Crystal Structure of the Mitotic Spindle Kinesin Eg5 Reveals a Novel Conformation of the Neck-linker*

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Success of mitosis depends upon the coordinated and regulated activity of many cellular factors, including kinesin motor proteins, which are required for the assembly and function of the mitotic spindle. Eg5 is a kinesin implicated in the formation of the bipolar spindle and its movement prior to and during anaphase. We have determined the crystal structure of the Eg5 motor domain with ADP-Mg bound. This structure revealed a new intramolecular binding site of the neck-linker. In other kinesins, the neck-linker has been shown to be a critical mechanical element for force generation. The neck-linker of conventional kinesin is believed to undergo an ordered-to-disordered transition as it translocates along a microtubule. The structure of Eg5 showed an ordered neck-linker conformation in a position never observed previously. The docking of the neck-linker relies upon residues conserved only in the Eg5 subfamily of kinesin motors. Based on this new information, we suggest that the neck-linker of Eg5 may undergo an ordered-to-disordered transition during force production. This ratchet-like mechanism is consistent with the biological activity of Eg5.

Prior to the separation of sister chromatids in anaphase, duplicated centrosomes are repositioned to opposite sides of the cell, forming the mitotic spindle as they move. Centrosome separation is dependent upon numerous proteins, including Eg5, a kinesin motor (1). Eg5 slides the microtubules of the developing spindle past each other, thereby pushing the centrosomes apart (2). This outward force is balanced by other kinesin motors that provide an inward force (3, 4).

Elucidation of the specific roles played by Eg5 in this process has been aided by the discovery of a small, cell-permeable molecule that selectively inhibits Eg5 activity. This compound was named monastrol because its presence causes the formation of a mono-astral spindle by inhibiting centrosome separation (5, 6). The addition of monastrol after bipolar spindle formation caused the spindle to collapse, indicating that a force is constantly required to maintain spindle integrity (6). In addition to its role as a cell biological reagent, monastrol and its derivatives may be useful in the clinical setting as anti-mitotic agents.

At least one Eg5 homologue has been found in every eukaryote (called BimC in Aspergillus (7), cut7 in Schizosaccharomyces pombe (8), cin8p in Saccharomyces cerevisiae (9), Klp61F in Drosophila (10) and Eg5 in Xenopus (11, 12) and humans (13)). These kinesins and other homologues (identified by sequence similarity) comprise the KinN2 kinesin subfamily (14). They share slow, plus end-directed, nonprocessive movement (15, 16), and a unique homotetrameric structure (17). Like conventional kinesin, two motor domains form a dimer via association of their stalks. However, in a KinN2 motor, two dimers interact, anti-parallel to each other, to form a rod with two motor domains at each end, a structure often referred to as “bipolar.” Both ends interact with microtubules, bundling and sliding past each other. The activity of these proteins is restricted to the mitotic spindle and is controlled, at least in part, by phosphorylation at a conserved C-terminal region by p34cdc2 (13, 18).

Recently, a general mechanism used by kinesins motors to produce motility has been proposed (19). This model is based upon alternating cycles of weak and strong microtubule binding that are dependent upon whether ATP or ADP is bound to the protein. When ATP is hydrolyzed to ADP, the affinity for the microtubule is weakened and the motor releases. When ATP re-binds, a series of conformational changes take place which trigger the movement of a mechanical element. This positions the motor closer to the next binding site on the microtubule, where it will bind tightly, allowing a step forward to take place. Although the same general scheme appears to be utilized by all kinesin motors, different kinesins perform numerous different activities in the cell. This wide variety of functions is the result of subtle alterations within the motor domain and the placement of the motor within the ultra-structure of the protein.

To better understand how Eg5 functions, we have determined the structure of its motor domain. The structure of the Eg5 motor revealed a unique conformation of the mechanical element. Unlike conventional kinesin, where the mechanical element is disordered in the APD state, the Eg5 mechanical element is structured in a position not observed in any other kinesin. This observation may help explain how Eg5 can work in arrays to efficiently slide microtubules and why Eg5 is not a processive motor.

EXPERIMENTAL PROCEDURES

Cloning and Purification—A fragment of Eg5 was amplified from human placenta cDNA (CLONTECH) by polymerase chain reaction using Pfu turbo (Stratagene). The region encoding residues 1–491 was

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The atomic coordinates and structure factors (code III6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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subcloned into pET23d (Novagen) containing a Myc epitope and a 6-His tag. The construct expressing untagged Eg5 (residues 1–368) used in this study was created by introducing a stop codon 3’ of the codon for K368 using QuickChange site-directed mutagenesis kit (Stratagene). Protein expression in Escherichia coli BL21(DE3) cells was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and cells were harvested after 4 h of growth at 25 °C. Frozen cells were thawed into 50 mM PIPES (pH 6.8), 2 mM MgCl2, 1 mM EGTA, 1 mM ATP, and 1 mM Tris-(2-carboxyethyl)phosphine (TCEP) HCl and lysed in a microfluidizer. The lysate was clarified by centrifugation and applied to a SP-Sepharose column (Amersham Pharmacia Biotech). Protein was eluted with a linear gradient of 0–250 mM NaCl. Eg5 was identified in fractions by SDS-polyacrylamide gel electrophoresis analysis, and the pooled fractions were applied to Mono-S and Mono-Q columns (Amersham Pharmacia Biotech), which were developed as the SP-Sepharose column. The final protein pool was >95% pure as judged by SDS-polyacrylamide gel electrophoresis, mass spectroscopy, and isoelectric focusing-electrophoresis. Microtubule-stimulated ATPase assays (data not shown) revealed our preparation to be as active as that described by Lockhart and Cross (15).

Crystallographic Data Collection

The structure of Eg5 was solved by molecular replacement methods with the KAR3 motor (22) as a model. Details of the data collection and refinement are presented in Table I. The refined Eg5 model revealed a protein with the general features expected of a kinesin motor, with six major β-sheets surrounded by six α-helices (Fig. 1A). The kinesin motor structure has been described as an arrowhead (23, 24) with a nucleotide binding site at the wider end of the arrowhead. In Eg5, the nucleotide binding site is occupied by Mg-ADP.

This structure of Eg5 is the first structure of a Kin N2 motor. However, it is the ninth structure of a motor from the kinesin superfamily. In analyzing the structure, we noticed many differences between Eg5 and other kinesin motors. Our challenge was to determine which of these features are important for Eg5 function in particular and which are important for kinesin motor function in general.

Here, we present a detailed comparison of Eg5 and one kinesin motor, KHC. We chose kinesin heavy chain (KHC) because it shares 40% identity with Eg5, the highest of all the motors with structures available (Fig. 1B). The comparison of divergent structures is facilitated by superimposing elements that are known to be conserved in the structures and then examining the differences this highlights in other regions. The phosphate binding region (P-loop) is conserved in all kinesin structures. Therefore, we used the P-loop region (Eg5 105–113, KHC 85–93) to align Eg5 with KHC. To more easily view the results of the comparison, Fig. 1, C—H, presents a single region of the overlapping structures at a time, with the Eg5 structure shown in pink and the KHC structure in blue.

After superimposing KHC and Eg5, it becomes apparent that the core β-sheets are almost identical in the two structures (Fig. 1C). However, there is a region of divergence near the tip of the protein, leading to the appearance of a slight tilting and lengthening of Eg5 with respect to KHC. The recently determined structure of the Kif1A motor shares this feature with Eg5 (25). It is believed that the tip of the kinesin arrowhead may play an important role in transient interactions with the microtubule during force production. Therefore, this structural alteration may be a factor in determining the affinity of kinesin motors for the microtubule rather than a transient change that occurs as part of the ATP hydrolysis cycle.

Of the six helices that surround the core β-sheets, helix α1 did not appear different in the Eg5 and KHC structures (not shown). However, there was a dramatic difference in helix α2. This helix is interrupted by a loop in all kinesins, and its function is not known. As seen in Fig. 1D, this loop is larger in Eg5 than in KHC. The size of the loop is variable among kinesin family members, but it is largest in the Kin N2 family (see Fig. 3A for a limited sequence comparison). This loop is located on the opposite face of the protein from that which binds to the microtubule and is in proximity to, but not a part of, the nucleotide binding site. One idea, which remains unsubstantiated, is that this loop may somehow regulate motor activity, perhaps by interacting with other proteins.

Kinesin motility is based upon nucleotide state sensing. In this way, small changes (the presence or absence of the γ-phosphate) can be transmitted to and amplified in other parts of the structure. This activity relies upon loop components of the switch I and switch II regions. When ATP binds, these loops make direct contact with the γ-phosphate of ATP and also form interactions with each other (22–26). These adjustments cause a cascade of secondary movements in the protein, including docking of the neck-linker mechanical element and increasing microtubule affinity. When ATP is hydrolyzed to ADP, the interactions among switch I, switch II, and the nucleotide are lost. This reverses the conformational changes that took place upon ATP binding, resulting in the release of the neck-linker and a decrease in microtubule affinity.

The switch I region is found at the end of helix α3. In KHC, switch I is a short α-helix, whereas in Eg5 switch I is a loop (Fig. 1E). Is this structural difference the basis for the func-

### Table I

| Data collection and refinement statistics |
|-----------------------------------------|
| Space group | P2₁ |
| a, b, c, β (°) | 53.08, 78.59, 94.15, 93.84 |
| Observed reflections | 236,822 |
| Unique reflections | 42,896 |
| Completeness (2.14–2.1 Å) | 94.9% (85%) |
| Rmerge (2.14–2.1 Å) | 12.5 (10.3) |
| Resolution (Å) | 9.4 |

- The structure was solved by molecular replacement methods with the KAR3 motor (22) as a model.
- Details of the data collection and refinement are presented in Table I.
- The refined Eg5 model revealed a protein with the general features expected of a kinesin motor, with six major β-sheets surrounded by six α-helices (Fig. 1A). The kinesin motor structure has been described as an arrowhead (23, 24) with a nucleotide binding site at the wider end of the arrowhead. In Eg5, the nucleotide binding site is occupied by Mg-ADP.
- This structure of Eg5 is the first structure of a Kin N2 motor. However, it is the ninth structure of a motor from the kinesin superfamily. In analyzing the structure, we noticed many differences between Eg5 and other kinesin motors. Our challenge was to determine which of these features are important for Eg5 function in particular and which are important for kinesin motor function in general.
- Here, we present a detailed comparison of Eg5 and one kinesin motor, KHC. We chose kinesin heavy chain (KHC) because it shares 40% identity with Eg5, the highest of all the motors with structures available (Fig. 1B). The comparison of divergent structures is facilitated by superimposing elements that are known to be conserved in the structures and then examining the differences this highlights in other regions. The phosphate binding region (P-loop) is conserved in all kinesin structures. Therefore, we used the P-loop region (Eg5 105–113, KHC 85–93) to align Eg5 with KHC. To more easily view the results of the comparison, Fig. 1, C—H, presents a single region of the overlapping structures at a time, with the Eg5 structure shown in pink and the KHC structure in blue.
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- The switch I region is found at the end of helix α3. In KHC, switch I is a short α-helix, whereas in Eg5 switch I is a loop (Fig. 1E). Is this structural difference the basis for the func-
FIG. 1. Structure of the Eg5 motor domain and its comparison to conventional KHC motor domain. A, the Eg5 motor with major $\beta$-sheets (gray) and $\alpha$-helices (gold) labeled as described for conventional kinesin by Kull et al. (23). One molecule of Mg-ADP is located in the nucleotide binding site. B, a sequence alignment of Eg5 and KHC motor domains. Structural elements are shown in gray for $\beta$-sheets and gold for $\alpha$-helices. Disordered regions are indicated by light gray lettering. C, overlay of the $\beta$-sheets of Eg5 (in pink) and KHC (in blue). The remainder of the structure is Eg5. D, overlay of helix $\alpha_2$, including a larger loop in Eg5 than in KHC. E, overlay of helix $\alpha_3$ and the switch I regions of Eg5 and Crystal Structure of Eg5.
tional differences between Eg5 and KHC? We think it is more likely that the two structures may represent two different states that all kinesin motors assume at some point during force generation. As mentioned above, the γ-phosphate acts to bring together switch I and switch II. However, both the Eg5 and KHC structures contain ADP and therefore no γ-phosphate. Therefore, switch I with ATP bound likely assumes the same conformation in all kinesins, whereas without the γ-phosphate “tether” this region is flexible. This prediction is supported by the many different positions of this region observed in other kinesin motors (22–26). However, it is also possible that the different switch I conformations may reflect real differences among kinesin family members. Additional structures and biochemical experiments should be able to answer this question in the future.

In addition to nucleotide sensing by switch I, the switch II region is also critical for nucleotide sensing and force production. Switch II consists of helix α4 (often referred to as the switch II helix or the relay helix) and a loop that interacts with the nucleotide. A portion of this loop is disordered and therefore not observed in the electron density map of Eg5 and most other kinesins (22–26). Helix α4 of Eg5 and KHC differ in two ways. In Eg5, helix α4 is one and one-half turns longer and slightly rotated with respect to helix α4 in KHC (Fig. 1F).

The helix extension observed in Eg5 is formed by ordering a region of the switch II loop that is disordered in the KHC structure. A longer α4 is also seen in the structure of Kif1A but only with ADP (not the ATP analogue AMP-PCP) bound (25). In that study, the length of helix α4 was found to be dependent upon the nucleotide state. However, by looking at all of the kinesin structures available, it becomes obvious that there is not a strict correlation with helix α4 length and nucleotide state (22–26). As a case in point, the two molecules of Eg5 in the asymmetric unit of the current crystal structure differ in the length of the α4 helix although they are nearly identical elsewhere (not shown). Therefore, it appears as though the length of α4 may change during ATP hydrolysis but that this change requires a low energy input in the absence of microtubules. In other words, crystals may trap structural intermediates that occur within motors in the absence of microtubules. This reflects a flexibility in this region that may be required for helix α4 to adjust its position in response to ATP binding.

In addition to the length of helix α4, the position of this helix is a key component in force generation. All known kinesin structures can be classified as switch II helix-up or switch II helix-down. Neck-linker docking is inhibited in the switch II helix-down position, which is believed to be reflective of the ADP-bound state. Although the position of α4 is slightly different in Eg5 and KHC, both of these structures are part of the switch II helix-down group (25). Again, we attribute the slightly different positions to trapping of these mobile elements in the crystal structures.

In addition to switch I and switch II, helix α5 and its neighboring loops undergo nucleotide-dependent rearrangements. Structural changes in this region likely affect microtubule binding, because this region is an important surface for interaction between the motor and the microtubule. The differences observed between Eg5 and KHC in this area (Fig. 1G) may again indicate flexibility of this region in the ADP-bound state in the absence of microtubules. Alternatively, this region may contribute to differences in microtubule binding observed in the two proteins.

Helix α6 is virtually identical in the two proteins (Fig. 1H). However, the region at the end of helix α6 is very different in the two structures. In the KHC model, there is no electron density in this region, whereas in Eg5 electron density is clearly visible. (Fig. 2). This region, termed the neck-linker, is a critical mechanical component of the force production cycle of kinesins and also serves to attach the motor to the coiled-coil stalks (27). In recent years, much attention has been paid to the neck-linker of kinesin motors. In all kinesin crystal structures except Eg5, the neck-linker is either disordered (as in KHC) or found docked to the motor, parallel to the longest motor edge (as in rat KHC). The neck-linker position directly correlates with the switch II helix position. The only exception is the Eg5 neck-linker, which adopts a position perpendicular to the long edge of the protein. The neck-linker position is not stabilized by crystal contacts but rather by a series of hydrogen bonds between the neck-linker and the β1/β2 lobe it docks against.

In addition to comparing the structure of Eg5 to the structures of other kinesin motors, we were interested in understanding how specific residues may play roles in specializing the activity of the different families of motor domains. In this analysis, we identified a subset of residues conserved among the KinN2 family that was not conserved in other kinesins. A sequence alignment of selected motor domains is presented in Fig. 2A, with Kin N2-specific residues highlighted in red. These residues were mapped onto the Eg5 structure and are shown in Fig. 2B.

Surprisingly, a number of the KinN2-conserved residues mapped to regions of Eg5 involved in neck-linker docking. Two residues in the neck-linker are specific to the KinN2 family, Lys-364 and Pro-365. These residues interact with residues Glu-49, and Thr-67, which are also conserved in the KinN2 family (Fig. 2C). The identification of these KinN2-conserved interactions lends more substance to the argument that the neck-linker conformation seen in Eg5 is not merely an experimental artifact but is a potential intermediate in the mechanochemical cycle.

This analysis identified other residues conserved specifically in the Kin N2 family that may be important for the interaction of the neck-linker with the core of the protein in other nucleotide states. Although we do not yet have structural information on the ATP bound state of Eg5, we can model where the neck-linker will go based on information available from other kinesin structures (22–26). In the ADP bound state, the parallel conformation of the neck-linker is precluded by the down position of the switch II helix. When ATP binds, the switch II helix moves up and the neck-linker is able to zip down the side of the motor core. This position is shown in Fig. 2C. Interestingly, some of the residues on Eg5 that would need to move to allow the neck-linker to dock are specifically conserved in the KinN2 family (Val-303, Arg-327, and Thr-328). Further down the predicted pathway for the neck-linker, another group of KinN2-specific residues is encountered at the “tip” of the protein (Gly-252, Glu-253, Glu-254). These may represent the last specific contact site the neck-linker makes with the motor core in the ATP bound state. Future experiments will determine the contribution of these conserved residues located in interesting regions.

DISCUSSION

The result of this analysis was that most of the differences between Eg5 and KHC are likely the result of capturing the
motors in slightly different stages of the movements that they go through during a force generation cycle. Kinesin motors are built to move and contain modules that move in a regulated and coordinated manner during force generation. Crystal structures are useful in that they capture one particular state that a motor may assume during force production. This information is valuable only in the context of understanding that other conformations did and will exist immediately before and immediately after the particular state a crystal has trapped.

By comparing Eg5 to all other kinesin structures, only one feature stood out as truly unique. This was the position of the neck-linker, docked perpendicular to the motor in the presence of ATP-PCP bound. The neck-linker was modeled after the position observed in rat KHC and Kif1A with AMP-PCP bound. By comparing Eg5 to all other kinesin structures, only one feature stood out as truly unique. This was the position of the neck-linker, docked perpendicular to the motor in the presence of ATP-PCP bound. The neck-linker was modeled after the position observed in rat KHC and Kif1A with AMP-PCP bound.
of Mg-ADP. This conformation was not seen to be stabilized by crystal contacts and involved conserved residues. Taken together, these observations suggest that perpendicular neck-linker docking may play a role in the force generation cycle of Eg5.

Although Eg5 contains a motor domain similar to that found in all other kinesins, it has evolved to perform a unique biological function. Unlike conventional kinesin, which walks along stationary microtubules, Eg5 has the job of putting microtubules into motion. A model highlighting possible differences in the mechanisms of these two types of kinesins is shown in Fig. 3. Eg5 works in arrays along the microtubule, and therefore to symbolize this, two motors are illustrated. However, for the sake of clarity, only one head is shown at either end of Eg5 on two microtubules that are oriented in opposite directions. Potential binding sites on the microtubule are labeled in gray and the neck-linker is red. Eg5 motors with ADP bound and neck-linker perpendicular to the motor may attach weakly to the microtubule. Upon exchanging ADP for ATP, a series of conformational changes takes place that cause the neck-linker to reorient itself parallel to the motor and the microtubule; this causes the microtubules to slide toward their minus ends. Note that the neck-linker is structured in both the ADP and ATP states. In contrast, the model for conventional kinesin movement along microtubules requires that the neck-linker be flexible in the ADP-bound state. This flexibility is illustrated by red arrows.

FIG. 3. A speculative model illustrating how the neck-linker may be utilized differently by Eg5 motors and processive walking motors. A, the activity of two Eg5 motors (in this figure, just one head is shown at either end of Eg5 on two microtubules that are oriented in opposite directions. Potential binding sites on the microtubule are labeled in gray and the neck-linker is red. Eg5 motors with ADP bound and neck-linker perpendicular to the motor may attach weakly to the microtubule. Upon exchanging ADP for ATP, a series of conformational changes takes place that cause the neck-linker to reorient itself parallel to the motor and the microtubule; this causes the microtubules to slide toward their minus ends. Note that the neck-linker is structured in both the ADP and ATP states. B, in contrast, the model for conventional kinesin movement along microtubules requires that the neck-linker be flexible in the ADP-bound state. This flexibility is illustrated by red arrows.

cause multiple motors bind the moving microtubules, randomization of the direction of the forces would cause a “canceling out” of the activities of many of the motors. Additionally, the structured nature of both the ADP and ATP bound states may help to maintain some of the rigidity that would be needed to slide microtubules efficiently. This could be referred to as a “ratcheting” mechanism, where the two binding sites are utilized to define the space through which the mechanical element can move and also to provide stability at both limits of its movement.

The model illustrated in Fig. 3A could be amended in a number of ways that may more accurately represent what occurs in cells. For example, how the four heads are coordinated with each other is not understood. Additionally, microtubules with the same polarity may interact with both ends of an Eg5 motor, resulting in microtubule bundling rather than sliding in opposite directions. Finally, it may be useful to envision an array of Eg5 motors, each moving the microtubule a distance less than that required for finding the next binding site. In this instance, movement of the microtubule by other motors may help microtubule release and facilitate the next encounter with the next binding site. The Eg5 structure presented here will allow these and other mechanistic details to be addressed.

Muscle myosin proteins use a similar ordered-to-ordered transition to generate directed movement (19). Like Eg5, myosin II acts in arrays which work together to move actin. Do other kinesins utilize an ordered-to-ordered neck-linker transformation? Crystal structures of other kinesins have revealed either a neck-linker in the zipped-down conformation or as disordered regions (22–27). Interestingly, electronmicroscopy studies of KHC have shown that although the neck-linker position was variable in the presence of ADP, a few positions were observed more frequently than others (28). One of these sites is consistent with the position of the neck-linker observed in the current structure of Eg5 (Fig. 4D in Ref. 28). Perhaps a stable neck-linker conformation in the presence of ADP is more transient in other kinesins, or it may not be as prone to forming as it is in Eg5.

Alternatively, a flexible neck-linker may be an adaptation required by two-headed “walking” kinesins as shown in Fig. 3B. Neck-linker flexibility may be important for the trailing motor to be propelled forward to the next binding site on the microtubule. The “stepping” or “hand-over-hand” processive mechanism proposed for conventional kinesin suggests that the neck-linker is flexible and can assume a number of different positions. Eg5 has been shown to be a nonprocessive motor (16). Therefore, neck-linker stability in both the ADP and ATP bound states of Eg5 may preclude this motor from being processive. Perhaps the need for controlled, efficient sliding of the mitotic spindle outweighs the benefits of processivity afforded by a flexible neck-linker. Alternatively, processivity may have been an evolutionary outgrowth of simpler, nonprocessive motors.

Recently, electronmicroscopy studies of Eg5 bound to microtubules revealed a decreased flexibility of the two heads when compared with similar experiments with KHC and other processive dimeric motors (29). This could reflect ordering of the neck-linker as discussed here. Future experiments, including electronmicroscopy labeling of the neck-linker, site-directed mutagenesis, and spectroscopic assays will determine whether the observed neck-linker ordering is truly critical for Eg5 activity.

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REFERENCES

1. Heald, R. (2000) *Cell* 102, 399–402
2. Sharp, D. J., McDonald, K. L., Brown, H. M., Matthies, H. J., Walczak, C., Vale, R. D., Mitchison, T. J., and Scholey, J. M. (1999) *J. Cell Biol.* 144, 125–138
3. Walczak, C., Vernos, I., Mitchison, T. J., Karsenti, E., and Heald, R. (1998) *Curr. Biol.* 8, 903–913
4. Sharp, D. J., Yu, K. R., Sisson, J. C., Sullivan, W., and Scholey, J. M. (1999) *J. Cell Biol.* 144, 125–138
5. Mayer, T. U., Kapoor, T. M., Haggerty, S. J., King, R. W., Schreiber, S. L., and Mitchison, T. J. (1999) *Science* 286, 971–974
6. Kapoor, T. M., Mayer, T. U., Coughlin, M. L., and Mitchison, T. J. (2000) *J. Cell Biol.* 150, 975–988
7. Enos, A. P., and Morris, N. R. (1990) *Cell* 60, 1019–1027
8. Hagan, I., and Yanagida, M. (1990) *Nature* 347, 563–566
9. Hoyt, M. A., He, L., Loo, K. K., and Saunders, W. S. (1992) *J. Cell Biol.* 118, 109–120
10. Heck, M. M., Peereira, A., Pesavento, P., Yannoni, Y., Spradling, A. C., and Goldstein, L. S. (1993) *J. Cell Biol.* 123, 655–679
11. LeGuellec, R., Parte, J., Couturier, A., Roghi, C., and Philippe, M. (1991) *Mol. Cell. Biol.* 11, 3395–3398
12. Swain, K. E., LeGuellec, R., Philippe, M., and Mitchison, T. J. (1992) *Nature* 359, 540–543
13. Blangy, A., Lane H. A., d’Herin, P., Harper, M., Kress, M., and Nigg, E. A. (1995) *Cell* 83, 1159–1169
14. Vale, R. D., and Fletterick, R. J. (1997) *Annu. Rev. Cell Dev. Biol.* 13, 745–777
15. Lockhart, A., and Cross, R. A. (1996) *Biochemistry* 35, 2365–2373
16. Crevel, I. M., Lockhart, A., and Cross, R. A. (1997) *J. Mol. Biol.* 273, 160–170
17. Kashina, A. S., Baskin, R. J., Cole, D. G., Wedman, K. P., Saxton, W. M., and Scholey, J. M. (1996) *Nature* 379, 270–272
18. Swain, K. E., and Mitchison, T. J. (1995) *Proc. Natl. Acad. U. S. A.* 92, 4289–4293
19. Vale, R. D., and Milligan, R. A. (2000) *Science* 288, 88–95
20. Otwinowski, Z., and Minor, W. (1997) in *Methods in Enzymology* (Carter, C. W., and Sweet, R. M., eds) Vol. 276, pp. 307–326, Academic Press, San Diego
21. Brunger, A. T., Kuriyan, J., and Karplus, M. (1987) *Science* 235, 458–466
22. Gulick A. M., Song, H., Endow, S. A., and Rayment, I. (1998) *Biochemistry* 37, 1769–1776
23. Kull, F. J., Sahlin, E. P., Lau, R., Fletterick, R. J., and Vale, R. D. (1996) *Nature* 380, 550–555
24. Sahlin, E. P., Kull, F. J., Cooke, R., Vale, R. D., and Fletterick, R. J. (1996) *Nature* 380, 555–559
25. Kikkawa, M., Sahlin, E. P., Fletterick, R. J., and Hirokawa, N. (2001) *Nature* 411, 439–445
26. Sack, S., Muller, J., Marx, A., Thormahlen, M., Mandelkow, E. M., Brady, S. T., and Mandelkow, E. (1997) *Biochemistry* 36, 16155–16165
27. Case, R. B., Rice, S., Hart, C. L., Ly, B., and Vale, R. D. (2000) *Curr. Biol.* 10, 157–160
28. Rice, S., Lin, A. W., Safer, D., Hart, C. L., Naber, N., Carragher, B. O., Cain, S. M., Pechatnikova, E., Wilson-Kubalek, E. M., Whittaker, M., Pate, E., Cooke, R., Taylor, E. W., Milligan, R. A., and Vale, R. D. (1999) *Nature* 402, 778–784
29. Hirose, K., Henningsen, U., Schliwa, M., Teyoshima, C., Shimizu, T., Alonso, M., Cross, R. A., and Ames, L. A. (2000) *EMBO J.* 19, 5308–5314