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Arabidopsis thaliana Protein, ATK1, is a Minus-End Directed Kinesin That Exhibits Non-Processive Movement

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The microtubule cytoskeleton forms the scaffolding of the meiotic spindle. Kinesins, which bind to microtubules and generate force via ATP hydrolysis, are also thought to play a critical role in spindle assembly, maintenance, and function. The *A. thaliana* protein, ATK1 (formerly known as KATA), is a member of the kinesin family based on sequence similarity and is implicated in spindle assembly and/or maintenance. Thus, we want to determine if ATK1 behaves as a kinesin *in vitro*, and if so, determine the directionality of the motor activity and processivity character (the relationship between molecular "steps" and microtubule association). The results show that ATK1 supports microtubule movement in an ATP-dependent manner and has a minus-end directed polarity. Furthermore, ATK1 exhibits non-processive movement along the microtubule and likely requires at least four ATK1 motors bound to the microtubule to support movement. Based on these results and previous data, we conclude that ATK1 is a non-processive, minus-end directed kinesin that likely plays a role in generating forces in the spindle during meiosis. Cell Motil. Cytoskeleton 52:144–150, 2002.

Key words: kinesin-like protein; meiosis; microtubule; processivity; motor; KATA

INTRODUCTION

In sexually reproducing eukaryotic organisms, the reduction from diploid to haploid chromosome number requires the proper formation and alignment of the meiotic spindle. Microtubules form the scaffold of the spindle and associated proteins aid in assembly, positioning, and maintenance of the spindle [Maccioni and Cambiazo, 1995; Lloyd and Hussey, 2001]. One family of proteins that has been implicated in these processes are the kinesins [for reviews see Endow, 1999; Sharp et al., 2000b]. This family of motor enzymes uses the energy produced from ATP hydrolysis to move itself unidirectionally along the microtubule towards either the plus-end or minus-end of the microtubule. The character of this directional movement can be either processive or non-processive. Processive movement (i.e., the ability to move multiple steps without detaching from the microtubule) is seen with conventional kinesin, which moves cargo long distances before disassociating from the microtubule [Howard et al., 1989; Block et al., 1990; Hackney, 1995]. Non-processive movement involves cargo transport for only short distances before the motor disassociates from the microtubule.

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When bound to microtubules kinesins may transport cargo that includes vesicles, chromosomes, or organelles. Additionally, kinesins may also exert forces on spindle microtubules themselves. An example of this is seen in mutational analysis experiments showing that plus- and minus-end directed motors generate counteracting forces in the spindle that serve to maintain spindle integrity [O'Connell et al., 1993; Saunders et al., 1997]. One interesting feature of some kinesins is their ability to crosslink microtubules by forming a homotetrameric structure with two motor domains at opposite ends of a central rod [Cole et al., 1994; Kashina et al., 1996]. Because of this unique structure, these kinesins may play a role in pushing the spindle poles apart, thereby creating the bipolar spindle array [Enos and Morris, 1990; Gordon and Roof, 1999; Sharp et al., 1999].

We are interested in understanding the roles that kinesins play in plant meiosis. Currently, five kinesins in the *A. thaliana* genome have been partially characterized and another 17 putative kinesins have been identified, based on sequence similarity to known kinesins [Liu and Lee, 2001; Reddy, 2001]. The best characterized plant kinesin, kinesin calmodulin binding protein (KCBP), is likely involved in trichome (tiny hair-like structures on the surface of leaves) development. [Oppenheimer et al., 1997; Reddy and Day, 2000; Reddy, 2001]. Furthermore, KCBP is the only *A. thaliana* motor that has been shown to support in vitro microtubule movement [Song et al., 1997].

Recently, an *A. thaliana* loss of function mutant in a putative plant kinesin was isolated [Chen et al., 2002]. This mutation, which consists of a *Ds* insertion in the *ATK1* gene (formerly known as *KATA*) that disrupts ATK1 expression, results in reduced male fertility and abnormal spindle structure during male meiosis. The putative kinesin, ATK1, which shares homology in its C-terminal motor domain to other known kinesins, was amplified from *A. thaliana* genomic DNA using degenerate primers to homologous regions on the motor domain of kinesin [Mitsui et al., 1993]. Previous experiments showed that ATK1 localizes to the mid-zone of the spindle apparatus during metaphase in cultured cells and sediments with microtubules in an ATP-sensitive manner [Liu et al., 1996; Liu and Palevitz, 1996]. Furthermore, time course immunolocalization studies using lysed tobacco cells show that the ATK1 antibody signal moves towards the poles, suggesting that ATK1 is a minus-end directed motor. However, to date, experiments showing that ATK1 is able to move microtubules in vitro have been unsuccessful.

To directly test whether ATK1 is a motile motor protein, the full-length ATK1 transcript was expressed in bacteria and the bacterial supernatant was used to perform motility assays. Our results show that ATK1 is a kinesin and can support in vitro microtubule movement in an ATP-dependent manner. Furthermore, additional experiments show that ATK1 is a minus-end directed motor that moves non-processively along microtubules. These results will be important in understanding the function of ATK1 in male meiosis.

MATERIALS AND METHODS

ATK1 Cloning

The full-length *ATK1* transcript was amplified using PCR from a floral cDNA library [Dwyer et al., 1994] using the forward primer 5′ GGGAATTCCATATGATGGCTTCTCGCAA 3′ and the reverse primer 5′ GGCCTCGAGGCCATAGCTTAAGC 3′. This amplified sequence was digested with EcoRI and XhoI and cloned into the peT-23b (+) expression vector. *Escherichia coli* strain BL21(DE3) (Novagen, Inc., Madison, WI) was transformed with the vector using heat shock.

ATK1 Expression

To express the protein, the transformed bacteria were grown overnight at 37°C in LB ampicillin. Approximately 5 ml of this initial culture was used to inoculate a larger volume culture that was grown to an O.D. of 0.6, then 0.5 mM isopropyl beta-D-thiogalactoside (IPTG; Sigma no. I6758) was added followed by an additional 10 h of induction at 27°C. The culture was then centrifuged at 6,000 RPM for 5 min and the bacterial pellet was resuspended in a lysis buffer containing lysozyme (1 mg/ml), 100 μM MgATP, 5 mM BME, 1 mM PMSF, 5 mM BAME, 5 mM TAME, and 10% glycerol in PM buffer (50 mM Pipes, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.9) for 30 min. The cells were sonicated using 30 pulses with a duration of 450 msec/pulse (Branson Sonifier 450, VWR Scientific). To remove cellular debris, the sonicated suspension was centrifuged at 100,000*g* in a TL-100 centrifuge (Beckman Corp., Irvine, CA) for 30 min at 4°C. The final supernatant was then frozen in liquid nitrogen. A single bacterial supernatant expressing ATK1 was used to produce the representative data presented in this paper. Three additional preparations were performed that yielded similar results.

Drosophila melanogaster kinesin heavy chain (DmKHC) was purified as previously described [Hancock and Howard, 1998].

Velocity and Directionality Assays

Motility assays were performed as described in Hancock and Howard [1998]. A flow cell was constructed using a no. 1 thickness coverslip placed above a glass slide (Fisher Scientific, Newark, DE) with spacers made from no. 1 coverslip glass. The chamber was sealed

with silicon grease and blocked for 1 min with a solution containing 0.5 mg/ml casein and 1 mM ATP, in PM buffer. Next, a solution containing ATK1 diluted into the blocking solution was injected into the flow cell and allowed to adsorb for 3 min. The flow cell was then washed twice with the blocking solution. Lastly, a solution containing rhodamine-labeled microtubules (~50 nM) with 10 μM taxol, 5 mM BME, 0.02 mg/ml glucose oxidase, 20 mM glucose, 0.008 mg/ml catalase, and 1 mM ATP in PM buffer was injected into the flow cell. Microtubules were then viewed using a 100X Neofluor lens (N.A. = 1.25) mounted on a Zeiss Axioskop (Zeiss Corp; Thornwood, NJ). The microscope was equipped with a 100-W mercury lamp, 500–540-nm excitation filter, 510-nm dichroic filter, and 570–610-nm emission filter. Images were captured using the time lapse feature on the Esee program developed for a Silicon Graphics Unix-based system (ISee Image Systems, Raleigh, NC). Images were taken in 1-sec intervals and the distance moved was then determined using the line draw function on Image Pro Plus (Media Cybernetics, MD).

To determine if ATK1 was a plus- or minus-end directed motor, motility assays were performed using rhodamine microtubules that have a brightly labeled minus end, using the method described in Henningsen and Schliwa [1997]. Directionality was then determined by noting which end of the microtubule (i.e., the plus-end or minus-end) was leading. At least 50 observations were made.

Processivity Assays

Processivity assays were performed in a similar manner to velocity assays. At least 12 time-lapse movies of 60-sec duration with 1-sec intervals were recorded for each data point. For landing assays, the number of microtubules that affixed to the surface of the coverslip, and moved, were noted for each 1-min time-lapse.

To determine the fraction of microtubules that move a distance greater than their length, a 60-sec time-lapse sequence was taken (as above) in at least three different regions on the coverslip at various dilutions of ATK1 or *Dm*KHC. Within each region, at least five microtubules were observed to determine if they moved a distance greater than their length.

Model for the Minimum Number of Motors Required for Motility

To calculate the minimum number of ATK1 motors required for microtubule movement, a model developed by Hancock and Howard [1998] was used to fit the microtubule landing rate versus motor density data. This model postulates that the probability of a microtubule landing and moving depends on the number of motors interacting with that microtubule. If an individual motor can move a microtubule (the definition of processivity), the landing rate falls proportionally with the motor density at limiting dilutions, but if multiple motors are necessary for movement, then the landing rate falls more steeply. To estimate the minimum number of motors required for movement, the landing rate, LR(ρ) was fit to the equation

$$LR(\rho) = LR_{max}(1 - e^{-\rho/\rho_o})^n$$

where ρ is the relative motor density $1/\rho_o$ is the surface area over which motors can associate with a microtubule, and LR_{max} is the maximal landing rate observed when the surface is saturated with motors. The parameter n is the number of motors required to move a microtubule, equal to 1 for a processive motor and >1 for a non-processive motor. To determine whether ATK1 is processive, the landing rate data was fit to the above equation for various values of n using SigmaPlot.

RESULTS

ATK1 Is a Kinesin

The ATK1 protein shares sequence homology with other kinesins in its putative carboxy-terminal motor domain and is, therefore, hypothesized to be a kinesin-like protein [Mitsui et al., 1993]. Until now, in vitro motility assays with ATK1 were unable to support this hypothesis, most likely due to protein degradation [Liu et al., 1996]. To determine if ATK1 is a microtubule motor, the full-length ATK1 protein was expressed in bacteria and the bacterial supernatant was used for motility assays. When the supernatant, along with ATP, was injected into the flow cell, microtubules moved across the coverslip. Experiments using a bacterial supernatant that did not express the ATK1 construct did not support microtubule movement (data not shown). Therefore, microtubule movement can only be attributed to the motor activity of ATK1.

To determine if ATK1 motility is dependent on ATP hydrolysis, the non-hydrolyzable ATP analog, AMP-PNP, was used instead of ATP. In these assays, microtubules were immobilized on the surface of the coverslip and no movement was observed, thus, supporting the hypothesis that ATP hydrolysis is required for motility. Similarly, in the absence of any nucleotide, microtubules were immobilized by ATK1 adsorbed to the coverslip.

ATK1 Is a Minus-End Directed Motor

The directionality of ATK1 was determined using minus-end labeled rhodamine microtubules. Because ATK1 is adsorbed to the coverslip in the flow cell, any force generated by ATK1 propels the microtubule for-

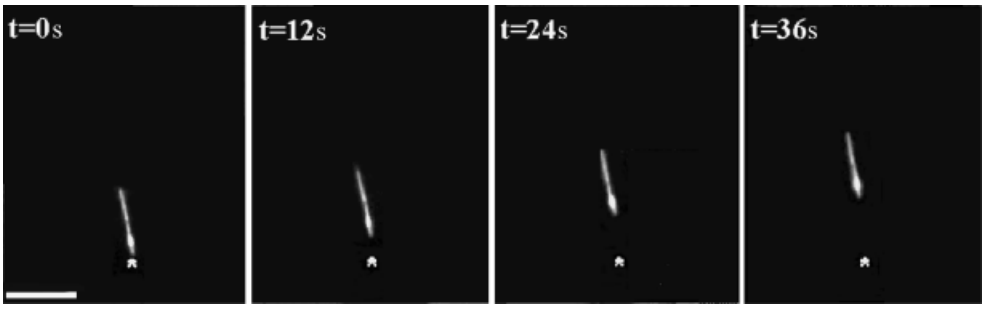

Fig. 1. Microtubule gliding assay using rhodamine-labeled microtubules that have a brightly marked minus-end. This 36-sec sequence shows a microtubule moving with its plus-end leading in the presence of ATK1. Because ATK1 is adsorbed to the coverslip, microtubules moving with their plus-end leading indicate that ATK1 is a minus-end directed motor. * represents a fixed point on the screen. Scale bar = 10 μm.

ward but not ATK1. Thus, if microtubules move with their plus-end leading, ATK1 must be a minus-end directed motor (i.e., the minus-end of the microtubule moves towards the motor in these assays). When these assays were performed with ATK1 and ATP, > 95% of the microtubules moved with their plus-end leading (Fig. 1). Since the bright end of these microtubules is usually located at the minus-end of the microtubule, this indicates that ATK1 is a minus-end directed motor. As a control, these same assays were performed with the plus-end motor, *Drosophila melanogaster* kinesin heavy chain (*Dm*KHC). Consistent with the well-characterized plus-end motility of this motor [Saxton et al., 1988], > 95% of the microtubules moved with their minus-end leading (data not shown).

ATK1 Velocity and Movement Character

The velocity of ATK1 movement was determined by measuring the rate of microtubule movement. The average velocity of ATK1 in the presence of ATP (at 25°C) was 0.16 ± 0.086 μm/sec (mean ± SD, n = 47). As a control, the average velocity of *Dm*KHC was 0.82 ± 0.21 μm/sec (mean ± SD, n = 49). These results are similar to the rates found by other investigators [Hancock and Howard, 1998].

To gain insight into the character of movement of ATK1, a dilution series was performed. The velocity of ATK1 was measured at varying dilutions of the bacterial supernatant, beginning at a 1:10 dilution and ending at 1:200. The velocity did not significantly change through the dilution series (Fig. 2). Dilutions beyond 1:200 did not support microtubule movement, though, in the presence of AMP-PNP, microtubules adhered but did not move at dilutions up to 1:600. At dilutions between 1:100 and 1:200, where motor densities are predictably low, microtubules did not pivot and exhibit translational movement. Importantly, control experiments with *Dm*KHC at low motor densities yielded some microtubules that swiveled about one point along the microtubule and underwent translational movement.

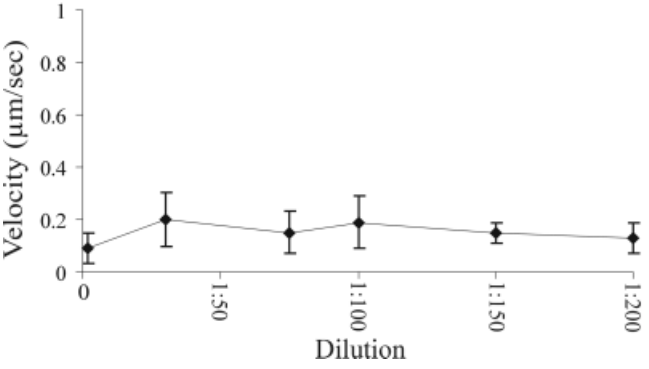

Fig. 2. The velocity of ATK1 as a function of dilution. Microtubule motility assays were performed with ATK1. Velocity was calculated as distance moved between two time points. As ATK1 became more dilute, the velocity did not change significantly ($P > 0.05$). Microtubule movement was not observed at dilutions over 1:200. Error bars represent standard deviation (n ≥ 12 for each point).

ATK1 Processivity

A processive motor is able to support microtubule gliding at low motor densities, while a non-processive motor, which remains attached to the microtubule for a relatively short period of time, will not. One method to determine whether a motor is processive is to measure the microtubule landing rate over a range of motor dilutions [Howard et al., 1989; Hancock and Howard, 1998]. In this analysis, the landing rate for a non-processive motor will decrease sharply when the minimum number of motors necessary to maintain motility is reached. Conversely, a processive motor will display a gradual decrease in landing rate as the motor density drops, due to the fact that it can support motility at low motor densities. To determine if ATK1 behaves processively, the landing rate (i.e., the number of microtubules that

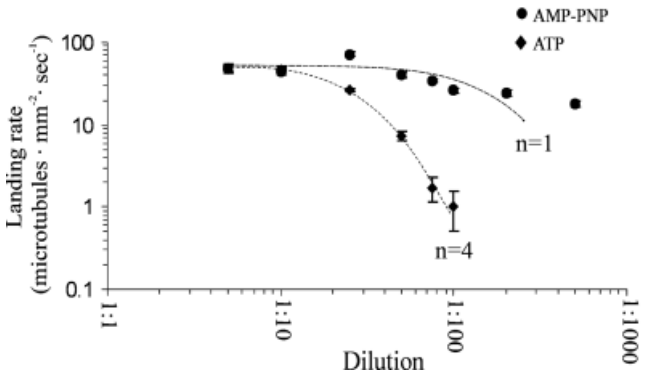

Fig. 3. Analysis of the landing rate of ATK1 in the presence of ATP (*diamonds*) or AMP-PNP (*circles*). In the presence of ATP, the landing rate of ATK1 drops quickly as ATK1 becomes more dilute. However, with AMP-PNP, the landing rate gradually decreases. The continuous curves are fits to the model predicting the minimum number of motors necessary for motility. In the presence of ATP, the landing rate fits a curve where n = 4 (landing $rate_{max}$ = 50.68, ρ_o = 0.02), while in the presence of AMP-PNP the landing rate was fit when n = 1 (landing $rate_{max}$ = 52.97, ρ_o = 0.01). Each data point represents the mean (± SE) of at least 12 assays done in 2 or more flow cells.

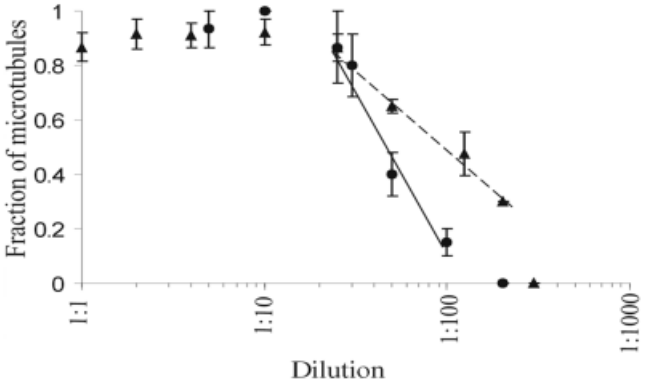

Fig. 4. The fraction of microtubules that moved a distance greater than their length in the presence of ATK1 (*circles*) or *Dm*KHC (*triangles*). Motility assays with ATK1 show that the fraction of microtubules that moved a distance greater than their length sharply drops as dilution increases when compared to the same assay done with *Dm*KHC. A best-fit line was calculated from the linear portion of each data set. Each data point represents the mean (± SE) of at least three different regions on the coverslip.

land on a unit area of the coverslip per unit time) was measured at a constant microtubule concentration and varying motor densities. In the presence of ATP, the landing rate of ATK1 drops steeply at high dilutions and eventually nears zero at a 1:200 dilution (Fig. 3), consistent with a non-processive motor. As a control, the same landing assay with ATK1 was performed with AMP-PNP instead of ATP. In this case, the landing rate decreases gradually as ATK1 becomes more dilute, showing that at very low motor densities, ATK1 can still bind tightly to microtubules in the presence of AMP-PNP. To estimate the minimum number of motors necessary for motility, a model derived by Hancock and Howard [1998] was used. This equation assumes that motors bind randomly to the surface of the microtubule and must interact with at least *n* motors to support microtubule movement *(*see Materials and Methods). In our case, the relative motor concentration (ρ) had to be substituted for the absolute motor density. In the presence of ATP, the landing rate best fit a curve where n = 4 (i.e., the calculated minimum number of motors necessary for motility is four) and the computer-defined parameters of landing rate $_{max}$ and ρ_o are equal to 50.68 and 0.02, respectively. To test the validity of this calculation, AMP-PNP was substituted for ATP; as expected, the landing rate best fit a curve where n = 1 (the landing rate $_{max}$ was equal to 52.97 and ρ_o was equal to 0.01).This result is consistent with only one ATK1 motor being required for landing in the presence of AMP-PNP.

A second assay used to determine if ATK1 is processive is to investigate the fraction of microtubules that move a distance greater than its length over a range of dilutions. A non-processive motor would have a sharp decrease in the number of microtubules that moved greater than their length as dilution increased, because multiple motors would likely be required to support microtubule movement. A processive motor, however, would have a gradual decrease in the number of microtubules that moved greater than their length, because presumably, one motor can support movement. At low dilutions of ATK1 (i.e., high motor density), most microtubules moved greater than their length, but as dilution increased (i.e., motor density decreased), the fraction of microtubules that moved greater than their length decreased at a faster rate than those from a control done with the processive motor *Dm*KHC (Fig. 4). Therefore, this second assay supports the hypothesis that ATK1 is a non-processive motor, requiring more than one motor to support microtubule movement.

DISCUSSION

Previous work has shown that ATK1 localizes to the mitotic apparatus in dividing cells, sediments with microtubules in an ATP-sensitive manner, and has homology to other C-terminal kinesins in its predicted motor domain [Mitsui et al., 1993; Liu et al., 1996; Liu and Palevitz, 1996]. Recently, a ATK1 mutant has been isolated that displays an aberrant male meiotic spindle, suggesting that ATK1 is involved in the assembly of the meiotic spindle [Chen et al., 2002]. These findings have prompted us to determine if ATK1 behaves as a kinesin in vitro, and if so, to investigate its directionality and processivity along the microtubule.

Multiple ATK1 Motors Bound to the Microtubule Are Necessary for Microtubule Movement

The results presented herein demonstrate that bacterially expressed ATK1 is able to support in vitro microtubule movement in an ATP-dependent manner and has a minus-end directed polarity. The gliding velocity of ATK1 was measured to be 0.16 μm/sec; similar to the velocities measured for two other minus-end directed motors (i.e., AtKCBP and ncd) [Moore et al., 1996; Song et al., 1997]. Furthermore, the velocity of ATK1 does not change as dilution is increased, although at dilutions over 1:200, microtubule movement ceases. An explanation for this discontinuance of microtubule movement simply could be that at dilutions over 1:200, ATK1 motor density is low, thus, resulting in an average of less than one motor per microtubule. However, this explanation is not supported by the observation that at dilutions over 1:200, in the presence of AMP-PNP instead of ATP, microtubules are tightly bound to the surface of the coverslip. Taken together, this indicates that ATK1 must be present on the coverslip at high enough concentrations to bind microtubules at these greater dilutions, but is unable to support microtubule movement. Therefore, we hypothesize that greater than one ATK1 motor is necessary for microtubule movement (i.e., two or more ATK1 motors act cooperatively to support movement).

ATK1 Is a Non-Processive Kinesin

Non-processive motors are thought to require more than one motor bound to the polymer to generate productive movement. For instance, the yeast myosin, myo2p, and the *Drosophila* kinesin, ncd, are non-processive and each needs several motors bound to the substrate (actin for the myosin motors and microtubules for ncd) to support in vitro movement [deCastro et al., 1999; Reck-Peterson et al., 2001]. Several results presented herein support the conclusion that ATK1 is also a non-processive motor. First, we observed that the landing rate of ATK1 drops sharply as dilution increases (Fig. 3). Non-processive motors have landing rates that decrease quickly once the density drops below the requisite number of molecules necessary for microtubule movement [Hancock and Howard, 1998; deCastro et al., 1999; Reck-Peterson et al., 2001]. Using a model that estimates the minimum number of motors required for motility, the ATK1 landing rate best fits a curve where at least four motors are necessary for microtubule movement. This is consistent with ATK1 acting in a cooperative manner to drive microtubule movement. Second, the fraction of microtubules that move greater than their length abruptly decreases as dilution is increased, as compared to the processive motor DmKHC (Fig. 4). This further supports the hypothesis that multiple ATK1 motors are required for microtubule movement. Third, we observed that at low ATK1 densities, microtubules that pivoted and exhibited translational movement were not seen. Experiments done with conventional kinesin have revealed that this pivoting point represents the attachment of one kinesin molecule to the microtubule [Howard et al., 1989]. If ATK1 was a processive motor, microtubules should have pivoted and exhibited translational movement because one motor would be capable of supporting movement, but this was not observed. Taken together, these data show that ATK1 exhibits non-processive motor qualities and likely requires at least four ATK1 molecules to support microtubule movement.

ATK1 Function

These results, in combination with previous data showing ATK1 cellular localization and phenotypic analysis of a ATK1 mutant, provide further insight into ATK1 function. Minus-end directed motors, like ATK1, are thought to stabilize the spindle by counteracting forces generated by plus-end directed motors. For example, mutations in the minus-end directed motors, KAR3 (*S. cerevisiae*) and KLPA (*A. nidulans*), suppress mutants in the plus-end directed motors *cin8/kip1* and bimc, respectively [O'Connell et al., 1993; Saunders, et al., 1997]. ATK1 has 35% identity to KLPA and 34% to KAR3, suggesting that ATK1 plays a similar role by counteracting forces produced by plus-end directed spindle motors. In support of this idea is the finding that ATK1 exhibits non-processive movement. The dynamic balance of forces in the spindle might be precisely controlled by many motors acting in a cooperative fashion [Sharp et al., 2000a,b]. Thus, a non-processive motor, like ATK1, could play a role by providing transient forces that can be more easily regulated than a processive motor, which would generate force over longer time periods. This would likely require that ATK1 can cross-link microtubules via its tail domain (either by the tail domain binding directly to the microtubule or binding to an intermediate protein that binds to the microtubule), similar to NCD in *Drosophila* [Karabay and Walker, 1999a,b]. A role in cargo transport is unlikely for ATK1; typically, processive motors are thought to serve this purpose by ensuring long distance transport of vesicles, small organelles, soluble mRNA, or protein particles, which only allow for one or a few motors to be bound [Carson et al., 1997; Prahlad et al., 1998; Hancock and Howard, 1999; Brendza et al., 2000; Verhey et al., 2001].

CONCLUSIONS

In summary, ATK1 was shown to behave as a kinesin in vitro by supporting microtubule movement in an ATP-dependent manner. Furthermore, directionality

experiments indicate that ATK1 is a minus-end directed kinesin that moves non-processively along the microtubule. Based on these results and previous experiments, we speculate that ATK1 might have a structural role in the meiotic spindle by providing forces that counteract those of plus-end directed motors.

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