Diffusion and Directed Movement

IN VITRO MOTILE PROPERTIES OF FISSION YEAST KINESIN-14 Pkl1*8

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Fission yeast Pkl1 is a kinesin-14A family member that is known to be localized at the cellular spindle and is capable of hydrolyzing ATP. However, its motility has not been detected. Here, we show that Pkl1 is a slow, minus end-directed microtubule motor with a maximum velocity of 33 ± 9 nm/s. The $K_m$ value of steady-state ATPase activity of Pkl1 was as low as 6.4 ± 1.1 nM, which is 20–30 times smaller than that of kinesin-1 and another kinesin-14A family member, Ncd, indicating a high affinity of Pkl1 for microtubules. However, the duty ratio of 0.05 indicates that Pkl1 spends only a small fraction of the ATPase cycle strongly associated with a microtubule. By using total internal reflection fluorescence microscopy, we demonstrated that single molecules of Pkl1 were not highly processive but only exhibited biased one-dimensional diffusion along microtubules, whereas several molecules of Pkl1, probably fewer than 10 molecules, cooperatively moved along microtubules and substantially reduced the diffusive component in the movement. Our results suggest that Pkl1 molecules work in groups to move and generate forces in a cooperative manner for their mitotic functions.

The functioning of mitotic spindles involves the coordinated activities of diverse microtubule (MT)$^2$-based motor proteins. Kinesin-related proteins play crucial roles in such functions as spindle assembly, maintenance, and chromosome segregation. They exert forces on MTs to translocate a cargo or MTs themselves (i.e. cross-link and slide MTs). Most members of the kinesin family move toward the plus ends of MTs, whereas several members that belong to the kinesin-14A (Kar3/Ncd) family exhibit minus end-directed motility. The minus end-directed kinesins are believed to have two roles for spindle functioning. The first role is to generate counteracting forces against plus end-directed motors to maintain the spindle (1–4). The second role is to organize spindle MTs at MT minus ends and focus them into spindle poles (5–7). However, despite much progress in recent years on spindle functioning of eukaryotic cells, the precise interpretation of the functions of motors remains complicated and controversial.

To elucidate the detailed mechanisms of spindle dynamics, it is necessary to characterize the motile properties of these motor proteins at a molecular level and to construct a model explaining the robust and dynamic spindle system. However, during the division of multicellular eukaryotes, it is difficult to specify the roles of each component due to the existence of a large number of potential contributors. In contrast, in the unicellular fission yeast Schizosaccharomyces pombe, there are nine kinesin family members, and among them, only five members are thought to participate in the mitotic functions (8–11); this offers an ideal model system to study mitotic processes because of its minimal motor constitution and because it has a lot of similarities with the mitotic processes of higher eukaryotes.

In the fission yeast, there are two members of the kinesin-14A family, Pkl1 and Klp2, both of which have been shown to be inessential (9). However, they have to affect MT organization. They are required for the normal function of the spindle poles and spindle MTs, although neither their in vitro motility nor their exact mitotic functions have been elucidated (12, 13). Pkl1 is localized at the cell nucleus during interphase and at the spindle and spindle poles during mitosis (8). Genetic studies have shown that Pkl1 deletion rescues the mutant phenotype of cut7, a fission yeast plus end-directed kinesin-5 family member, and the overexpression of Pkl1 results in phenotypes similar to those observed in cut7 mutants (8, 9). These observations suggest that Pkl1 provides counteracting forces against Cut7 in the spindle. It has also been shown that Pkl1 is functionally related to $\gamma$-tubulin in the microtubule-organizing center (14), which has been thought to contribute to spindle bipolarity. Most recently, direct association of Pkl1 motor domain with $\gamma$-tubulin has been demonstrated (15). These studies suggest that Pkl1 affects the MT organization via direct interactions with both spindle pole and microtubule-organizing center complexes to regulate spindle bipolarity.

The motor domains of Pkl1 are similar in sequence to the Saccharomyces cerevisiae kinesin-14A motor Kar3 (16), which is a well studied minus end-directed motor and also contributes to mitotic processes (17, 18). As reported for Kar3 (19), Pkl1 is expected to be motile, but a previous study reported that motil-
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FIGURE 1. Pkl1 constructs used in this study. A, schematic representation of Pkl1 constructs. The probability of coiled-coil formation is plotted against the amino acid number using the COILS program (47) at the bottom. B, SDS-PAGE analysis of Pkl1 proteins performed on a 7.5% polyacrylamide gel (left) and a 10% polyacrylamide gel (right) that were stained with Coomassie Brilliant Blue R-250 after electrophoresis. Arrowheads, purified proteins; asterisks, tubulin that was added in the MT-affinity purification process (see “Experimental Procedures”). The proteins were >88% pure as assessed by densitometry of gels, except for GST-Pkl1N (60%), and SNAP-Pkl1F (6.4%) and AviTag-Pkl1F (2.2%), which contained major amounts of tubulin.

ity was not detected, although it had ATPase and MT binding activities (8).

In this study, we show that Pkl1 is in fact an MT minus end-directed motor protein. To obtain further insights into the motile properties of Pkl1, we focused on its processivity: an ability to perform several stepping cycles without detaching from a filament. Processive movements have been reported for kinesin-1 (20–23) and some other kinesin-related proteins, which move a long distance without dissociating from MTs. Nonprocessive movement has been described for several kinesin-related proteins, which work in small groups or in an array of motors like muscle myosin. Because little is known about the molecular mechanisms of kinesin-14A family members other than Ncd and Kar3, it is important to identify whether the motor domains of these members share a low duty ratio, nonprocessive motility. By using a combination of biochemical and single-molecule techniques, we demonstrated that Pkl1 is a low duty ratio motor that has features both similar to and distinct from those of Ncd. Moreover, we compared the motility of Pkl1 in single- and multiple-molecule assays, reporting potentially important properties for its mitotic functions.

EXPERIMENTAL PROCEDURES

Cloning—The DNA encoding the N-terminal part (amino acids 1–430) of S. pombe Pkl1 was amplified by PCR as two separate parts to remove the intron of S. pombe genomic DNA (53 nucleotides). The two sets of forward and reverse primers are as follows: 5’-GGGGGATCCATGTTAATTGAGAATACAAAAGGACATTTCCATA-3’ containing a BamHI site (underlined) and 5’-TATCTTGCTTGCCCTTTTC-3’; 5’-TTTATTGAAAAGGAGAATCCTTT-3’ and 5’-CCGAATCTGGCCATTATTTTCTTT-3’ containing an EcoRI site (underlined). These fragments were ligated to the BamHI/EcoRI sites of pUC119 (Takara bio). To construct GST-Pkl1N, the N-terminal fragment of pkl1 was ligated to the BamHI/EcoRI sites of pGEX-2T (GE Healthcare).

The DNA encoding full-length Pkl1 (Pkl1F) was constructed with the pUC119/pkl1H plasmid and the N-terminal fragment using the BamHI/MscI sites. SNAP-Pkl1F and AviTag-Pkl1F were constructed by ligating the BamHI/EcoRI fragment from the pUC119/pkl1F plasmid to a modified pET-32a vector (Novagen) containing an N-terminal SNAP tag (Covalys) or AviTag (Avidity). The linker amino acid sequence between SNAP tag and Pkl1 is GS, and that between AviTag and Pkl1 is PRDIGS.

To construct GFP-Pkl1H and AviTag-Pkl1H, the pkl1H fragment was digested with NruI/EcoRI and ligated into the Smal/MfeI sites of a modified pET-14b vector (Novagen) containing an N-terminal His6 tag followed by green fluorescent protein (GFP; F64L/S65T/A206K variant) or ligated into the NruI/EcoRI site of a modified pET-32a vector containing an N-terminal AviTag. The linker amino acid sequence between GFP and Pkl1 is GPE, and that between AviTag and Pkl1 is PREL. To construct Pkl1H, the pkl1H fragment was PCR-amplified from pUC119/pkl1H vector with the forward and reverse primers, 5’-GGGGGATCCATCGATATCATATCATATCATCACAACGCTGCAAAGGAGAATATCGAGTTG-3’, containing a BamHI site (underlined) and a His6 tag, and 5’-GGGGAATTCTTTAGTTTATAAATG-3’, containing an EcoRI site (underlined). The PCR products were ligated into the BamHI/EcoRI sites of pET-17b expression vector (Novagen).

To construct GFP-Pkl1S and Pkl1S, the forward and reverse PCR primers, 5’-GGGGGATCCATCGATATCATATCATATCATCACAACGCTGCAAAGGAGAATATCGAGTTG-3’ and 5’-GGGGGAATTCTTTAGTTTATAAATG-3’, containing Smal and EcoRI sites (underlined), were used. The pkl1S fragment was cloned into pET-14b (Novagen) or pET-14b/GFP vector, as described for Pkl1H. In the resultant GFP-Pkl1S construct, the linker amino acid sequence between GFP and Pkl1 is GPG. The sequences were verified by DNA sequencing.

Expression and Purification—Plasmids were transformed into BL21 Star (DE3) (Invitrogen) or BL21-CodonPlus (DE3) RIL (Stratagene) host cells for expression in bacteria. Cells were grown in LB medium supplemented with 50 µg/ml ampicillin. Expression was induced at A600 = 0.7 with 0.1 mM isopropyl-β-D-thiogalactopyranoside (expressed as final concentration).
Cells were grown for a further 4–6 h at 20 °C and then harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at −80 °C.

Proteins were purified as described previously (24), except that the gel filtration process was performed on an NAP-5 column (GE Healthcare) and that the final MT affinity purification step was modified for SNAP-Pkl1F and AviTag-Pkl1F due to the low recovery rate; proteins were cosedimented with MTs, and the precipitate was resuspended and placed on ice for use in further assays. GST-Pkl1N was purified by GST-bind resin (catalog number 70541; Novagen). SNAP-vitro 647 labeling of SNAP-Pkl1F was performed at a mixing ratio of 1:3 (Pkl1F head/SNAP-vitro 647) for 30 min at room temperature.

Preparation of Tubulin and Polarity-marked MTs—Tubulin was purified from porcine brain (25). Polarity-marked MTs labeled with X-rhodamine succinimidyl ester (catalog number C-1309; Invitrogen) were prepared according to Hyman et al. (26) and stabilized with 20 μM paclitaxel. The Cy5 (catalog number PA25001; GE Healthcare) or BODIPY-FL (catalog number D-6102; Invitrogen) labeling of tubulin were performed as described (24).

Sucrose Density Gradient Centrifugation—The sedimentation coefficient ($s_{20,w}$) of Pkl1 in assay buffer (12 mM PIPES-KOH, 2 mM MgCl$_2$, 1 mM EGTA, pH 6.8) supplemented with 1 mM dithiothreitol was determined by using 5–25 or 5–40% (w/v) sucrose gradient centrifugation, as previously described (27). Gel filtration chromatography was performed with the buffer described above, with the addition of 300 mM potassium acetate to prevent nonspecific adsorption (TSKgel G3000SWXL; Tosoh). Stokes radii were determined from a plot of elution volumes vs. the radii of standard proteins (28). Molecular weights were calculated according to Equation 1,

$$M = 6\pi N \eta_0 \times r_{Stokes} \times s_{20,w}(1 - \nu p)$$  
(Eq. 1)

where $\eta_0$ is the viscosity of the solvent (1.0 × 10$^{-2}$ g/cm/s$^2$), $N$ is Avogadro’s number, $\nu$ is the average partial specific volume of the sample (0.725 cm$^3$/g), and $p$ is the density of the solvent (1.0 g/cm$^3$) (29).

Steady-state ATPase Measurement—The steady-state ATPase activity of GFP-Pkl1 was assayed with the EnzChek phosphate assay kit (catalog number E6646; Invitrogen) in assay buffer containing 1 mM dithiothreitol, 0.7 mg/ml casein, and 1% (v/v) Tween 20 at 25 °C. MTs were sedimented through a 25% sucrose cushion (200,000 × g for 15 min) and resuspended with assay buffer supplemented with 20 μM paclitaxel to remove excess GTP. MTs were recentrifuged at 16,000 × g for 2 min to remove aggregates and used within 2 h. The kinetic parameters were determined by hyperbolic regression ($y = V_{max} \times [S]/(S + K_m)$, where $S$ is either ATP or MT concentration).

MT Gliding Assays—MT gliding assays were performed in assay buffer supplemented with ATP and 10 μM paclitaxel under a dark field microscope, as described previously (24). For assays using AviTag, the flow chamber was first coated with streptavidin (catalog number 192-11644; Wako) to immobilize biotinylated AviTag-fused proteins. The gliding velocity of MTs that moved in a continuous manner for at least 30 s was measured.

MT Bundling Assays—The samples were prespun at 16,000 × g for 10 min to remove any protein aggregates. MT bundling assays were performed in assay buffer with 0.3–3 μM MTs, 0.05–0.1 μM proteins, 10 μM paclitaxel, and 0–1 mM ATP. The mixture was rocked at room temperature for 30 min and then observed under a dark field microscope.

Single-molecule Motility Assays—The movements of individual GFP

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**TABLE 1**

**Oligomeric state of Pkl1 proteins**

| Construct | $r_{Stokes}$ (nm) | $s_{20,w}$ (M$^{-1}$ cm$^{2}$ s$^{-1}$) | Estimated Polypeptide | $M_r$ (g/mol) | $M_w$ (g/mol) | Ratio | Oligomeric state |
|-----------|------------------|--------------------------------------|----------------------|----------------|----------------|-------|-----------------|
| SNAP-Pkl1F | 8.7              | 7.6                                 | 273,379              | 117,141        | 2.3            | Dimer |
| Pkl1H     | 5.4              | 5.1                                 | 113,000              | 58,850         | 1.9            | Dimer |
| GFP-Pkl1H | 5.9              | 5.9                                 | 145,000              | 85,282         | 1.7            | Dimer |
| Pkl1S     | 2.9              | 3.9                                 | 46,500               | 46,567         | 1.0            | Monomer |
| GFP-Pkl1S | 4.0              | 4.4                                 | 72,500               | 73,652         | 1.0            | Monomer |

**FIGURE 2. Physical properties and fluorescence intensities of Pkl1 proteins.** A, fluorescence intensity distributions of GFP-Pkl1H (top) and GFP-Pkl1S (bottom) moving on MTs. The insets show example traces of photobleaching behavior. A, arbitrary units. B, fluorescence intensity distributions of kinesin-GFP (black bar) and GFP-Pkl1H (gray bar) measured simultaneously on MTs. C, fluorescence intensity distribution of SNAP-Pkl1F moving on MTs. The inset shows an example trace of photobleaching behavior.
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fusion proteins were observed with a total internal reflection fluorescence microscope as described previously (24) with the following modifications. Briefly, the chamber was coated twice with anti-α-tubulin monoclonal antibody (T-6199; Sigma) for 5 min and blocked with ~7 mg/ml dephosphorylated casein (catalog number C-8032; Sigma) for 5 min. Cy5-MTs or BODIPY-FL-MTs were then introduced and incubated for 5 min. After washing with assay buffer, the chamber was filled with assay buffer containing 100–500 pM Pkl1 protein, 10 μM paclitaxel, 0.7 mg/ml casein (catalog number C-8032; Sigma), 0.2 mg/ml casein (catalog number 2242; Merck), 0.1% (v/v) Tween 20, 0.2% (w/w) glucose, 1% (v/v) 2-mercaptoethanol, 85 units/ml glucose oxidase (catalog number G-2133; Sigma), 1,300 units/ml catalase (catalog number 106810; Roche Applied Science), and 5 mM ATP. After the movements were recorded, the polarity of the MTs was determined by observing the direction of the movement of kinesin-GFP or kinesin-Al exa647. Photobleaching behavior was analyzed by integrating intensities from 9 × 9 pixels and subtracting the background. The photobleaching rates of GFP-Pkl1H and SNAP-Pkl1F were 0.031 and 0.020 s⁻¹, respectively (Fig. S1). These values were slower than the detachment rate of GFP-Pkl1H and SNAP-Pkl1F (0.072–0.10 s⁻¹). The mean duration was determined by using nonlinear fitting of the cumulative probability distribution \(1 - \exp((-t_{0} - t)/\tau)\) (30). The fit parameters used were \(t_{0}\) and \(\tau\). The run length is defined as the distance between the appearance and disappearance of a spot on the MT. A minus symbol refers to the polarity of the MT. The instantaneous velocity was calculated from the pairwise distances for window size 2.0 s (31). MSD \(\rho(\tau)\) was plotted by averaging the squared displacement for nonoverlapping intervals \(\tau\) (32). The diffusion constant and the drift velocity were determined by fitting \(\rho(\tau)\) with \(\rho(\tau) = 2D\tau + \nu^2\tau^2\) (where \(D\) represents a diffusion coefficient and \(\nu\) is drift velocity) to the first six time intervals of the obtained MSD plots.

Multiple Molecule Motility Assays—The movements of quantum dot (Qdот) were observed with a total internal reflection fluorescence microscope as described above. Qdot-Pkl1H was prepared by mixing 0.8–8 μM AviTag-Pkl1H (dimer concentration) and 0.16 μM Qdot605-streptavidin conjugate (catalog number Q10101MP; Invitrogen) for 15 min at room temperature. The sample was then diluted with ~7 mg/ml casein (C-8032; Sigma) and placed on ice.

RESULTS

Oligomeric State of the Pkl1 Proteins—Pkl1 has a predicted coiled-coil region at amino acid residues 196–481, suggesting that the native form of Pkl1 is at least dimeric (Fig. 1A). To evaluate the oligomeric state of Pkl1, the physical properties of full-length Pkl1 (Pkl1F) fused with SNAP tag were analyzed by sucrose density gradient centrifugation and gel filtration analysis. The calculated molecular mass shows that SNAP-Pkl1F forms a dimer in solution (Table 1). Table 1 also shows that both Pkl1H, a HindIII fragment containing the coiled-coil region (8), and GFP-Pkl1H form dimers, and both Pkl1S and GFP-Pkl1S exist as monomers in solution, indicating that a monomeric variant of GFP does not affect the oligomeric state of Pkl1.

We also evaluated the oligomeric states of Pkl1 proteins by measuring the fluorescence intensities of fluorescent spots with a total internal reflection fluorescence microscope. We wished to express all constructs in a GFP-fused form, but full-length Pkl1 (Pkl1F) protein was degraded in a variety of host cells and expression conditions (not shown). Therefore, the GFP moiety of GFP-Pkl1F was replaced by SNAP tag that covalently binds a fluorescent substrate, which yielded stable protein capable of being imaged in a single-molecule experiment.

GFP-Pkl1H spots were photobleached generally in a one-step or a two-step photobleaching process, whereas GFP-Pkl1S spots were photobleached exclusively in a one-step process (Fig. 2A). The distribution of the fluorescence intensities supports that these spots of GFP-Pkl1H and GFP-Pkl1S correspond to single molecules of Pkl1 dimer and monomer, respectively. Another experiment clearly showed that we were observing single molecules of GFP-Pkl1H dimers; kinesin-GFP, well characterized rat kinesin-1 dimeric construct (amino acids 1–430) fused with GFP, and GFP-Pkl1H were mixed, and then their fluorescence intensities were measured simultaneously on MTs (Fig. 2B). The GFP spots can be readily classified according to their manner of moving along MTs. Two distributions appear to be essentially identical, demonstrating that GFP-Pkl1H is dimeric. The fluorescence intensities of SNAP-Pkl1F
spots were also measured (Fig. 2C). It should be noted that the labeling ratio of fluorophore to SNAP-Pkl1F was not very high (15–20%) in this particular case. Since this ratio directly affects the distribution of the fluorescence intensities, the distribution obtained here does not necessarily prove the argument that this protein is dimeric; the data only show that obvious aggregates were not present in our experiment. Collectively, we conclude that SNAP-Pkl1F and GFP-Pkl1H are dimeric, and GFP-Pkl1S is monomeric.

MT Gliding Driven by Pkl1—To detect the motility of Pkl1, we first performed an in vitro MT gliding assay (Fig. 3). Unlike in a previous study (8), surface-bound Pkl1H molecules supported MT gliding with a maximum average velocity of 33 ± 9 nm/s (mean ± S.D.) in the presence of 5 mM ATP (Fig. 4A and Table 2), demonstrating that Pkl1 is an MT motor protein. The MT gliding velocity of GFP-Pkl1H showed a hyperbolic dependence on ATP concentration with a maximum velocity of 42 ± 4 nm/s and half-maximal activation constant of 1,100 ± 240 μM (Fig. 4B).

Polarity-marked fluorescent MTs were translocated with the brightly marked minus end lagging, indicating that Pkl1 is a minus end-directed motor protein. As a control, the plus end-directed motor kinesin-1 (RK430-AviTag) was used in the same assay. In contrast to Pkl1, MTs moved with the brightly marked Pkl1 concentration, indicating that Pkl1 is nonprocessive. To test whether Pkl1 is processive, we estimated the duty ratio of the dimer construct GFP-Pkl1H. The duty ratio is defined as the fraction of the time that each motor spends in its strongly attached state. The ratio of the $K_{m,ATP}$ of the steady-state ATPase activity and the MT gliding velocity gives an estimation of the duty ratio (37). Fig. 4C and 4D, shows that the kinetic constant $K_{m,ATP}$ of ATPase activity of GFP-Pkl1H was 59 ± 12 μM (mean ± S.E. of the fitted parameter), and $K_{m,ATP}$ of gliding velocity was 1,100 ± 240 μM. The duty ratio was thus calculated to be 0.05, indicating that Pkl1 dimers spend only a small fraction (5%) of the ATPase cycle strongly associated with the MT. Because processive movement requires a duty ratio of at least 0.5 based on a hand-over-hand mechanism of the two-headed motor, we assume that Pkl1 is nonprocessive. This value is similar to those measured for nonprocessive motors: 0.05 for myosin II (38), 0.1 for Ncd (39), and 0.03 for NcK1n3 (40).

Single-molecule Motility Assays—To clarify whether Pkl1 is processive, the behavior of single molecules of SNAP- or GFP-fused Pkl1 was directly observed using a total internal reflection fluorescence microscope in assay buffer containing 5 mM ATP (Fig. 5). All constructs did not show smooth, unidirectional movement but exhibited one-dimensional diffusion along MTs.

MT gliding velocities of AviTag-Pkl1F and GFP-Pkl1S are summarized in Table 2.

MT Activation of Steady-state ATPase Activity—Fig. 4C shows that MTs stimulated the ATPase rate of GFP-Pkl1H to a maximum of 2.0 ± 0.1 s$^{-1}$ ($k_{cat}$). The $k_{cat}$ value was calculated assuming that the two heads equally hydrolyze ATP, and it is expressed per head. As expected from MT gliding velocity, the $k_{cat}$ value was smaller than that reported for kinesin-1 (~50 s$^{-1}$) (33, 34) but was similar to those of Ncd, Kar3, and Eg5. A notable feature of Pkl1 kinetics was $K_{m,ATP}$, the MT concentration required for half-saturation of the stimulation by MTs. The value was as low as 6.4 ± 1.1 nM, comparable to those reported for BimC and KIF1A (2.5 and 16 nM, respectively) (35, 36). The low $K_{m,ATP}$ value suggests that Pkl1 has a high association rate with MTs and/or kinetic processivity in which Pkl1 hydrolyzes multiple ATP molecules per encounter with an MT.

Duty Ratio—Our preliminary experiment showed that the landing rate of MTs to the Pkl1-coated surface dropped steeply upon lowering minus end leading (not shown). MT gliding velocities of AviTag-Pkl1F and GFP-Pkl1S are summarized in Table 2.

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TABLE 2
Summary of single-molecule motility assays on MTs
Instantaneous velocities were directly calculated from the raw data (not from fitting) and shown as means ± S.E.; values in parentheses are p values for the probability of being wrong in concluding that the mean is not zero (null hypothesis). The minus symbol refers to the polarity of the MTs. Diffusion coefficients and drift velocities were determined from MSD plots; values in parentheses are p values for null hypothesis. n is the number of runs that were scored for each construct from 5–10 different flow chambers. MT gliding velocities ± S.D. were determined from 30–80 MTs in the presence of 5 mM ATP.

| Construct       | Instantaneous velocity | Run length | Duration | Diffusion coefficient | Drift velocity | n       | MT gliding velocity |
|-----------------|------------------------|-----------|----------|-----------------------|----------------|---------|---------------------|
| SNAP-Pkl1F      | −2.2 ± 1.5 (0.1539)    | −69 ± 126 | 9.6 ± 0.1 | 5.5 ± 0.3 (0.9180)    | 26 ± 77         | 134     | 17 ± 2              |
| GFP-Pkl1H       | −5.8 ± 1.1 (<0.0001)   | −98 ± 37  | 13.7 ± 0.1| 0.34 ± 0.04 (0.0263)  | 45 ± 22         | 126     | 33 ± 9              |
| GFP-Pkl1S       | 0.7 ± 0.3 (0.0536)     | 13 ± 18   | 14.1 ± 0.7| 0.18 ± 0.01           | 10 ± 14         | 125     | 2.1 ± 0.8           |

MT gliding velocity of SNAP-Pkl1F is represented by that of AviTag-Pkl1F.

To quantify the bias of the movements toward the minus end of MTs, we calculated the instantaneous velocity from the pairwise distance (Fig. 5, D–F) (31). We found that GFP-Pkl1H dimers had a clear bias toward the minus end of the MTs in the presence of ATP (the p value for the hypothesis that the mean equals zero was <0.0001; Student’s t test), whereas it had no bias in the absence of ATP (p = 0.24). SNAP-Pkl1F and GFP-Pkl1S also showed diffusive movement along MTs in the presence of ATP. However, we cannot conclude that these velocities had a bias (p = 0.1539 and p = 0.0536 for SNAP-Pkl1F and GFP-Pkl1S, respectively), although they exhibited minus end-directed MT gliding (Table 2 and Fig. S2).

It is clear that the motor domains of Pkl1 are capable of introducing a bias in the diffusive movement along MTs, as shown by GFP-Pkl1H. The directional bias of GFP-Pkl1S may well be too small to emerge from bidirectional diffusion along MTs, whereas the failure to detect the directional bias of SNAP-Pkl1F is likely to be caused by the heterogeneity in the binding mode: one is by the motor domain, and the other is by the N-terminal tail domain. Unlike Ncd, the observed traces of SNAP-Pkl1F did not contain stationary molecules that remain attached to MTs in long periods. Instead, a large population of SNAP-Pkl1F molecules showed association-dissociation events without moving along MTs, and is apparent at near zero velocity in the instantaneous velocity histogram (Fig. 5D). This is consistent with the fact that the histograms were much better fitted by the sum of two Gaussians as compared with the single Gaussian. This may also explain the insufficient fitting of the
duration data to an exponential decay function (Fig. 5G, inset). The result suggests that the N-terminal tail domain of Pkl1 can weakly interact with MTs but cannot act as a stable MT binding domain. In fact, GST-Pkl1N, a construct containing only the N-terminal domain, did not show any MT bundling (not shown), in contrast to the N-terminal domains of Ncd (41).

Qdot Movement Driven by Pkl1—The single molecules of Pkl1 thus can only show diffusive movement along MTs. Given the smooth MT gliding of Pkl1, we expected that multiple molecules of Pkl1 can move cooperatively along MTs. To test this, we observed the movement of Qdot bound with multiple molecules of Pkl1. At a mixing ratio of 5:1 (Pkl1/Qdot), Qdot showed biased Brownian motion toward the minus end of MTs in a similar manner as single molecules of GFP-Pkl1H. In contrast, at a mixing ratio of 50:1, Qdot exhibited both processive and diffusive traces (Fig. 6A). Fig. 6, B and C, clearly shows that the Pkl1H-Qdot (50:1) moved farther along MTs and had a lower diffusion coefficient (Table 3). According to the manufacturer, 5–10 streptavidins are attached on the surface of Qdot, and therefore the number of Pkl1 molecules that transport the Qdot was estimated to be fewer than 10 molecules. These results demonstrate that several molecules of Pkl1, probably fewer than 10 molecules, can dramatically increase the smooth, processive runs along MTs.

DISCUSSION

A previous study revealed that Pkl1 is capable of binding MTs and hydrolyzing ATP but has not demonstrated any MT gliding with the addition of ATP (8). In this study, we revealed that Pkl1 is a slow MT-based motor protein, and movement is minus end-directed. Our study provides the first direct evidence of motility for spindle kinesins from fission yeast. The slow velocity of the Pkl1 is consistent with the velocity of cellular processes, such as spindle pole movements and chromosome movements.

Our preliminary work suggested that the motor domain of Pkl1 tends to adsorb on a glass surface, presumably leading to unsuccessful MT gliding. To circumvent this problem, we refined the in vitro MT gliding assay by optimizing the surface immobilization method. To immobilize Pkl1 onto a glass surface, we first generated a Pkl1 construct tagged with both GST and BCCP (biotinylated protein from Escherichia coli). This protein was immobilized via either anti-GST antibody or streptavidin. Both of these methods were successful in detecting the motility of Pkl1 (data not shown); however, these methods somehow yielded a low ratio of moving fraction of MTs. This problem was also present in the MT gliding of AviTag-Pkl1F (Fig. S2A). We finally found that GFP tag and anti-GFP antibody provided more stable MT gliding, thereby yielding reliable statistical data to calculate the duty ratio.

The direct observation of the individual molecules of Pkl1 showed that Pkl1 is only weakly processive; Pkl1H had bias toward the minus end of the MTs but is accompanied by one-dimensional diffusion. This type of processivity has been reported for both plus and minus end-directed kinesins, such as KIF1A (36), Eg5 (42, 43), Ncd (24), and CENP-E (44). Considering that the duty ratio of Pkl1 is quite low, we hypothesize that individual Pkl1 molecules are weakly tethered to MTs during its weak binding state in the ATPase cycle; during the weak binding state that dominates the ATPase cycle of Pkl1 (95%), single molecules of Pkl1 are subject to thermal forces and would exhibit diffusive movement along the MT if the strength of the interactions between Pkl1 and the MT is moderate enough to prevent its sticking to and dissociating from the MT. Although the exact motif that causes this tethering interaction is unknown, at least one such motif must exist in the motor domain. In fact, a monomeric construct containing almost only the motor domain (GFP-Pkl1S) showed a long duration on MTs with an average of 14.1 s (Fig. 5f and Table 2), which is longer than that of the dimeric Ncd construct measured under the same condition (24).

![FIGURE 6. Qdot movement driven by AviTag-Pkl1H](image)

**TABLE 3**

Summary of Qdot movement on MTs driven by Pkl1

| Pkl1/Qdot | Instantaneous velocity | Run length | Duration | Diffusion coefficient | Drift velocity |
|-----------|------------------------|------------|----------|-----------------------|---------------|
|           | mm/s                   | mm         | s        | $10^4$ mm$^2$/s       | mm/s          |
| 5:1       | $-3.1 \pm 0.4 (0.0001)$ | $-160 \pm 80$ | $59 \pm 1$ | $0.88 \pm 0.02$       | $28 \pm 16 (0.0330)$ | 120 |
| 50:1      | $-5.8 \pm 0.2 (0.0001)$ | $-740 \pm 90$ | $139 \pm 2$ | $0.13 \pm 0.01$       | $23 \pm 13 (0.0252)$ | 124 |
The concept of the duty ratio was originally based on a simple assumption that a motor is either attached to or detached from a filament. The low duty ratio of Pkl1 obtained herein was calculated from traditional assays but does not rely on the above assumption. The calculation relies only on the difference in time to wait for ATP binding between the process of ATP hydrolysis and that of mechanical force generation (37). Thus, the low duty ratio obtained by this method indicates that Pkl1 spends a large fraction of the ATPase cycle “not strongly” attached with an MT, which does not necessarily imply that Pkl1 should be completely detached from the MT.

The ratio of the \( k_{\text{on}} \) value in the ATPase assay, and the dissociation rate from MTs in the single-molecule motility assay gives an estimation of the average number of ATP molecules hydrolyzed per encounter with an MT. The ratio for Pkl1H was 2.0 s\(^{-1}\)/0.073 s\(^{-1}\) = 27 (i.e. 27 ATP molecules per MT encounter) (the dissociation rate 0.073 s\(^{-1}\) was given by the inverse of duration). Assuming that Pkl1 moves steadily toward the minus end of the MT with 8-nm steps and without any backward steps, the average run length will be 27 \times 8 \text{ nm} = 220 \text{ nm}. On the other hand, the average run length measured was 98 nm. The inconsistency should be attributed to the frequent interruption by diffusive steps, which introduces backward steps into the movement. This suggests that Pkl1 is weakly processive in the sense that Pkl1 hydrolyzes multiple ATP molecules per encounter with an MT; however, the movement contains a large number of diffusive steps that are driven by thermal fluctuation and therefore does not require ATP hydrolysis.

The diffusion coefficient of Pkl1F was much larger than those of Pkl1H and Pkl1S. We speculate that the weak, nonspecific interactions between the N-terminal domain of Pkl1F and an MT allow repeated rebinding to the MT, resulting in the larger diffusion coefficient. However, interactions between the N-terminal domain of Pkl1 and the MT should be rather weak compared with Ncd, which may make a difference in processivity we observed; full-length Ncd has been shown to move processively (24), whereas Pkl1 did not. On the other hand, the difference between double-headed Pkl1H and single-headed Pkl1S could be simply explained by the difference in velocity between Pkl1H and Pkl1S, assuming that two heads of Pkl1H equally contribute to the directional bias. However, the distribution of the instantaneous velocity of Pkl1H toward both the minus and plus ends of MTs was clearly larger than that of Pkl1S (Fig. 5, E and F). This raises the possibility that two-headed Pkl1H can jump farther toward both directions along MTs than can one-headed Pkl1S, because the two heads of Pkl1H allow effective rebinding to MTs without dissociating from MTs. Thus, we surmise that the diffusion coefficient is dependent on the number of MT interaction domain per molecule as well as the strength of the interaction between each domain and the MT.

We showed that several molecules of Pkl1 reduced the diffusion coefficient and drove smooth movement toward the minus end of MTs (Fig. 6). The result implies that each motor in groups acts as a tether to the MT and reduces the unproductive diffusion along the MT, as discussed previously (36, 45). In this mechanism, it will be advantageous for each motor to have a low duty ratio so as not to interrupt the movement of the whole system.

The tethering interaction of the Pkl1 motor domains has some implications for \textit{in vivo} functions. This property may allow Pkl1 to diffuse along MTs, thereby increasing the probability to interact with the other mitotic factors on spindle MTs. Furthermore, the diffusive nature of Pkl1 may increase local Pkl1 concentration on spindle MTs. Low duty ratio motors in the spindle may well be designed to generate and degenerate tension of spindle MTs and may therefore be better suited to rapidly affect the dynamics of the spindle MTs than processive motors. These motors are required to work in groups to generate forces in the spindle. Our results suggest that the motor domains of Pkl1 mediate sustained interactions with MTs, which may allow Pkl1 to form an array of motors on spindle MTs and thus are useful for generating forces in a cooperative manner for mitotic functions. Although the precise role of Pkl1 remains uncertain, the minus end-directed motility of Pkl1 reinforces the model that the kinesin-14 antagonizes the force generated by the plus end-directed kinesin of the kinesin-5 subfamily (Cut7) in regulating spindle assembly and maintenance. However, unlike Ncd (24, 41, 46), the tail domain of Pkl1 is unlikely to act as a second MT binding domain to cross-link two MTs. It is possible that other factors allow MT cross-linking by mediating association of the tail domain of Pkl1 with the MT. Alternatively, direct interactions with \( \gamma \)-tubulin may anchor the multiple molecules of Pkl1 to the minus ends of MTs and thus allow force generation to affect MT organization. Further studies are required to address these possibilities.
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