed in RFL-6 cells. The microtubule cutting ability of GFP-katanin p60^{MIS} which distribute in whole-cell was stronger than GFP-katanin p60 which distribute in nuclear. Therefore, the cutting function on microtubule of katanin p60 depends on its distribution, and the whole cell distribution is beneficial to katanin p60 in cutting microtubule.

Keywords: Katanin p60, Microtubule Cutting, RFL-6 Cell, Protein Distribution

1. Introduction

Katanin is a heterodimeric hydrolase belonging to the AAA protein family [1]. It is ubiquitously expressed in plants and animals, mainly cutting tubulin. According to the molecular weight of the subunit, the smaller catalytic subunit named p60 is 60KDa, and its role is cutting microtubules; the larger catalytic subunit is 80KDa, named p80, the role of katanin p80 assisted p60 cutting microtubules [2-4]. Katanin plays an important role in cell division, neural development, cell migration, and the formation of motor organelles [5]. There are three Katanin-cut microtubule models, one of which is the combination of dynein and microtubule, which causes the microtubule structure to change, becoming "S" or "V" shape and thus is recognized as Katanin for cutting [6, 7]; Second models include dynamic instability mechanisms and the phenomenon of "size-dependent mobilization"[8, 9]; the third model is Katanin cutting post-translationally modified microtubules¹⁰. But, whether the distribution of katanin effect

on its cutting function is not clear. Therefore, we constructed GFP-p60 and GFP-p60^{MIS} eukaryotic expression plasmids which distribute in nuclear and whole cell in RFL-6 cell to investigate their cutting function on microtubule, which provided a model basis for the subsequent overexpression of p60 gene cleavage research and its mechanism.

2. Materials and Methods

2.1. Materials

RFL-6 Cell was purchased from the shanghai Cell Bank. The types and manufactures of materials purchased are as follow: Dulbecco's Modified Eagle Medium (GIBCO), fetal bovine serum (GIBCO), Rabbit polyclonal antibody to GFP (abcam), rat monoclonal antibody to tubulin (abcam), Alexa Fluor 549 Donkey anti rat (abcam). EcoR I and Bgl II Restriction enzyme, DNA marker, Homologous recombinant ligase, 2x Taq Plus Master Mix (TaKaRa).

2.2. Methods

2.2.1. Construction of GFP-p60, GFP-p60^{MIS} Recombinant Plasmid

According to Rat p60 gene sequence in Gene Bank, designing primers for PCR of GFP-p60 in table 1. Rat cDNA as the template, the conditions for PCR were as follows: pre-denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 90 s, and a final extension at 72°C for 5 min. Recycling the PCR products were used to harvest katanin p60 and p60^{MIS}, Bgl II and EcoR I restriction endonucleases were used to treat pEGFP-C1. In the presence of Homologous recombinant ligase, katanin p60 and p60^{MIS} were connected at 37°C for 30 min and transfected into E coli. Identification of these plasmids was done by digestion with restriction endonucleases and subsequent sequencing.

Table 1. Primers of constructed plasmids.

Plasmid	Sequence	Sequence
GFP-Katanin p60	Forward	5'-TACAAGTCCGGACTCAGATCTA TGAGTCTCTAATGATTACTGAGA ATGTA-3'
	Reverse	5'-GTACCGTCGACTGCAGAATTCT TACATTCTCAGTAATCATTAGAA ACTCA-3'
GFP-Katanin p60 ^{MIS}	Forward	5'-TACAAGTCCGGACTCAGATCTT CCAGTCTCTAATGATTACTGAGA ATGTA-3'
	Reverse	5'-GTACCGTCGACTGCAGAATTCT TACATTCTCAGTAATCATTAGAA ACTCA-3'

2.2.2. Culture and Transfection of RFL-6 Cell

RFL-6 cell were routinely cultured. GFP、GFP-katanin p60 and GFP-katanin p60^{MIS} were used to transfect RFL-6 cell with Lipo2000. Immunofluorescence staining and western blot assay were performed.

2.2.3. Detection of Protein Expression by Western Blot Assay

At 48h after transfection with GFP、GFP-katanin p60 and GFP-katanin p60^{MIS}, RFL-6 cells were harvested, washed with cold PBS twice, lysed in lysis buffer on ice for 10 min, and then centrifuged at 12 000 r/min for 15 min at 4°C. The supernatant was collected and protein quantification was done by using coomassie brilliant blue. The protein concentration was calculated. Then, total proteins were subjected to 10% SDS-PAGE (50µg/lane) and then transferred onto 0.45µm NC membrane which was subsequently blocked, treated with anti-GFP antibody and horseradish peroxidase conjugated secondary antibody. Visualization was done with ECL, and protein bands were scanned.

2.2.4. Fluorescent Immunocytochemistry

After calcium phosphate mediated transfection, cells were harvested, fixed in 4% paraformaldehyde, treated with perforating agent twice (5 min for each), and blocked in 5% donkey serum for 1 h at room temperature. Then, these cells

were incubated with primary antibody at 4°C overnight and washed with TBST (0.1%TritonX-100 in TBS). Following incubation with secondary antibody, cells were washed with TBST (0.1%TritonX-100 in TBS). After DAPI staining, the coverslips containing cells were air-dried and mounted, and then observed under a laser scanning confocal microscope. Image-Proplus software was used to detect the content of microtubules in RFL-6 cells.

2.2.5. Statistical Analysis

The images of RFL-6 cells were collected by laser confocal microscope, and the images of Hela cells were transferred into Image J for processing. Each group was set up with double blindness and repeated three times independently. At least 30 cells were counted in each group of RFL-6 cells, and all the data were expressed in mean ±SD. SPSS 17.0 software and One-way ANOVA method were used to carry on the correlation statistical analysis, *representing P < 0.05, the difference was statistically significant.

3. Results

3.1. Construction, Identification and Sequencing of Two Recombinant Plasmids GFP-katanin p60 and GFP-katanin p60^{MIS}

According to design primers get katanin p60 and katanin p60^{MIS}, and then connected to pEGFP-C1 which treated by Bgl II and EcoR I. The positive monoclonal culture was selected from the culture plate and incubated over-night. The plasmids were extracted by enzyme digestion (Figure 1) and sequenced to ensure that the recombinant plasmids were not mutated, deleted or mislocated.

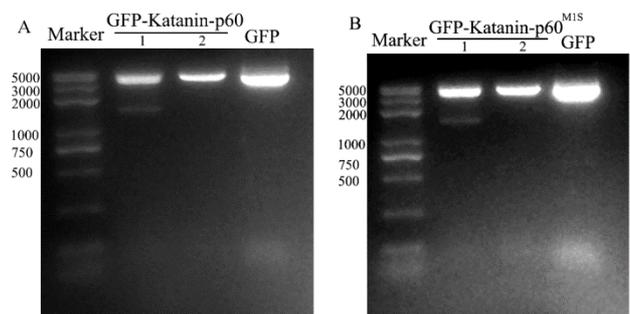


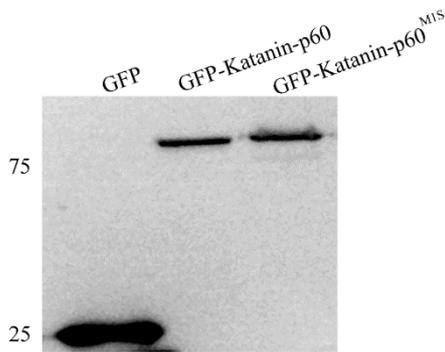
Figure 1. Identification of eukaryotic plasmids of GFP-katanin p60 and GFP-katanin p60^{MIS}.

A: GFP-p60 recombinant plasmids; B: GFP-p60^{MIS} recombinant plasmids. Lane 1: DNA Marker; Lane 2: Results of double enzyme digestion; Lane 3: Single enzyme digestion; Lane 4: Empty plasmid digestion.

3.2. Expression of GFP-katanin p60 and GFP-katanin p60^{MIS}

RFL-6 cell were routinely cultured and transfected with GFP, GFP-katanin p60 and GFP-katanin p60^{MIS}. In control group, cell transfected with GFP. But, in the experimental group, cell transfected with GFP-katanin p60 and GFP-katanin p60^{MIS} respectively. After 48h transfection, according

to western blot detecting those proteins (Figure 2).



Line 1 GFP; Line2 GFP-p60; Line 3 GFP-p60^{MIS}

Figure 2. Expression of GFP- katanin p60 and GFP- katanin p60^{MIS}.

3.3. Microtubule Cutting of RFL-6 Cell by GFP-katanin p60 and GFP-katanin p60^{MIS}

To observe the microtubule cutting efficiency of RFL-6 cells by GFP-katanin p60 and GFP-katanin p60^{MIS}, we transfected them into RFL-6 cells respectively, and cultured for 24h. After stained with immunofluorescence, RFL-6 cell observed under confocal microscope. The microtubules of RFL-6 cells in the experimental group of GFP-katanin p60^{MIS} were depolymerized and cut into small pieces, but the other groups were not. In addition, the fluorescence intensity of the microtubules of the RFL-6cells in the GFP-katanin p60^{MIS} group was significantly weaker than GFP- katanin p60 group. The results showed that overexpression of GFP-p60^{MIS} had a remarkable microtubule cutting effect on RFL-6 cells (Figure 3).

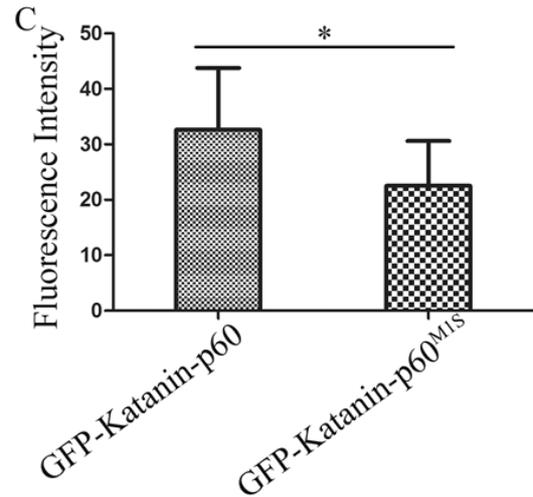
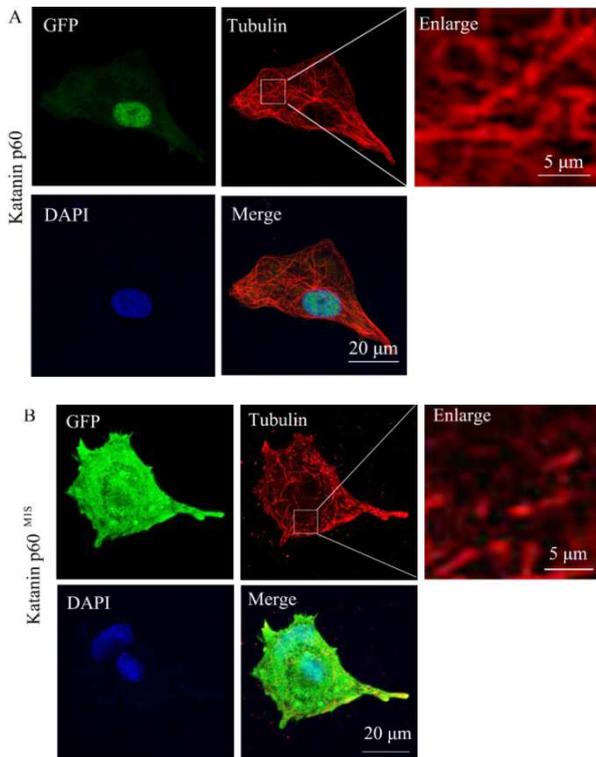


Figure 3. Analysis of overexpression GFP-katanin p60, GFP-katanin p60^{MIS} in RFL-6 cell by immunofluorescence.

(A) and (B) Immunostaining for GFP (green), tubulin (red) and DAPI (blue) in katanin p60 and katanin p60^{MIS} in RFL-6 cell. Scale bar 10um and 2um. (C) Quantification of microtubule intensity for the experiment shown in (A), microtubule intensity in 20 cell were measured per condition. Data represent mean ± SD, P<0.05.

4. Discussion

Katanin, spastin, and fidgetin are all microtubule-cleaved proteins [11, 12]. At present, two kinds of microtubule cutting proteins including katanin and spastin have been studied widely. These two proteins are mainly bound to stable microtubules, which are primarily regulated by acetylated or poly-glutamylated post-translational modified tubulin [13-15]. Fidgetin mainly combines with deacetylated microtubule which was first discovered in sea urchins in 1993 [16]. It can be used to cleave microtubule by ATP, katanin consists of katanin p80 and katanin p60 which is capable of catalyzing the hydrolysis of ATP to cleave microtubules [2-4]. To study the distribution of katanin p60 in cells and the ability to cleave microtubules, the katanin p60 gene was amplified by PCR, and then the katanin p60 gene was transferred into the eukaryotic vector. The results showed that the size of the product after PCR was consistent with katanin p60. And, the sequencing results showed that the katanin p60 gene had no mutation. The above results show that the recombinant plasmid of katanin p60 was successfully constructed.

In order to confirm that the recombinant plasmid can correctly express p60 protein, the control vector plasmid GFP, recombinant plasmid GFP-katanin p60, GFP-katanin p60^{MIS} were transiently transfected RFL-6 cell for 24h, and western blot results showed two recombinant plasmids both expressed the specific target band at 85 kDa, but the control vector had only one target band at 25 kDa. Katanin p60 is encoded by 419 amino acids and has a molecular weight of approximately 60 kDa. Therefore, the results indicate that both recombinant plasmids can accurately express katanin p60 protein.

In order to confirm the distribution and function of katanin p60 in cells, the control vector GFP, recombinant plasmid

GFP-katanin p60, GFP-katanin p60^{MIS} were transiently transfected into RFL-6 cells for 24 h. The results showed that wild-type katanin p60 was mostly located in the nucleus, protein expression was mainly concentrated in the nucleus, and another recombinant plasmid expressed katanin p60^{MIS} protein in whole cells. The reason for the difference in distribution may be that this study mutated the initiation codon ATG to TCC. It has been reported in the literature that the initiation codon mutation does not affect the expression and function of p60 protein [17]. Similar to the control vector in whole cell distribution, the ability to cleave microtubules showed that the whole cell distribution of the recombinant plasmid was significantly stronger than the wild type katanin p60. The katanin p60 has a weaker cutting ability than spastin. The katanin p60 protein accumulated in the nucleus does not destroy the cytoskeleton efficiently. However, the whole cell-distributed katanin p60 protein is more likely to destroy the cytoskeleton.

5. Conclusion

In summary, as a member of the microtubule-cleaving protein family, the cutting function on microtubule of katanin p60 depends on its distribution. The katanin p60 accumulated in the nucleus does not destroy the cytoskeleton efficiently, but the whole cell distribution is beneficial to katanin p60 in cutting microtubule.

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