Kinesin Kar3Cik1 ATPase Pathway for Microtubule Cross-linking*

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Kar3Cik1 is a Saccharomyces cerevisiae kinesin-14 that functions to shorten cytoplasmic microtubules (MTs) during yeast mating yet maintains mitotic spindle stability by cross-linking anti-parallel interpolar MTs. Kar3 contains both an ATP- and a MT-binding site, yet there is no evidence of a nucleotide-binding site in Cik1. Presteady-state and steady-state kinetic experiments were pursued to define the regulation of Kar3Cik1 interactions with the MT lattice expected during interpolar MT cross-linking. The results reveal that association of Kar3Cik1 with the MT occurs at 4.9 μM⁻¹ s⁻¹, followed by a 5-s⁻¹ structural transition that limits ADP release from the Kar3 head. Mant-ATP binding occurred at 2.1 μM⁻¹ s⁻¹, and the pulse-chase experiments revealed an ATP-promoted isomerization at 69 s⁻¹. ATP hydrolysis was observed as a rapid step at 26 s⁻¹ and was required for the Kar3Cik1 motor to detach from MT. The conformational change at 5 s⁻¹ that occurred after Kar3Cik1 MT association and prior to ADP release was hypothesized to be the rate-limiting step for steady-state ATP turnover. We propose a model in which Kar3Cik1 interacts with the MT lattice through an alternating cycle of Cik1 MT collision followed by Kar3 MT binding with head-head communication between Kar3 and Cik1 modulated by the Kar3 nucleotide state and intramolecular strain.

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2The abbreviations used are: MT, microtubule; ipMT, interpolar MT; AMPNP, adenosine-5’-(β,γ-imido)triphosphate; ATPS, adenosine-5’-(γ-thio)triphosphate; mant, 2’(3’)-O-(N-methylanthraniloyl).

MT shortening at the MT plus end in an ATP-dependent manner (8). In contrast, Kar3Vik1 association with MTs was cooperative, saturating the lattice of one MT with other nearby MTs showing no evidence of Kar3Vik1 binding (19). This cooperative binding behavior also appeared to stabilize the MT lattice and inhibit Kar3Vik1-promoted MT depolymerization (19).

Because kinesin-14s have their motor domains at the carboxyl terminus, Allingham et al. (19) crystallized the carboxyl-terminal globular domain of Vik1 to determine its structural relationship to the Kar3 catalytic domain (19, 20). Surprisingly, this Vik1 domain exhibited the fold of a kinesin motor head even though the Vik1 motor homology domain (Vik1MHD) did not bind nucleotide or reveal a site for nucleotide binding. These studies also showed that Kar3Vik1 promoted MT minus end-directed MT gliding, and the Vik1MHD could bind MTs in the absence of Kar3. These results indicated that the Kar3MD (Kar3 motor domain) and Vik1MHD must communicate to coordinate their interactions with the MT (19).

Based on these results, we hypothesized that Kar3Cik1 for its ipMT cross-linking function (11) may share mechanistic characteristics in common with Kar3Vik1. To test this hypothesis, we pursued a mechanistic analysis of the MT-Kar3Cik1 ATPase using paclitaxel to stabilize the MTs from depolymerization. The results were also evaluated in the context of Drosophila melanogaster Ncd, a homodimeric kinesin-14 that has been extensively studied (21–35). The Kar3Cik1 results presented here indicate that both Kar3 and Cik1 interact with the MT and that a single ATPase cycle requires head-head coordination mediated by both nucleotide state and intramolecular strain.

EXPERIMENTAL PROCEDURES

Experimental Conditions—All experiments were performed at 25 °C in the ATPase buffer; 20 mM Heps, pH 7.2, with KOH, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate, 1 mM dithiothreitol, 5% sucrose. The MT concentrations reported throughout the study resulted from bovine tubulin that was cold-depolymerized, clarified, polymerized with 1 mM MgGTP, and stabilized with 40 μM paclitaxel. At this concentration of paclitaxel, the MTs are stable from depolymerization by Kar3Cik1 (8, 19).

Kar3Cik1—The Kar3Cik1 motor protein used in this study was characterized previously (19). The truncated Kar3 gene was cloned into plasmid pET24d and when expressed yields amino acid residues Met-Ala-Lys⁶²⁶-Lys⁷²⁹ with a predicted Mr = 52,833. The truncated version of Cik1 (encoding Lys⁵²⁵–Asp⁵⁹⁴) was cloned into plasmid pET15b and when expressed yields residues MGSSH₆SSGGLVPGRGSHMet-Lys⁵²⁵-Asp⁵⁹⁴ with pre-
dicted $M_e = 43,059$. Both plasmids were transformed and coex-pressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL, yielding a Kar3Cik1 heterodimer that formed through native coiled-coil interactions and with the predicted $M_e = 95,892$.

Kar3Cik1 was purified with ADP tightly bound at the Kar3 active site (8, 19). Before each experiment, Kar3Cik1 was clari-fied (Beckman Coulter TLA 100 rotor, 3 min, 90,000 rpm, 4 °C), and the concentration was determined by the Bio-Rad protein assay using IgG as a standard. Protein concentrations were expressed as the Kar3Cik1 heterodimer with the ATP site concentration at one per Kar3Cik1.

**Steady-state ATPase Kinetics**—The steady-state kinetics for Kar3Cik1 were determined by following [α-32P]ATP turnover to [α-32P]ADP, as described previously (36). The rates of ATP turn-over as a function of MgATP (Fig. 1A) were fit to the Michaelis-Menten equation. The MT concentration dependence was fit to quadratic Equation 1, which was required because the enzyme concentration was not 10-fold less than the $K_{i,MT}$.

$$
\text{Rate} = 0.5 \cdot k_{cat} \cdot ((E_0 + K_{1/2,MT} + MT_0)^2 - (4E_0S_0)^{1/2}) \quad \text{(Eq. 1)}
$$

Rate is the concentration of ADP, formed per s/μM Kar3Cik1; $k_{cat}$ is the maximum rate constant of steady-state turnover; $E_0$ is the Kar3Cik1 concentration in μM; $K_{1/2,MT}$ is the concentration of tubulin polymer that provides one-half the maximum rate of steady-state ATP turnover, and $MT_0$ is the tubulin polymer concentration.

**MT-Kar3Cik1 Cosedimentation Assays**—Reactions of 200 μl with MTs (0–10 μM tubulin and 40 μM paclitaxel) were incubated for 30 min with 2 μM Kar3Cik1 plus 1 mM or 100 μM MgADP, 1 mM MgAMPPNP, or 100 μM MgADP followed by apyrase treatment (described below) to generate the nucleotide-free state. The incubation was followed by centrifugation at 25 °C (Beckman Coulter TLX Ultracentrifuge, 90,000 rpm, 30 min). For each reaction, the supernatant was collected (top 100 of 200 μl). The MT pellet was resuspended in 100 μl of 5 mM CaCl2 in buffer and incubated at 4 °C for 10 min. This step was repeated, combining the two 100-μl aliquots. Laemmli 2× sample buffer was added to the supernatant (100 μl + 100-μl sample buffer) and pellet (200 μl + 200-μl sample buffer) samples, and these were subjected to SDS-PAGE, loading equal volumes, followed by staining with Coomassie Brilliant Blue R-250. The protein was quantified using Image J (National Institutes of Health), and the data were plotted as the fraction of Kar3Cik1 that partitioned to the pellet as a function of increasing MT concentration (Figs. 3 and 10). The data were fit to quadratic Equation 2,

$$
\frac{(MT \cdot E)}{(E)} = 0.5 \times \left[\left(E_0 + K_d + MT_0\right)^2 - (4E_0S_0)^{1/2}\right] \quad \text{(Eq. 2)}
$$

where $MT \cdot E$ is the fraction of Kar3Cik1 that sediments with the MT pellet; $E_0$ is the total Kar3Cik1, and $K_d$ is the apparent dissociation constant.

**Formation of Nucleotide-free MT-Kar3Cik1 Complex**—Nucleotide-free Kar3Cik1 was generated by preforming the MT-Kar3Cik1 complex in the presence of 100 μM MgADP (15 min), followed by addition of apyrase (0.02 unit/ml, grade VII, Sigma), and incubated for 90 min. This experimental design assumes that the Kar3 head with ADP tightly bound will partition off of the MT but remain tethered to the MT by Cik1 (E1 intermediate, Fig. 11). The apyrase VII isoform preferentially selects for ADP to convert to AMP, which binds so weakly to the Kar3 active site that Kar3Cik1 is essentially nucleotide-free (data not shown). The concentration of apyrase used is sufficiently low that it does not compete with Kar3Cik1 for nucleotide during the presteady-state kinetic experiments. Control experiments with 100 μM [α-32P]ATP show complete conversion of [α-32P]ADP to [α-32P]AMP after the 90-min incubation ($k_{obs} = 0.0008 \pm 0.0001$ s$^{-1}$). We hypothesize that this protocol results in the formation of the E3 intermediate with the Cik1 head bound to the MT and the Kar3 head ahead of Cik1 at the next MT-binding site toward the minus end (E3, Fig. 11).

**Kinetics of MT Association and ATP-promoted MT-Kar3Cik1 Dissociation**—The SF-2003 KinTek stopped-flow instrument was used to measure the kinetics of Kar3Cik1 association with MTs (Figs. 2 and 4) and ATP-promoted dissociation of the MT-Kar3Cik1 complex (Fig. 9) by monitoring the change in turbidity at 340 nm. The association data were fit to the linear function as shown in Equation 3,

$$
k_{obs} = k_{+4}(\text{tubulin}) + k_{-4} \quad \text{(Eq. 3)}
$$

where $k_{obs}$ is the observed rate of the initial exponential phase of the averaged transients; $k_{+4}$ represents the second-order rate constant for MT association, and $k_{-4}$ defines the rate constant of motor dissociation as provided by the y intercept (scheme at top of Table 1). The concentration of tubulin represents the MT polymer stabilized with paclitaxel.

For the ATP-promoted dissociation kinetics (Fig. 9), the transients show an initial phase of rapid increase in turbidity for the first ~100 ms, followed by a decrease in turbidity correlated with motor detachment from MT. The transients were normalized such that all began at maximum turbidity at ~100 ms. The observed rates of the exponential decrease in turbidity plotted as a function of increasing MgATP concentrations were fit to a hyperbolic function, yielding $k_{max}$ and corresponding to the rate constant of ATP-promoted Kar3Cik1 detachment from the MT.

**Kinetics of MT-activated Mant-ADP Release and Mant-ATP Binding**—The MT-activated mant-ADP release (Fig. 5) and mant-ATP binding kinetics (Fig. 6) were measured by following the fluorescence change of the nucleotide analogs mant-ATP and mant-ADP (Invitrogen), which were excited at 360 nm and detected at 450 nm via a 409 nm long pass filter (Semrock, Inc.). These nucleotide analogs have been shown to exhibit similar kinetics to ATP and ADP for kinesin motor proteins, although typically the apparent $K_{i,\text{mant-ATP}}$ is 2–3-fold weaker because of the fluorescent group (37). The mant-ADP release data were fit to a hyperbolic function, which provided the intrinsic rate constant (Table 1). The observed rates of the exponential phase of fluorescence enhancement (Fig. 6) associated with mant-ATP binding to the active site of Kar3Cik1 were plotted as a function of increasing mant-ATP concentration. These data were fit to the linear function as shown in Equation 4,
where $k_{\text{obs}}$ is the observed rate associated with the initial exponential phase of the fluorescence increase; $k_{s_{1}}$ defines the second-order rate constant for mant-ATP binding, and $k_{\text{off}}$ corresponds to the observed rate constant of mant-ATP dissociation as provided by the $y$ intercept.

**ATP Hydrolysis by Acid Quench**—The presteady-state kinetic experiments to study the time dependence of ATP hydrolysis (Fig. 8) were performed using a rapid chemical quench-flow instrument (RQF-3, KinTek Corp.). The nucleotide-free MT-Kar3Cik1 complex was preformed as described above and reacted with increasing concentrations of $[\alpha-32\text{P}]$ATP plus 200 mM KCl (syringe concentration) for times ranging from 5 to 500 ms, followed by the MgATP chase for 6 s ($>10$ turnovers). The reaction was expelled from the instrument into a 1.5-ml tube that contained 100 $\mu$M of 22 $\mu$M formic acid to terminate the reaction and release nucleotide on the active site. During the 6-s chase with unlabeled MgATP, any unbound or loosely bound $[\alpha-32\text{P}]$ATP was diluted; therefore, only the stably bound $[\alpha-32\text{P}]$ATP proceeded forward to ATP hydrolysis. The products $[\alpha-32\text{P}]$ADP $+$ P$_{i}$ were separated from $[\alpha-32\text{P}]$ATP, and the concentration of $[\alpha-32\text{P}]$ADP product formed was plotted as a function of time. The transients were fit to the burst equation (Equation 5), where $A_{0}$ is the amplitude of the exponential burst phase, representing the concentration of ATP bound to the active site that proceeded through ATP hydrolysis; $k_{b}$ is the rate of the exponential burst phase, and $t$ is the time in seconds, and $k_{s_{\text{slow}}}$ is the rate of the linear phase ($\mu$M$^{-1}$s$^{-1}$), which corresponds to steady-state turnover at 100 mM KCl. The observed rate (Fig. 8B) and the amplitude of the exponential burst phase (Fig. 8C) were plotted as a function of MgATP concentration, and each data set was fit to a hyperbolic function.

**Pulse-Chase Experiments**—To determine the kinetics of ATP binding (Fig. 7), the rapid chemical quench-flow instrument was used with the quench syringe containing 30 mM MgATP (syringe concentration). The nucleotide-free MT-Kar3Cik1 complex was preformed as described above and reacted with increasing concentrations of $[\alpha-32\text{P}]$ATP for times ranging from 5 to 500 ms, followed by the MgATP chase for 6 s ($>10$ turnovers). The reaction was expelled from the instrument into a 1.5-ml tube that contained 100 $\mu$M of 22 $\mu$M formic acid to terminate the reaction and release nucleotide on the active site.

**RESULTS**

The scheme at the top of Table 1 is the Kar3Cik1 ATPase pathway used for the design of the presteady-state kinetic experiments with the constants determined in Table 1. Fig. 11 presents the model of Kar3Cik1 interactions with the MT based on these rate and equilibrium constants (Table 1).
MT-Kar3Cik1 Association Kinetics—Kar3Cik1 was rapidly mixed with MTs in the stopped-flow instrument, and an increase in turbidity as a function of time was monitored as the signal that represented the formation of the MT-Kar3Cik1 complex (Figs. 2 and 4). The observed rate of the initial fast phase increased linearly as a function of increasing MT concentration (Fig. 2B), and the fit of the data provided an apparent second-order rate constant, $k_{\text{obs,0}} = 5.1 \, \mu M^{-1} \, s^{-1}$ (Table 1). However, in this simple experimental design, there may be an equal probability of either Cik1 or Kar3 colliding with the MT first. We hypothesized that if the experiment were performed in a high ADP environment, we may prejudice MT association through Cik1 collision because the Kar3-ADP state is weakly bound to the MT. To test this hypothesis, the MT association kinetics were repeated in the presence or absence of 1 mM MgADP (Fig. 2C), and the fits of the data provided an apparent second-order rate constant of 4.9 $\mu M^{-1} \, s^{-1}$, which was comparable with the 5.1 $\mu M^{-1} \, s^{-1}$ measured in the absence of MgADP at the no additional ADP condition (Fig. 2B and Table 1). For the initial exponential phase to be similar in the presence or absence of MgADP, the rate of MT collision by Kar3 or Cik1 must be very similar, consistent with the transients in Fig. 2C and the second-order rate constants reported in Figs. 2B and 4B. The observed rate of the second exponential
phase increased with increasing MT concentrations (Fig. 4B),
and the fit of the data provided a maximum rate of 5 s$^{-1}$, which
was similar to the steady-state $k_{cat}$.

**Mant-ADP Release from Kar3Cik1 Is Biphasic**—For kinesins,
MT association dramatically activates ADP release from the
active site (27, 29, 37–40). To measure these kinetics (Fig. 5),
Kar3Cik1 was incubated with mant-ADP at a 1:6 ratio to exchange ADP at the active site with mant-ADP. Subsequently,
the Kar3Cik1-Mant-ADP complex was rapidly mixed in the
stopped-flow instrument with MTs plus 1 mM MgATP. The high concentration of ATP acts to dilute mant-ADP once released from the active site and effectively prevents its rebind-
ing to Kar3Cik1. The transients shown in Fig. 5A reveal an immediate rapid fluorescence decrease followed by a slower second phase. The observed rates of the initial fast phase were plotted as a function of MT concentration, and the fit of the data provided a maximum rate constant of 110 s$^{-1}$ (Fig. 5B and Table 1). The second slow phase of mant-ADP release also increased as a function of MT concentration, and the fit of the data provided a maximum rate of 5 s$^{-1}$. This rate was compa-
rable with steady-state turnover ($k_{cat} = 5$ s$^{-1}$) and the rate of the second slow phase of the MT association kinetics in the presence of MgADP (Fig. 4B). Note too the absence of an initial lag in the mant-ADP transients in Fig. 5A.

Because of the absence of a lag in the mant-ADP release trans-
sients, we propose that the initial exponential fluorescence
decrease is due to a population of motors where the Kar3 heads collided with the MT first activating mant-ADP release. There-
fore, the maximum rate at 110 s$^{-1}$ represents the intrinsic rate constant of mant-ADP release. We attribute the second slow phase of mant-ADP release to be due to the population of Kar3Cik1 motors where Cik1 collided with the MT first, followed by Kar3 MT association, and then mant-ADP release.
The nucleotide-free MT were also determined using the natural substrate MgATP (Fig. 7). The nucleotide-free MT in the presence of MgADP was rapidly mixed in the stopped-flow instrument with increasing concentrations of MTs. Final concentrations are as follows: 1.25 μM Kar3Cik1 for 1.25–4 μM MTs, 2.5 μM Kar3Cik1 for 2.5–12.5 μM MTs, and 1 mM MgADP. A, representative transients at varying MT concentrations were each fit to a double exponential function with the observed rates reported in B. Relative amplitudes were obtained from the fit of the data for phase 1 and 2, respectively: 5 μM MT transient, 0.04 and 0.12; 6 μM MT transient, 0.05 and 0.15; 8 μM MT transient, 0.07 and 0.18. B, observed rates from the initial exponential phase (●) were plotted as a function of MT concentration, and the data were fit to Equation 3 to yield $k_{obs} = 4.9 \pm 0.1 \text{ s}^{-1}$. Inset, the observed rates of the second slow phase (●) were plotted as a function of MT concentration, and the fit of the data to Equation 1 provided $k_{max} = 5.0 \pm 0.8 \text{ s}^{-1}$; $K_{1/2, MT} = 6.6 \pm 2.1 \text{ μM}$.

because of the ADP and that apyrase treatment results in the nucleotide-free state of Kar3 that binds the MT. Subsequently, the nucleotide-free MT-Kar3Cik1 complex was rapidly mixed in the stopped-flow instrument with mant-ATP, and the transients reveal a fluorescence enhancement as mant-ATP enters the more hydrophobic environment of the active site (Fig. 6A). The observed rates of the initial exponential phase increased linearly as a function of mant-ATP concentration, and the fit of the data provided the second-order rate constant of 2.1 μM$^{-1}$ s$^{-1}$ with the y intercept predicting a dissociation rate of 16.6 s$^{-1}$.

ATP Binding by Pulse-Chase—The kinetics of ATP binding were also determined using the natural substrate MgATP (Fig. 7). The nucleotide-free MT-Kar3Cik1 complex was preformed and rapidly mixed in the rapid quench instrument with [$\alpha$-$\text{32P}$]ATP for times ranging from 5 to 500 ms and subsequently mixed with an excess of unlabeled MgATP. The high concentration of MgATP effectively dilutes [$\alpha$-$\text{32P}$]ATP free in solution or weakly bound at the active site. Therefore, this experimental design only detects the MT-Kar3Cik1-[\alpha-$\text{32P}$]ATP complex that proceeds through ATP hydrolysis.

The individual transients (Fig. 7A) were fit to Equation 5. The initial exponential phase of each transient represents the first ATP turnover, and the plot of the observed rates as a function of increasing ATP concentration is presented in Fig. 7B. The observation that the exponential rate of the first ATP turnover saturates at increasing ATP concentrations reveals that a rate-limiting ATP-promoted isomerization occurs that generates the MT-E$^*$-ATP intermediate, poised for ATP hydrolysis (scheme at top of Table 1). The observed exponential rates were plotted as a function of ATP concentration, and the fit of the data to Equation 6 provided the maximum rate constant of the ATP-promoted isomerization, $k_{\text{ATP}} = 69 \text{ s}^{-1}$ with the apparent $K_{d,\text{ATP}} = 8.3 \text{ μM}$ (Table 1). The amplitude of the initial exponential phase, $A_{np}$ represents the formation of product on the active site during the first ATP turnover and can be related to the concentration of active sites in the experiment. The hyperbolic fit of these data in Fig. 7C revealed a maximum burst amplitude of 0.94 per Kar3Cik1, indicative that every Kar3 head was bound to the MT and competent to bind and hydrolyze ATP during the first ATP turnover. ATP turnover in the absence of MTs is exceedingly slow (0.014 s$^{-1}$) (8, 19); therefore, Kar3 heads not bound to the MT would not be detected on the time scale of the pulse-chase experiments. The linear phase of the transients represents subsequent ATP turnovers, and the
The observed rates of the initial exponential phase were similar to the rates measured by steady-state experiments.

**ATP Hydrolysis by Acid Quench**—The presteady-state kinetics of ATP hydrolysis were pursued by rapidly mixing the preformed, nucleotide-free MT-Kar3Cik1 complex with varying concentrations of $[^{32}\text{P}]\text{ATP}$, followed by a formic acid quench to terminate the reaction and release nucleotide from the active sites. Representative transients fit to Equation 5 (Fig. 8A) reveal an initial exponential phase representing a burst of product formation on the active site during the first ATP turnover, followed by a linear phase representing product formation during subsequent ATP turnovers. Because at high ATP concentrations ATP binding no longer limits ATP hydrolysis, this experimental design measures the kinetics of ATP hydrolysis. The observed rates of the initial exponential burst phase were plotted as a function of increasing ATP concentrations, and the hyperbolic fit of the data provided the rate constant of ATP hydrolysis, $k_{\text{cat}} = 26 \text{ s}^{-1}$ with the apparent $K_{d,\text{ATP}} = 36 \mu\text{M}$ (Fig. 8B and Table 1). Fig. 8C shows the plot of the burst amplitude data as a function of increasing ATP concentrations, and the hyperbolic fit provided the maximum burst amplitude of 0.56 per Kar3Cik1 with the apparent $K_{d,\text{ATP}} = 9.8 \mu\text{M}$. The burst amplitude observed was significantly less than the full burst amplitude expected for the Kar3Cik1 concentrations used in the experiment. However, the steady-state $K_{m,\text{ATP}}$ at 26 $\mu\text{M}$ and the apparent $K_{d,\text{ATP}}$ constants determined by the pulse-chase experiments (Table 1) suggest that weak ATP binding cannot account for the reduced burst amplitude. Furthermore, the full burst amplitude in the pulse-chase kinetics provided evidence that Kar3Cik1 was fully active, indicating that there were not technical reasons to account for the decreased burst amplitude. We pursued analysis of the ATP binding and hydrolysis kinetics using KinTek Global Kinetic Explorer software (41). The results from this analysis were consistent with the hypothesis that ATP hydrolysis is a reversible step. The computational

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**FIGURE 6.** Mant-ATP binding to nucleotide-free MT-Kar3Cik1. The apyrase-treated, nucleotide-free MT-Kar3Cik1 complex was rapidly mixed in the stopped-flow instrument with increasing concentrations of MgMant-ATP. Final concentrations are as follows: 10 $\mu\text{M}$ Kar3Cik1, 25 $\mu\text{M}$ MTs, and 1.25–35 $\mu\text{M}$ MgMant-ATP. A, representative transients at 5, 10, and 20 $\mu\text{M}$ MgMant-ATP, and each was fit to a single exponential function followed by a linear phase. B, observed rates of the initial exponential phase from each individual transient were plotted as a function of Mant-ATP concentration. The data were fit to Equation 4, which provided the second-order rate constant for Mant-ATP binding, $k_{+1} = 2.1 \pm 0.06 \mu\text{M}^{-1} \text{s}^{-1}$ and $k_{\text{off}} = 16.6 \pm 1.0 \text{s}^{-1}$.

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**FIGURE 7.** Pulse-chase kinetics of ATP binding. The apyrase-treated, nucleotide-free MT-Kar3Cik1 complex was rapidly mixed in the chemical quench-flow instrument with increasing concentrations of $[^{32}\text{P}]\text{ATP}$ plus KCl and chased with unlabeled MgATP. Final concentrations are as follows: 10 $\mu\text{M}$ Kar3Cik1/25 $\mu\text{M}$ MTs for 5–200 $\mu\text{M}$ $[^{32}\text{P}]\text{MgATP}$, 5 $\mu\text{M}$ Kar3Cik1/25 $\mu\text{M}$ MTs for 5–80 $\mu\text{M}$ $[^{32}\text{P}]\text{MgATP}$ plus 100 mM KCl. A, representative transients show an exponential burst of $[^{32}\text{P}]\text{ADP}$ product formation followed by a linear phase of product formation. B, observed exponential rates of the presteady-state burst phase were plotted as a function of MgATP concentration and fit to Equation 6, which provided $K_k, k_{+1} = 8.4 \pm 0.02 \mu\text{M}^{-1} \text{s}^{-1}, K_k = 0.12 \pm 0.01 \mu\text{M}^{-1}$, and $k_{-1} = 69 \pm 1.4 \text{s}^{-1}$, and the apparent $K_{d,\text{ATP}} = 8.3 \mu\text{M}$. C, amplitude of each burst phase was normalized to the fraction of Kar3Cik1 active sites and plotted as a function of MgATP concentration. The data fit to a hyperbolic function provided the maximum burst amplitude at 0.94 $\pm 0.04$ per Kar3Cik1 heterodimer and apparent $K_{d,\text{ATP}} = 28.2 \pm 4.6 \mu\text{M}$. B and C include data from multiple experiments.
results and discussion will be presented in a future paper with the phosphate release kinetics as these steps are linked in the first ATP turnover.

Dissociation of the MT-Kar3Cik1 Complex—The preformed nucleotide-free MT-Kar3Cik1 complex was rapidly mixed with increasing concentrations of MgATP plus KCl. Final concentrations are as follows: 10 μM Kar3Cik1/25 μM MTs for 8–300 μM Mg[α-32P]ATP, 15 μM Kar3Cik1/30 μM MTs for 170–450 μM Mg[α-32P]ATP, plus 100 mM KCl. A, individual transients fit to Equation 5 show an exponential burst of [α-32P]ADP-Pi product formation followed by a linear phase of product formation. B, observed exponential rates of the presteady-state burst phase were plotted as a function of MgATP concentration and fit to a hyperbola, defining the rate constant of ATP hydrolysis $k_{\text{cat}} = 26 \pm 0.8 \text{s}^{-1}$ and apparent $K_{\text{cat}} = 35.9 \pm 4.6 \text{μM}$. C, amplitude of each burst phase was normalized to the fraction of Kar3 active sites and plotted as a function of MgATP concentration. The hyperbolic fit provides a maximum burst amplitude at 0.56 ± 0.02 per Kar3Cik1 heterodimer and the $K_{\text{cat}} = 9.8 \pm 2.5 \text{μM}$. B and C include data from multiple experiments.

motor head will detach. Fig. 9A shows representative transients with an initial phase of rapid increase in turbidity for the first ~100 ms, followed by a decrease in turbidity correlated with motor detachment from the MT. The initial turbidity increase is thought to be due to a mixing artifact from the MTs because the rate of this exponential rise was not correlated with increasing ATP concentrations. The observed rates of the exponential turbidity decrease, correlated with Kar3Cik1 detachment from MTs, were plotted as a function of increasing MgATP concentration, and the hyperbolic fit of the data provided $k_{\text{cat}} = 11.5 \text{s}^{-1}$ with $K_{\text{cat}} = 17.6 \text{μM}$. To test the hypothesis that ATP hydrolysis was required for Kar3Cik1 detachment from the MT, a common characteristic of kinesins (30, 39, 42–45),

FIGURE 8. Acid quench kinetics of ATP hydrolysis. The apyrase-treated, nucleotide-free MT-Kar3Cik1 complex was rapidly mixed in the rapid quench instrument with increasing concentrations of Mg[α-32P]ATP plus KCl. Final concentrations are as follows: 10 μM Kar3Cik1/25 μM MTs for 8–300 μM Mg[α-32P]ATP, 15 μM Kar3Cik1/30 μM MTs for 170–450 μM Mg[α-32P]ATP, plus 100 mM KCl. A, individual transients fit to Equation 5 show an exponential burst of [α-32P]ADP-Pi product formation followed by a linear phase of product formation. B, observed exponential rates of the presteady-state burst phase were plotted as a function of MgATP concentration and fit to a hyperbola, defining the rate constant of ATP hydrolysis $k_{\text{cat}} = 26 \pm 0.8 \text{s}^{-1}$ and apparent $K_{\text{cat}} = 35.9 \pm 4.6 \text{μM}$. C, amplitude of each burst phase was normalized to the fraction of Kar3 active sites and plotted as a function of MgATP concentration. The hyperbolic fit provides a maximum burst amplitude at 0.56 ± 0.02 per Kar3Cik1 heterodimer and the $K_{\text{cat}} = 9.8 \pm 2.5 \text{μM}$. B and C include data from multiple experiments.

FIGURE 9. ATP-promoted dissociation kinetics of the MT-Kar3Cik1 complex. The apyrase-treated, nucleotide-free MT-Kar3Cik1 complex was preformed and rapidly mixed in the stopped-flow instrument with increasing concentrations of MgATP plus KCl. A, representative transients at 12.5, 50, and 75 μM MgATP. B, observed exponential rates of the decreasing turbidity phase of each transient were plotted as a function of MgATP concentration and fit to a hyperbola, providing $k_{\text{cat}} = 11.5 \pm 0.3 \text{s}^{-1}$, $K_{1/2,\text{ATP}} = 17.6 \pm 2.5 \text{μM}$. C, comparison of the dissociation kinetics promoted by a slowly hydrolyzable ATP analog, MgATP-γS (green), or a nonhydrolyzable ATP analog, MgAMPPNP (orange), buffer (black), MgADP (blue), and MgATP (red).
the dissociation experiments were repeated using the slowly hydrolyzable ATP analog, ATPγS, the nonhydrolyzable analog AMPPNP, buffer, ADP, or ATP. The results in Fig. 9C indicate that only MgATP promoted the exponential decrease in turbidity expected for dissociation of the MT-Kar3Cik1 complex, consistent with the interpretation that Kar3Cik1 detachment from the MT occurs after ATP hydrolysis (ADP dissociation kinetics discussed below).

**Alternating Cycle of Cik1 MT Collision Followed by Kar3 MT Association**—Fig. 11 presents a model in which Cik1 collides with the MT, followed by Kar3 binding and rapid ADP release. The E3 intermediate that is formed has both heads bound to the MT; Kar3 is nucleotide-free, and strain is generated between the heads because of the 8 nm distance between the two MT-binding sites. Based on this model, ADP cannot promote Kar3Cik1 detachment (Fig. 9) because ADP would bind to the active site of Kar3 and generate the Kar3-ADP state that is weakly bound to the MT, yet Kar3Cik1 would remain tethered to the MT through Cik1 (E3 to E2 to E1 intermediate, Fig. 11). This result is in striking contrast to homodimeric Ncd and Eg5 where the ADP-promoted dissociation kinetics mimicked the ATP-promoted dissociation kinetics (30, 39). The ADP cosedimentation data in Fig. 3 were consistent with this interpretation because Kar3Cik1 exhibited saturation binding to the MTs with the stoichiometry of Kar3Cik1 binding to MTs at 1:2, i.e. one Kar3Cik1 per two tubulin subunits. The 1:2 stoichiometry suggests that the Kar3 head of the E1 intermediate is sufficiently close to the MT to prevent occupancy by Kar3Cik1 at every tubulin subunit.

We hypothesized that ATP binding would generate the ~75° rotation of the coiled-coil neck/stalk observed for Ncd (31–33) and act to pull the Cik1 head off of the MT (E4 intermediate, Fig. 11). This rotation would result in the Cik1 head being more distant from the MT, and we designed an experiment to test this hypothesis (Fig. 10). We repeated the MT-Kar3Cik1 cosedimentation experiments but this time in the presence of 1 mM MgAMPPNP to mimic the ATP state. The results showed that at these conditions 2 μM Kar3Cik1 exhibits saturation binding at 2 μM tubulin polymer, i.e. a 1:1 stoichiometry. These results indicate that every tubulin-binding site is now occupied by Kar3Cik1, which is only possible if the Cik1 head does not obscure the β-tubulin-binding site for Kar3. This 1:1 stoichiometry is consistent with results observed for the Kar3MD (46, 47) and Ncd in AMPPNP (31, 33). Another prediction of the proposed model is that the nucleotide-free state (E3 intermediate, Fig. 11) should also show a stoichiometry of 1:2 because both heads are bound to the MT. The data in Fig. 10, C and D, reveal 2 μM Kar3Cik1 exhibits saturation binding at 4 μM tubulin polymer, i.e. a 1:2 stoichiometry as observed for ADP (Fig. 3).

**DISCUSSION**

**Experimental Strategies**—Based on our earlier studies, we assumed that Cik1 would also be able to bind the MT independently of Kar3 as observed for the Vik1MHD (19). However, attempts to purify the carboxyl-terminal domain of Cik1 for direct experiments proved difficult because of protein insolubility during expression. We also recognized that to pursue a mechanistic analysis required that we be able to generate MT-Kar3Cik1 complexes that were in the same intermediate state at the start of the experiment. By preforming the MT-Kar3Cik1 complex in a high ADP environment, we reasoned that the Kar3-ADP head would partition off of the MT but be tethered to the MT by Cik1, and ADP could be removed.
Kinesin-14 Kar3Cik1 Microtubule Interactions

subsequently by apyrase treatment. These approaches enabled us to pursue the experiments reported here.

**Cik1 Binds the MT Independent of Kar3**—Multiple experiments provide evidence that Cik1 binds the MT lattice independently of Kar3. First, the equilibrium binding experiments at 1 mM MgADP (Fig. 3) and the nucleotide-free state (Fig. 10) show saturation binding of 2 μM Kar3Cik1 at 4 μM tubulin polymer, a stoichiometry of one Kar3Cik1 per two αβ-tubulin subunits. This stoichiometry suggests that both Kar3 and Cik1 require a MT-binding site and that in the ADP state (**E1 intermediate, Fig. 11**) the Kar3-ADP head may sterically prevent another Kar3Cik1 from binding. In contrast, the results for the 1 mM MgAMPPNP experiment (Fig. 10) reveal a stoichiometry that is now 1:1, i.e., one Kar3Cik1 per αβ-tubulin subunit. The AMPPNP results for Kar3Cik1 are consistent with the results obtained for Ncd (31–33), which indicated that the rotation of the coiled-coil neck/stalk by ~75° moved the partner head more distant from the MT.

Second, the MT association kinetics changed dramatically when performed in the presence of MgADP (Figs. 2C and 4) revealing a distinct second phase of MT association at 5 s⁻¹. Although the results indicated that the initial MT collision event was similar in the presence or absence of MgADP, the dramatic ADP-promoted second phase suggested that the initial collision event was through Cik1 followed by Kar3 MT binding. We reasoned that if this hypothesis were true, then the mant-ADP release kinetics should also reveal a slow phase of mant-ADP release at ~5 s⁻¹ associated with the Kar3-mant-ADP head binding the MT and release of mant-ADP to the solution. The mant-ADP release kinetics in Fig. 5 support this interpretation. Furthermore, this experiment places the 5-s⁻¹ rate-limiting step in the pathway, based on steady-state ATP turnover, after MT collision and prior to mant-ADP release because the intrinsic rate constant of mant-ADP release from the Kar3 head is exceedingly fast at 110 s⁻¹ (Fig. 5). Therefore, we propose that Cik1 head collision with the MT leads to a structural transition that promotes Kar3-ADP head binding followed by ADP release, thus forming an intermediate state with both heads bound to the MT with the Kar3 head positioned ahead of Cik1 toward the MT minus end by 8 nm (Fig. 11).

Third, the MT-Kar3Cik1 dissociation experiments also indicate that Cik1 binds to the MT independently of Kar3 (Fig. 9C). For homodimeric kinesins, MgADP drives the dissociation kinetics comparable with MgATP because the ADP intermediate is most weakly bound to the MT (25, 30, 37, 39, 42, 44, 45). In contrast, the transients in Fig. 9C show that whereas MgATP promotes Kar3Cik1 detachment from the MT, MgADP does not. In fact, the MgADP transient is comparable with the MgATPγS, MgAMPPNP, and buffer transients. This result is only possible if Cik1 were able to tether Kar3 to the MT. These data were interpreted as MgADP binding the Kar3 head leading to the Kar3-ADP state, which is weakly bound to the MT. This head detaches from the MT, but Kar3Cik1 remains bound to the MT through Cik1 (**E1 intermediate, Fig. 11**).

Therefore, although any one experiment may be subject to alternative interpretations, it is the cumulative results of all of these experiments that lead to the conclusion that the

**FIGURE 11. Model of Kar3Cik1 stepping.** The cycle begins as Cik1 collides with the MT to establish the MT minus end-directed orientation of Kar3Cik1 (**E1 intermediate**) with the Kar3-ADP head detached and pointed toward the minus end of the MT. MT binding by Cik1 triggers a conformational change that leads to the Kar3-ADP head binding to the next MT-binding site toward the MT minus end and initially as a weak binding state (**E2**). Association of Kar3-ADP results in ADP release leading to a state in which both Kar3 and Cik1 are bound to the MT and strained because the heads are now separated by 8 nm (**E3**). ATP binding initiates the rotation (bent arrow) of the coiled-coil neck/stalk, and this rotation pulls the Cik1 head from the MT (**E4**). Subsequent ATP hydrolysis followed by Pᵢ release results in Kar3Cik1 detachment from the MT (**E5**). Kar3Cik1 transitions to its solution conformation (**E0**) and can rebind the MT for another ATPase cycle. This model assumes that Kar3Cik1 is a nonprocessive kinesin as observed for kinesin-14 motors (49, 50).
carboxyl-terminal globular domain of Cik1 can bind the MT independently of Kar3.

**Alternating Cycle of Cik1-Kar3 MT Binding**—The model presented in Fig. 11 proposes that Cik1 collides with the MT and establishes the orientation of Kar3Cik1 on the MT with Cik1 bound to the MT and Kar3-ADP detached but pointed to the MT minus end as observed for Ncd (26, 31–33, 48). We propose that the Cik1 binding event signals a structural transition that results in the Kar3-ADP head binding to the MT followed by ADP release to form the E3 intermediate with intramolecular strain generated through the necks because Kar3 and Cik1 are now separated by 8 nm on the MT lattice. What is remarkable about these initial steps (Fig. 11, $E1–E3$) is that Cik1 dramatically slows the structural transition required for Kar3 MT association and ADP release. The MT association kinetics (Figs. 2 and 4) and mant-ADP release kinetics (Fig. 5) clearly show that intrinsically these are very rapid events.

Subsequently, ATP binds to the Kar3 head, generates the $\sim 75^\circ$ rotation observed for Ncd, and pulls Cik1 away from the MT. ATP hydrolysis occurs, followed by P$_i$ release, and Kar3Cik1 detachment from the MT. We propose that once in solution, Kar3Cik1 returns to the conformation that is poised to bind to the MT for another ATPase cycle. Note that Fig. 11 presents a model in which Kar3 and Cik1 bind to the same protofilament. However, based on the Kar3Vik1 structural studies from Allingham et al. (19), Kar3 and Cik1 may bind to side-by-side protofilaments, and experiments are planned to test this hypothesis.

As an anti-parallel MT-MT cross-linker of ipMTs in the overlap zone, this model assumes that multiple motors are involved and that Kar3Cik1 acts as a nonprocessive motor consistent with results for Ncd (49, 50). Although the amino-termini of Kar3Cik1 is not present in our expressed motor protein, we assume that this domain contains a second MT-binding site that is not regulated through the nucleotide state consistent with results for Ncd (50–53).

Kar3Cik in Comparison with Kar3Vik1 and Ncd—Earlier studies revealed that both Kar3Vik1 and Ncd exhibit cooperative binding to the MT lattice (19, 31). In contrast, however, Kar3Cik1 at low protein concentrations was targeted to the MT plus end by Cik1, and it was the MT plus end localization that was critical for ATP-promoted MT shortening (8, 19). The experiments here clearly show that Kar3Cik1 can saturate the MT-binding sites, indicating that Kar3Cik1 can bind the MT lattice as expected for its function to cross-link anti-parallel ipMTs. Cik1 also contains a pivot point glycine, Gly$^{376}$, in a region of high amino acid similarity to Vik1 Gly$^{374}$ and Ncd Gly$^{375}$. The pivot point glycine has been associated with the ATP-promoted rotation of the Ncd neck coiled-coil that is believed to be the force-producing conformational change that drives minus end-directed motility (31–33). Therefore, these similarities suggest that Kar3Cik1 will share mechanistic features in common with Kar3Vik1 and Ncd for its MT-MT cross-linking function.

The cryo-electron microscopy studies for MT-Ncd complexes suggested that only one head of the Ncd homodimer interacted with the MT at a time, i.e. the unbound head was not required to generate MT movement. These results were based on the failure to trap a two-head-bound state but also from motility experiments. Endres et al. (33) showed that Ncd heterodimers that lacked one head promoted MT gliding at a rate similar to the Ncd homodimer, and the steady-state ATPase was also similar. Kocik et al. (35) extended this analysis by generating Ncd heterodimers with one head fully active and the partner head carrying a point mutation in switch I or switch II rendering it inactive. Interestingly, these mutant heterodimers, which carried one wild type head, showed a dramatic reduction in the rate of MT gliding. These observations were in striking contrast to their one-headed Ncd heterodimer that exhibited wild type MT gliding as observed previously by Endres et al. (33). Therefore, the Kocik et al. (35) results indicate that both heads of the Ncd dimer are required for MT minus end-directed movement, and there is head-head communication. Additional support of the hypothesis that both Ncd heads interact with the MT during the ATPase cycle came from the mechanistic studies from Foster and Gilbert and Foster et al. (29, 30), where they showed that in pulse-chase kinetic studies comparable with the experiments shown in Fig. 7, two ATP molecules were hydrolyzed, one for each motor head, prior to Ncd detachment from the MT.

The results for Kar3Vik1 and Kar3Cik1 indicate that both Vik1 and Cik1 bind the MT, and the motility generated requires Kar3 and Vik1 or Kar3 and Cik1 for robust motility. At this time, we do not fully understand the differences in the three motors to understand force generation for parallel and anti-parallel MT-MT cross-linking. In addition, we do not know whether Kar3Cik1 uses a novel mechanism to promote MT shortening during karyogamy. These are questions to explore in future experiments. However, the results presented here clearly indicate that Kar3Cik1 interacts with the MT lattice through an alternating cycle of Cik1 MT collision followed by Kar3 MT binding with head-head communication between Kar3 and Cik1 modulated by Kar3 nucleotide state and intramolecular strain.

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