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

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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, 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.
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 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 $D = 1,000 \text{ nm}^2/\text{s}$ without KCl to $D = 3,800 \text{ nm}^2/\text{s}$ 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).

To systematically and quantitatively analyze the dependence of Eg5-GFP motility on ionic strength, we kept buffer concentration and pH constant (70 mM Pipes, pH 6.8) and added increasing amounts of KCl. For each buffer condition, >60 trajectories of individual Eg5-GFP tetramers were extracted from the recordings, and both the mean squared displacement (MSD) and the mean displacement (MD) were calculated for increasing time intervals, $\tau$ (Fig. 1, E–M; Kwok et al., 2006). The velocities and diffusion constants obtained from a second-order polynomial fit to MSD($\tau$) and from linear fits to MD($\tau$) and to the variance of the MD($\tau$) are summarized in Table I. The 1D diffusion constant increased with increasing salt concentration (from $D = 0.63 \pm 0.06 \times 10^3 \text{ nm}^2/\text{s}$ at 0 mM KCl to $D = 3.6 \pm 0.3 \times 10^3 \text{ nm}^2/\text{s}$ at 80 mM KCl), whereas the mean velocity dropped (from $v = 9 \pm 10 \text{ nm/s}$ at low salt to 0 nm/s at higher salt concentrations). Furthermore, we found that the average duration of motile events decreased from 34 ± 3 s with no KCl added to 16 ± 2 s in the presence of an additional 60 mM KCl (Fig. 1 N). These results show that upon addition of more salt, Eg5-GFP’s motility changed from a combination of directional motion and diffusion (<20 mM KCl) to purely diffusive motion (>40 mM KCl). The exact ratio of diffusive and 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 $\sim 1,000 \text{ nm}^2/\text{s}$ without KCl to $\sim 3,800 \text{ nm}^2/\text{s}$ in the presence of 60 mM KCl (Fig. 1, I–M; and Table I).

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

<table>
<thead>
<tr>
<th>Buffer (70 mM Pipes + KCl added)</th>
<th>ATP</th>
<th>ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD</td>
<td>MSD</td>
<td>n</td>
</tr>
<tr>
<td>0 mM</td>
<td>8.9 ± 0.1</td>
<td>0.67 ± 0.02</td>
</tr>
<tr>
<td>20 mM</td>
<td>10.1 ± 0.3</td>
<td>1.34 ± 0.04</td>
</tr>
<tr>
<td>40 mM</td>
<td>0.0 ± 0.40</td>
<td>2.38 ± 0.07</td>
</tr>
<tr>
<td>60 mM</td>
<td>0.3 ± 0.6</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>80 mM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>60 mM (axonemes)</td>
<td>23.0 ± 0.4</td>
<td>2.39 ± 0.07</td>
</tr>
</tbody>
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NA, not applicable.

*a* 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 GPPs 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).

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 nm²/s (green) and D > 1,500 nm²/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.

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 Eg5 (Fig. 5 A). Trajectories of 94 motors between 11 overlapping and sliding microtubule pairs (mean velocity ± SD = 33 ± 6 nm/s) could now be traced. Analysis of motor motility in the overlap region is complicated by not knowing whether motors at any given time interact with just one or with both microtubules. For example, Eg5 molecules that appear to move directionally could actually be freely diffusing on the sliding microtubule, being transported along with it. In addition, motors can switch between modes of interaction. The three possible motor populations can, in principle, be distinguished based on their average speeds and diffusion constants. Motors diffusing on the transported microtubule should exhibit both a high speed and a high diffusion constant, whereas motors driving microtubule sliding are expected to move at half the translocated microtubule’s speed with a lower diffusion constant.

To distinguish, without bias, intervals corresponding to active, directional motility from passive diffusion (along either the immobile or the sliding microtubule), we calculated the MSD and MD over 15-s intervals to obtain short-term diffusion constants and velocities (Douglass and Vale, 2005). Fig. 5 (B–D) shows scatter plots of the short-term velocity versus short-term...
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 \( (D < 1,500 \text{ nm}^2/\text{s}; \text{Fig. 5 F}) \) is narrow and peaks around 15 nm/s (mean velocity \( \pm \) SD = 12 \pm 8 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 microtubules, 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 513 amino acids was amplified by PCR, fused in frame to GFP, and inserted into the bacterial expression vector pRSET. The linker sequence GSSGGGGSGGGGSGGGGS was inserted between Eg5 and GFP, and a tobacco etch virus protease-cleavable polyhistidine tag was added to the C terminus. Eg5-513-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 = 2Dt + v^2). Velocity and diffusion constants were also determined from the MDs using the relations MD = v + and variance of the MD = 2Dv. MDs were calculated in the same way, but without the squaring of displacements. For the calculation of D from the variance of the MD, the standard error of the variance was used as error bars (Taylor, 1997).

In contrast to the MSD calculations, the MD calculations require knowledge of the polarity of the microtubules because in an average over randomly oriented microtubules, the MD would always average to zero. In our experiments, the orientation of the microtubules could be inferred from the overall direction of motion of the motors. In the experiments at high ionic strengths, there was no obvious overall direction of motion, but the orientation of about half of the microtubules could be inferred from a few clearly aggregated clumps of motor (high fluorescence intensity) that still moved directionally (~4% of total events; intensity at least four times that of single motors).

For calculation of the short-term MSD and MD in Fig. 5, traces were merged into one array, and the MSD and MD were calculated for a sliding subarray of size 15 (Douglas and Vale, 2005). D and v were then obtained from a linear fit to the first three points of the MSD (MSD = 2Dt) and MD (MD = v + ) respectively, including zero excluded. For Fig. 5 E and F, Dv pairs were sorted based on D to yield a distribution of velocities for D > 1,500 nm/s^2 (Fig. 5 E) and D < 1,500 nm/s^2 (Fig. 5 F). The same analysis for trajectories on individual microtubules (Fig. 5, C and D) classified 20% of data points above 1,500 nm/s^2 at low ionic strength and 25% below this cut-off at high ionic strength. The average velocity of high D data points in Fig. 5 C is 16 nm/s. The average velocity of the low D data points in Fig. 5 D is 0.75 nm/s.

Online supplemental material

Fig. S1 shows biochemical and fluorescence characterization of Eg5-513-GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801145/DC1.

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