Kinesin from the plant pathogenic fungus *Ustilago maydis* is involved in vacuole formation and cytoplasmic migration

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SUMMARY

A gene encoding the heavy chain of conventional kinesin (*kin2*) has recently been identified in the dimorphic fungus *Ustilago maydis* (Lehmler et al., 1997). From the phenotype of *kin2* null-mutants it was concluded that Kin2 might be involved in vesicle traffic towards the tip. However, this model did not explain why *kin2*-null mutant hyphae were unable to create empty cell compartments that are normally left behind the growing tip cell. Here we present a re-investigation of the function of Kin2 in hyphae and sporidia. We provide evidence that suggests a different and unexpected role of this kinesin motor in hyphal growth of *Ustilago maydis*. In addition, Kin2 was partially purified from *U. maydis* and in vitro properties were investigated. Isolated kinesin supported in vitro microtubule gliding at speeds of up to 1.8 μm/second, and showed motility properties and hydrodynamic behavior similar to those described for kinesin from *N. crassa*. It appears to be the product of the *kin2* gene. Compared with wild-type sporidia, the *kin2*-null mutant sporidia grew normally but were defective in accumulation of Lucifer Yellow in their vacuoles, which were smaller than normal and often misplaced. The dikaryotic hyphae, produced by the fusion of two *kin2*-null sporidia, showed tip growth, but unlike wild-type hyphae, these structures lacked the large, basal vacuole and contain significantly more 200-400 nm vesicles scattered over the hole hypha. This defect was accompanied by a failure to generate regular empty cell compartments that are left behind in wild-type tip cells as the hyphae grow longer. These results suggest that Kin2 is a microtubule-dependent motor enzyme which is involved in the formation of vacuoles. The accumulation of these vacuoles at the basal end of the tip cell might be crucial for the formation of the empty sections and supports cytoplasmic migration during the growth of dikaryotic hyphae.

Key words: Kinesin, Null mutant, Endocytosis, Organelle transport, Fungus, Smut, Dimorphism, Tip growth, Molecular motor, *Ustilago maydis*

INTRODUCTION

Mechanoenzymes of the kinesin type convert chemical energy into movement along microtubules (for reviews see Schroer and Sheetz, 1991; Bloom and Endow, 1994). The founding member of this large superfamily of motor proteins, 'conventional' kinesin, was first isolated from animal sources (Vale et al., 1985; Brady, 1985; Scholey et al., 1985) and has been intensely investigated over the last decade. Several lines of evidence suggest a crucial role for conventional kinesin in organelle transport (Vale et al., 1985; Brady, 1985; Schroer et al., 1988; Marks et al., 1994; reviewed by Schroer and Sheetz, 1991), although its cargo and precise function are still matters of debate (Nakata and Hirokawa, 1995; reviewed by Bloom and Endow, 1994).

For several reasons, filamentous fungi are well suited for the study of molecular motors: (1) hyphal growth occurs at the tip (reviewed by Gow, 1995a) and requires vectorial transport of vesicles along filamentous components of the cytoskeleton to the apex, which is the actively growing region (for reviews see McKerracher and Heath, 1987; Heath, 1995), (2) up to several grams of material can be cultivated easily, so native fungal motors can be purified and biochemically characterized (Steinberg and Schliwa, 1995, 1996; Steinberg, 1997), and (3) genetic techniques can be used to study the function of motors in vivo (Plamann et al., 1994; Xiang et al., 1994; Lehmler et al., 1997; Seiler et al., 1997; Wu et al., 1998; Yamashita and May, 1998b; overview in Yamashita and May, 1998a; Steinberg, 1998).

The dimorphic basidiomycete *Ustilago maydis* is a plant pathogen. Under laboratory conditions haploid, yeast-like sporidia can be propagated and are accessible to both genetic and molecular methods (reviewed by Banuett, 1995). On maize leafs two haploid sporidia of different mating type can fuse to form a filamentous dikaryotic hypha. This form invades the plant tissue and induces tumors in meristematic tissues. Fungal

proliferation in these tumors is followed by karyogamy, meiosis, and spore formation (Snetselaar and Mims, 1993, 1994; reviewed by Banuett, 1995). Cell fusion and dikaryon formation can be induced on charcoal nutrient plates (Day and Anagnostakis, 1971). However, outside of a plant these dikaryotic form grows for a limited time only. Typical for smut fungi, dikaryotic mycelium consists of a single binucleated tip cell that leaves empty-looking, septated cell wall compartments behind while it migrates to the locus of invasion (reviewed by Fischer and Holton, 1957).

Recently, the *kin2* gene was identified and cloned from *Ustilago maydis* (Lehmler et al., 1997). Its predicted amino acid sequence displayed a high sequence similarity with Nkin, the conventional kinesin from *N. crassa* (Steinberg and Schliwa, 1995). The subsequent investigation of *kin2-null* strains revealed several defects in hyphal growth and organization, but no changes in kinesin-deficient sporidia were found (Lehmler et al., 1997). Of these defects the inability to form empty sections at the distal end of the cell was the most obvious one, and it was suggested that this impairment results in reduced pathogenincity. On the other hand, GFP-localization studies and electron microscopy of the tip region suggested a role in apical vesicle transport (Lehmler et al., 1997). Therefore, the authors deduced that Kin2 might be involved in membrane traffic towards the hyphal tip, a conclusion that was in line with results from Nkin-deficient strains of *N. crassa* (Seiler et al., 1997).

A role for Kin2 in apical organelle transport might be in agreement with an expected role for organelle motors in supporting the growing tip region. However, it remained unexplained how a motor for the apical transport could participate in the formation of empty sections at the opposite end of the elongated tip cell. The present study was undertaken to extend our understanding of kinesin's function in membrane traffic and growth in *Ustilago maydis*. The behavior of *kin2-null* mutants indicates that kinesin takes part in the organization and formation of vacuoles in sporidia and hyphae of *Ustilago maydis*. The formation of basal vacuoles might be necessary for the appearance of the empty compartments and, thereby, Kin2 is supporting the migration of the cytoplasm within the cell wall tube toward the hyphal tip.

MATERIALS AND METHODS

Strains and culture conditions

U. maydis strains FB1 (*a1b1*), FB2 (*a2b2*), and diploid FBD11 (*a1a2b1b2*) have been described (Banuett and Herskowitz, 1989). The haploid null-mutant strains in the *U. maydis* kinesin, FB1Δ*kin2*, FB2Δ*kin2*, as well as the diploid strain CLD12 (*a1a2b1b2ΔΔkin2*) were described by Lehmler et al. (1997). The cells were stored on nutrient plates containing 2.5% (w/v) potato dextrose (Difco) and 2% (w/v) bacto-agar (Difco; further named PD-plates); mating plates were supplemented with 1% (w/v) activated charcoal (Sigma, 9157). Liquid cultures of yeast-like sporidia were grown in 1% (w/v) yeast extract, 0.4% (w/v) bacto-peptone, and 0.4% (w/v) sucrose (YEPS, modified from Tsukuda et al., 1988) or 3% (w/v) glucose (YEPG), or a minimal medium with 5% (w/v) glucose and 1 mM phosphate (modified from Holliday, 1974) at 26-31°C with gentle shaking. For mating, cells were grown overnight in CM/1% glucose (Holliday, 1974; Banuett and Herskowitz, 1989), centrifuged at 10,000 *g* for 2 minutes and washed with 2 volumes of sterile water. Then about the same number of cells of each mating type was mixed and spread out on PD plates, containing 1% charcoal (Sigma). After 19-21 hours of incubation at room temperature the resulting dikaryons were used for further experiments.

Preparation of microtubules and assay of in vitro motility

Microtubule protein was prepared from fresh pig brain, using three cycles of polymerization and depolymerization according to the method of Shelanski et al. (1973). Tubulin was purified from this mixture by ion exchange chromatography, using phosphocellulose according to the method of Weingarten et al. (1975) with modifications previously described (Steinberg and Schliwa, 1995). Microtubules (MTs) were polymerized from pig brain tubulin at a concentration of 2-6 mg/ml in the presence of 10% DMSO and 1 mM GTP for 30 minutes at 32°C. For gliding assays MTs were diluted to 0.5-0.7 mg/ml in AP100 (100 mM Pipes, pH 6.9, 2 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml Nα-*p*-tosyl-L-arginine-methylester (TAME), 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 10 μg/ml pepstatin, 25 μg/ml leupeptin) containing 7 μM taxol, briefly vortexed, and stored at room temperature for 1-4 days. MT gliding activity was measured as described by Steinberg and Schliwa (1995). To assure the results would be comparable with previously determined values for the *Neurospora* kinesin, care was taken to comply with all criteria previously described for this assay (Steinberg and Schliwa, 1996).

Isolation of kinesin and determination of its hydrodynamic behavior

U. maydis cells were grown in YEPS overnight as described above. Cells were centrifuged at 1,000 *g* for 10 minutes and the pellets were resuspended in AP100 buffer. The cells were disrupted in a French Press at 24,000 PSI, and this homogenate was depleted of cell debris and organelles by centrifugation at 10,000 *g* for 20 minutes followed by a centrifugation at 120,000 *g* for 1 hour. The protein concentration of the high speed supernatant (S2) was measured according to the method of Bradford (1976; catalase as a standard) and diluted with AP100 (see above) to 7 mg/ml. Further purification of kinesin was performed as described by Steinberg and Schliwa (1996). Briefly, S2 was supplemented with taxol-stabilized MTs in the presence of 0.5 mM adenosine-5′-[β,γ-imido]-triphosphate (AMPPNP) and apyrase to deplete the endogenous ATP. MTs with associated proteins were pelleted by centrifugation, resuspended in AP100; the attached motor enzymes were released with 5 mM-10 mM MgATP and separated from the MTs by centrifugation. In preparation for kinesin purification from hyphae, cells were mated on charcoal plates and grown for 24 hours. The hyphae were disrupted by grinding with quartz-sand in the presence of buffer, as described previously (Steinberg and Schliwa, 1995). As seen by light microscopy, this treatment did not disrupt contaminating sporidia, which were always present in the material harvested from agar plates.

The Stokes radius and sedimentation coefficient of Kin2 were determined with protein from 3 independent isolations, following previously described procedures (Steinberg and Schliwa, 1996). As Kin2 was a minor protein in the ATP-release (S5), the peak fraction from gel filtration or sucrose density centrifugation was determined by western blotting using the antibody NKC, which was raised against a 17 amino acid sequence region in the C terminus of the *N. crassa* kinesin (Steinberg, 1997) that is almost identical to the comparable sequence stretch of Kin2 (Lehmler et al., 1997). The native molecular mass and the axial ratio was calculated as described (Steinberg and Schliwa, 1996).

Gel electrophoresis and western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) with modifications according to Garfin (1990). The polyclonal NKC was affinity purified essential as described by Olmsted (1986), and western blots were done as previously described (Steinberg and Schliwa, 1995).

Determination of growth rates

The influence of *kin2* deletion on the growth of sporidia was investigated using liquid cultures at 31°C. After inoculation of 3×10 ml YEPS, YEPG or minimal medium/5% glucose/1 mM phosphate from an overnight culture, the turbidity at 595 nm was monitored over 9-24 hours. The mean value from 2-3 cultures was determined at each time and the doubling time was estimated by non linear regression using the program PRISM (GraphPad, SanDiego, USA). To determine the survival rate on agar plates the same number of cells was spread out on PD-plates, and colonies were counted after 2 days at 25°C. For determination of rates of tip-growth and vacuole expansion, sporidia were mated on charcoal plates. After 12-14 hours a colony of young hyphae was placed on poly-L-lycine coated coverslips and grown for an additional 10 hours. Elongation rates were then assessed by 5-15 minutes observation in water. For growth under different osmotic conditions, hyphae were grown on poly-L-lysine for 10 hours, followed by embedding in 0.5% low melting agarose (precooled to 30-35°C), containing 50 mM, 250 mM or 500 mM sucrose or sorbitol. After 40 hours the distance of single hyphae from the edge of the colony was measured, using the MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA, USA). Statistical analysis of data was done using appropriate programs in the PRISM package (GraphPad Inc., San Diego, USA). Values are given as mean ± standard deviation and sample size, and data sets were compared using unpaired two-tailed *t*-tests.

Endocytosis assays and vacuole staining

Endocytotic activity was observed using Lucifer Yellow, an inert, water soluble marker that is internalized with the fluid phase (Riezman, 1985) and FM4-64 (Molecular Probes), a vital dye that was used to monitor the endocytotic pathway in yeast cells (Vida and Emr, 1995). Lucifer Yellow did not pass the cell wall of *Ustilago* sporidia, so protoplasts were prepared by incubation in 20 mM sodium citrate, pH 5.8, 1 M sorbitol and 3 mg/ml novozyme (Novolabs). After 30 minutes at 30°C the protoplasts were washed in YEPS, 1 M sorbitol, pH 5.8, and incubated in 100 µl fresh YEPS, 1 M sorbitol, 3 mg/ml Lucifer Yellow-CH (Sigma) for 1-2 hours at 30°C. This was followed by washing the protoplasts with ice-cold sodium acetate, pH 5.6, 1 M sorbitol. The cells were embedded in 0.7% low melting agarose and observed by epifluorescence microscopy. For FM4-64 staining growing cells were incubated with 1 µg/ml FM4-64 in YEPS at room temperature for 15 minutes. This solution was replaced by YEPS, and cells were kept at room temperature under gentle shaking for additional 30 minutes, and observed by epifluorescence microscopy.

The acidic compartments of sporidia were stained using Neutral Red (Sigma), Acridine Orange (Sigma), and a yeast vacuole marker kit (Molecular Probes), which included CellTracker™blue CMAC (7-amino-4-chloromethyl-coumarin), and carboxy-DCFDA (5-[and-6]-carboxy-2′,7′-dichloro-fluorescein diacetate). Cells were sedimented at 500 *g* for 2 minutes, resuspended in 50 mM sodium citrate, pH 5.0, with 2% glucose (for carboxy-DCFDA staining) or Hepes, pH 7.4, with 5% glucose for other dyes, then incubated in 3-10 µM buffered dye for 15-30 minutes at 30°C. After two washes with buffer, the cells were embedded in buffered 0.7% low melting agarose precooled to 30-35°C, and analyzed by fluorescent light microscopy. The staining of vacuoles in protoplasts was accomplished with CellTracker™blue CMAC in YEPS, pH 7.4, as described for Lucifer Yellow above. Unfortunately, all attempts to stain the vacuoles in hyphae failed, so vacuoles in theses cells were identified by their characteristic appearance under differential interference contrast (DIC) optics and by electron microscopy.

Indirect immunofluorescence of MTs and polymerization assays

For indirect immunofluorescence of MTs in hypha, diploid sporidia were grown overnight in CM as described above. Cells were sedimented at 1,000 *g* and resuspended in water. After 12 to 24 hours in water on poly-L-lysine coated coverslips the cells formed long hyphae. The cells were washed with water and fixed for 20 to 45 minutes in 50 mM Pipes, pH 6.8, 25 mM EGTA, 5 mM MgSO4, 1% (v/v) formaldehyde, and 0.125% (v/v) glutaraldehyde. After rinsing 3 times with PEM (50 mM Pipes, pH 6.8, 5 mM EGTA, 5 mM MgSO4) the cell wall was digested for 60 minutes at room temperature with 3 mg/ml novozyme in PEM with 1 mM PMSF, 10 µg/ml TAME, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml aprotinin, 1 µg/ml pepstatin. This was followed by rinsing 3 times with PBS, pH 7.2 and 2 minutes in PBS with 1% Triton X-100 and 5 mM EGTA. The glutaraldehyde was quenched with 2 mg/ml borohydride in PBS for 10 minutes followed by a PBS wash. The cells were incubated with primary antibody (mouse anti-tubulin; N356, Amersham) for 45 minutes at room temperature. This was followed by three washes with PBS, a 45 minute incubation with the secondary antibody (Cy2-conjugated goat anti-mouse), and additional PBS treatment. In sporidia best staining of MTs was achieved by adding formaldehyde to a final concentration of 6% to a logarithmic growing culture. After 30 minutes incubation the cells were washed three times with PBS and fixed onto poly-L-lysine coverslips. Further treatment was done as described above, exept that cell wall digestion was done for 20 minutes at room temperature, antibody incubation was done for 1-3 hours at 36°C and borohydride treatment was left out.

For depoymerization/repolymerization experiments diploid FBD11 cells and dicaryotic hyphae were grown on poly-L-lysine coverslips and incubated in 10 µM nocodazole in PBS for 30 minutes at room temperature. Immediately after release from nocodazole by incubation in PBS for 5 to 30 minutes cells were fixed and stained for MTs.

Electron microscopy

3 ml of YEPS were innoculated with 100 µl of an overnight grown liquid culture of diploid sporidia (FBD11, CLD12) and grown at 27°C until they reached an OD_{595} of 0.4-0.6. Cells were sedimented at 1,000 *g* for 2 minutes and resuspended in an equal volume of water. 100 µl of this suspension was placed on poly-L-lysine coated coverslips and hyphae were grown overnight in a humid chamber. The cells were fixed in 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer, pH 7.2, for 4 hours, followed by 3 rinses with buffer and a postfixation with 2% (w/v) in $KMnO_4$ in water for 1 hour. After extended washing, the cells were stained with 0.5% uranyl acetate overnight and dehydrated in several acetone/water dilution steps over 1.5 hours. These preparations were infiltrated with Spurr's resin over 2 days. The plastic polymerized at 70°C, then sections were cut, stained with lead citrate and uranyl acetate and investigated using a Philips CM-10 electron microscope at 80 kV. Quantifications were done on prints at a magnification of ×32,500. Vesicles were counted in 3 µm², using the magnification available from calibrations with a replica geating.

RESULTS

Kin2 from *Ustilago maydis* shares properties with kinesin from *Neurospora crassa*

Kin2 protein was partially purified from several haploid strains, as well as from dikaryotic mycelia, as described in Materials and Methods. Our isolation procedure yielded a fraction (S5) with robust MT-gliding activity. MTs moved at ~1.5 µm/second, but after fractionation of this material on sucrose gradients, the in vitro velocity increased to ~1.9 µm/second. SDS-PAGE of the S5 fraction revealed a polypeptide of 110 kDa, a size expected for the product of *kin2* (Lehmler et al., 1997). This polypeptide was missing from fractions prepared by identical methods from strain FB∆*kin2* and these extracts showed no gliding activity (not shown). A polypeptide of this apparent molecular mass co-migrated with the MT-gliding
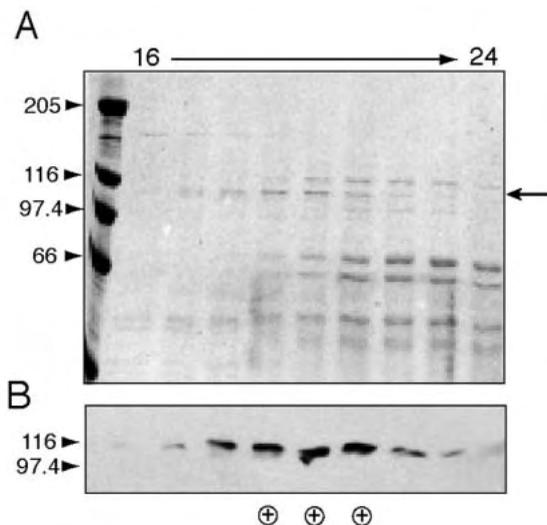
Fig. 1. Analysis of protein extracts (S5) from wild-type (wt) and mutant (Δkin) sporidia of *Ustilago maydis*. (A) Coomassie stained SDS-PAGE-gels of fractions from a 5-20% sucrose density gradient separation of the S5 fraction from wild-type sporidia. The proteins released by ATP from exogenous taxol-stabilized MTs, which were incubated in extracts from wild-type sporidia, contained a band at 110 kDa and supported movement of microtubules in a gliding assay (marked by plus) (B) A corresponding western blot, probed with an affinity purified polyclonal antibody against a peptide from the tail of fungal kinesins (NKC). The band of 110 kDa (arrow) co-purifies with the MT-gliding activity (plus). This activity correlates with a reaction against NKC. Numbers of ~150 μl gradient fractions are given above, and the bottom of the gradient is to the left. Molecular mass markers (in kDa) are shown at left.

activity in sucrose gradients (Fig. 1A,B). Motility was supported by several nucleotides (ATP>GTP>CTP>UTP), but not by ITP (Table 1), and the activity was sensitive to AMP-PNP. The polyclonal antibody NKC, which was raised against a synthetic 17 amino acid polypeptide (Steinberg, 1997) that is common to the predicted amino acid sequences of Kin2 and Nkin (Lehmler et al., 1997; Steinberg and Schliwa, 1995),

Table 1. Comparison of biochemical and biophysical properties of Kin2 from *U. maydis* and Nkin from *N. crassa*

		Kin2	Nkin
$V_{MT\text{-gliding}}$	(μm s^{-1})	1.5-1.9	2.1-3.8
ATP	(%)	100	100
GTP	(%)	58-63	50
CTP	(%)	20-28	9-19
UTP	(%)	17-29	12-21
ITP	(%)	0	0
AMPPNP inhibition	AMPPNP:ATP	<0.2	<0.3
Stokes radius	(nm)	8.07±0.41	6.27±0.53
Dw20	(×10^{-7} cm^2 s^{-1})	2.66±0.13	3.34±0.20
Sw20	(×10^{13} s)	7.74±0.30	8.80±0.15
MW in SDS-PAGE	(kg mol^{-1})	110	105/108
MW native	(kg mol^{-1})	~257 (230-290)	~227 (199-260)
Axial ratio	a:b	18.2	11.5

Motility data from several experiments of at least 2 isolations, biophysical data from at least 3 isolations; data for Nkin from Steinberg and Schliwa (1996).
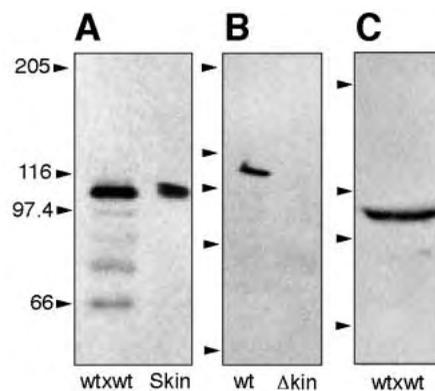
Fig. 2. Immunological characterization of polypeptides from *Ustilago maydis* probed with NKC antibodies. (A) The antibody recognizes a band of 110 kDa in the ATP-release (S5) from wild-type hyphae (wtxwt), as well as the kinesin from *Syncephalastrum racemosum* (Skin). (B) In S5 from wild-type (wt) sporidia Kin2 is clearly recognized, but the motor is undetectable in S5 from *kin2*-null cells. (C) Protoplasts from 20 hour old wild-type hyphae were lysed with Triton X-100 and the remaining membranes sedimented. The antibody NKC recognized a single band at 110 kDa. Molecular mass markers (in kDa) are shown at left.

recognized a 110 kDa polypeptide on immunoblots of crude extracts from *U. maydis* sporidia (Lehmler et al., 1997). This antibody recognized the partially purified 110 kDa polypeptide well as a purified kinesin-like protein of 115 kDa called Skin (Fig. 2A) from the zygomycete *Syncephalastrum racemosum* (Steinberg, 1997). No signal appeared in identical prepared extracts from Δ*kin2*-sporidia (Fig. 2B). Moreover, the insoluble fraction of lysed protoplasts generated from dikaryotic hyphae, which contained mostly membranes, gave a clear signal in immunoblots with NKC (Fig. 2C), and preparations from sporidia and hyphae of *U. maydis* displayed the same motility characteristics. From all these data we conclude that the 110 kDa polypeptide represents a 'conventional' kinesin that is the product of the *kin2* gene.

The hydrodynamic behavior of Kin2 was determined from its behavior on gel filtration columns and sucrose gradients, using protein from three independent isolations. The Stokes radius of 8.07±0.41 nm and sedimentation coefficient of 7.74±0.30×10^{-13} predicted a molecular mass for the native protein of ~260 kDa (range: 230-290 kDa), with an axial ratio of 18.2 (summarized in Table 1). Native Kin2 therefore appears to be a dimer without associated polypeptides.

Deletion of *kin2* affects vacuole formation and endocytosis but has no effect on the growth of sporidia under laboratory conditions

The acidic compartments of sporidia could be stained by the established vacuole dyes, Neutral Red and Acridine Orange (Anderson and Orci, 1988) as well as by carboxy-DCFDA (Roberts et al., 1991), and a new coumarin-based probe, CellTracker^{TM}blue CMAC (Haughland, 1996). All of these dyes stained the same spherical structures (data not shown), which probably represent the large storage vacuoles.

After staining sporidia of the wild-type strains FB1 and FB2 with CMAC, ~80% of all cells contain 2-3 vacuoles >1.5 μm in diameter, which were regularly spaced (Fig. 3A,B). In
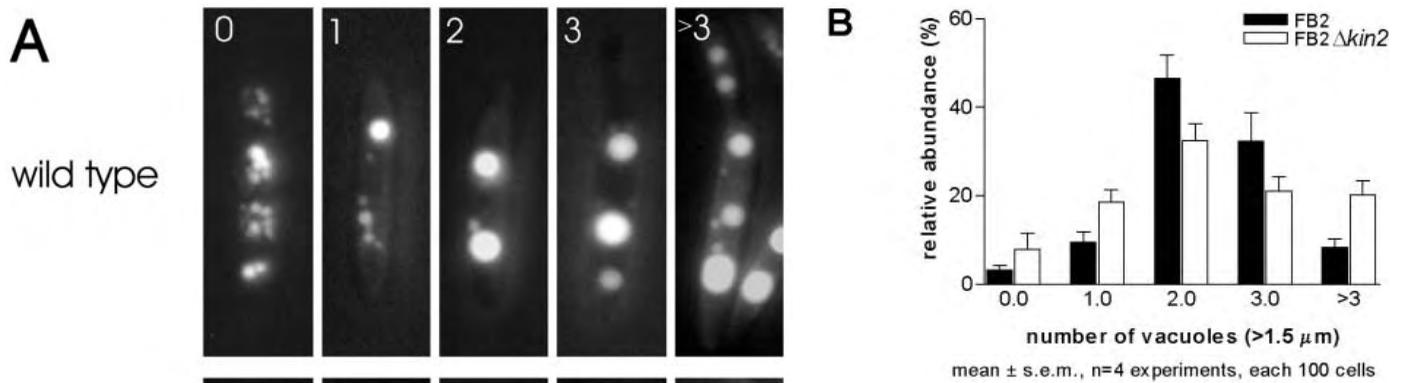
Fig. 3. Vacuole organization of wild-type and *kin2-null* mutant sporidia. (A) Overlays of phase contrast and fluorescence images after staining of acidic compartments with CellTracker[TM]blue CMAC. Representatives of typical wild-type cells are shown. In addition to the large, regularly-positioned vacuoles, small acidic organelles were occasionally visible. Kinesin-deficient mutant sporidia contained more small vacuoles, and the main acidic compartments were smaller and irregularly positioned. White numbers refers to the number of vacuoles >1.5 μm in diameter. Bar, 5 μm. (B) Over 80% of all wild-type cells contained two or three vacuoles with a diameter >1.5 μm. In contrast, *kin2*-null sporidia showed a broader distribution with many cells containing >3 or <2 big vacuoles.

contrast, FB1Δ*kin2* and FB2Δ*kin2* cells contained significantly more cytoplasmic compartments that stained by CMAC (Fig. 3A,B). Most of these compartments were small, but even cells with bigger vacuoles contained more than usual, and these compartments were scattered irregularly (Fig. 3A, Fig. 5). Even these comparatively larger vacuoles, however, were significantly smaller than the vacuoles in wild-type cells (Table 2). By counting the number and measuring the diameter of the stained compartments in >100 FB2 and FB2Δ*kin2* cells, we estimated the total vacuole volumes to be ~11.7 μm^3 and ~10.8 μm^3, respectively. Given the uncertainties of this estimation, these numbers are probably not significantly different.

Intact sporidia and hyphae did not accumulate the endocytosis marker dye Lucifer Yellow, but showed a strong staining of the cell wall (not shown). After the wall had been removed by digestion with novozyme, the stable protoplasts internalized Lucifer Yellow. In 4 independent experiments with wild-type (FB1, FB2), and kinesin-deficient mutant strains (FB1Δ*kin2*, FB2Δ*kin2*), 50-70% of the wild-type protoplasts took up the dye (*n*>300). After 1 to 2 hours several small vacuoles had accumulated the Lucifer Yellow and gave a strong fluorescent signal (Fig. 4A-C). In contrast, <2% of the kinesin-deficient mutant cells (*n*>400) incorporated dye, and only occasionally a single vacuole showed bright staining (Fig. 4D,E). This defect was not due to the absence of vacuoles in mutant cells, as 50-60% of FB2Δ*kin2* and FB2 protoplasts showed vacuolar staining with CellTracker[TM]blue CMAC under the same conditions (*n*>100 cells; data not shown).

Table 2. Comparison of wild-type and kinesin-deficient mutant cells of *U. maydis*

|		Wild type		*kin2*-null		Different?*
Sporidia						
Number of vacuoles‡		4.6±2.0	(50)	6.3±3.1	(50)	Yes (*P*=0.0025)
Diameter of vacuoles§	(μm)	2.3±0.4	(150)	2.1±0.3	(150)	Yes (*P*<0.0001)
Doubling in YEPG, 32°C	(min)	55.9±1.4	(2)	54.7±2.4	(2)	No (*P*=0.6041)
Doubling in YEPS, 32°C	(min)	98.6±2.8	(2)	102.2±1.6	(2)	No (*P*=0.2544)
Doubling in minimal, 32°C	(min)	348.7±54.2	(4)	354.1±11.25	(2)	No (*P*=0.8578)
Viability on plates, 25°C	(%)	102.7±9.7	(6)	73.2±13.6	(6)	Yes (*P*=0.0015)
Hyphae						
Number of vesicles in hyphae¶		2.7±1.6	(20)	6.3±2.3	(12)	Yes (*P*<0.0001)
Number of vesicles in sporidiae¶		3.1±2.0	(11)	9.5±1.7	(12)	Yes (*P*<0.0001)
Length of tip cell	(μm)	152.2±9.6	(20)	99.0±10.5	(20)	Yes (*P*<0.0001)
Tip growth rate**	(μm min^{-1})	1.48±0.33	(20)	0.07±0.05	(10)	Yes (*P*<0.0001)

*Means ± s.d. (sample size) are listed; means were compared using unpaired two-tailed *t*-tests (α: 0.05).
‡Only coumarin stained organelles bigger than 1.5 μm.
§Only the biggest vacuole per cell was measured.
¶Per 3 μm^2 cytoplasm in EM-sections.
**Grown on charcoal-agar plates observed in water: 24-29 hours old.
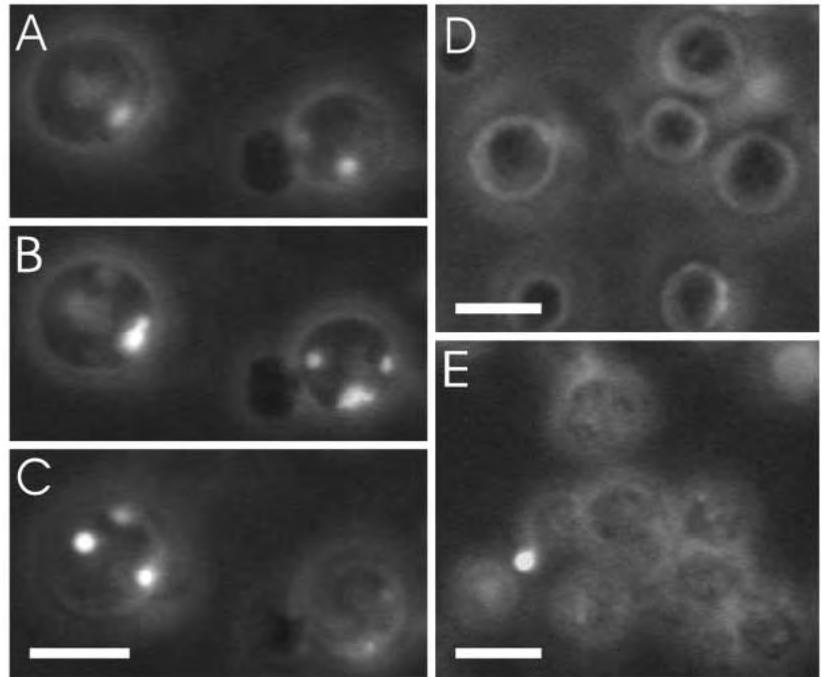
Fig. 4. Accumulation of Lucifer Yellow in the vacuoles of protoplast from *U. maydis* sporidia. (A-C) Optical sections through wild-type protoplasts shown as overlays of phase contrast and fluorescence images. In four independent experiments 50-70% of the cells accumulated the marker dye in several small vacuoles. The dimensions and locations of the vacuoles are clearly visible. (D,E) Protoplasts prepared from *kin2*-null sporidia look normal, but only 2% of these cells showed vacuole staining (E). Bar, 5 μm.

Surprisingly, the uptake of FM4-64, another vital dye for endocytosis (Vida and Emr, 1994) was not inhibited in kinesin-deficient mutant strains (Fig. 5). 5 minutes after incubation in FM4-64 the dye did stain the plasma membrane of wild-type and mutant cells, and after 30 minutes all of the marker dye was localized in the tonoplast. However, staining vacuoles with FM4-64 confirmed a irregular vacuole morphology as described above. Unfortunately, dikaryotic hyphae were not assessable to FM4-64 or Lucifer Yellow staining.

To examine whether the deletion of *kin2*, and its associated phenotypes, had an influence on growth-rates, sporidia were grown in sucrose-medium (YEPS), glucose-medium (YEPG) and low phosphate minimal medium. In several experiments the cells grew completely normally in all these media (Table 2). Interestingly, however, the deletion of *kin2* reduced the survival rate of colonies on PD-plates. In 2 independent experiments using 6 agar-plates, the viability of wild-type strains was ~100% after 2 days at 25°C, whereas mutant sporidia formed significantly fewer colonies (Table 2).

The growth of wild-type hyphae is accompanied by large basal vacuoles that are absent from the mutant hyphae

After the mating of wild-type sporidia on charcoal plates, the resulting hyphae consisted of a tip cell, ~150 μm long, which formed large basal vacuoles near its septum as it grew (Fig. 6A,B). Young colonies of wild-type hyphae were placed on poly-L-lysine coated coverslips, and hyphae grew on this surface to extend out of the colony. These hyphae consisted of a tip cell that left empty-looking compartments behind; these compartments were bounded by cell wall and regularly separated by septa (Figs 6B, 7A). The tip cells continued their movement on the glass surface for ~5 days, without the addition of nutrients. The velocity of hyphal expansion in water was ~1.5 μm/minute for hyphae only 24 hours old, but it dropped to ~0.003 μm/minute after 5 days. 40 hours after mating, growth under low osmotic conditions (50 mM sorbitol) led to 20-40 cells per image (×20) moving as far as 590 μm (490±65 μm, *n*=9) from the edge of the colony. In 500 mM sorbitol only 3-15 cells were found ~400 μm (406±76 μm, *n*=8)
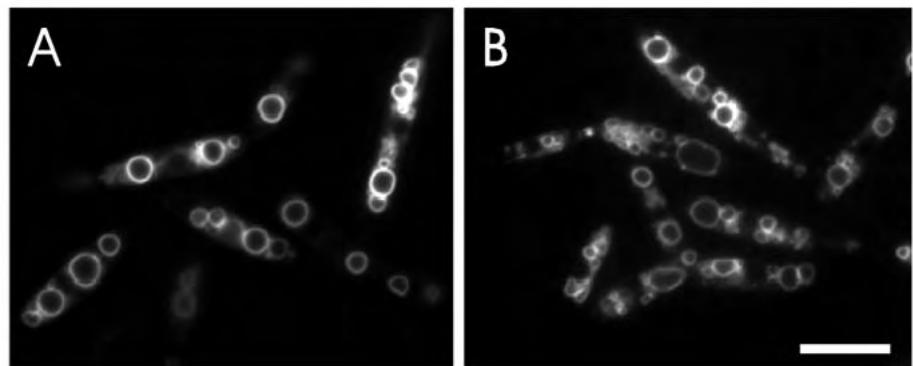
Fig. 5. Endocytosis of FM4-64 into the tonoplast of hapolid sporidia. 30 minutes after incubation the marker dye is incorporated into both the tonoplast of wild-type (A) and *Δkin2* cells (B). Note that mutant cells contain more but often smaller vacuoles. Bar, 10 μm.
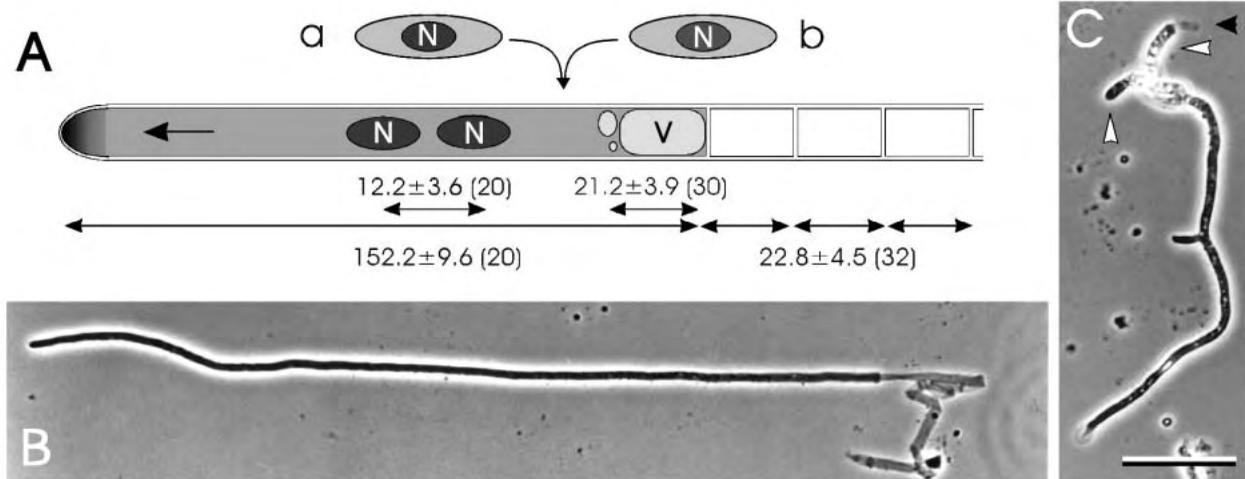
Fig. 6. Dikaryotic hyphae of *U. maydis* grown on charcoal agar-plates. (A) Schematic summary of the organization of a wild-type hypha. Two haploid sporidia of different mating types (a, b) fuse and give rise to a single tip cell that contains two nuclei (N) and performs tip growth (shaded, direction of growth marked with arrow). The cells are characterized by basal vacuoles (V) that fuse and expand to approximately the same dimension as the empty sections. Average dimensions ± s.d. and sample size are given below. (B) Phase contrast image of a wild-type hypha, 26 hours after mating. Note the empty compartments to the right of the elongated tip cell. (C) Phase contrast image of a 26 hour old *kin2*-null hypha. The irregular hypha is still in contact with two sporidia (white arrowheads) and shows a small empty section (black arrowhead). Bar, 20 μm for B and C.

from the colony. The effect of osmotic stress on the hyphae was confirmed using sucrose instead of sorbitol; the hyphae grew slower in sucrose. In contrast, *kin2*-null hyphae grown under the same conditions did not leave the colony.

In wild-type cells we found a surprisingly good correlation between the maximum length of the basal vacuoles (~21 μm), and the dimension of the apparently empty sections left behind by the growing tip cell (~23 μm, Fig. 6A). To examine the relationship between the vacuoles and the empty sections we monitored the genesis of basal vacuoles in 32 hours-old hyphae. While the tip was growing at a rate of 0.81±0.09 μm/minute (*n*=3), small vacuoles accumulated near the septum, fused, and gave rise to the basal vacuoles that expanded along the cell axis at a rate of 0.974±0.01 μm/minute for 20-30

minutes. After 30-40 minutes a new septum appeared and separated the vacuolar part from the cytoplasm of the cell (Fig. 7A-E).

In contrast to wild-type hyphae, 24 hour-old dikaryon cells obtained from the cross of FB1Δ*kin2* and FB2Δ*kin2* showed a dramatically reduced tip growth rate on agar plates (Table 2). The mutant hyphae were often curved, and the average length of a fully grown hypha at 24-28 hours was ~100 μm (Fig. 6C). The mycelium grown on agar plates almost failed to create empty compartments and only occasionally very small empty compartments were seen (Fig. 6C, black arrowhead). Interestingly, these cells tended to form additional septa, which were never accompanied by large basal vacuoles (Fig. 7F).

These light microscopic observations were confirmed by an

Fig. 7. Relationship between septa and vacuoles in wild-type (A-E) and kinesin-deficient hyphae (F). (A) DIC image of empty cell sections that were left behind while the tip cell moved to the right (arrow). The tube of cell wall, partitioned by septa, contains no visible cytoplasm, though it is divided into regular sections. Bar, 10 μm for A-E. (B-E) Different stages of vacuole formation. Vacuoles (V) are separated from the empty portion of the hypha (D) by a concave septum (S). Note the lack of refractile particles in the region where the septum will soon appear (E, arrow). (F) Mutant hyphal cell (FB1Δ*kin2*xFB2Δ*kin2*) which contains an additional septum (S) that is not accompanied by vacuoles. Bar, 3 μm.
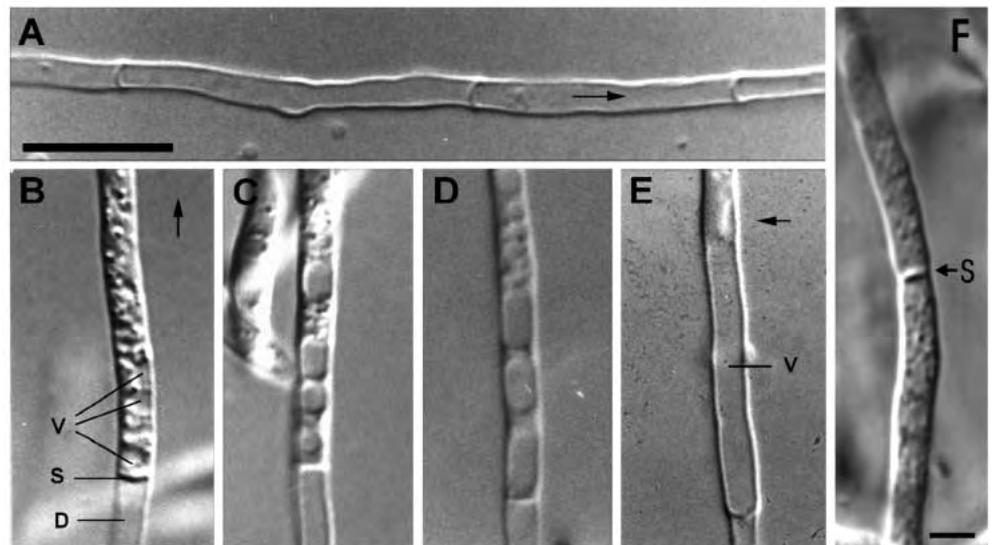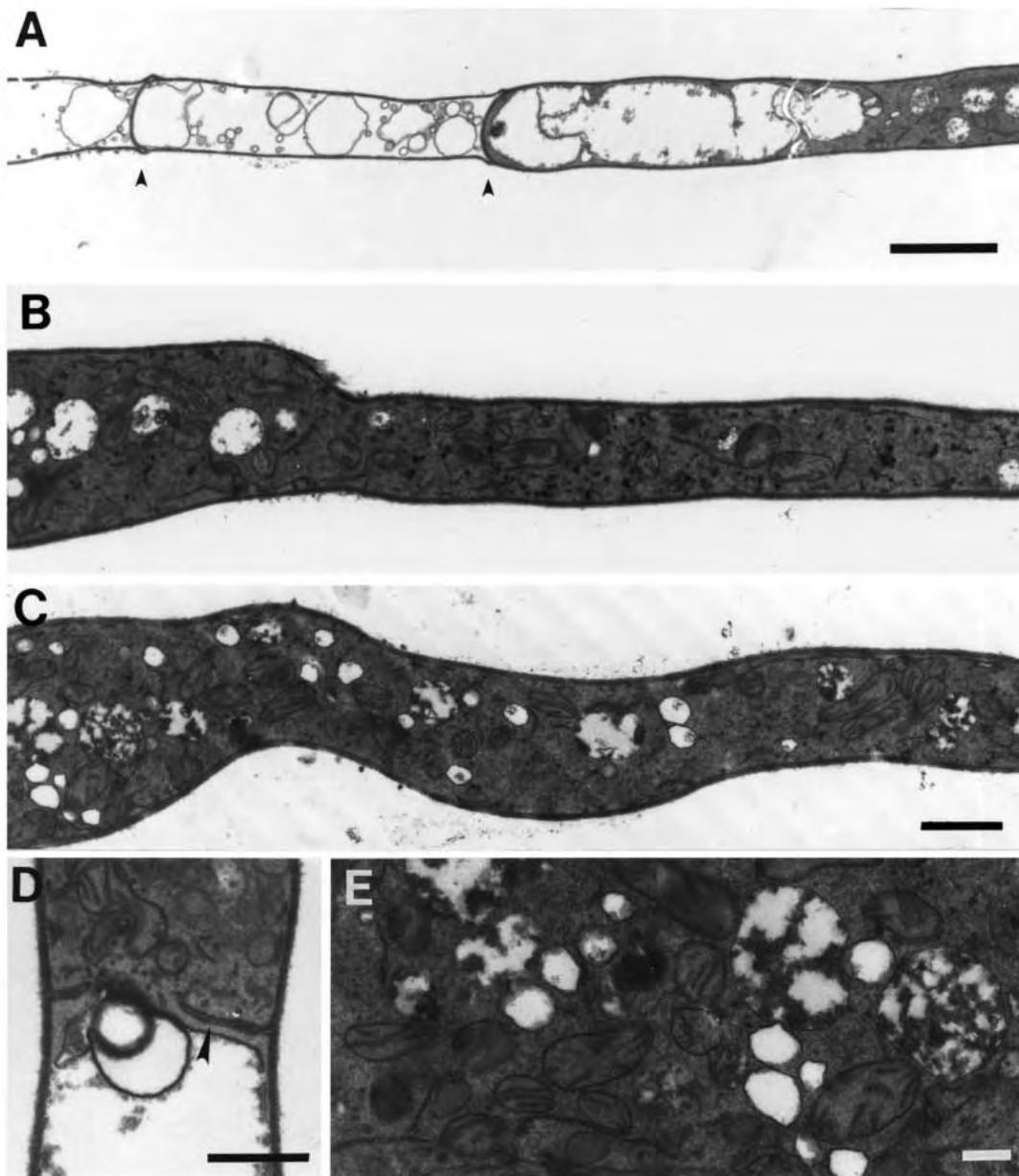
Fig. 8. Electron micrographs of hyphae grown on poly-L-lysine coverslips. (A) Large vacuoles accumulate at the basal part of the hypha. These organelles are separated from the dead compartments by septa (arrows). Note the membrane residues in these sections. Bar, 2 μm. (B,C) Comparison of a young wild-type hypha (B) and a kinesin deficient mutant hypha (C). The wild-type hypha grows straight and big vesicles, filled with fuzzy material, accumulate in the basal sporidia (left). Mutant hyphae are thicker, curved and filled with many vesicles. Bar: 1 μm. (D) A septum (arrowhead) forms directly above the large basal vacuole in a wild-type cell, excluding almost all cytoplasm from the dead sections. Bar, 1 μm. (E) Beside the large vesicles that are filled with electron dense material, mutant hyphae contain a smaller fraction of vesicles which appear almost empty. Bar, 0.3 μm.

electron microscopic analysis of *Ustilago maydis* hyphae grown from FBD11 and CLD12 in a water drop on poly-L-lysine coated coverslips. In wild-type hyphae (FBD11) large basal vacuoles were found closely associated with the basal septum (Fig. 8A), which appeared directly above the vacuole (Fig. 8D). The following dead segments contained membrane residues but no cytoplasm (Fig. 8A). Vesicles ~400 nm in diameter (378 ± 90 nm, $n=37$) were scattered over the whole length of the tip cell. In the chemically fixed cells these organelles were usually filled with fuzzy, electron-dense material; they accumulated and enlarged at the basal region of growing hyphae or outgrowing sporidia (Fig. 8B). The kinesin-deficient CLD12 hyphae were thicker, curved and contained vesicles of the same appearance and size (409 ± 109 nm, $n=38$). In addition, smaller vesicles were scattered over the entire length of the mutant cell (Fig. 8C,E). These organelles appeared almost empty and showed a significantly smaller

diameter (226 ± 49 nm, $n=50$) than the vesicles found in wild-type hyphae. The total number of vesicles in CLD12 hyphae was 3 times higher than in wild-type hyphae (Table 2). In agreement with the observations by light microscopy, large basal vacuoles were almost never found in Δ*kin2*-cells.

MTs nucleate at both poles of the cell

Wild-type cells contained numerous MTs (Fig. 9A,I). The deletion of kin2 did not alter the MT-cytoskeleton (Lehmler et al., 1997; own results, not shown), as well as the polar distribution of actin patches at one or both poles of the sporidia (data not shown). Treatment with 10 μM nocodazole for 30 minutes did completely depolymerize MTs in diploid sporidia and hyphae (not shown). 8 to 10 minutes after release from nocodazole short MTs appeared at one or both poles of sporidia (Fig. 9B,C). In hyphae ~40% of the tip cells ($n=50$) showed short microtubules near their apex (Fig. 9D,E) and in ~40% of
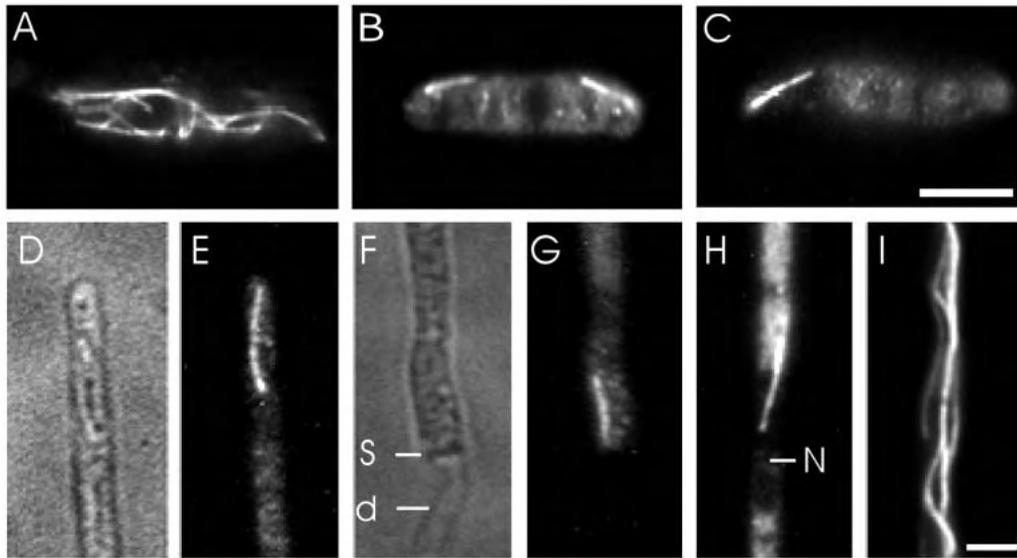
Fig. 9. Microtubule staining in diploid wild-type sporidia and hyphae of *U. maydis*. (A-C) Wild-type sporidia contain long microtubules running through the length of the cell (A). After release from nocadazole tratment short MTs appear at one or both poles of the cells (B,C). Bar, 5 μm for A-C. (D-I) 10 minutes after release from nocodazole hypal cells contain short MTs at their tip (D: phase contrast, E: immunofluorescence) and at the basal region (F: phase contrast, G: immunofluorescence). Note that the MT is located close to the septum (S), which separates the cytoplasm from the dead portion (d). Occasionally, a single MT is found close to the diploid nucleus (N in Fig. H). Repolymerization results in overlapping MTs (I). Bar, 2 μm for D-I.

all cells an additional microtubule appeared at the opposite pole of the hyphal tip cell (Fig. 9F,G). In the remaining 20% a third MT was visible that occasionally was located near a nucleus in the middle of the cell (Fig. 9H). At later time points the microtubules increased in length, resulting in a meshwork of overlapping MTs (Fig. 9I).

DISCUSSION

The kinesin motor from *U. maydis* is similar to that from other fungi

The *kin2* gene of *U. maydis* contains an open reading frame whose predicted amino acid sequence is 40-70% identical with the 'conventional' kinesin from *N. crassa* (Lehmler et al., 1997). Here we describe the partial isolation and characterization of a 110 kDa polypeptide from *U. maydis* that has MT-dependent motor activity. The apparent molecular mass of this polypeptide in SDS-PAGE corresponded with the molecular mass predicted from the *kin2* gene sequence (Lehmler et al., 1997), and the 110 kDa band was recognized by an antibody raised against a 17 amino acid peptide in the C-terminal tail region of the predicted sequence of conventional kinesin from *N. crassa* (Steinberg and Schliwa, 1995; Steinberg, 1997). Moreover, the 110 kDa band was undetectable in preparations from *kin2*-null cells, either by Coomassie staining or by western blot analysis. These data strongly imply that the 110 kDa polypeptide is the product of the *kin2* gene.

Like Nkin from *N. crassa* and a kinesin from the zygomycete *Syncephalasytum racemosum* (Skin), Kin2 supported in vitro gliding that was faster than movements based on kinesins from animal sources, and it showed an unusual nucleotide specificity for movement (Steinberg and

Schliwa, 1996; Steinberg, 1997). All these fungal kinesins bind the antibody NKC (Steinberg, 1997; this study), and moreover, the calculated molecular mass of native Kin2 suggests that it, like Nkin and Skin, lacks the light chains found with all native conventional kinesins that have been purified from animal sources (summarized by Bloom and Endow, 1994). Because highly similar kinesins are found in three major classes of fungi, but not in the genome of budding yeast (for Internet addresses see Goffeau et al., 1996), it seems plausible that at least some of the unique characteristics of these enzymes result from their specialized function in elongated hyphal cells.

Kin2 takes part in endocytosis and vacuole formation in haploid sporidia

Based on its predicted amino acid sequence, Kin2 belongs to the protein family of conventional kinesins (Lehmler et al., 1997). These motors are thought to be involved in organelle transport (summarized by Schroer and Sheetz, 1991; Bloom and Endow, 1994), but their specific functions in living cells have remained elusive. The deletion of *kin2* from *U. maydis* did not affect the growth and morphology of its haploid sporidia (Lehmler et al., 1997) and growth remained normal under changed media composition. Δ*kin2* cells did, however, show a reduced viability on plates, which implies an impaired resistance to environmental stress. We therefore tested the importance of kinesin for endocytosis using physiological assays (Riezman, 1985; Vida and Emr, 1994) and several vacuole staining procedures (Haugland, 1996). The Δ*kin2* strains showed clear defects in the transport of Lucifer Yellow into vacuoles, and it displayed an altered organization of acidic compartments, with a tendency to form more and smaller vacuoles that were often misplaced within the cell. Since total vacuole volume was essentially unchanged, while vacuole morphology and organization was altered, we infer that Kin2

plays a role in the formation and positioning of vacuoles in *U. maydis* sporidia. As endocytosis might contribute to vacuole formation in fungi (Klionsky et al., 1990), maybe via a transport vesicle shuttle from late endosomes to the lysosomal compartment (Storrie and Desjardins, 1996), these defects may reflect the disruption of some aspects of the MT-dependent membrane traffic towards the lysosomal-like vacuole. However, Δ*kin2* strains are not impaired in growth and no inhibition of endocytosis of the marker dye FM4-64 was observed, suggesting that endocytotic membrane traffic is not completely inhibited by the deletion of *kin2*.

Kinesin may contribute to cytoplasmic migration during hyphal growth in *U. maydis*

Tip growth in fungi is accomplished by an osmotic force that expands the flexible hyphal apex (reviewed by Wessels, 1986; Harold et al., 1995), a process that requires a continuous transport of secretory vesicles to supply the growing tip with additional wall material and exoenzymes (reviewed by Heath, 1995; Gow, 1995a). At the apex these organelles, in combination with cytoskeletal elements, form a characteristic structure of accumulated vesicles called the Spitzenkörper (Girbardt, 1957), which is believed to support hyphal tip growth (reviewed by Bartnicki-Garcia, 1996). It has been suggested that fungal kinesins are involved in these secretory process (Seiler et al., 1997) and thereby support the expansion of the apex. Consistent with this view, kinesin-deficient mutant hyphae of *U. maydis* (Lehmler et al., 1997) and *N. crassa* (Seiler et al., 1997) show morphological defects and a lack of vesicle accumulation that is normally seen in wild-type hyphae. However, the deletion of a conventional kinesin in *Nectria haematoccoca* (Wu et al., 1998), which displays over ~70% and ~90% identity with the motor region of Kin2 and Nkin, respectively, did not severely change the appearance of the Spitzenkörper. Studies on the Spitzenkörper in living cells demonstrate that this structure is fairly dynamic and unstable (López-Franco and Bracker, 1996). General inhibition of tip growth by glucan synthesis inhibitors (Kurtz et al., 1994) as well as environmental stresses (Girbardt, 1957; Grove and Bracker, 1970; López-Franco and Bracker, 1996) will all affect the localization and even the existence of the Spitzenkörper, so Girbardt (1957) concluded that the Spitzenkörper is a sensitive indicator of perturbations to the fungal cell. Therefore, the absence of these structures from mutant hyphae of *Ustilago maydis* might be an indirect consequence of such a disturbance due to the deletion of *kin2*.

Deletion of the kinesin gene clearly affects the hyphal cell. *Kin2*-null hyphae of *Ustilago maydis* were not able to leave the conventional, non-cellular compartments behind (Lehmler et al., 1997), though this process was not completely stopped, as small membrane filled compartments were occasionally found. Hyphae did, however, grew up to ~100 μm in length and reached 93.5±14% of the volume of wild-type hyphae (*n*=5, measured after 20-25 hours on agar plates). These results indicate that tip growth is not blocked by the *kin2* deletion, even though the function of Kin2 becomes crucial late in the hyphal growth process. This later stage is characterized by the migration of cytoplasm towards the growing tip, while empty-looking compartments are left behind at the basal end of the cell. Our electron microscopic data confirm that membranes, but not cytoplasm, are left behind while the tip cell is migrating

forward. For wild-type hyphae, we found a significant correlation between the length of the largest basal vacuoles and the length of empty-looking sections separated from the hyphal tip cell by septae. This correlation implies a functional relationship between the two structures. Given that infective hyphae of smut fungi must grow on the epidermis of a host without any access to nutrients until they can invade the host tissue (summarized by Fischer and Holton, 1957) hyphal extension by vacuole formation is an economical way to save cytoplasm, and this process might even contribute to the osmotic pressure that forces the tip growth process (discussed by Koch, 1994).

The absence of basal vacuoles from the *kin2*-null mutant hyphae, the increased number of vacuole-like vesicles that are scattered over the entire length of the hyphae, and in addition the vacuole phenotype of *kin2*-null sporidia all support the idea that kinesin plays a role in vacuole formation. We suggest that the deletion of kinesin disturbs the formation of basal vacuoles and blocks the normal pathway by which tip cells form empty-looking compartments and thereby save their cytoplasm. The reduced growth, decreased pathogenicity and the disturbance of the Spitzenkörper-like structure of Δ*kin2* mutant strains (Lehmler et al., 1997) could thus be viewed as an indirect results from the disruption of this pathway.

This model is supported by several observations. First, the formation of a basal vacuole should require long distance transport of membranes towards the septum. Consistent with a function as a long distance organelle transporter, kinesin's deletion does not affect the growth of yeast-like sporidia but is crucial for the growth of the dikaryotic hyphae (Lehmler et al., 1997). Second, studies in *Uromyces phaseoli* and *Sordaria macrospora* suggest that in fungal hypha, MTs are oriented with their plus-ends distal to the growing tips (Hoch and Staples, 1985; Thompson-Coffe and Zickler, 1992). The depolymerisation/repolymerisation experiments demonstrated in this study suggest that MTs in *Ustilago* hypha might be oriented in an antiparallel fashion, with a majority nucleating at the hyphal tip. As all fungal conventional kinesins so far studied move their cargos towards the plus-end of the MTs (Steinberg and Schliwa, 1995; Steinberg, 1997), Kin2 could use the MTs that nucleate at the hyphal tips to serve as a motor for the formation of a basal vacuole. Third, drug studies imply that in filamentous fungi vacuole formation and movement is MT-dependent (Herr and Heath, 1982; Steinberg and Schliwa, 1993).

In this model Kin2 of *U. maydis* would be a plus-end directed motor that moves organelles back from the growing tip. These organelles may be lysosome-like vesicles, since they are acidic, and vacuoles are thought to be the fungal equivalent of the lysosomal compartment of higher eukaryotes (Klionsky et al., 1990). In vertebrate cells the movement of lysosomes is also thought to be MT-dependent (Swanson et al., 1987; Heuser, 1989), and several studies describe a fundamental role for conventional kinesin in the transport of these organelles (Hollenbeck and Swanson, 1990; Burkhart et al., 1993; Feiguin et al., 1994; Nakata and Hirokawa, 1995). Another possibility is that kinesin might transport small vesicles that bud off late endosomes and move towards the lysosomal compartment, as suggested by the vesicle shuttle model (reviewed by Storrie and Desjardins, 1996). This transport is also part of the endocytotic pathway and a defect in some vesicle transport from

endosomes to the lysosomal compartment might be responsible for the endocytosis defect observed in mutant sporidia. Thus a fundamental function of conventional kinesin in membrane traffic might be conserved from animals to fungi. Our results add an interesting perspective on the growth of a hypha; they suggest that a retrograde movement of vesicles contributes to a process that is thought to occur mainly at the apical region of the wall. Studies aimed to identify the cargo of Kin2 and the polarity of transport will be necessary to test this model.

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