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

James J. Hartman,[†] Jeff Mahr,[‡] Karen McNally,[‡] Katsuya Okawa,[§] Akihiro Iwamatsu,[§] Susan Thomas,[‡] Sarah Cheesman,[‡] John Heuser,[‖] Ronald D. Vale,[*†] and Francis J. McNally[‡#]

[*]Howard Hughes Medical Institute
[†]Department of Cellular and Molecular Pharmacology
University of California
San Francisco, California 94143
[‡]Section of Molecular and Cellular Biology
University of California
Davis, California 95616
[§]Central Laboratories for Key Technology
Kirin Brewing Company
1-13-5 Fukuura
Kanazawa-ku Yokahama, Kanagawa
Japan
[‖]Department of Cell Biology
Washington University School of Medicine
St. Louis, Missouri 63110

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 play 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 (Mitchison and Kirschner, 1984; Horio and Hotani, 1986). 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 that 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 (Vale, 1991; Shiina et al., 1992, 1994; McNally and Vale, 1993). 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

#To whom correspondence should be addressed.

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* egg 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 sequences 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 (aa) sequences of the cDNA clones contained 139 aa and 306 aa 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 aa polypeptide (Figure 1A). A BLAST search with the predicted p60 protein sequence revealed that this polypeptide contains a C-terminal domain (aa 231–447) that is highly conserved in the AAA ATPase superfamily (Figure 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 aa 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% aa identify, Figure 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 amino acid 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 aa polypeptide that contains six "WD40" repeat motifs extending from residues 1 to 256 (Figure 2A). An alignment of these repeats with two unrelated WD40 repeat–containing proteins is shown in Figure 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 aa protein with 61% aa identity in the WD40 domain (aa 1–256) (Figure 2B), 23% aa identity in the central 187 residues, and 54% aa identity in the C-terminal 164 aa 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 an N-terminal His[6] tag), and purified the expressed proteins using metal affinity chromatography (Figure 3A). When p60 and p80 were coexpressed, 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 coimmunoprecipitation of equal quantities of p60 and p80 (Figure 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 platinum (Heuser, 1983, 1989). The platinum-shadowed p60 appeared as a 14–16 nm ring punctuated in the center by a 3–5 nm opening, often with

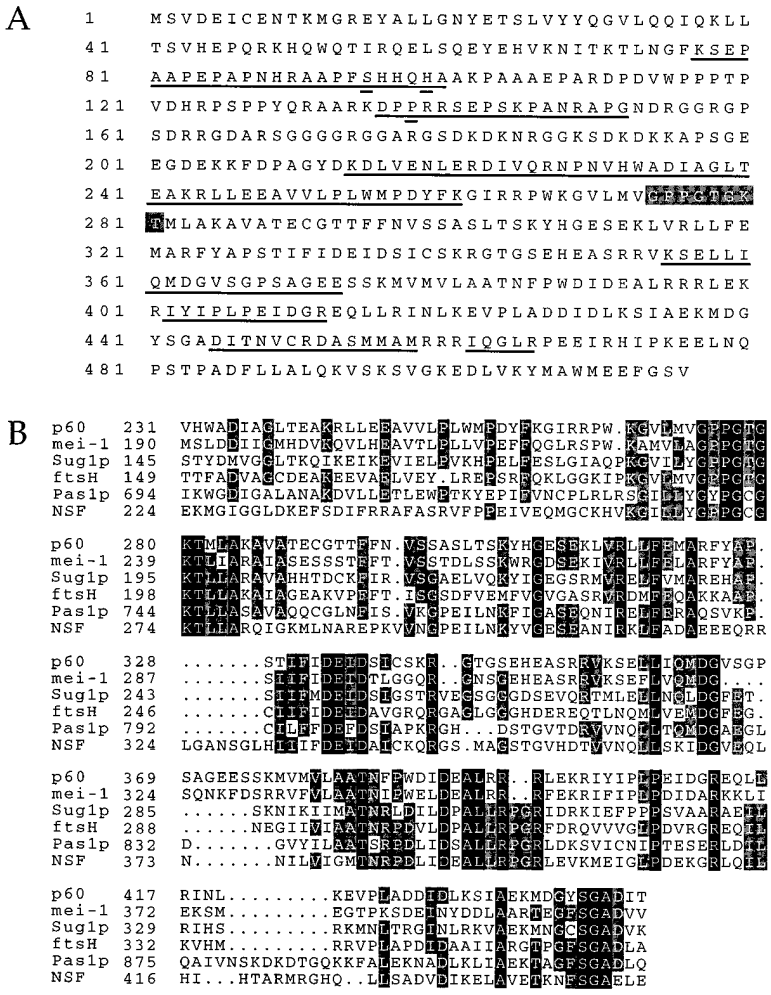

Figure 1. Sequence Analysis of p60 Katanin

(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.

what appeared 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 (Figure 4B). Rings were also seen for p60/p80 complexes (Figures 4C and 4D) 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 (Figure 4D), and smaller rings of the size of p60 alone with several p80-sized particles radiating from the central ring (Figure 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 coexpressed p60/p80 heterodimer displayed an ATP turnover rate of 0.3 ATP/s/heterodimer; this activity was stimulated ~10-fold by microtubules (Figure 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 (Figure 5A). p80 itself had no detectable ATPase activity. The activation of ATPase activity by microtubules displayed an atypical, nonhyperbolic 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 (Figure 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 (Figure 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

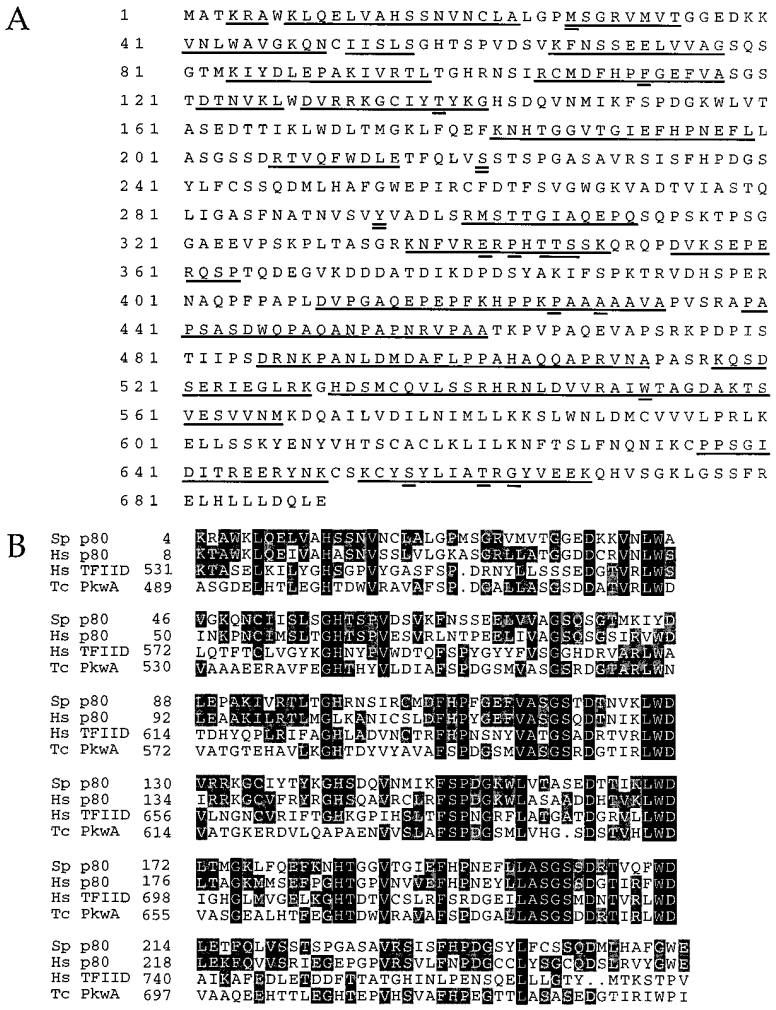

Figure 2. Sequence Analysis of p80 Katanin

(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 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.

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 (Figure 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 (Figure 5C). The loss of microtubule polymer was confirmed by centrifugation studies, which showed an increase in nonsedimentable 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 (Figure 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

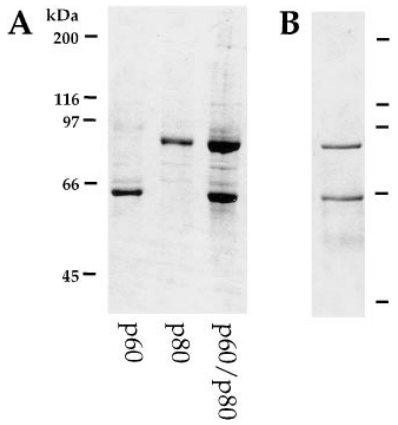

Figure 3. Expression and Purification of Recombinant Katanin Subunits

(A) Coomassie-stained SDS-PAGE analysis of expressed katanin subunits. 6×His-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 cross-linked 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.

polypeptides were cotranslated in a rabbit reticulocyte system. The truncated p80 (Δ1–302) was coimmunoprecipitated by the anti-p60 antibody only in the presence of p60 and just as efficiently as full-length p80 (Figure 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 coimmunoprecipitate with p60 (Figure 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 coimmunoprecipitate with p60 (Figure 6). These findings suggest that the C-terminal 130 aa 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 colocalized with γ-tubulin staining (Figure 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 (aa 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 colocalized with γ-tubulin staining was observed 2–4 hr after transfection in addition to diffuse cytoplasmic fluorescence (Figure 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 colocalize with γ-tubulin, and later, massive aggregates several micrometers 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 τ (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

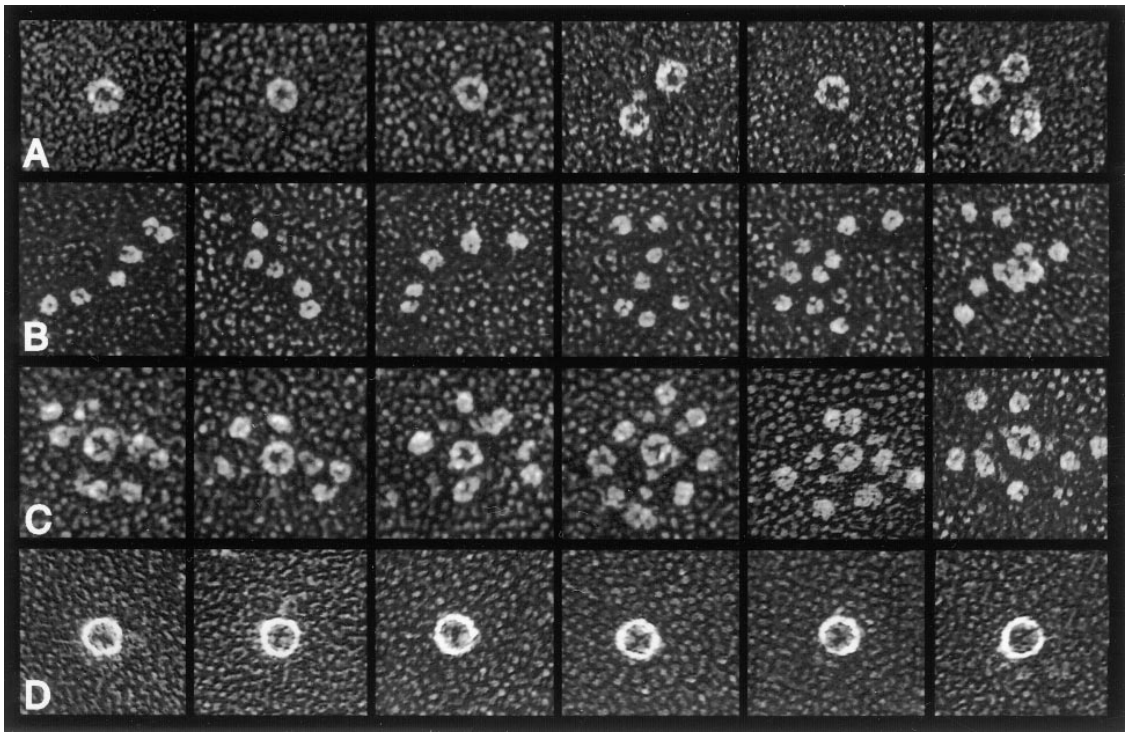

Figure 4. Structure of Katanin as Visualized by Rotary-Shadowing Electron Microscopy

(A) Rings (14–16 nm diameter) observed in preparations of recombinant p60.

(B) Single particles of recombinant p80; occasional aggregates are seen (right-most picture) but rings are never observed.

(C and D) Different rings observed in recombinant p60/p80 preparations. (C) shows a "splayed" complex, consisting of a central p60-like ring surrounded by a halo of particles that resemble p80. In (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 300,000×. The dimensions indicated above include the platinum shadowing, which typically adds 2 nm of material to the protein surface.

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/s and maximal microtubule-stimulated rate of 3 ATP/katanin/s are comparable to values of 1 ATP/s for p97 (Peters et al., 1992) and 0.08 ATP/s for recombinant NSF (Morgan et al., 1994). NSF ATPase is also stimulated 2-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 ($<2\ \mu$M), 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 cross-linking 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-cross-linked 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

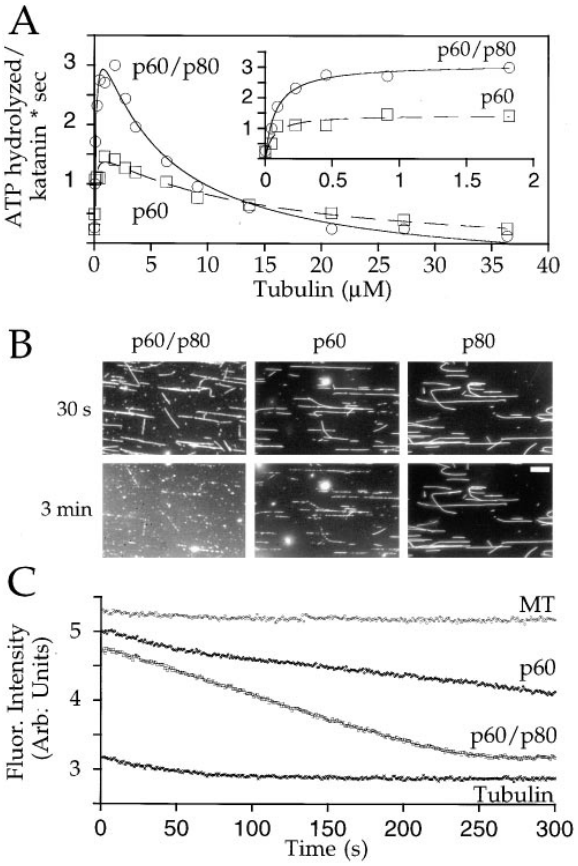

Figure 5. Activities of Recombinant Katanin Subunits

(A) ATPase activities of 0.04 μM p60 katanin (squares) and coexpressed p60/p80 (circles) 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. Taxol-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 coexpressed 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.

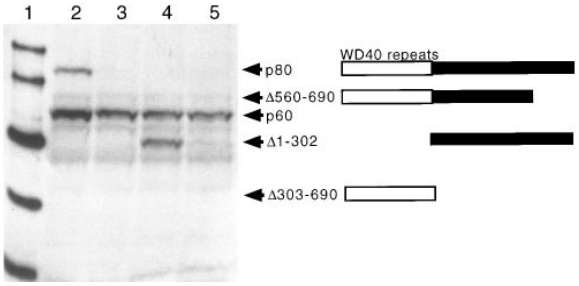

Figure 6. The WD40 Repeats of p80 Katanin Are Not Required for Interaction with p60 Katanin

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, M_r: 100,000, 75,000, 50,000, 35,000, and 25,000; (lane 2) p60 cotranslated with full-length p80; (lane 3) p60 cotranslated with the Δ560–690 derivative of p80; (lane 4) p60 cotranslated with the Δ1–302 derivative of p80; (lane 5) p60 cotranslated 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).

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

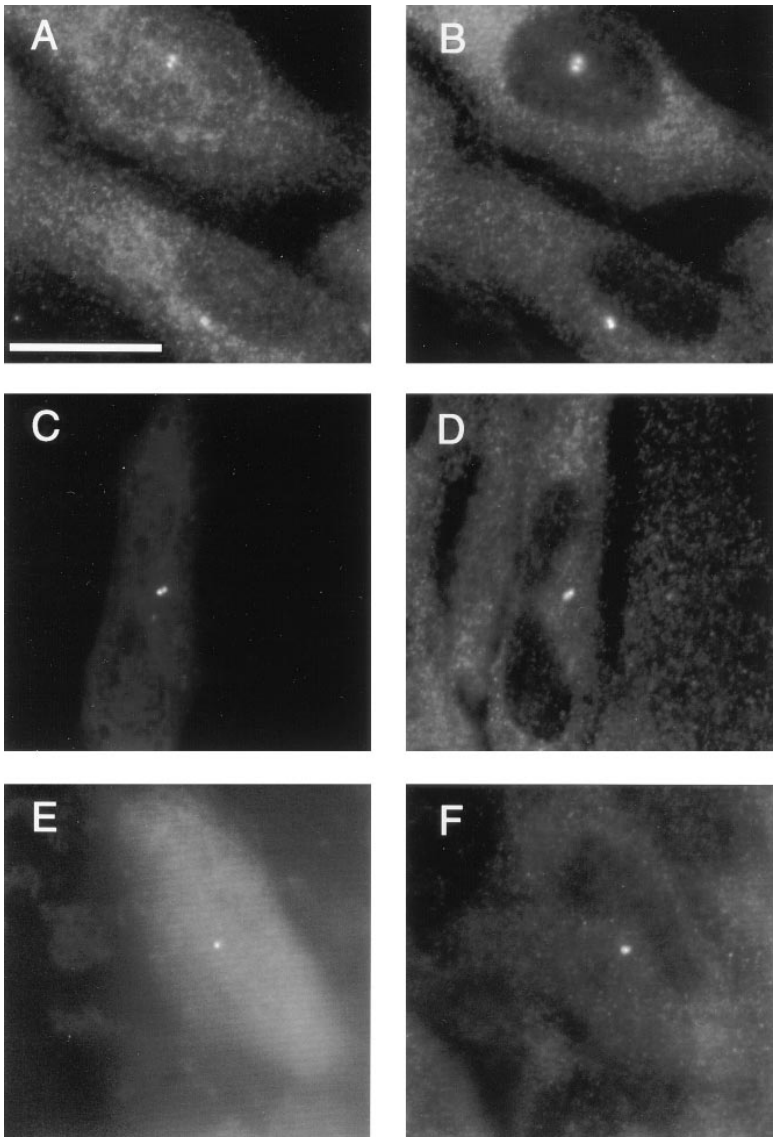

Figure 7. Human p80 Katanin and a Fusion Protein of the Human p80 WD40 Domain with GFP Colocalize with γ-Tubulin at Centrosomes of MSU1.1 Human Fibroblasts

(A and B) Colocalization of immunofluorescence staining by a human p80 katanin-specific antibody (A) and a γ-tubulin-specific antibody (B).

(C–F) Colocalization of GFP fluorescence (C and E) with staining by a γ-tubulin-specific antibody (D and F). Colocalization to two centrosomes is seen in (C) and (D) while colocalization 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.

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 β propeller structure, as first determined for the β subunits of transducin and G_i (Wall et al., 1995; Sondek et al., 1996). However, each WD40 domain exhibits very specific heterophilic protein interactions; exposed residues in the β subunit of G_i interact with the α subunit (Wall et al., 1995), whereas the corresponding residues in the WD40 transcription factor TUP1 mediate binding to a second transcription factor α2 (Komachi and Johnson, 1997). Since the G protein β subunits interact with multiple partner proteins (Wall et al., 1995; Gaudet et al., 1996), 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.

Experimental Procedures

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 aa 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 #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 amino acid 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 cross-linked to protein A Sepharose using 20 mM dimethylpimilidate (Harlow and Lane, 1988). After equilibration in TBST, 20–40 μl of antibody beads was 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 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 6×His 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 100× antibiotic/antimycotic (Life Technologies) to 0.5× 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 postinfection 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 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/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-mercaptoethanol, 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, 1983, 1989). 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 300,000×.

ATPase Assays

ATPase activity was measured by a modified malachite green method (Kodama et al., 1986). ATPase reactions of 50–100 μl 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 (Williams and Lee, 1982; Hyman et al., 1990). After assembly, microtubules were sedimented (230,000 × 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 extinction coefficient of 1.03 $ml \cdot mg^{-1} \cdot cm^{-1}$ (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 μg/ml), catalase (36 μg/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 containing 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 μg/ml pyruvate kinase (Boehringer Mannheim), and 1 mg/ml BSA. The reaction volume was 80 μl, 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 Coimmunoprecipitation

In order to facilitate the nonradioactive 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 aa 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 coimmunoprecipitation assays, p60 and p80 constructs were usually coexpressed. 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 aa 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 aa 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 0.5× PBS, 3.7% formaldehyde, 75% methanol at 22°C for 10 min followed by rehydration in TBST. Antibody labeling was carried out in TBST containing 4% BSA. Images were captured with a Nikon Microphot SA microscope, 100× Plan Fluor 1.3 objective, Photometrics Quantix camera, and IP Lab Spectrum software (Scanalytics).

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