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Cloning and expression of kinesins from the thermophilic fungus *Thermomyces lanuginosus*

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Abstract

The motor domain regions of three novel members of the kinesin superfamily TLKIF1, TLKIFC, and TLBIMC were identified in a thermophilic fungus *Thermomyces lanuginosus*. Based on sequence similarity, they were classified as members of the known kinesin families Unc104/KIF1, KAR3, and BIMC. TLKIF1 was subsequently expressed in *Escherichia coli*. The expression level was high, and the protein was mostly soluble, easy to purify, and enzymatically active. TLKIF1 is a monomeric kinesin motor, which in a gliding motility assay displays a robust plus-directed microtubule movement up to 2 μm/s. The discovery of TLKIF1 also demonstrates that a family of kinesin motors not previously found in fungi may in fact be used in this group of organisms.

Keywords: intracellular motility; kinesin; microtubules; motor protein; thermomyces

Kinesins constitute a diverse superfamily of motor proteins essential for many cellular functions including organization and maintenance of mitotic and meiotic spindles and transport of vesicles and organelles (Barton & Goldstein, 1996; Hirokawa, 1998; Goldstein & Philp, 1999). Intense effort to characterize the cellular functions of kinesins has been complemented by substantial progress in deciphering the mechanism of movement (Vale & Fletterick, 1997). A common feature of all members of the kinesin superfamily is the presence of a mechanochemical motor domain (Vale & Fletterick, 1997), which is necessary for binding to microtubules, movement, and force generation fueled by hydrolysis of ATP. The atomic structure of the kinesin motor domain was recently determined by X-ray crystallography (Kull et al., 1996; Sablin et al., 1996), opening the way to mechanistic analysis of motility. Further progress in the structural and kinetic studies of this group of proteins is made difficult by the somewhat fragile nature of kinesin enzymes. Many kinesins have been difficult, if not impossible to express in active form in bacteria, which severely limits the amount of pure protein available for studies.

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Abbreviations: ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA; ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FPLC, fast protein liquid chromatography; 1PTG, isoprophyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

For many other classes of proteins, stability problems were overcome by identifying, cloning, and expressing counterparts from a thermophilic organism (Kiefer et al., 1998). Enzymes from thermophiles are often more stable, express better in bacterial systems, and serve as a robust scaffold for mutagenesis studies. Because this approach was unexplored for kinesins, we isolated representative kinesins from a thermophilic organism. Even though members of the kinesin superfamily are restricted to the eukaryotic kingdom, which inhabits ecological niches much more tame than Archea and Procaryota, there are, nevertheless, eukaryotes whose enzymatic machinery operates at substantially elevated temperatures. One example is the thermophilic fungus *Thermomyces lanuginosus*, which tolerates temperatures up to 60 °C, has a growth optimum of 50 °C, and will not grow below 30 °C (Deacon, 1997). *T. lanuginosus* was previously explored as a source of thermostable lipases (Berg et al., 1998), xylanases (Schlacher et al., 1996), and glucoamylases (Basaveswara Rao et al., 1981). It was also used as a source of ribosomal subunits for electron microscopic studies (Harauz & Flannigan, 1990). The demonstrated ability of *T. lanuginosus* to encode thermostable enzymes, and its relatively extreme thermophilicity (for a eukaryote) made *T. lanuginosus* an ideal candidate for a possible source of thermostable kinesins.

Results and discussion

Our initial polymerase chain reaction (PCR)-based screen discovered three novel kinesin-like proteins in *T. lanuginosus*, which we named TLKIF1, TLKIFC, and TLBIMC. Based on sequence homology in recovered regions of the motor domain (Fig. 1), these predicted proteins were most similar to motors from three different

A TLKIFC

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cggaagtgggaagacctacacgatgccttctgaagatggcatgattccacgagccgtaca
 G  S  G  K  T  Y  T  M  P  S  E  D  G  M  I  P  R  A  V  H

tcagatcttcgagacggccaagagtctcgaggagaaagggtggacgtatcgaattgaggg
 Q  I  F  E  T  A  K  S  L  E  E  K  G  W  T  Y  R  I  E  G

ggagtttgtcgaggtgtacaacgaaaacctcaatgacctcctcggtcgggcagacgatct
 E  F  V  E  V  Y  N  E  N  L  N  D  L  L  G  R  A  D  D  L

tgacaagaaaaagcacgagattcggcatgacatgcagagatgcaagaccaccatcaccga
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 R  N  R  S  V  A  A  T  K  A  N  E  R  S  S  R  S  H  S  V

gttcattctccgcctagttggtcacaacagcgtcacgggagagagcagcgagggcacatt
 F  I  L  R  L  V  G  H  N  S  V  T  G  E  S  S  E  G  T  L

gaattt
 N
```

TLBIMC

```
tggcacgggcaagacatacacgatgtctggcgacatgacggacaccttggggatcttgtc

 G  T  G  K  T  Y  T  M  S  G  D  M  T  D  T  L  G  I  L  S

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 D  N  A  G  I  I  P  R  V  L  Y  R  L  F  Q  K  L  E  D  T

cgaacataccgtcaagtgctcgttcattgagctatacaatgaggagctgcgggatcttct
 E  H  T  V  K  C  S  F  I  E  L  Y  N  E  E  L  R  D  L  L

agcgtacgacgagaaatcaaacctcaagatctacgacaatgagaacaagaagggccaagc
 A  Y  D  E  K  S  N  L  K  I  Y  D  N  E  N  K  K  G  Q  A

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 N  S  T  M  V  Q  G  M  E  E  T  Y  I  D  S  A  S  A  G  I

caaattgcttcagaagggcagtcatgcacgccaggtagccgcaacaaaatgcaatgacct
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gagctcgcgaagtcatactatttttcactatcacaacacaagtgaagcgaacaacggatac
 S  S  R  S  H  T  I  F  T  I  T  T  Q  V  K  R  T  T  D  T

tggcgaggagtacatctgccagggcaagctaaacttg
 G  E  E  Y  I  C  Q  G  K  L  N  L
```

B TLKIF1 sequence

```
atgtcgggcggtggaaatatcaaggtggtggtcgggtacgcccgttcaacgcccgagaa
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I  D  R  G  A  K  C  I  V  R  M  E  G  N  Q  T  I  L  T  P

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P  P  G  A  E  E  K  A  R  K  S  G  K  T  I  M  D  G  P  K

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A  F  A  F  D  R  S  Y  W  S  F  D  K  N  A  P  N  Y  A  R

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N  N  C  I  F  A  Y  G  Q  T  G  S  G  K  S  Y  S  M  M  G

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E  L  Q  K  D  K  N  L  T  C  T  V  E  V  S  Y  L  E  I  Y

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cacccgtcgaccggcccctacgtggaggacttggcgaagctggtcgtgcgatcattccaa
H  P  S  T  G  P  Y  V  E  D  L  A  K  L  V  V  R  S  F  Q

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E  I  E  N  L  M  D  E  G  N  K  A  R  T  V  A  A  T  N  M

aacgagacatccagtcgatcccacgccgtcttcactttgaccttgacgcaaaagtggcat
N  E  T  S  S  R  S  H  A  V  F  T  L  T  L  T  Q  K  W  H

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```
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P  G  R  H  D  R  T  L  S  K  A  G  S  D  A  D  G  D  S  R

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V  D  V  E  E  L  R  Q  Q  Q  A  Q  M  E  E  A  L  K  T  A

aagcaggaattcgatatc
K  Q  E  F  D  I
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Fig. 1. Sequences of *Thermomyces* kinesins. **A:** Sequence fragments of the motor domain regions of TLKIFC and TLBIMC obtained by degenerate PCR with a predicted protein translation. **B:** Sequence of the longest cloned fragment of TLKIF1.

classes of kinesins (Table 1). TLKIFC and TLBIMC were most similar to *Emericella nidulans* KLPA (O'Connell et al., 1993) and BimC (Enos & Morris, 1990), respectively. For TLKIF1, we were unable to obtain a full-length clone even after substantial effort. However, the partial sequence clearly indicated that TLKIF1 was a member of the UNC104/KIF1 family of kinesins. This finding was unexpected because no other member of this family had been discovered outside of the animal kingdom. The homology between TLKIF1 and its closest homologue, KIF1B from mouse (Nangaku et al., 1994), extended over 600aa, well beyond the canonical motor domain. A Blast (Altschul et al., 1997) homology search of the nonredundant protein database returned 11 protein sequences that share both the motor domain and the so-called "UNC104 box" (Ponting, 1995) homology. This group of proteins includes motors involved in axonal transport of vesicle precursors (UNC104/KIF1A) (Okada et al., 1995), transport of mitochondria (KIF1B) (Nangaku et al., 1994), cytokinesis (KLP38B) (Ohkura et al., 1997), undefined neural functions (KIN-73) (Li et al., 1997), and several others of yet unknown function. Even though the sequence of TLKIF1 is, overall, most similar to mouse KIF1B, a phylogenetic tree generated by ClustalW (Fig. 2) indicates that the fungal motor is so divergent that it does not belong to any particular group of UNC104/KIF1 motors (Fig. 2). Apart from the kinesins the only other proteins that have an UNC104 box homology region are the *Drosophila* protein Canoe and its homologues from human and rat, the ras-binding proteins AF-6 (Prasad et al., 1993) and afadin (Mandai et al., 1997), respectively. The function of this region is unknown.

Although TLKIF1 and other members of the UNC104/KIF1 family have two short predicted coiled coil regions, KIF1A and KIF1B, whose sequence is most similar to TLKIF1, were previously demonstrated to be monomeric (Nangaku et al., 1994). Consistent with that finding, bacterially expressed constructs of TLKIF1 also behaved as monomers in sucrose density gradient centrifugation (data not shown) and size exclusion chromatography (Table 2).

When expressed in bacteria, two different constructs of TLKIF1 yielded large quantities of soluble protein (Fig. 3). The proteins were active as demonstrated by a very robust plus directed in vitro gliding motility (Fig. 4). The shorter construct, TLKIF1-597, moved microtubules at 0.3 μm/s. The longer TLKIF1-784 demonstrated a dramatic increase in gliding velocity, moving microtubules with speeds up to 2 μm/s. These rates are similar to those seen in some other fast fungal motors such as kinesins from *Neurospora* (Steinberg & Schliwa, 1995) and *Syncephalastrum* (Grummt et al., 1998).

Despite the high motility velocities, the rate of ATP turnover by TLKIF1-784 was found to be relatively low, $\sim$8 s^{-1}. This observation suggests that the fast motility of TLKIF1 is not a result of more rapid stepping or ATP-hydrolysis by the motor. A reasonable explanation for the discrepancy between the turnover rate and the motility is that the TLKIF1 motor is not processive, and the rate observed in the motility assay is generated by a collection of molecules interacting with a microtubule in succession (Spudich, 1990). This interpretation is in agreement with the behavior of the TLKIF1 relative, UNC104, which can generate microtubule movement in a multiple molecule gliding assay, but in single molecule motility assays no movement of UNC104 was observed (Pierce & Vale, 1998). Both of these findings are in conflict with a recent report that some monomeric variants of the related KIF1A can move as single molecules (Okada & Hirokawa, 1999).

Evaluation of thermostability revealed that TLKIF1 expressed in bacteria remains stable at temperatures up to 45 °C (Fig. 5). This value is lower then expected because in vivo this motor is expected to function at temperatures up to 60 °C. This discrepancy may be a result of truncation and modification of the construct used in our

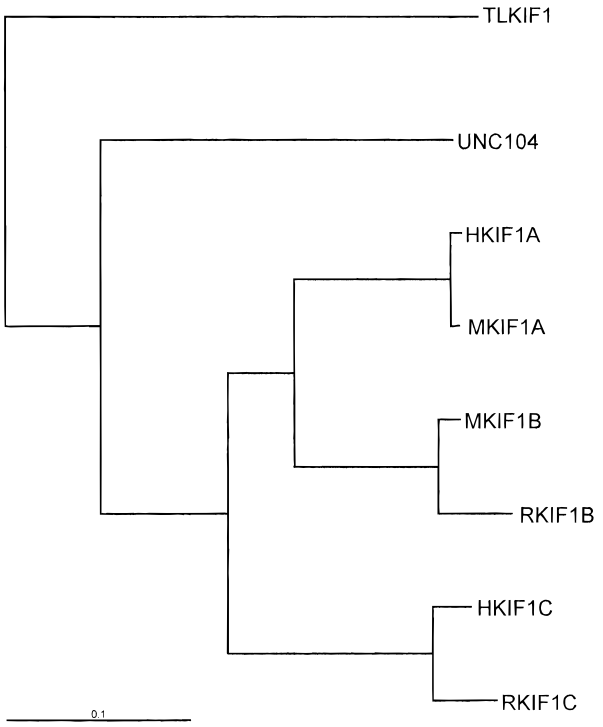

Fig. 2. Phylogeny tree for Kif1/Unc104 family members. Proteins were aligned within the longest common stretch of sequence, using ClustalW. The tree was displayed using TreeView. Scale bar corresponds to 0.1 amino acid substitutions per site.

Table 1. *T. lanuginosus kinesins*

Protein (length of homology[a])	Closest relative	Identity (%)	Similarity (%)
TLKIF1 (615)	Kif1B	52	69
TLKIFC (291)	KlpA	81	88
TLBIMC (155)	BimC	83	89

[a]Number of amino acids.

Table 2. *Size exclusion chromatography*

Protein	MW (kDa)	Elution volume
β-Amylase	200	38.4 ± 0.2
Alcohol dehydrogenase	150	43.2 ± 0.1
Bovine serum albumin	66	51.1 ± 0.4
Carbonic anhydrase	29	67.1
TLKIF1-597	68	45.8 ± 0.2
TLKIF1-784	89	50.4 ± 0.6

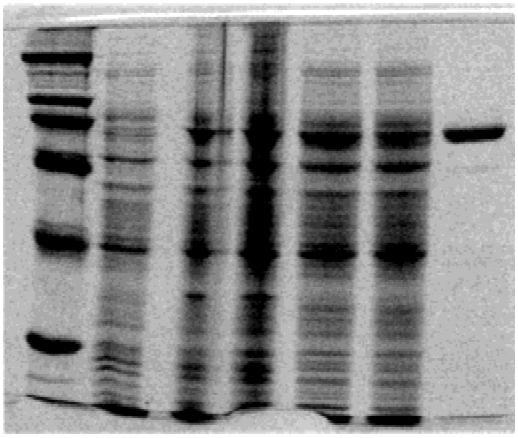

Fig. 3. Purification of TLKIF1-597. Samples collected during purification of TLKIF1-597 were collected and analyzed on SDS PAGE. Lanes represent, respectively, uninduced and IPTG-induced bacterial cells, cell lysate, cleared cell lysate, agarose-NTI column flowthrough, and 200 mM imidazole elution.

study. Alternatively, increased stability in the fungus may be caused by specific protein modification, associated proteins, or other processing occurring within the natural host.

Our discovery of TLKIF1 reveals the existence of a well-known family of kinesins in fungi for the first time. Because no true homologue of TLKIF1 is known in other organisms, we can only speculate about its possible role in fungi. In animals, several members of the UNC104/KIF1 family are involved in axonal transport of vesicles and organelles (Hirokawa, 1998). A similar role for a fast motor such as TLKIF1 in hyphal transport seems reasonable. Such a role could explain why motors of this family are absent from the first completely sequenced fungal genome of *Saccharomyces cerevisiae* (Cherry et al., 1997), which does not form long hyphae with cytoplasmic continuity and does not use microtubules for cytoplasmic transport (Jacobs et al., 1988). Further work will be required to understand the in vivo function of TLKIF1.

TLKIF1 will be a useful tool for the study of motor proteins. The very efficient and easy to reproduce in vitro motility of TLKIF1 was already employed successfully in a search for inhibitors of kinesin motility (Sakowicz et al., 1998). The cellular role of TLKIF1 will be better addressed in a more genetically amenable system. In this regard we expect that there will be homologues of TLKIF1 present in *E. nidulans*. Sequence information from our study should facilitate the cloning of the *E. nidulans* counterpart of TLKIF1, because the two other kinesin-like proteins (TLKIFC and TLBIMC) we identified in *T. lanuginosus* have more than 80% similarity to their *E. nidulans* relatives.

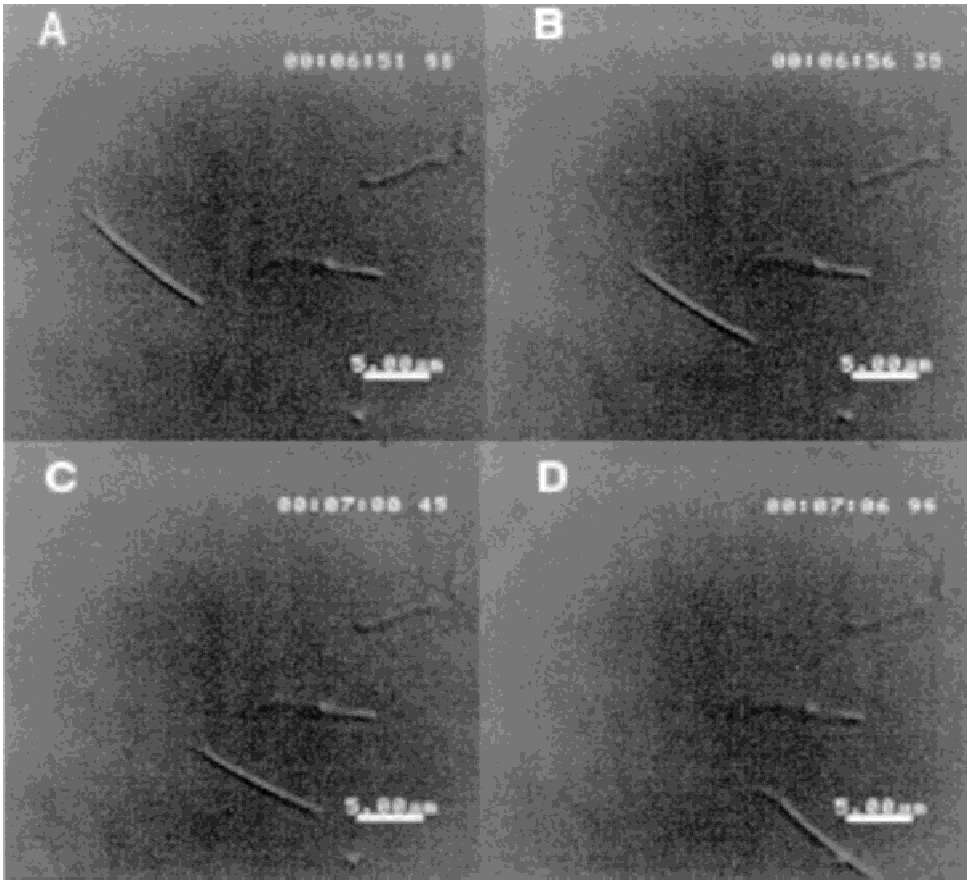

Fig. 4. In vitro motility assay of TLKIF1. Demembranated *Chlamydomonas* axonemes are shown gliding on a coverslip coated with TLKIF1-784. The forked (plus) end is trailing indicating that TLKIF1 is a plus end directed motor.

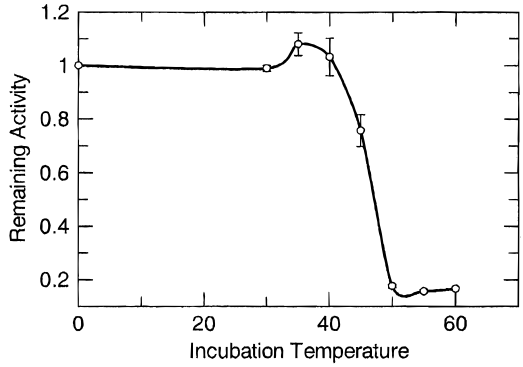

Fig. 5. Thermostability of TLKIF1. Aliquots of TLKIF1-597 were heated for 10 min at the indicated temperature, and then tested for remaining MT-stimulated ATPase activity. The activity of an unheated sample stored on ice was taken as 100%.

Materials and methods

Fungal growth

A culture of *T. lanuginosus* was purchased from the American Type Culture Collection (ATCC-22083). Fungus was initially grown on YsPs agar plates [4 g yeast extract, 1 g K_2HPO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 15 g soluble starch, 20 g agar per 1,000 mL water −25% tap/75% MiliQ:(Emerson, 1941)] for three days at 55 °C until plates were uniformly covered with fungal mycelium. Dices of those culture plates were subsequently used to inoculate four flasks with 500 mL YsPs liquid medium (same as YsPs agar but without agar) and grown for three days at 50 °C with agitation. Fungal mycelia were recovered by filtration on a Buchner funnel, and collected cakes were frozen in liquid nitrogen.

Cloning of T. lanuginosus kinesins

Total genomic DNA and total RNA were purified from the frozen mycelium of *T. lanuginosus* according to published procedures (Sambrook et al., 1989). Degenerate oligonucleotides homologous to highly conserved regions of the kinesin motor domain were used in PCR reactions to amplify members of the kinesin superfamily from total genomic DNA of *T. lanuginosus*. The forward primers used were: 5′-GCGCGGATCCAT(T/C)TT(T/C)GC(T/C/A)TA (T/C)GG(T/C/A/G)CA(A/G)AC, which corresponds to the amino acid sequence IFAYGQT. The reverse primer was: 5′-GCGCGAATTCTC(A/G/T)GA(A/G/C/T)CC(A/G/T)GC(A/C/G)A(G/A)(G/A)TC (A/G/C/T)AC, which corresponds to the amino acid sequence VDLAGS.

The PCR products were separated on an agarose gel and the band of appropriate size was excised, purified, digested with EcoRI and BamHI restriction enzymes, and subcloned into the pBS-SK vector. Vectors containing inserts encoding three different putative kinesin-like proteins (named TLKIF1, TLKIFC, and TLBIMC) were identified by sequencing. The presence of these sequences in the fungal genome was confirmed by Southern blotting.

A fragment encoding part of the motor domain of TLKIF1 was used to probe a *T. lanuginosus* genomic DNA library (Nazar et al., 1987). The DNA of an isolated phage carrying the TLKIF1 genomic sequence was digested with EcoRI to release the insert. The 3 kb insert was purified from an agarose gel and subcloned into EcoRI digested pBS-SK and sequenced. A set of primers deduced from the genomic sequence was used to amplify TLKIF1 from total fungal RNA by RT-PCR. The primers used were: 5′-GGAA TTCCATATGTCGGGCGGTGGAAATATC-3′ and 5′-GCGAAG CTTCTTCCGGATGGTTGAATC-3′.

The amplified fragment was gel purified and subcloned into NdeI-HindIII cut pET23b. After subcloning, the HindIII site was recut, blunted with mung bean nuclease and religated, putting the coding sequence in frame with the hexahistidine tag to give pET-TLKIF1-597. In addition, because the subcloned fragment of genomic DNA had only one intron at the 5′-end of the gene and an open reading frame untill the EcoRI site, TLKIF1-597 was extended by adding a SacI-EcoRI fragment of the original genomic isolate giving pET-TLKIF1-784.

Despite repeated efforts, we were unable to obtain a full-length clone of TLKIF1, neither by the phage library hybridization, nor by RT-PCR from the fungal RNA.

Protein purification

Escherichia coli BL21(DE3) carrying pET-TLKIF1 (597 or 784) were grown to OD600∼1 and induced with 0.5 mM IPTG for 3 h at 37 °C. Cells were harvested, resuspended in PEM80 buffer (80 mM PIPES pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA) with 1 mM DTT, 1 mM PMSF, and 0.1 mM ATP. Cells were lysed by three passages through a French Press. Cell membranes and insoluble protein were removed by ultracentrifugation (45 min, 35,000 rpm, 4 °C, TI1270). Supernatant was batch bound to Ni-NTA-Agarose (Qiagen, Hilden, Germany), loaded on a column, and washed extensively with lysis buffer and with lysis buffer plus 50 mM imidazole pH 6.8. Protein was eluted with lysis buffer plus 200 mM imidazole pH 6.8.

Oligomeric state determination

The apparent molecular weight of the bacterially expressed TLKIF1597 and TLKIF1784 proteins was determined by gel filtration and glycerol gradient centrifugation. For gel filtration a Sephacryl S-200 column (Pharmacia, Uppsala, Sweden) connected to an FPLC system (Pharmacia) was used. One hundred micrograms of protein was loaded in running buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.01% NP-40, 1 mM DTT, 0.1 mM MgATP) and run at 0.4 mL/min. The elution profile of TLKIF1 proteins was compared to the elution of a set of molecular weight standards consisting of beta-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

For glycerol gradient sedimentation protein samples with molecular weight standards were layered on top of 5 mL of 15–35% glycerol gradient and centrifuged in an AH-650 swinging bucket rotor (Sorvall, Newtown, Connecticut) at 40,000 rpm (14,9000 g), for 38 h at 4 °C. After centrifugation, twenty 270 μL fractions were collected from each tube, TCA precipitated, and their protein content analyzed by SDS-PAGE.

Motility assays

Inverted gliding motility assays were performed according to standard protocols (Cohn et al., 1993). A typical experiment was carried out on an uncleaned coverslip in a 10 μL volume in 80 mM

PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 10 μM taxol, 5–0.25 mM MgATP, 1 mg/mL TLKIF1 (597 or 784), and 1 μg/mL microtubules. For the directionality measurements, demembranated *Chlamydomonas* axonemes were used in place of microtubules.

ATPase measurements

The ATPase activity of kinesins was determined by a radioactive assay. MgATP used in the reaction mix was spiked with gamma^{32}PATP. Aliquots (1 μL) of the reaction mix were spotted on PEI-cellulose thin-layer chromatography plates. After drying, the plates were developed in 0.5 M sodium phosphate pH 3.5, and the amounts of ATP and Pi determined on a Phosphoimager (Molecular Devices, Sunnyvale, California).

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