Microtubule cross-linking triggers the directional motility of kinesin-5

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Although assembly of the mitotic spindle is known to be a precisely controlled process, regulation of the key motor proteins involved remains poorly understood. In eukaryotes, homotetrameric kinesin-5 motors are required for bipolar spindle formation. Eg5, the vertebrate kinesin-5, has two modes of motion: an adenosine triphosphate (ATP)-dependent directional mode and a diffusive mode that does not require ATP hydrolysis. We use single-molecule experiments to examine how the switching between these modes is controlled. We find that Eg5 diffuses along individual microtubules without detectable directional bias at close to physiological ionic strength. Eg5’s motility becomes directional when bound between two microtubules. Such activation through binding cargo, which, for Eg5, is a second microtubule, is analogous to known mechanisms for other kinesins. In the spindle, this might allow Eg5 to diffuse on single microtubules without hydrolyzing ATP until the motor is activated by binding to another microtubule. This mechanism would increase energy and filament cross-linking efficiency.

Introduction

During cell division, the bipolar mitotic spindle is assembled to orchestrate equal segregation of the genetic material into two daughter cells. Shape, size, and function of the mitotic spindle depend on the motile properties of microtubule-based motor proteins (Sharp et al., 2000; Wittmann et al., 2001). Eg5, an evolutionarily conserved member of the kinesin-5 family, plays a key role in organizing microtubules into a bipolar spindle (Savin et al., 1992; Cole et al., 1994). The homotetrameric configuration of kinesin-5 is crucial for its in vivo function in budding yeast (Hildebrandt et al., 2006) and is arranged such that one pair of motor domains is located at each end of a central stalk (Kashina et al., 1996). Consistent with this structure, it has been demonstrated that Eg5 can cross-link two microtubules and slide them apart (Kapitein et al., 2005).

In earlier work, we have explored the motility characteristics of Eg5 using single-molecule fluorescence experiments (Kwok et al., 2006). The movement of single homotetrameric Eg5 molecules includes an ATP hydrolysis-independent diffusive component in addition to directional processive runs. Similar 1D diffusion along microtubules has been found for other microtubule-based motor proteins and is believed to play functional roles in cells (Vale et al., 1989; Culver-Hanlon et al., 2006; Helenius et al., 2006).

It is also known that the ATPase cycle of kinesins is allosterically controlled by microtubule binding (Kuznetsov and Gelfand, 1986). This prevents unproductive ATP hydrolysis when the motor is not interacting with a microtubule. Furthermore, kinesin-1 switches from an inhibited, microtubule unbound state to a motile state upon cargo binding (Hackney et al., 1992; Stock et al., 1999). Thus, for kinesin-1, motor activity requires binding both a microtubule and cargo. For the homotetrameric Eg5, it turns out that microtubules are both track and cargo. Therefore, the motor would need to differentiate between interactions with one and two microtubules. Such a regulatory mechanism for Eg5 had not been identified.
Results and discussion

Eg5’s motility on single microtubules switches from directional to diffusive upon increasing ionic strength

Ionic strength is known to influence motor–microtubule interactions (Okada and Hirokawa, 2000). To explore Eg5 regulation, we used in vitro single-molecule fluorescence motility assays to examine Eg5-GFP motility on individual microtubules in buffers with various ionic strengths. We found that at ionic strengths close to physiological conditions, full-length Eg5 diffused along single microtubules, whereas a dimeric construct did not show persistent microtubule interactions. Full-length Eg5 switched to directed motion when bound between two microtubules, resulting in relative sliding of microtubules. This suggests a track/cargo interaction-based regulatory mechanism that allows Eg5 to move processively only when cross-linking two microtubules.

Here, we have examined in single-molecule experiments whether Eg5 is regulated via switching between the directional and diffusive modes. We found that for full-length Xenopus laevis Eg5, diffusive and directional motility on single microtubules can be modulated by changing the ionic strength. Significantly, we found that at ionic strengths close to physiological conditions, full-length Eg5 diffused along single microtubules, whereas a dimeric construct did not show persistent microtubule interactions. Full-length Eg5 switched to directed motion when bound between two microtubules, resulting in relative sliding of microtubules. This suggests a track/cargo interaction-based regulatory mechanism that allows Eg5 to move processively only when cross-linking two microtubules.
directional motion depends on ionic strength, with diffusive motion predominating under close to physiological conditions.

We previously showed that diffusive motion of Eg5-GFP on microtubules did not require ATP hydrolysis, and, in the presence of ADP, only the diffusive mode of motion was observed (Kwok et al., 2006). Here, we found that the dependence of the diffusion constant on ionic strength in the presence of ADP was very similar to that in the presence of ATP; it increased about fourfold, from $D = 0.63 \pm 0.06 \times 10^3 \text{nm}^2/\text{s}$ without KCl to $3.8 \pm 0.3 \times 10^3 \text{nm}^2/\text{s}$ in the presence of 60 mM KCl (Fig. 1, I–M; and Table I).

Diffusive motility at near-physiological ionic strength is a property of full-length Eg5

Recently, it was shown in optical trapping experiments that a truncated, dimeric human Eg5 motor construct is capable of only very short processive runs (Valentine et al., 2006). No diffusive motility was reported. However, in a similar assay, full-length Eg5 moved processively over longer distances, but in an irregular manner, indicating diffusive periods (Korneev et al., 2007). To explore the motility of single dimeric Eg5 motors under various ionic conditions, we generated a C-terminal GFP fusion with the N-terminal 513 amino acids of *X. laevis* Eg5 based on the dimeric construct of human Eg5 previously reported (Valentine et al., 2006). The bacterial expression of this protein resulted in a mixture of monomeric and dimeric forms (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200801145/DC1).

Table I. Summary of speed and diffusion constants measured for kinesin-5 under different experimental conditions

| Buffer (70 mM Pipes + KCl added) | ATP | ADP |
|----------------------------------|-----|-----|
|                                  | MD  | MSD | MD  | MSD |
| v (nm/s)                         | D ($10^3 \text{nm}^2/\text{s}$) | n   | v (nm/s) | D ($10^3 \text{nm}^2/\text{s}$) | n |
| 0 mM                             | 8.9 ± 0.1 | 0.67 ± 0.02 | 77 | 9.1 ± 0.6 | 0.63 ± 0.06 | 77 |
| 20 mM                            | 10.1 ± 0.3 | 1.34 ± 0.04 | 62 | 11 ± 1 | 1.2 ± 0.2 | 62 |
| 40 mM                            | 0.0 ± 0.40 | 2.38 ± 0.07 | 50 | 0 ± 3 $\times 10^4$ | 2.6 ± 0.2 | 81 |
| 60 mM                            | 0.3 ± 0.6 | 3.3 ± 0.1 | 48 | 0 ± 1 $\times 10^4$ | 3.8 ± 0.3 | 123 |
| 80 mM                            | 3.1 ± 8.1 | 3.6 ± 0.3 | 60 | 3.3 ± 0.3* | 3.8 ± 0.2 | 103 |
| 60 mM (axonemes)                 | 23.0 ± 0.4 | 2.39 ± 0.07 | 42 | 26.0 ± 1.3 | 1.8 ± 0.3 | 42 |

NA, not applicable.

*Fit to first 5 s.

Figure 2. Dimeric Eg5 exhibits persistent microtubule association only at ionic strength well below physiological conditions.

(A) Kymograph of the displacement of 230 pM Eg5-513-GFP dimers versus time in 80 mM Pipes buffer with 0.2 mM ATP. A large majority of binding events last two frames or less ($< 2$ s). Similar results were obtained at 2 mM ATP (not depicted).

(B) Kymographs showing processive runs by 14 pM Eg5-513-GFP dimers in 20 mM Pipes with 2 mM ATP. Bars, 2 μm.
Size exclusion chromatography was used to isolate the dimeric form, which was then used in imaging experiments. We confirmed that the purified dimer contains two GFPs under all assayed conditions based on its fluorescent properties (Fig. S1, B–G). Motility assays showed that under high ionic strength conditions, motors only remained microtubule bound for very short periods (<2 s), and motility could not be resolved (Fig. 2 A). To exclude the possibility that this construct was inactive, we tested motility at low ionic strength (20 mM Pipes) and observed persistent directional runs (Fig. 2 B). These data suggest that a homotetramer-specific diffusive mode keeps full-length Eg5 attached to a microtubule for longer times at near physiological conditions. It is also possible that this diffusive mode involves interactions between the C terminus of Eg5 and microtubules, as suggested in microtubule-bundling experiments with Drosophila melanogaster kinesin-5 constructs (Tao et al., 2006).

**Figure 3. Full-length Eg5 motility at high ionic strength is directional on microtubule bundles (axonemes).** (A) Cartoon of a possible interaction geometry of Eg5 with microtubule bundles. Note that Eg5 has a length of <80 nm (Kashina et al., 1996), and an axoneme has a diameter of 200 nm, with microtubule doublets being <70 nm apart (Alberts et al., 2002). (B) Kymographs of Eg5-GFP motility versus time on an axoneme in the presence of ATP. (C) MD calculated from motility recordings in the presence of ATP. Fit for ATP represents MD = vτ + Dτ [v = 23 nm/s; D = 1.8 × 10^3 nm^2/s]. Fit for ADP represents MD = 2Dτ [D = 1.3 × 10^3 nm^2/s]. Error bars represent SD. Bar, 1 μm.

Eg5 moves directionally on microtubule bundles at high ionic strength

We have shown previously that Eg5 can cross-link two microtubules and drive their relative movement by moving toward both plus ends in an ATP-dependent manner even at high ionic strength (Kapitein et al., 2005). This appears to be inconsistent with the diffusive behavior of Eg5-GFP observed here under these conditions. One explanation is that the simultaneous interaction of individual Eg5 tetramers with two microtubules enhances directional motion. To test this, we examined the motion of Eg5-GFP on bundles of microtubules (axonemes), which might allow Eg5 molecules to interact with more than one microtubule at the same time (Fig. 3 A). On axonemes, single Eg5 motors made directional runs of several micrometers (Fig. 3 B), even at salt concentrations (70 mM Pipes plus 60 mM KCl) at which Eg5’s motility on single microtubules was purely diffusive (Fig. 1, H–L). MD and MSD analyses indicate that the motility of Eg5 on axonemal microtubule bundles has an ATP-dependent directional component in addition to 1D diffusion, even at a high ionic strength (Fig. 3, C and D). This result is consistent with directionality being caused by the interaction of Eg5 with two microtubules, although we cannot rule out an influence of particular properties of axonemal microtubules.

Eg5 can switch from diffusive to directional motion upon binding a second microtubule

To directly test whether Eg5 motion is regulated through interactions with a second microtubule, we used Eg5-GFP in a microtubule–microtubule sliding assay (Fig. 4 A; Kapitein et al., 2005). Microtubules were immobilized on the glass surface,
In these experiments, microtubule sliding events with sufficiently sparse motors to map individual trajectories were rare. To overcome this problem, we performed experiments at lower concentrations of Eg5-GFP, but in the presence of unlabeled tetrameric Eg5. Below, the corresponding kymograph of Eg5-GFP shows directional runs between the overlapping microtubules (region marked with two red dotted lines). B–F Analysis of Eg5 motility during relative sliding. B Scatter plot of all pairs of short-term velocity and diffusion constant determined for a window of 15 s moving over the composite position-time trace of 94 Eg5 motors traced in the overlap zone of 11 microtubule pairs (2,335 points obtained from 2,349 s of total time). The horizontal dotted line indicates the average velocity of sliding microtubules (33 nm/s), and the vertical dotted line indicates the threshold used to discriminate slow and fast diffusion. C and D Similar analyses for Eg5 moving on individual microtubules at low ionic strength [C, 70 mM Pipes; Fig. 1; 4,266 points] and high ionic strength [D, data pooled from 70 mM Pipes + 60 mM KCl and 70 mM Pipes + 80 mM KCl; 2,478 points]. E Position-time traces. Black, fraction of the composite trace used for B. Green and red, sorted time points with a short-term diffusion constant; $D < 1,500 \text{ nm}^2/\text{s}$ (green) and $D > 1,500 \text{ nm}^2/\text{s}$ (red). F Histograms of the short-term velocities as obtained from the time points in the green and red trace in E. The arrow indicates the average microtubule sliding velocity. G Graph summarizing Eg5 behavior under various conditions. Bar, 2 μm.
diffusion constant for Eg5 in between sliding microtubules at high ionic strength (Fig. 5 B), Eg5 on individual microtubules (Fig. 1) at low ionic strength (70 mM Pipes; Fig. 5 C), and high ionic strength (70 mM Pipes + 60/80 mM KCl; Fig. 5 D). These data reveal that Eg5 motility between sliding microtubules is a mixture of the two types of motility observed on single microtubules at low and high ionic strength (directional and non-directional; diffusive). We next sorted all time points into two categories: one corresponding to all time points with a short-term diffusion constant \(< 1,500 \text{ nm}^2/\text{s}\), and the second one corresponding to diffusion constants \(> 1,500 \text{ nm}^2/\text{s}\) (Fig. 5, B and E).

The velocity distribution of the first class (\(D < 1,500 \text{ nm}^2/\text{s}\); Fig. 5 F) is narrow and peaks around 15 nm/s (mean velocity \(\pm SD = 12 \pm 8 \text{ nm/s}\)). The velocity distribution of the second class (\(D > 1,500 \text{ nm}^2/\text{s}\)) is much wider and peaks around 30 nm/s, similar to the velocity of sliding microtubules.

From this analysis, we conclude that, for individual Eg5 tetramers in the overlap zone of two sliding microtubules, there is a strong correlation between the diffusion constant and the velocity. Some of the motors move for part of the time with a velocity of \(\sim 15 \text{ nm/s}\) and with a relatively low diffusion constant, which is analogous to Eg5-GFP’s motility along single microtubules at low salt concentrations (Fig. 1). A second class has a higher diffusion constant and a velocity corresponding to that of the sliding microtubule. This second class consists of motors that are (occasionally) diffusing on the sliding microtubule and get transported with it. Collectively, these results demonstrate that Eg5 can switch from diffusive motility to directional motility upon binding to a second microtubule (Fig. 5 G).

**Potential regulatory mechanisms for homotetrameric Eg5**

We have shown that full-length Eg5’s motility comprises an unbiased, diffusive mode independent of ATP hydrolysis and a plus end-directed processive mode that requires ATP hydrolysis. The balance of these modes depends on ionic strength, cross-link geometry, and, as we have shown previously, monastrol concentration (Fig. 5 G; Kwok et al., 2006). At high ionic strengths, full-length Eg5’s motility on single microtubules is predominantly diffusive, whereas dimeric Eg5 associates only very briefly, suggesting that domains (motor or nonmotor) in the full-length homotetramer contribute to the interaction that mediates diffusion. Decreasing the ionic strength enhances the directionality of full-length Eg5 and increases the processive run length of dimers. The run length of a processive motor is determined by the ability to keep the nucleotide states of its two motor domains out of phase. Runs terminate when both domains are in the ADP-bound state. We speculate that with ADP on all heads, dimeric Eg5 detaches from the microtubule, whereas full-length Eg5 enters its diffusive mode. For full-length Eg5, the similar effect of monastrol and increasing salt can be understood if both inhibit ADP release, thereby promoting an ADP-ADP diffusive state. Indeed, kinetic studies on Eg5 inhibition indicate that monastrol stabilizes the ADP-bound state (DeBonis et al., 2003; Cochran et al., 2005). In addition, a strong decrease in microtubule-stimulated ATPase activity at increased ionic strength has been reported for a monomeric construct of human Eg5 (DeBonis et al., 2003).

We have also demonstrated that homotetrameric Eg5’s binding to a second microtubule alters the balance between diffusive and directional motion. The question remains how a signal affecting mechanochemistry gets transmitted from one end of the homotetrameric molecule to the other end. Electron micrographs of Eg5 show very straight conformations of the stalk, without evidence for hinges (Kashina et al., 1996). It is thus unlikely that Eg5’s regulation uses a large-scale hinge motion. This would be in contrast to kinesin-1, in which the cargo-inhibited folding of the tail onto the motor domains turns off the motor (Friedman and Vale, 1999; Stock et al., 1999). On the other hand, the tail domain of the opposing dimer of Eg5 is likely located close to where the folded tail domain of a kinesin-1 dimer would be in the inhibited state. It could thus play a regulatory role with only relatively small conformational changes. Another possibility is that changes in thermal motions of the distal motor domains are coupled to fluctuations in the stalk, which, in turn, control the balance between diffusive modes and processive bursts in the proximal motor domains. Such a mechanism has been hypothesized for molecular motors such as dynein (Bray and Duke, 2004; Hawkins and McLeish, 2006). Further experimental work, possibly using optical traps to apply well-controlled loads, will be needed to explore how a mechanical signal on one end of the molecule can switch the motility of homotetrameric Eg5.

In summary, our results provide evidence for a functional specialization of Eg5 that is thus far unique among the kinesins, namely the capability to switch between different modes of motion on microtubules in response to binding another microtubule. Our data suggest that ATP-dependent directional motility is suppressed when Eg5 interacts with only one microtubule and is activated upon binding a second microtubule. This could equip a homotetrameric kinesin with cargo sensitivity and increase its energy efficiency. In addition, nonspecific attachment to and ATP-independent diffusion along single microtubules enhances the probability of capturing another microtubule. After cross-linking, Eg5 switches to directional motility and drives the sorting of these microtubules. These findings provide an important step toward understanding the complex regulation of Eg5 and its contribution to bipolar spindle assembly during cell division.

**Materials and methods**

**Protein constructs**

A recombinant full-length *X. laevis* Eg5-GFP construct was expressed and purified as described previously (Kwok et al., 2006). To generate dimeric GFP-labeled Eg5, a fragment of the *X. laevis* Eg5 gene coding for the N-terminal S13 amino acids was amplified by PCR, fused in frame to GFP, and inserted into the bacterial expression vector pRSET. The linker sequence GSSGGGSSGGGSSGGGGS was inserted between Eg5 and GFP, and a tobacco etch virus protease-cleavable polyhistidine tag was added to the C terminus. Eg5-S13-GFP was expressed in BL21 Escherichia coli and purified as described for the full-length Eg5-GFP with the following modifications (Kwok et al., 2006). Removal of the polyhistidine tag by tobacco etch virus protease was performed at 4°C for 12 h. Purification by size exclusion chromatography was performed with a Superdex-200 column (GE Healthcare). Axonemes (from sea urchin sperm) and Cy5-labeled tubulin (from porcine brain) were prepared according to published procedures (Gibbons and Frank, 1979; Hyman, 1991; Kapitein et al., 2005).
Diffusion constant and speed were determined from the MSD as described previously [Kwok et al., 2006]. The MSD for purely diffusive motion is a straight line, reflecting the linear increase in positional variance typical for diffusive motion (MSD = 2Dt). In the case of a directional bias, the MSD will show an additional quadratic component proportional to the velocity (thus, MSD = 2D + v^2). Velocity and diffusion constants were also determined from the MDs using the relations MD = D + v. For simultaneous observation of GFP and Cy5, emulsion light was first filtered with a triple bandpass filter (Z488/532/633M; Chroma Technology Corp.), separated with a dichroic beam splitter (S65DCXR; Chroma Technology Corp.), and finally redirected onto the tube lens at slightly different angles, resulting in two separate images on the camera chip (Micromax; Roper Scientific). Data were taken with continuous excitation (20–30 W/cm^2) and a 14-s integration time, unless stated otherwise. Experiments with mixtures of Eg5-GFP and wild-type Eg5 (Fig. 5) were performed on a total internal reflection fluorescence (TIRF) microscope (Eclipse TE2000E; Nikon) with a 100 × NA 1.49 plan Apo objective (Carl Zeiss, Inc.), and an EM CCD camera (QuantEM; Roper Scientific) for detection.

Single-molecule motility and photobleaching experiments involving the shorter Eg5-S13-GFP construct were performed on another instrument, which was described previously [Kwok et al., 2006], with the following modifications. The inverted microscope (Axiovert; Carl Zeiss, Inc.) was equipped with a laser TIRF slider (Carl Zeiss, Inc.), a 100 × NA 1.45 Alpha Plan-Fluar objective (Carl Zeiss, Inc.), and an EM CCD camera (iXon DU-897; Andor Technology). Data were taken with TIRF illumination from a 491-nm laser source [Cobalt Calypso 50; Solarem Technology] with a 0.4-s exposure time at a frame rate of 1 s.^1

Coverslips were cleaned by ultrasonication in −0.2 M potassium hydroxide followed by three rounds of ultrasonication in ultrapure water. Coverslips were rendered positively charged by aminoisolationization with 3-(2-aminoethy lamino)ethanolpropyltrimethoxysilane (DETA; SigmaAldrich). In some experiments, coating with poly-lysine (SigmaAldrich) was used for the same purpose. Sample chambers were first incubated with Cy5-labeled microtubules (in some experiments, rhodamine-labeled microtubules were used; Fig. 5 A) or axonemes for 10 min followed by 5–10-min incubation with 0.2 mg/ml casein. Finally, chambers were perfused with ~100–300 μM of motors in motility buffer. For relative sliding experiments, free microtubules were added to the motility buffer, and the motor concentration was increased three to five times using Eg5-GFP (Fig. 4) or a mixture of 1 nM Eg5-GFP and 2 nM of unlabeled full-length Eg5 (Fig. 5) to enhance landing rates of microtubules.

Motility buffer
Pipes was used as buffering agent. This buffer has two acid groups, and buffering at the pKa [6.8] requires an equimolar amount of HPipes and Pipes. Buffering at 100 mM Pipes (acid form of Pipes) required the addition of 105 mM KOH, whereas buffering the basic form (K2Pipes) required the addition of 35 mM HCl. In the latter case, however, 140 mM K+ are contributed by the buffer. The lowest concentration of Pipes used was 70 mM because the buffering capacity at lower concentrations was not sufficient to maintain a constant pH after adding the various other components [Eg5-GFP, ATP, etc.]. Consistently, experiments performed with K2Pipes buffer (70 mM K2Pipes; 140 mM K+; in total yielded results similar to those made with H2Pipes in the presence of an additional 60/80 mM KCl [Fig. 5]. In addition to the variable amount of KCl, the motility buffers contained 1 mM EGTA, 3 mM MgCl2, 2 mM ATP or ADP, 14 mM DTT, 10 μM paxtacel, 25 mM glucose, 20 μg/ml glucose oxidase, and 35 μg/ml catalase.

Data analysis
Motility data were acquired using WinView (Roper Scientific) or MetaMorph (MDS Analytical Technologies) and were analyzed with custom-written routines in LabVIEW (National Instruments). The x-y coordinates of moving fluorescence spots were determined by fitting a 2D Gaussian to the observed intensity profile in each frame. Mainly nanoconstricting trajectories were analyzed; occasionally a trajectory was traced until it crossed another trajectory. Spots with an intensity four times larger than the average were excluded as well as trajectories shorter than four frames or immobile for more than three frames. The microtubule position was determined from fitting a straight line to all of the motor coordinates of a run. The motor coordinates were then transformed into coordinates along and perpendicular to the microtubule by projection.

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