

A kinesin-like mechanoenzyme from the zygomycete *Syncephalastrum racemosum* shares biochemical similarities with conventional kinesin from *Neurospora crassa*

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The kinesin superfamily consists of mechanoenzymes that convert chemical energy, stored in nucleoside triphosphates, into movement along microtubules. The founding member of this protein superfamily, the so-called conventional kinesin, was only known from animal sources until the recent description of kinesin from the filamentous fungus *Neurospora crassa* (G. Steinberg, M. Schliwa, *Mol. Biol. Cell* 6, 1605–1618 (1995)). To determine whether similar motors with comparable features are common in other filamentous fungi, a kinesin from a zygomycete, *Syncephalastrum racemosum*, was purified. Here, the isolation and characterization of this motorensyme is described. The purified protein consisted of a doublet at 112 kDa and 115 kDa with no additional polypeptides. This was consistent with a calculated molecular mass of ~240 kDa and suggests that the motor is a dimer with a more globular shape than conventional kinesin from animal sources. In gliding assays the enzyme moved microtubules at 2.5 to 3.4 $\mu\text{m/s}$ and had a nucleotide specificity similar to the *Neurospora* kinesin motor. Peptide antibodies against conserved regions in the head and the tail domain of conventional kinesins cross-reacted with the *Syncephalastrum* motor. In vitro, the enzyme was able to drive the microtubule-dependent movement of vesicles isolated from *Syncephalastrum racemosum*, as well as *Neurospora crassa*, and *Aspergillus nidulans*. In summary, the *Syncephalastrum* motor has many of the unique features in common with the conventional kinesin from the ascomycete *Neurospora crassa* and probably shares a similar function in living hyphae.

Abbreviations. AMPPNP Adenosine-5'-[α,β -imido]-triphosphate. – DIC Differential enhanced contrast. – MTs Microtubules. – Nkin *Neurospora* kinesin. – PBS Phosphate-buffered saline. – Synkin *Syncephalastrum* kinesin.

Introduction

Organelle transport is an important feature of all eukaryotic cells and has fascinated scientists for more than 200 years (reviewed in [40]). In particular, in elongated axons the molecular basis of intracellular movement of vesicles and mitochondria has been intensively studied (reviewed in [11]). With the purification of the microtubule (MT)-dependent mechanoenzyme kinesin from neuronal tissue [10, 50] and sea urchin eggs [41], a potential motor for plus-end directed movements was discovered. Numerous studies confirmed a major role for kinesin in organelle transport processes in several animal cell types (reviewed in [8, 42]), presumably in combination with minus-end directed cytoplasmic dynein which was first found in neurons [36] and was subsequently described for other cell types (reviewed in [24]). In addition, recent studies indicate a more complex situation in the axon, as it is now commonly accepted that cytoplasmic myosins [6, 13, 29] and several kinesin-like motors (e.g. [2, 17, 38], reviewed in [8, 23]) may also take part in the transport of membranous organelles.

Intracellular movement of organelles in fungi is found in single cells like yeast [26], but the process is more prominent and obvious in the elongated cells of filamentous fungi (reviewed in [20, 34]). The molecular basis of this process is still not completely understood. Inhibitor experiments and electron microscopy studies implicate MTs [1, 18, 21, 46], as well as actin filaments [22, 27] as the underlying tracks for organelle transport (reviewed in [19, 20]). Recent genetic results gave insight into the molecular motors that may be involved in intracellular transport in fungi. They established an important function for cytoplasmic dynein in nuclear migration in the ascomycetes *Aspergillus nidulans* [52], *Neurospora crassa* [39], and *Saccharomyces cerevisiae* [12]. Moreover, it turns out that an actomyosin system might be responsible for the movement of mitochondria in yeast [31, 45]. Interestingly, mitochondria and vesicle movements in *Neurospora crassa* are found to depend only on MTs [46], and the Neuro-

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spora kinesin is a good candidate for a mechanoenzyme that may take part in organelle motility [47]. This motor enzyme from *N. crassa* displayed several unique features, which were not found for animal kinesins. These include the absence of light chains, a far more globular shape, unusually fast in vitro motility, and the requirement of ATP for the stabilization of the gliding activity. This study was undertaken to address the question whether comparable kinesins are present in other filamentous fungi, and, if so, whether these motors share some of the unusual features with Nkin (*Neurospora* kinesin). The filamentous fungus *Syncephalastrum racemosum* was chosen as a typical representative of the ancient class of zygomycetes which are believed to be an ancestor group for asco- and basidiomycetes [3]. Here, I describe the purification and characterization of a putative mechanoenzyme from this species. MT-motility experiments, along with biochemical and the immunological data, suggest that this motor represents a "conventional" kinesin, with remarkable similarities to the *Neurospora* motor. Probably kinesins of this type exist in all filamentous fungi, and they might be responsible for at least some of the MT-dependent organelle transport processes in hyphae.

Materials and methods

Chemicals

Unless otherwise noted, reagents and enzymes were obtained from Sigma (Deisenhofen/Germany), salts were supplied by Merck (Darmstadt/Germany), and growth media ingredients by Difco (Augsburg/Germany).

Cells and culture conditions

Syncephalastrum racemosum, strain CBS 349.35 (provided by Dr. R. Agerer, Munich/Germany) was originally obtained from the Centraalbureau voor Schimmelcultures, Baarn/The Netherlands. Cultures were stored on nutrient agar plates containing SM-medium (16 mM KH_2PO_4 , 6 mM K_2HPO_4 , 4 mM MgSO_4 , 1% glucose, 2% bacto-agar, 1% peptone, 1% yeast extract). For preparation of conidia solutions, agar-plate cultures were grown for 7 to 10 days at room temperature under dim light. 5 to 10 ml of sterile water was placed on top of the mycelium, and the conidia were harvested with a sterile microscope slide. The number of conidia in solution was counted in a Neubauer chamber (usually $\sim 10^7$ conidia/ml), then 50 $\mu\text{g}/\text{ml}$ kanamycin was added, and the stock was stored at 4°C for further use within the following two weeks. For microscopic analysis and purification of kinesin 1000 ml liquid medium (3.5 mM Na_2HPO_4 , 3.6 mM KH_2PO_4 , 50 mM maltose, 1.4% peptone, 0.7% yeast extract) was inoculated with 9×10^6 conidia, and cells were grown under continuous illumination and gentle shaking (80–150 rpm) for 40 h at 21°C. Under these conditions the average yield from a 1000 ml liquid culture was 15 g hyphae.

Staining of the cell wall

Syncephalastrum conidia were grown in culture medium on poly-L-lysine-coated coverslips overnight in a humid chamber. After rinsing the coverslip with phosphate-buffered saline (PBS: 8 mM Na_2HPO_4 , 14.5 mM KH_2PO_4 , pH 7.2, 0.5 mM MgCl_2 , 2.7 mM KCl, 137 mM NaCl) the sample was fixed with 3% (v/v) formaldehyde, washed several times with an excess of fresh buffer. The cell wall was stained with 5 $\mu\text{g}/\text{ml}$ calcofluor in PBS for 5 min, followed by 3 rinses with PBS.

Analysis of organelle motility in vivo

Twelve to twenty hours old *Syncephalastrum* cells were taken from liquid cultures, placed in a flow-through chamber (see above) and analyzed in PBS using video-enhanced DIC microscopy. Thin parts of the branched hyphae with active organelle transport were selected and,

after flushing in 200 to 400 μg PBS, movements of highly refractile vesicles were monitored. The number of organelles crossing lines in hyphae was counted and used as the 100% reference value. This was followed by replacing the PBS with 500 μl of 10 μM nocodazole, benomyl (Dupon, Muenster/Germany) or cytochalasin D in PBS, and after 30 min and additional replacement of the solution organelle movements were measured. This experimental procedure, as well as dimethyl sulfoxide (DMSO; Fluka, Neu-Ulm/Germany) alone, had no influence on organelle transport as tested by control experiments.

Preparation of MTs

Tubulin was purified from fresh pig brain using three cycles of polymerization and depolymerization according to the method of Shelanski et al. [44] with modifications as previously described [47]. MTs were polymerized from pig brain tubulin at a concentration of 2 to 6 mg/ml in the presence of 10% DMSO and 1 mM GTP for 30 min at 32°C. The MTs were diluted to 0.5 to 0.7 mg/ml in 100 mM PIPES, pH 6.9, 2 mM MgCl_2 , 1 mM ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 1 mM EDTA, containing 7 μM taxol, briefly vortexed, and stored at room temperature for 1 to 4 days.

Isolation of kinesin from *S. racemosum* and determination of biophysical properties

S. racemosum cells were grown for 40 h as described above and ground with quartz-sand in the presence of the buffer AP100 (100 mM PIPES, pH 6.9, 2 mM MgCl_2 , 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM, phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ tosyl-L-arginine methyl ester, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 $\mu\text{g}/\text{ml}$ leupeptin) and the kinesin was isolated according to a previously described protocol [48]. The resulting supernatant after ATP release of the motor molecules from the taxol-stabilized pig brain microtubules (S5) was tested in gliding assays. The pellet was resuspended in half of the S5 volume, centrifuged, and the resulting S6 was pooled with the S5. The motor containing S5/S6, which was usually 230 μl from 15 g hyphae (1000 ml liquid culture), was stored on ice and stayed active for several days. Additional purification, determination of the Stokes radius and sedimentation coefficient and the calculation of the molecular weight and axial ratio was done according to previously described procedures [48].

Gel electrophoresis and ATPase assay

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Laemmli [30] with modifications according to Garfin [14]. Gels were stained with Coomassie Blue or silver nitrate according to Blum et al. [9]. The ATPase activity was measured following the procedure of Itaya and Ui [25] with modifications previously described [48].

Antibody preparation and Western blot analysis

Corresponding to a conserved region in the tail sequence of the *Neurospora* kinesin [47] a 17 amino acid peptide (FLERNLEQLTQVQRQLV) was synthesized by Eurogentec (Seraing/Belgium). The peptide was coupled to hemocyanin and two female New Zealand white rabbits were immunized with this construct (Eurogentec, Seraing/Belgium). The antibody, named NKC, was purified according to Olmsted [35], and subsequent Western blots were done as previously described [47].

Motility assays

MT gliding activity was measured as described in Steinberg and Schliwa [47]. To guarantee that the observations were comparable with those made for the *Neurospora* kinesin, care was taken to comply with all criteria previously described for this assay [47]. As the kinesin from *Syncephalastrum* was unstable after gel filtration, most measurements were done using a flow-through chamber or density gradient material. This had no influence on the motility behavior of the motor enzymes. *Syncephalastrum* kinesin showed a high sensitivity to the absence of ATP. Therefore, the motor was stabilized with 0.05 mM ATP, and the

influence of this nucleotide on motility assays was determined as negligible in several control experiments.

To determine the polarity of movement, *Chlamydomonas* axonemes were isolated according to Witman [51], salt extracted [28] and placed on poly-L-lysine coverslips. One μ l of carboxylated latex beads (Polysciences, Warrington, PA/USA) in AP10 (composition like AP100 but 10 mM Pipes) was mixed with 2 μ l 5 mg/ml casein and 2 μ l motor-containing supernatant (S5) or column-purified peak fraction. After 15 min on ice, 1 μ l of this mixture was added to the axonemes in the presence of 2 mM MgATP. Single beads were grasped with a laser trap and placed on the axonemes, and after release from the trap they moved along the axonemes towards the plus-end of the polar structure.

For in vitro organelle motility experiments, an unspecified membrane fraction was prepared. Cells were ground with quartz-sand in the presence of AP100 and protease inhibitors as described above, and centrifuged for 30 min at 10000g. The upper layer of the pellet was resuspended in protease-containing buffer. The membranes were stripped with 600 mM KCl and sedimented through a 15% sucrose cushion of equal volume. This solution contained organelles of different sizes, with a major portion of mitochondria as confirmed by rhodamine 123 staining. As described for the bead assay, a mixture of kinesin solution, casein and organelles was incubated on ice for 15 min and added to stabilized MTs, and organelle movements were monitored using video-enhanced DIC light microscopy [5]. Vertebrate organelles were taken from the first pellet of the tubulin isolation procedure (pig vesicles) or were isolated from rat liver. Statistical analysis was done using two-tail t-tests within the program PRISM (GraphPad, San Diego, CA/USA).

Results

Transport of unclassified vesicles in the hyphae is MT based

Syncephalastrum racemosum grows as elongated, highly branched hyphae of variable diameter, ranging from ~ 5 to 12 μ m. The cells are surrounded by a cell wall and are not divided up by septae (Fig. 1), as is typical for zygomycete fungi [3]. As a result, hyphae are filled with numerous nuclei that are distributed over the length of the cells, as seen both by DAPI staining and by contrast-enhanced video-microscopy (data not shown). The cytoplasm contains a continuously moving system of tubes and vacuoles and long MTs, which are oriented along the long axis of the hypha (data not shown). Mitochondria could not be identified with certainty and therefore were not investigated. Small refractile vesicles of unknown function perform saltatory movement at rates of 2 to 2.5 μ m/s. Interestingly, unlike *Neurospora crassa*, the fre-

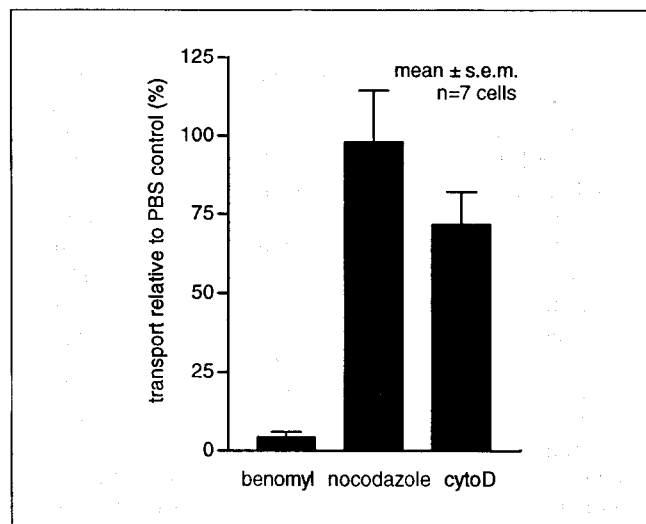


Fig. 2. Influence of cytoskeleton affecting inhibitors on the organelle transport in *S. racemosum*. The number of refractile vesicles that cross a line in 30 s after a 30 min treatment with 10 μ M of benomyl, nocodazole or cytochalasin D was counted and compared to the transport rate of organelles in the same cell under PBS treatment. Cytochalasin D has only a minor influence on the organelle transport, but benomyl dramatically reduces the transport rate. Nocodazole did not inhibit organelle motility (values are given as mean $\pm$ s.e.m., $n = 7$ cells).

quency of intracellular transport in *S. racemosum* decreased rapidly, while the specimen was observed under light microscopy. This could be immediately restored when fresh liquid, culture medium, PBS, or degassed water was flushed into the chamber. The frequency of transport and the movements decreased again within 1 to 2 min after the flow ended. 100 μ M Gd^{3+} , a reagent that affects mechanosensitive cation channels in fungi [15, 53] had no effect on this phenomenon. The subsequent investigation of organelle transport rates took the requirement for a continuous flow of buffer into account.

To clarify the role of the cytoskeleton in the movement of refractile vesicles, the cells were treated with inhibitors that affect the MT-cytoskeleton (nocodazole, benomyl) or F-actin (cytochalasin D). Placed in PBS, the hyphae lived for over 1 h without dramatic change of their internal organization (e.g.



Fig. 1. Morphology of *S. racemosum*. Calcofluor staining of a branched hypha grown on a poly-L-lysine-coated coverslip. As is typ-

ical for zygomycetes, the fungus grows coenocytic from a single conidium (arrow). – Bar 50 μ m.

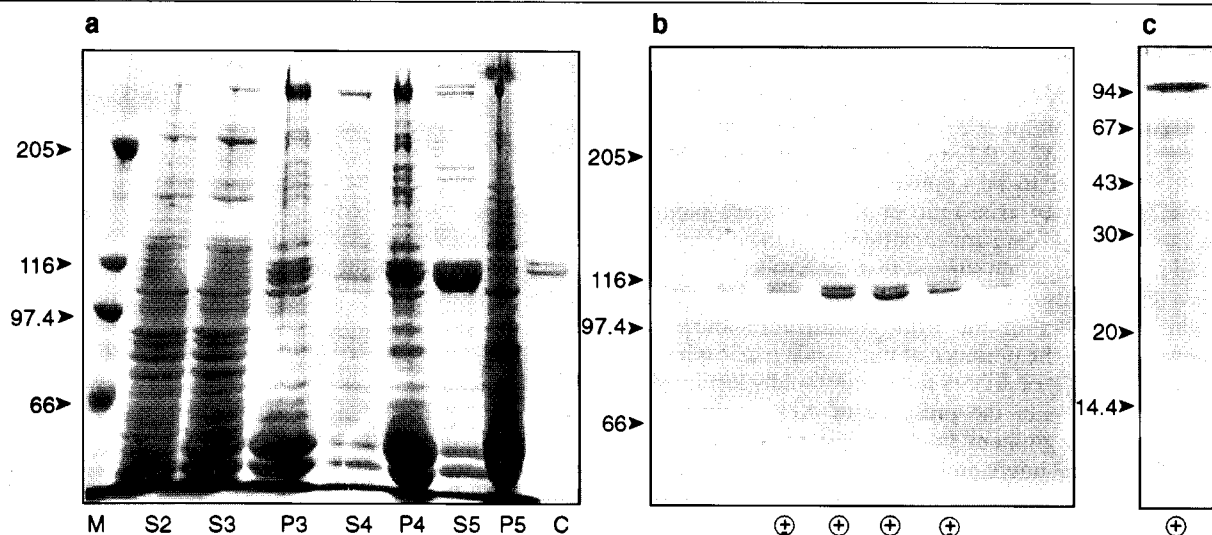


Fig. 3. Isolation of the *Syncephalastrum* kinesin. – **a.** A cytoplasmic extract of *S. racemosum* (S2) was incubated with taxol-stabilized pig brain MTs. In the absence of ATP the enzyme bound to the polymers and cosedimented with the MTs (P3). The pellet was resuspended in fresh buffer, salt washed and sedimented again (P4). The release of the kinesin with MgATP-containing buffer yields a very pure supernatant (S5), which was further purified by a gel-filtration run (C). – **b.** Gel

filtration of 200 μ l kinesin containing S5. Coomassie stain of a 7% polyacrylamide-gel showing fraction 25 to 32 (500 μ l) after running over a superose-6-column. The 112/115 kDa doublet comigrated with the MT-gliding activity (plus and plus/minus). – **c.** Silver stain of the fraction number 29 in a 15% polyacrylamide gel. Beside the 112 kDa and 115 kDa polypeptides no additional light chains or contaminants are detected.

size of vacuoles), and still showed apparently unchanged vesicle transport. Incubation with 10 μ M nocodazole had no effect on the movements, and although 10 μ M cytochalasin D reduced the transport rate to $\sim 70\%$ of the control, this change was not statistically significant ($\alpha:0.05$; $P:0.192$; Fig. 2). In contrast to nocodazole, 10 μ M benomyl significantly reduced the intracellular transport of refractile vesicles within 15 to 30 min (Fig. 2). This effect was in part reversible, but the increase of vesicle movement by the replacement of benomyl solution with fresh PBS was accompanied by the appearance of additional vacuoles, and the cytoplasm congealed before the cells died.

***S. racemosum* cells contain an abundant dimeric mechanoenzyme**

To isolate a potential organelle motor, a previously successful protocol for isolating kinesins from filamentous fungi was applied [47, 48], which is based on the "fishing" of motor molecules with exogenous MTs in an ATP-depleted extract, followed by an ATP release [10, 50]. The isolation procedure led to a supernatant containing a double band at 112 and 115 kDa (Fig. 3a). Beside these polypeptides, only some exogenous pig tubulin and low amounts of contaminating polypeptides are found in the ATP release (S5). Further purification by sucrose density gradient (not shown) or gel filtration

Tab. I. Comparison of the biophysical and motility properties of kinesin from *S. racemosum*, *N. crassa* and animal sources.

		Synkin	Nkin ^a	Kinesin ^b
V MT-gliding	(μ m s ⁻¹)	2.1– 3.4	2.1– 3.8	0.6 – 0.8
MgATP	(%)	100	100	100
MgGTP	(%)	36 –46	50	78 –100
MgCTP	(%)	13 –17	9 –19	0 – 31
MgUTP	(%)	20 –24	12 –21	0 – 38
MgITP	(%)	0 – 3	0	27 – 50
AMPPNP inhibit.	AMPPNP:ATP	< 1.2– 1.5	< 0.3	< 0.5
Stokes radius	(nm)	7.04 $\pm$ 0.43	6.27 $\pm$ 0.53	9.64 $\pm$ 0.87*
D _{w20}	($\times 10^{-7}$ cm ² s ⁻¹)	3.13 $\pm$ 0.18	3.34 $\pm$ 0.20	2.24 $\pm$ 0.21*
S _{w20}	($\times 10^{13}$ s)	8.37 $\pm$ 0.13	8.80 $\pm$ 0.15	9.56 $\pm$ 0.34* (6.5**)
MW in SDS-PAGE	(kg mol ⁻¹)	112/115	105/108	124,64*
MW native	(kg mol ⁻¹)	$\sim$ 240 (220–262)	$\sim$ 227 (199–260)	$\sim$ 379 (294–494)*
axial ratio	a:b	12.3	11.5	19.5* (40**)

Motility data from several experiments of at least 2 isolations, biophysical data from at least 3 isolations. – ^a Data from [48]. – ^b Native isolated kinesin from several animal sources (summarized in [48]). – * Folded conformation, data from [7]. – ** Unfolded conformation, data from [16].

yielded pure motor (Fig. 3b), which migrated with the gliding activity as tested in a MT-motility assay. Additional light chains were never detected in any isolation as confirmed by silver staining of gels (Fig. 3c). Using the estimated Stokes radius (7.04 ± 0.43 nm; $n = 3$) and the sedimentation coefficient, estimated as 8.37 ± 0.13 nm ($n = 3$), the native molecular mass was found to be approximately 240 kDa (range 220–262 kDa), with an axial ratio of 12.3 (data are summarized in Tab. I). These data are consistent with the absence of additional polypeptides and suggest that the *Syncephalastrum* motor is a dimer in its native form.

The gliding activity of the *Syncephalastrum* motor was highly dependent on the presence of nucleoside triphosphates. In the absence of ATP or ATP γ S the motor rapidly lost its MT-gliding activity. Even in the presence of ATP, purified gel filtration or sucrose gradient fractions lost their MT-gliding activity within several hours on ice. Occasionally, gel filtration led to protein which was unable to promote MT-gliding, but still showed a highly variable ATPase activity, ranging from 0.088 to 0.207 μ mol/min/mg in the presence of 1 mg/ml MTs, and variable MT activation of 2.5 to 15-fold.

Synkin shares immunological homology with conventional kinesin from *Neurospora*

The peptide antibody MMR44.1 (provided by Dr. M. A. McNiven, Rochester, MN/USA) raised against the HIPYR-region of the kinesin head sequence [32] recognized recombinant *Drosophila* kinesin (provided by U. Henningsen from a plasmid of Dr. L. S. B. Goldstein) and showed a strong affinity to both bands of the 112 kDa/115 kDa doublet of the kinesin from *S. racemosum* (Synkin; Figs. 4a, b). An antibody against a peptide region of the C-terminal tail of the conventional

kinesin from *Neurospora crassa*, termed NKC, recognized both polypeptides from *Syncephalastrum* and the *Neurospora* control protein, but did not bind to recombinant *Drosophila* kinesin (Fig. 4c) or isolated bovine brain kinesin (data not shown). In crude extracts of *S. racemosum* (S2, see above), NKC clearly recognized a polypeptide of the same relative molecular mass as the isolated kinesin (Fig. 4d). Unfortunately, this antibody could not be used for immunofluorescence, as it gave a vesicular staining in wild type hyphae of *Ustilago maydis*, but showed the same staining in null mutants that contain no kinesin on Western blots (data not shown).

The *Syncephalastrum* mechanoenzyme supports in vitro motility

The purified enzyme was able to transport taxol-stabilized MTs polymerized from pig tubulin in vitro. In several experiments from more than 10 isolations, it supported MT-gliding at a rate of 2.1 to 3.4 μ m/s, and no difference was found whether S5, sucrose density or gel filtration fractions were investigated. For in vitro motility the *Syncephalastrum* motor had different efficiencies for different nucleotides (ATP > GTP > CTP > UTP). This gliding activity could only be inhibited by a relatively high amount of AMPPNP. At a ratio of 1.2 to 1.5 AMPPNP:ATP the MT-gliding activity was $\sim 50\%$ inhibited (all motility data are summarized in Tab. I). The direction of motility was determined using unipolar *Chlamydomonas* axonemes in a modified in vitro motility assay [37]. Latex beads that were coated with low amount of kinesin from density gradient fractions or S5 always moved to the fan-shaped end of *Chlamydomonas* axonemes with 1.5 to 1.7 μ m/s (Figs. 5a–d). As this part of the axoneme represents the end with a higher polymerization rate (plus-end) of the MTs [4] the kinesin from *S. racemosum* is a plus-end directed motor.

Kinesin containing S5 and, to a lower extent, purified *Syncephalastrum* kinesin was able to move unidentified vesicles from a crude organelle preparation along MTs in vitro. After incubating salt-extracted membranes with motor and casein, organelles of different refractive index and size moved unidirectionally along MTs in a saltatory manner at a velocity of 1.7 to 1.8 μ m/s (Figs. 5e–h). This was done using the S5 fraction, and the same motility occurred when using density gradient material, although to a much lower extent. The motility of membranes could easily be monitored in video-enhanced DIC-light microscopy and was clearly different from the Brownian motion of unattached vesicles. In some experiments this movement was very prominent, other samples just showed occasional movements or did not transport vesicles at all, while supporting normal MT-gliding. In several control experiments salt-treated or untreated membranes alone were not able to move along MTs in the presence of ATP.

Interestingly, vesicles from *Syncephalastrum* were moved by *Neurospora* kinesin and vice versa. In several experiments salt-treated organelles were transported by the *Neurospora* motor at velocities of 1.43 ± 0.19 μ m/s (mean $\pm$ s.d.; $n = 4$). This motility was not observed when untreated membranes were used. On the other hand, *Syncephalastrum* kinesin supported motility of untreated *Neurospora* organelles (1.30 ± 0.17 μ m/s, $n = 20$). After incubation of these membranes with 500 mM KCl the velocity was significantly higher (2.37 ± 0.15 μ m/s, $n = 8$; $\alpha:0.05$; $P < 0.0001$). Comparable results were found when using *Aspergillus nidulans* organelles. In these experiments *Syncephalastrum* kinesin moved small vesicles before and after salt treatment at 2.25 ± 0.73 μ m/s

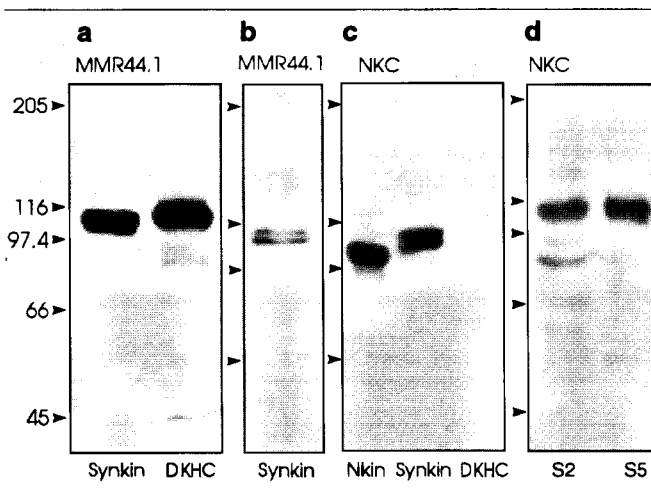


Fig. 4. Immunological characterization of the kinesin from *S. racemosum*. – **a.** A peptide antibody against a conserved peptide in the globular domain of the kinesin heavy chain (MMR44.1) recognized the *Syncephalastrum* kinesin (Synkin) as well as recombinant kinesin from *Drosophila melanogaster* (DKHC). – **b.** Both bands of the 112/115 kDa doublet were stained by this antibody. – **c.** An affinity-purified polyclonal antibody against a sequence region in the C-terminal tail of kinesin from *Neurospora crassa*, termed NKC, clearly recognized *Neurospora* kinesin (Nkin) and the kinesin from *S. racemosum* (Synkin), but not *Drosophila* kinesin (DKHC). – **d.** In protein extracts of *S. racemosum* (S2) the antibody gave a strong signal at approximately the same molecular mass as in S5.

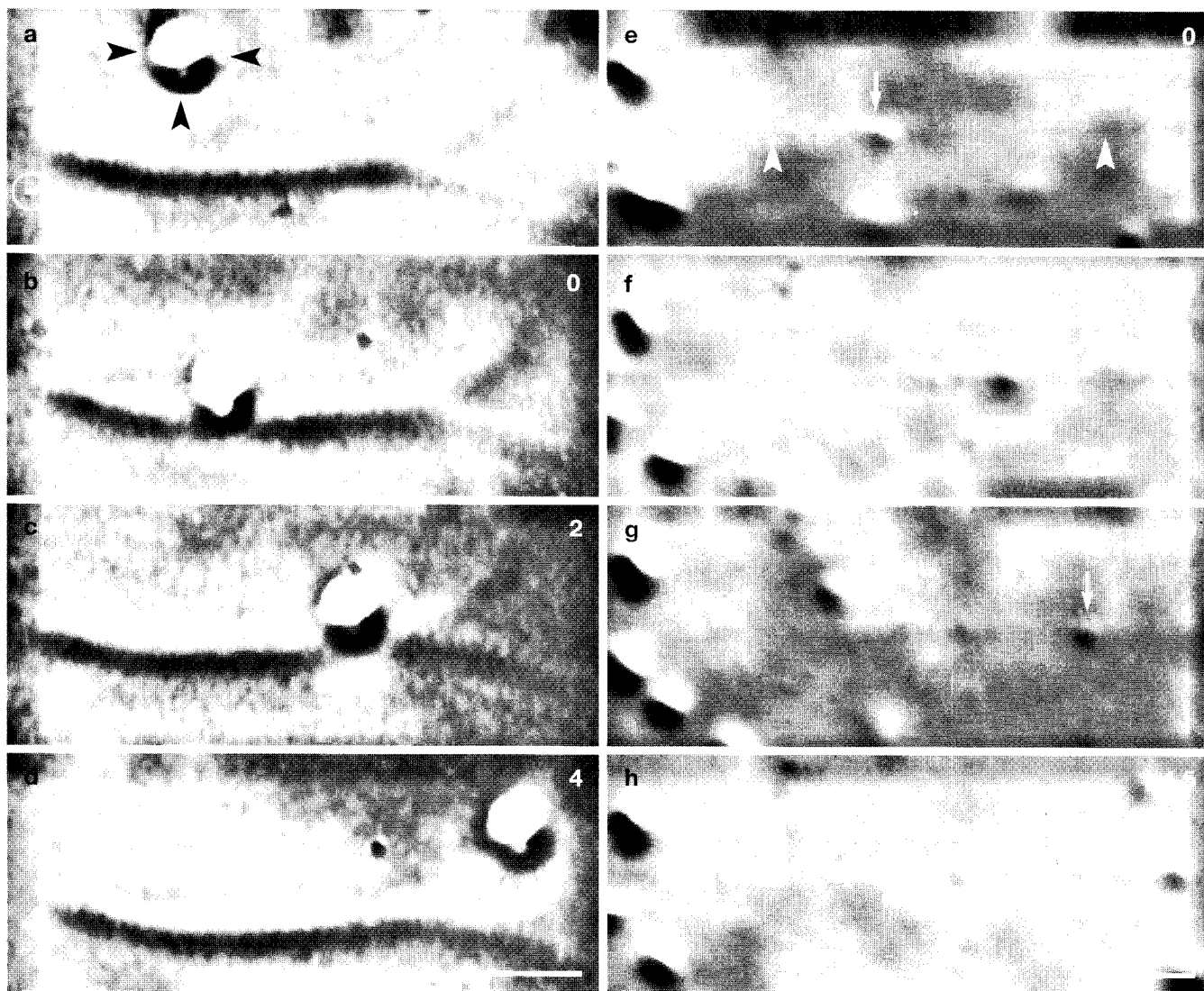


Fig. 5. In vitro motility performed by the *Syncephalastrum* kinesin. – **a to d.** Latex beads were coated with kinesin, grasped by a laser trap (arrowheads) and placed on a stationary bipolar axoneme isolated from *Chlamydomonas* flagella. After the trap was switched off, motor molecules on the surface moved the beads to the fan-shaped end of the

axoneme that consists of the plus-ends of the MTs (marked with *plus*); time in second is referred to by *white numbers*. – **e to h.** Movement of a small vesicle (arrows) treated with *S. racemosum* kinesin along a taxol-stabilized MT (arrowheads). White numbers indicate the time in seconds. – Bars 3 μm.

($n = 20$) and $3.07 \pm 0.27 \mu\text{m/s}$ ($n = 7$), respectively (significantly different, $\alpha:0.05$; $P:0.0082$). In control experiments neither rat liver mitochondria, nor freshly prepared vesicles from pig brain were transported by the *Syncephalastrum* or *Neurospora* motor. These observations suggest that fungal vesicle motors and vesicles are mutually compatible, but more studies are needed to clarify this point.

Discussion

The recently described kinesin from the filamentous ascomycete *Neurospora crassa* showed unusual features that were not found for conventional kinesins from animal sources [47, 48]. These properties and the loss of sequence similarity in the rod portion of the *Neurospora* motor clearly set apart this kinesin from other conventional kinesins. However, the assumed

function as an organelle motor, the relative high abundance, and significant sequence similarities in the head and tail portion confirmed Nkin to be a fungal representative of this kinesin family [47]. This study was undertaken to ask whether comparable kinesins are present in the ancient group of zygomycetes. The purified kinesin from *S. racemosum* displayed a surprisingly high similarity with the *Neurospora* kinesin and confirmed some of the unusual features that are probably typical for kinesin from filamentous fungi. Moreover, vesicles and motor enzymes of both species were found to be interchangeable which suggests a common function in living cells.

For purification of the motor enzyme a previously successful protocol was applied. The main purification step, the binding to taxol-stabilized MTs, followed by a ATP-release, is based on a tight binding (rigor state) of kinesin on MTs in the absence of nucleotides [10, 33, 41, 50]. Applied to *S. racemosum* this procedure yielded a remarkably pure supernatant that con-

tained a high amount of a 112/115 kDa polypeptide doublet. These proteins were identified to be kinesin motors, probably isoforms of true kinesin from *Syncephalastrum*, as confirmed by antibodies against the conserved region in the tail of the conventional kinesin from *Neurospora* and the peptide antibody MMR44.1 [32]. The further characterization of the *Syncephalastrum* kinesin (Synkin) revealed remarkable similarities with the kinesin from *Neurospora crassa*. The purification of Synkin yielded no additional polypeptides and the motor seems to be a dimer in its native state. Light chains, which are known from all "conventional" kinesins (summarized in [8]), were also not found for the *N. crassa* kinesin. This still does not rule out the existence of highly protease sensitive light chains that were degraded over the course of the purification (discussed in [48]). Nevertheless, it seems unlikely that such polypeptides were lost in several purifications from two different fungal species. This is further supported by the results from the characterization of a kinesin from a basidiomycete that also seems to lack light chains (Steinberg et al., manuscript in preparation). These findings are consistent with the results from a polymerase chain reaction approach where primers against conserved sequence stretches of kinesin light chains in combination with RNA from *Syncephalastrum* and *Neurospora* did not yield appropriate products for the fungi, but were successfully used in a mouse library (E. Granderath-Kube, unpublished). In addition, probing S2 and S5 from *Neurospora* and *Syncephalastrum* with anti-light-chain antibodies as well as with recombinant light chains did not indicate the existence of real light chains (F. Goeyda, unpublished). Although typical light chains might not exist in fungi, this clearly leaves the opportunity for other cofactors that are necessary for a proper function of the motor. The instability of purified *S. racemosum* kinesin might be due to the loss of such cofactors rather than be a result of protein denaturation or partial degradation.

The remarkable similarities of both fungal kinesins from phylogenetically distant classes might be due to a similar overall organization of these molecules, which is different from kinesins from animal sources [8, 48]. Whether or not there is some relationship between high in vitro motility, the absence of light chains, and/or the compact native conformation of fungal kinesins is not known. Although animal kinesins move organelles in vitro with a velocity of 1 to 2 $\mu\text{m/s}$ [8] their MT-gliding velocity is 2 to 4 times lower. This might be due to assay conditions (e.g. interaction with the glass surface or an interaction of the enzymatic active heads with the light chains). Therefore, it seems reasonable to assume that a different molecular organization of fungal kinesins might overcome some of these problems and yield a gliding velocity that reflects the in vitro motility of organelles.

Nothing is known about the in vivo function and properties of the refractile vesicles, and it remains open whether Synkin is involved in the MT-dependent intracellular movements described in this paper. Like other conventional kinesin ([45, 49], summarized in [8]), both fungal kinesins were able to move fungal organelles in vitro. The kinesin from *S. racemosum* moved vesicles from *Neurospora crassa* but not vertebrate membranes from two different sources, which argues for a specific interaction with the *N. crassa* organelles. After salt stripping of the fungal organelles, no difference in velocity was seen for the homogeneous system, but Synkin accelerated the transport of *Neurospora* organelles to near the maximum velocity observed in in vitro gliding assays. Further experi-

ments addressing the binding of fungal kinesins to organelles will give insight into the underlying mechanism, which might involve salt-sensitive polypeptide on the membrane that control the in vitro velocity.

In conclusion, the purified kinesins from the zygomycete *S. racemosum* and the ascomycete *N. crassa* share remarkable biochemical and motility similarities. The existence of highly similar motors in distant fungal classes suggests that comparable kinesins are probably present in all filamentous fungi, and their resemblance might be a result of an adaptation to unique functions or regulations in the filamentous hyphae. From its high abundance in *S. racemosum* and *N. crassa* and the in vitro motility of vesicles one can conclude that kinesin might be involved in vectorial transport of abundant vesicles, although kinesins cargo in fungi is still a matter of debate ([31a, 43a]; Steinberg et al., manuscript in preparation). Genetic and physiological studies should elucidate kinesins' role in tip growth or secretion processes of filamentous fungi.

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References

- [1] Aist, J. R., C. J. Bayles: Organelle motility within mitotic asters of the fungus *Nectaria haematococca*. *Eur. J. Cell Biol.* **56**, 358-363 (1991).
- [2] Aizawa, H., Y. Sekine, R. Takemura, Z. Zhang, M. Nangaku, N. Hirokawa: Kinesin family in the murine central nervous system. *J. Cell Biol.* **119**, 1287-1296 (1992).
- [3] Alexopoulos, C. J., C. W. Mims, M. Blackwell: *Introductory mycology*. 4th ed. John Wiley & Sons. New York, Chichester, Brisbane, Toronto, Singapore 1996.
- [4] Allen, C., G. G. Borisy: Structural polarity and directional growth of microtubules of *Chlamydomonas* flagella. *J. Mol. Biol.* **90**, 381-402 (1974).
- [5] Allen, R. D., N. S. Allen, J. L. Travis: Video-enhanced contrast, differential interference contrast (AVEC-DIC). Microscopy: A new method capable of analyzing microtubule related movement in the reticulopodial network of *Allogromia laticollaris*. *Cell Motil.* **1**, 291-302 (1981).
- [6] Bähler M., R. Kroschewski, H.-E. Stöfler, T. Behrmann: Rat myr 4 defines a novel subclass of myosin I: Identification, distribution, localization, and mapping of calmodulin-binding sites with differential calcium sensitivity. *J. Cell Biol.* **126**, 375-389 (1994).
- [7] Bloom, G. S., M. C. Wagner, K. K. Pfister, S. T. Brady: Native structure and physical properties of bovine brain kinesin and identification of the ATP-binding subunit polypeptide. *Biochemistry* **27**, 3409-3416 (1988).
- [8] Bloom, G. S., S. A. Endow: Motor proteins. 1: Kinesins. *Protein Profile* **1**, 1059-1105 (1994).
- [9] Blum, H., H. Beier, H. J. Gross: Improved silver staining of plant proteins, RNA, and DNA in polyacrylamid gels. *Electrophoresis* **8**, 93-99 (1987).

- [10] Brady, S. T.: A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* **317**, 73–75 (1985).
- [11] Brady, S. T.: Molecular motors in the nervous system. *Neuron* **7**, 521–533 (1991).
- [12] Eshel, D., L. A. Urrestarazu, S. Vissers, J.-C. Jauniaux, J. C. van Vliet-Reedijk, R. J. Plants, I. R. Gibbons: Cytoplasmic dynein is required for normal nuclear segregation in yeast. *Proc. Natl. Acad. Sci. USA* **90**, 11172–11176 (1993).
- [13] Espreafico, E. M., R. E. Cheney, M. Matteoli, A. A. Nascimento, P. V. De Camilli, R. E. Larson, M. S. Mooseker: Primary structure and cellular localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. *J. Cell Biol.* **119**, 1541–1557 (1992).
- [14] Garfin, D. E.: One-dimensional gel electrophoresis. *Methods Enzymol.* **182**, 425–441 (1990).
- [15] Garrill, A., S. L. Jackson, R. R. Lew, I. B. Heath: Ion channel activity and tip growth: tip-localized stretch-activated channels generate an essential Ca^{2+} gradient in the oomycete *Saprolegnia ferax*. *Eur. J. Cell Biol.* **60**, 358–365 (1993).
- [16] Hackney, D. D., J. D. Levitt, J. Suhan: Kinesin undergoes a 9 S to 6 S conformational transition. *J. Biol. Chem.* **267**, 8696–8701 (1992).
- [17] Hall, D. H., E. M. Hedgecock: Kinesin-related gene *unc-104* is required for axonal transport of synaptic vesicles in *C. elegans*. *Cell* **65**, 837–847 (1991).
- [18] Heath, I. B.: Evidences against a direct role for cortical actin arrays in saltatory organelle motility in hyphae of the fungus *Saprolegnia ferax*. *J. Cell Sci.* **91**, 41–47 (1988).
- [19] Heath, I. B.: The roles of actin in tip growth of fungi. *Int. Rev. Cytol.* **123**, 95–127 (1990).
- [20] Heath, I. B.: The cytoskeleton. In: N. A. R. Gow, G. M. Gadd (eds.): *The Growing Fungus*. pp. 99–134. Chapman and Hall. London 1995.
- [21] Heath, I. B., M. C. Heath: Microtubules and organelle movements in the rust fungus *Uromyces phaseoli* var. *vignae*. *Cytobiologie* **16**, 393–411 (1978).
- [22] Heath, I. B., S. G. W. Kaminskyj: The organization of tip-growth related organelles and microtubules revealed by quantitative analysis of freeze-substituted oomycete hyphae. *J. Cell Sci.* **93**, 41–52 (1989).
- [23] Hirokawa, N.: Organelle transport along microtubules – the role of KIFs. *Trends Cell Biol.* **6**, 135–141 (1996).
- [24] Holzbaur, E. L. F., R. B. Vallee: Dyneins: Molecular structure and cellular function. *Annu. Rev. Cell Biol.* **10**, 339–372 (1994).
- [25] Itaya, K., M. Ui: A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta* **14**, 361–366 (1996).
- [26] Jones, H. D., M. Schliwa, D. G. Dubrin: Video microscopy of organelle inheritance and motility in budding yeast. *Cell Motil. Cytoskeleton* **25**, 129–142 (1993).
- [27] Kaminskyj, S. G. W., K. S. Yoon, I. B. Heath: Cytoskeletal interactions with post-mitotic migrating nuclei in the oyster mushroom fungus, *Pleurotus ostreatus*: evidences against a force-generating role of astral MTs. *J. Cell Sci.* **94**, 663–674 (1989).
- [28] King, S. M., T. Otter, G. B. Witman: Purification and characterization of *Chlamydomonas* flagellar dyneins. *Methods Enzymol.* **134**, 291–306 (1986).
- [29] Kuznetsov, S. A., G. M. Langford, D. G. Weiss: Actin-dependent organelle movement in squid axoplasm. *Nature* **356**, 722–725 (1992).
- [30] Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970).
- [31] Lazzarino, D. A., I. Boldogh, M. G. Smith, J. Rosand, L. A. Pon: Yeast mitochondria contain ATP-sensitive, reversible actin-binding activity. *Mol. Biol. Cell* **5**, 807–818 (1994).
- [31a] Lehmler, C., G. Steinberg, K. Snetselaar, M. Schliwa, R. Kahmann, M. Bölker: Identification of a motor protein required for filamentous growth in *Ustilago maydis*. *EMBO J.*, in press.
- [32] Marks, D. L., J. M. Larkin, M. A. McNiven: Association of kinesin with the Golgi apparatus in rat hepatocytes. *J. Cell Sci.* **107**, 2417–2426 (1994).
- [33] McCaffrey, G., R. D. Vale: Identification of a kinesin-like microtubule-based motor protein in *Dictyostelium discoideum*. *EMBO J.* **8**, 3229–3234 (1989).
- [34] McKerracher, L. J., I. B. Heath: Topical review: Cytoplasmic migration and intracellular organelle movements during tip growth of fungal hyphae. *Exp. Mycol.* **11**, 79–100 (1987).
- [35] Olmsted, J. B.: Analysis of cytoskeletal structures using blot-purified homospecific antibodies. *Methods Enzymol.* **134**, 467–472 (1986).
- [36] Paschal, B. M., R. B. Vallee: Retrograde transport by the microtubule-associated protein MAP 1C. *Nature* **330**, 181–183 (1987).
- [37] Paschal, B. M., R. B. Vallee: Microtubule and axoneme gliding assays for force production by MT motor proteins. *Methods Cell Biol.* **39**, 65–74 (1993).
- [38] Pesavento, P. A., R. J. Stewart, L. S. B. Goldstein: Characterization of the KLP68D kinesin-like protein in *Drosophila*: Possible roles in axonal transport. *J. Cell Biol.* **127**, 1041–1048 (1994).
- [39] Plamann, M., P. F. Minke, J. H. Tinsley, K. S. Bruno: Cytoplasmic dynein and actin related protein Arp1 are required for normal nuclear distribution in filamentous fungi. *J. Cell Biol.* **127**, 139–149 (1994).
- [40] Schliwa, M.: Mechanisms of intracellular organelle transport. In: J. W. Shay (ed.): *Cell and Muscle Motility*. Vol. 5: *The Cytoskeleton*. pp. 1–82. Plenum Press. New York 1984.
- [41] Scholey, J. M., M. E. Porter, P. M. Grissom, J. R. McIntosh: Identification of kinesin in sea urchin eggs and evidence for its localization in the mitotic spindle. *Nature* **318**, 483–486 (1985).
- [42] Schroer, T. A., M. P. Sheetz: Functions of microtubule-based motors. *Annu. Rev. Physiol.* **53**, 629–652 (1991).
- [43] Schroer, T. A., B. J. Schnapp, T. S. Reese, M. P. Sheetz: The role of kinesin and other soluble factors in organelle movement along MTs. *J. Cell Biol.* **107**, 1785–1792 (1988).
- [43a] Seiler, S., F. E. Nargang, G. Steinberg, M. Schliwa: Kinesin is essential for cell morphogenesis and polarized secretion in *Neurospora crassa*. *EMBO J.*, in press.
- [44] Shelanski, M. L., F. Gaskin, C. R. Cantor: Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA* **70**, 765–768 (1973).
- [45] Simon, V. R., T. C. Swayne, L. A. Pon: Actin-dependent mitochondrial motility in mitotic yeast and cell-free systems: Identification of a motor activity on the mitochondrial surface. *J. Cell Biol.* **130**, 345–354 (1995).
- [46] Steinberg, G., M. Schliwa: Organelle movement in the wild type and wall-less fz; sg; os-1 mutants of *Neurospora crassa* are mediated by cytoplasmic microtubules. *J. Cell Sci.* **106**, 555–564 (1993).
- [47] Steinberg, G., M. Schliwa: The *Neurospora* organelle motor: A distant relative of conventional kinesin with unconventional properties. *Mol. Biol. Cell* **6**, 1605–1618 (1995).
- [48] Steinberg, G., M. Schliwa: Characterization of the biophysical and motility properties of kinesin from the fungus *Neurospora crassa*. *J. Biol. Chem.* **271**, 7516–7521 (1996).
- [49] Urrutia, R., M. A. McNiven, J. P. Albanesi, D. B. Murphey, B. Kacher: Purified kinesin promotes vesicle motility and induces active sliding between microtubules in vitro. *Proc. Natl. Acad. Sci. USA* **88**, 6701–6705 (1991).
- [50] Vale, R. D., T. S. Reese, M. P. Sheetz: Identification of a novel force-generation of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* **42**, 39–50 (1985).
- [51] Witman, G. B.: Isolation of *Chlamydomonas* flagella and flagellar axonemes. *Methods Enzymol.* **134**, 280–290 (1986).
- [52] Xiang, X., S. M. Beckwith, R. Morris: Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. USA* **91**, 2100–2104 (1994).
- [53] Zhou, X.-L., M. A. Stumpf, H. C. Hoch, C. Kung: A mechanosensitive cation channel in membrane patches and in whole cells of the fungus *Uromyces*. *Science* **253**, 1415–1417 (1991).