Cloning and functional expression of a 'fast' fungal kinesin

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Abstract Conventional kinesins are molecular motors that move towards the plus end of microtubules. In animal species, they have been shown to be remarkably conserved in terms of both their primary sequence and several physiological properties, including their velocity of movement. Here we report the cloning of Synkin, a homologue of conventional kinesin from the zygomycete fungus *Syncephalastrum racemosum* [Steinberg, Eur. J. Cell Biol. 73 (1997) 124–131] that is 4–5 times faster than its animal counterparts. Expression in bacteria yields a fully functional motor that moves at the same speed as the native motor isolated from fungal hyphae and has similar hydrodynamic properties. Its sequence is most closely related to that of two other fungal kinesins from *Neurospora* and *Ustilago*, and shares several biochemical properties with the *Neurospora* motor. Fungal kinesins therefore seem to form a conserved subfamily of conventional kinesins distantly related to animal kinesins. They may help to identify sequence features important for determining motor velocity.

Key words: Kinesin; Fungus; Sequence analysis; Protein expression; Peptide antibody

1. Introduction

The kinesin superfamily of microtubule motor proteins is comprised of conventional kinesin heavy chains and kinesin-like proteins. These microtubule motors are involved in organelle transport, spindle formation and chromosome movement during cell division [1–4]. The members of the kinesin superfamily share a highly conserved motor domain of about 340 amino acid residues that contains binding sites for both ATP and microtubules. This domain is believed to generate the force for motility. Conventional kinesins from various animal sources transport organelles towards the plus end of microtubules. They consist of two heavy chains (KHC, ~120 kDa) and two light chains (KLC, ~60 kDa) [5,6]. The heavy chains, which are sufficient for force generation, possess three distinct domains [7]: the globular amino-terminal motor domain, a stalk domain that forms an α-helical coiled-coil, and a carboxy-terminal tail domain which interacts with the light chains. The latter are believed to be involved in organelle binding [1,8].

Recently, kinesins have been purified in our laboratory from two different fungal sources, the ascomycete *Neurospora crassa* (Nkin) and the zygomycete *Syncephalastrum racemosum* (Synkin). These two motor proteins share several interesting features [10]. First, they are unusually fast. Conventional kinesins from higher eukaryotes move microtubules at 0.5–0.7 μm/s in gliding assays whereas the two fungal kinesins from *N. crassa* and *S. racemosum* produce microtubule gliding at an approximately five times higher rate (around 2.7 μm/s). Second, despite their high rate of movement, they have a much lower affinity for ATP. Third, both lack light chains and are therefore homodimers. Despite the lack of light chains, they apparently are able to bind organelles and to transport them along microtubules.

To determine which regions might be important for the high velocity of the fungal kinesins and the binding to organelles we cloned the cDNA encoding Synkin and characterized the biophysical and motile properties of the expressed protein. Like Nkin [10] or Kin2 from *Ustilago maydis* [11] this motor is a distant relative of the conventional kinesins from animal species. Our data show that both fungal kinesins are closely related, suggesting a structural basis for some of the uncommon motile and physiological properties of these motors.

2. Materials and methods

2.1. Isolation of S. racemosum cDNA

S. racemosum was grown as described before [8]. The hyphae were ground in liquid nitrogen in a sterile mortar. The powder was used for isolation of total RNA with the RNeasy total RNA kit (Qiagen). The poly(A)$^+$ RNA was enriched with the Oligotex mRNA mini kit (Qiagen). cDNA synthesis was performed with the StrataScript RT-PCR kit (Stratagene) and poly(A) tailing with terminal transferase (Boehringer Mannheim). The tailed cDNA was purified using a PCR purification kit (Qiagen) before it was used as a template for PCR.

2.2. Cloning of Synkin

Three rounds of nested PCR with degenerate primers were used to amplify a fragment of the Synkin cDNA. The first PCR was performed with the primers SK1 and SK4, the second PCR with SK1 and SK3, the third PCR with SK3 and SK5 (SK1: 5′-GCG AAT TCG GI[A,T] [G,C][I,C]T A[C,T]G C[I,C]T T[C,T]G A[C,T]A A-3′, SK3: 5′-GCG GAT CC[G,T] [A,G]T T[C,T]T T[I,C]T C[C,T]T C[A,G]T GNA C-3′, SK4: 5′-GCG GAT CC[C,T] TC[I,C] A[A,G][C,T] TCI AT[A,G] TC[C,T] TT-3′, SK5: 5′-GCG AAT TCG TT[C,T] GCN TA[C,T] GGN CA[A,G] AC-3′). The deduced amino acid sequence of the amplified fragment was highly homologous to Nkin.

The complete cDNA of Synkin was cloned by rapid amplification of cDNA ends (RACE [12]). The 5′-RACE was performed with cDNA which was synthesized with a Synkin-specific primer (5′-GGA AGG TTC TCC GAC GGG-3′). After adding a poly(A) tail to the 5′-end of the cDNA, PCR was performed with 1 μM R_N (5′-AAG GCT CCG TCG GCA TCG ATC GCG CGA CTC TTT TTT TTT TTT TT[AGC]-3′), 0.08 μM R_O (5′-AAG GCT CCG TCG GCA TCG-3′) and 1 μM of the outer Synkin-specific primer (5′-GGA TTC AGA AGG TCT CTG A-3′). The annealing temperature in the first cycle was 45°C. The second PCR was carried out with the inner Synkin-specific primer (5′-CCT TGA CAG TAA ACT CGA G-3′) and R_I (5′-GCA TCG ATC GCG CGA CTC-3′).

The 3′-RACE was performed with oligo dT primed cDNA. The Synkin-specific primer for the first round of PCR had the sequence 5′-GGT AAA ACC TTC ACG ATG A-3′, the Synkin-specific primer for the nested PCR 5′-GGT GCC GAT ATC GAT GAC G-3′.

A full-length fragment for expression was amplified with the pri-

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mers 5′-GGT GGA TCC CAT ATG TCG GGC AAC AAT A-3′ and 5′-TCC CCC GGG CTC TAT CGT GAG TTC AT-3′. The product was digested with *Nde*I and *Sma*I and cloned in the bacterial expression vector pT7-7 (Tabor, 1990) which was digested with the same enzymes. Sequence ambiguities were clarified by sequencing of clones from independent PCR reactions. The sequences were analyzed with the programs from the University of Wisconsin Genetics Computer Group (UWGCG; Devereux et al., 1984).

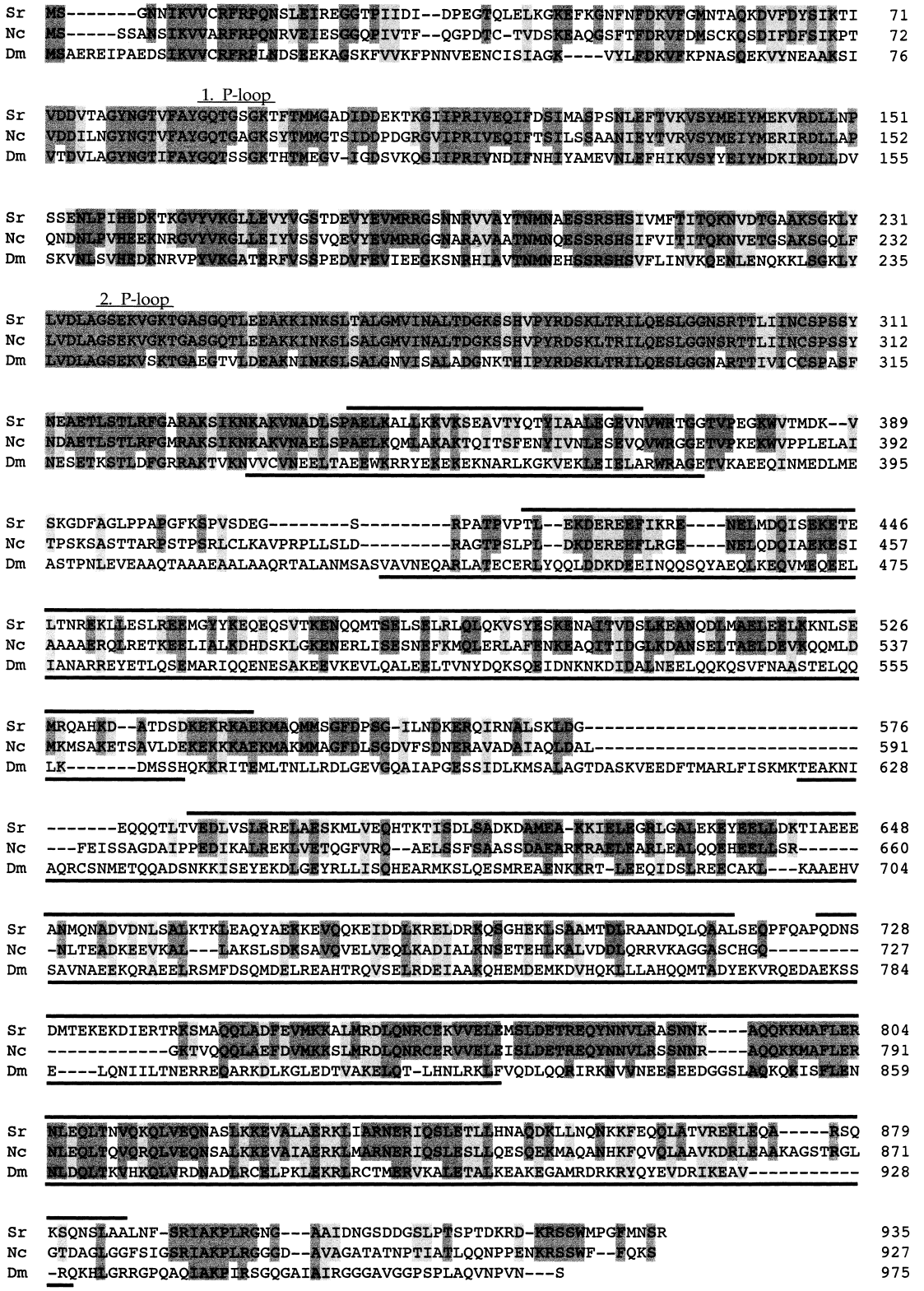

Fig. 1. Sequence comparison of Synkin, Nkin and DmKHC. The amino acid sequences of conventional kinesin from *S. racemosum* (Sr), *N. crassa* (Nc) and *Drosophila melanogaster* (Dm) are shown. Identical amino acids are marked in dark gray, conserved residues in light gray. The bars indicate regions with a high coiled-coil probability (>85%, window size 28 aa), those above the alignment for Synkin, those below for DmKHC.

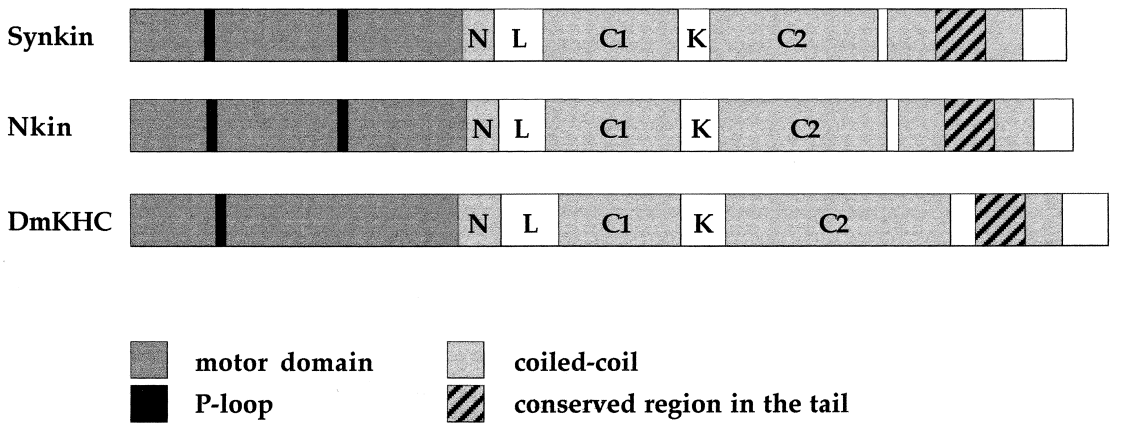

Fig. 2. Schematic comparison of the domain organization of Synkin, Nkin and DmKHC. The motor domain is followed by the stalk, which consists of several subdomains: neck (N), linker (L), coil1 (C1), kink (K) and coil2 (C2).

2.3. Preparation of microtubules

Tubulin was purified from pig brain using three cycles of polymerization and depolymerization according to the method of Shelanski et al. [13] with modifications previously described [14]. Microtubules were polymerized at a concentration of 1–15 mg/ml in the presence of 1 mM GTP and 10% dimethyl sulfoxide.

2.4. Bacterial expression and purification of Synkin

The vector Synkin-pT7 was transformed into *E. coli* strain BL21. 500 ml bacterial culture was induced overnight with 0.2 mM IPTG at 20°C. The bacteria were harvested (Sorvall GSA rotor; 6000 rpm; 10 min; 4°C) and resuspended in 12 ml of AP100 (100 mM PIPES, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) containing a cocktail of protease inhibitors (100 μM Pefabloc SC, 10 μg/ml tosyl-arginine-methyl ester, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin), 1 mM dithiothreitol and 10 μM ATP. After sonication (4×30 s, output 4, Branson Sonifier 250) the lysed cells were centrifuged (Beckman rotor 42.1; 42 000 rpm; 60 min; 4°C) and the supernatant (S2) was collected. After addition of 7 μM taxol, taxol-stabilized microtubules (0.3–0.5 mg/ml S2), 200 μM AMP-PNP and apyrase (5 U/ml S2), the mixture was incubated on ice for 15–60 min. The rest of the purification and the determination of the hydrodynamic behavior and of the gliding velocity were performed as described previously [10].

3. Results and discussion

3.1. Synkin sequence and structural organization

The purification of kinesin from the zygomycete *S. racemosum* by microtubule affinity resulted in two polypeptides with apparent molecular weights of 112 kDa and 115 kDa [8]. Tryptic fragments of this material [8] were microsequenced. The five peptide sequences TGASGQTLEEAK (1), EATGSYAFDK (2), ISEK (3), SGRALTFSRIAK (4) and IELEGRLGALEK (5) thus obtained showed homology to the sequence of Nkin. Degenerate primers encoding two of the peptides (2+5) and two conserved motifs of the kinesin motor domain (VHE[E,D]KNR and FAYGQT) were used to amplify a fragment of the Synkin cDNA. Using a PCR-based approach we then cloned the complete cDNA of Synkin. This cDNA is approximately 3400 bp in length and contains one long open reading frame of 2883 bp which starts at position 120 of the nucleotide sequence (accession number at EMBL nucleotide sequence database: AJ225894). The predicted protein is 935 amino acids long (Fig. 1), has a calculated molecular mass of 104 684 Da and a p*I* of 5.44. The amino acid sequence of Synkin is highly homologous to the sequence of Nkin. It displays 75% sequence identity to Nkin in the N-terminal motor domain (aa 1–336) and 40% and 63% identity in the stalk and tail (aa 741–935) domains, respectively. The overall organization of Synkin and Nkin is similar to that of other conventional kinesins (Fig. 2).

We cloned the cDNA of Synkin to look for regions which are conserved between Synkin and Nkin. Such regions could be responsible for the unconventional properties of these members of the family of conventional kinesins. The N-terminal motor domain contains all sequence motifs of kinesins (Fig. 1). Interestingly, both fungal kinesins possess a second P-loop motif in addition to the P-loop present in all kinesins. This second P-loop motif is the result of a single amino acid exchange in a region which is highly conserved among animal conventional kinesins. The underlined serine residue in the sequence VDLAGSEKVSKT is replaced in the fungal kinesins by a glycine residue. The VDLAGSE motif is located in the 11 [15,16] on the surface of the kinesin head. It is unlikely that this single motif can bind a second ATP molecule. However, the single amino acid exchange which leads to the formation of the second P-loop motif is in the context of a region starting at the VDLAGSE motif and spanning 123 aa (i.e. approximately the last third of the motor domain) which is highly conserved between Synkin and Nkin (97% identity). This region could represent a subdomain which is responsible for the elevated velocity of these fungal kinesins.

The stalk of the fungal kinesins shows a similar domain

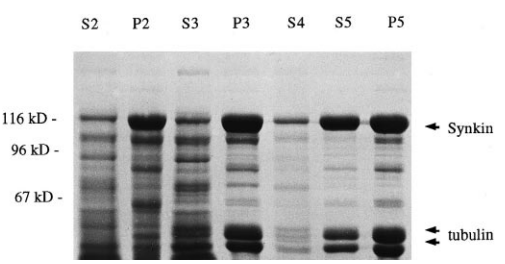

Fig. 3. Purification of bacterially expressed Synkin by microtubule affinity. Coomassie-stained 7.5% SDS-polyacrylamide gel. S2: bacterial extract (100 000×*g* supernatant); P2: pellet after the 100 000×*g* centrifugation; S3, P3: supernatant and pellet after incubation with taxol-stabilized microtubules, apyrase and AMP-PNP; S4: supernatant of a washing step; S5, P5: supernatant and pellet after the ATP elution step.

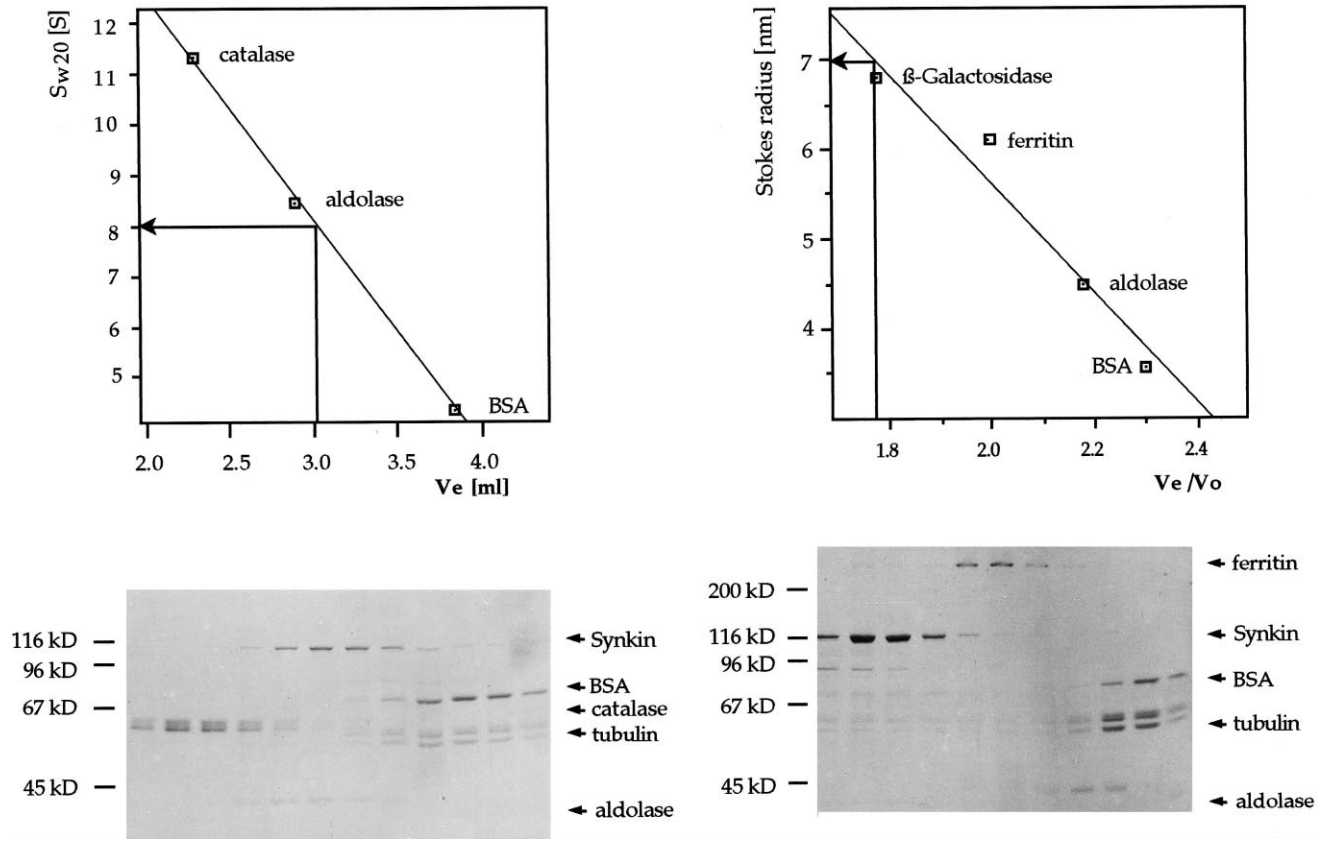

Fig. 4. Hydrodynamic behavior of bacterially expressed Synkin. Globular calibration proteins were separated on a 5–20% sucrose density gradient (n=2) or by gel filtration on a Superose-6B column (n = 3). The mean elution volume (n = 6) or the average Ve/Vo (n = 10) of bacterially expressed Synkin was used to determine its Stokes radius and sedimentation coefficient. The lower part of the figure shows experiments where Synkin was loaded together with calibration proteins on the density gradient or gel filtration column. The peaks of the calibration proteins lie directly below the corresponding points in the calibration curves shown in the upper part of the figure. Therefore, it is possible to determine the Stokes radius and S_{w20} directly from this figure. BSA, bovine serum albumin.

organization as DmKHC (see [17] for a review; Fig. 2). The motor domain is followed by a neck region (N, aa 342–370) which is predicted to form a coiled-coil [18]. The linker domain (L) corresponds to a break in the predicted coiled-coil (aa 371–419) and is believed to act as a flexible hinge. The bulk of the stalk consists of two regions which are predicted to form coiled-coils. These two regions, coil1 (C1, aa 420–545) and coil2 (C2, aa 584–716), are interrupted by the kink (K).

The beginning of the neck region is part of the highly conserved region described above. The predicted coiled-coil starts 10 aa later in Synkin and is 14 aa shorter than in animal kinesins. It is conceivable, therefore, that the transition between the head and the stalk has a different architecture in fungal kinesins, which could be important for their high velocity. A striking feature of the linker region of fungal kinesins is that it contains many proline residues (Synkin eight, Nkin nine). However, the possibility that this high proline content is responsible, at least in part, for the increased velocity of Synkin and Nkin has been ruled out (Grummt and Schliwa, unpublished results).

The degree of sequence homology in coil1, kink, and coil2 is generally low, but a stretch of approximately 60 amino acids in the middle of the stalk (aa 501–558 in Synkin) containing the anterior part of the kink region is highly conserved among fungal kinesins. It has been speculated that kinesin is inactive in a closed conformation where the tail binds near the motor domains [19,20]. This bent conformation can only be adopted if the kink region is highly flexible. Changing the elastic properties of the kink would be a way to regulate the activity of a kinesin. This could be achieved by binding of a

Table 1
Comparison of biophysical and motile properties of kinesins from different sources

	Synkin bacterial	Synkin native[a]	Nkin native[b]	Bovine brain kinesin[c]
Gliding velocity [μm/s]	2.1–3.6	2.1–3.4	2.1–3.8	0.6–0.8
Stokes radius [nm]	7.03 ± 0.17	7.04 ± 0.43	6.27 ± 0.53	9.64 ± 0.87
Sedimentation coefficient (S_{w20}) [S]	8.01 ± 1.23	8.4 ± 0.13	8.8 ± 0.15	9.56 ± 0.34
Calculated molecular weight [kDa]	~232	~240	~230	~380
Apparent molecular weight in SDS-polyacrylamide gel electrophoresis [kDa]	110	112/115	105/108	125+70

[a]Data from [8].
[b]Data from [9].
[c]Data from [5].

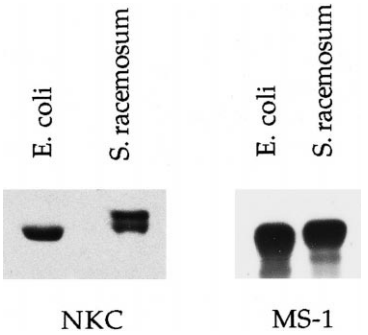

Fig. 5. Comparison of the immunological behavior of bacterially expressed and native Synkin. Immunoblots reacted with antibody NKC (left) against a conserved motif in the tail of fungal kinesins and antibody MS-1 (right) against a peptide from the Synkin linker region. Both antibodies recognize bacterially expressed Synkin. NKC reacts with both the larger and smaller form of native Synkin isolated from *S. racemosum*, whereas MS-1 recognizes only the smaller form of native Synkin. The difference in the electrophoretic mobility of the lower band between native and bacterially expressed Synkin (112 kDa and 110 kDa, respectively) may be due to post-translational modification of native Synkin.

protein near this region or by posttranslational modification. The conserved stretch of the fungal kinesins at this position could therefore be involved in the regulation of the activity of the fungal kinesins.

At amino acid 743 a region starts which is highly conserved both among fungal and animal kinesins (aa 795 in DmKHC). In accordance with [21] we define this position as the beginning of the tail domain. It includes a stretch of 60 amino acids containing a leucine zipper motif that is highly conserved (aa 794–848). A data library search has shown that this region is specific for conventional kinesins. The possible role of this region has been discussed [10].

3.2. Bacterial expression of Synkin

Bacterial expression of the coding region of the Synkin cDNA yielded high amounts of a soluble polypeptide with a relative mass of about 112 000. This polypeptide could be purified by a microtubule affinity step in the presence of apyrase and the non-hydrolyzable ATP analogue AMP-PNP (Fig. 3). The material released from microtubules by ATP (S5) consisted almost exclusively of the 112 000 polypeptide, with contaminations of tubulin. Thus this polypeptide exhibited the ATP-dependent microtubule binding and release behavior typical of microtubule motors.

The biophysical properties of bacterially expressed Synkin in comparison with native Synkin, Nkin and kinesins from animal sources are summarized in Table 1. The Stokes radius (Fig. 4) was determined by gel filtration on a Superose-6 column and yielded a value of 7.03 ± 0.17 nm (n=10). Five independent measurements of the sedimentation coefficient (Fig. 4) by density gradient centrifugation on a 5–20% sucrose gradient gave a value of 8.01 ± 1.23 S. Using the Svedberg equation, the native molecular weight was calculated to be 232 kDa. This fits well with the expected molecular weight of a Synkin dimer.

A microtubule gliding assay [10] was used to determine the motile properties of bacterially expressed Synkin. Both the high-speed supernatant (S2) of bacteria expressing Synkin and the ATP eluate from the microtubules (S5) supported fast microtubule gliding in vitro (2.6 ± 0.4 μm/s, range 2.1–3.6 μm/s; 11 independent isolations, at least six microtubules per isolation). Therefore, the bacterially expressed Synkin behaved very like Synkin purified from *S. racemosum* (Table 1) [8].

3.3. Immunological characterization

The identity of bacterially expressed Synkin was investigated in immunoblots using two polyclonal antibodies (Fig. 5). The antibody NKC was raised against a peptide that is part of a conserved motif in the tail (the MAFLERN motif, aa 781–804 in Nkin), which is found only in conventional kinesins. The antibody NKC is specific for fungal kinesins [8]. It recognizes both the smaller and larger isoforms of Synkin purified from *S. racemosum*. The antibody MS-1 is directed against a peptide in the linker domain of Synkin (aa 399–417) and is specific for the smaller form of Synkin only. The bacterially expressed Synkin is recognized by both antibodies. It is conceivable, therefore, that the higher (115 kDa) polypeptide represents another member of the kinesin superfamily which is highly homologous to Synkin in the tail domain, but not in the linker domain. Presumably, it is another member of the family of conventional kinesins, since it contains the conserved motif in the tail.

Two other lines of evidence also point to the existence of a second similar kinesin in *S. racemosum*. First, a Southern blot analysis with genomic DNA from *S. racemosum* was performed with several restriction enzymes. In all cases, we obtained more bands than expected if there existed only a single Synkin gene (results not shown). Second, two of the peptide sequences that were obtained by microsequencing were similar, but not identical to the Synkin sequence. The peptide **EATGSYAFDK** is similar to EFKGNFNFDK (aa 43–52, conserved amino acids are indicated by bold letters), **SGRALTFSRIAK** is homologous to SLAALNFSRIAK (aa 884–895). The three other peptides were identical to the sequence of Synkin reported here. Thus, it seems likely that the two divergent peptide sequences are derived from a different, yet closely related, polypeptide.

In animal species, two conventional kinesins have been described so far only for neuronal tissues, where a neuronal form of conventional kinesin has been found in addition to the ubiquitous form [22,23]. Possibly, only elongated cell types such as hyphae of syncytial fungi or neurons need more than one conventional kinesin for the transport of organelles over great distances.

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