RESEARCH COMMUNICATION

Functional evidence for *in vitro* microtubule severing by the plant katanin homologue

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Temporal and spatial assembly of microtubules in plant cells depends mainly on the activity of microtubule-interacting proteins, which either stabilize, destabilize or translocate microtubules. Recent data have revealed that the thale cress (*Arabidopsis thaliana*) contains a protein related to the p60 catalytic subunit of animal katanin, a microtubule-severing protein. However, effects of the plant p60 on microtubule assembly are not known. We report the first functional evidence that the recombinant *A. thaliana* p60 katanin subunit, Atp60, binds to microtubules and severs them in an ATP-dependent manner *in vitro*. ATPase activity of Atp60 is stimulated by low tubulin/katanin ratios, and is inhibited at higher ratios. Considering its properties *in vitro*, several functions of Atp60 *in vivo* are discussed.

Key words: AAA ATPase, ATPase activity, cytoskeleton, video microscopy.

INTRODUCTION

The microtubular cytoskeleton of higher-plant cells is composed of highly dynamic and ordered arrays. In both mitotic and differentiated cells, these arrays continuously reorganize in response to internal and external stimuli, in correlation with the acquisition of specific functions [1]. During the cell cycle and differently from animal cells, at least five different microtubule arrays come into succession. In interphase cells, cortical microtubules surround the cell. In G_2 phase, perinuclear microtubules assemble from the nuclear periphery, which is a microtubule nucleation site. Concomitantly, the preprophase band forms under the plasmalemma at the site of the future division plane. During prophase, while the preprophase band disassembles, perinuclear microtubules rearrange to build the mitotic spindle. Finally, in late anaphase through telophase, short antiparallel microtubules form the phragmoplast in the spindle mid-zone, where they participate in cell-plate formation. All these plant microtubule arrays are highly dynamic, and this dynamicity allows very rapid transitions from one microtubule array to another. For example, in G_0-phase differentiated cells, the whole cortical microtubule cytoskeleton can reorganize from an orientation perpendicular to the elongation axis into a longitudinal configuration within very short time periods [2].

Our knowledge on proteins that regulate the reorganization of the plant microtubule cytoskeleton is still limited. Assumptions made on the basis of data obtained in animal cells predict that fast and accurate reorganization of plant microtubule arrays requires the activity of microtubule-interacting proteins. These proteins may act either as microtubule dynamic effectors by stabilizing or destabilizing microtubules or as motors that translocate microtubules [3,4]. To date, two plant microtubule-associated proteins and one microtubule-binding protein have been cloned [5–7]. One of them has been purified, and was shown to promote microtubule assembly [5]. However, plant microtubule-destabilizing proteins remain unknown. Only recently, an *Arabidopsis thaliana* (thale cress) gene possessing significant homology with the gene of animal katanin catalytic subunit, a microtubule-severing protein, has been identified [8–10]. The goal of the present study was therefore to demonstrate that the protein encoded by this gene is a plant microtubule-severing protein.

In animal cells, katanin is a heterodimer composed of a regulatory subunit of 80 kDa (called p80) and a catalytic subunit of 60 kDa (called p60), which hydrolyses ATP to sever microtubules [11]. p60 has a short N-terminal conserved domain that interacts with microtubules, and a large C-terminus ATPase domain [12]. A recent model for p60-severing functions proposes that ring-shaped p60 hexamer binds to microtubules. Subsequent ATP hydrolysis would induce a conformational change in the p60 hexamer that could weaken tubulin dimer interactions and lead to the severing of microtubules [13]. *In vivo*, katanin might be responsible for the release of microtubules from the centrosomes in neurons [14], as well as during mitosis in non-neuronal cells [15]. Katanin might also have an essential role in the depolymerization of spindle microtubules at their minus-ends [16,17].

In the present study, we have functionally characterized the *A. thaliana* orthologue of the katanin catalytic subunit (Atp60; name derived from *A. thaliana* p60). We have shown that, *in vitro*, recombinant Atp60 severs microtubules in an ATP-dependent manner, and that its ATPase activity is modulated by its binding to microtubules.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies were raised against the first 20 amino acids of Atp60 and purified against the antigenic peptide.

Abbreviations used: Atp60, *Arabidopsis thaliana* katanin catalytic subunit; DAPI, 4,6-diamidino-2-phenylindole; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported are deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AF358779 and AF048706.

RNA isolation and cDNA cloning of *A. thaliana* katanin

Purification of total RNA from *A. thaliana* seedlings (ecotype Columbia) was performed using the RNeasy Plant Mini-Preps (Qiagen, Hilden, Germany). Purification of mRNA from the total RNA fraction was performed using the mRNA Direct Kit (Dynal, Oslo, Norway). Construction of the *A. thaliana* cDNA library was achieved using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, U.S.A.). Rapid amplification of cDNA ends (RACE)–PCR experiments were performed using the Advantage cDNA Polymerase Mix (Clontech). The katanin gene specific primers were synthesized on the basis of the genomic sequence found in the database (BAC F5I6; GenBank® accession number AC018848). The full-length cDNA was obtained from RACE–PCR performed with primers Ukat18 (GTGGCTTCCATCTTCTACTTTGT) and Rkat16 (GCAGATCCAAACTCAGAGAGCCAC), in combination respectively with the adaptor primer 1 (CCATCCTAATACGACTCACTATAGGGC) and the nested adaptor primer 2 (ACTCACTATAGGGCTCGAGCGGC) provided with the Marathon kit. Fragments were inserted in the pBluescript KS Vector (Stratagene Europe, Amsterdam, The Netherlands), and sequenced from both ends.

Baculovirus expression and purification of katanin

Full-length Atp60 cDNA was amplified by PCR using primers 5H (CGCGGATCCGTGGGAAGTAGTAAT) and 3H (CCGGAATTCCGTTAAGCAGATCCAAACTC), and cloned into the pBacPAK-His-1 transfer vector (Clontech) at the *Bam*HI and *Eco*RI cloning sites downstream of a hexahistidine tag. Recombinant baculoviruses expressing Atp60 fused to His_6 at its N-terminus were used to infect Sf9 insect cells at a multiplicity of infection of ten plaque-forming units per cell. Sf9 cells were grown as monolayer cultures in Sf900-II SFM medium (Life Technologies, Cergy-Pontoise, France) at 27 °C. Infected cells were collected 72 h after infection by low-speed centrifugation (2500 ***g*** for 5 min). Recombinant Atp60 was prepared as described previously [13]. Cells were suspended in lysis buffer [50 mM Tris/HCl (pH 8.5)/300 mM NaCl/2 mM $MgCl_2$/20 mM imidazole/10 mM 2-mercaptoethanol/0.05 % (v/v) Nonidet P40/1 mM ATP/0.1 mM PMSF] supplemented with Complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), frozen in liquid nitrogen and stored at −80 °C. Thawed cells were sonicated and centrifuged (40000 ***g*** at 4 °C for 45 min). The cleared lysate was incubated in batch with Ni^{2+}-nitriloacetate resin (Qiagen) for 1 h at 4 °C. Resin was washed with washing buffer [20 mM Tris/HCl (pH 8.0)/1 M NaCl/2 mM $MgCl_2$/40 mM imidazole/0.02 % (v/v) Triton X-100/10 mM 2-mercaptoethanol/0.5 mM ATP/0.1 mM PMSF] and His_6-tagged bound proteins were eluted using elution buffer [20 mM Tris/HCl (pH 8.0)/0.1 M NaCl/2 mM $MgCl_2$/0.1 M imidazole/0.02 % (v/v) Triton X-100/10 mM 2-mercaptoethanol/0.25 mM ATP/10 % (v/v) glycerol]. Purified His_6-tagged Atp60 was frozen in liquid nitrogen and stored at −80 °C. Under our experimental conditions, recombinant Atp60 purified from *Escherichia coli* was not functional.

Microtubule-severing assays

Microscope-based severing assays were performed as described previously [18], but with a few modifications. Microtubule-associated-protein-free tubulin was purified from bovine brains and coupled with tetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, OR, U.S.A.), as described previously [19]. Bovine neural microtubule-associated proteins were eluted from the anion-exchange column used to purify tubulin with 0.6 M NaCl in BRB80 [80 mM Pipes (pH 6.9)/1 mM $MgCl_2$/1 mM EGTA], dialysed against BRB80 and stored in liquid nitrogen. Perfusion cell chambers (approx. volume of 10 μl) were prepared by sticking a cover-glass on a coverslip using double-sided tape. Chambers were flushed for 5 min with microtubule-associated proteins (1 mg/ml), washed with 10 cell-vols of K-buffer [20 mM Hepes (pH 7.4)/2 mM $MgCl_2$], quenched for 5 min with 0.2 mg/ml casein, and washed again with K-buffer. Taxol-stabilized, labelled microtubules were perfused and allowed to attach for 5 min. Cells were washed with 10 volumes of freshly prepared severing buffer, consisting of K-buffer supplemented with 1 mM ATP and an oxygen-scavenger system [12]. Images were captured using a Zeiss Axioplan 2 microscope and a Hamamatsu CCD (charge-coupled-device) orca1 camera.

DAPI (4,6-diamidino-2-phenylindole)-severing assays were performed as described previously [12]. Taxol-stabilized microtubules or tubulin (2 μM) were incubated in a solution containing 10 μM DAPI, 1 mM ATP, 10 mM phosphoenolpyruvate, 250 μg/ml pyruvate kinase, 1 mg/ml BSA in 20 mM Hepes, pH 7.5, 2 mM $MgCl_2$ and 0.1 mM EGTA. Reactions took place at 22 °C in 200 μl samples, and started with the addition of Atp60. Fluorescence intensity was measured (excitation wavelength 360 nm; emission wavelength 460 nm) using a FluoroMax spectrofluorimeter (Spex, Edison, NJ, U.S.A.).

ATPase assays

ATPase assays were performed using a continuous coupled assay with ATP regeneration [20]. Reactions (200 μl) took place at room temperature in ATPase buffer [25 mM Aces (pH 6.9)/2 mM magnesium acetate/2 mM potassium-EGTA/0.1 mM potassium-EDTA/1 mM 2-mercaptoethanol/1 mM ATP/2 mM phosphoenolpyruvate/0.25 mM NADH/5 μg/ml pyruvate kinase/3 μg/ml lactate dehydrogenase], and started by the addition of Atp60. The degree of stimulation shown in Figure 5(B) below was calculated by dividing the ATPase activity of Atp60 in the presence of microtubules by its basal ATPase activity in the absence of microtubules.

Co-sedimentation assays

Microtubule-binding assays were performed as described previously [18]. Taxol-stabilized microtubules and Atp60 were incubated for 30 min at 22 °C in binding buffer [20 mM Hepes (pH 7.5)/2 mM $MgCl_2$/0.1 mM EGTA/20 μM taxol/0.5 mg/ml lysozyme]. Reaction mixtures (50 μl) were spun through a glycerol 'cushion' [30 % (v/v) glycerol in binding buffer] for 5 min at 100000 ***g***. Supernatants and pellets were analysed by SDS/PAGE, and Western blots were probed with anti-Atp60 antibodies.

RESULTS

Cloning, expression and purification of the *A. thaliana* p60 subunit

The *Arabidopsis* full-length cDNA encoding for a protein related to the animal katanin catalytic subunit p60 was cloned. This encodes a protein of 523 amino acids (termed Atp60). The initiating methionine residue was identified by sequencing an in-frame stop codon upstream of the first amino acid. Atp60 shows high homology with animal p60 (47 % identity/62 % similarity with human p60). The domain organization of

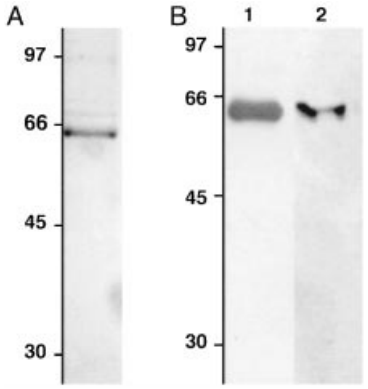

Figure 1 Purification of recombinant Atp60

(**A**) A Coomassie-Blue-stained SDS/polyacrylamide gel of purified His_6-tagged Atp60 is shown. (**B**) A Western blot of purified His_6-tagged Atp60 (lane 1) and a total protein extract from *A. thaliana* (lane 2) probed with Atp60 antibodies. In both samples, a unique polypeptide of 62 kDa was detected. Molecular-mass markers are indicated (in kDa) on the left of the gels.

Atp60 is similar to that of other p60s, i.e. a conserved N-terminal domain of approx. 30 amino acids (54% identity/83% similarity with human p60), a less conserved central domain (26% identity/37% similarity with human p60) and a highly conserved C-terminal domain of 320 amino acids corresponding to the ATPase domain. While this work was in progress, the sequence of Atp60 was deposited in the GenBank® database (accession number AF358779 [9]; accession number AF048706 [10]).

For biochemical characterization of Atp60, we prepared His_6-tagged Atp60 from insect cells. After purification, the His_6-tagged protein sample contained a major protein of an apparent molecular mass of 62 kDa (Figure 1A) that was recognized by an affinity-purified, polyclonal anti-Atp60 antibody on a Western blot (Figure 1B, lane 1). In total-protein extract from *Arabidopsis* and tobacco BY-2 cells, the anti-Atp60 antibodies detected a unique polypeptide of a similar molecular mass to Atp60 (Figure 1B, lane 2, and results not shown). In control Western blotting experiments, neither the purified His_6-tagged Atp60 nor the endogenous Atp60 were detected by the pre-immune serum (results not shown). The recombinant Atp60 was used for all the functional assays described below.

Atp60 severs microtubules *in vitro*

Microtubule-severing properties of Atp60 were analysed using time-lapse video microscopy of fluorescent taxol-stabilized microtubules attached to a coverslip via animal microtubule-associated proteins. After several minutes of incubation with Atp60 and 1 mM ATP, microtubules fragmented and appeared as dotted lines (Figure 2A). In most experiments, nearly all microtubules were undetectable within 15 min after the perfusion with Atp60 and ATP. We also observed microtubule shortening. The mean length of microtubules was shortened from 7.2 ± 3.0 μm to 2.4 ± 1.5 μm within 10 min after perfusion with Atp60 and ATP (Figure 2B). In control experiments where taxol-stabilized microtubules were incubated in the absence of Atp60 or ATP, neither of these two events, i.e. severing and shortening of microtubules, were observed (results not shown).

Microtubule disassembly by Atp60 was also analysed in a DAPI-based quantitative assay. In this assay, the fluorescence

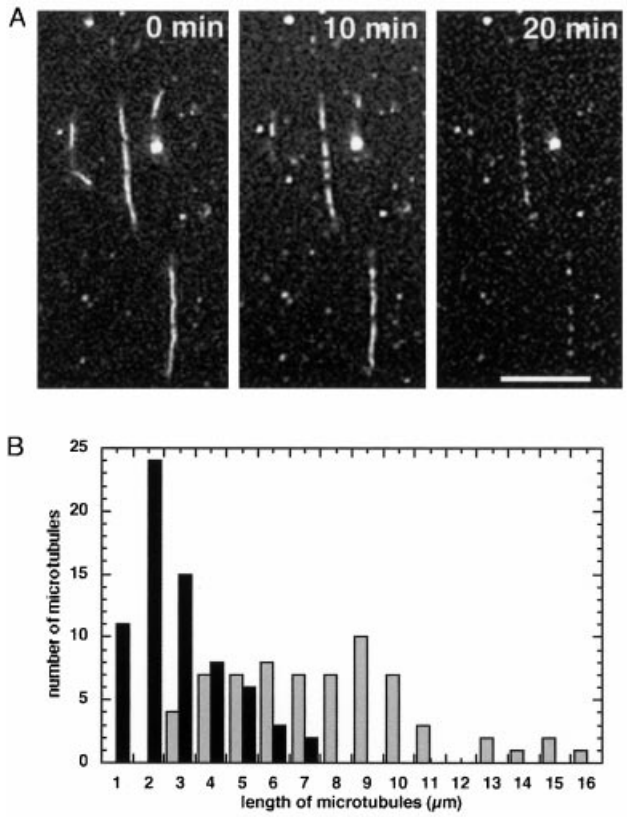

Figure 2 Microtubule-severing activity of Atp60 observed by video microscopy

(**A**) Time-lapse video microscopy of fluorescent taxol-stabilized microtubules is shown. After addition of Atp60 and ATP, the microtubules fragmented. The scale bar represents 5 μm. (**B**) Histogram showing the lengths of microtubules measured just after the addition of Atp60 and ATP (grey bars) and 10 minutes later (black bars). The means of three independent video-microscopy experiments are shown for each bar.

intensity of microtubule-bound DAPI is greater than that of tubulin-dimer-bound DAPI. When taxol-stabilized microtubules were incubated with Atp60 (0.12 μM), ATP (1 mM) and DAPI (2 μM), the fluorescence intensity decreased linearly over time, indicating the fragmentation of the microtubules (Figure 3). In contrast, the fluorescence intensity of DAPI remained constant when Atp60 was omitted. A similar experiment was performed by measuring the turbidity of a solution of taxol-stabilized microtubules at 350 nm. At this wavelength, solutions of polymerized microtubules show a peak of turbidity, whereas a solution of unpolymerized tubulin does not [21]. Under these conditions, the turbidity of taxol-stabilized microtubules remained constant in both the presence and the absence of Atp60 and ATP (results not shown). This result suggests that some tubulin oligomers were present in the final reaction of Atp60-induced microtubule severing. From these data, we concluded that Atp60 is able to fragment microtubules *in vitro* in an ATP-dependent manner.

Atp60 binds to microtubules

We examined the ability of Atp60 to interact with microtubules using co-sedimentation assays. As shown in Figure 4, approx. 50–70% of Atp60 co-sedimented with taxol-stabilized microtubules in the absence of ATP (lane 3). Upon addition of

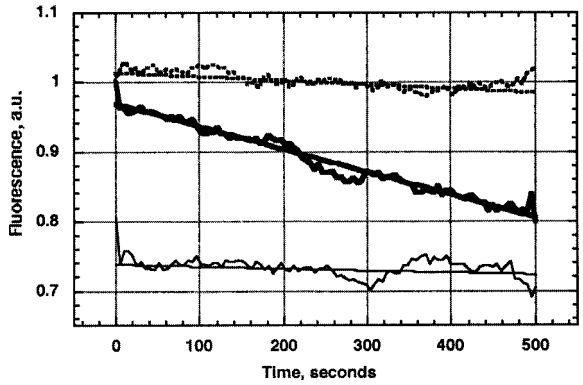

Figure 3 Microtubule-severing activity of Atp60 detected by fluorimetry

Time-course disassembly of taxol-stabilized DAPI-labelled microtubules in the presence (emboldened line) or in the absence (dotted line) of Atp60 and ATP. Under the same conditions, the fluorescence intensity of tubulin-bound DAPI was low and constant (shown by the thin line). Data obtained from three independent experiments were smoothed out and fitted using Kaleidagraph. a.u., arbitrary units.

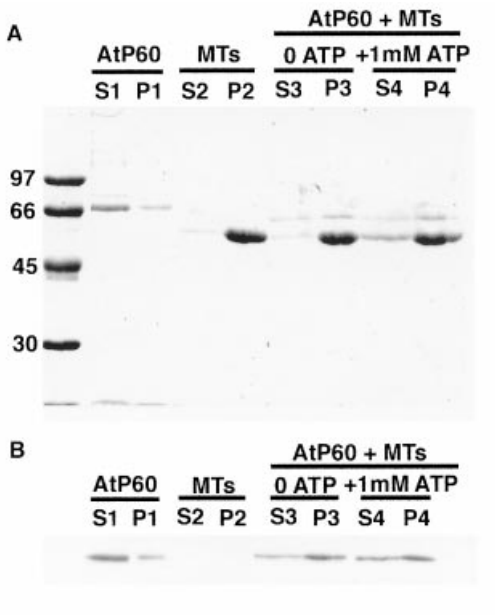

Figure 4 Binding of Atp60 to microtubules *in vitro*

Atp60 (lanes S1 and P1) and taxol-stabilized microtubules (Mts) (lanes S2 and P2) were centrifuged alone on a glycerol cushion, or incubated together in the absence (lanes S3 and P3) or presence (lanes S4 and P4) of ATP. (**A**) Analysis of the supernatants (S) and the pellets (P) on a Coomassie-Blue-stained SDS/polyacrylamide gel. In (**B**) is shown the corresponding Western blot, probed with anti-Atp60 antibodies. Masses of molecular markers are indicated on the left in kDa.

1 mM ATP, tubulin was found in the supernatant, together with some Atp60 (lane 4). These results suggest that, in the presence of ATP, Atp60 severed some microtubules and then dissociated from them. Control experiments showed that, under our experimental conditions, Atp60 remained soluble in the absence of microtubules (lane 1), and that microtubules did not depolymerize on their own (lane 2). Therefore Atp60 directly interacts with microtubules, and this interaction is ATP-independent.

Atp60 has a microtubule-stimulated ATPase activity

The ATPase domain of Atp60 is highly homologous with other animal p60 proteins. We investigated the ability of Atp60 to hydrolyse ATP *in vitro* (Figure 5). Under our experimental conditions, the rate of hydrolysis of ATP was calculated as one ATP molecule/Atp60 molecule per second. The basal ATPase activity of Atp60 was stimulated by microtubules. A maximum stimulation of 6-fold for the ATPase activity of Atp60 was observed with 1 μM tubulin, corresponding to an Atp60/tubulin molar ratio of 0.04. On the other hand, higher concentrations of microtubules inhibited the ATPase activity of Atp60. No detectable ATPase activity was observed with either the microtubules in the absence of Atp60 (results not shown) or microtubules and Atp60 in the absence of ATP (Figure 5A). Taken together, these results prove that the ATPase activity of Atp60 conforms to a non-hyperbolic microtubule stimulation.

DISCUSSION

Using two different assays, we have shown that recombinant Atp60 severs microtubules *in vitro*. In both assays, the severing of microtubules was completed within 10 to 15 min. This efficiency of the microtubule-severing activity of Atp60 is comparable with that of animal recombinant p60. From these results, we conclude that Atp60 is the plant homologue of the animal katanin. Atp60 is the first plant protein described so far having a microtubule-severing activity.

We observed that the ATPase activity of Atp60 is dependent upon binding to microtubules. ATP hydrolysis was stimulated at a low tubulin/Atp60 molar ratio, and inhibited at higher ratios. A comparable biphasic microtubule-dependent ATPase activity has been observed for sea-urchin katanin [12]. Hartman and Vale [13] proposed an attractive model that could account for the peculiar properties of animal katanins. In this model, p60 binds microtubules as a hexamer. Low tubulin/katanin molar ratios would favour katanin subunit interactions, whereas at high tubulin/katanin ratios, katanin subunits preferentially interact with microtubules and p60 oligomerization is impaired. The observed ATPase and microtubule-binding properties of Atp60 could fit to this model. Preliminary data suggest that Atp60 can oligomerize, and it will be important to determine the final size of the Atp60 oligomer and the roles of microtubules and nucleotide hydrolysis in the oligomerization process.

Atp60 interacts directly with microtubules. Animal katanins bind microtubules via their N-termini (amino acids 1–210) [13]. However, with the exception of the first 30 amino acids, the N-terminal part of p60 is not well conserved between the Plant and Animal Kingdoms. Furthermore, the microtubule-binding activity of animal p60 is regulated by p80, a katanin regulatory subunit of 80 kDa [18]. Although the sequencing of the *Arabidopsis* genome has nearly been completed, sequences having significant levels of homology with the specific conserved domains of p80 have yet to be found in the databases. Characterization of the plant katanin regulatory subunit, if it exists, and the identification of Atp60's interacting domains for both microtubules and other Atp60 monomers, will be essential to elucidate the regulatory mechanisms of Atp60 microtubule-severing properties, and will provide new insights into the properties of both plant and animal katanins.

The next challenge will be to investigate the physiological functions of Atp60. During the time course of this work, two

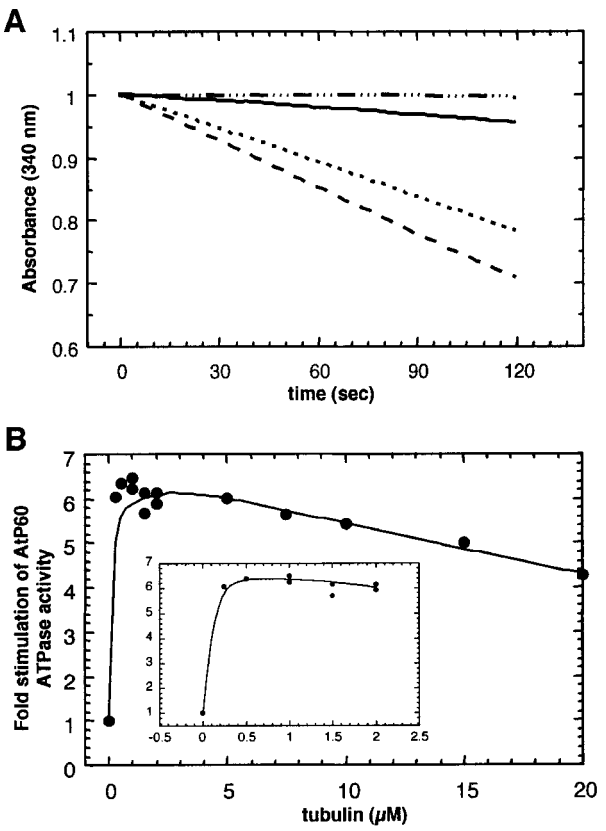

Figure 5 ATPase activity of Atp60

ATPase activity of Atp60 (0.04 μM) was stimulated by low tubulin concentrations and inhibited at higher concentrations. (**A**) ATPase activity of Atp60 (0.04 μM) was monitored at 340 nm in a steady-state coupled assay in the absence of microtubules (continuous line) or in the presence of 1 μM (broken line) or 20 μM (dotted line) of taxol-stabilized microtubules. In the absence of ATP, no ATPase activity was detected (dot–dashed line). (**B**) Dependence of the ATPase activity of Atp60 on the concentration of tubulin. ATPase activity of Atp60 (0.04 μM) was highly stimulated by low tubulin concentrations (up to 2 μM) and inhibited at higher concentrations. The inset shows stimulation of ATPase activity at low tubulin concentrations (0–2 μM).

A. thaliana mutants mutated in the gene of Atp60 have been described [8,9]. These mutants were selected for alterations in cell-wall biosynthesis (*fra2*) and cell-elongation defects (*botero1*). Careful analysis of the mutant cells revealed that, in both mutants, reorganization of microtubules during mitosis occurred normally, whereas cortical microtubules failed to reorientate transversely as cells leave the mitotic zone. Correlatively, these cells do not switch from an isotropic to an anisotropic phase of growth. Considering the functional properties of Atp60 described here, we surmise that Atp60 could sever cortical microtubules and have an important role in the control of the length of cortical microtubules, and, consequently, in their translocation and reorientation. Atp60 could also have an essential role in the regulation of the extensive microtubule redistribution that occurs during mitosis. Atp60 could free microtubule minus-ends from their nucleation site, as has been suggested for animal cells [17]. During the G_2/M transition, a burst of microtubule assembly occurs at the nuclear periphery, which is a plant microtubule-nucleating site. Atp60 could release these nucleus-associated microtubules so that the nucleation sites can be used for the assembly of new microtubules. In support of this hypothesis is the observation that anti-katanin antibodies stain the perinuclear region in prophase and in late telophase of *Arabidopsis* root-tip cells [10]. Furthermore, in the *fra2* katanin *Arabidopsis* mutant, the disassembly of the perinuclear microtubule array is delayed at the end of mitosis [9].

Finally, Atp60 could also have a role in the disassembly of microtubule arrays at specific stages of the cell cycle, such as at the preprophase band and/or the spindle poles during anaphase. Because *botero1* and *fra2* mutant cells seem to divide normally and show no dramatic defect in their mitotic microtubular networks, it has been suggested that katanin is not involved in the control of the microtubule rearrangements that occur during plant mitosis. However, we do not know whether Atp60 mutant cells divide as fast as wild-type cells. It is possible that other as-yet-unknown proteins compensate for the lack of katanin-severing activity in the mutants. Regarding the essential role played by animal katanin in the severing of microtubules in mitotic extracts [16], we favour this latter hypothesis. In this context, experiments such as micro-injection of katanin or anti-katanin antibodies in cycling cells will be very useful in elucidating the functions of katanin in plant mitosis.

In conclusion, we report in the present study the functional characterization of the first-described plant microtubule-severing protein, Atp60. Exciting questions remain to be elucidated, such as: (i) what are the molecular mechanisms of Atp60 microtubule-severing activity? (ii) what are the functions of Atp60 during the cell cycle? and (iii) how is Atp60 activity regulated?

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