Title
Biochemical studies of katanin

Author
Hartman, James J.

Publication Date
1999

Peer reviewed|Thesis/dissertation

Biochemical Studies of Katanin: A Microtubule-Severing ATPase

by

James J. Hartman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

I dedicate this thesis to my father, Robert Hartman,
whose patient answering of many years of questions
helped me learn how to ask them.

ACKNOWLEDGMENTS

I will begin by thanking my advisor, Dr. Ronald Vale, for his support with this project. In addition to making the initial observations on microtubule severing that made this thesis possible, he has always been an excellent source of ideas and experiments. His laboratory has been a good place to be a scientist not simply because of the abundant facilities, but more importantly because he is interested in maintaining a group of people that work well together. Finally, I think his visual way of thinking and explaining is very powerful, and quite useful for communicating in this era of increasing scientific specialization. I hope that at least a little of that ability to see an experiment in terms of a single key image stays with me.

The second biggest acknowledgment probably belongs to my undergraduate advisor at the University of Pittsburgh, Dr. Lewis Jacobson, without whom I never would have ended up at UCSF. The one-on-one apprenticeship I received from him was a deciding factor in my going to graduate school and continuing in science. His insistence on accuracy, precision, and terseness in explanations and discussion were important lessons. And his willingness to let me fumble for answers when I needed to was an incredibly important experience.

I also want to thank the members of my thesis committee: Tim Mitchison (who had to be replaced because of moving to Harvard), Peter Walter, and Dave Morgan. When we had meetings, which was undoubtedly not frequent enough, they were supportive of the experiments I had done and very helpful and straightforward about future directions.

Dr. Frank McNally has been an excellent collaborator on this project, and I owe him a debt of gratitude for doing the crucial experiments that

isolated katanin and allowed me to do this thesis. Yes, I thought he was crazy when I first came to the lab, but graduate school has made me realize that he may be one of the few people who aren't. His complete honesty about the relevance and quality of experiments has been refreshing, and useful. The fact that he never spares his own work from the same critical treatment keeps it honest. And he always had good stories for the lab.

The members of the Vale lab have been irreplaceable, most likely because they are all such unique characters. I thank them all for putting up with me ranting and raving about my many pet peeves, including but not limited to: bleached paper towels, single sided copies, evil cars, the Trilateral commission, broken machinery, crashed computers, people who didn't clean up the lunch room, and much, much more.

Specific acknowledgments begin with Geno de Hostos and Josh Niclas, who have been major influences in my life and excellent friends. Together with Jim Wilhelm, the circle of darkness was complete. But that darkness was important, because comic relief is vital to keeping your sanity. Geno has been an excellent running partner, glove-snapper, and karmic advisor. The tongue scraping and self-flagellation he recommended has been so helpful. And meeting the beta reticulans was the thrill of a lifetime. I owe Josh for many things, especially getting me hooked on single ended triode amplifiers, the Mermen, the 20 Tank brewery, and for being an all-around great guy. I don't have to thank Jim Wilhelm (but I will), because he has more-or-less shared my brain for many years, and he knows how important that has been. We can usually agree on one thing, and that concerns the fundamental character of people and the role of irony in the universe. But regardless of what he says, Jim is an optimist inside otherwise he would never be able to work so hard at science. Hiding optimism behind a veneer of cynicism seems

to be a trademark of a certain subset of Vale lab members. I think a recent addition, Beth, will be continuing the bloodline.

In a different category, Dara Friedman has been a great person to work with because her enthusiasm is so blatant and contagious. If you can be that excited about lentil soup and pesto, you can be excited about anything! Dara has been a great person to chat, vent, complain, and laugh with, and I'm really going to miss her. Cindy Hart has been a great friend as well, and I think she deserves a Ph.D. for just putting up with all of us in the Vale lab. I'll miss the access to all those great CDs, and for many long lunches featuring #7 and #38 or #27. Both Cindy and Nora-Hom Booher have been great coworkers, and maybe I have been able to laugh with them so much because I'm one of the few people in the lab for whom they HAVEN'T made a mutant. Of course, this project probably wouldn't have taken so long if I wasn't so stubborn and had tapped into their talents! Pete Takizawa, the recent father, has been great to work with because he is so darn normal and socially well-adjusted. In the world of science, it is nice to work with someone who actually knows the score of last night's game. I'm glad that he reminded me it was fun to go see a baseball game, to drink lots of coffee in the afternoon, maybe to have a beer or two, and maintain sanity.

My lab neighbors, Nira Pollock and Sarah Rice, deserve special thanks for putting up with my lab decorations, listening to KUSF with me (whether they wanted to or not), and not getting TOO upset when I put stuff in their space or "borrowed" their pipetman. Former neighbors Laura Romberg and Gunther Woehlke deserve similar acknowledgment. In addition, Nira has been an excellent source of Bach, while Sarah makes great brats.

I really need to thank my very close friends Martha Arnaud, Sarah Mutka, and Paul Peluso for allowing me to maintain enough sanity to

graduate. Sarah and I have spent many hours together, peering inside the computers of HSE10. Neither of us has yet answered why we didn't just go into web design. And I don't want to begin to add up the number of hours I spent having coffee with Martha (always discussing science, of course). I miss our frequent sessions people-watching, buying CDs, and just talking. As for Paul, while our best work was most likely done over a pint of Guinness and talking about music, we had a lot of fun together playing with all the local UCSF technology and gizmos. Martha, Sarah, and Paul: I will miss you all greatly.

I don't think anyone can survive graduate school without an emotional support system. My longtime partner Sharon (now my wife) has somehow managed NOT to kill me in spite of many years of graduate school whining. I have no idea how. I thank her for being supportive, sympathetic, and just generally wonderful. She always falls asleep whenever I start to talk about work. I think that's good. Finally, I very much want to thank my parents, Robert and Connie Hartman, for emphasizing the importance of education, and for always being supportive of my interests and goals. Without their words of encouragement, reality checks, and recommendations of persistence, I would have quit graduate school several times over. My father deserves special thanks for answering the deluge of questions I've asked him about science, and other things, for as long as I can remember. His influence was why I went into science, and why I'm still at it. Thanks!

Biochemical Studies of Katanin, A Microtubule Severing ATPase

by

James J. Hartman

ABSTRACT

Katanin is a microtubule-stimulated ATPase that can disassemble stable microtubules *in vitro*, and may function in cells to promote microtubule reorganization during mitosis. Microtubules are stabilized by multiple interactions between tubulin subunits, forming a highly cooperative lattice. Katanin disrupts this stable structure, creating breaks in the microtubule.

To investigate how katanin couples ATP hydrolysis to the disruption of tubulin contacts, we identified cDNAs encoding the p60 and p80 subunits of katanin. p60 is a new member of the AAA ATPase superfamily, while p80 is a unique protein containing N-terminal WD40 repeats. Baculovirus expression revealed that p60 is sufficient for both microtubule-stimulated ATPase and microtubule-severing activities. Transfection experiments demonstrated that the p80 WD40 repeats were sufficient for targeting to the centrosome, where katanin is concentrated *in vivo*. This suggests katanin is organized into an enzymatic subunit (p60) and a targeting subunit (p80).

Rotary-shadowing electron microscopy revealed 15-20 nm rings in p60 preparations, but hydrodynamic analysis indicated p60 is monomeric in the presence of both ATP and ADP. However, in the presence of ATP and a mutation (E334Q), p60 formed a stable 15 S oligomer. These results suggest that wildtype p60 can undergo cycles of oligomerization coupled with

nucleotide hydrolysis. We used fluorescence resonance energy transfer (FRET) between p60 fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to show that both microtubules and ATP combine to promote oligomerization of wildtype katanin. Oligomerization closely resembled the non-hyperbolic activation of katanin ATPase activity by microtubules, suggesting that microtubules stimulate katanin's ATPase activity by promoting p60-p60 interactions. Nucleotide state affects microtubule binding, causing tight binding with ATP-γ-S and weak binding with ADP. This data, combined with data on other AAA ATPases, suggests a model in which an oligomeric ring of katanin makes multiple contacts with the microtubule. Coupling ATP hydrolysis to shape changes in the microtubule-bound ring provides a possible mechanism for mechanically straining tubulin-tubulin contacts, and disassembly of the ring in the ADP state suggests a way to reduce microtubule affinity and enhance the release of severed products.

Peter Walter, Ph.D.
Committee Chairman

Ronald Vale, Ph.D.
Adivsor

TABLE OF CONTENTS

INTRODUCTION.....1

CHAPTER 1.....16

Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit

CHAPTER 2.....58

A Nucleotide-Dependent Oligomerization Cycle by the AAA Enzyme Katanin Drives Microtubule Disassembly

CHAPTER 3.....84

Biochemical analysis of native and recombinant katanin ATPase activities

CONCLUSIONS.....127

REFERENCES.....140

LIST OF TABLES

CHAPTER 2

Table 1:

Effect of nucleotide state and microtubules on CFP-p60:YFP-p60 FRET.....75

CHAPTER 3

Table 1:

Nucleotide specificity of microtubule severing by katanin.....103

LIST OF FIGURES

INTRODUCTION

Figure 1:

Possible roles for katanin function *in vivo*.....14

CHAPTER 1

Figure 1:

Sequence analysis of p60 katanin.....44

Figure 2:

Sequence analysis of p80 katanin.....46

Figure 3:

Expression and purification of recombinant katanin subunits.....48

Figure 4:

Structure of katanin as visualized by rotary-shadowing electron microscopy.....50

Figure 5:

Activities of recombinant katanin subunits.....52

Figure 6:

The WD40 repeats of p80 katanin are not required for interaction with p60 katanin.....54

Figure 7:
Human p80 katanin and a fusion protein of the human p80 WD40 domain with GFP colocalize with γ-tubulin at centrosomes of MSU1.1 human fibroblasts.....56

CHAPTER 2

Figure 1:
Oligomerization of p60 katanin.....76

Figure 2:
Nucleotide-dependent binding of p60 katanin to microtubules.....78

Figure 3:
Effect of microtubules on p60 oligomerization, ATPase, and microtubule severing activities.....80

Figure 4:
Model for microtubule severing by katanin.....82

CHAPTER 3

Figure 1:
Time course and enzyme concentration dependence of ATP hydrolysis by native katanin.....105

Figure 2:
Microtubule activation of native sea urchin katanin ATPase activity.....107

Figure 3:
ATP dependence of katanin's microtubule-stimulated ATPase activity.....109

Figure 4:
Salt dependence of the ATPase activity of native katanin.....111

Figure 5:
pH dependence of the basal ATPase activity of recombinant p60 katanin.....113

Figure 6:
Inhibition of the ATPase activity of native katanin by ADP.....115

Figure 7:
Inhibition of native katanin ATPase activity by nucleotide analogs.....117

Figure 8:
Effect of nonpolymeric nocodazole tubulin on the ATPase activity of native katanin.....119

Figure 9:
Effect of nonpolymeric colchicine-tubulin and GDP-tubulin on the ATPase activity of recombinant p60 katanin.....121

Figure 10:
Stimulation of recombinant p60 katanin ATPase activity by poly-glutamic acid.....123

Figure 11:
Stimulation of recombinant p60 katanin ATPase activity by anti-p60 antibodies.....125

Return-Path: <jhartman@sirius.com>
To: editor@cell.com
From: jhartman@sirius.com
Subject: Permission to include paper in dissertation
Date: Thu, 1 Apr 99 03:54:40 +0000

To whom it may concern:

I would like permission to include in my Ph.D. dissertation a copy of the paper cited below, which was published in Cell. The dissertation will be microfilmed by University Microfilms Incorporated, and they request permission to supply single copies on demand.

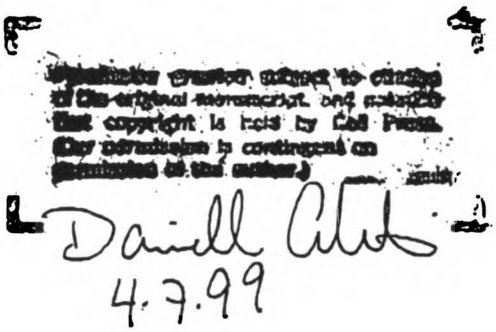
Hartman, JJ; Mahr, J; McNally, K; Okawa, K; Iwamatsu, A; Thomas, S; Cheesman, S; Heuser, J; Vale, RD; McNally, FJ. Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. Cell, 1998 Apr 17, 93(2):277-87.

Please respond by FAX to 415-476-5233, or by email to jhartman@sirius.com.

Thank you for your help.

Sincerely,

James Hartman



Copyright stamp reads as follows:

"Permission granted subject to citation of the original manuscript and notation that copyright is held by Cell Press. (Our permission is contingent on permission of the author.)"

INTRODUCTION

This thesis describes the biochemical characterization of katanin, an ATPase that disassembles stable microtubules. When this project was started, katanin had just been purified and its basic activities characterized (McNally and Vale, 1993). The experiments described here were intended to clarify the mechanism by which katanin uses the energy of ATP hydrolysis to catalyze microtubule disassembly. A first step was cloning and expressing the katanin subunits, which is described in Chapter 1. In the process, we learned that katanin is a member of the AAA superfamily of ATPases. Interestingly, while katanin is the only AAA ATPase that has been shown to disassemble microtubules, other members of this family possess the ability to disrupt multiprotein complexes. The work in Chapter 2 focuses on the oligomeric nature of p60, and how transient association of p60 subunits during the ATPase cycle may be important for the mechanism of severing. The high sequence conservation within the AAA family, together with the oligomeric nature of many AAA proteins, suggests that this information will be useful for understanding how this entire class of proteins work. Finally, Chapter 3 describes a series of experiments characterizing native and baculovirus-expressed katanin ATPase activities, work that may be helpful in future experiments with this enzyme. In this introduction, I present a brief review of microtubule rearrangements, focusing on severing and the role katanin might play in modulating microtubule behavior *in vivo*.

Microtubule Rearrangements

The microtubule cytoskeleton, a polymer network composed of α/β tubulin subunits, participates in many cellular functions. In mitotic cells, microtubules in the mitotic spindle enable accurate segregation of chromosomes, while in nondividing cells they organize cytoplasmic

organelles and function in morphogenesis and cell motility. To carry out these diverse functions, the microtubule network must be capable of major reorganization under different cellular conditions.

A well-studied mechanism of plasticity is dynamic instability, a property inherent to the tubulin polymer. Pure microtubules, even at steady state, undergo periods of growth and shrinkage via gain and loss of tubulin dimers from the microtubule ends. Differential interference contrast (DIC) microscopy of single microtubules shows that individual microtubules stochastically switch from growing to shrinking (termed catastrophe) or from shrinking to growing (termed rescue). *In vitro*, a population of microtubules contains many growing slowly, with a few undergoing rapid shortening. (Mitchison and Kirschner, 1984; Walker et al., 1988) Because of this interesting *in vitro* behavior, considerable experimentation has been done to determine if this process is relevant in cells. Fluorescence photobleaching studies show that tubulin subunits readily exchange between assembled microtubules and the pool of cytoplasmic tubulin (Salmon et al., 1984; Sammak et al., 1987). Microtubules regrow heterogenously after polymer assembly is blocked by the drug nocodazole, as would be expected for a population that grows and shrinks randomly (Cassimeris et al., 1986). In addition, exogenous labeled tubulin injected into cells becomes incorporated both at the distal plus ends of preexisting microtubules, as well as throughout the length of microtubules newly polymerized from centrosomes (Hyman and Mitchison, 1991). Taken together, these results suggest that dynamic instability is important *in vivo*, as well as *in vitro*.

Structural basis for dynamic instability

The precise mechanism that causes microtubules to switch from growing to shrinking is still unclear (Desai and Mitchison, 1997), but GTP hydrolysis has a profound effect on microtubule stability. Hydrolysis of GTP bound to the β-tubulin subunits occurs either during or soon after microtubule polymerization (Carlier et al., 1989), and the resulting GDP-tubulin lattice is less stable than the GTP-tubulin lattice. In contrast, microtubules polymerized with the nonhydrolyzable GTP analog GMPCPP are quite stable to time and dilution (Caplow et al., 1994; Hyman et al., 1994). A cap of GTP-tubulin subunits is thought to stabilize the microtubule lattice and protect the microtubule from depolymerization; exposure of GDP-tubulin subunits destabilizes the polymer and results in depolymerization (Desai and Mitchison, 1997). Supporting this model, protofilaments of depolymerizing GDP microtubules have a more curved appearance than those of GMPCPP microtubules, suggesting the tubulin dimer adopts different conformations in the GDP versus GTP state (Mandelkow et al., 1991; Muller-Reichert et al., 1998). In addition, the free energy of GTP hydrolysis by microtubules is low, suggesting that the energy of nucleotide hydrolysis is stored as mechanical energy (strain) in the GDP-tubulin lattice that comprises most of the length of microtubules (Caplow et al., 1994). In this model, depolymerization is energetically driven by strain, released when GDP-tubulin subunits become exposed at the ends of microtubules and protofilaments lose contacts with their neighbors.

Proteins that affect the intrinsic dynamics of tubulin

The dynamics of microtubules in cells can be quite different than that observed for pure tubulin *in vitro* (Lieuvin et al., 1994; Schulze and

Kirschner, 1987), and many proteins have been identified that can modulate the stability of microtubules. Microtubule-associated proteins (MAPs), such as tau, stabilize microtubule polymers. Tau binds specifically to the polymeric form of tubulin and increases the rate, length, and extent of microtubule polymerization (Cleveland et al., 1977). Real-time observation of individual microtubules has shown that tau acts by increasing the on-rate of tubulin dimers at the growing ends of microtubules, as well as by lowering the rate of growth to shrinkage transitions (catastrophe frequency) (Drechsel et al., 1992). Furthermore, phosphorylation of tau by MAP2 kinase lowers its affinity for microtubules, providing a pathway for posttranslationally controlling tau's stabilization of microtubules *in vivo* (Drechsel et al., 1992).

While MAPs are stabilizing factors, other classes of proteins destabilize microtubules and promoting depolymerization. Two examples are the KinI kinesins and OP18. Both of these proteins increase catastrophe frequency, but do so through different mechanisms. The KinI kinesins, such as XKCM1, are ATP-dependent catalysts of microtubule destabilization. XKCM1 binds preferentially to microtubule ends, triggering a destabilizing conformational change that leads to catastrophe. Nucleotide hydrolysis is not required for destabilization, since XKCM1 induces splaying of protofilaments at the ends of microtubules in the presence of the nonhydrolyzable ATP analog AMPPNP. XKCM1 forms a stable complex with the tubulin dimers released from the microtubule, and the energy of ATP hydrolysis is necessary to disrupt this stable kinesin/tubulin complex, allowing for multiple cycles of disassembly (Desai et al., 1999). Interestingly, XKCM1 can destabilize microtubules polymerized with the nonhydrolyzable GTP analog GMPCPP, suggesting it is able to change the structure of tubulin independent of the tubulin-bound nucleotide (Desai et al., 1999). Electron micrographs of

GMPCPP microtubules being depolymerized by XKCM1 show protofilament peels similar to those seen in rapidly depolymerizing GDP microtubules (Mandelkow et al., 1991), even though the tubulin is still in a GTP-like state.

OP18 also destabilizes microtubules, but there is some controversy as to the precise mechanism of action. OP18 was identified as a catastrophe-promoting factor using *in vitro* microtubule polymerization assays (Belmont and Mitchison, 1996), and analytical ultracentrifugation experiments subsequently demonstrated that it forms a stable complex with two tubulin dimers (Jourdain et al., 1997). This suggested that OP18 promotes catastrophe by simply sequestering tubulin and making it unavailable for polymerization, a model supported by the demonstrated tubulin-binding activity of OP18 (Belmont and Mitchison, 1996; Jourdain et al., 1997). However, recent experiments show that the tubulin sequestering and catastrophe-promoting activities are separable by truncation and changes in pH (Howell et al., 1999). OP18 promotes microtubule catastrophe at pH 7.5, even though it binds tubulin dimers only weakly at this pH (Howell et al., 1999). This suggests that OP18 can destabilize microtubules by a mechanism other than sequestration, possibly by interacting directly with the microtubule ends and causing a change in tubulin structure. This structural change is likely to be different from that caused by the KinI kinesins, however, since GMPCPP microtubules can be destabilized by XKCM1 (Desai et al., 1999) but not OP18 (Howell et al., 1999).

Severing proteins

All of the previously discussed proteins modulate microtubule stability by influencing the rate of tubulin addition/loss specifically from microtubule ends. Additionally, microtubule stability could be decreased by severing

proteins acting on the microtubule wall, creating internal breaks in the microtubule polymer. Several actin filament severing proteins have been identified, a well-studied example being gelsolin which severs actin filaments in a Ca^{2+} and PIP_2-regulated fashion (Vandekerckhove, 1990). Gelsolin binds both monomeric and polymeric actin, and the structures of full length plasma gelsolin (Burtnick et al., 1997) and the gelsolin segment 1-actin complex (McLaughlin et al., 1993) are known in atomic detail. This structural data has suggested a mechanism for severing in which the gelsolin filament binding domain interacts with the side of F-actin, positioning the G-actin-binding domain (segment 1) where it can bind to and destabilize the contacts between two adjacent actin monomers within a single strand of F-actin (McLaughlin et al., 1993).

The need for severing was less clear for microtubules than actin. The high off rate of tubulin monomers, at least *in vitro*, enables microtubules to depolymerize much faster than actin. However, the lack of dynamics in certain populations of cellular microtubules (Schulze and Kirschner, 1987), the stability of the interphase microtubule array in certain cell types (Lieuvin et al., 1994), and the observation that free microtubule minus ends are stable *in vivo* (Nicklas et al., 1989) made a role for microtubule severing proteins plausible. Severing proteins have been suggested to be involved in flagellar resorption in Chlamydomonas, as well as in the rapid microtubule depolymerization and contraction of heliozoans such as *Reticulomyxa* (Chen and Schliwa, 1990) and *Actinocoryne* (Febvre-Chevalier and Febvre, 1992). To achieve the millisecond contraction of its microtubule-filled stalk, the latter would require an estimated tubulin dissociation rate of $3x10^7$ dimers$^*s^{-1}$, 10^4 faster than generally observed. This strongly suggested the involvement of

factors that can sever or fragment microtubules in order to accelerate depolymerization.

Microtubule severing was first directly observed in extracts of *Xenopus* eggs, evident by the rapid fragmentation and eventual disappearance of artificially-stabilized fluorescent microtubules that would otherwise be stable for several days (Vale, 1991). Incubation with Xenopus extracts caused an increase in the number of microtubules, while their average length commensurately decreased, and real-time observations showed individual microtubules being broken at random sites along their length. This was quite distinct from dynamic instability, where gain and loss of subunits occurs strictly at the ends. *Xenopus* extracts disrupted microtubules stabilized either by treatment with the microtubule-binding drug paclitaxel or by polymerization with the nonhydrolyzable GTP analog GMPCPP, suggesting that severing was neither caused by displacing paclitaxel from microtubules, nor was dependent on the nucleotide state of tubulin dimers in the microtubule lattice. Severing was dependent on the addition of ATP and inhibited by apyrase and EDTA, suggesting a direct or indirect requirement for nucleotide energy in the severing reaction. Interestingly, severing activity was seen in mitotic but not interphase *Xenopus* egg extracts, consistent with a role for severing in the microtubule rearrangements that remodel the interphase cytoskeleton at the onset of and throughout mitosis. The susceptibility of severing to inactivation by trypsin, heat, or N-ethylmaleimide suggested the involvement of one or more proteins, but difficulties with purifying the Xenopus severing activity led to the search for other sources of microtubule-severing activity.

Katanin, a microtubule-severing protein from sea urchin eggs

Katanin was purified from eggs of the sea urchin *Stronglyocentrotus purpuratus* on the basis of its ability to sever paclitaxel-stabilized microtubules, using a microscope-based assay (McNally and Vale, 1993) similar to that used for characterizing *Xenopus* severing activity (Vale, 1991). Katanin is composed of 60 kDa and 80 kDa polypeptide subunits, present in a 1:1 ratio based on Coomasie-stained SDS-PAGE gels of purified protein. The subunits are thought to be in a heterodimeric complex with a predicted native molecular weight (Siegel and Monty, 1966) of 105 kDa, based on its measured 4.1S sedimentation coefficient and 51Å Stokes radius (McNally and Vale, 1993). Katanin requires Mg^{2+}ATP to sever microtubules, and interaction with microtubules activates katanin's basal ATPase activity. Furthermore, the energy derived from ATP hydrolysis is required for microtubule severing, since the slowly-hydrolyzed ATP analog ATP-γ-S inhibits the ATPase activity of katanin but does not support microtubule severing (McNally and Vale, 1993). Katanin can act enzymatically, with 1 molecule releasing a 75-fold excess of tubulin over the course of a 50 min incubation (McNally and Vale, 1993). The products of katanin severing are likely to be tubulin dimers or small oligomers, since fluorescence resonance energy transfer experiments indicate that mixed polymers of fluorescein- and rhodamine-labeled tubulin fail to transfer energy after severing (McNally and Vale, 1993). And tubulin released from microtubules by katanin is capable of repolymerization, eliminating the possibility that katanin acts by proteolyzing or post-translationally modifying its substrate (McNally and Vale, 1993).

In addition to katanin, two other microtubule-severing proteins have been reported. Both proteins, p56 (Shiina et al., 1992) and EF-1α (Shiina et al., 1994), were purified from *Xenopus* egg extracts. Neither protein requires ATP

for activity, in contrast with both katanin (McNally and Vale, 1993) and the initial microtubule-severing activity characterized in *Xenopus* egg extracts (Vale, 1991). Recently, a *Xenopus* homolog of p60 katanin has been identified, and antibodies raised against this protein indicate p60 katanin is present in many different vertebrate cell and tissue types (McNally and Thomas, 1998). Importantly, the almost complete lack of severing in *Xenopus* extracts treated with anti-p60 antibodies demonstrates that katanin, not p56 or EF-1α, is responsible for microtubule severing in mitotic *Xenopus* egg extracts (McNally and Thomas, 1998).

Possible function of katanin *in vivo*

The precise function(s) of katanin *in vivo* are still unclear, but immunofluorescence experiments show that katanin is concentrated at the centrosomes of sea urchin (McNally et al., 1996), *Xenopus* (McNally and Thomas, 1998), and human cells (Hartman et al., 1998). This localization, together with the observed activation of *Xenopous* katanin during mitosis, suggests several possible roles for microtubule severing *in vivo*: disassembling microtubule minus ends in the mitotic spindle and releasing microtubules from the centrosome.

In the mitotic spindle, tubulin subunits undergo a net movement toward the centrosome, termed poleward flux (Mitchison, 1989). This process has been suggested to be important for maintaining spindle structure (Waters et al., 1996), and microtubule motor proteins are likely to supply the force drawing microtubules into the centrosome. Microtubules arise by polymerization from nucleating sites in the pericentriolar material (Moritz et al., 1995; Zheng et al., 1995), and microtubules attached to their sites of polymerization do not have minus ends available for depolymerization.

Flux requires that microtubule minus ends get released to allow minus-end depolymerization and further poleward movement. Katanin is both activated in mitosis and localized to the correct place, the pericentriolar material, to act as a minus end destabilizing factor in the process of poleward flux (Fig. 1A).

Centrosomal katanin may also be involved in the release of cytoplasmic microtubules from the centrosome at the onset of mitosis. The number of noncentrosomal microtubules increases at the G2->M transition in sea urchin eggs (Harris et al., 1980; Hollenbeck and Cande, 1985) and Dictyostelium cells (Kitanishi-Yumura and Fukui, 1987). In addition, centrosomal release is greater in mitotic versus interphase extracts of *Xenopus* eggs. As with flux, the localization and regulation of katanin are consistent with it being involved with this process (Fig. 1B).

Is there a role for katanin in nondividing cells? Neurons, skeletal muscle cells, and certain epithelial cell types all contain large arrays of noncentrosomal microtubules in interphase (reviewed by (Cassimeris, 1993)). Keating et al. (1997) used a combination of time-lapse fluorescence microscopy and laser marking to demonstrate that noncentrosomal microtubles arise primarily by nucleation at and subsequent release from centrosomes in interphase rat PtK_1 cells. The released microtubule minus ends were either stable or depolymerized, but were never seen to grow (Keating et al., 1997). This is consistent with a model in which katanin releases microtubules from the centrosome, followed by either disassembly or stabilization depending on the activity of minus end capping proteins or other regulators of dynamic instability. Neurons, in particular, might use katanin to release microtubules from their centrosomal nucleation sites as part of the mechinery for

transporting preformed microtubules down the growing axon (Baas and Yu, 1996) (Fig. 1C).

Interest in the mechanism of severing

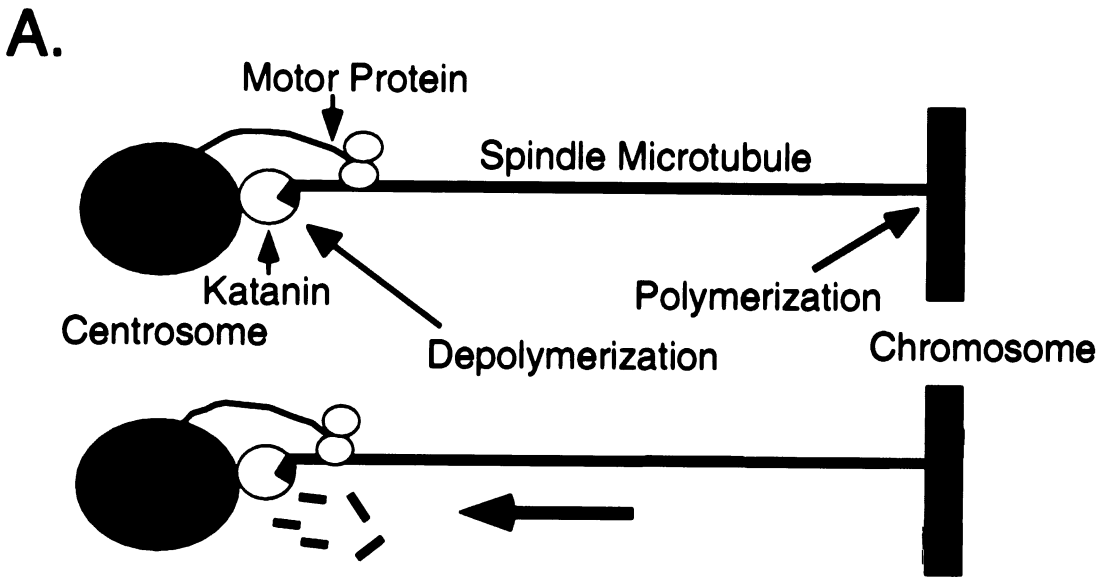
While microtubule ends can be very dynamic, the microtubule wall is not. Experiments with end-stabilized microtubules indicate that subunits dissociate from the wall at ~10^{-8} dimers*s^{-1}, roughly 10^{11} more slowly than from the ends (Dye et al., 1992). This enormous difference in stability arises because of an additional longitudinal tubulin-tubulin contact made by a wall versus an end-exposed tubulin dimer. This is similar to what has been observed with actin. Fragmenting an actin filament requires disrupting three interactions between actin monomers (two longidudinal, one lateral), while endwise loss requires disrupting only two (one longidudinal, one lateral). Actin depolymerization occurs 10^8 faster than the fragmentation, demonstrating the enormous impact of a single additional protein-protein contact on the stability of protein complexes (Erickson, 1989). Paclitaxel-stabilized microtubules, normally stable for several days, are disrupted less than a minute after addition of katanin and ATP. Katanin is able to use the energy of ATP hydrolysis to disrupt the stable protein complex of the microtubule wall, making it unique among the proteins studied thus far that interact with microtubules.

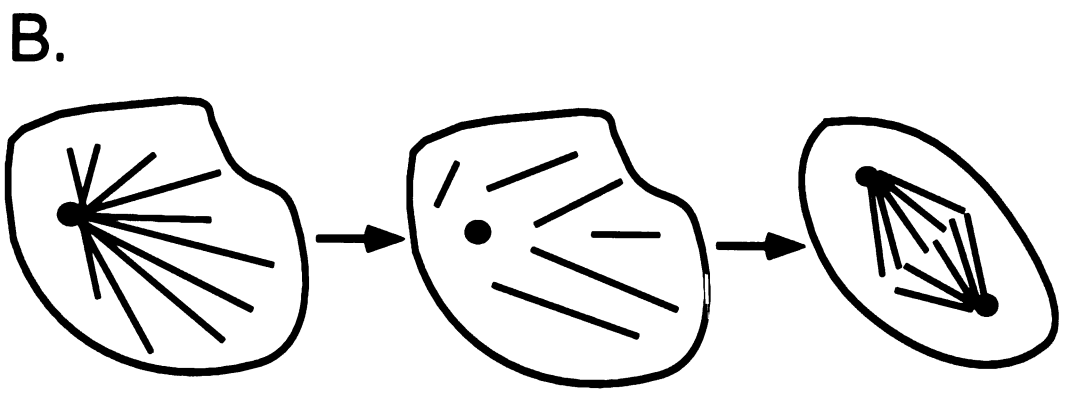
As an initial step in studying the mechanism of katanin-mediated severing, we isolated cDNAs encoding the p60 and p80 subunits of katanin. Our discovery that p60 katanin is a member of the large superfamily of AAA ATPases is exciting, especially since other members of this family disrupt multiprotein complexes. We showed that the p60 subunit alone has microtubule-stimulated ATPase activity and severing activity, indicating that

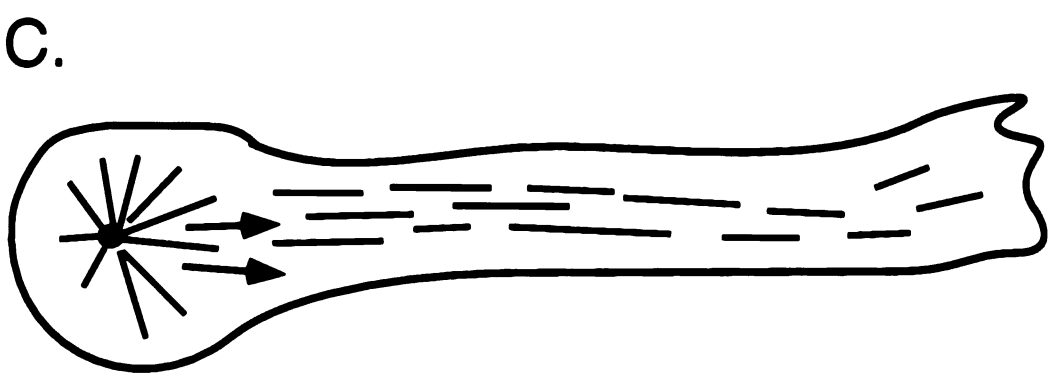
it is the enzymatic subunit of the complex. Electron micrographs of p60 and p60/p80 katanin show the presence of 15-20 nm rings, suggesting that p60 can oligomerize as has been reported for other AAA ATPases. This was initially confusing, since katanin behaves like a heterodimer in hydrodynamic experiments (McNally and Vale, 1993). This question has been addressed by studies using a fluorescence resonance energy transfer assay, together with a p60 mutant defective in ATP hydrolysis, to demonstrate that katanin undergos cycles of reversible oligomerization in the presence of ATP and microtubules. It is likely that many AAA ATPases will employ similar cycles of oligomerization, and the well-characterized nature of katanin's substrate (the microtubule) makes this system attractive for further study of the mechanism of the AAA ATPases.

Figure 1: Proposed roles for katanin function *in vivo*.

(**A**) Centrosomal katanin may be involved in the poleward flux of tubulin subunits in the mitotic spindle. (**B**) Centrosomal katanin may also be involved in the release of microtubules from the centrosome observed at the onset of mitosis. (**C**) A possible function for katanin in nonmitotic cells is in the creation of noncentrosomal arrays of microtubules by release from the centrosome. One example is the release of centrosomal microtubules for transport down the elongating axon of developing neurons.


A.
Motor Protein
Spindle Microtubule
Katanin
Centrosome
Depolymerization
Polymerization
Chromosome


B.


C.

CHAPTER 1

Katanin, a Microtubule Severing Protein, is a Novel AAA ATPase that Targets to the Centrosome Using a WD40-Containing Subunit

The work presented in Chapter 1 is the result of a collaboration between Dr. Frank McNally and myself. The cloning of p60 katanin, the expression of both katanin subunits, and the biochemical assays of katanin ATPase and microtubule severing activity were my work. Frank McNally, and others in his lab, were responsible for cloning p80, as well as identifying the region of p80 responsible for heterodimerization and demonstrating that the WD40 repeats of p80 are responsible for targeting of katanin to the centrosome. This chapter is reproduced from Cell, 1998, Vol. 93, pp. 277-287 by copyright permission of Cell Press.

SUMMARY

Microtubule disassembly at centrosomes is involved in mitotic spindle function. The microtubule severing protein katanin, a heterodimer of 60 and 80 kDa subunits, was previously purified and shown to localize to centrosomes in vivo. Here we report the sequences and activities of the katanin subunits. p60 is a new member of the AAA family of ATPases, and we show that expressed p60 has microtubule-stimulated ATPase and microtubule severing activities in the absence of p80. p80 is a novel protein containing WD40 repeats, which are frequently involved in protein-protein interactions. The p80 WD40 domain does not participate in p60 dimerization, but localizes to centrosomes in transfected mammalian cells. These results indicate katanin's activities are segregated into a subunit (p60) that possesses enzymatic activity and a subunit (p80) that targets the enzyme to the centrosome.

INTRODUCTION

Microtubules, polymers of α/β tubulin heterodimers, help to organize membranous organelles during interphase and comprise the mitotic spindle of a dividing cell. Microtubule networks are highly dynamic and undergo dramatic reorganizations during the cell cycle. Upon entry into mitosis, the interphase microtubule array rapidly disassembles and then reorganizes into the mitotic spindle (Zhai et al., 1996). Microtubule dynamics also plays an important role in mitotic spindle function. Coordination of microtubule growth at kinetochores with microtubule disassembly at centrosomes is thought to be important for maintaining spindle structure (Waters et al., 1996). Coordinated disassembly of microtubules at their kinetochore attachment sites also may be a major driving force in chromosome separation during anaphase A (Desai and Mitchison, 1995).

Some of the dynamic properties of microtubules observed in vivo can be accounted for by the intrinsic properties of the α/β tubulin heterodimer. Even at steady state, microtubules composed of pure tubulin undergo periods of prolonged growth and shrinkage and can switch rapidly between these two states (Horio and Hotani, 1986; Mitchison and Kirschner, 1984). This process, known as dynamic instability, is thought to result from GTP hydrolysis by tubulin; a cap of GTP-containing tubulin subunits at the microtubule end is believed to stabilize the lattice, while exposure of tubulin-GDP at ends destabilizes the polymer (Caplow and Shanks, 1996; Desai and Mitchison, 1997).

However, the properties of pure tubulin cannot account for many behaviors of microtubules in living cells. A variety of proteins have been purified which modulate the dynamic behavior of microtubules, and are thought to be vital for controlling changes in the microtubule cytoskeleton in

vivo. Microtubule-associated proteins (MAPs) bind to the microtubule wall and promote microtubule polymerization by enhancing the rate of microtubule growth and suppressing transitions from a growing to a shrinking state (termed catastrophe) (Drechsel et al., 1992; Kowalski and Williams, 1993). Conversely, other proteins such as OP18 (Belmont and Mitchison, 1996) and XKCM1 (Walczak et al., 1996) increase the frequency of catastrophes and thus promote disassembly of microtubules from their ends.

In contrast to OP18 and XKCM1, microtubule-severing proteins promote the disassembly of microtubules by generating internal breaks within a microtubule (McNally and Vale, 1993; Shiina et al., 1994; Shiina et al., 1992; Vale, 1991). Katanin, a heterodimer of 60 kDa and 80 kDa subunits purified from sea urchin eggs, is unique among the known microtubule and actin severing proteins in that it disrupts contacts within the polymer lattice by using energy derived from ATP hydrolysis (McNally and Vale, 1993). Katanin acts substoichiometrically, as one molecule of katanin can release several tubulin dimers from a microtubule. Katanin does not appear to proteolyze or modify tubulin, since the tubulin released from the disassembly reaction is capable of repolymerizing (McNally and Vale, 1993). The mechanism of microtubule severing by katanin, however, is not understood.

Katanin-catalyzed microtubule severing and disassembly could potentially be involved in several changes in the microtubule cytoskeleton observed in vivo. Recent studies have shown that katanin is concentrated at the centrosome in a microtubule-dependent manner in sea urchin embryos (McNally et al., 1996). One phenomenon that could require disassembly of microtubules at the centrosome is the poleward flux of tubulin in the mitotic spindle (Mitchison, 1989). The disassembly of microtubule minus ends at the spindle pole during poleward flux could be driven by katanin, or katanin

could simply allow depolymerization by uncapping microtubule minus ends that are docked onto γ–tubulin ring complexes (Moritz et al., 1995; Zheng et al., 1995). Another possible role for katanin at the centrosome is in promoting the release of microtubules from their centrosomal attachment points. Microtubules are nucleated from γ-tubulin ring complexes at the centrosome (Joshi et al., 1992; Moritz et al., 1995), but release of microtubule minus ends has been observed indirectly in Dictyostelium (Kitanishi-Yumura and Fukui, 1987) and directly in PtK1 cells (Keating et al., 1997) and Xenopus eggs extracts (Belmont et al., 1990). Finally, katanin could accelerate the rapid disassembly of the interphase microtubule network at the G2/M transition (Zhai et al., 1996) by severing cytoplasmic microtubules, which would increase the number of free microtubule ends from which depolymerization could occur.

To further understand the mechanism and biological roles of katanin, it is essential to establish the molecular identities and activites of the two katanin subunits. Here, we report the sequences of the p60 and p80 subunits and characterize the activities of the individual subunits. Our results indicate that p60 serves the enzymatic function of the katanin complex, while p80 acts as a centrosome targeting subunit.

RESULTS

To begin dissecting the functional domains of katanin, we isolated cDNA clones for the p60 and p80 subunits from cDNA derived from sea urchin (Strongylocentrotus purpuratus) egg mRNA. After first obtaining peptide sequence of several proteolytic fragments from the two sea urchin katanin subunits, cDNA clones were isolated using a combination of degenerate PCR, cDNA library screening, and anchor-ligated PCR (see Experimental Procedures). The predicted amino acid sequences of the cDNA clones contained 139 a.a. and 306 a.a. of peptide sequences obtained by direct microsequencing of p60 and p80 respectively.

p60 is a Novel Member of the AAA ATPase Superfamily

Sequence analysis of the p60 cDNA clone revealed an open reading frame that encodes a 516 a.a. polypeptide (Fig. 1A). A BLAST search with the predicted p60 protein sequence revealed that this polypeptide contains a C-terminal domain (a.a. 231 - 447) that is highly conserved in the AAA ATPase superfamily (Fig. 1B) (Confalonieri and Duguet, 1995). This ~220 amino acid region contains the "Walker A" (P-loop) and "Walker B" motifs found in many ATPases (Walker et al., 1982). AAA proteins, which contain either one or two of these 220 a.a. ATP-binding modules, constitute a large superfamily whose members have been implicated in a variety of cellular functions (Confalonieri and Duguet, 1995).

Of the AAA domains entered into sequence data bases, mei-1, a C. elegans protein required for meiosis (Clark-Maguire and Mains, 1994a), is most closely related to p60 (55% a.a. identify, Fig. 1B). Mei-1 was discovered in a genetic screen as a protein that is required for meiotic spindle formation, but disappears during subsequent mitotic divisions. Interestingly, both p60 (McNally et al., 1996) and mei-1 (Clark-Maguire and Mains, 1994b) are

localized to spindle poles in a microtubule-dependent manner. However, the N-terminal half of p60 has no significant homology to mei-1, suggesting that p60 and mei-1 may not be orthologs. BLAST searches with p60 sequences, however, revealed several human ESTs (expressed sequence tags) that have strong a.a. identity outside of the AAA domain, suggesting the existence of vertebrate homologs of p60.

p80 Contains WD40 Repeats

Sequence analysis of the sea urchin p80 cDNA clone revealed a predicted 690 a.a. polypeptide that contains six "WD40" repeat motifs extending from residues 1-256 (Fig. 2A). An alignment of these repeats with two unrelated WD40 repeat-containing proteins is shown in Fig. 2B. The WD40 repeats in several proteins have been documented to participate in protein-protein binding interactions (Komachi et al., 1994; Wall et al., 1995). The C-terminal region of p80 (residues 257-690) did not exhibit significant amino acid identity to any previously described protein. However, significant identity of sea urchin p80 was observed with several human EST clones. The sequences of these clones were used to isolate a full-length human p80 katanin homolog by PCR (see Experimental Procedures). The human cDNA encodes a predicted 655 a.a. protein with 61% a.a. identity in the WD40 domain (a.a. 1-256) (Fig. 2B), 23% a.a. identity in the central 187 residues, and 54% a.a. identity in the C-terminal 164 a.a. with S. purpuratus p80 katanin (latter two regions are not shown).

Baculovirus Expression and Molecular Structure of the Katanin Subunits

Deciphering the roles of the two katanin subunits is essential for understanding the enzyme's mechanism and biological activities. However,

separation of the native sea urchin p60/p80 subunits requires denaturing conditions. We therefore sought to express the two subunits together and separately and then test their enzymatic activities. Bacterial expression of p60 produced largely insoluble protein, and the small amount of soluble p60 had no microtubule-stimulated ATPase activity (data not shown). However, using the baculovirus expression system, we obtained soluble p60, p80, and the p60/p80 complex (each expressed with a N-terminal $His_{(6)}$ tag), and purified the expressed proteins using metal affinity chromatography (Fig. 3A). When p60 and p80 were co-expressed, the stoichiometry of the two subunits in the purified protein was approximately equal (1.0:0.9 p60:p80 molar ratio, as determined by Coomassie staining). Moreover, immunoprecipitation with an anti-p60 antibody led to co-immunoprecipitation of equal quantities of p60 and p80 (Fig. 3B). These results indicate that baculovirus -expressed p60 and p80 heterodimerize, as observed with native katanin (McNally and Vale, 1993).

To examine katanin's structure, baculovirus-expressed p60, p80, or p60/80 was adsorbed onto mica chips, and the chips were subsequently frozen, etched, and rotary shadowed with planinum (Heuser, 1989; Heuser, 1983). The platinum-shadowed p60 appeared as a 14-16 nm ring punctuated in the center by a 3-5 nm opening, often with what appears to be cracks radiating outward (Figure 4A). p80, on the other hand, appeared as ~11 nm particles and occasional unstructured protein aggregates; rings were not observed (Fig. 4B). Rings were also seen for p60/p80 complexes (Fig. 4C,D) and native sea urchin katanin (data not shown). Interestingly, two types of p60/p80 complexes were visible: large ~20 nm diameter rings with bright edges, which is suggestive of taller complexes that extend upward from the mica (Fig. 4D), and smaller rings of the size of p60 alone with several p80 sized particles

radiating from the central ring (Fig. 4C). The large and small rings might represent closed and "splayed" versions of the p60/p80 complex, respectively, which could be produced if the complex dissociates upon mica adsorption. Both p60 and p60/p80 structures resemble the rings observed for the AAA ATPases NSF and p97, whose dimensions are 15-17 nm (Hanson et al., 1997).

p60 Katanin has Microtubule-Stimulated ATPase and Severing Activity

With the availability of isolated p60 and p80, we then examined whether the individual subunits have ATPase activity. The co-expressed p60/p80 heterodimer displayed an ATP turnover rate of 0.3 ATP/sec/heterodimer; this activity was stimulated ~10-fold by microtubules (Fig. 5A). This basal activity and the fold stimulation by microtubules are similar to that observed for native sea urchin katanin (data not shown). Consistent with the finding of an AAA domain in its sequence, p60 alone displayed a microtubule-stimulated ATPase activity. Surprisingly, the maximal basal and microtubule-stimulated ATPase rates of p60 were only 2-fold lower than those of the p60/p80 heterodimer (Fig. 5A). p80 itself had no detectable ATPase activity. The activation of ATPase activity by microtubules displayed an atypical, non-hyperbolic behavior. ATP turnover by p60 and p60/p80 was stimulated at low concentrations of microtubules (peak at ~2 μM tubulin), but then decreased at higher microtubule concentrations (Fig. 5A). This same complex pattern of microtubule stimulation was also observed for native sea urchin katanin (data not shown).

We then tested the microtubule severing activity of p60, p80, and p60/p80 using a fluorescence microscopy assay (McNally and Vale, 1993). Both p60 and p60/p80 severed microtubules in this assay (Fig. 5B). Broken microtubules were observed within 1 min after introducing 0.1 μM p60 or

p60/p80, and microtubules were completely disassembled after 5 min. The reaction appeared somewhat slower with p60 alone. Microtubules remained intact if ATP was omitted from the reaction (not shown). In contrast, p80 was unable to sever microtubules at concentrations 5-fold higher than those used for p60 (Fig. 5B). These experiments demonstrate that p60 alone can carry out all of the steps necessary for coupling ATP hydrolysis to microtubule disassembly.

To better compare the microtubule severing activities of p60 and p60/p80, we developed a quantitative microtubule disassembly assay based upon a previous finding that DAPI fluorescence intensity is higher when this dye is bound to polymerized versus free tubulin (Heusele et al., 1987). When katanin and ATP were incubated with DAPI-labeled microtubules, a linear decrease in fluorescence intensity was observed as a function of time, reflecting the conversion of microtubules to tubulin (Fig. 5C). The loss of microtubule polymer was confirmed by centrifugation studies, which showed an increase in non-sedimentable tubulin with a similar time course (data not shown). The fluorescence decrease induced by these enzymes reached a steady-state level that was slightly higher than pure, monomeric tubulin, suggesting that some tubulin oligomer may exist at steady state. The rate of fluorescence decrease was proportional to p60 or p60/p80 concentration over a 10-fold range (data not shown). When the rates of microtubule disassembly were compared, p60 was half as active as p60/p80 (Fig. 5C). This slower rate of microtubule disassembly is consistent with the previously described 2-fold decrease in ATPase activity of p60 compared with p60/p80.

The p80 WD40 Domain Targets to the Centrosome

The finding that p60 by itself can sever microtubules left open the question of the function of the p80 katanin subunit. At least two functional domains of p80 could be postulated. First, since katanin is a heterodimer (McNally and Vale, 1993), some part of p80 must be involved in heterodimerization with p60. Second, because previous studies have shown that katanin is concentrated at centrosomes in vivo (McNally et al., 1996), p80 could contain a domain that interacts with a centrosomal protein to allow targeting of the katanin holoenzyme. Because WD40 repeats have been implicated in heterophilic protein-protein interactions (Komachi et al., 1994; Wall et al., 1995), the six WD40 repeats in p80 represented a good candidate domain for participating in either dimerization or centrosome targeting.

In order to test whether the WD40 repeats of p80 are required for heterodimerization with p60, we deleted the entire WD40 domain and examined whether the truncated p80 (p80Δ1-302) interacted with p60 when the two polypeptides were co-translated in a rabbit reticulocyte system. The truncated p80 (Δ1-302) was co-immunoprecipitated by the anti-p60 antibody only in the presence of p60 and just as efficiently as full length p80 (Fig. 6). This finding indicates that the WD40 repeats are not required for dimerizing the two katanin subunits. Nevertheless, it remained possible that the WD40 domain was one of multiple, redundant p60-interacting domains. However, a C-terminal truncation of p80 (p80Δ303-690) containing only the WD40 domain did not co-immunoprecipitate with p60 (Fig. 6). These results indicate that the p80 WD40 repeats are neither necessary nor sufficient for dimerization with p60. To determine which region of p80 is required for interaction with p60, a p80 deletion lacking the C-terminal 130 amino acids (p80Δ560-690) was constructed and was found not to co-immunoprecipitate

with p60 (Fig. 6). These findings suggest that the C-terminal 130 a.a. of p80, but not the WD40 repeat domain, are involved in the dimerization with p60.

To examine whether the p80 WD40 repeats bind to a protein in the centrosome, we tested whether these repeats can target a heterologous protein (green fluorescent protein, GFP) to the centrosome after transient transfection in the human fibroblast cell line, MSU1.1 (Lin et al., 1995). The WD40 domain of human p80 katanin was used, because it was more likely that the human protein would interact with centrosomal proteins in this human cell line. Immunofluorescence of MSU1.1 cells with an antibody specific for human p80 katanin (see Experimental Procedures) showed labeling of the cytoplasm and more concentrated staining at one or two spots that co-localized with γ–tubulin staining (Fig. 7), confirming that endogenous katanin is concentrated at centrosomes in fibroblasts as it is in sea urchin embryos (McNally et al., 1996). In contrast to the localization in sea urchin embryos, the concentration of p80 at centrosomes in fibroblasts remained after complete depolymerization of microtubules with nocodazole (data not shown), suggesting that katanin is bound to the pericentriolar material. When a fusion protein consisting of the six WD40 repeats of human p80 katanin (a.a. 1-263) appended to the N-terminus of green fluorescent protein (GFP) was expressed in MSU1.1 cells, one or two foci of green fluorescence that co-localized with γ–tubulin staining was observed 2-4 hr after transfection in addition to diffuse cytoplasmic fluorescence (Fig. 7). Identical results were obtained in transfections of Hela cells (not shown). In contrast to these findings, cells transfected with GFP alone never revealed foci of green fluorescence at centrosomes (not shown). After longer periods of expression (8-24 hr) of p80 WD40-GFP, numerous heterogeneously-sized bright foci of green fluorescence appeared that did not co-localize with γ–tubulin, and later,

massive aggregates several mm in diameter were observed (not shown). These results indicate that the WD40 repeats of human p80 katanin are sufficient to target GFP to the centrosome and suggest that once the centrosome binding sites are saturated, the additional fusion protein aggregates in the cytoplasm.

DISCUSSION

Katanin is a unique enzyme that couples ATP hydrolysis to the dissociation of tubulin subunits from the microtubule lattice (McNally and Vale, 1993). Other than the motor proteins kinesin and dynein, katanin is the only known microtubule-associated ATPase. In this study, we have determined the primary structure of the p60 and p80 katanin subunits and examined the roles of the two subunits in microtubule severing and the cellular localization of the enzyme.

Mechanism of Katanin-Mediated Microtubule Severing

Sequence analysis of p60 katanin revealed that it is a novel member of the AAA family of ATPases. This finding suggested that p60 might be responsible for the previously reported ATPase activity of the native katanin dimer (McNally and Vale, 1993). However, neither p60 nor p80 contained an identifiable microtubule binding sequence, such as those found in tau (Butner and Kirschner, 1991) or MAP1B (Noble et al., 1989), and therefore it was not possible to ascribe the microtubule binding and severing activities of katanin to either subunit based upon sequence information alone. By measuring the activities of the p60 and p80 subunits purified individually and together as a dimer, we have found that katanin's p60 subunit exhibits both microtubule-stimulated ATPase activity and microtubule-severing activity in the absence of the p80 subunit. Since p60 has all elements required for functional interactions with microtubules, future structure-function studies on the mechanism of microtubule severing can be focused on this single subunit. Furthermore, we have found that p60 katanin can form rings, the dimensions and appearence of which are similar to those reported for the AAA proteins NSF and p97 (Hanson et al., 1997). The comparison of p60 with

other AAA proteins provides clues as to how katanin disassembles microtubules, as discussed below.

The ATPase properties of katanin show both similarities and differences with other AAA family members. Katanin's basal ATPase activity of 0.3 ATP/katanin/sec and maximal microtubule-stimulated rate of 3 ATP/katanin/sec are comparable to values of 1 ATP/sec for p97 (Peters et al., 1992) and 0.08 ATP/sec for recombinant NSF (Morgan et al., 1994). NSF ATPase is also stimulated two-fold upon binding to its target protein, α- or γ–SNAP (Morgan et al., 1994). However, katanin's ATPase activity displays a complex stimulation by microtubules. At low microtubule concentrations (<2 μM), ATPase activity increases with increasing microtubule concentration, but at higher microtubule concentrations, ATPase activity decreases until it eventually approaches basal levels. In contrast, stimulation of kinesin ATPase by microtubules (Gilbert and Johnson, 1993) displays typical hyperbolic curves that reach saturation.

At least two potential explanations could account for the unusual ATPase behavior of katanin. One possibility is that katanin binds microtubules at two sites, which could elevate the local microtubule concentration by crosslinking and thereby stimulate katanin's ATPase activity. At higher microtubule concentrations, however, the ratio of katanin to microtubules would be lower, resulting in a less-crosslinked network and less stimulation of ATPase activity. In support of this idea, bundling of microtubules by katanin has been observed by microscopy (unpublished observations). This behavior has been seen in another cytoskeletal-polymer stimulated ATPase, Acanthamoeba myosin I, which has two discrete actin binding sites: a low affinity catalyic site and a higher affinity site not involved in catalysis (Lynch et al., 1986).

A second explanation for katanin's complex enzymatic behavior could involve katanin oligomerization into rings. Rotary-shadowing EM images show oligomeric ring structures in katanin preparations; however hydrodynamic experiments with both native (McNally and Vale, 1993) and recombinant katanin (data not shown) suggest that the majority of the protein is monomeric. One hypothesis is that microtubules promote p60-p60 oligomerization, and that the assembly of p60 monomers into a higher order structure on the microtubule stimulates ATPase activity. According to this idea, low microtubule concentrations would facilitate multimerization, since p60 monomers would be more likely to bind near one another on the microtubule. High microtubule concentrations, on the other hand, would inhibit p60 assembly by sequestering p60 monomers at noncontiguous sites on the lattice. Self assembly into rings also has been suggested as the cause of dynamin's biphasic stimulation of GTPase activity (Tuma and Collins, 1994; Warnock et al., 1996). Cryo-electron microscopy studies of the p60-microtubule complex will hopefully provide a means of testing this hypothesis.

Based upon studies of other AAA family members, katanin oligomers/rings may prove to be important in the severing mechanism. Although serving diverse functions, many AAA proteins appear to share a common function as nucleotide-dependent molecular chaperones that disassemble protein complexes (Confalonieri and Duguet, 1995). The best studied AAA member is NSF, which binds to and induces the disassembly of ternary SNARE complexes after hydrolysis of ATP (Hanson et al., 1995; Hayashi et al., 1995). This reaction plays a role either in vesicle fusion and/or recycling of components in membrane trafficking pathways. Recently, electron microscopy studies have revealed that the NSF ring structure adopts

extended and compact conformations in the ATP-γ–S and ADP states, respectively (Hanson et al., 1997). If attached at several points to a protein complex, this transition could break apart bonds in the SNARE complex (Hanson et al., 1997). Katanin may work in an analagous fashion. A ring of katanin's dimensions could potentially contact multiple tubulin sites on a microtubule, and a structural change during ATP hydrolysis could shift the positions of tubulin binding sites with respect to one another, which would disrupt the microtubule lattice. Another possibility is that katanin acts more like an ATP-regulated version of actin severing proteins, which are thought to compete for sites at protein-protein interfaces within the polymer. In this type of mechanism, the AAA domain could serve as an ATP-dependent protein clamp that binds tightly to and disrupts tubulin-tubulin interfaces during particular steps in the ATPase cycle.

Targeting of Katanin to Centrosomes

Our studies show that p80 does not constitute an essential element of katanin's enzymatic mechanism. The finding that p80 is not required for microtubule-severing activity was somewhat surprising, because all of the p60 immunoprecipitates with p80 from sea urchin cytosol (unpublished observations). However, experiments reported here have uncovered a role for p80 in targeting katanin to centrosomes in vivo. This conclusion is based upon the finding that the WD40 domain of p80 can target GFP to the centrosome in cultured human cell lines. Because the WD40 domain cannot dimerize with endogenous p60, the centrosomal localization must be due to direct interaction of the WD40 domain with one or more resident centrosomal proteins. WD40 domains are thought to form a conserved beta propeller structure, as first determined for the beta subunits of transducin and

G_i (Sondek et al., 1996; Wall et al., 1995). However, each WD40 domain exhibits very specific heterophilic protein interactions; exposed residues in the beta subunit of G_i interact with the alpha subunit (Wall et al., 1995), whereas the corresponding residues in the WD40 transcription factor TUP1 mediate binding to a second transcription factor a2 (Komachi and Johnson, 1997). Since the G protein beta subunits interact with multiple partner proteins (Gaudet et al., 1996; Wall et al., 1995), it is also possible that the p80 katanin WD40 domain can interact with more than one protein in vivo. p80 is the only known centrosomal protein with a WD40 motif. The findings that katanin has an entire subunit devoted to centrosome localization and that this subunit is conserved between mammals and echinoderms suggest an important role for katanin at the centrosome.

The WD40 domain of p80 katanin represents the first example of a structural motif that targets a protein to the centrosome in mammals, although a centrosome-targeting domain has been defined for the Drosophila protein CP190 (Oegema et al., 1995). This provides an opportunity to identify the centrosomal component(s) responsible for anchoring katanin. Further information on the docking of katanin to the centrosome may provide clues regarding katanin's role in microtubule disassembly at this organelle.

MATERIALS AND METHODS

Peptide Microsequencing

Katanin was purified from extracts of S. purpuratus eggs essentially as described previously (McNally and Vale, 1993), except that the hydroxyapatite chromatography was carried out using a Pharmacia HR10/30 column packed with 20 μm ceramic hydroxyapatite beads (American International Chemical, Natick, MA). Internal peptide sequences of the p60 and p80 subunits were obtained from native sea urchin katanin as described (Iwamatsu, 1992). Two additional p80 peptides were obtained from Chris Turck (Howard Hughes Medical Institute, UCSF): DASMMAM and IQGLR.

p60 Cloning

A cDNA encoding a 400 bp fragment of the p60 subunit (corresponding to a.a. 214-374) was cloned from S. purpuratus first strand cDNA using nested PCR with degenerate oligonucleotides. This fragment was then used to screen a lambda ZAP-Express cDNA library made from S. purpuratus unfertilized egg mRNA by hybridization. Several independent positive clones were isolated. One clone was completely sequenced (GENBANK accession number AF052191).

p80 Cloning

An initial partial cDNA clone of p80 katanin was obtained by screening an S. purpuratus unfertilized egg cDNA library (Wright et al., 1991) with an antibody specific for p80 katanin, anti-p81^{aff} (McNally et al., 1996). The insert of the initial clone was used to isolate a longer cDNA clone (pFM18) from the same library by plaque hybridization. A cDNA clone encoding the 5′ end of p80 katanin (pFM23) was obtained by anchor-ligated PCR (Apte and Siebert, 1993) using primers derived from pFM18 sequences and reverse transcription reactions utilizing S. purpuratus unfertilized egg mRNA as template. A full-

length p80 cDNA (GENBANK accession #AF052433) was generated by joining the inserts of pFM18 and pFM23 at a common BstX1 site.

BLAST searches of GENBANK with p80 sequences revealed homology with a human infant brain cDNA (GENBANK accession#: T16102) which was obtained from Dr. James Sikela (University of Colorado Health Sciences Center) and sequenced. Sequences obtained from the T16102 clone were used to obtain multiple 3′ end cDNA clones by 3′ RACE from HT1080 (human fibrosarcoma) total RNA. An overlapping cDNA clone (pFM54) containing the translation start site was obtained by PCR amplification from an adult human hippocampal cDNA library (Stratagene, Inc.). Sequence analysis of partial cDNAs PCR amplified from HT1080 total RNA or from the hippocampal library were over 98% identical in predicted a.a. sequence. The complete DNA sequence of human p80 katanin is available from GENBANK (accession #AF052432).

Antibody Production and Immunoprecipitation

The full-length S. purpuratus p60 cDNA coding sequence was inserted into pMAL-C2 (New England Biolabs) and expressed as a C-terminal fusion to maltose binding protein in E. coli. Soluble MBP-p60 fusion protein was purified on an amylose affinity column, eluted with maltose, and injected into rabbits (antiserum production by BABCO, Berkely, CA). To select p60-specific antibodies that do not react with other AAA members, antibodies recognizing the N-terminal non-AAA domain of p60 were affinity purified on an Affi-Gel column coupled with the N-terminal residues 1-152 of p60 fused to MBP (Harlow and Lane, 1988). The resulting affinity-purified antibody recognized a single 60 kDa polypeptide in immunoblots of S. purpuratus unfertilized egg extract.

To prepare a specific antibody to human p80 katanin, the full length human p80 cDNA was ligated into the E. coli expression vector, pET-28a$^+$ (Novagen) as a BamH1-XhoI fragment. The protein was expressed and then purified in a denatured state in 8M urea by nickel chelate chromatography on His-Bind Resin (Novagen). Rabbits were immunized with polyacrylamide slices containing SDS-PAGE resolved human p80 katanin. Resulting serum was affinity purified with CNBr Sepharose-coupled, bacterially-expressed human p80 katanin. The resulting affinity purified antibody recognized a single 80 kDa polypeptide in immunoblots of SDS-solubilized Hela cells (not shown).

For immunoprecipitations used to demonstrate association of baculovirus-expressed S. purpuratus p60 and p80, affinity purified anti-p60 antibodies were covalently crosslinked to protein A Sepharose using 20 mM dimethylpimilidate (Harlow and Lane, 1988). After equilibration in TBST, 20-40 ml of antibody beads were added to katanin samples diluted in TBST containing 1-2 mg/ml soybean trypsin inhibitor (SBTI). The immunoprecipitations were incubated at 4° C for 1-2 hr, washed five times with 1 ml of ice-cold TBST, and eluted in SDS-containing sample buffer.

Baculovirus Expression and Purification of Katanin

Katanin subunits were expressed using the Bac-to-Bac^{TM} baculovirus expression system (Life Technologies), a commercial version of the site-specific transposition system for making recombinant baculovirus (Luckow et al., 1993). p60 and p80 cDNA coding sequences were each PCR amplified (Expand polymerase, Boehringer Mannheim) and then subcloned separately into pFastBac HT, which resulted in the fusion of a 6xHis Ni^{2+} binding sequence to the N-terminus of both p80 and p60. A p60-p80 coexpression virus was made by cloning the complete p60-FastBac HT and p80-FastBac HT

coding regions into the transfer vector, pDual. Recombinant baculovirus were prepared according to the Life Technologies protocol.

Sf9 cells were grown in SFM-900 II SFM (Life Technologies) supplemented with 100X antibiotic/antimycotic (Life Technologies) to 0.5X using the shaker culture method (Weiss et al., 1995). Expression of katanin subunits was performed in 1 l flasks containing 200-300 ml of media using a multiplicity of infection of 0.5-1.0 pfu/cell. The cells were harvested at approximately 72 hr post infection by low speed centrifugation and resuspended in lysis buffer (50 mM Tris pH 8.5, 300 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM ATP, 1 mg/ml pepstatin, 1 mg/ml leupeptin, 1 mg/ml aprotinin) before freezing in liquid nitrogen, and storage at -80°C.

To purify the expressed subunits, frozen cells were thawed and DNA was sheared by two passes through a Bio-Neb Cell Disrupter [100 psi helium, 13 l/min]. Cell debris was removed by centrifugation (40,000 g for 45 min). Subunits were bound in batch to Ni^{2+}-NTA Superflow (QIAGEN), washed [20 mM Tris pH 8.0, 1 M NaCl, 2 mM $MgCl_2$, 40 mM imidazole, 0.02% Triton X-100, 10 mM 2-mercapotethanol, 0.5 mM ATP] and eluted [20 mM Tris pH 8.0, 100 mM NaCl, 150 mM imidazole, 2 mM $MgCl_2$, 0.02% Triton X-100, 10 mM 2-mercaptoethanol, 100 μM ATP], followed by freezing in liquid nitrogen. Additional purification was sometimes performed by anion-exchange chromatography. Katanin concentrations were estimated by comparison with BSA standards using either a commercial Bradford reagent (Bio-Rad) or by densitometric analysis of Coomassie-stained SDS-PAGE gels with NIH-IMAGE after image capture on a CCD-based imaging system (Foto/Analyst, Fotodyne).

Electron Microscopic Imaging

Proteins were adsorbed to mica, freeze-dried, and platinum replicated according to established procedures (Heuser, 1989; Heuser, 1983). Sample preparation and imaging were similar to that used in the imaging of NSF (Hanson et al., 1997), except that mica flakes were washed with a buffer consisting of 10 mM K-HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM nucleotide (ATP or ATP-γ–S). Images were processed using Adobe Photoshop and displayed at 300,000X.

ATPase Assays

ATPase activity was measured by a modified malachite green method (Kodama et al., 1986). ATPase reactions of 50-100 ml were carried out in a buffer previously used for measuring the ATPase activity of native katanin [20 mM K-HEPES pH 8.0, 25 mM K-Glutamate, 2 mM $MgCl_2$, 10% glycerol (v/v), 0.02% Triton X-100 (w/v), 1 mg/ml BSA] (McNally and Vale, 1993), except that soybean trypsin inhibitor (SBTI) was replaced by BSA as a carrier protein because SBTI increased background phosphate contamination. An ATP regenerating system consisting of 0.5-1.0 mM phospho-enol pyruvate and 2U pyruvate kinase was included to minimize the inhibition by ADP observed previously for native katanin (McNally and Vale, 1993) . Microtubules were prepared from bovine brain tubulin (Hyman et al., 1990; Williams and Lee, 1982). After assembly, microtubules were sedimented (230,000 x g; 10 min), resuspended in ATPase buffer lacking BSA, and the polymers were resuspended by repeated passage through a 27 gauge needle. Microtubule concentration was determined by measuring the absorbance at 275 nm in 6M guanidine HCl by using a molecular mass of 110 kDa and an extintion coefficient of 1.03 $ml*mg^{-1}*cm^{-1}$ (Hackney, 1988). ATPase reactions were carried out at room temperature, and were initiated by addition of katanin.

Severing Assays

Microscope-based severing assays were performed using previously published procedures (McNally and Vale, 1993), except that microtubules were immobilized by first perfusing flow cells with a bacterially expressed kinesin mutant that binds strongly to microtubules but is unable to hydrolyze ATP (K560, G234A mutant; R. Vale and E. Taylor, unpublished results). Assays were performed in 20 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mM ATP with an oxygen scavenger system consisting of glucose oxidase (220 mg/ml), catalase (36 mg/ml), glucose (22.5 mM), and 2-mercaptoethanol (71.5 mM). Images were captured using a cooled, slow-scan CCD (Photometerics) and processed using Adobe Photoshop.

DAPI severing assays were performed using conditions where the change in fluorescence intensity was linear with the amount of tubulin polymer added (Heusele et al., 1987). Severing reactions contained 2 μM microtubules (polymerized and resuspended in ATPase buffer as above) were incubated with 10 μM DAPI, along with 1 mM ATP, 10 mM phospho-enol pyruvate, 250 mg/ml pyruvate kinase (Boehringer Mannheim), and 1 mg/ml BSA. The reaction volume was 80 ml, and fluorescence intensity was measured by exciting at 370 nm and measuring the emission at 450 nm using a model 8100 fluorimeter (SLM Instruments) in photon counting mode.

In vitro Translation Co-Immunoprecipitation

In order to facilitate the non-radiactive detection of in vitro translated p60 and p80, each cDNA was ligated into the vector pCITE-4a+ (Novagen) such that the proteins would be translated in frame with a 37 a.a. N-terminal S-Tag. In vitro synthesis of proteins directly from plasmid DNAs was accomplished using the Single Tube Protein System 2, T7 (Novagen). For co-immunoprecipitation assays, p60 and p80 constructs were usually co-

expressed. However, identical results were obtained if the constructs were expressed separately and then incubated together for 30 min at room temperature. For immunoprecipitations, lysates were incubated on ice with Pansorbin (Calbiochem)-antibody complexes, washed in NET buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA (pH 8.0), 0.25% gelatin 0.02% sodium azide), then resuspended in SDS-PAGE sample buffer. In vitro translation products in both the pellets and supernatants from the immunoprecipitations were resolved by SDS-PAGE, transferred to nitrocellulose, probed with S-protein HRP conjugate (Novagen) and detected by chemiluminescence.

Cell Culture and Transfections and Immunofluorescence

To allow transient expression of a human p80 WD40-GFP fusion protein in HeLa cells, a DNA fragment containing a.a. 1-263 of human p80 katanin was generated by PCR amplification, placing a BamH1 site and a Kozak consensus at the predicted translation start and an EcoR1 site after the codon for a.a. 263. This BamH1-EcoR1 fragment was ligated into the GFP fusion vector pEGFP-N1 (Clontech).

Both MSU1.1 and Hela cells were grown on 18 mm glass coverslips in Optimem medium (Life Technologies) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Plasmids were transfected using Superfect Reagent (Qiagen) for 2 hr after which coverslips were washed with PBS and placed in fresh culture medium at 37°C with 5% CO_2 for 1-24 hr.

For imaging of GFP-fluorescence and immunofluorescence with the human p80 katanin antibody or with the γ–tubulin antibody, monoclonal GTU88 (Sigma Chemical), cells on coverslips were fixed either in -20°C methanol or in 0.5X PBS, 3.7% formaldehyde, 75% methanol at 22°C for 10 min followed by rehydration in TBST. Antibody labelling was carried out in

TBST containing 4% BSA. Images were captured with a Nikon Microphot SA microscope, 100x Plan Fluor 1.3 objective, Photometrics Quantix camera and IP Lab Spectrum software (Scanalytics).

ACKNOWLEDGMENTS

We thank C. Walczak for helpful comments on the manuscript. This work was supported in part by grants GM53060 and GM49752 from the N.I.H. to F.J.M. and R.D.V. respectively. J.J.H. was partially supported by a NSF predoctoral fellowship.

Figure 1: Sequence analysis of p60 katanin.

(**A**) Predicted protein sequence of the S. purpuratus katanin p60 subunit (GENBANK AF052191). Sequences obtained by direct peptide microsequencing are underlined. Differences between the predicted peptide sequence and that obtained by direct sequencing are indicated by doubled underlines (S95 was reported as F, H99 was reported as P, and P138 was reported as T). The Walker A (P-loop) motif is shaded. (**B**) Amino acid sequence alignment of the p60 AAA domain with AAA members mei-1 (C. elegans, GenBank L25423), Sug1p (S. cerevisiae, GenBank X66400), ftsH (E. coli, GenBank M83138), Pas1p (S. cerevisiae, GenBank M58676), and NSF (C. longicaudatus, GenBank X15652). Identical residues are shaded black, residues conserved in >60% of the shown members are shaded gray. Left hand numbering indicates the amino acid residue in the corresponding sequence. Alignment was performed using PILEUP (Genetics Computer Group) and the output was shaded using MACBOXSHADE.

A

```
1    MSVDEICENTKMGREYALLGNYETSLVVYYQGVLQQIQKLL
41   TSVHEPQRKHQWQTIRQELSQEYEHVKNITKTLNGFKSEP
81   AAPEPAPNHRAAPFSHHQHAAKPAAAEPARDPDVWPPPTP
121  VDHRPSPPYQRAARKDPPRRSEPSKPANRAPGNDRGGRGP
161  SDRRGDARSGGGGRGGARGSDKDKNRGGKSDKDKKAPSGE
201  EGDEKKFDPAGYDKDLVENLERDIVQRNPNVHWADIAGLT
241  EAKRLLEEAVVLPLWMPDYFKGIRRPWKGVLMV
281  MLAKAVATECGTTFFNVSSASLTSKYHGESEKLVRLLFE
321  MARFYAPSTIFIDEIDSICSKRGTGSEHEASRRVKSELLI
361  QMDGVSGPSAGEESSKMVMVLAATNFPWDIDEALRRRLEK
401  RIYIPLPEIDGREQLLRINLKEVPLADDIDLKSIAEKMDG
441  YSGADITNVCRDASMMAMRRRIQGLRPEEIRHIPKEELNQ
481  PSTPADFLLALQKVSKSVGKEDLVKYMAWMEEFGSV
```

B

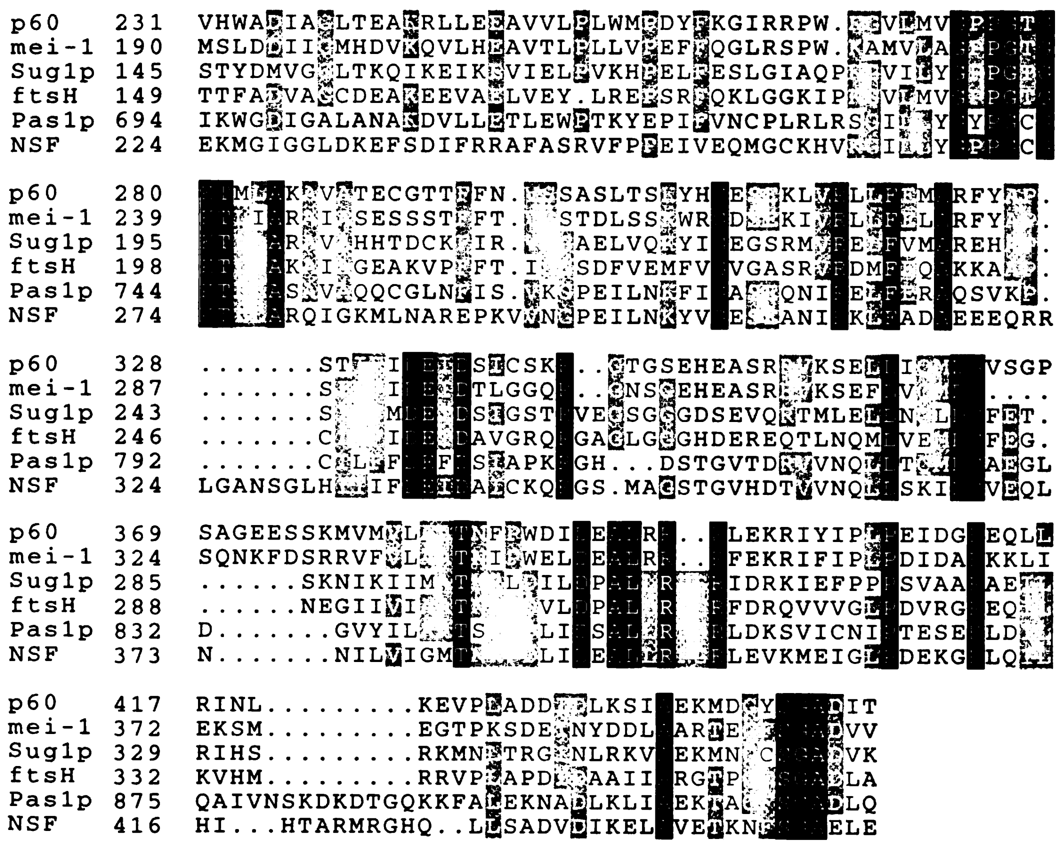


Figure 2: Sequence analysis of p80 katanin.

(**A**) Predicted protein sequence of the S. purpuratus katanin p80 subunit (GENBANK AF052433). Sequences obtained by direct peptide microsequencing are underlined. Differences between the predicted peptide sequence and that obtained by direct peptide sequencing, or differences found between 2 different p80 cDNA clones are indicated by double underlines. (**B**) Amino acid sequence alignment of the WD40 repeat region of p80 with a putative human ortholog of p80 (Hs p80, GenBank AF052432), TFIID (Homo sapiens, GenBank U80191), and putative serine/threonine kinase PkwA (Thermomonospora curvata, GenBank P49695). Identical residues are shaded black, residues found in at least 2 sequences are shaded in grey. Left hand numbering indicates the amino acid residue in the corresponding sequence. Alignment was performed using PILEUP (Genetics Computer Group) and the output was shaded using MACBOXSHADE.

A

```
1     MATKRAWKLQELVAHSSNVNCLALGPMSGRVMVTGGEDKK
41    VNLWAVGKQNCIISLSGHTSPVDSVKFNSSEELVVAGSQS
81    GTMKIYDLEPAKIVRTLTGHRNSIRCMDFHPFGEFVASGS
121   TDTNVKLWDVRRKGCIYTYKGHSDQVNMIKFSPDGKWLVT
161   ASEDTTIKLWDLTMGKLFQEFKNHTGGVTGIEFHPNEFLL
201   ASGSSDRTVQFWDLETFQLVSSTSPGASAVRSISFHPDGS
241   YLFCSSQDMLHAFGWEPIRCFDTFSVGWGKVADTVIASTQ
281   LIGASFNATNVSVYVADLSRMSTTGIAQEPQSQPSKTPSG
321   GAEEVPSKPLTASGRKNFVRERPHTTSSKQRQPDVKSEPE
361   RQSPTQDEGVKDDDATDIKDPDSYAKIFSPKTRVDHSPER
401   NAQPFPAPLDVPGAQEPEPFKHPPKPAAAAAVAPVSRAPA
441   PSASDWQPAQANPAPNRVPAATKPVPAQEVAPSRKPDPIS
481   TIIPSDRNKPANLDMDAFLPPAHAQQAPRVNAPASRKQSD
521   SERIEGLRKGHDSMCQVLSSRHRNLDVVRAIWTAGDAKTS
561   VESVVNMKDQAILVDILNIMLLKKSLWNLDMCVVVLPRLK
601   ELLSSKYENYVHTSCACLKLILKNFTSLFNQNIKCPPSGI
641   DITREERYNKCSKCYSYLIATRGYVEEKQHVSGKLGSSFR
681   ELHLLLDQLE
```

B

```
Sp p80      4
Hs p80      8
Hs TFIID  531
Tc PkwA   489

Sp p80     46
Hs p80     50
Hs TFIID  572
Tc PkwA   530

Sp p80     88
Hs p80     92
Hs TFIID  614
Tc PkwA   572

Sp p80    130
Hs p80    134
Hs TFIID  656
Tc PkwA   614

Sp p80    172
Hs p80    176
Hs TFIID  698
Tc PkwA   655

Sp p80    214
Hs p80    218
Hs TFIID  740
Tc PkwA   697
```

Figure 3: Expression and purification of recombinant katanin subunits.

(**A**) Coomassie-stained SDS-PAGE analysis of expressed katanin subunits. 6xHis-tagged katanin subunits were purified from lysates of baculovirus-infected insect cells by binding to Ni^{2+}-NTA Superflow followed by elution with imidazole, as described in the Experimental Procedures. Cells were infected with either p60 virus alone, p80 virus alone, or coinfected with equal amounts of p60 and p80 viruses. (**B**) Immunoprecipitation was performed on extracts of insect cells coinfected with p60- and p80-expressing baculoviruses using affinity-purified p60 antibody crosslinked to protein A agarose. Proteins bound to the resin were analyzed by SDS-PAGE followed by staining with Coomassie. This immunoprecipitate shows that baculovirus-expressed p60 and p80 form a complex with equal stoichiometry.

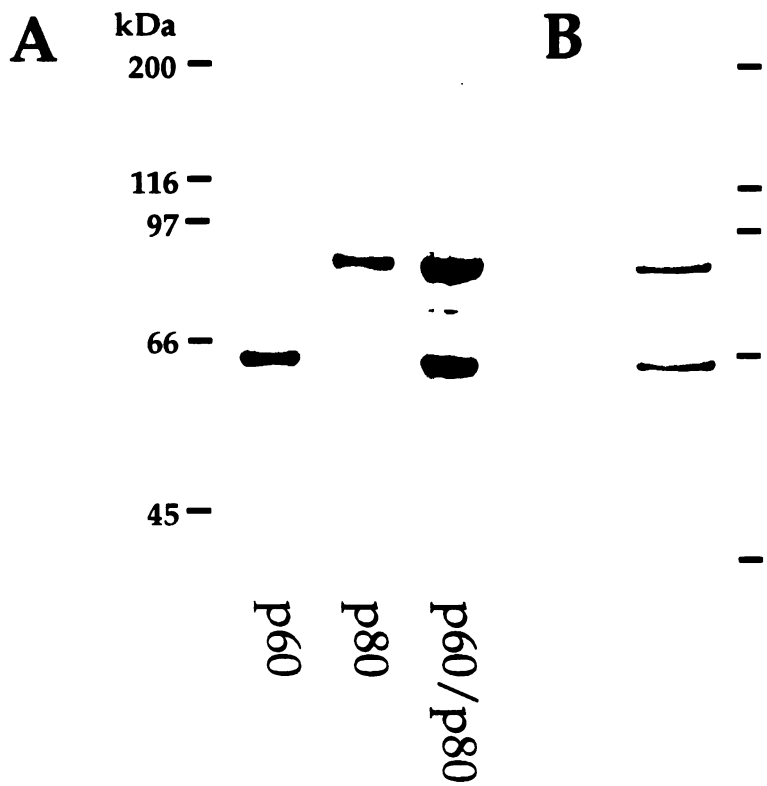

A
kDa
200
116
97
66
45
p60
p80
p60/p80
B

Figure 4: Structure of katanin as visualized by rotary-shadowing electron microscopy.

Panel A shows 14-16 nm diameter rings observed in preparations of recombinant p60. **Panel B** shows single particles of recombinant p80; occasional aggregates are seen (rightmost picture) but rings are never observed. **Panel C and D** shows different rings observed in recombinant p60/p80 preparations. Panel C shows a "splayed" complex, consisting of a central p60-like ring surrounded by a halo particles that resemble p80. In **panel D**, intact 20 nm diameter rings are seen with bright edges, suggesting they extend >10 nm above the mica surface. All images are shown at 300,000X. The dimensions indicated above include the platinum shadowing, which typically adds 2 nm of material to the protein surface.

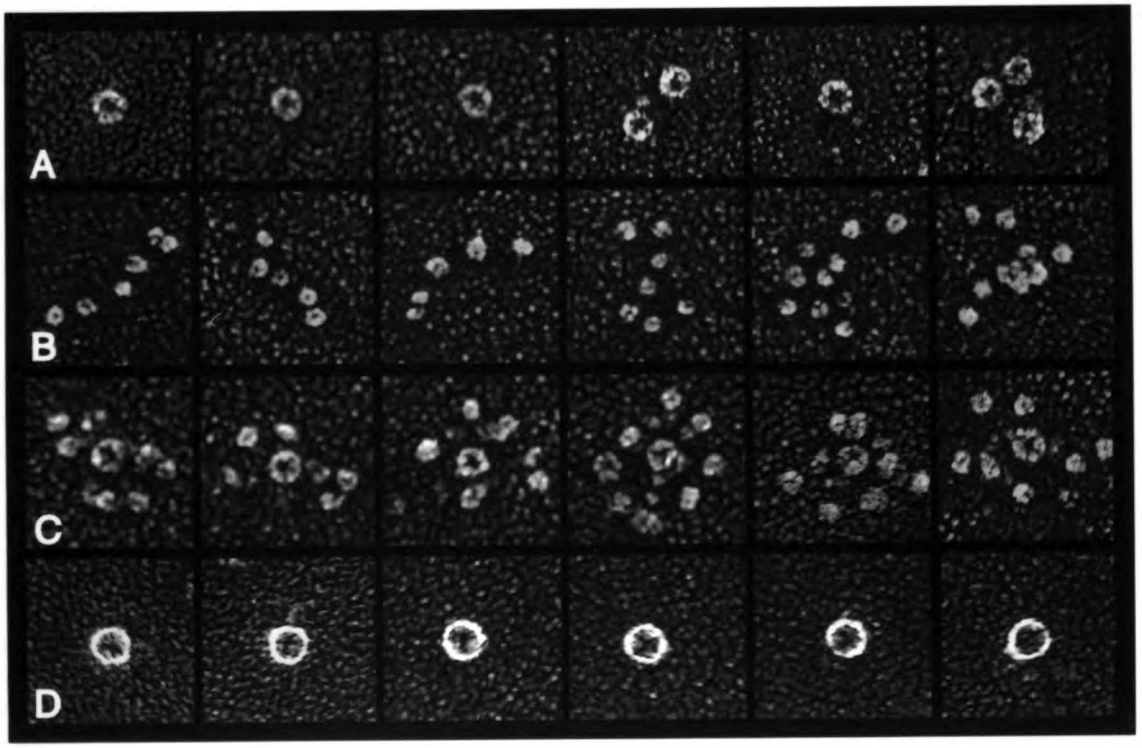

A
B
C
D

Figure 5: Activities of recombinant katanin subunits.

(**A**) ATPase activities of 0.04 μM p60 katanin (9) and co-expressed p60/p80 (7) were determined at various microtubule concentrations as described in the Experimental Procedures. Both p60 katanin and p60/p80 show similar patterns of microtubule stimulation, with p60 katanin having approximately one half of the maximally-stimulated ATPase activity of p60/p80. The insert in the upper right shows the stimulation of ATPase activity at low (0-2 μM) microtubule concentration. (**B**) Microtubule severing activity of recombinant katanin subunits. Paclitaxel-stabilized, rhodamine-labeled microtubules were adsorbed onto the surface of a microscope perfusion chamber, and then recombinant katanin subunits were introduced. The time elapsed after perfusing p60/p80 (0.1 μM), p60 (0.1 μM), or p80 (0.5 μM) is shown. The recombinant co-expressed p60/p80 and p60, but not p80, can sever and disassemble microtubules. Scale bar, 10 μm. (**C**) Quantitative measurement of microtubule disassembly using a DAPI fluorescence assay. MT indicates microtubules (2 μM) without added protein, and tubulin indicates microtubules that had been depolymerized by treatment with 10 mM $CaCl_2$ on ice for 1.5 hr. p60 katanin and p60/p80 were added at 0.2 μM concentration, and the fluorescence change as a function of time after protein addition is shown. p80 did not cause a change in fluorescence that was different from that shown for microtubules alone.

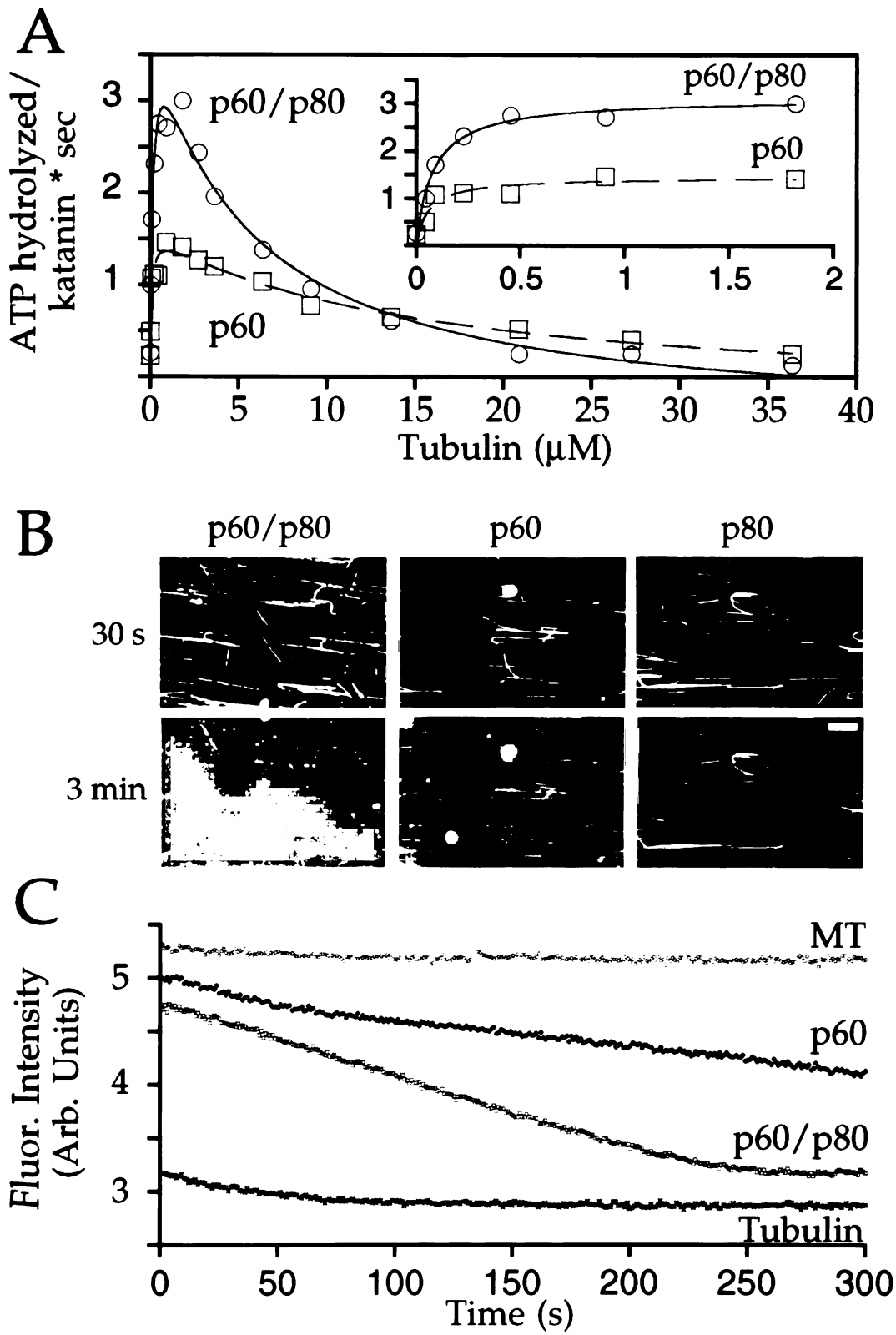

A
ATP hydrolyzed/ katanin * sec
p60/p80
p60
Tubulin (μM)
B
p60/p80
p60
p80
30 s
3 min
C
Fluor. Intensity (Arb. Units)
MT
p60
p60/p80
Tubulin
Time (s)

Figure 6: The WD40 repeats of p80 katanin are not required for interaction with p60 katanin.

Epitope-tagged derivatives of p80 and p60 were synthesized in vitro in a combined transcription-translation reaction. p60 and interacting proteins were immunoprecipitated with a p60-specific antibody and the resulting immunoprecipitates were resolved by SDS-PAGE and blotted to nitrocellulose. In vitro translated proteins were detected by chemiluminescence as described in Experimental Procedures. Lane 1: molecular weight standards Mr: 100,000, 75,000, 50,000, 35,000 and 25,000; lane 2: p60 co-translated with full-length p80; lane 3: p60 co-translated with the Δ560-690 derivative of p80; lane 4: p60 co-translated with the Δ1-302 derivative of p80; lane 5: p60 co-translated with the Δ303-690 derivative of p80. The structure of each deletion derivative of p80 is shown at right. The Δ560-690 and Δ303-690 translation products were detected in the supernatants of the immunoprecipitations (not shown).

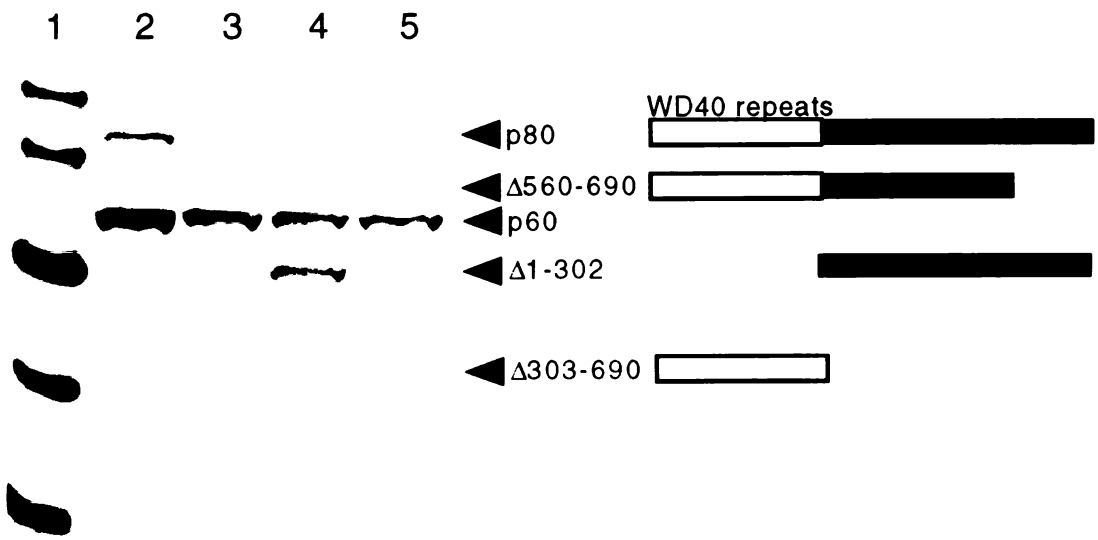
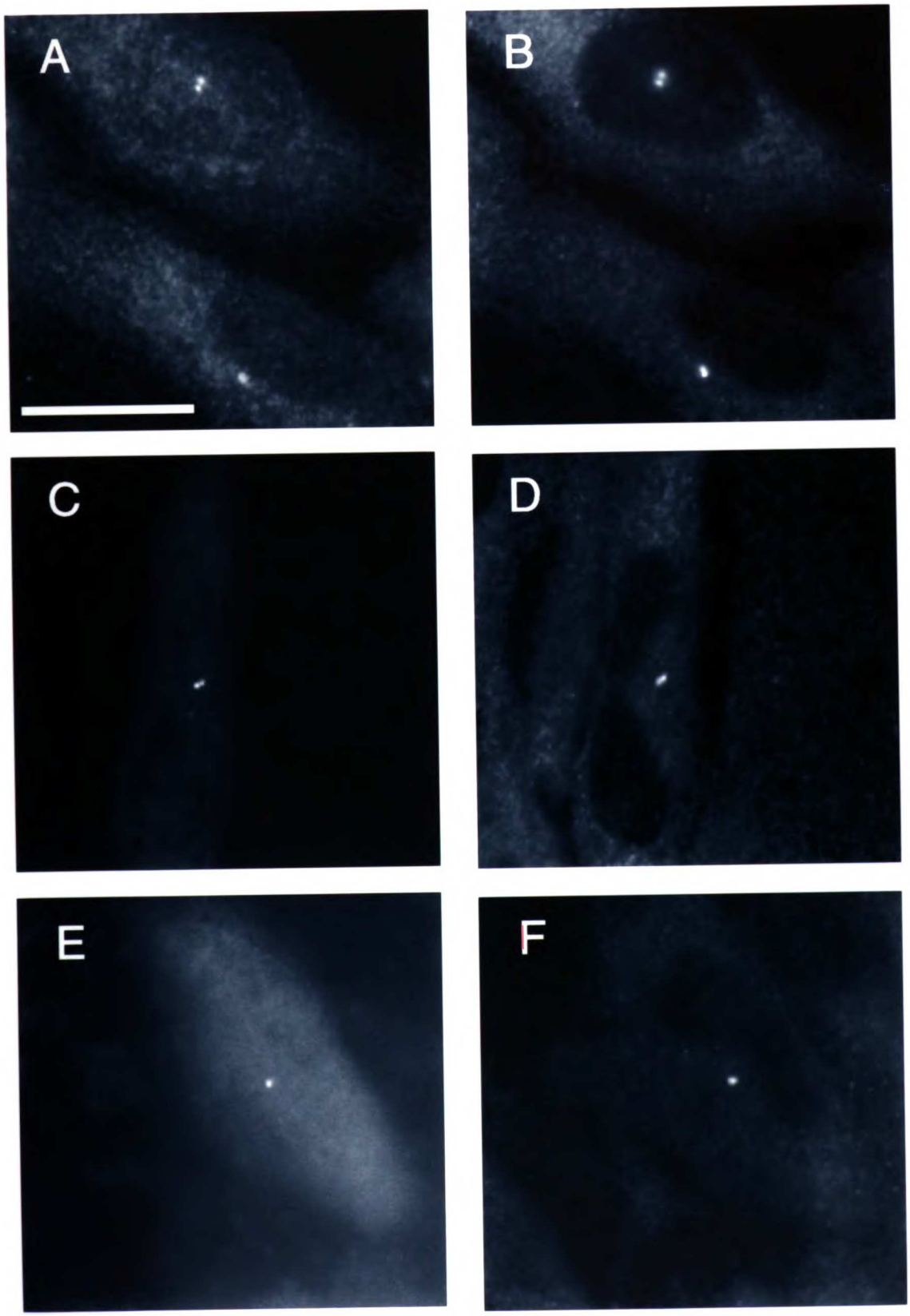

1
2
3
4
5
WD40 repeats
p80
Δ560-690
p60
Δ1-302
Δ303-690

Figure 7: Human p80 katanin and a fusion protein of the human p80 WD40 domain with GFP co-localize with γ–tubulin at centrosomes of MSU1.1 human fibroblasts.

A and B: Co-localization of immunofluorescence staining by a human p80 katanin-specific antibody (**A**) and a γ–tubulin specific antibody (**B**). **C-F:** Co-localization of GFP fluorescence (**C** and **E**) with staining by a γ-tubulin-specific antibody (**D** and **F**). Co-localization to two centrosomes is seen in C and D while co-localization to a single centrosome is seen in E and F. The apparently higher background of cytoplasmic green fluorescence in E relative to C is a display artifact. The fluorescence intensity of the centrosomes in C is at least 5 fold greater than that of the centrosome in E. The p80 antibody was detected with an Oregon Green 488 second antibody and the γ–tubulin antibody was detected with a Texas Red-X second antibody. Fluorescence signals were separated with fluorescein and Texas Red filter sets (Chroma Technologies). Bar = 14 μm.


A
B
C
D
E
F

CHAPTER 2

A Nucleotide-Dependent Oligomerization Cycle by the AAA Enzyme Katanin Drives Microtubule Disassembly

ABSTRACT

Katanin, a member of the AAA ATPase superfamily, uses nucleotide hydrolysis energy to sever and disassemble microtubules. Many AAA enzymes also are involved in disassembling stable protein-protein complexes, but their mechanism is not well understood. Using a fluorescence resonance energy transfer assay, we show that p60 oligomerizes in an ATP- and substrate (microtubule)-dependent manner. Oligomerization both increases katanin's affinity for microtubules and activates its ATPase activity. ATP hydrolysis and phosphate release by microtubule-bound katanin oligomers cause tubulin disassembly followed by the dissociation of katanin to free monomers. Coupling of a nucleotide-dependent oligomerization cycle to the disassembly of a target protein complex may be a general feature of ATP-hydrolyzing AAA domains.

INTRODUCTION

Microtubules, polymers of a and b tubulin subunits, form the mitotic spindle and organize membranous organelles in interphase cells. To accomplish these disparate functions, the microtubule cytoskeleton must be capable of rapidly organizing into different configurations. The ability of pure microtubules to undergo spontaneous growth and shrinkage at their ends, even at steady state, contributes to the cellular rearrangements of these polymers (Desai and Mitchison, 1997; Mitchison and Kirschner, 1984). In addition to end dynamics, the microtubule wall can be disrupted by the severing enzyme katanin (McNally and Vale, 1993). Potential *in vivo* roles for katanin-mediated microtubule severing include releasing microtubules from centrosomes (Keating et al., 1997), depolymerizing microtubule minus ends in the mitotic spindle as a component of poleward flux (Mitchison, 1989), and accelerating microtubule turnover at the G2/M transition by creating unstable microtubule ends (Zhai et al., 1996).

Katanin is a microtubule-stimulated ATPase (McNally and Vale, 1993), and ATP hydrolysis is required for katanin-mediated severing. ATP energy is needed for this reaction, since the microtubule wall is inherently stable. Each tubulin dimer forms longitudinal bonds with other dimers in a single protofilament, and lateral bonds between dimers hold adjacent protofilaments together (Nogales et al., 1999; Nogales et al., 1998). As a result, subunit loss occurs ~10^{11} more slowly from the microtubule wall than from the ends (Dye et al., 1992). In the presence of katanin and ATP, however, the microtubule wall can be disassembled within a minute, even when stabilized by paclitaxel or nonhydrolyzable GTP analogs (McNally and Vale, 1993). The

released tubulin subunits are not denatured or proteolyzed, since they are competent to reassemble (McNally and Vale, 1993).

Katanin is a heterodimer organized into an enzymatic subunit (p60) that carries out the ATPase and severing reactions and a targeting subunit (p80) that localizes katanin to the centrosome (Hartman et al., 1998). The sequence of p60 reveals that it belongs to the AAA ATPase superfamily, members of which share one or two copies of a conserved 230 amino acid ATPase domain (Confalonieri and Duguet, 1995; Lenzen et al., 1998; Yu et al., 1998). AAA proteins have been implicated in a myriad of cellular processes as diverse as membrane targeting (e.g. NSF, VPS4, p97), organelle biogenesis (PAS1p), proteolysis (SUG1), and transcriptional regulation (TBP1) (Patel and Latterich, 1998). One hypothesis unifies the AAA proteins as a family of nucleotide-dependent chaperones that can disassemble specific protein complexes or unfold polypeptides (Confalonieri and Duguet, 1995). However, little is known about how changes in the nucleotide state of the AAA domain are coupled to alterations in the structure of the protein targets. Katanin constitutes a good model system for understanding AAA-mediated disassembly, since it can be expressed in a functional form and its target (the microtubule) has been well characterized.

RESULTS

Previous work on the AAA protein NSF indicated that it forms a hexameric ring (Fleming et al., 1998; Hanson et al., 1997), and that conformational changes in the shape of the ring may be important for its disassembly of SNARE complexes (Hanson et al., 1997). p60 katanin preparations also contain 14-16 nm rings that are similar in shape and dimension to NSF (Hartman et al., 1998). However, while electron microscopy data suggested that p60 could self-associate, we found that GFP-p60 (Fig. 1A) and p60 (data not shown) migrated primarily as monomers (4S) in 10-35% glycerol gradients in ATP, ADP, and ATP-γ–S. GFP-p60 also migrated as monomeric species by gel filtration (Stokes radius 66Å). As a control, the NSF D2 AAA domain migrated at 8S, which is the size expected for a hexamer of 30 kDa AAA subunits (Fleming et al., 1998; Hanson et al., 1997). Therefore, in contrast to NSF, p60 does not form stable hexameric rings. Rather, these data, taken together with the prior electron microscopy, suggest that p60 monomers and oligomers exist in a reversible equilibrium and that the population of rings may not be stable to the time/dilution effects of sedimentation and gel filtration.

To examine the dynamics of katanin ring formation in solution as well as in the presence of its microtubule substrate, we developed a new fluorescence resonance energy transfer (FRET) based assay. To achieve stoichiometric labeling at a defined location on the p60 molecule, we prepared p60 fusions with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as the donor-acceptor pair (Miyawaki et al., 1997). The half-maximal energy transfer distance, R_0, for the CFP and YFP pair is ~5 nm (R. Tsien, personal communication), which is similar to the intrasubunit

distances within the AAA ring (Lenzen et al., 1998; Yu et al., 1998). To test this FRET assay, we prepared a DEAD box mutant (E334Q) of CFP-p60 and YFP-p60 which was designed to block nucleotide hydrolysis and trap the enzyme in the ATP state. An equivalent mutation abolishes the ATPase and membrane fusion activities of NSF (Whiteheart et al., 1994) and promotes oligomerization of VPS4, a single AAA domain protein involved in vacuolar targeting (Babst et al., 1998). We confirmed that $p60^{E334Q}$ has no detectable ATPase activity (data not shown). When CFP- and YFP-$p60^{E334Q}$ fusion proteins were mixed together in the presence of ADP, no energy transfer occurred and the emission of the CFP/YFP mixture was identical to the proteins tested separately (data not shown). However, in solutions containing ATP, the CFP/YFP-$p60^{E334Q}$ mixture showed a reduced CFP emission and correspondingly enhanced YFP emission, which is indicative of FRET (Fig. 1B). This result indicates that p60 subunits can associate into an oligomeric complex when ATP is present in the active site.

To confirm the conclusion from the above FRET experiment, the oligomeric state of CFP-$p60^{E334Q}$ was determined by hydrodynamic analysis. In the presence of ATP, CFP-$p60^{E334Q}$ sedimented at 4S and 15S in glycerol gradients (Figure 1A). The 15S complex dissociated to 4S monomers when the enzyme was incubated with 2 mM ADP. To determine the oligomeric state of the 15S complex, gel filtration of CFP-$p60^{E334Q}$ was performed in the presence of ATP, which yielded a major peak with a Stokes radius of 8.6 nm. The Stokes radius and sedimentation coefficient predict a molecular weight of 520 kDa, which is consistent with CFP-$p60^{E334Q}$ forming a hexamer of 90 kDa subunits in the presence of ATP. Thus, the FRET assay accurately reports on the nucleotide-dependent hexamer formation of $p60^{E334Q}$.

Using the FRET assay, we sought to determine whether microtubules and/or ATP analogs could promote oligomerization of wildtype p60. ATP-γ-S, a poorly hydrolyzable nucleotide, was used to mimic the ATP state, since it inhibits katanin ATPase activity (McNally and Vale, 1993), and since both ATP and ATP-γ-S supported similar levels of FRET in $p60^{E334Q}$ (data not shown). Little or no energy transfer occurred in the absence of microtubules regardless of the nucleotide present (Table I). However, we observed a FRET signal when p60 katanin was incubated with microtubules and ATP-γ–S but not ADP (Table I). The no nucleotide state (apyrase added) produced a similar result to that of ADP (not shown). We therefore conclude that both nucleotide (ATP) and substrate (microtubules) cooperate in stimulating the oligomerization of p60.

What are the consequences of hexameric ring formation for the katanin enzymatic cycle and its interaction with microtubules? Using a microtubule cosedimentation assay, we found that GFP-p60 bound to microtubules with high affinity (K_d~0.9 μM) in the presence of ATP-γ–S, whereas the affinity was reduced 20-fold (K_d~18 μM) in the presence of ADP (Fig. 2). High affinity binding may require the microtubule polymer, since oligomeric CFP-$p60^{E334Q}$ did not co-elute with monomeric tubulin on gel filtration columns (data not shown). To assess how the AAA domain influences microtubule binding by p60 katanin, we expressed and analyzed the microtubule affinity of the N-terminal domain of p60 lacking the AAA domain, fused to the N-terminus of GFP (p60ΔAAA). Based upon prior results with NSF (Nagiec et al., 1995), we postulated that this N-terminal region would constitute the microtubule binding portion of p60. Indeed, p60ΔAAA bound to microtubules in a nucleotide-insensitive manner (data not shown) and with an intermediate affinity between the GFP-p60 ATP and

ADP states. Thus, the AAA domain appears to affect the binding affinity of the adjacent microtubule binding domain. In conclusion, p60 cycles between tight and weak microtubule-binding states by undergoing nucleotide-dependent transitions in the AAA domain; tight binding occurs in nucleotide states (ATP or ATP analogs) that stabilize p60 rings.

Native katanin (data not shown) and baculovirus-expressed p60 and p60/p80 display an unusual microtubule-stimulated ATPase reaction in which the activity peaks at 2-10 μM microtubules and then decreases as the microtubule concentration is further increased (Hartman et al., 1998). This differs from the expected Michaelis-Menten hyperbolic stimulation that, for example, is typical of microtubule motor proteins (Gilbert and Johnson, 1993). One explanation for this behavior is that ATPase activation is driven by hexamer formation and that the degree of oligomerization is determined by a competition between p60-p60 and p60 monomer-MT associations. To test this idea, we simultaneously measured the FRET and ATPase activities of p60 as a function of microtubule concentration. Both ATPase activity and FRET signal increased together as the microtubule concentration was raised, and then declined in a similar manner at higher microtubule concentrations (Fig. 3A). In agreement with the ATPase measurements, we also found that microtubule disassembly by katanin was inhibited at a high microtubule to katanin ratio (Fig. 3B). These results strongly suggest that microtubules stimulate the activity of p60 by facilitating p60-p60 interactions. Conversely, high microtubule concentrations reduce ATPase and severing activity by preventing p60-p60 associations through the sequestration of p60 monomers at discontiguous, low affinity sites on the lattice.

DISCUSSION

The above results suggest a model for how katanin disrupts tubulin contacts within a microtubule wall (Fig. 4). Katanin-ADP is monomeric, but nucleotide exchange for ATP enhances p60-p60 affinity. Oligomerization is most efficient, however, in the presence of its protein substrate, suggesting that microtubules act as a scaffold for promoting oligomerization. The p60 ring is then bound to microtubules with high affinity, potentially as a result of forming multiple tubulin contacts. However, microtubule binding energy alone is insufficient to destabilize the microtubule lattice, since katanin in the presence of ATP-γ–S binds tightly to microtubules but does not sever or release tubulin subunits (data not shown). Once katanin oligomers assemble on the microtubule, their ATPase activity is stimulated, thereby enabling the enzymatic reaction to be coupled to the disassembly of its microtubule substrate. Nucleotide hydrolysis and subsequent phosphate release appear to serve two functions. First, these events may change the conformation of the katanin ring, leading to mechanical strain and destabilization of tubulin-tubulin contacts. Consistent with this idea, large conformational changes have been observed for NSF in its ATP and ADP states (Hanson et al., 1997). Second, following this conformational transition, the p60-p60 and p60-tubulin interactions weaken, dissociating tubulin and releasing p60-ADP to begin a new round of disassembly. This proposed cycle has similarities to that of dynamin, which self-assembles into a spiral pattern on endocytic membranes tubules, changes conformation after GTP hydrolysis in manner that vesiculates the tubule, and then disassembles in the GDP state (Sweitzer and Hinshaw, 1998).

The oligomerization cycle described for katanin may also occur in many other AAA enzymes. VPS4, a single AAA domain protein, oligomerizes when ATP is present and the protein concentration is elevated in vitro (Babst et al., 1998). It has been proposed that oligomerization of VPS4 could be facilitated physiologically by an as yet unidentified membrane-associated target (Babst et al., 1998), and this could now be tested using the FRET-based assay described in this study. Unlike katanin and VPS4, NSF is a constitutive hexamer (Fleming et al., 1998), due to the presence of an additional AAA domain (D2) that binds but does not hydrolyze ATP (Whiteheart et al., 1994). Our results, however, raise the possibility that the ATP-hydrolyzing AAA domains (D1) may undergo cycles of tight and weak interactions while remaining tethered in the NSF complex via their D2 domains. Thus, nonhydrolyzing AAA domains may serve as anchors to keep the enzymatic subunits in close proximity throughout the hydrolytic cycle.

AAA proteins have been suggested to act as chaperones (Confalonieri and Duguet, 1995; Leonhard et al., 1999), and certain mechanistic similarities are apparent with the chaperone GroEL. The stable GroEL ring complex binds misfolded polypeptides at multiple hydrophobic sites within its cavity, undergoes large interdomain motions that strain the bound polypeptide upon ATP binding, and then releases the polypeptide for refolding in the ADP state (Shtilerman et al., 1999; Xu et al., 1997). Similarly, AAA oligomers may undergo a concerted conformational change that dramatically alters polypeptides bound to the exterior of the ring. The disassembly of a protein complex by AAA proteins and the refolding of a polypeptide by GroEL also both appear to require the hydrolysis of many NTP molecules (50 ATPs per tubulin released by katanin (see Fig. 3 legend) and 50-150 ATPs per renaturation of a misfolded protein by GroEL (Sigler et al., 1998)). Measuring

the conformational state of tubulin during katanin-mediated disassembly may provide insight into whether AAA proteins transiently unfold a portion of their target proteins.

MATERIALS AND METHODS

Microtubules and tubulin

Bovine brain tubulin was prepared by two cycles of polymerization followed by chromatography on phosphocellulose P-11 (Whatman, Ltd.) (Williams and Lee, 1982) before freezing in liquid nitrogen and storage at -80°C. Thawed tubulin was precleared by centrifugation at 337,000 x g (10 min, 4°C) to remove aggregates. Microtubules were polymerized by addition of DMSO to 10% and GTP to 1 mM, followed by incubation at 37°C for 30-60 min. For most experiments, microtubules were stabilized by addition of paclitaxel to 20 μM, followed by an additional 15-30 min incubation at 37°C, before sedimentation at 267,000 x g (10 min, 25°C) through a 60% glycerol cushion in ATPase buffer (20 mM K-HEPES pH 7.5, 25 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 10% glycerol) containing 10 μM paclitaxel. Microtubules were resuspended in ATPase buffer containing 20 μM paclitaxel. Microtubule concentration was measured by absorbance at 275 nm in 6M guanidine HCl, using an extinction coefficient of 1.030 ml•mg-1•cm-1 (Hackney, 1988).

Expression constructs

Katanin p60 was cloned into the BamHI site of pFastBac HT B (Life Technologies, Inc.). The resulting baculovirus construct expresses p60 or p80 with the following additional N-terminal amino acids: MSYYHHHHHHDYDIPTTENLYFQGS. The GFP mut2 [B. P. Cormack et al., *Gene*, **173**, 33 (1996)] coding sequences were amplified with primers containing KasI sites at both ends, and inserted into the KasI site in pFastBac HT B. The resulting baculovirus constructs express GFP fused to the N-terminus of p60. ECFP (Clonetech, Inc.) and EYFP (Clonetech, Inc.) coding

sequences were similarly inserted into the pFastBac-p60 expression plasmid. The E334Q mutant was prepared by overlap extention amplification [S. N. Ho et al., *Gene* **77**, 51 (1989)] of the wildtype p60 cDNA, followed by cloning of the mutant sequence into pFastBac HT B and sequencing to check for amplification errors. The p60ΔAAA construct was prepared by amplifying the region coding for a.a. 1-210 of p60 and inserting this into pFastBac HT B at the BamHI and XhoI sites. The GFP coding sequence was amplified and inserted at KpnI and HindIII sites, resulting in a baculovirus that expresses p60 (a.a. 1-210) with the same additional N-terminal amino acids as wildtype p60, fused to the N-terminus of GFP.

Expression and purification of recombinant katanin

Recombinant katanin subunits were expressed by infection of Sf9 cells with baculovirus, followed by purification on Ni-NTA agarose as described (Hartman et al., 1998). Expressed katanin was frozen in liquid nitrogen and stored at -80°C. Inclusion of ATP at 250 μM before freezing was found to improve protein stability.

ATPase assays

ATPase activity was measured using either a radioactive assay following the release of ^{32}P from [γ–^{32}P]ATP (for native katanin) or a modified malachite green assay (for recombinant katanin)(Kodama et al., 1986). Reactions of 50-100 μl were carried out at 22-24°C in ATPase buffer (20 mM K-HEPES pH 7.5, 25 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 10% glycerol) containing 1 mg/ml BSA, as well as 20 μM paclitaxel unless otherwise indicated. DEAE-purified ATP was used at 0.5 or 1.0 mM, and reactions were started by addition of katanin. Aliquots were removed at

various times and quenched by addition of perchloric acid to a final concentration of 0.3M and stored on ice.

For radioactive assays, DEAE-ATP was supplemented with [γ-^{32}P]ATP (2 Ci/mmol, NEN) to yield ~40,000-80,000 total cpm per time point by Cherenkov counting. In addition, the PCA quench contained 10-20 mM KPO_4 to prevent the nonspecific loss of counts on the charcoal during separation. Unhydrolyzed ATP was removed by vortexing the quenched reactions with a 50 µl slurry of activated charcoal (Norit A, Sigma, Inc.) previously prepared by boiling in 1 M HCl, followed by extensive water washing. The charcoal was removed by centrifugation at 14,000 x g (3 min, 4°C) and the supernatant was Cherenkov counted after addition of ~2 ml of water. Total hydrolysis was limited to 10-20%, to minimize product inhibition.

For nonradioactive assays, an ATP regenerating system consisting of 0.5-1.0 mM phospho-enol pyruvate and 2U pyruvate kinase was usually included to minimize the inhibition by ADP previously reported for native katanin (McNally and Vale, 1993). Phosphate concentration was measured by addition of an equal volume of malachite green reagent (Kodama et al., 1986), followed by incubation at 25°C for 35 min and subsequent measurement of absorbance at 650 nm. The phosphate standards were prepared by dilution of 0.65 mM KPO_4 (Sigma Diagnostics, Inc.).

Severing assays

Microtubule severing rate was measured using a fluorescence-based assay for DAPI binding described as previously described (Hartman et al., 1998). To calculate the amount of tubulin dimers released, the rate of change

of fluorescence (ΔF/sec) was divided by ($(FL_{unsevered})$-$(FL_{ca2+depolymerized})$/MT concentration, μM).

Hydrodynamic analysis

Glycerol gradient centrifugation was modified from previous methods (Hanson et al., 1997). Gradients were prepared by layering equal steps of 10%, 16.3%, 22.5%, 28.8%, and 35% glycerol in gradient buffer (20 mM K-HEPES pH 8.0, 75 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 1 mM DTT), with 2 mM of the indicated nucleotide and equimolar $MgCl_2$; gradients were allowed to form overnight at 4°C. Proteins in Ni-NTA elution buffer were diluted ~1:1 with gradient buffer (final concentration ~5 μM) and incubated with 2 mM of the appropriate nucleotide and 2 mM $MgCl_2$ for 15 min on ice prior to centrifugation. NSF D2 domain was expressed and purified (Hanson et al., 1997)) and handled as described above. BSA (4.1S), catalase (11.2S), and thyroglobulin (19.4S) were used as standards. Gradients were centrifuged for 12 hr at 285,000 x g in a SW60 Ti rotor (Beckman Instruments, Inc.) at 4°C. GFP-tagged p60 was quantitated by fluorescence (λ_{ex} 470, λ_{em} 508) using either an SLM 8100 in photon counting mode (Spectronics, Inc.) or a Perkin-Elmer LS-5B (Perkin-Elmer, Inc.). Standards and NSF D2 were quantitated by Bradford. Gel filtration chromatography was performed on ~15 μg of $p60^{E334Q}$ using a 0.78x30 cm TSK-4000SWXL column (TosoHaas, Inc.) equilibrated with 20 mM K-HEPES pH 7.8, 75 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 5% glycerol, and 1 mM ATP. Standard proteins were BSA (Stokes radius 35Å), catalase (52Å), and thyroglobulin (86Å). Molecular weight estimates were made using the measured Stokes radius and S-value [L. M. Siegel and K. J. Monty, Biochem. Biophys. Acta **112**, 346 (1966)]

FRET measurements

Separately purified CFP-p60 and YFP-p60 were preincubated together in the indicated ratios at 10X assay concentration for 30-60 min on ice. The CFP/YFP-p60 mixture was then diluted into reactions containing 2 mM nucleotide and equimolar $MgCl_2$ in ATPase buffer (20 mM K-HEPES pH 7.5, 25 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 10% glycerol, 20 μM paclitaxel). Reactions contained 1 mg/ml BSA to prevent surface adsorption. 20 mM glucose and 0.1 mg/ml hexokinase, which converted residual ATP to ADP, were used to measure FRET in the ADP state. For FRET measurements with microtubules, p60 was preincubated with the nucleotide mixture for 10 min at 22°C before microtubules were added. After a 10-15 min incubation with microtubules, energy transfer was quantitated by exciting the sample at 433 nm and measuring the fluorescence at 480 nm and 535 nm. The ratio of emission at 535 nm/480 nm was used as the measure of energy transfer (Miyawaki et al., 1997). For emission scans, the sample was excited at 433 nm, and the fluorescence emission measured between 450 and 600 nm using excitation and emission slit sizes of 1 nm.

Microtubule binding

GFP-p60 (0.4 μM final) was mixed with paclitaxel-stabilized microtubules and 1 mg/ml BSA in ATPase buffer and incubated at 22°C for 20 min. 5 mM glucose and 0.1 mg/ml hexokinase, which converted residual ATP to ADP, was used for measuring binding in ADP. Samples (50 μl) were incubated at 22°C for 20 min prior to loading onto a 50 μl cushion of 66% glycerol in ATPase buffer, followed by centrifugation for 5 min at 436,000 x g (20°C). The supernatant and cushion were removed and diluted 1:1 with water to reduce sample viscosity. The microtubule pellets were

depolymerized by incubation on ice for 30 min in ATPase buffer containing 5 mM $CaCl_2$, followed by dilution 1:1 with water. Bound and free GFP-p60 were determined by fluorescence (λ_{ex} 470, λ_{em} 508).

Table 1:

Effect of nucleotide state and microtubules on CFP-p60:YFP-p60 FRET.

Protein	Nucleotide	Microtubules	FRET signal (% of ADP)
$p60^{E334Q}$	ADP	-	100 ± 1.2
$p60^{E334Q}$	ATP	-	124 ± 0.9
$p60^{wt}$	ADP	-	100 ± 0.8
$p60^{wt}$	ADP	+	102 ± 0.2
$p60^{wt}$	ATP-γ-S	-	98 ± 0.1
$p60^{wt}$	ATP-γ-S	+	119 ± 0.2

Measurements of p60 E334Q FRET were made at 0.13 μM total CFP/YFP-p60 using a mixture of 1 donor CFP:5 acceptor YFP molecules. Experiments with wildtype p60 were performed at 0.5 μM total CFP/YFP-p60 using a mixture of 1 donor CFP:2 acceptor YFP molecules. MgATP, MgADP, or MgATP-γ-S were present at 2 mM, and microtubules were included at 5 mM where indicated. FRET values are normalized using the ADP value as 100% (1.13 for $p60^{E334Q}$, 0.46 for $p60^{wt}$) . The mean and standard deviation of two measurements ($p60^{wt}$) or three measurements ($p60^{E334Q}$) are shown. FRET in the presence of ADP was not observed over a wide range of microtubule concentrations (data not shown).

Figure 1: Oligomerization of p60 katanin.

(**A**) Hydrodynamic analysis. Sedimentation profile of GFP-p60 katanin through 10-35% glycerol gradients in the presence of 2 mM MgATP (●) or MgADP (○). Both sediment as a single species of ~4S. A DEAD box mutant of p60, GFP-p60^{E334Q} (21), was tested in the presence of 2 mM MgATP (▲) and MgADP (Δ). GFP-p60^{E334Q} sediments as a mixture of 4S and 15S species in the presence of ATP, but as a single species of 4S in the presence of ADP. As a control for AAA oligomerization, E. coli expressed NSF D2 domain was sedimented through gradients containing 2 mM MgATP and the sedimentation peak is indicated by an arrow.

(**B**) Fluorescence Resonance Energy Transfer (FRET) of a 1:9 mixture of CFP-p60^{E334Q} (donor) and YFP-p60^{E334Q} (acceptor) in the presence of 2 mM MgATP (●) or 2 mM MgADP (○). FRET is indicated by the decreased emission at 480 nm and the increased emission at 535 nm in the presence of MgATP. The MgADP emission profile is identical to that calculated for CFP-p60^{E334Q} and YFP-p60^{E334Q} measured separately (data not shown).

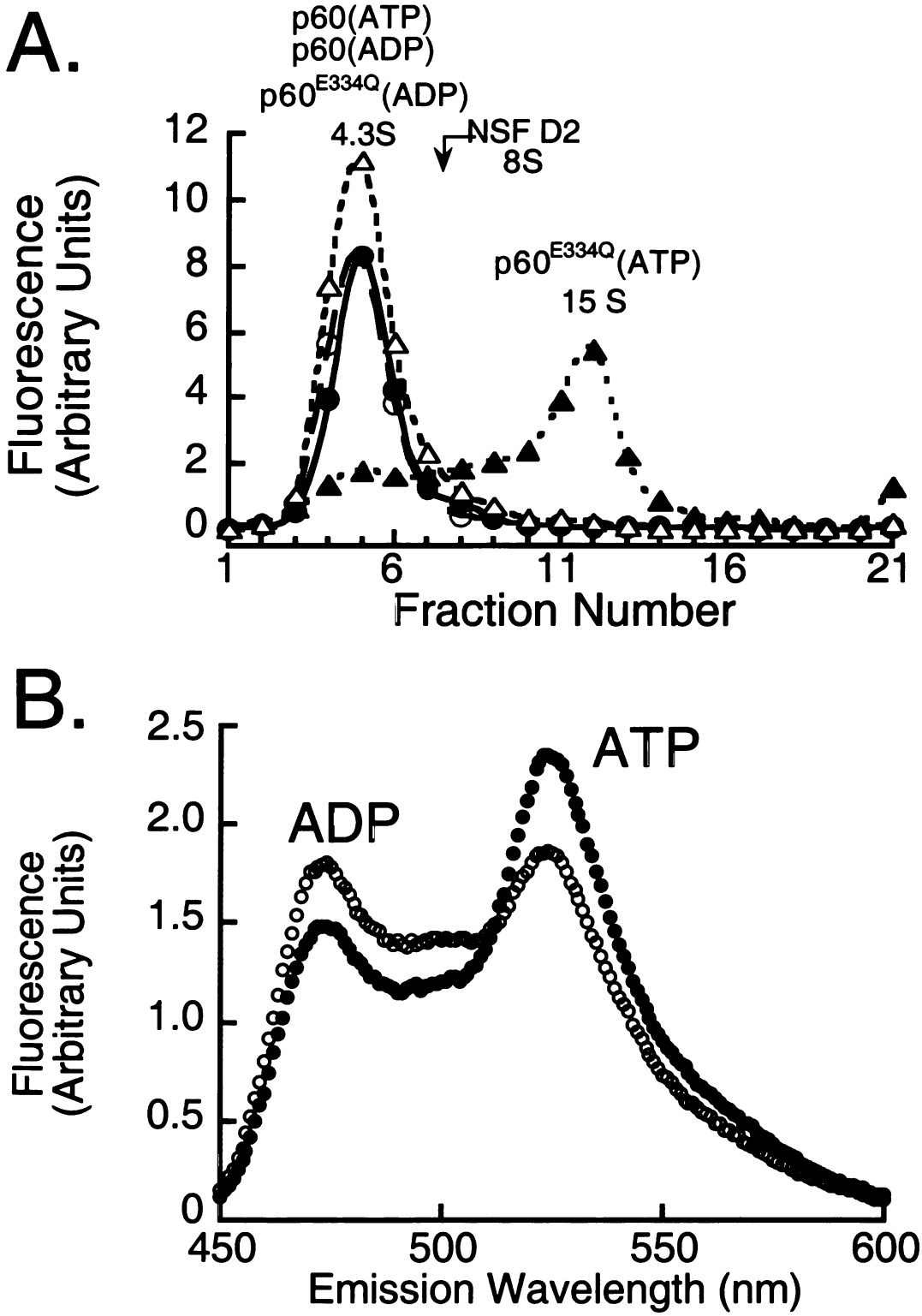

A.
p60(ATP)
p60(ADP)
p60E334Q(ADP)
4.3S
NSF D2
8S
p60E334Q(ATP)
15 S
Fluorescence (Arbitrary Units)
Fraction Number
B.
ATP
ADP
Fluorescence (Arbitrary Units)
Emission Wavelength (nm)

Figure 2: Nucleotide-dependent binding of p60 katanin to microtubules.

Cosedimentation of GFP-p60 and microtubules were tested in the presence of 2 mM MgATP-γ–S (●; K_d~0.9 μM) or MgADP (○; K_d~18 μM). Also shown is a truncated p60 that lacks the C-terminal AAA domain (p60ΔAAA; ▲; K_d~6 μM). Binding of this protein was not affected by nucleotide (data not shown). Binding is expressed as the fraction of GFP-p60 that cosedimented with microtubules, and the best fit to a hyperbolic curve is shown.

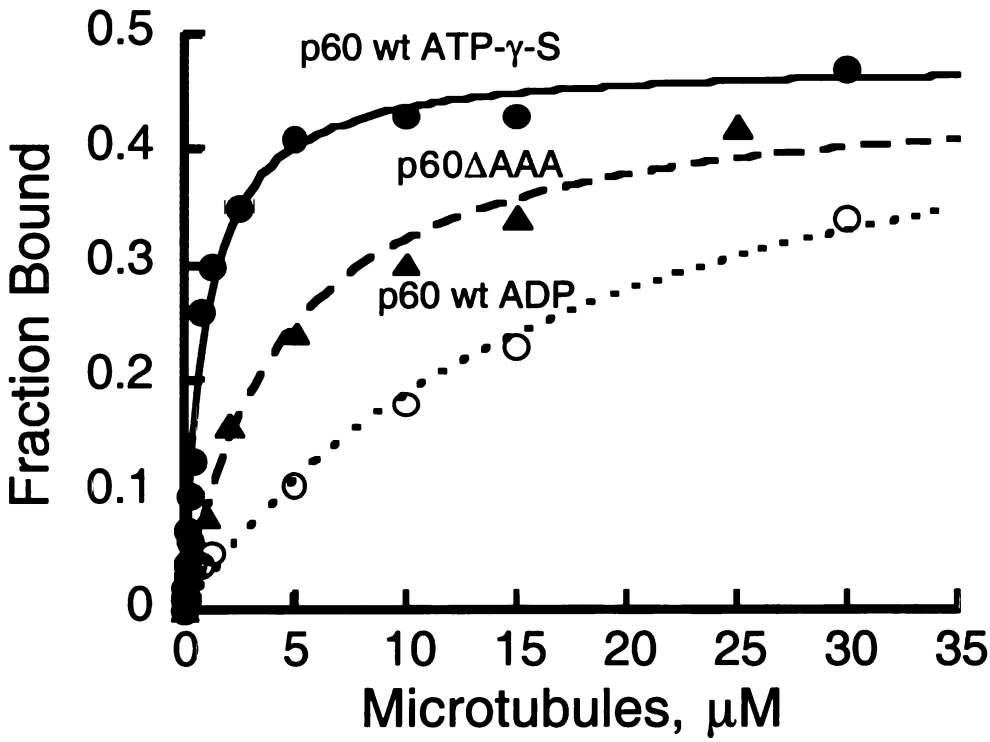

0.5
0.4
0.3
0.2
0.1
0
Fraction Bound
p60 wt ATP-γ-S
p60ΔAAA
p60 wt ADP
0
5
10
15
20
25
30
35
Microtubules, μM

Figure 3: Effect of microtubules on p60 oligomerization, ATPase, and microtubule severing activities.

(A) Oligomerization and ATPase activity as a function of microtubule concentration. ATPase activity (●) and FRET (○) were measured in a 0.2 μM, 1:5 mixture of CFP-p60 and YFP-p60. Note the similar pattern of increasing ATPase and oligomerization at 0-10 μM microtubules, followed by decreasing signal as microtubule concentration is raised above 10 μM. Values have been normalized to the percentage of the maximum observed FRET or ATPase signal. A curve fit shown is for two competing Michaelis-Menten reactions (((A*[tubulin])/(B+[tubulin]))-((C*[tubulin])/(D+[tubulin]))).
(B) Comparison of ATPase (hatched bars) and microtubule-disassembly activity (solid bars) of 0.2 μM p60 at 5 and 10 μM microtubules. ATPase activity begins to decline above ~2 μM microtubules for untagged p60 (Hartman et al., 1998), versus above ~10 μM microtubules for CFP-p60/YFP-p60 (Fig. 3a). Untagged p60 was used for this assay because microtubule concentrations >10 μM are not compatible with fluorescence microtubule disassembly assay. Activities have been normalized to the activity at 5 μM, and error bars indicate the standard deviation of 2 measurements. Maximum activity was 1.9 ATP $p60^{-1}$ sec^{-1} and 0.04 tubulin dimer $p60^{-1}$ sec^{-1}, yielding a coupling ratio of ~50 ATP/tubulin dimer removed from the microtubule.

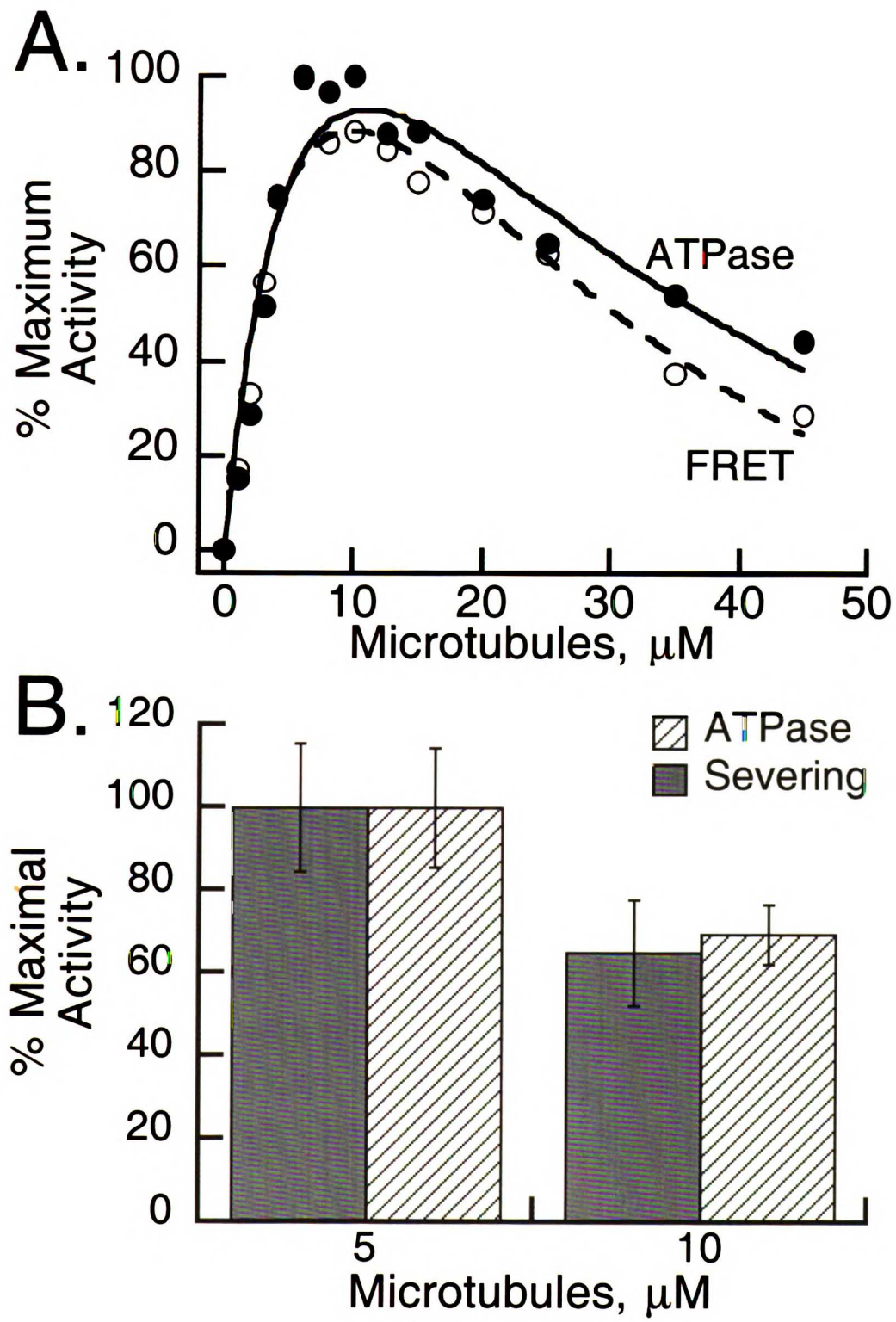
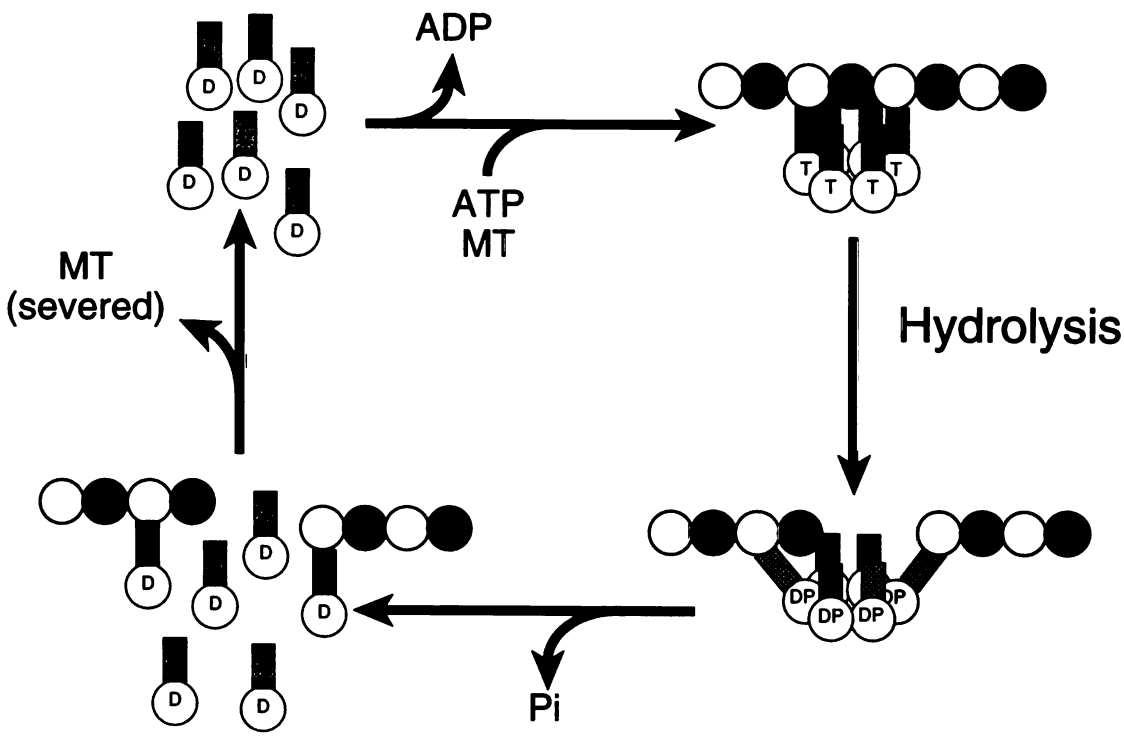

A.
100
80
60
40
20
0
% Maximum Activity
ATPase
FRET
0
10
20
30
40
50
Microtubules, μM
B.
120
100
80
60
40
20
0
% Maximal Activity
ATPase
Severing
5
10
Microtubules, μM

Figure 4: Model for microtubule severing by katanin.

See text for details. Only a single protofilament of the microtubule is shown for simplicity. T, DP, and D represent ATP, ADP•P_i, and ADP states, respectively. The conformational change is shown to occur with γ–phosphate bond cleavage, although this could alternatively occur as a result of γ-phosphate release.


ADP
ATP
MT
Hydrolysis
Pi
MT
(severed)
D
T
DP

CHAPTER 3

Biochemical Characterization of the ATPase Activity of Native and Recombinant Katanin

The work presented in Chapter 3 is the result of a long collaboration with Dr. Takashi Shimizu. Over the course of several years, Dr. Shimizu shared his intuition and expertise at designing and performing ATPase experiments. In addition, he helped provide me with a wonderful opportunity to visit his laboratory in Japan.

INTRODUCTION

Katanin couples the hydrolysis of ATP to the disruption of contacts in the microtubule lattice. Understanding the mechanism of microtubule severing will require knowledge about how katanin hydrolyzes ATP and uses the resulting free energy to do work. As a first step, we report some of the basic parameters of katanin's ATPase activity. In this chapter, we document the complex stimulation of katanin's ATPase activity by microtubules. This is very similar to what has been observed for baculovirus-expressed katanin subunits (Hartman et al., 1998), demonstrating that the non-Michaelis-Menten kinetics are not an artifact of expression system, but rather are an inherent property of the enzyme. We also explore the inhibition of katanin's ATPase activity by ADP, as well as by several nonhydrolyzable nucleotide analogs. In addition, we have tested the ability of a nucleotide analog library to support severing, and suggest several analogs that might be useful for further study. Finally, we present some preliminary data on the stimulation of katanin ATPase activity by alternative activators, including several types of non-polymeric forms of tubulin and anti-katanin antibodies. Together, these results provide a starting point for a pre-steady-state study of this interesting ATPase.

RESULTS

Understanding the mechanism of katanin-mediated microtubule severing requires first understanding the interactions of katanin with its substrates, ATP and microtubules. Katanin hydrolyzes ATP at a reasonably linear rate up to at least 30 minutes, and the rate of ATP hydrolysis increases several fold when ~1 μM paclitaxel-stabilized microtubules are included in the reaction (Fig. 1A). It has been suggested that microtubule severing is cooperative (McNally and Vale, 1993), but katanin's basal and microtubule-stimulated ATPase activities are reasonably proportional to enzyme concentration in the limited regime tested (25-300 nM, Fig. 1B). Low concentrations of microtubules are quite effective at activating katanin, with 0.1 μM stimulating the ATPase activity to nearly the same extent as 0.9 μM. Interestingly, the lowest enzyme concentration tested (~12 nM) falls below the linear curve fits for the microtubule-stimulated reactions, suggesting that cooperativity may be occurring at low enzyme concentrations, in the presence of microtubules. Alternatively, this could be explained by katanin's tendency to adsorb nonspecifically on surfaces at low concentrations (data not shown).

When we determined a full dose-response curve for activation of katanin ATPase by microtubules, we were surprised to find that katanin's ATPase activity is stimulated in a complex, non-Michaelis-Menten fashion (Fig. 2). ATPase activity increased sharply as microtubule concentration was increased from 0-0.5 μM (Fig. 2B), but then declined as microtubule concentration was further increased (Fig. 2A). This is quite distinct from the hyperbolic stimulation of kinesin's ATPase activity by microtubules (Gilbert and Johnson, 1993), and suggests a complex interaction between katanin and the microtubule substrate. Previous experiments indicated that subtilisin-

treated microtubules, lacking the negatively-charged C-termini of α- and β-tubulin, could stimulate the ATPase activity of katanin without being severed (McNally and Vale, 1993). We find that subtilisin-treated microtubules stimulate the ATPase activity of katanin with a similar dose-response, and to a similar extent, as paclitaxel-treated microtubules (Fig. 2). This suggests the interactions between katanin and tubulin C-termini, while necessary for severing, are not required for full stimulation of katanin's ATPase activity by microtubules. Importantly, this demonstrates that microtubule activation of katanin's ATPase activity can be uncoupled from microtubule severing.

In addition to the interaction with microtubules, we were interested in examining katanin's interaction with its nucleotide substrate, ATP. Using a coupled enzyme ATPase assay that maintains a constant ATP/ADP ratio, we measured the effect of ATP concentration on microtubule-stimulated ATPase activity (Fig. 3). The ATP dependence of katanin's microtubule-stimulated ATPase activity is hyperbolic, suggesting it behaves like a Michaelis-Menten enzyme with respect to nucleotide binding and hydrolysis. In the presence of ~1 μM microtubules, we observe an apparent K_m for ATP of ~50 μM ATP. How does this compare with the data on other AAA proteins? ATPase experiments with recombinant NSF, a AAA protein involved in membrane trafficking , documented a K_m for ATP of 650 μM (Tagaya et al., 1993), considerably higher than what we observe. Recent direct binding experiments, however, indicate that the weaker, hydrolytic D1 domain of NSF binds ATP with a K_d of ~15-20 μM (Matveeva et al., 1997), which is more similar to katanin's K_m for ATP.

We also examined how salt and pH affect katanin's ATPase activity. Strong salt dependence can be indicative of ionic interactions between the enzyme and its substrate. The microtubule-stimulated ATPase activity of

kinesin, for example, is highly salt dependent, suggesting the importance of ionic interactions between it and its microtubule substrate. This has been supported by a mutational analysis of kinesin, showing that mutation of several different basic residues to alanine results in lower microtubule affinity (Woehlke et al., 1997). We tested the sensitivity of both the basal and microtubule-stimulated ATPase activities to increasing concentrations of potassium glutamate, ranging from 25 mM (standard in our ATPase assays) up to 500 mM (Fig. 4). We observed that microtubule-stimulated ATPase activity was similar at 25 and 50 mM, but dropped by 25% at 100 mM and 75% at 200 mM K-glutamate. The rate of microtubule-stimulated ATP hydrolysis was similar to the basal, unstimulated rate at 200 mM potassium glutamate. Contrary to the sharp salt dependence of the microtubule-stimulated rate, the basal ATPase rate declined more gradually over the concentration range tested, suggesting a smaller contribution of charged residues to the interaction of katanin with ATP (Fig. 4).

The pH optima of enzymes reflects the ionization of groups on the enzyme important for both protein stability and chemical catalysis. Previous results suggest that katanin aggregates below ~pH 6.5 (F. McNally, personal communication). We observed maximal basal ATPase activity at pH 8.0, with activity declining at both higher and lower pH (Fig. 5). This experiment also suggests that katanin is more active in K-EPPS pH 7.5 buffer than in the K-HEPES pH 7.5 buffer usually employed for severing and ATPase experiments, but this has not been carefully examined to rule out impurities in the buffer preparations. Reactions are generally carried out at pH 7.5, a compromise between the higher katanin activity but lower microtubule stability at higher pH. Our data on katanin fits with the limited biochemical data available on other AAA ATPases, which indicate a relatively high pH optimum.

Recombinant NSF ATPase, for example, revealed a 3-fold higher ATPase activity at pH 9.0 versus pH 7.0 (Tagaya et al., 1993).

ATPase experiments can be very useful for measuring the interaction of other nucleotides and nucleotide analogs with ATP-hydrolyzing enzymes. Previous experiments indicated that ADP inhibits the ATPase activity of katanin (McNally and Vale, 1993), but a dose-response curve was not determined. We tested the ability of 0.1-1.0 mM ADP to inhibit the basal and microtubule-stimulated ATPase activities of katanin in the presence of 0.5 mM ATP, and estimate an IC_{50} of 0.3 mM and 0.6 mM for these reactions, respectively (Fig. 6). The ability of ADP to inhibit katanin's ATPase activity at concentrations roughly equal concentrations to ATP suggests that both nucleotides bind with similar affinities to the active site of katanin. The fits well with direct binding experiments that have been published using NSF, which indicate the hydrolytic D1 domain of NSF has a comparable affinity for ATP and ADP, with a K_d for ATP of 40 μM and a K_d for ADP of 140 μM (Matveeva et al., 1997).

When we tested the ability of artificial nucleotide analogs to inhibit katanin's ATPase activity, we found that the nonhydrolyzable ATP analog AMPPNP had little effect on katanin ATPase activity at concentrations up to 1 mM, in the presence of 0.5 mM ATP (Fig. 7A). However, the slowly-hydrolyzed ATP analog ATP-γ-S inhibited both the basal and microtubule-stimulated ATPase activity of katanin with IC_{50}s of 0.5 and 0.1 mM, respectively (Fig. 7B). This is consistent with previous reports that ATP-γ–S, but neither AMPPNP nor AMPPCP, inhibits katanin's ATPase and microtubule-severing activities (McNally and Vale, 1993). ATP-γ–S can also interact with NSF, as evidenced by its ability to promote formation of a tight complex between NSF, α–SNAP, and GST-syntaxin 1a (Hanson et al., 1995),

and its ability to stabilize conformation of NSF different from MgATP, ATP/EDTA, or ADP as imaged by rotary shadowing electron microscopy (Hanson et al., 1997). The inability to observe inhibition of katanin ATPase activity by AMPPNP is somewhat surprising, since Lenzen et al. (1998) have determined the structure of the NSF D2 domain complexed with this nonhydrolyzable analog. One explanation is that the nonhydrolyzing NSF D2 domain makes different contacts with nucleotide substrates than does the hydrolyzing D1 domain of NSF, which is more closely related to the AAA domain found in katanin.

Nucleotide analogs were also tested for their ability to support microtubule severing by native katanin. Table I compares katanin microtubule-severing activity to microtubule translocation by kinesin and 14S dynein in the presence of various nucleotides and nucleotide analogs. 2'deoxyATP, 3'deoxyATP, and dideoxyATP are all quite active in supporting severing and microtubule gliding by kinesin and dynein, while PRTP supports poor severing and kinesin gliding. The 8'bromo- and 8'azido-derivatives of ATP support poor severing, which may explain the failure of previous experiments to localize the ATP binding site to p60 katanin by crosslinking of ^{32}P-labeled 8'azido ATP (data not shown).

One of the purposes of testing nucleotide analogs was to identify substrates that might be useful for pre-steady-state kinetic studies. For these purposes, fluorescent derivatives that change their spectral properties are very useful. Importantly, the fluorescent derivatives formycin-TP and etheno-ATP both caused microtubule severing (Table I). Unfortunately, initial experiments using formycin-TP, etheno-ATP, and mant-ATP showed little fluorescence change when added to p60 (data not shown). This may be due, however, to poor nucleotide binding at the <10 μM concentrations of

nucleotide tested. Katanin appears to precipitate in the absence of nucleotide (data not shown), complicating the experiment further. For future experiments, availability of more concentrated katanin preparations will hopefully enable use of higher nucleotide concentrations, approaching the estimated K_m for ATP of 50 μM.

It is currently unknown whether katanin interacts directly with free tubulin dimers during its chemical cycle. Surface-adsorbed tubulin significantly inhibits microtubule severing in mitotic Xenopus egg extracts (Vale, 1991), and recent experiments indicate that katanin is primarily responsible for this activity (McNally and Thomas, 1998). This suggests an interaction between katanin and tubulin capable of competing with the katanin-microtubule interaction, but the conformation and folding of surface-adsorbed tubulin is unknown and difficult to control. We used our ATPase activity to look for direct interactions between katanin and tubulin dimers, by testing if several forms of nonpolymeric tubulin could stimulate the basal ATPase activity of katanin. Preliminary results indicate that tubulin treated with the microtubule-depolymerizing drug nocodazole, and then centrifuged to remove tubulin oligomers and aggregates could stimulate the ATPase of native katanin. Stimulation was up to ~3-fold at 10 μM tubulin, but the dose-response was quite different than what is observed with paclitaxel-stabilized microtubules, showing an almost linear increase of ATPase activity between 1-10 μM nocodazole-tubulin (Fig. 8). One explanation for this non-hyperbolic increase could be the presence of an ATPase activity inherent to the nocodazole-tubulin substrate, which was not accounted for in this preliminary experiment. Alternatively, nocodazole-tubulin could have a much higher $K_{0.5}$ than paclitaxel-stabilized microtubules, due to a weak interaction between the enzyme and tubulin substrate. Another explanation

is that the nocodazole-tubulin preparation is contaminated with a low proportion of oligomeric or aggregated tubulin molecules capable of stimulating katanin's ATPase activity. A more interesting possibility would be the stimulation of katanin ATPase activity by a unique conformation of nocodazole-tubulin. For that reason, we wanted to test the effect of tubulin that had been prevented from polymerizing by other methods.

We tested the effect of colchicine-tubulin and GDP-tubulin on the ATPase activity of recombinant p60 katanin, rather than native sea urchin katanin, because of its greater abundance and ease of preparation. Treatment of tubulin with colchicine prevents polymerization, due to the formation of a stable complex of the drug with tubulin dimers (Andreu and Timasheff, 1982). Initial results suggest that colchicine-tubulin also stimulates the basal ATPase activity of p60 katanin, and the dose-response is quite different from activation by microtubules (Fig. 9A). This activation does not appear hyperbolic in the range of 1-20 μM tubulin, and could be either roughly linear or weakly sigmoidal with an inflection point around 10 μM. GDP-tubulin is very difficult to polymerize, and should represent a more physiological preparation of nonpolymeric tubulin. We found no significant ATPase activation up to ~15 μM, however, suggesting katanin is not activated by all forms of nonpolymeric tubulin (Fig. 9B). This difference suggests that either the drug-treated tubulin substrates are in different conformations than GDP-tubulin, or that the drug-treated samples contain small populations of alternative forms of tubulin that can stimulate katanin. The strong tendency of tubulin to aggregate and form small oligomers is a difficult complication of experiments with putatively non-polymeric tubulin. Eliminating this caveat will require analysis of the aggregation state of tubulin in parallel with

ATPase activity, to verify that nocodazole- and colchicine-tubulin can indeed interact with and activate katanin's ATPase activity.

One suggestion for how microtubules stimulate the ATPase activity of katanin is that they promote oligomerization of p60, and increased p60-p60 interactions are responsible for accelerated hydrolysis of ATP. This idea is strengthened by the high correlation of ATPase activity and oligomerization as measured by fluorescence resonance energy transfer (this thesis, Chapter 2). In addition, the recent determination of the atomic structure of the hexameric NSF AAA D2 domain indicates that while virtually all nucleotide contacts are made within a single protomer, one residue of an adjacent protomer (Lys-639) interacts with the γ-phosphate oxygen of the nucleotide (Lenzen et al., 1998). While this residue is not well ordered in the crystal structure, and Lys-639 is not conserved in the NSF ortholog Sec18p (Lenzen et al., 1998), this observation suggests that protomer-protomer contacts are important for complete interaction of AAA proteins with their nucleotide substrates and provides a physical basis for how oligomerization could affect ATP hydrolysis.

To examine if non-microtubule substrates could stimulate p60's ATPase activity, we tested the ability of poly-glutamic acid to act as a surrogate for microtubules in stimulating the ATPase activity of p60 katanin. Microtubules are known to have negatively-charged, glutamic acid-rich C-termini (Sackett et al., 1985), and these tails are required for microtubule severing, since their removal by the protease subtilisin prevents severing but not ATPase activation (McNally and Vale, 1993). Poly-glutamic acid, average molecular weight 14 kDa, stimulates the basal ATPase activity of p60 approximately 2-fold at 2 μM, compared with the 4-fold stimulation seen with equal concentrations of paclitaxel-stabilized microtubules (Fig. 10). One possibility for how this amino acid polymer can activate p60 is by

concentrating katanin monomers via ionic interactions with the basic (pI~10) N-terminus of p60. Glutamic acid residues (pKa=4.1) will be negatively charged at the reaction pH of 7.5, and could act similarly to an ion exchange matrix, concentrating p60 monomers and increasing the local concentration, thereby promoting oligomerization. Interestingly, ATPase activity was less stimulated when the poly-glutamic acid concentration was raised to 20 μM (data not shown), similar to what is observed for stimulation of katanin's ATPase activity by high concentrations of microtubules. This is consistent with a model in which high substrate concentrations are less able to stimulate katanin's ATPase activity because p60 monomers become segregated at noncontiguous sites (Hartman et al., 1998).

Finally, we used anti-p60 antiserum to bring p60 monomers in close proximity and tested whether this stimulates p60's ATPase activity. We found that whole anti-p60 antiserum increased the ATPase activity of p60 >10-fold, 2-fold higher than 2 μM microtubules (Fig. 11). Addition of microtubules and serum together were similar to serum alone, suggesting that stimulation was via the same rather than additive or synergistic pathways (Fig. 11). Serum alone had only minimal ATPase activity (data not shown). Also, addition of anti-p80 antiserum did not stimulate p60's ATPase activity, nor did it prevent activation of ATPase activity by microtubules (Fig. 11). This suggests that stimulation of p60 interactions can, in fact, promote ATP hydrolysis. More conclusive experiments will require the use of purified or monoclonal antibodies, and careful comparison of ATPase activation by bivalent and monovalent antibody preparations.

DISCUSSION

Initial observations of microtubule severing in Xenopus extracts suggested the importance of ATP in the severing reaction (Vale, 1991), and subsequent purification of the sea urchin microtubule severing-protein katanin revealed it to be a microtubule-stimulated ATPase (McNally and Vale, 1993). This study extends that work by further characterizing the biochemical properties of katanin's ATPase activity. The ATPase activity of native sea urchin katanin has a unusual dose-response for activation by microtubules, just as has been reported for recombinant, baculovirus-expressed p60 and p60/p80 katanin (Hartman et al., 1998). ATPase activity peaks at ~1 μM microtubules, and then declines at higher microtubule concentrations. The observation of this phenomena with both native and recombinant protein, purified in different ways, suggests that it is an inherent property of katanin's ATPase activity and not the result of a peculiarity of either the native or recombinant proteins. This stimulation differs from the Michaelis-Menten stimulation of kinesin ATPase by microtubules (Gilbert and Johnson, 1993), suggesting katanin interacts with microtubules very differently than kinesin and that this interaction may be worthy of further study. Subtilisin-treated microtubules may be an important tool for probing the interaction of katanin with microtubules, since they are unique in being able to uncouple the ATPase and microtubule severing activities of p60.

ATPase experiments have furthered our understanding of how katanin interacts with its nucleotide substrate, ATP. Nucleotide binding may be similar in different members of the AAA ATPases superfamily, given the similarities of the Kd for ATP binding to the D1 domain of NSF (20 μM) (Jourdain et al., 1997) and the half-maximal ATPase activity of katanin (50

μM). Furthermore, our experiments indicate that ADP is an inhibitor of katanin ATPase activity (IC_{50}=0.3-0.6 mM), and this consistent with the demonstration that NSF D1 has similar affinity for ATP and ADP (Jourdain et al., 1997). The slowly-hydrolyzed ATP analog ATP-γ–S is able to interact with the AAA domain of multiple AAA ATPases, since it inhibits the ATPase activity of katanin and stabilizes substrate interactions in katanin (this thesis, Chapter 2) and NSF (Jourdain et al., 1997). In contrast, AMPPNP appears to be a poor inhibitor of katanin ATPase activity.

Interaction of katanin with tubulin substrates is more complex than with nucleotides, and considerably more experimentation remains to be done in this area. Our initial observations that nocodazole-tubulin and colchicine-tubulin can stimulate katanin's ATPase activity suggests some interaction with nonpolymeric forms of tubulin. Careful analysis of the structure of the tubulin substrate during the ATPase reaction will be required to rule out tubulin aggreagates or oligomers as the cause of ATPase activation. The inability of nonpolymeric GDP-tubulin to stimulate the ATPase activity of p60, together with the ability of several AAA ATPases to specifically disaggregate proteins (Confalonieri and Duguet, 1995), makes this a strong possibility. Similarly, the ability of surface-adsorbed tubulin to inhibit Xenopus severing (Vale, 1991) could be attributed to competition of katanin binding to microtubules and adsorbed aggregates.

Our results that anti-p60 antiserum, and to a lesser extent poly-glutamic acid, stimulates the ATPase activity of p60 supports the suggestion that p60-p60 interactions are necessary for ATP hydrolysis. Bringing p60 monomers together, either by binding to neighboring tubulin sites on a microtubule or by artificial means like binding to adjoining sites on a bivalent antibody, stimulates ATPase activity. There is increasing evidence that

oligomerization is either required for or coupled with ATPase activity in multiple members of the AAA superfamily, including katanin (this thesis, Chapter 2), VPS4 (Babst et al., 1998), and p97 (Peters et al., 1992). The recently determined atomic structure of the hexameric NSF D2 domain reveals one of the contacts between the protein and the nucleotide substrate comes from a neighboring subunit (Lenzen et al., 1998), suggesting a physical basis for how this regulation could be achieved. The ability to drive oligomerization of p60 katanin by addition of pure substrate (microtubules), as well as by artificial means like antibodies or polyamino acids, make katanin attractive for studying how oligomerization affects nucleotide binding and hydrolysis in the entire AAA ATPase superfamily. Pre-steady-state analysis will hopefully provide details about the reaction mechanism by which microtubules promote p60 oligomerization and ATP hydrolysis.

MATERIALS AND METHODS

Microtubules and tubulin

Bovine brain tubulin was prepared by two cycles of polymerization followed by chromatography on phosphocellulose P-11 (Whatman, Ltd.) (Williams and Lee, 1982) before freezing in liquid nitrogen and storage at -80°C. Thawed tubulin was precleared by centrifugation at 337,000 x g (10 min., 4°C) to remove aggregates. Microtubules were polymerized by addition of DMSO to 10% and GTP to 1 mM, followed by incubation at 37°C for 30-60 min. For most experiments, microtubules were stabilized by addition of paclitaxel to 20 μM, followed by an additional 15-30 min incubation at 37°C, before sedimentation at 267,000 x g (10 min, 25°C) through a 60% glycerol cushion in ATPase buffer (20 mM K-HEPES pH 7.5, 25 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 10% glycerol) containing 10 μM paclitaxel. Microtubules were resuspended in ATPase buffer containing 20 μM paclitaxel. Microtubule concentration was measured by absorbance at 275 nm in 6M guanidine HCl, using an extinction coefficient of 1.030 ml•mg-1•cm-1 (Hackney, 1988).

Nocodazole-tubulin was prepared by adding nocodazole (10 mM in DMSO) to precleared tubulin at 60 μM, followed by incubation on ice for ~30 minutes and centrifugation at 337,000 x g (10 min, 4°C). Colchicine-tubulin was prepared by addition of 1.25 mM colchicine to precleared tubulin from a 125 mM stock in ethanol, followed by incubation at 22°C for 30 min. Buffer exchange was performed using a G-25 Sephadex (Pharmacia, Inc.) spin column equilibrated in ATPase buffer. The tubulin-colchicine eluate was supplemented with colchicine to 1.25 mM and stored on ice. GDP-tubulin was prepared by polymerization of microtubules as above, without addition

of paclitaxel. Microtubules were sedimented through a 60% glycerol cushion in ATPase buffer without paclitaxel by centrifugation at 337,000 x g (10 min, 37°C). The cushion was washed several times with water, before resuspending the microtubule pellet in ATPase buffer without paclitaxel. The microtubules were depolymerized on ice for 30 min, and then precleared by centrifugation at 337,000 x g (10 min, 4°C) to remove aggregates and residual microtubules. GDP-tubulin was stored on ice before addition to ATPase reactions.

Purification of native sea urchin katanin

Native katanin was purified from extracts of *Stonglyocentrotus purpuratus* (North Coast Invertebrate Collectors, Bodega Bay, CA) eggs essentially as previously described (McNally and Vale, 1993), except that hydroxyapetite chromatography was performed using a HR 10/30 column (Pharmacia, Inc.) packed with 20 μm ceramic hydroxyapetite beads (American International Chemical, Natick, MA). Mono Q (Pharmacia, Inc.) purified native katanin was frozen in liquid nitrogen and stored at -80°C.

Expression constructs

Katanin p60 and p80 were cloned into pFastBac HT B (Life Technologies, Inc.). The resulting baculovirus constructs expresses p60 and p80 katanin with the following additional N-terminal amino acids: MSYYHHHHHHDYDIPTTENLYFQGS.

Expression and purification of recombinant katanin

Recombinant katanin subunits were expressed by infection of Sf9 cells with baculovirus, followed by purification on Ni-NTA agarose as described

(Hartman et al., 1998). Expressed katanin was frozen in liquid nitrogen and stored at -80°C. Inclusion of ATP at 250 μM before freezing was found to improve protein stability.

ATPase assays

ATPase activity was measured using either a radioactive assay following the release of ^{32}P from [γ–^{32}P]ATP for native katanin, or a modified malachite green assay for recombinant katanin (Kodama et al., 1986). A radioactive assay was necessary for native katanin samples, because of the presence of contaminating phosphate in the enzyme preparation. Reactions of 50-100 μl were carried out at 22-24°C in ATPase buffer (20 mM K-HEPES pH 7.5, 25 mM K-glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 10% glycerol) containing 1 mg/ml BSA or 0.25 mg/ml soybean trypsin inhibitor, as well as 20 μM paclitaxel unless otherwise indicated. DEAE-purified ATP was used at 0.5 or 1.0 mM, and reactions were started by addition of katanin. Aliquots were removed at various times and quenched by addition of perchloric acid to a final concentration of 0.3M and stored on ice.

For radioactive assays, DEAE-ATP was supplemented with [γ–^{32}P]ATP (2 Ci/mmol, NEN) to yield ~40,000-80,000 total cpm per time point by Cherenkov counting. In addition, the PCA quench contained 10-20 mM KPO_4 to prevent the nonspecific loss of radioactivity on the charcoal during separation. Unhydrolyzed ATP was removed by vortexing the quenched reactions with a 50 μl slurry of activated charcoal (Norit A, Sigma, Inc.) previously prepared by boiling in 1 M HCl, followed by extensive water washing. The charcoal was removed by centrifugation at 14,000 x g (3 min, 4°C) and the supernatant Cherenkov counted after addition of ~2 ml of water. Total hydrolysis was limited to 10-20%, to minimize product inhibition.

For nonradioactive assays, an ATP regenerating system consisting of 0.5-1.0 mM phospho-enol pyruvate and 2U pyruvate kinase was usually included to minimize the inhibition by ADP previously reported for native katanin (McNally and Vale, 1993). Phosphate concentration was measured by addition of an equal volume of malachite green reagent (Kodama et al., 1986), followed by incubation at 25°C for 35 minutes and measurement of absorbance at 650 nm. The phosphate standards were prepared by dilution of 0.65 mM KPO_4 (Sigma Diagnostics, Inc.).

Microtubule severing assays

Microtubule severing assays were carried out essentially as described elsewhere (McNally and Vale, 1993).

Table I:

Nucleotide specificity of microtubule severing by katanin.

Microtubule severing was assayed as previously described (McNally and Vale, 1993), and scored according to a qualitative scale. ++++ represents no microtubules remaining after ~3 min, +/- represents little or no change visible in a similar field after ~3 min. Kinesin and 14S dynein data were previously published (Shimizu et al., 1991). - indicates no movement or microtubule severing observed.

Table I:

Nucleotide specificity of microtubule severing by katanin.

	Microtubule Severing	Microtubule Translocation (% of ATP-driven velocity)	
Nucleotide	Katanin	Kinesin	14S Dynein
ATP	++++	100%	100%
2'dATP	+++	91%	99%
3'dATP	++++	72%	77%
ddATP	++	69%	67%
ADP	-	n.d.	n.d.
AMPPNP	-	n.d.	n.d.
Monomethyl ATP	++++	47%	17%
Dimethyl ATP	++	20%	-
PRTP	+	1.8%	-
8'bromo ATP	+	3.1%	-
8'azido ATP	+/-	-	27%
FTP	++++	8.8%	2.0%
Etheno ATP	+++	13%	-
ATPαS(Sp)	++	18%	28%
ATPαS(Rp)	+/-	-	-
ATPβS(Sp)	+/-	-	-
ATPβS(Rp)	+/-	-	-
ATPγS	+/-	2.2%	12%

Figure 1: Time course and enzyme concentration dependence of ATP hydrolysis by native katanin.

(**A**) Time course of ATP hydrolysis by native, sea urchin katanin. Reactions containing 0.5 mM ATP and ~14 nM katanin, either alone (●) or together with 0.9 μM paclitaxel-stabilized microtubules (❑), were incubated at 22°C for 0-30 min. ATPase activity was measured by release of ^{32}P from [γ-^{32}P]ATP as described in Materials and Methods. Dotted lines indicate linear curve fits, with an estimated rate of ATP hydrolysis of ~1.4 ATP katanin^{-1} sec^{-1} without added microtubules and 3.2 ATP katanin^{-1} sec^{-1} in the presence of 0.9 μM microtubules.

(**B**) Enzyme concentration dependence of the katanin ATPase reaction. Indicated concentrations of katanin were incubated with 0.5 mM ATP, either alone (●) or in the presence of 0.1 μM (○) or 0.9 μM (❑) paclitaxel-stabilized microtubules for 12 min at 22°C. The best fit to a straight line is indicated.

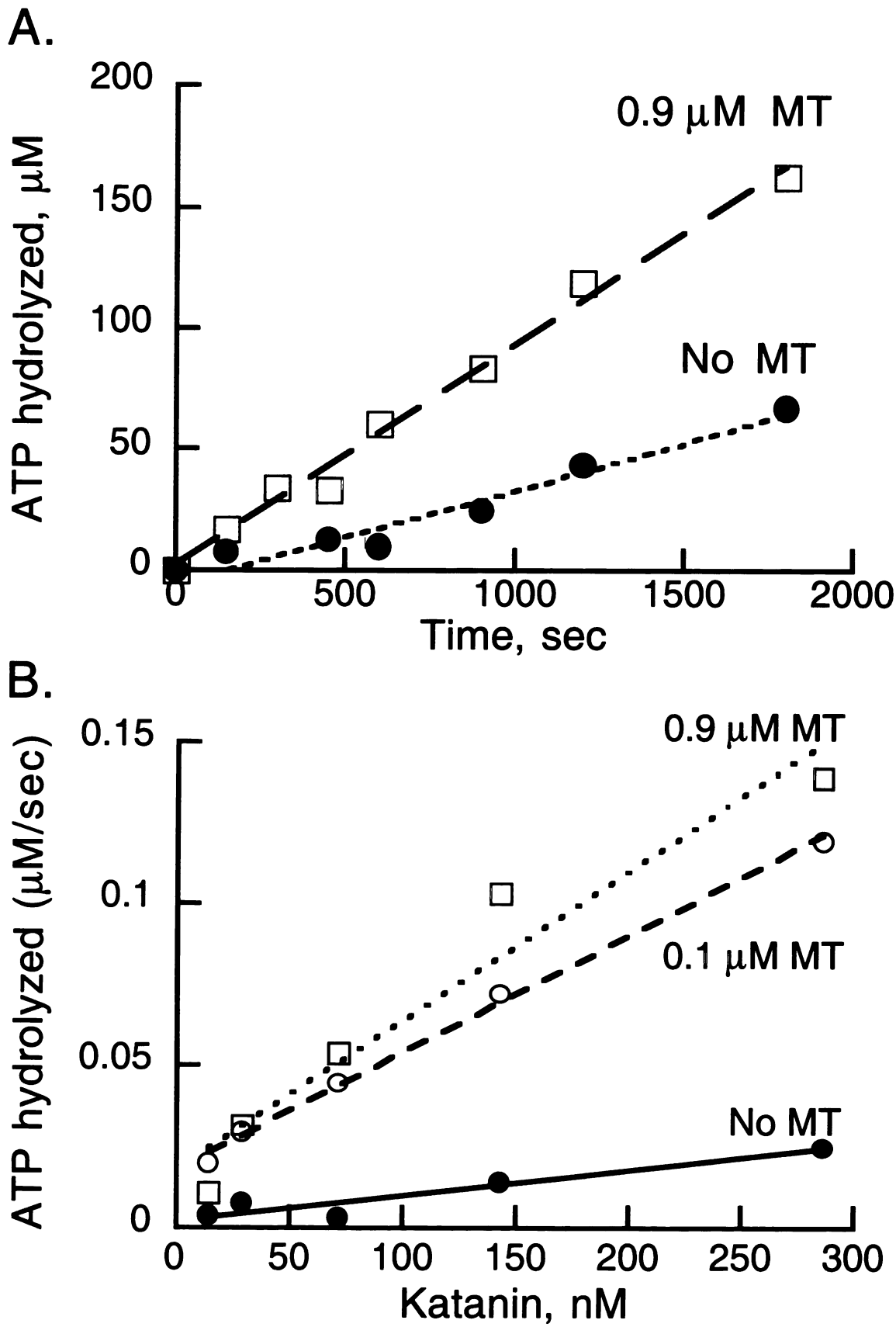

A.
200
150
100
50
0
ATP hydrolyzed, μM
0.9 μM MT
No MT
0
500
1000
1500
2000
Time, sec
B.
0.15
0.1
0.05
0
ATP hydrolyzed (μM/sec)
0.9 μM MT
0.1 μM MT
No MT
0
50
100
150
200
250
300
Katanin, nM

Figure 2: Microtubule activation of native sea urchin katanin ATPase activity.

(**A**) ATPase activity of native, sea urchin katanin incubated with varying concentrations of paclitaxel-stabilized microtubules (●) or subtilisin-treated, paclitaxel-stabilized microtubules (❑). Reactions contained 28 nM katanin, and were incubated at 22°C with time points taken at 0, 10, 20, and 30 min. ATPase activity was measured by release of ^{32}P from [γ–^{32}P]ATP as described in Materials and Methods. A curve fit shown is for two competing Michaelis-Menten reactions $(((V_{max1}*[tubulin])/(K_{m1}+[tubulin]))-((V_{max2}*[tubulin])/(K_{m2}+[tubulin])))$.

(**B**) Expanded view of ATPase activity at low microtubule concentrations (0-2 μM).

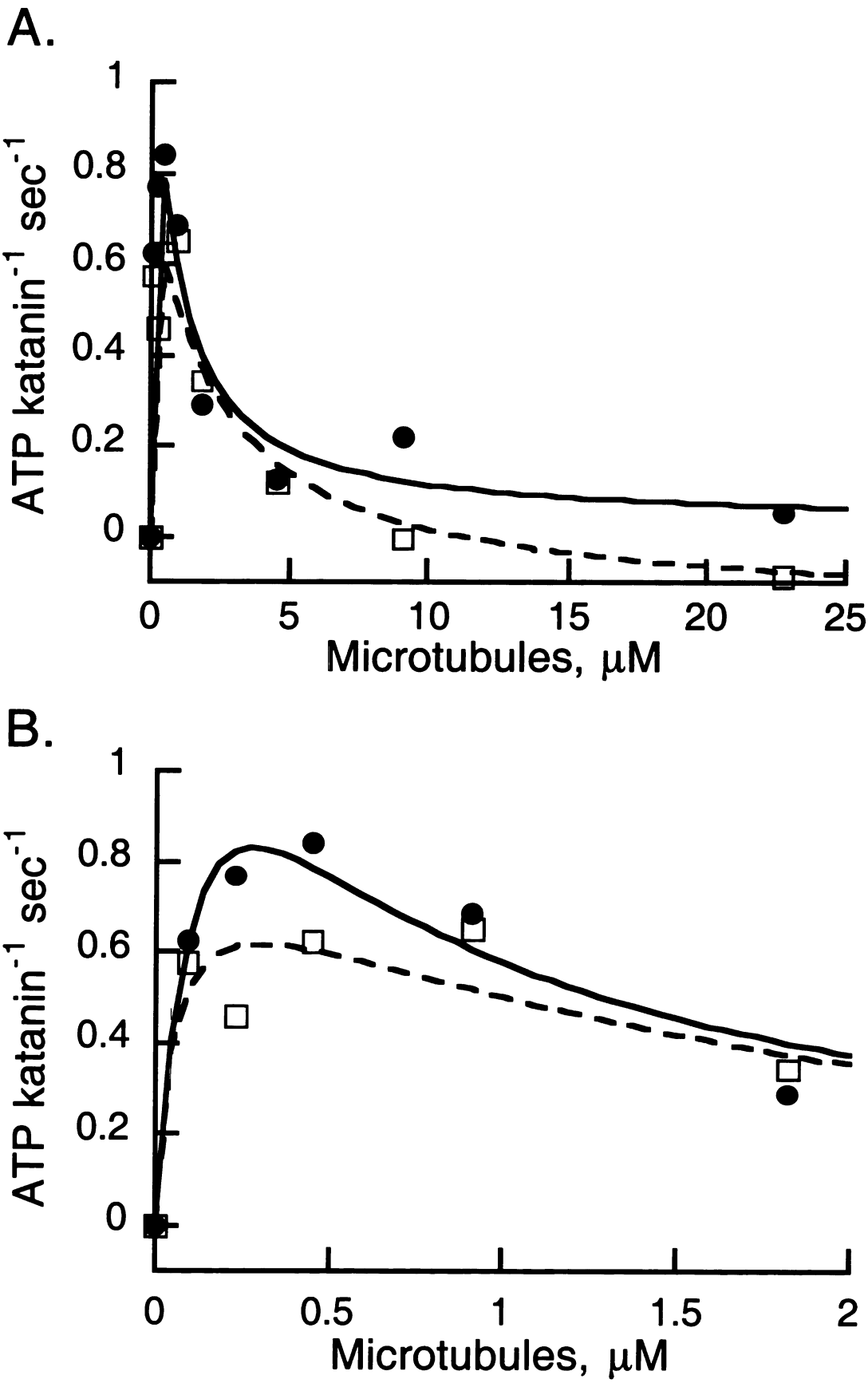

A.
ATP katanin^{-1} sec^{-1}
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Microtubules, μM
B.
ATP katanin^{-1} sec^{-1}
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
Microtubules, μM

Figure 3: ATP dependence of katanin's microtubule-stimulated ATPase activity.

The ATPase activity of ~0.2 μM native katanin was measured at 22°C in the presence of 1 μM paclitaxel-stabilized microtubules using the pyruvate kinase and lactate dehydrogenase coupled assay described in Materials and Methods. The dotted line shows the best fit to a hyperbola, yielding a K_m for ATP of 46 μM.

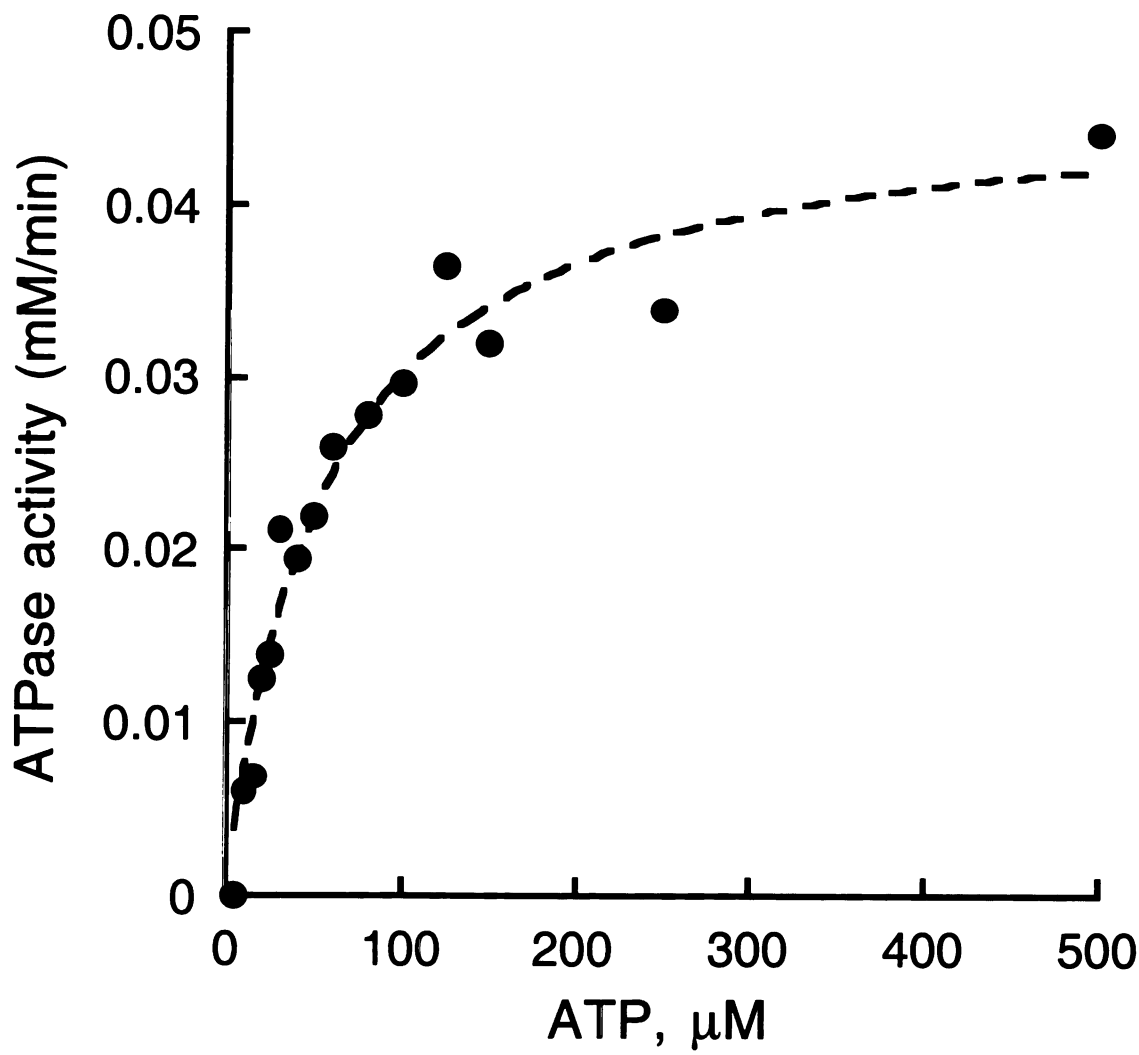

0.05
0.04
0.03
0.02
0.01
0
ATPase activity (mM/min)
0
100
200
300
400
500
ATP, μM

Figure 4: Salt dependence of the ATPase activity of native katanin.

Native katanin (~50 nM) was incubated at 22°C alone (○), or together with 1.8 μM paclitaxel-stabilized microtubules (❑) for either 30 min (no microtubules) or 20 min (with microtubules) in the presence of 1 mM ATP and varying concentrations of potassium glutamate. ATPase activity was measured by release of ^{32}P from [γ–^{32}P]ATP as described in Materials and Methods.

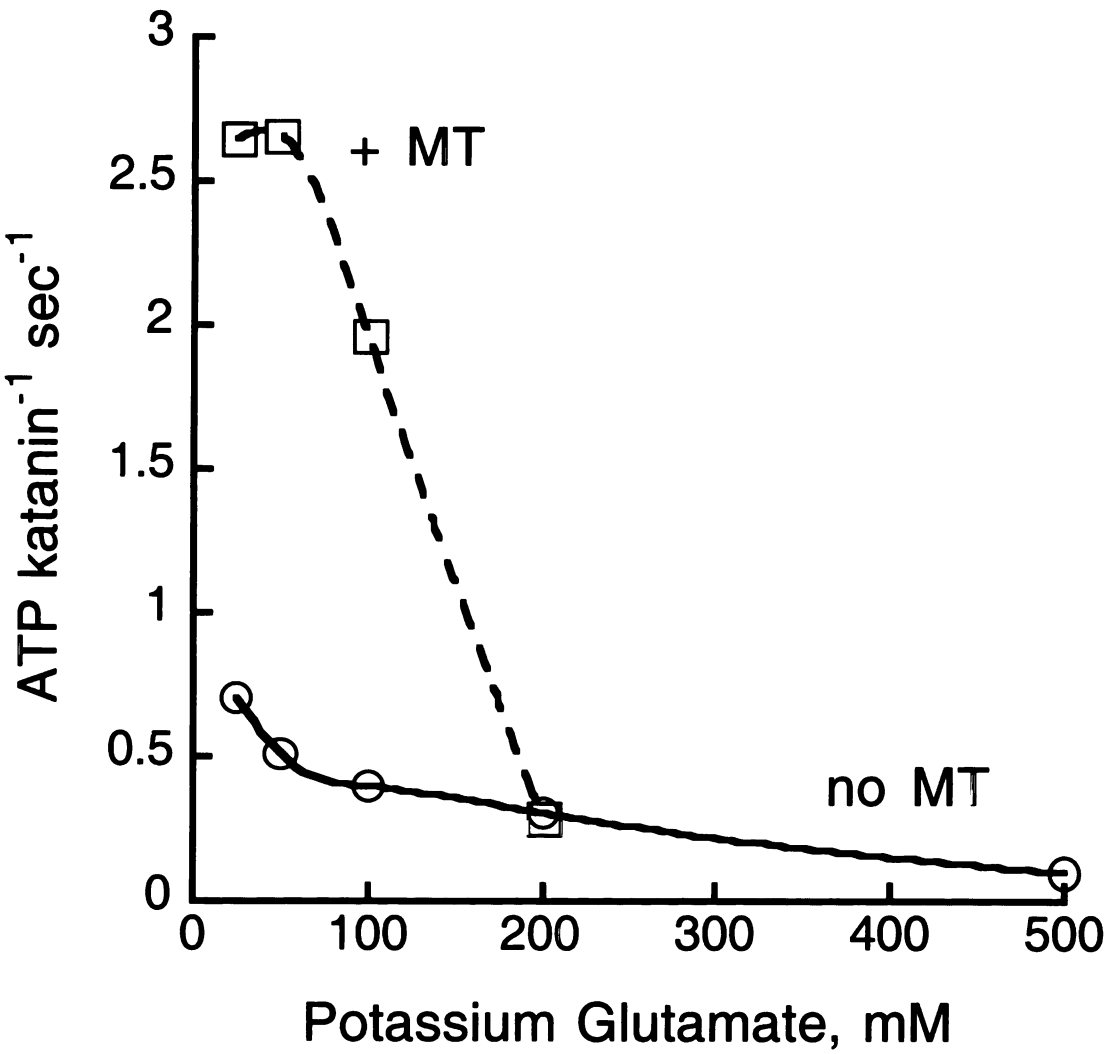

3
2.5
2
1.5
1
0.5
0
ATP katanin^{-1} sec^{-1}
+ MT
no MT
0
100
200
300
400
500
Potassium Glutamate, mM

Figure 5: pH dependence of the basal ATPase activity of recombinant p60 katanin.

Recombinant, baculovirus-expressed p60 katanin (0.2 μM) was incubated at 22°C for 40 min in the presence of 1 mM ATP and ATPase reaction buffer containing 20 mM K-EPPS of varying pH. The standard ATPase buffer, containing 20 mM K-HEPES pH 7.5, had an ATPase activity of 0.09 ATP katanin^{-1} sec^{-1} (data not shown). ATPase activity was measured using the malachite green method, with a pyruvate kinase and phospho(enol)pyruvate regenerating system included to prevent product inhibition, as described in Materials and Methods.

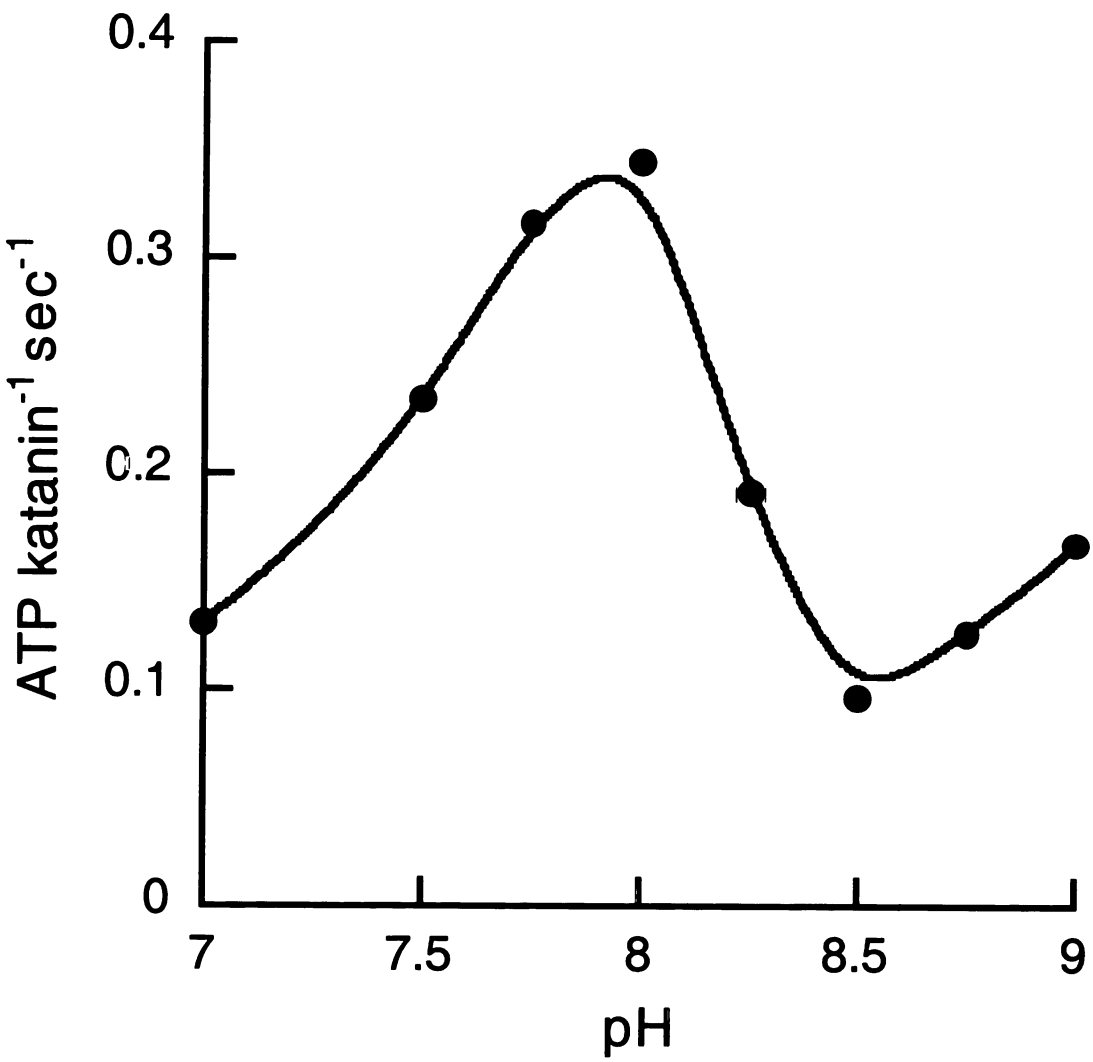

0.4
0.3
0.2
0.1
0
ATP katanin^{-1} sec^{-1}
7
7.5
8
8.5
9
pH

Figure 6: Inhibition of the ATPase activity of native katanin by ADP.

Native, sea urchin katanin (~ 50 nM) was incubated with 0.5 mM ATP for 10 min at 22°C alone (●), or in the presence of 0.9 μM paclitaxel-stabilized microtubules (❑). ATPase activity was measured by release of ^{32}P from [γ-^{32}P]ATP as described in Materials and Methods. The curve fit shown is the best fit to a straight line, yielding an IC_{50} for ADP of 0.3 mM (without microtubules) and 0.6 mM ADP (with microtubules).

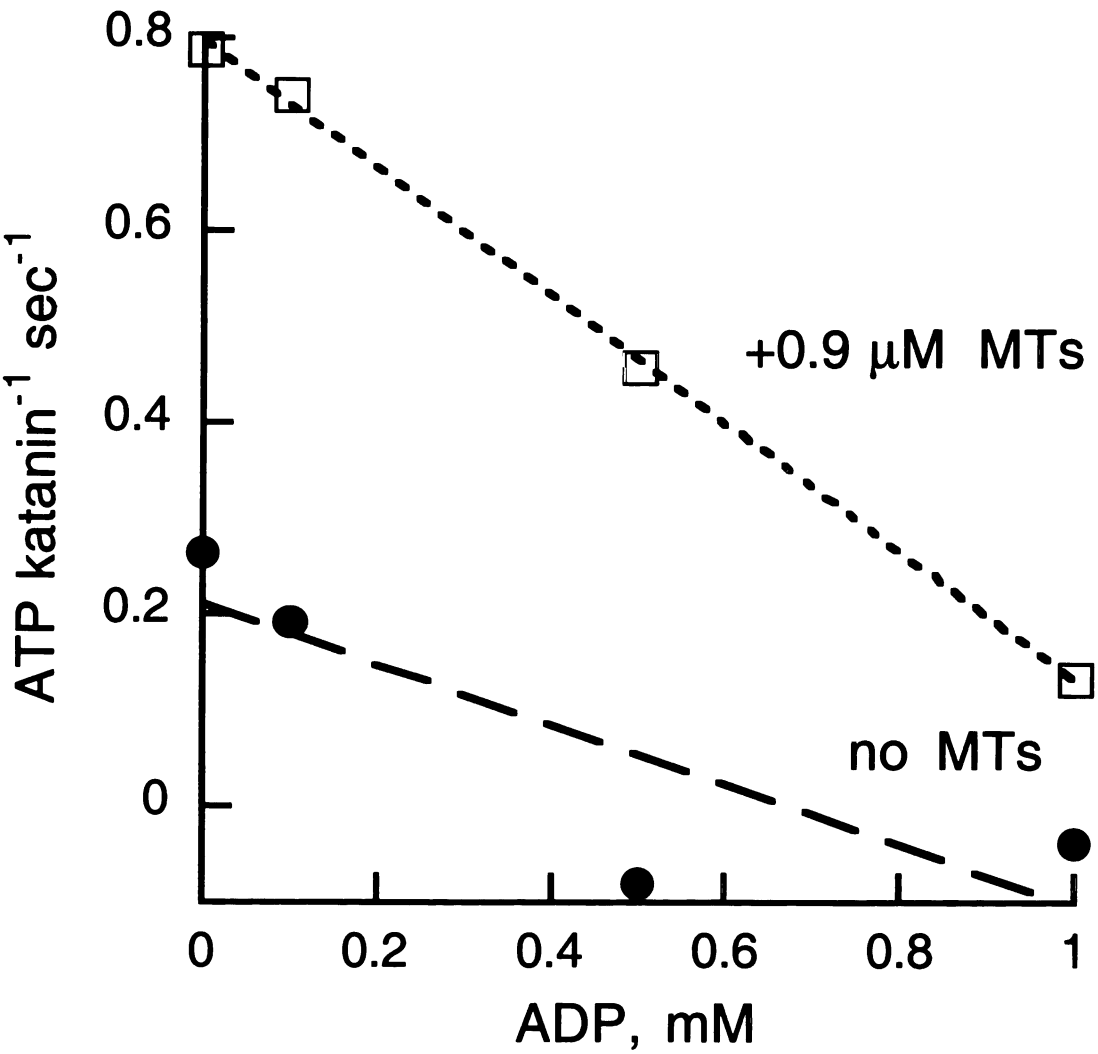

0.8
0.6
0.4
0.2
0
ATP katanin^{-1} sec^{-1}
+0.9 μM MTs
no MTs
0
0.2
0.4
0.6
0.8
1
ADP, mM

Figure 7: Inhibition of native katanin ATPase activity by nucleotide analogs.

(**A**) The nonhydrolyzable ATP analog AMPPNP does not inhibit the ATPase activity of native katanin at concentrations below 1 μM. Native katanin (~50 nM) was incubated with 0.5 mM ATP at 22°C alone (●), or in the presence of 0.9 μM paclitaxel-stabilized microtubules (❑) together with 0.1 to 1.0 mM AMPPNP. ATPase reactions were carried out for 20 min (without microtubules) or 8 min (with microtubules) and ATPase activity was measured by release of ^{32}P from [γ–^{32}P]ATP as described in Materials and Methods. Best fit lines are indicated.

(**B**) Native katanin is inhibited by the slowly hydrolyzable ATP analog ATP-γ-S. Native katanin (~50 nM) was incubated with 0.5 mM ATP at 22°C alone (●), or in the presence of 0.9 μM paclitaxel-stabilized microtubules (❑) together with 0.2 to 1.0 mM ATP-γ-S. ATPase reactions were carried out for 20 min (without microtubules) or 8 min (with microtubules) and ATPase activity was measured by release of ^{32}P from [γ-^{32}P]ATP as described in Materials and Methods. Curve fitting is a best fit line (no microtubules) or interpolated line (with microtubules).

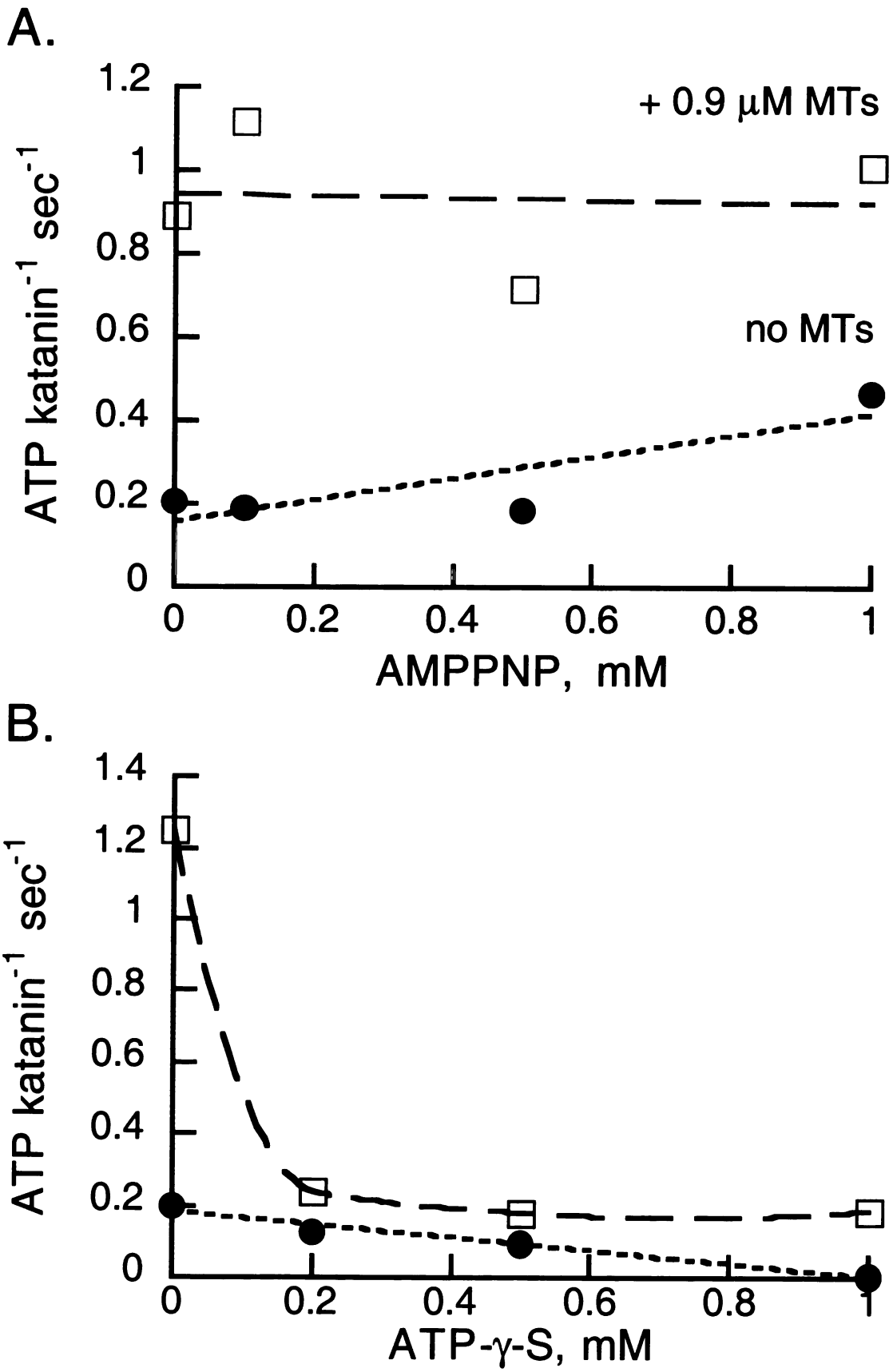

A.
1.2
1
0.8
0.6
0.4
0.2
0
ATP katanin^{-1} sec^{-1}
+ 0.9 μM MTs
no MTs
0
0.2
0.4
0.6
0.8
1
AMPPNP, mM
B.
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ATP katanin^{-1} sec^{-1}
0
0.2
0.4
0.6
0.8
1
ATP-γ-S, mM

Figure 8: Effect of nonpolymeric, nocodazole tubulin on the ATPase activity of native katanin.

Native katanin (~100 nM) was incubated with varying concentrations of nocodazole-tubulin in the presence of 0.5 mM ATP and 15 μM nocodazole. Reactions were carried out for 10 min at 22°C, and ATPase activity was measured by release of ^{32}P from [γ-^{32}P]ATP as described in Materials and Methods. The best fit to a straight line is indicated.

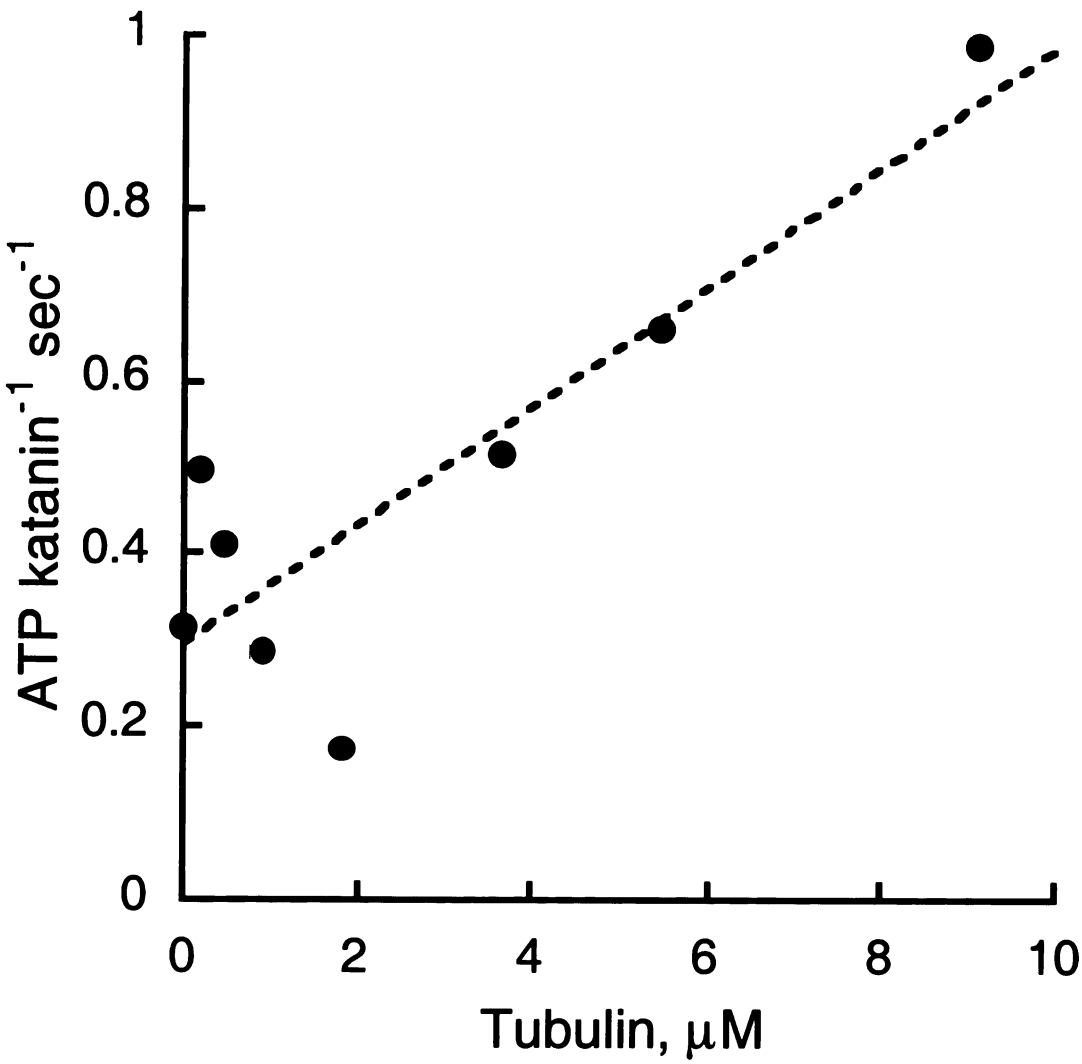

1
0.8
0.6
0.4
0.2
0
ATP katanin^{-1} sec^{-1}
0
2
4
6
8
10
Tubulin, μM

Figure 9: Effect of nonpolymeric colchicine-tubulin and GDP-tubulin on the ATPase activity of recombinant p60 katanin.

(**A**) Colchicine-tubulin stimulation of baculovirus-expressed p60 katanin ATPase activity. p60 katanin (~100 nM) was incubated for 15 min at 22°C in the presence of 1 mM ATP, 1.25 mM colchicine, and varying concentrations of colchicine-tubulin. A phospho(enol)pyruvate and pyruvate kinase regenerating system was included, to minimize inhibition by ADP. ATPase activity was measured by the malachite green method, as described in Materials and Methods. The best fit to a straight line is indicated.

(**B**) GDP-tubulin stimulation of baculovirus-expressed p60 katanin ATPase activity. p60 katanin (~100 nM) was incubated for 15 minutes at 22°C in the presence of 1 mM ATP and varying concentrations of GDP-tubulin. A phospho(enol)pyruvate and pyruvate kinase regenerating system was included, to minimize inhibition by ADP. ATPase activity was measured by the malachite green method, as described in Materials and Methods.

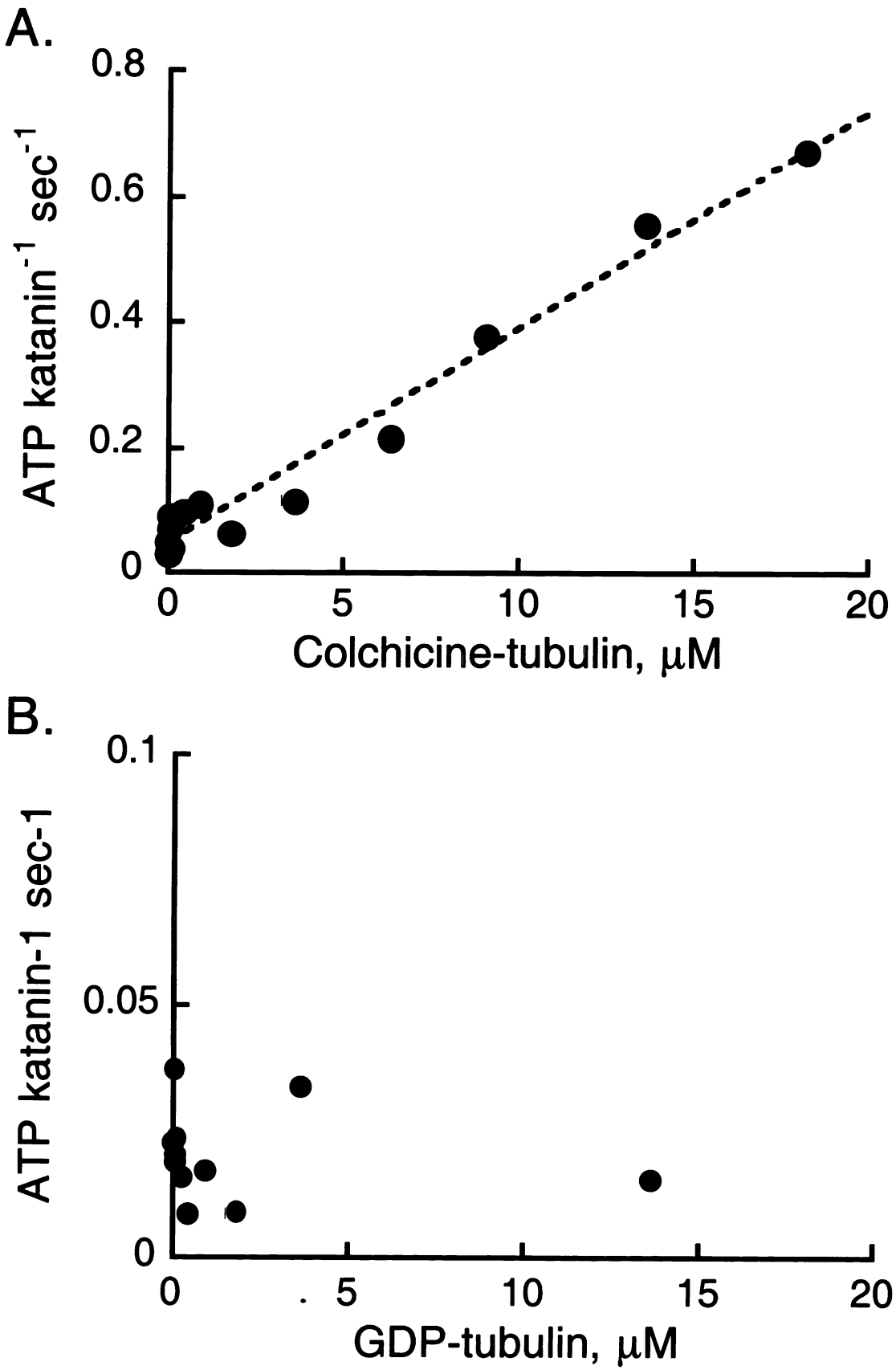

A.
0.8
0.6
0.4
0.2
0
ATP katanin^{-1} sec^{-1}
0
5
10
15
20
Colchicine-tubulin, μM
B.
0.1
0.05
0
ATP katanin-1 sec-1
0
. 5
10
15
20
GDP-tubulin, μM

Figure 10: Stimulation of recombinant p60 katanin ATPase activity by poly-glutamic acid.

p60 katanin (~40 nM) was incubated with 1 mM ATP either alone (●), with 2 μM paclitaxel-stabilized microtubules (❑), or with 2 μM poly-glutamic acid (avg. molecular weight 14 kDa, Sigma)(○). Reactions were carried out at 22°C, and aliquots were removed at 0, 10, 20, and 30 min. ATPase activity was measured using the malachite green method, as described in Materials and Methods. Best fits to straight lines are indicated.

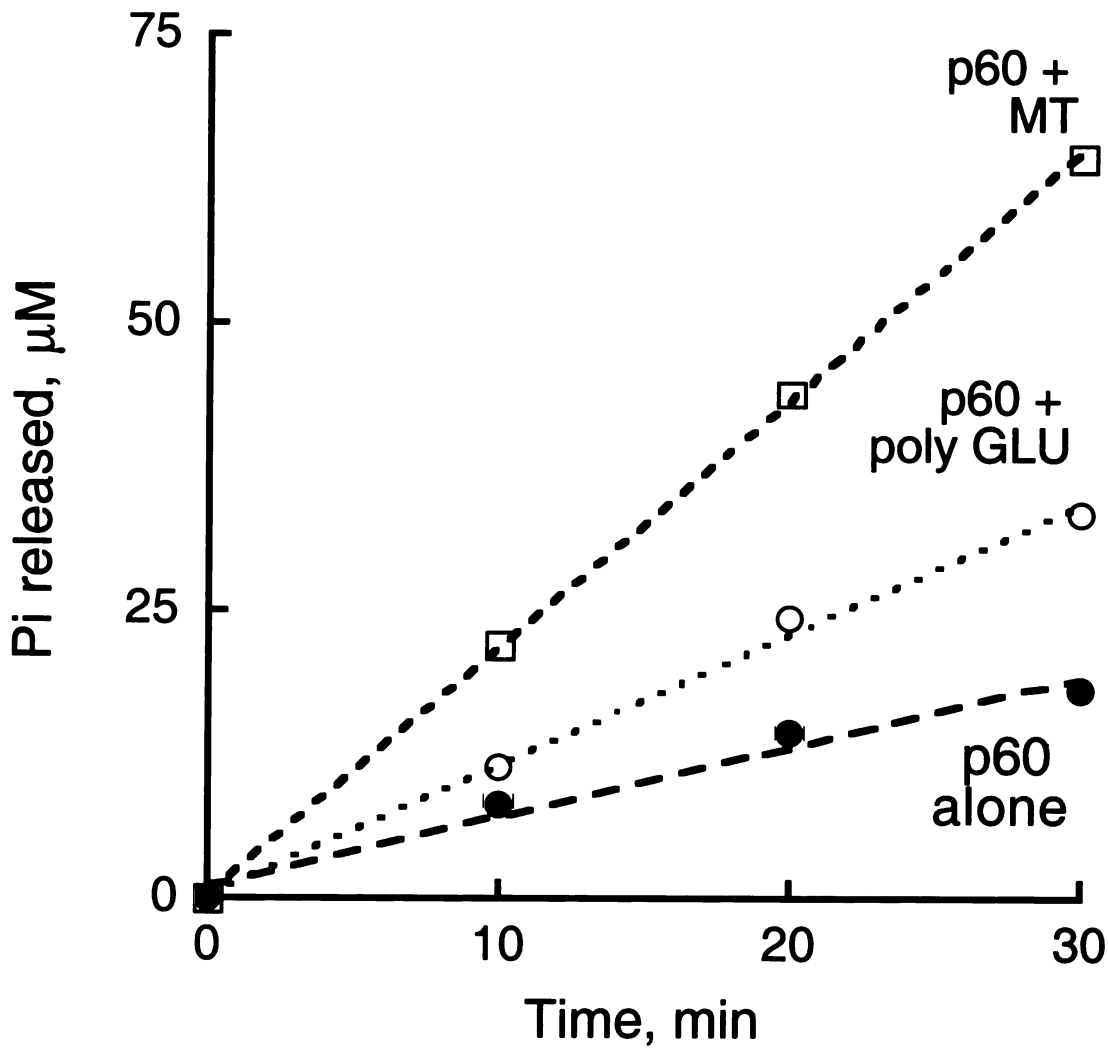
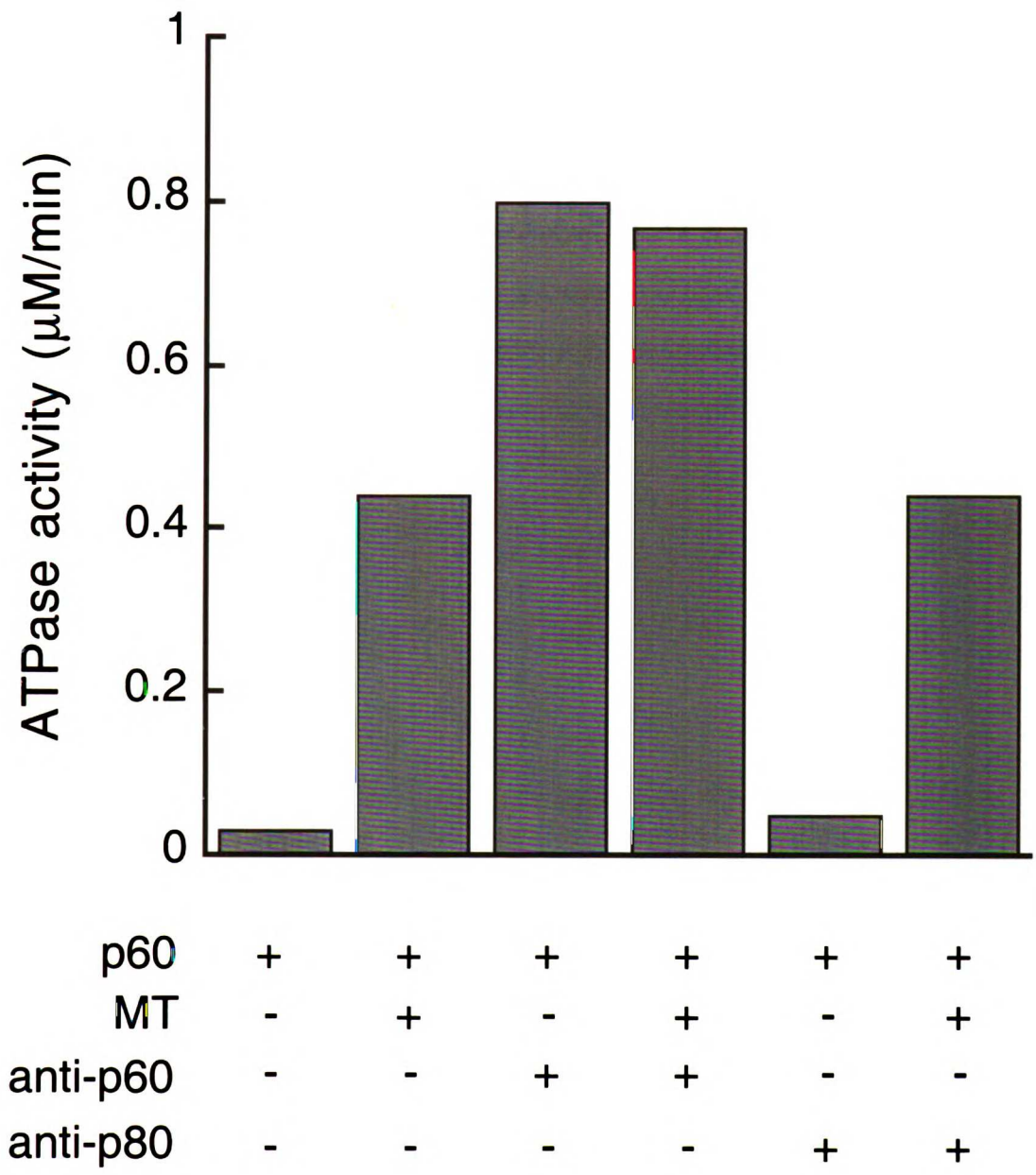

75
50
25
0
Pi released, µM
0
10
20
30
Time, min
p60 +
MT
p60 +
poly GLU
p60
alone

Figure 11: Stimulation of recombinant p60 katanin ATPase activity by anti-p60 antibodies.

p60 katanin (~100 nM) was preincubated for 20 min on ice together with either 2 µl whole serum (anti-p60 or anti-p80) or 2 µl ATPase buffer. ATPase reactions were then carried out at 22°C, in the presence of 1 mM ATP and 2 µM paclitaxel-stabilized microtubules (where indicated). Aliquots were removed at 0, 10, 20, 40, and 60 min, and ATPase activity measured by the malachite green method as described in Materials and Methods. The indicated rates of ATP hydrolysis were estimated by linear regression.


ATPase activity (μM/min)
1
0.8
0.6
0.4
0.2
0
p60 + + + + + +
MT - + - + - +
anti-p60 - - + + - -
anti-p80 - - - - + +

CONCLUSIONS

One of the most important findings in this work is that p60 katanin belongs to the AAA (ATPases Associated with a variety of cellular Activities) superfamily of ATPases, a growing group of proteins sharing one or two copies of a highly conserved 230 amino acid sequence module (Confalonieri and Duguet, 1995). Contrary to their extensive sequence conservation, AAA ATPases have been connected with many seemingly disparate functions in cells, including homotypic (p97) and heterotypic (NSF) vesicle fusion, organelle biogenesis (PAS1), intramitochondrial protein sorting (YME1), and disaggregation/proteolysis of misfolded proteins (numerous 26S proteosome regulatory subunits). The discovery of this homology is important for two reasons. First, it allows identification of sequences that have been highly conserved throughout the superfamily, suggesting they are functionally important to the many different reactions carried out by AAA ATPases. Second, the high liklihood of mechanistic similarity between different family members enables exchange of information about how AAA proteins work between the large number of laboratories working on these proteins.

Common features of AAA ATPases

What similarities do p60 and other AAA ATPases share that might be important for understanding microtubule severing? For one, several AAA ATPases have been demonstrated to be oligomeric. NSF (Fleming et al., 1998), p97 (Peters et al., 1992), and VAT (Rockel et al., 1999) all form stable hexameric complexes, and this has been attributed to the presence of a nonhydrolyzing AAA domain (D2) that anchors the complex together. NSF is oligomeric in the presence of either ATP-γ–S or ADP, but adopts different conformations in these two nucleotide states as revealed by rotary shadowing electron microscopy (Hanson et al., 1997). Few single AAA domain proteins

like p60 have been studied biochemically, but current results suggest that they can oligomerize as well. DEAD box mutants of p60 and Vps4, $p60^{E334Q}$ (this thesis, Chapter 2) and $Vps4^{E233Q}$ (Babst et al., 1998), bind but do not hydrolyze ATP and form high molecular weight complexes. Furthermore, $p60^{E334Q}$ and $Vps4^{E233Q}$ oligomers are stable in the presence of ATP but not ADP. From this, it is tempting to speculate that all AAA ATPases share the ability to oligomerize at some point in the ATPase cycle, and they may either change oligomerization state and/or shape depending on the nucleotide bound to the AAA domain.

This ability to oligomerize appears to be inherent to the AAA domain. Removal of the nonhydrolyzing D2 AAA domain prevents stable oligomerization of NSF (Hanson et al., 1997), while the isolated D2 domain can form stable hexamers (Fleming et al., 1998). We postulate that the N-terminal, unique half of p60 contains the substrate (microtubule) binding site, based on similar findings with NSF (Nagiec et al., 1995). This is supported by our findings that p60ΔAAA, a truncated form of p60 completely lacking the AAA domain and thus presumably unable to oligomerize, can still bind microtubules in a nucleotide-insensitive manner and with affinity intermediate to the ATP and ADP states of full-length p60. From this, we predict that oligomerization is not essential for at least weak binding of p60 to microtubules. Consequently, oligomerization of p60 has the potential for taking multiple, independent microtubule binding sites and locking them together in a multivalent complex.

What are the implications of this nucleotide-dependent multimerization of microtubule binding sites for the severing mechanism? First, multimerization has the ability to dramatically increase the affinity of enzymes for their substrates, based on the chelate effect (Jencks, 1981).

Bivalent antibodies, for example, bind their targets ~1000 times stronger than univalent Fab fragments, due to loss of entropy upon binding at the first site (Erickson, 1989; Hornick and Karush, 1972). This is a significant enhancement of affinity, but does not approach the maximum enhancement predicted (estimated $\sim 10^{11}$ for antibodies) because the linkages between the binding sites in antibodies are flexible (Erickson, 1989). Reduced flexibility between binding sites can result in higher affinity, provided the sites can contact their substrate, because a greater amount of entropy is lost at the initial binding event. And loss of entropy is a major driving force that makes cooperative, polymeric structures like actin filaments and microtubules stable (Erickson, 1989). In the case of microtubules, the formation of a single additional tubulin-tubulin contact makes subunit loss $\sim 10^{11}$ slower from the wall then at the ends (Dye et al., 1992), demonstrating the profound influence of cooperativity in establishing stable macromolecular complexes. With the proper coupling, the energy released by binding a multimer to the microtubule could be applied to destabilizing tubulin-tubulin contacts in the microtubule.

In addition to ensuring tight binding, an oligomer of independent binding sites also has the potential for moving sites with respect to each other, providing a source of strain and a mechanical model for severing. Differences in the shape of NSF hexamer in the presence of ATP-γ–S and ADP have already been seen by electron microscopy, strengthening this assertion for the AAA family (Hanson et al., 1997). These two principles, oligomerization-enhanced binding and mechanical strain driven by shape changes, suggest several non-exclusive models for how katanin disassembles microtubules.

Microtubule severing by binding-induced strain

One possibility is that katanin severs microtubules by using binding energy to induce strain in the microtubule lattice. Tau stabilization of microtubules is an example of using binding at multiple sites to do work on microtubules. While tau is not multimeric, it does contains an array of four short amino acid repeats separated by flexible linkers. Each repeat binds microtubules weakly when tested separately (K_d > 200 μM tubulin), but linked together in the same polypeptide, the free energy of binding is additive and results in much tighter binding (K_d < 1 μM) (Butner and Kirschner, 1991). Binding of tau specifically to polymeric tubulin, not tubulin dimers, enhances the stability of microtubules, increasing the rate and extent of tubulin polymerization *in vitro* (Drechsel et al., 1992).

Conversely, proteins that bind tightly to unstable conformations of tubulin would be expected to destabilize microtubules. This has been seen with the Kin I kinesins, which bind to microtubule ends and induce a lattice-destabilizing conformational strain into the tubulin subunits, causing splaying of the protofilaments and eventual depolymerization (Desai et al., 1999). Binding of XKCM1 to microtubules in the presence of nonhydrolyzable AMPPNP is sufficient to cause this structural change and cause depolymerization; the energy of ATP hydrolysis only appears to be necessary for switching XKCM1 into a weak binding state that releases product and enables another cycle of action (Desai et al., 1999). Gelsolin appears to sever actin polymers using a cooperative, binding-energy driven mechanism as well. The minimal gelsolin construct that can sever actin consists of an N-terminal actin capping domain, linked to an actin filament side-binding domain. Based on crystallographic analysis of this minimal severing protein complexed with actin, the side-binding domain docks gelsolin onto the

filament and positions the capping domain where it can occlude an essential actin-actin contact . This mechanism is absolutely dependent on the ability of the side-binding domain to tether the capping domain on the filament, increasing the local concentration of actin and driving the binding of the capping domain to the monomer interface. Without the tethering activity, incredible amounts of the isolated capping domain would be required to achieve the same binding.

In the case of p60, the coalescing of multiple microtubule binding domains into a ring complex has the potential to increase dramatically the affinity of p60 for microtubules, providing the energy to strain the microtubule. Our data suggests p60(ATP-γ–S) binds microtubule with a K_d~0.9 μM, similar to the affinity of full-length tau for microtubules (Gustke et al., 1994) but not exceptionally tight. Our FRET assay suggests that microtubule-bound p60(ATP-γ–S) is oligomeric, but a molecule with ~6 binding sites has the potential for much tighter binding than we observe. One explanation is that the full enhancement of binding is not experienced because of flexible linkages between the binding sites, similar to what precludes the tightest binding to bivalent antibodies as described above. Alternatively, the ATP-γ–S state may not represent the tightest binding conformation of the enzyme. Since ATP hydrolysis is necessary for severing (McNally and Vale, 1993), a transient intermediate in the ATPase cycle, like p60(ADP•Pi), might bind microtubules more tightly, but be difficult to measure in our steady-state assay. Another possibility is that all binding sites might not be able to contact the microtubule simultaneously in the ATP-γ-S state. Our measurements might then reflect only partial occupancy of the p60 ring with microtubule ligand.

A hexameric ring of p60, the oligomeric structure suggested for p60 by hydrodynamics and by comparison with other AAA proteins, would have very different symmetry than the tubulin subunits in a microtubule. Tight binding of multiple tubulin subunits to the ring could impart significant strain on the microtubule, providing a mechanism for disrupting tubulin-tubulin contacts. Unlike the actin severing protein gelsolin, katanin is known to carry out multiple cycles of severing, requiring release of the tubulin tightly bound to the ring. ATP hydrolysis supplies energy that could be used in this model to lower the affinity of p60 for tubulin, stimulating product release and enabling multiple rounds of severing. This model predicts large changes in microtubule binding affinity in different nucleotide states, with very tight binding to a strained microtubule conformation occurring at come point in the ATPase cycle. It does not require any large conformational changes, however, although some structural rearrangements might comprise the mechanism for switching between tight and weak binding states.

Microtubule-severing by mechanical strain induced by shape changes

The potential for multiple binding sites in a katanin ring, together with the shape changes observed in the NSF ring (Hanson et al., 1997), suggests a mechanism whereby movement of tubulin-binding domains relative to one another strains the microtubule lattice. In this model, a katanin ring in the ATP state first binds the microtubule in a "relaxed" conformation, making contacts with several neighboring tubulin subunits. Then, hydrolysis of ATP causes p60 to adopt a different, "strained" conformation that moves the tubulin binding domains apart from each other. If tubulin subunits were tightly bound to the p60 subunits, this conformational change would be able

to mechanically force the tubulin subunits apart, disrupting tubulin-tubulin contacts and weakening the microtubule. Finally, in the ADP state p60 has been shown to have lower affinity for itself and tubulin, promoting disassembly of the katanin ring and release of the severed tubulin products. This is similar to the proposed mechanism for dynamin-catalyzed vesiculation of membrane tubules. In the absence of guanine nucleotides, dynamin self-assembles into an ordered helical lattice on charged membranes, elongating them into membrane tubules (Sweitzer and Hinshaw, 1998). Hydrolysis of GTP by lipid-bound dynamin results in the constriction of membrane tubules and vesiculation (Sweitzer and Hinshaw, 1998). If p60 severs microtubules by a similar mechanism, it should be possible to observe significant structural changes in the position of the microtubule binding domain in different nucleotide states, in order to provide the mechanical strain to break the tubulin bonds. This model would also predict the formation of very tight bonds between p60 and tubulin subunits in the microtubule, tighter than tubulin-tubulin contacts in the microtubule, because if the tubulin-p60 interaction were weak during the relaxed-tight conformational change, the p60 ring would simply fall off the microtubule instead of severing it. Another possibility is that the shape change in p60 induces a change in tubulin structure, lowering the affinity of the bound tubulin for its neighbors in the microtubule lattice. In either case, the p60-tubulin linkage would have to be reasonably rigid, otherwise the energy released by the change in ring shape would just deform the tubulin binding domain of p60, rather than the tubulin-tubulin contacts. The energetics of multivalent p60-tubulin binding could also be a key feature in this mechanical model, since the coordinated formation of multiple p60-

tubulin bonds could allow the breaking of a single, stronger tubulin-tubulin bond.

Microtubule-severing by partial unfolding of tubulin subunits

Another possibility for microtubule severing involves the p60-catalyzed unfolding of a tubulin subunit in the microtubule wall. Like a chaperone, p60 could unfold certain structural elements that hold one tubulin subunit in contact with its neighbors in the microtubule (Nogales et al., 1999), allowing it to refold into an active form after it has been removed from the microtubule wall. Previous experiments demonstrating the polymerizability of tubulin subunits released from severed microtubules (McNally and Vale, 1993), together with the complexity of the tubulin folding pathway (Tian et al, 1996), suggest that p60 does not fully unfold tubulin during the severing reaction. This does not rule out the localized unfolding of one or two structural elements, especially if p60 remains in contact with the tubulin subunit, preventing aggregation until the tubulin reaches its native state. Interestingly, the AAA domain of the yeast mitochondiral inner membrane protease Yme1 has been shown to enhance the refolding of DHFR and rhodanase *in vitro*, suggesting it has chaperone activity (Leonhard et al., 1999) and lending some support to this mechanism. Furthermore, the unfolding process may involve mechanical forces similar to those proposed in the previous model. Experiments with the GroEL chaperonin suggest that ATP binding is coupled to the forceful unfolding substrate proteins (Shtilerman et al., 1999). This fits with structural studies that indicate a significant change in the shape of the GroEL/GroES complex upon ATP binding, moving suspected substrate binding domains with respect to one another (Xu et al., 1997). Distinguishing between a chaperone model and

simple mechanical displacement will require evidence for transient structural changes in tubulin, such as protease susceptibility or the temporary loss of α–helical or β-sheet regions involved in tubulin-tubulin contacts.

Role of the C-terminal tail in the severing reaction

Finally, the C-terminal tail of tubulin might play an essential role in the severing mechanism. Subtilisin-treated microtubules are not severed by katanin (McNally and Vale, 1993), even though they stimulate katanin ATPase activity in a manner very similar to uncleaved microtubules (this thesis, Chapter 3). Subtilisin removes a negatively charged, glutamic acid rich ~2 kDa peptide from the C-terminus of both α– and β-tubulin (Sackett et al., 1985). The product, tubulin S, has lower overall negative charge, and has been shown to have a critical concentration 10-fold lower than untreated tubulin, polymerizing more readily and to a larger extent (Sackett et al., 1985). The C-terminal domain of tubulin has been implicated in binding of several MAPs to microtubules. For example, subtilisin treatment of tubulin abolishes MAP2 binding (Serrano et al., 1984), and a peptide that corresponds to the region removed by subtilisin treatment abolishes MAP2-stimulated tubulin assembly (Maccioni et al., 1986). While direct binding assays have not been performed with subtilisin-treated microtubules and katanin, the available ATPase data suggest that p60 is capable of making reasonably tight bonds with microtubules in the absence of the negatively-charged C-terminal tails, and these microtubules are capable of promoting the oligomerization of p60 as evidenced by the ability of subtilisin microtubules to stimulate p60's ATPase activity.

One possibility is that p60 makes two types of contacts with the microtubule, first oligomerizing via one kind of contact, then pulling on the

C-terminal tubulin tails to actually promote severing. In this mechanism, the charged tails could be thought of as handles that p60 holds onto, pulling the tubulin subunits along when the p60 ring undergoes concerted structural changes. An alternative possibility is that p60 actually restructures the C-terminal tail, using it like a wedge to disrupt tubulin-tubulin contacts. The increased polymerizability of tubulin lacking the charged C-terminal tails suggests that it exerts at least some destabilizing effect on the tubulin polymer. Furthermore, the high density of negative charges in the C-terminus suggests that it adopts an extended conformation (Sackett et al., 1985). Binding of katanin could reposition this region in a location where it disrupts a tubulin-tubulin interface, such as the primarily electrostatic lateral interactions between tubulin subunits (Nogales et al., 1999), leading to microtubule destabilization. This appears to be the only case, thus far, of a multiprotein substrate that can uncouple the ATPase and mechanical activity of an AAA ATPase, and it may be quite useful for future study of the mechanism of the family.

Future experiments

Given this abundance of possible mechanisms for how katanin physically disrupts microtubules, what experiments can be done to better define the microtubule severing reaction? First, electron microscopy of katanin bound to microtubules could provide considerable information about the severing reaction. We predict that p60 forms a hexamer on the microtubule, but EM reconstruction would provide definitive information about the active form of p60 bound to microtubules, and possibly indicate how many tubulin subunits are contacted. Electron microscope reconstructions of the p60-microtubule complex in the presence of different

nucleotides will enable the accurate description of what structural components of tubulin are being contacted by katanin, as well as provide snapshots of how this pattern changes during the ATPase cycle. Crystallographic structural studies will undoubtedly provide the highest resolution information about changes in the shape of p60 during the ATPase cycle, but difficulties with obtaining large amounts of katanin, together with the complications of a transient oligomerization cycle, could make obtaining high quality protein crystals difficult. Other possibilities include using fluorescent probes, as well as circular dicroism and protease sensitivity to study the folding state of tubulin during the severing reaction. Alternatively, isotope exchange experiments similar to those used to show unfolding of substrates by GroEL/ES (Shtilerman et al., 1999), could be applied to the microtubules to test if p60 forcibly unfolds structural elements of tubulin in order to remove it from the microtubule lattice.

Closing

Multiple cooperative interactions stabilize the microtubule wall, and cooperativity may be essential to the mechanism of any protein that can disassemble them. The targets of other AAA proteins, like the SNAP-SNARE complex disassembled by NSF, could be considered cooperative structures. Even protein aggregates could also be considered cooperative, because of the contribution of many weak, nonspecific hydrophobic contacts. It may be unlikely that a single polypeptide can provide enough binding energy to either directly disrupt protein-protein contacts or to stay bound to the substrate throughout a cycle of mechanical straining. The AAA domain may be an "oligomerization engine", providing the cooperativity necessary to achieve tight coordinated binding and to link the hydrolysis of multiple ATP molecules with either large structural movements or to the release of a tight binding state. Cooperative hydrolysis of multiple ATP molecules may function to provide a large block of free energy, which can be coupled to overcoming the inherent stability of many different of macromolecular complexes.

REFERENCES

Andreu, J. M., and Timasheff, S. N. (1982). Biochemistry, 6465-6476.

Apte, A. N., and Siebert, P. D. (1993). Anchor-ligated cDNA libraries: a technique for generating a cDNA library for the immediate cloning of the 5' ends of mRNAs. Biotechniques *15*, 890-893.

Baas, P. W., and Yu, W. (1996). A composite model for establishing the microtubule arrays of the neuron. Mol. Neurobiol. *12*, 145-161.

Babst, M., Wendland, B., Estepa, E. J., and Emr, S. D. (1998). The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome fusion. EMBO J. *17*, 2982-2993.

Belmont, L. D., Hyman, A. A., Sawin, K. E., and Mitchison, T. J. (1990). Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. Cell *62*, 579-589.

Belmont, L. D., and Mitchison, T. J. (1996). Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. Cell *84*, 623-631.

Burtnick, L. D., Koepf, E. K., Grimes, J., Jones, E. Y., Stuart, D. I., McLaughlin, P. J., and Robinson, R. C. (1997). The crystal structure of plasma gelsolin: implications for actin severing, capping, and nucleation. Cell *90*, 661-670.

Butner, K., and Kirschner, M. (1991). Tau protein binds to microtubules through a flexible array of distributed weak sites. J. Cell Biol. *115*, 717-730.

Caplow, M., Ruhlen, R. L., and Shanks, J. (1994). The free energy of hydrolysis of a microtubule-bound nucleoside triphosphate is near zero: all of the free energy for hydrolysis is stored in the microtubule lattice. J. Cell Biol. *127*, 779-788.

Caplow, M., and Shanks, J. (1996). Evidence that a single monolayer tubulin-GTP cap is both necessary and sufficient to stabilize microtubules . Mol. Biol. Cell *7*, 663-675.

Carlier, M.-F., Didry, D., Simon, C., and Pantaloni, D. (1989). Mechanism of GTP hydrolysis in tubulin polymerization: characterization of the kinetic intermediate microtubule-GDP-Pi using phosphate analogs. Biochemistry *28*, 1783-1791.

Cassimeris, L. (1993). Regulation of microtubule dynamic instability. Cell Motil. Cytoskel. *26*, 275-281.

Cassimeris, L. U., Wadsworth, P., and Salmon, E. D. (1986). Dynamics of microtubule depolymerization in monocytes. J. Cell Biol. *102*, 2023-2032.

Chen, Y.-T., and Schliwa, M. (1990). Direct observation of microtubule disassembly in Reticulomyxa: unusually rapid length changes and microtubule sliding. Cell Motil. Cytoskel. *17*, 214-226.

Clark-Maguire, S., and Mains, P. E. (1994a). mei-1, a gene required for meiotic spindle formation in Caenorhabditis elegans, is a member of a family of ATPases. Genetics *136*, 533-546.

Clark-Maguire, S., and Mains, P. E. (1994b). Localization of the mei-1 gene product of Caenorhabditis elegans, a meiotic-specific spindle component. J. Cell Biol. *126*, 199-209.

Cleveland, D. W., Hwo, S.-Y., and Kirschner, M. W. (1977). Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. J. Mol. Biol. *116*, 227-247.

Confalonieri, F., and Duguet, M. (1995). A 200-amino acid ATPase module in search of a basic function. BioEssays *17*, 639-650.

Desai, A., and Mitchison, T. J. (1995). A new role for motor proteins as couplers to depolymerizing microtubules . J Cell Biol *128*, 1-4.

Desai, A., Verma, S., Mitchison, T. J., and Walczak, C. E. (1999). Kin I kinesins are microtubule-destabilizing enzymes. Cell *96*, 69-78.

Desai, A. B., and Mitchison, T. J. (1997). Microtubule polymerization dynamics. In Ann. Rev. Cell Dev. Biol., J. A. Spudich, J. Gerhart, S. L. McKnight and R. Schekman, eds. (Palo Alto, CA: Annual Reviews, Inc.), pp. 83-117.

Drechsel, D. N., Hyman, A. A., Cobb, M. H., and Kirschner, M. W. (1992). Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. Mol. Biol. Cell. *3*, 1141-1154.

Dye, R. B., Flicker, P. F., Lien, D. Y., and Williams, R. C. J. (1992). End-stabilized microtubules observed in vitro: stability, subunit interchange, and breakage. Cell Motility and the Cytoskeleton *21*, 171-186.

Erickson, H. P. (1989). Co-operativity in protein-protein association. J. Mol. Biol. *206*, 465-474.

Febvre-Chevalier, C., and Febvre, J. (1992). Microtubule disassembly in vivo: intercalary destabilizatoin and breakdown of microtubules in the heliozoan Actinocoryne contractilis. J. Cell Biol. *118*, 585-594.

Fleming, K. G., Hohl, T. M., Yu, R. C., Muller, S. A., Wolpensinger, B., Engel, A., Engelhardt, H., Brunger, A. T., Sollner, T. H., and Hanson, P. I. (1998). A revised model for the oligomeric state of the N-ethylmaleimide-sensitive fusion protein, NSF. J. Biol. Chem. *273*, 15675-15681.

Gaudet, R., Bohm, A., and Sigler, P. B. (1996). Crystal structure at 2.4 Å resolution of the complex of transducin bg and its regulator, phosducin. Cell *87*, 577-588.

Gilbert, S. P., and Johnson, K. A. (1993). Expression, purification, and characterization of the Drosophila kinesin motor domain produced in Escherichia coli. Biochemistry *32*, 4677-4684.

Gustke, N., Trinczek, B., Biernat, J., Mandelkow, E.-M., and Mandelkow, W. (1994). Domains of tau protein and interactions with microtubules. Biochemistry *33*, 9511-9522.

Hackney, D. O. (1988). Kinesin ATPase: Rate-limiting ADP release. Proc. Natl. Acad. Sci. U.S.A. *85*, 6314-6318.

Hanson, P. I., Otto, H., Barton, N., and Jahn, R. (1995). The N-ethylmaleimide-sensitive fusion protein and α–SNAP induce a conformational change in syntaxin. J. Biol. Chem. *270*, 16955-16961.

Hanson, P. I., Roth, R., Morisake, H., Jahn, R., and Heuser, J. E. (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. Cell *90*, 523-535.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. (Cold Spring Harbor: Cold Spring Harbor Laboratory).

Harris, P., Osborn, M., and Weber, K. (1980). Distribution of tubulin-containing structures in the egg of the sea urchin Stronglyocentrotus purpuratus from fertilizatoin through first cleavage. J. Cell Biol. *84*, 668-679.

Hartman, J. J., Mahr, J., McNally, K., Okawa, K., Iwamatsu, I., Thomas, S., Cheeseman, S., Heuser, J., Vale, R. D., and McNally, F. J. (1998). Katanin, a

microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. Cell *93*, 277-287.

Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T., and Neimann, H. (1995). Disassembly of the reconstituted synaptic vesicle membrane fusion complex in vitro. EMBO J. *14*, 2317-2325.

Heusele, C., Bonne, D., and Carlier, M. F. (1987). Is microtubule assembly a biphasic process? A fluorimetric study using 4',6-diamidino-2-phenylindole as a probe. Eur. J. Biochem. *165*, 613-620.

Heuser, J. (1989). Procedure for 3-D visualization of molecules on mica via the quick-freeze, deep-etch technique. J. Elect. Micro. Tech. *13*, 244-263.

Heuser, J. E. (1983). Procedure for freeze-drying molecules adsorbed to mica flakes. J. Mol. Biol. *169*, 155-195.

Hollenbeck, P. J., and Cande, W. Z. (1985). Microtubule distribution and reorganization in the first cell cycle of fertilized eggs of Lytechinus pictus. Eur. J. Cell Biol. *37*, 140-148.

Horio, T., and Hotani, H. (1986). Visualization of the dynamic instability of individual microtubules by dark-field microscopy. Nature *321*, 605-607.

Hornick, C. L., and Karush, F. (1972). Antibody affinity. 3. The role of multivalance. Immunochemistry *9*, 325-340.

Howell, B., Larsson, N., Gullberg, M., Cassimeris, L. (1999). Dissociation of the tubulin-sequestering and microtubule catastrophe-promoting activities of oncoprotein 18/stathmin. Mol. Biol. Cell *10*, 105-118.

Hyman, A., Dreschel, D., Kellogg, D., Salser, S., Sawin, K., Steffen , P., Wordeman, L., and Mitchison, T. (1990). Preparation of modified tubulins. In Methods in Enzymology: Academic Press, Inc., pp. 303-319.

Hyman, A. A., and Mitchison, T. J. (1991). Two different microtubule-based motor activites with oppostie polarities in kinetochores. Nature *351*, 206-211.

Hyman, A. A., Salser, S., Drechsel, D. N., Unwin, N., and Mitchison, T. J. (1994). Role of GTP hydrolysis in microtubule dynamics: information from a slowly hydrolyzable analog, GMPCPP. Mol. Biol. Cell *3*, 1155-1167.

Iwamatsu, A. (1992). S-carboxymethylation of proteins transferred onto polyvinylidene difluoride membranes followed by in situ protease digestion and amino acid microsequencing. Electrophoresis *13*, 142-147.

Jencks, W. P. (1981). Proc. Nat. Acad. Sci. *78*, 4046-4050.

Joshi, H. C., Palacios, M. J., McNamara, L., and Cleveland, D. W. (1992). Gamma-tubulin is a centrosomal protein required for cell cycle-dependent . Nature *356*, 80-3.

Jourdain, L., Curmi, P., Sobel, A., Pantaloni, D., Carlier, M.-F. (1997). Stathmin: a tubulin-sequestering protein which forms a terniar T2S complex with two tubulin molecules. Biochemistry *36*, 10817-10821.

Keating, T. J., Peloquin, J. G., Rodionov, V. I., Momcilovic, D., and Borisy, G. G. (1997). Microtubule release from the centrosome. Proc. Natl. Acad. Sci. U.S.A. *94*, 5078-5083.

Kitanishi-Yumura, T., and Fukui, Y. (1987). Reorganization of microtubules during mitosis in Dictyostelium: dissociation from MTOC and selective assembly/disassembly in situ. Cell Motil. Cytoskel. *8*, 106-117.

Kodama, T., Fukui, K., and Kometani, K. (1986). The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 as studied by a modified malachite green method for determination of inorganic phosphate. J. Biochem. *99*, 1465-1472.

Komachi, K., and Johnson, A. D. (1997). Residues in the WD repeats of Tup1 required for interaction with alpha-2. Mol. Cell Biol. *17*, 6023-6028.

Komachi, K., Redd, M. J., and Johnson, A. D. (1994). The WD repeats of Tup1 interact with the homeo domain protein alpha 2. Genes Dev. *8*, 2857-2867.

Kowalski, R. J., and Williams, R. J. (1993). Microtubule-associated protein 2 alters the dynamic properties of microtubule assembly and disassembly. J Biol. Chem. *268*, 9847-9855.

Lenzen, C. U., Steinmann, D., Whiteheart, S. W., and Weis, W. I. (1998). Crystal structure of the heximerization domain of N-ethylmaleimide-sensitive fusion protein. Cell *94*, 525-536.

Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. (1999). Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. Nature *398*, 348-351.

Lieuvin, A., Labbe, J.-C., Doree, M., and Job, D. (1994). Intrinsic microtubule stability in interphase cells. J. Cell Biol. *124*, 985-996.

Lin, C., Maher, V., and McCormick, J. (1995). Malignant transformation of human fibroblast strain MSU-1.1 by v-fes requires an additional genetic change. Int. J. Cancer *63*, 140-147.

Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993). Efficient generation of infections recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. J. Virol. *67*, 4566-4579.

Lynch, T. J., Albanesi, J. P., Korn, E. D., Robinson, E. A., Bowers, B., and Fujisaki, H. (1986). ATPase activities and actin-binding properties of subfragments of Acanthamoeba myosin IA.. J Biol Chem *261*, 17156-17162.

Maccioni, R. B., Serrano, L., Avila, J., and Cann, J. R. (1986). Characterization and structural aspects of the enhanced assembly of tubulin after removal of its carboxyl-terminal domain. Eur. J. Biochem. *156*, 375-381.

Mandelkow, E. M., Mandelkow, E., and Milligan, R. A. (1991). Microtubule dynamics and microtubule caps: a time-resolved cryo-electron microscopy study. J. Cell Biol. *114*, 977-991.

Matveeva, E. A., He, P., and Whiteheart, S. W. (1997). N-ethylmaleimide-sensitive fusion protein contains high and low affinity ATP-binding sites that are functionally distinct. J. Biol. Chem. *272*, 26413-26418.

McLaughlin, P. J., Gooch, J. T., Mannherz, H.-G., and Weeds, A. G. (1993). Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. Nature *364*, 685-692.

McNally, F., Okawa, K., Iwamatsu, A., and Vale, R. (1996). Katanin, the microtubule-severing ATPase, is concentrated at centrosomes. J. Cell Sci. *109*, 561-567.

McNally, F. J., and Thomas, S. (1998). Katanin is responsible for the M-phase microtubule-severing activity in Xenopus eggs. Mol. Biol. Cell *9*, 1847-1861.

McNally, F. J., and Vale, R. D. (1993). Identification of katanin, an ATPase that severs and disassembles stable microtubules. Cell *75*, 419-429.

Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. Nature *312*, 237-242.

Mitchison, T. J. (1989). Polewards microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. J Cell Biol *109*, 637-652.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J., Adams, J., Ikura, M., and Tsien, R. (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. Nature *388*, 882-887.

Morgan, A., Dimaline, R., and Burgoyne, R. D. (1994). The ATPase activity of N-ethylmaleimide-sensitive fusion protein (NSF) is regulated by soluble NSF attachment proteins. J. Biol. Chem. *269*, 29347-29350.

Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B., and Agard, D. A. (1995). Microtubule nucleation by gamma-tubulin-containing rings in the centrosome. Nature *378*, 638-640.

Muller-Reichert, T., Chretien, D., Severin, F., and Hyman, A. A. (1998). Structural changes at microtubule ends accompanying GTP hydrolysis: information from a slowly hydrolyzable analog of GTP, guanylyl (α,β)methylenediphosphonate. Proc. Natl. Acad. Sci. USA *95*, 3661-3666.

Nagiec, E. E., Bernstein, A., and Whiteheart, S. W. (1995). Each domain of the N-ethylmaleimide-sensitive fusion protein contributes to its transport activity. J. Biol. Chem. *270*, 29182-29188.

Nicklas, R. B., Lee, G., Reider, C., and Rupp, G. (1989). Mechanically cut mitotic spindles: clean cuts and stable microtubules. J. Cell Sci. *94*, 415-423.

Noble, M., Lewis, S., and Cowan, N. (1989). The microtubule binding domain of microtubule-associated protein MAP1B contains a repeated sequence motif unrelated to that of MAP2 and tau. J. Cell Biol. *109*, 3367-76.

Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999). High-resolution model of the microtubule. Cell *96*, 79-88.

Nogales, E., Wolf, S. G., and Downing, K. H. (1998). Structure of the alpha beta tubulin dimer by electron crystallography. Nature *391*, 199-203.

Oegema, K., Whitfield, W. G. F., and Alberts, B. (1995). The cell cycle-dependent localization of the CP190 centrosomal protein is determined by the coordinate action of two separable domains. J. Cell Biol. *131*.

Patel, S., and Latterich, M. (1998). The AAA team: related ATPases with diverse functions. Trends Cell Biol. *8*, 65-71.

Peters, J., Harris, J., Lustig, A., Muller, S., Engel, A., Volker, S., and Franke, W. (1992). Ubiquitous soluble Mg(2+)-ATPase complex. A structural study. J Mol Biol *223*, 557-71.

Rockel, B., Walz, J., Hegerl, R., Peters, J., Typke, D., and Baumeister, W. (1999). The structure of VAT-a CDC48/p97 homologue from a archaeon thermoplasma acidophilum studied by electron microscopy. Presented at: Cellular Functions of AAA Proteins, The Salk Institute, San Diego, April 1999.

Sackett, D. L., Bhattacharyya, B., and Wolff, J. (1985). Tubulin subunit carboxyl termini determine polymerization efficiency. J. Biol. Chem. *260*, 43-45.

Salmon, E. D., Leslie, R. J., Saxton, W. M., Karow, M. L., and McIntosh, J. R. (1984). Spindle microtubule dynamics in sea urchin embryos: analysis using a fluorescein-labeled tubulin and measurements of fluroescent redistribution after laser photobleaching. J. Cell Biol. *99*, 2165-2174.

Sammak, P. J., Gorbsky, G. J., and Borisy, G. G. (1987). Microtubule dynamics in vivo: a test of mechansims of turnover. J. Cell Bio. *104*, 395-405.

Schulze, E., and Kirschner, M. W. (1987). Dynamic and stable populations of microtubules in cells. J. Cell Biol. *104*, 277-288.

Serrano, L., Avila, J., and Maccioni, R. B. (1984). Controlled proteolysis of tubulin by subtilisin: localization of the site for MAP2 interaction. Biochemistry *23*, 4675-4681.

Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A., and Nishida, E. (1994). Microtubule severing by elongation factor-1α. Science *266*, 282-285.

Shiina, N., Gotoh, Y., and Nishida, E. (1992). A novel homo-oligomeric protein responsible for an MPF-dependent microtubule-severing activity. Embo J. *11*, 4723-4731.

Shimizu, T., Furusawa, K., Ohashi, S., Toyoshima, Y., Okuno, M., Malik, F., Vale, R. D. (1991). Nucleotide specificity of the enzymatic and motile activities of dynein, kinesin, and heavy meromyosin. J. Cell Biol. *112*, 1189-1197.

Shtilerman, M., Lorimer, G. H., and Englander, S. W. (1999). Chaperonin function: folding by forced unfolding. Science *284*, 822-825.

Siegel, L. M., and Monty, K. J. (1966). Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation: application to crude preparations of sulfite and hydroxylamine reductases. Biochem. Biophys. Acta *112*, 346-362.

Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A., and Horwich, A. L. (1998). Structure and function in GroEL-mediated protein folding. Ann. Rev. Biochem. *67*, 581-608.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996). Crystal structure of a G_A protein bg dimer at 2.1Å resolution. Nature *379*, 369-374.

Sweitzer, S. M., and Hinshaw, J. E. (1998). Dynamin undergoes a GTP-dependent conformational change causing vesiculation. Cell *93*, 1021-1029.

Tagaya, M., Wilson, D. W., Brunner, M., Arango, N., and Rothman, J. E. (1993). Domain structure of an N-ethylmaleimide-sensitive fusion protein involved in vesicular transport. J. Biol. Chem. *268*, 2662-2666.

Tian, G., Huang, Y., Rommelaere, H., Vandekerckhove, J., Ampe, C., Cowan, N. J., (1996). Pathway leading to correctly folded beta-tubulin. Cell *86*, 287-296.

Tuma, P. L., and Collins, C. A. (1994). Activation of dynamin GTPase is a result of positive cooperativity. J Biol Chem *269*, 30842-30847.

Vale, R. D. (1991). Severing of stable microtubules by a mitotically activated protein in Xenopus egg extracts. Cell *64*, 827-839.

Vandekerckhove, J. (1990). Actin-binding proteins. Curr. Opin. Cell Biol. *2*, 41-50.

Walczak, C. E., Mitchison, T. J., and Desai, A. (1996). XKCM1: A Xenopus kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. Cell *84*, 37-47.

Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). Distantly related sequences in the a and b-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide fold. EMBO J. *1*, 945-951.

Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. F., Voter, W. A., Erickson, H. P., and Salmon, E. D. (1988). Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. J. Cell Biol. *107*, 1437-1448.

Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995). The structure of the G protein heterotrimer Giα1β1γ2. Cell *83*, 1047-1058.

Warnock, D. E., Hinshaw, J. E., and Schmid, S. L. (1996). Dynamin self-assembly stimulates its GTPase activity. TA - J Biol Chem . J Biol Chem *271*, 22310-22314.

Waters, J. C., Mitchison, T. J., Rieder, C. L., and Salmon, E. D. (1996). The kinetochore microtubule minus-end disassembly associated with flux produces a force that can do work. Mol Biol Cell *7*, 1547-1558.

Waters, J. C., Skibbens, R. V., and Salmon, E. D. (1996). Oscillating mitotic newt lung cell kinetochores are, on average, under tension and rarely push. J Cell Sci., *109*, 2823-2831

Weiss, S. A., Godwin, G. P., Gorfien, S. F., and Whitford, W. G. (1995). Insect Cell Culture in Serum-Free Media. In Baculovirus Expression Protocols, C. D. Richardson, ed. (Totowa, New Jersey: Humana Press Inc.), pp. 79-95.

Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Rothman, J. E. (1994). N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. J. Cell Biol. *126*, 945-954.

Williams, R. C. J., and Lee, J. C. (1982). Preparation of Tubulin from Brain. In Methods in Enzymology: Academic Press, Inc., pp. 376-385.

Woehlke, G., Ruby, A. K., Hart, C. L., Ly, B., Hom-Booher, N., Vale, R. D. (1997). Microtubule interaction site of the kinesin motor. Cell *90*, 207-216.

Wright, B. D., Henson, J. H., Wedaman, K. P., Willy, P. J., Morand, J. N., and Scholey, J. M. (1991). Subcellular localization and sequence of sea urchin kinesin heavy chain: Evidence for its association with membranes in mitotic apparatus and interphase cytoplasm. J. Cell Biol. *113*, 817-833.

Xu, Z., Horwich, A. L., and Sigler, P. B. (1997). The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex. Nature *388*, 741-750.

Yu, R. C., Hanson, P. I., Jahn, R., and Brunger, A. T. (1998). Structure of the ATP-dependent oligomerization domain of N-ethylmaleimide sensitive factor complexed with ATP. Nature Struct. Biol. *5*, 803-811.

Zhai, Y., Kronebusch, P. J., Simon, P. M., and Borisy, G. G. (1996). Microtubule dynamics at the G-2-M transition: Abrupt breakdown of cytoplasmic microtubules at nuclear envelope breakdown and implications for spindle morphogenesis. J. Cell Biol. *135*, 201-214.

Zheng, Y., Wong, M. L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring . Nature *378*, 578-583.


UC
San Francisco
LIBRARY
UNIVERSITY OF CALIFORNIA · UNIVERSITY OF CALIFORNIA · UNIVERSITY OF CALIFORNIA