

Genetic evidence for a microtubule-destabilizing effect of conventional kinesin and analysis of its consequences for the control of nuclear distribution in *Aspergillus nidulans*

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Summary

Conventional kinesin is a microtubule-dependent motor protein believed to be involved in a variety of intracellular transport processes. In filamentous fungi, conventional kinesin has been implicated in different processes, such as vesicle migration, polarized growth, nuclear distribution, mitochondrial movement and vacuole formation. To gain further insights into the functions of this kinesin motor, we identified and characterized the conventional kinesin gene, *kinA*, of the established model organism *Aspergillus nidulans*. Disruption of the gene leads to a reduced growth rate and a nuclear positioning defect, resulting in nuclear cluster formation. These clusters are mobile and display a dynamic behaviour. The mutant phenotypes are pronounced at 37°C, but rescued at 25°C. The hyphal growth rate at 25°C was even higher than that of the wild type at the same temperature. In addition, kinesin-deficient strains were less sensitive to the microtubule destabilizing drug benomyl, and disruption of conventional kinesin suppressed the cold sensitivity of an α -tubulin mutation (*tubA4*). These results suggest that conventional kinesin of *A. nidulans* plays a role in cytoskeletal dynamics, by destabilizing microtubules.

This new role of conventional kinesin in microtubule stability could explain the various phenotypes observed in different fungi.

Introduction

Kinesins are microtubule-dependent motor proteins involved in a wide variety of cellular processes (Hirokawa, 1998). The prototype of kinesin, now referred to as conventional kinesin, was first isolated from squid brain (Vale *et al.*, 1985). It uses energy derived from ATP hydrolysis to move processively towards the microtubule-plus end (Vale and Milligan, 2000). All conventional kinesins are believed to be involved in the long-range transport of vesicles and organelles. In view of this function it is not surprising that representatives of this family of kinesins have also been identified in several filamentous fungi, which heavily rely on tip-directed transport to support fast apical growth. In contrast, conventional kinesin is not present in the much smaller yeast *Saccharomyces cerevisiae*, in which vesicular transport is either accomplished by diffusion or mediated by myosin motors (Lillie and Brown, 1994). Interestingly, fungal conventional kinesins which, to date, include representatives from four species in three different classes of fungi (Steinberg and Schliwa, 1995; Lehmler *et al.*, 1997; Grummt *et al.*, 1998a; Wu *et al.*, 1998) are the fastest kinesins known. With a rate of movement of about 2.5 $\mu\text{m}/\text{sec}$, they are about four times faster than their animal counterparts. This and additional favourable features of fungal kinesins prompted further studies that contributed to our knowledge of kinesin mechanobiology (Steinberg and Schliwa, 1996; Henningsen and Schliwa, 1997; Grummt *et al.*, 1998b; Crevel *et al.*, 1999; Woehlke and Schliwa, 2000).

For the study of kinesin function, filamentous fungi offer additional, distinct advantages over animal species. Deletion mutants are viable and result in informative phenotypes, whereas such mutants in *Caenorhabditis elegans* (Patel *et al.*, 1993), *Drosophila melanogaster* (Saxton *et al.*, 1991) and the mouse (Tanaka *et al.*, 1998) are embryonic lethal. Thus, deletion mutants of *Neurospora crassa* (Seiler *et al.*, 1997), *Ustilago maydis*

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(Lehmler *et al.*, 1997) and *Nectria haematococca* (Wu *et al.*, 1998) generally grow slower and exhibit changes in hyphal morphology which may be directly linked to altered vesicular and vacuolar transport. This view is supported by the analysis of mutant kinesin transformed into the null strain of *N. crassa* which, in addition, helped identify domains of the kinesin motor responsible for cargo binding, regulation and intramolecular folding (Kirchner *et al.*, 1999a; Seiler *et al.*, 2000). Other phenotypic changes appear to be more species-specific. Thus, for example, in *N. crassa* nuclear distribution is less regular, a feature previously believed to be linked solely to defects in the molecular motor dynein (Morris *et al.*, 1995; Fischer, 1999; Suelmann and Fischer, 2000b). In *U. maydis*, vacuole formation is impaired (Steinberg *et al.*, 1998), whereas the distribution of mitochondria near the hyphal tip is altered in *N. haematococca* (Wu *et al.*, 1998).

The variation in the phenotypic expression of kinesin defects cautions against generalizations and calls for more detailed analyses of kinesin function in additional model organisms. It is for this reason that we have embarked on an analysis of conventional kinesin in *A. nidulans*, a well-established model system for the study of cell growth, polarity, mitosis and nuclear migration. Here we show that mutation of the kinesin gene *kinA* not only affects polarized growth, but also has a pronounced effect on nuclear positioning. Unexpectedly, several of the cellular functions previously described to be affected in other species remained unaltered, and the growth defect was alleviated, even reverted, at lower temperature. Most interestingly, however, we provide evidence that kinesin affects microtubule stability. This influence might explain several of the mutant kinesin phenotypes and offers new insights into the function of this motor protein.

Results

Molecular cloning of the conventional kinesin gene kinA

We took advantage of the *A. nidulans* sequencing project at Cereon Genomics LLC (Cambridge, USA) and screened the existing genomic database for kinesin-like proteins. We identified one partial sequence with high homology to the N-terminus of known fungal conventional kinesin motor domains. The 1200 bp DNA-sequence information was used to isolate a cosmid containing the entire gene from the pKBY library (Yelton *et al.*, 1984). This cosmid, a 5.2 kb *HindIII* (pEW21) and a 5.3 kb *BamHI*

(pRF647) subclone were used as templates for the determination of the gene sequence using synthetic oligonucleotides as primers (Fig. 1). Three corresponding partial cDNAs which covered the coding region of *kinA*, were polymerase chain reaction (PCR)-amplified after reverse transcription of RNA, subcloned and sequenced. Comparison of genomic DNA with the cDNA sequences revealed the presence of four introns, 67, 63, 70 and 117 bp in length. Three are located at the 5' end of the gene and one at the 3' end. The position of the last intron is conserved in *N. crassa* and *N. haematococca*. After removal of the intron sequences an open reading frame of 927 amino acids was deduced that encodes a putative protein with a calculated molecular mass of 103 kDa. The protein is hydrophilic with four predicted coiled-coil domains (Fig. 1). The sequence of *A. nidulans kinA* is available in the EMBL database under the accession number AJ291452. Pair-wise comparison with other fungal conventional kinesins revealed 72% similarity to the *N. haematococca* protein, 71% to *N. crassa*, 52% to *S. racemosum* and 51% to *U. maydis*. Several motifs important for proper function of the motor are conserved in *A. nidulans*.

One striking feature of *N. crassa* conventional kinesin is the high velocity with which it migrates along microtubules. In order to determine the speed of this motor from *A. nidulans* we purified the protein from wild type (FGSC26) (Steinberg and Schliwa, 1996). In a gliding assay (see *Experimental procedures*) the motor moved polymerized microtubules at a rate of 2.1 $\mu\text{m}/\text{sec}$. As the native protein was rather unstable, we expressed the motor in *E. coli* and purified the recombinant protein. We determined the gliding velocity to be 2.01 $\mu\text{m}/\text{sec}$ ($\pm 0.32 \mu\text{m}$; $n=50$) and the ATPase activity k_{cat} (s^{-1}) was 55.4. This demonstrates that *A. nidulans* KinA not only shares sequence similarity with other fungal conventional kinesins but also possesses similar biochemical properties (Steinberg and Schliwa, 1996).

KinA is required for polarized growth and is involved in nuclear migration and positioning

To gain insights into the biochemical function of conventional kinesin in *A. nidulans*, we constructed a null mutant by homologous recombination. We PCR-amplified a 0.9 kb fragment upstream and a 1 kb fragment downstream of the translational start codon. The two PCR fragments were cloned up- and downstream of the *pyr4*

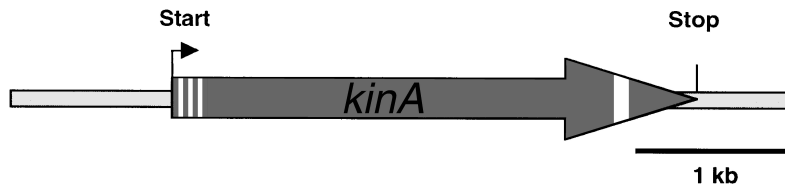
Fig. 1. Characterisation of *A. nidulans* conventional kinesin *kinA*.

A. Scheme of the *kinA* gene locus. The open reading frame is indicated by the arrow and introns are marked as grey boxes within the arrow.

B. Alignment of *A. nidulans kinA* with conventional kinesin sequences of *N. haematococca*, *N. crassa*, *S. racemosum* and *U. maydis*. The alignment was done with DNASTar using Megalign with a window size of 5 and a gap penalty of 10.

C. Coiled-coil prediction according to Lupas *et al.* (1992). The sequence of *A. nidulans kinA* is available under the accession number AJ291452.

A



B

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1  MASSTSNPSTIKVVARFRPQNKVELASGGQPIVFEFENDET-----CSINSKEA  SGSFTFDRVFPMDSKQTDVFNYSI  Aspergillus
1  MSSA-----NSIKVVARFRPQNKVELASGGMPVVSFDGEDT-----CSLDSKEA  QGSFTFDRVFDMAACKQDIFDPSI  Nectria
1  MSSA-----NNIKVVARFRPQNKVELASGGQPIVFEFENDET-----GTVDSKEA  QGSFTFDRVFDMSCKQSDIFDPSI  Neurospora
1  MS-----NNIKVVARFRPQNKVELASGGQPIVFEFENDET-----GKEFGKFN  FPKVFGMNTAQKDVFDVYSI  Syncephalastrum
1  MS-----NNIKVVARFRPQNKVELASGGQPIVFEFENDET-----GTVDSKEA  QGSFTFDRVFDMAACKQDIFDPSI  Ustilago

74  APTVDDILNGYNGTVFAYGQGTGAGKSYIMMGTSIDDDVEVGRGIIPRIVEQIFASILTSPSNIEYTVRLLSYMEIYMERIRDL  Aspergillus
69  RSTVDDILNGYNGTVFAYGQGTGAGKSYIMMGTSIDDDVEVGRGIIPRIVEQIFASIMSSPGTIEYTVRVRVSYMEIYMERIRDL  Nectria
70  KPTVDDILNGYNGTVFAYGQGTGAGKSYIMMGTSIDDDVEVGRGIIPRIVEQIFASILTSPSNIEYTVRLLSYMEIYMERIRDL  Neurospora
69  KTVVDDVLAGYNGTVFAYGQGTGAGKSYIMMGTSIDDDVEVGRGIIPRIVEQIFASILTSPSNIEYTVRLLSYMEIYMERIRDL  Syncephalastrum
73  KTVVDDVLAGYNGTVFAYGQGTGAGKSYIMMGTSIDDDVEVGRGIIPRIVEQIFASILTSPSNIEYTVRLLSYMEIYMERIRDL  Ustilago

154  LVPQNDNLPVHHEEKSROVYVVKOLLEIVYSSVQEVYEVNRRGCGNARAVAAATNMNQESSRSHSIFVITVTQKNLETGSAKSG  Aspergillus
149  LAPQNDNLPVHHEEKNRQVYVVKOLLEIVYSSVQEVYEVNRRGCGNARAVAAATNMNQESSRSHSIFVITVTQKNLETGSAKSG  Nectria
150  LVPQNDNLPVHHEEKNRQVYVVKOLLEIVYSSVQEVYEVNRRGCGNARAVAAATNMNQESSRSHSIFVITVTQKNLETGSAKSG  Neurospora
149  LNPSSNLPVHHEEKNRQVYVVKOLLEIVYSSVQEVYEVNRRGCGNARAVAAATNMNQESSRSHSIFVITVTQKNLETGSAKSG  Syncephalastrum
153  LAPQNDNLPVHHEEKNRQVYVVKOLLEIVYSSVQEVYEVNRRGCGNARAVAAATNMNQESSRSHSIFVITVTQKNLETGSAKSG  Ustilago

234  QLPFLVDLAGSEKVGKTKGASGQTLLEEAKKINKLSALGMVINALTDGKSSHIPYRDSKLTRELQESLGGNSRTLLIINCSP  Aspergillus
229  QLPFLVDLAGSEKVGKTKGASGQTLLEEAKKINKLSALGMVINALTDGKSSHIPYRDSKLTRELQESLGGNSRTLLIINCSP  Nectria
230  QLPFLVDLAGSEKVGKTKGASGQTLLEEAKKINKLSALGMVINALTDGKSSHIPYRDSKLTRELQESLGGNSRTLLIINCSP  Neurospora
229  KLYLVDLAGSEKVGKTKGASGQTLLEEAKKINKLSALGMVINALTDGKSSHIPYRDSKLTRELQESLGGNSRTLLIINCSP  Syncephalastrum
233  NLYLVDLAGSEKVGKTKGASGQTLLEEAKKINKLSALGMVINALTDGKSSHIPYRDSKLTRELQESLGGNSRTLLIINCSP  Ustilago

314  SSYNDAETVSTLRFPGVRAKAIKKNKAKVNAELSPAELEKQLLRRRAQSQVTSFENYISALESEVSSWRSGETVPREKWTTPARN  Aspergillus
309  SSYNDAETLSTLRFPGVRAKAIKKNKAKVNAELSPAELEKQLLRRRAQSQVTSFENYISALESEVSSWRSGETVPREKWTTPARN  Nectria
310  SSYNDAETLSTLRFPGVRAKAIKKNKAKVNAELSPAELEKQLLRRRAQSQVTSFENYISALESEVSSWRSGETVPREKWTTPARN  Neurospora
309  SSYNDAETLSTLRFPGVRAKAIKKNKAKVNAELSPAELEKQLLRRRAQSQVTSFENYISALESEVSSWRSGETVPREKWTTPARN  Syncephalastrum
313  CVYNDAETLSTLRFPGVRAKAIKKNKAKVNAELSPAELEKQLLRRRAQSQVTSFENYISALESEVSSWRSGETVPREKWTTPARN  Ustilago

394  AEAVRGAKAEARGP-RPSTPSRLHDVSRSETRPDSS--RIGDRSSTPS-IVLEKDEREEFLRRENELEQDQIAEKESHIAT  Aspergillus
389  TEAVARTKADARSSTRPSTPS-LIAESRSETPA-----ISERAGTPS-LPLDKDEREEFLRRENELEQDQIAEKESQATA  Nectria
389  ELAITSKAS-ASTARPSTPSRLDPEASRAETPA-----ISERAGTPS-LPLDKDEREEFLRRENELEQDQIAEKESIAA  Neurospora
388  ---KVSQDPAFLPPAPGPKSPVSD-----GSRPAETVP-TLEKDEREEFLRRENELEQDQIAEKESIAA  Syncephalastrum
392  ---KVVVGGQAPIAATPTLAATQSRPLTPAVENLRELSRPEPTPSAVSIDKDEREEFLRRENELEQDQIAEKESIAA  Ustilago

470  IERGLREARDELRTLKENSVARSARGKDNKELNAEVNLELRMHLEKVSYESKEAAITMDSUREANSELTAELDEVKQQLLVVRM  Aspergillus
461  AEKQLRETKEELVLYLKEHDSKVDKENEKLLTEVNEFKMQLERLTFESKEAQTMDTLKLEANTELTELDLDDVQKQLLVVRM  Nectria
461  AERQLRETKEELVLYLKEHDSKVDKENEKLLTEVNEFKMQLERLTFESKEAQTMDTLKLEANTELTELDLDDVQKQLLVVRM  Neurospora
450  REKLLLELREEMGYRKEQEQSVTKENQMTSELSLELQLQVSYESKENAITVDSLKEANQDLMAELEELKKNLSLMAEQ  Syncephalastrum
468  AESALREATQELAFYREQEQATLSKDNKSNMSEINEIKLQLEERVTYEHKDATITLIDLKQKNDLSALELEELKKNLSLMAEQ  Ustilago

550  KAKETTAAALDEKKEKKAEMKMAKMAQFDLGGVFSNDRHKLQNLITQRVDSLHOIAEAGEVITAPDDLELRAESSETQGF  Aspergillus
541  SAKETGAALDEKKEKKAEMKMAKMAQFDLGGVFSNDRHKLQNLITQRVDSLHOIAEAGEVITAPDDLELRAESSETQGF  Nectria
541  SAKETSAVLDEKKEKKAEMKMAKMAQFDLGGVFSNDRHKLQNLITQRVDSLHOIAEAGEVITAPDDLELRAESSETQGF  Neurospora
530  AHKDATD--SDKKEKKAEMKMAQMSGDFPSG-ILNDRKQIRNALSCLD---GEQQTLTVDLVSRLRELESKMLV  Syncephalastrum
548  TVKDPSQ--EKKEKKAEMKMAQMSGDFPSG-IVSEKKEQIRNALSCLD---AGSTGGALSAADDLSTLREQLLESQSL  Ustilago

630  KQAEELTVNDRSELNLVLDQSRMMELEQKLANLEQDYESLLARNLGEBD-----LAETKERLEKVYIAKKNLVEELAAA  Aspergillus
621  KQAEELSMYTTSSA--DSRRRQBLEARLEAVQGEYEVVLRNLPEBD-----VEVKARLENAPANRQTAQSPV  Nectria
621  KQAEELSSFAASDA--EARKRAELEALQEHLEELLSRNLPEAD-----KEVKALLAKSLSDSAVQVEL  Neurospora
602  EQHTKTSIDLASADKDEMAEKKILEGRGLGALKEVEYELLDKTITAEBAFNMQNADVDNLSALRKLKLEAQYAEKKEVQKEI  Syncephalastrum
622  REQSERVVPQSLAENNELHQRRQELSERFATLEAEYELLDKSISKAEBANHVVDVT-IQDLKAKLELQYASXRDAQTSEI  Ustilago

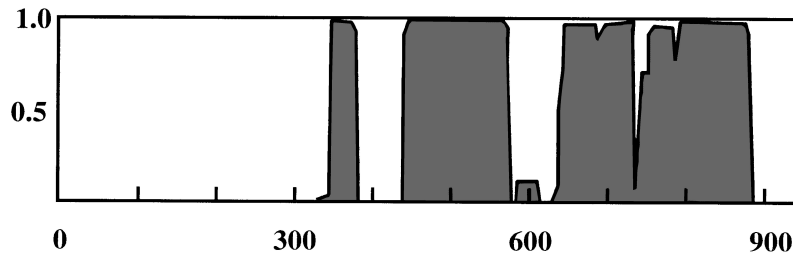
700  SELQQQLARKEDELTKLQQLSADSSQSRG-----STNGAA-----S-----KSLQQQIAEFDAMKKSLSMRD  Aspergillus
690  DELKADITKAAABENTRMKTLIDDLQQRV-----KAGAAA-PMANG-----KTIQQQIAEFDVMMKKSLSMRD  Nectria
690  EQLKADITKAAABENTHEHLKALVDDLQRRV-----KAGAGVAMANG-----KTVOQQIAEFDVMMKKSLSMRD  Neurospora
682  DDLKRELDKQSGHEKLSAAMTDLRAANDQLQAALSEQPFAQVQDNMDTEKEDIETRSKMAQQQLAEFDVMMKKSLSMRD  Syncephalastrum
701  NDLKROLEPKAKETIAITLSTNNUNLKSNEELKRAFAVTT-AGVEGGRNLAESARELERVRKTMAAQSEFDVMMKKSLSMRD  Ustilago

755  LQNRCEVVELEISLDDAREQYNNVLRSSNNRAQQKMAFLERNLEQLTHVQRQLVEQNSLKKKEVAIAERKLIARNERI  Aspergillus
749  LQNRCEVVELEISLDDAREQYNNVLRSSNNRAQQKMAFLERNLEQLTHVQRQLVEQNSLKKKEVAIAERKLIARNERI  Nectria
750  LQNRCEVVELEISLDDAREQYNNVLRSSNNRAQQKMAFLERNLEQLTHVQRQLVEQNSLKKKEVAIAERKLIARNERI  Neurospora
762  LQNRCEVVELEISLDDAREQYNNVLRSSNNRAQQKMAFLERNLEQLTHVQRQLVEQNSLKKKEVAIAERKLIARNERI  Syncephalastrum
790  LQNRCEVVELEISLDDAREQYNNVLRSSNNRAQQKMAFLERNLEQLTHVQRQLVEQNSLKKKEVAIAERKLIARNERI  Ustilago

835  SLEALLQESQKLLTOANHRFEAQLTAVKERLEAAKGGSTKGLPGMDSNGGPF--AGSRTAKPLRGGGGGNEPVAAVQS  Aspergillus
829  QLESLLQDSQKMAAANHHKFEVQLAAVKKERLELAKAGSTRGL--NPSGGFSPFAGSRTAKPLRGGGGG--DVAPAIP  Nectria
830  QLESLLQDSQKMAAANHHKFEVQLAAVKKERLELAKAGSTRGL--GTDAGLGGFSGSRTAKPLRGGGGDAVAGATANP  Neurospora
842  QSLETLHNAQDKLLNQNKKFEQQLATVRRERLEQARSQKSON-SLAA-----NFS--RIAKPLRGGGAAID-----  Syncephalastrum
860  QALEQLHMDSDQKMLLQNRKFEQQLAVKERLHHAQMRKASV-AGSAPGPLGGFEG--RIAKPLRGGGGTSGPQVKSVA  Ustilago

913  -----QDTGTRKTSW---FFDRR  Aspergillus
904  TIQNL-----HQTEGNSGSSNRKASW---FFNKS  Nectria
907  TIATL-----QQ---NPPENKRSW---FFQKS  Neurospora
906  -----NGSDDGSLPTSPDKDKRSSWMPGMNSR  Syncephalastrum
936  ARQAVPFGSVAGASLGENSPKARSSW---FFSSK  Ustilago
    
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C



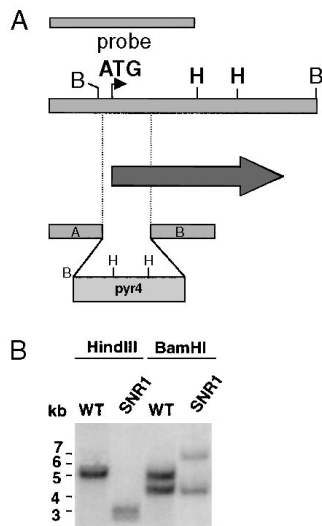


Fig. 2. Disruption of the *kinA* gene.

A. Scheme of the disruption construct. Two DNA fragments (A and B) were PCR-amplified and cloned up- and downstream of the nutritional marker *pyr4*. Homologous integration of the construct leads to a deletion of the translational start site of *kinA* and a disruption of the open reading frame. The probe used in the Southern blot is shown above the scheme of the genomic locus.

B. Southern blot analysis. Genomic DNA of a wild type (FGSC26) and the kinesin disruptant strain (SNR1) was isolated, restricted with *Hind*III (= H) (left) and *Bam*HI (= B) (right), separated on a 1% agarose gel, blotted and hybridized with the probe indicated in A.

gene of *N. crassa* (see *Experimental procedures*). Homologous integration of the construct (pRF645) in *A. nidulans* would lead to a deletion of both the region required for initiation of translation and 45 amino acids of the KinA polypeptide. In addition, the *pyr4* gene disrupts the coding region for *kinA*. The disruption construct was digested with *Pvu*II and the whole digest transformed into the uracil-auxotrophic *A. nidulans* SRF200 strain. Colony-purified uracil-prototrophic transformants were tested using PCR for a homologous integration event at the *kinA* locus. In two out of 27 strains, PCR suggested disruption of the wild-type copy (Fig. 2). However, faint bands of the wild-type *kinA* situation were still detectable indicating a heterokaryotic rather than a pure deletion strain. Therefore, one of them was crossed with a uracil-auxotrophic strain (SAS7) and progenies were tested for the integration event by Southern blotting. All uracil-prototrophic strains harboured the knock-out situation, whereas all uracil-auxotrophic strains displayed a wild-type band pattern (Fig. 2).

One strain (SNR1) was chosen for further comparison with a wild type (SNR3) obtained in the same cross (Fig. 3). The *kinA* deletion strain grew slower at 37°C and formed compact colonies. Surprisingly, growth of the mutant was better than wild type when compared at 25°C (Fig. 3). As deletion of conventional kinesin had an

impact on hyphal morphology, branching, chitin deposition, or vacuole and mitochondrial distribution in other fungi, we analysed these parameters in a wild-type and a $\Delta kinA$ strain. Unexpectedly, we could not observe a significant change in any of these parameters (results not shown). However, a pronounced difference in nuclear distribution was evident (Fig. 4). To analyse this effect in more detail, the $\Delta kinA$, *argB*⁻ strain (SNR1) was selected from the progeny of the above cross and transformed with a GFP construct targeting GFP to nuclei (SNR5) (Suelmann *et al.*, 1997). A wild-type strain, also derived from the above cross (SNR3), was transformed with the same construct (SNR6). In both germinating conidia and older hyphae, nuclei appeared clustered rather than evenly distributed (Fig. 4). This phenotype resembled the nuclear distribution phenotype of *aps* mutants (Suelmann *et al.*, 1998). In addition, the mean distance of the leading nucleus from the hyphal tip was shorter in the wild type (8 μ m) than in the kinesin mutant (16 μ m). To study an effect of the kinesin deletion on nuclear migration we followed nuclear division and distribution *in vivo*. Conidia were germinated on microscope slides and observed by video fluorescence microscopy. Pictures were taken every 5 min and then assembled into movie sequences (see *Supplementary material*). In the wild type, each conidium contained one nucleus which divided once or twice until the germ tube started to extend. The leading nucleus followed the growing hyphal tip at the same speed as the tip elongates (about 1 μ m per min at 37°C). The basal nuclei also migrated towards the tip, but paused on their way or even moved backwards for a short time period. This phenomenon is more pronounced in the filamentous fungus *Ashbya gossypii*, in which it is described as

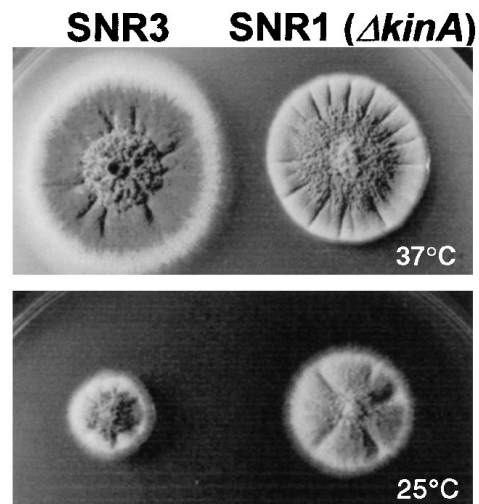


Fig. 3. Phenotype of *kinA* disruptant strains. One original *kinA* mutant strain was crossed with a wild-type strain. A wild type (SNR3) and a kinesin mutant strain (SNR1) were selected for comparison. Strains were grown at 37°C for 5 days or at 25°C for 3 days.

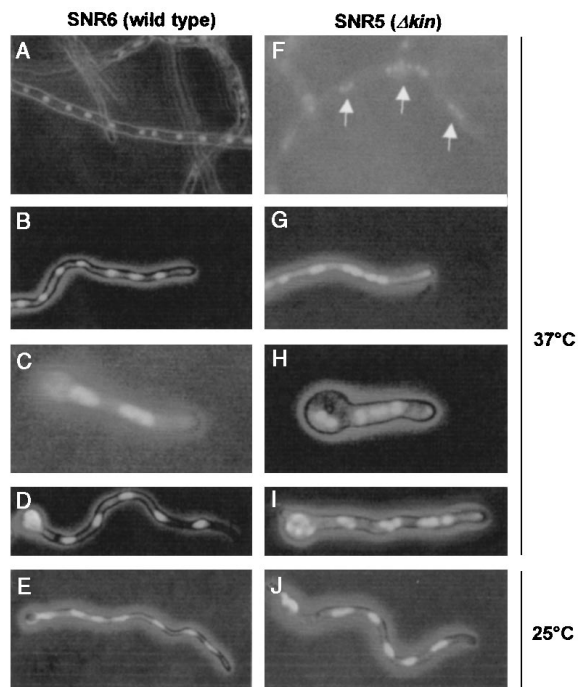


Fig. 4. Nuclear distribution in a wild type and a *kinA* mutant strain. A wild-type strain (SNR3) and a kinesin mutant strain (SNR1) were co-transformed with a sGFP construct, targeted to nuclei (pRS31) and a nutritional marker gene plasmid (pDC1). Fluorescing strains were selected and compared for nuclear distribution. Left = wild type (SNR6); right = $\Delta kinA$ strain (SNR5).

A, F. Nuclear distribution in older hyphal compartments.
B, G. Nuclei close to the hyphal tip.
C, H. Young germlings after 7 h of growth at 37°C.
D, I. Germlings after 9 h of growth.
E, J. Germlings of wild type and mutant strain at 25°C.

'oscillations' (Alberti-Segui *et al.*, 2001). As the germtube elongated further, nuclei multiplied nearly synchronously and redistributed evenly thereafter. Once eight nuclei were generated, the first septum was formed at the base of the germtube. In the kinesin mutant, nuclei divided two or three times before a germtube emerged. Small germlings therefore already contained many nuclei, whereas the wild type only contained two or four. Sometimes the entire cluster of nuclei moved out of the conidium and left the spore empty. Oscillations of nuclei still occurred. As the germtube extended, nuclei migrated towards the hyphal tip and appeared clustered rather than evenly distributed. As the slower growth phenotype of the mutant is rescued by incubation at lower temperature, we also analysed nuclear behaviour at 25°C. We found no significant differences between mutant and wild type with respect to the velocity of nuclear migration, distribution or oscillations (Table 1).

Conventional kinesin localizes to the cytoplasm

To determine the subcellular localisation of conventional kinesin in *A. nidulans* we constructed a C-terminal fusion

protein with GFP. The chimeric protein was expressed under the control of the inducible *alcA* promoter. *A. nidulans* GR5 was transformed with the construct pRF917 and pRG1 to uracil auxotrophy and transformants were screened for GFP fluorescence on the colony level after growth of the strains on threonine-containing plates. Positive strains were inoculated on glycerol-containing media to induce the *alcA* promoter to a medium level. Under these conditions the kinesin-GFP fusion protein was still visible in the fluorescence microscope and localized mainly to the cytoplasm (Fig. 5). However, in less than 1% of the germlings was a tubular structure visible, resembling the microtubule arrangement in the cell (Fig. 5B). Likewise it was shown that, in *U. maydis*, after treatment of the cells with CCCP (carbonylcyanide-*m*-chlorophenylhydrazine), the kinesin-GFP protein bound to microtubules (Lehmler *et al.*, 1997).

Dynein–kinesin double mutants display an additive phenotype

Dynein has been shown to be essential for nuclear migration in *A. nidulans* and, in addition, displays an effect on microtubule stability (Xiang *et al.*, 1994; Willins *et al.*, 1995). To study a possible interaction of dynein and conventional kinesin, we constructed a double mutant. A temperature-sensitive dynein strain (XX3) was crossed to the kinesin deletion mutant (SNR1) and three double mutants selected from the progeny. The correct genotype was confirmed through back-crosses and by Southern blotting (results not shown). The three double mutants

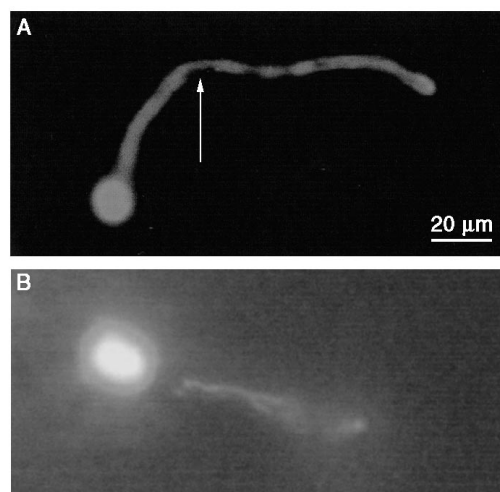


Fig. 5. Subcellular localisation of conventional kinesin as a KinA-GFP fusion protein.

A. The fusion protein is evenly distributed in the cytoplasm and vacuoles are not stained (arrow).
B. In some germlings a filamentous structure resembling microtubule filaments was observed.

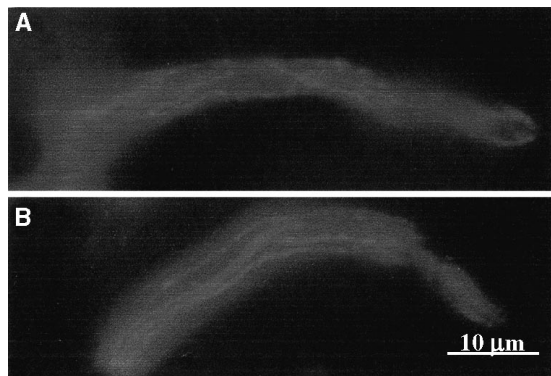


Fig. 8. Microtubules in hyphae of SNR3 (A) and SNR1 (B). Germlings were fixed and processed for secondary immunofluorescence as described (see *Experimental procedures*).

These results suggest that conventional kinesin mutation stabilizes microtubules. Despite these results, there was no apparent effect on the number or the integrity of cytoplasmic microtubules in hyphae of the kinesin mutant compared with wild type using immunofluorescence microscopy (Fig. 8). However, subtle differences in the arrangement and changes in microtubule dynamics would not be detectable using this method.

To further probe a potential link between conventional kinesin and microtubule stability *in vivo*, we studied the effect of the kinesin deletion on the cold sensitivity of a *tubA4* α -tubulin mutant. Strains carrying the *tubA4* allele

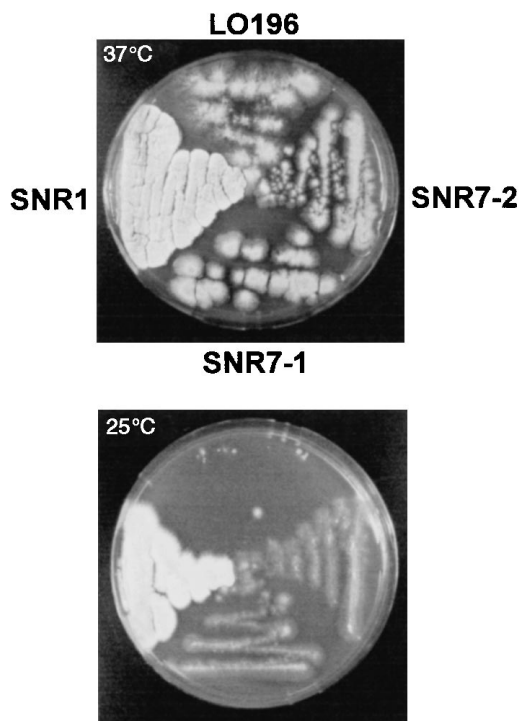


Fig. 9. Growth of a kinesin mutant (SNR1), a cold sensitive α -tubulin mutant (LO196) and two kinesin/tubulin double mutants (SNR7) at 37°C and 25°C.

are cold-sensitive but the effect can be rescued in the presence of the microtubule-stabilizing agent deuterium oxide (Oakley *et al.*, 1987). We crossed the $\Delta kinA$ strain (SNR1) with the tubulin mutant strain LO196. As the *tubA4* strain is also affected in conidiospore formation and appears brown on agar plates, mutant colonies can easily be distinguished from wild type. Therefore, we selected strains from the progeny which combined the phenotype of the two parents, namely compact colonies at 37°C and no conidiospores (SNR7). We confirmed the double-mutant genotype through back-crosses to a wild-type strain and by Southern blot (result not shown). Interestingly, the kinesin mutation rescued the cold sensitivity of the α -tubulin defect (Fig. 9).

Discussion

Polarized cells such as neurons or filamentous fungi are attractive models for the study of long-range organelle movement. In this context, the motor protein conventional kinesin appears to play a role in the anterograde transport of vesicles (Hirokawa, 1998; Kirchner *et al.*, 1999b). Whereas in neurons one of the cargoes for conventional kinesin has just been identified as scaffolding proteins for the JNK signalling pathway (Bowman *et al.*, 2000; Verhey *et al.*, 2001), in the case of filamentous fungi, cell wall precursors or enzymes involved in cell wall metabolism might be transported. We analysed the function of conventional kinesin in the filamentous fungus *A. nidulans* and found that it supports hyphal growth and contributes to nuclear distribution, conceivably by affecting microtubule stability.

To allow hyphal extension in fungi, vesicles first travel towards an organelle close to the hyphal apex, called the 'Spitzenkörper' (Grove and Bracker, 1970; Riquelme *et al.*, 1998). This organelle has been studied in great detail in *N. crassa* and *Aspergillus niger*. The location of this structure determines the growth direction and its size correlates with the speed of tip extension (Riquelme *et al.*, 2000). Thus, in conventional kinesin mutants of *N. crassa*, which grow slower than wild type, the Spitzenkörper has a reduced size and newly deposited cell wall material was detected as patches along the hyphae rather than at the hyphal tip, suggesting a fusion of vesicles along hyphae. The authors concluded from these results that the conventional kinesin motor protein is directly involved in vesicle transport towards the Spitzenkörper (Seiler *et al.*, 1997). In *A. nidulans*, the absence of kinesin also provokes a reduction in hyphal growth rate but we did not observe any deposition of cell wall material along hyphae. This could be as a result of the different growth rates of both fungi. *N. crassa* grows faster than *A. nidulans* and hence also has a larger Spitzenkörper, reflecting a very active secretion process. As both fungi still grow in the absence

of the conventional kinesin motor protein, it is probable that other motors are able to take over at least part of the function. This, in combination with a reduced secretion in *A. nidulans*, might explain the absence of cell wall patches along the hyphae in contrast to *N. crassa*.

Interestingly, a temperature drop rescued the growth defect of *A. nidulans* kinesin mutants, which grew even better than wild-type strains under these conditions. If the only role of conventional kinesin is to bind the cargo and transport it along microtubules, it is unlikely that a lower temperature will restore the phenotype. This led us to consider that conventional kinesin could play an additional role in microtubule stability and that a certain degree of stability is required for efficient hyphal tip elongation. Two experiments supported this hypothesis. First, kinesin mutants were less sensitive towards the microtubule-destabilizing drug benomyl, and, second, disruption of the motor gene suppressed a mutation in the α -tubulin gene, *tubA4*. Oakley *et al.* (1987) found that the microtubule-stabilizing agent deuterium oxide, which acts by reinforcing hydrophobic interactions between tubulin molecules, counteracts the mutation in the *tubA4* allele. As the kinesin loss-of-function mutation also rescued the *tubA4* defect, we speculate that kinesin destabilizes microtubules by weakening the hydrophobic forces between tubulin subunits. Whether microtubules are destabilized at the ends, as recently it was described for KIN1, remains to be determined (Desai *et al.*, 1999).

A second striking phenotype of kinesin-deficient *A. nidulans* strains is the misdistribution of nuclei in germlings and in hyphae. Nuclear migration is dependent on microtubules and the motor protein dynein (reviewed in Fischer, 1999; Morris, 2000). In dynein mutant strains, most nuclei do not migrate out of the conidiospore. Because some nuclei still move, a kinesin motor was speculated to contribute to nuclear migration (Xiang *et al.*, 1995). We observed that, in the conventional kinesin mutants, nuclei do migrate out of the spore albeit in a different way than in wild type. Nuclei sometimes moved in a cluster, reversed or became clustered as the germ tube extended. This phenotype was temperature dependent and rescued at 25°C, again suggesting changes in microtubule stability. There is an increasing body of evidence that microtubule stability is important for nuclear migration and positioning. Thus, in *S. cerevisiae* different kinesin motors were found to be involved in certain steps of nuclear distribution and affect microtubule stability. Kip2 stabilizes and Kip3 destabilizes microtubules (Cottingham and Hoyt, 1997; Miller *et al.*, 1998). Another mutation in *S. cerevisiae* which disturbs microtubule stability is *num1* (= nuclear migration). *NUM1* encodes a large PH-domain containing protein that localizes in a cell-cycle dependent manner to the cortex. In *num1* deletion strains, binucleate mother cells are produced and astral microtubules are

very long (Kormanec *et al.*, 1991; Farkasovsky and Küntzel, 1995). Recently, it was shown that the protein interacts with the dynein intermediate chain (Farkasovsky and Küntzel, 2001). Interestingly, mutation of a homologue of Num1p in *A. nidulans*, *ApsA*, affects nuclear positioning in a similar way to the conventional kinesin mutation does, i.e. nuclei do migrate but they are clustered in hyphae (Fischer and Timberlake, 1995; Suelmann *et al.*, 1998). Whether *ApsA* indeed affects microtubule stability in *A. nidulans* has not yet been demonstrated. Another example of a link between nuclear movement and microtubule dynamics in *A. nidulans* is the phenotype of the nuclear migration mutants, *nudA* and *nudF*. *nudA* encodes the heavy chain of the dynein motor and deletion of the gene blocks nuclear migration (Xiang *et al.*, 1995). The effect can be reversed by addition of benomyl to the media and through a mutation in α -tubulin (Willins *et al.*, 1995). This is very similar to the findings with conventional kinesin reported here. Similarly, *NUDF* is also involved in the maintenance of microtubule stability (Han *et al.*, 2001). A human homologue of this protein, *LIS1*, which is required for brain development, was shown recently to reduce microtubule catastrophe events (Sapir *et al.*, 1997).

An important question to consider is how microtubule dynamics might affect nuclear positioning. In *S. cerevisiae*, growth and shrinkage of microtubules contributes to the movement of the nucleus within the mother cell and the positioning at the bud neck prior to mitosis. After mitosis, astral microtubules elongate into the bud cell and search the cortex. Once they make contact, they start to shrink and the daughter nucleus migrates into the bud (Carminati and Stearns, 1997; Shaw *et al.*, 1997). It is not surprising that mutations changing the stability of microtubules influence these movements. In filamentous fungi the contribution of microtubule dynamics to nuclear positioning has not yet been studied in detail. However, Plamann *et al.* (1994) have suggested an attractive model for how nuclear movement could occur, which is similar to a model proposed for nuclear migration in the pre-blastoderm of *Drosophila* (Baker *et al.*, 1993). According to this model, nuclei are connected through overlapping, antiparallel microtubules, emanating from two adjacent nuclei. The first nucleus requires anchoring to the growing hyphal tip. A microtubule sliding mechanism would lead to movement of the nuclei. If forces pulling on each side of a nucleus would be equal, no movement would occur and the nucleus would remain at its position. In this case, the position of the nucleus would be rather labile and could be easily disturbed, e.g. by changing the stability of the connecting microtubules. This model could explain the oscillations of single nuclei we have observed in our experiments for both wild type and the kinesin mutant. Experiments of McKerracher and Heath (1986) seem to further prove this model. They disturbed the microtubules

by UV microbeam irradiation in the fungus *Basidiobolus magnus* and found that anterior irradiation (ahead of the nucleus) caused the nucleus to stop or move backwards, and posterior irradiation caused acceleration of the nuclear velocity (McKerracher and Heath, 1986). However, other mechanisms for nuclear migration in filamentous fungi have been proposed, which also require the integrity of the microtubule cytoskeleton. According to a second model, nuclei could move along microtubules with dynein associated to the nuclear envelope, analogous to vesicle movement. This situation has been described for female pronuclear migration in fertilized *Xenopus* egg cells (Reinsch and Gönczy, 1998). However, dynein has not yet been detected at the nucleus but at the hyphal tip and as moving comet-like structures in the cytoplasm (Xiang *et al.* 2000). GFP-labelled versions of dynein and of microtubules are now available and should allow the discrimination between different models of nuclear migration in *A. nidulans* (<http://www2.umdj.edu/rmlabweb/index.html>) (Xiang *et al.*, 2000, Han *et al.*, 2001). It will be the challenge for further studies to determine the exact contribution of microtubule stability to nuclear migration and polarized growth.

Experimental procedures

Strains, plasmids and culture conditions

Supplemented minimal and complete media for *A. nidulans* were prepared as described, and standard strain construction procedures were used (Käfer, 1977). A list of *A. nidulans* strains used in this study is given in Table 2. Standard laboratory *Escherichia coli* strains (XL-1 blue, BL21, Top 10 F') were used. Plasmids and cosmids are listed in Table 3.

Molecular techniques

Standard DNA transformation procedures were used for

A. nidulans (Yelton *et al.*, 1984) and *E. coli* (Sambrook *et al.*, 1989). For polymerase chain reaction (PCR) experiments, standard protocols were applied using a capillary Rapid Cycler (Idaho Technology) for the reaction cycles. DNA sequencing was done commercially (MWG Biotech). Genomic DNA was extracted from the fungus using the DNeasy Plant Mini Kit (Qiagen). RNA was isolated with TRIzol[®] from GibcoBRL according to the manufactures protocol (GibcoBRL Life technologies). DNA and RNA analyses (Southern and Northern hybridizations) were performed as described (Sambrook *et al.*, 1989).

cDNA cloning. After cDNA generation using the SMART kit (Clontech) *kinA*-specific partial cDNAs were amplified with the following oligonucleotides: AnKin5'For (5'-ATACCCGTCCAGTCCTGG-3') plus AnKin5'Rev (5'-ATACGACTTTCCTGCACCGG-3'), AnKinStart1 (5'-GATGTAAAACCTGATACATTC-3') plus AnKin4a (5'-CTTCGGATTCCAGTGC-3'), AnKin5 (5'-CCGCCACGGAGAGGC-3') plus AnKin6 (5'-CTAGAGATCTCGCTCGAC-3').

Expression of *KinA* in *E. coli*. A complete *KinA* encoding cDNA was constructed by amplifying one 5' fragment including the ATG and one 3' fragment including the stop codon with cDNA as template and proofreading Expand polymerase (Boehringer). The middle part of the gene, which does not contain any intron, was introduced as a 1.8 kb *Bgl*II fragment between the 5' and 3' fragments by standard cloning. The primers for cloning were for the 5' fragment: AnKin5'For and AnKin5'Rev (see above). For the 3' fragment: Ankin6 (see above) and Ankinstop (5'-TCATCGTCTATCAAAAACCAACT-3'). The complete cDNA was amplified with primers adding *Nde*I and *Mfe*I restriction sites to the ends for cloning into the *E. coli* expression vector pT7-7. The final clone (pT7-7AnKin) was sequenced and transfected into *E. coli* BL21. The cells were grown in the presence of 100 µg ml⁻¹ ampicillin at 37°C and expression induced by adding IPTG (3 mM) and incubation at 21°C for 15 h as described in Kallipolitou *et al.* (submitted). In brief, cells were harvested, broken by sonication and cell debris removed by centrifugation at 100 000 *g* for 35 min at 4°C. Further purification was achieved with SP-sepharose using a NaCl step gradient in a phosphate buffer (0.1 M Na₂HPO₄/0.1 M NaH₂PO₄, pH 7.4).

Table 2. *A. nidulans* strains used in this study.

Strain	Genotype	Source
FGSC26	<i>biA1; veA1</i>	FGSC, Kansas, USA
SRF200	<i>pyrG89; ΔargB::trpCΔB; pyroA4; veA1</i>	Karos and Fischer (1996)
SAS7	<i>pyrG89, pabaA1, yA2; veA1</i>	A. Singh, Marburg
GR5	<i>pyrG89; wA3; pyroA4; veA1</i>	Waring <i>et al.</i> (1989)
LO196	<i>pabaA1, proA1, adE20, yA2; wA3; pyroA4; tubA4, veA1</i>	N.R. Morris, Piscataway, USA
SNR1	<i>yA2; ΔargB::trpCΔB; pyroA4; veA1; kinA::pyr4</i> (SRF200 transformed with pRF645 crossed to SAS7)	This study
SNR3	<i>pyrG89, yA2; ΔargB::trpCΔB; veA1</i> (SRF200 transformed with pRF645 crossed to SAS7)	This study
XX3	<i>pyrG89; nudA1, chaA1, veA1</i>	N. R. Morris, Piscataway, USA
SNR5	SNR1 transformed with pRS31 and pDC1	This study
SNR6	SNR3 transformed with pRS31 and pDC1	This study
SNR7	<i>wA3; pyroA4; tubA4, veA1; kinA::pyr4</i> cross between LO196 and SNR1	This study
SNR8	<i>yA2; ΔargB::trpCΔB; pyroA4; veA1, nudA1; kinA::pyr4</i>	This study
SNR9	SNR8 transformed with pRS31 and pDC1	This study
SRF479	GR5 transformed with pRG1 and pRF917	This study

Table 3. Plasmids used in this study.

Cosmids/Plasmids	Construction	Source
PUC18	Cloning vector	MBI Fermentas, St. Leon-Rot
PCR 2.1 TOPO	PCR Cloning vector	Invitrogen (NV Leek, The Netherlands)
pDC1	<i>A. nidulans argB</i> gene in pIC20R	Aramayo <i>et al.</i> (1989)
pEW15	<i>kinA</i> -containing cosmid from pKBY library	This study
pEW21	5.2 kb <i>HindIII</i> fragment of pEW15 cloned into PUC18	This study
pRF647	4.3 kb <i>BamHI</i> fragment of pEW15 cosmid cloned into PUC18	This study
pRF595	<i>pyr4</i> gene PCR amplified and cloned into PCR 2.1 TOPO	This study
pT7-7AnKin	cDNA of <i>kinA</i> cloned in <i>E. coli</i> expression vector pT7-7	This study
pRF917	<i>alcA(p)::kinA::sgfp</i> fusion construct	This study
pNR637	<i>kinA</i> upstream fragment A cloned into PCR 2.1 TOPO	This study
pNR636	<i>kinA</i> downstream fragment B cloned into PCR 2.1 TOPO	This study
pRF645	Two fragments upstream (A) and downstream (B) of <i>kinA</i> start codon cloned around <i>pyr4</i>	This study
pRS31	sGFP fusion protein targeted to nuclei	Suelmann <i>et al.</i> (1997)

The conventional kinesin protein eluted between 0.1 and 0.2 M NaCl.

Cloning of the *kinA* disruption construct (pRF645). Two DNA fragments located upstream and downstream of the *kinA* coding start codon were PCR amplified with the following primers: fragment A (0.9 kb), AnKin1a (5'-GTCCCACTTATG ATGTCGC-3') plus AnKin2 (5'-CGGGCGTTGCAGGAACAC-3') and fragment B (1 kb), AnKin3 (5'-AGCGCCGCCA CTACGTATCTGGC-3') with a *NotI* site added plus AnKin4 (5'-AGGTACCCGGTTTACCACACTGC-3') with an added *KpnI* site. The two fragments have been cloned around the *N. crassa* marker gene *pyr4* cloned into TOPO (SRF595). The final plasmid was cut with *PvuII*, which releases the entire construct plus some vector border sequences, and transformed into *A. nidulans* SRF200.

GFP-labelling of *KinA* (pRF917). Amplification of the *KinA* open reading frame using primers AnKin7 (5'-CTCGATA TCCTTCAAAAACCGCTGG-3') and AnKin8 (5'-CGTCTAT CAAAAACCAACTTGTGC-3'), Expand polymerase (Boehringer) and pEW15 as template was carried out. The PCR fragment was cloned into TOPO. The stop codon was not included into the primer as translation continues into the polylinker. The *alcA* promoter was cloned upstream of *kinA* as a *BamHI* fragment and *sgfp* as a *XbaI* restriction fragment downstream into the polylinker of TOPO.

Fluorescence microscopy. Observation of germinating spores in the fluorescence microscope was essentially done as described earlier (Hoepfner *et al.*, 2000). Vacuoles were stained with the fluorescent dye Oregon green as described. Mitochondria were visualized by GFP as shown in a previous paper (Suelmann and Fischer, 2000a).

ATPase assay. A coupled ATPase assay was used as described (Grummt *et al.*, 1998b). The assay was carried out with microtubule concentrations varying between 0 and 15 μ M. The assay mixture contained 20 μ M taxol and 1 mM ATP in ACES buffer (12.5 mM ACES/KOH, pH 6.8, 2 mM Mg-acetate, 3 mM MgCl₂, 0.5 mM EGTA). The assay was performed at 25°C at a protein concentration of 26.5 nM. Protein concentration was determined with the Bradford assay (Bradford, 1976). k_{cat} values were determined by fitting a hyperbolic curve to a plot of microtubule concentration against the ATPase rate.

Gliding assay. The gliding velocity of microtubules moved by *A. nidulans* kinesin attached to the surface of a coverslip

was determined essentially as described (Steinberg and Schliwa, 1996).

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Supplementary material

The following material is available from <http://www.blackwell-science.com/products/journals/suppmat/mole/mole2609/mmi2609sm.htm>

Movies showing the effects of kinesin deletion on nuclear migration.

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